Correct regionalisation of a tissue primordium is essential for coordinated morphogenesis

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Abstract

During organ development, tubular organs often form from flat epithelial primordia. In the placodes of the forming tubes of the salivary glands in the *Drosophila* embryo, we previously identified spatially defined cell behaviours of cell wedging, tilting and cell intercalation that are key to the initial stages of tube formation. Here we address what the requirements are that ensure the continuous formation of a narrow symmetrical tube from an initially asymmetrical primordium whilst overall tissue geometry is constantly changing. We are using live-imaging and quantitative methods to compare wild-type placodes and mutants that either show disrupted cell behaviours or an initial symmetrical placode organisation, with both resulting in severe impairment of the invagination. We find that early transcriptional patterning of key morphogenetic transcription factors drives the selective activation of downstream morphogenetic modules, such as GPCR signalling that activates apical-medial actomyosin activity to drive cell wedging at the future asymmetrically-placed invagination point. Over time, transcription of key factors expands across the rest of the placode and cells switch their behaviour from predominantly intercalating to predominantly apically constricting as their position approaches the invagination pit. Misplacement or enlargement of the initial invagination pit leads to early problems in cell behaviours that eventually result in a defective organ shape. Our work illustrates that the dynamic patterning of the expression of transcription factors and downstream morphogenetic effectors ensures positionally fixed areas of cell behaviour with regards to the invagination point. This patterning in combination with the asymmetric geometrical set-up ensures functional organ formation.
Introduction

Complex three-dimensional organs arise from simple tissue primordia, and in many cases these primordia are flat polarised epithelial sheets. Early in development the expression of the first patterning genes broadly sets up embryonic regions. This is followed by the activation of gene regulatory networks that specify the fate of tissue primordia in defined locations (Gilmour et al., 2017; Sidor and Röper, 2016). The patterning and fate determination gene products do not directly affect morphogenetic changes, but rather instruct the expression of downstream morphogenetic effectors that drive a tissue primordium down a path of defined physical changes. In many well-studied cases of tissue morphogenesis, such as mesoderm invagination or germband extension in the fly, many morphogenetic effectors are induced evenly across the tissue primordium. Regional differences in the physical changes or ‘behaviours’ of cells observed in these primordia can arise due to physical effects and feedback or interference either from cells within the primordium or from surrounding tissues (Chanet et al., 2017; Collinet et al., 2015; Lye et al., 2015). A further layer of control and complexity is added as the primordia themselves can also be patterned to guide the differential behaviour and changes of groups of cells within a primordium. Such pre-patterning of morphogenetic events within a single tissue primordium is much less understood.

We use the formation of a narrow-lumen tube in the Drosophila embryo as a model system to identify key requirements for successful organ formation. The symmetrical tubes of the embryonic salivary glands form from a flat, nearly circular epithelial primordium, the salivary gland placode (Fig. 1A). We recently uncovered a patterning of key cell behaviours that drive the initiation and earliest stages of the tube budding process (Gillard et al., 2021; Sanchez-Corrales et al., 2018). Interestingly, the point of invagination from the circular placode primordium is not in the centre of the placode, but in an asymmetric, eccentric position in the dorsal-posterior corner (Fig. 1A). Using quantitative morphometric methods we showed that the early apical constriction and associated cell wedging at the position of the invagination pit initiates the tissue bending (Booth et al., 2014; Sanchez-Corrales et al., 2018). Isotropic constriction driven by apical-medial actomyosin is concentrated near the forming invagination pit (Fig.1A’, A’’), but at a distance to the pit a second cell behaviour dominates: cell intercalation. Driven by a polarised junctional accumulation of actomyosin, cell intercalation events such as T1 exchanges and rosette formation and resolution help to elongate the tissue radially towards the invagination pit and contract it circumferentially, via circumferential neighbour gains (Fig. 1A’) (Sanchez-Corrales et al., 2018).

This asymmetric set-up of the placode raises two questions: firstly, is the asymmetry required for successful organ formation or could a symmetrical set-up not lead to the same
result? An indication that the asymmetry of the salivary gland placode is important for wild-type tube formation is given by the reports that in certain mutant situations where a more central invagination appears to form, the overall morphogenesis is disrupted and the invaginated structure that forms is sack-like and irregular. This is true for embryos lacking the transcription factor Hkb that is expressed early in the placode (Myat and Andrew, 2000b; 2002), and could therefore potentially implicate Hkb in establishing early placode patterning.

Secondly, how is this asymmetry of the placode established prior to morphogenesis commencing and then maintained throughout the process? Previous studies have shown that several proteins known to be expressed in the placode can be detected at the mRNA level initially in the dorsal posterior region of the placode. These include the transcription factor Forkhead (Fkh) (Myat and Andrew, 2000a), the dynein-associated protein Klar (Myat and Andrew, 2002), as well as the kinase Btk29 (Chandrasekaran and Beckendorf, 2005). Defined expression of these and other factors at the dorsal-posterior corner could set up the initiation of cell shape changes in this region. In agreement with this, we previously found that fkh\(^{-}\) mutants specifically fail to apically constrict in the dorsal-posterior corner, though they do show intercalation behaviour, and these mutants do not invaginate a tube (Sanchez-Corrales et al., 2018). Following on from the early regionalised behaviours that drive the initial tissue bending and tube invagination (Sanchez-Corrales et al., 2018), it is thus far unclear what mechanisms continue to drive the sustained invagination until all secretory cells have internalised. This could be due to continued transcriptional patterning and activation of downstream effectors, and/or could be due to mechanical signalling and feedback.

Here, we investigate how the asymmetry is set-up and maintained during salivary gland tube formation, in particular beyond the initial stages. We found that apical constriction is always strongest near the invagination pit at any moment in time. As more and more coronae of cells approach the region near the pit, they switch behaviour from predominantly intercalating to predominantly apically constricting/wedging. This continued switch to apical constriction leads to a smooth continued invagination process akin to a ‘standing wave’ of apical constriction, through which the placodal cells flow. The continued near-pit constriction is driven by apical-medial myosin, whose peak intensity and peak pulsatile strength is tracking in a fixed position close to the pit over time. Using live-imaging of endogenous fluorescently-tagged versions of Hkb and Fkh, we show that their expression levels pre-pattern where initial constriction is occurring. Both are upstream of a dynamic patterning of the GPCR ligand Fog, that in turn activates apical-medial myosin. Loss of the correct pre-patterning, such as in hkb\(^{-}\) mutants or when Fog is overexpressed, leads to loss of the symmetrical final organ shape with a narrow lumen and instead gives rise to expanded and sack-like glands with widened lumena.
Our work establishes that after the tissue is patterned at the posterior corner, cells follow dynamic rules of behaviour as they approach the pit. Symmetrical tube morphogenesis thus relies on a regionalisation and patterning of the primordium long before cells show distinctive behaviours. Establishing general mechanisms that modulate the orderly and repetitive behaviour of cells to form correctly shaped simple model organs such as the salivary glands will assist the understanding of the morphogenesis of other more complex tubular organs in mammals.
Results

**Patterned apical constriction remains fixed around the pit over time**

The initial morphogenetic change that occurs during the budding of the tube of the salivary glands in the *Drosophila* embryos is the apical constriction of cells at the point of the future site of invagination, the pit (Fig. 1 A, A') (Girdler and Röper, 2014; Myat and Andrew, 2000a; b; Sanchez-Corrales et al., 2018). We recently provided the first quantitative morphometric analysis of the very early stages of this process, and revealed that, firstly, isotropic apical constriction near the future pit commences long before changes at the tissue level are apparent, and that, secondly, the apical constriction is indicative of a 3D cell behaviour of wedging of cells at the pit (Fig. 1A'). Importantly, we uncovered a strong regionalisation of cell behaviours during the early stages of tube budding: cells near the invagination point predominantly showed apical constriction behaviour while cells at a distance from the pit predominantly showed intercalation behaviour (Sanchez-Corrales et al., 2018)(Fig.1A). What is unclear is how the early tube initiation via isotropic apical constriction of cells, combined with directional intercalation of the cells still positioned away from the forming pit, evolves over time. The process of tube formation is characterised by the continuous invagination of cells and the formation of a symmetrical narrow-lumen tube from an asymmetric placode primordium. We can imagine two scenarios: in one, cell behaviour is fixed at early stages of budding, i.e. apical cell constriction is restricted to the pit and only apparent during early stages. In a second scenario, cell behaviour depends on a cell’s position relative to the invagination pit and thus cell behaviours change as cells move towards the invagination point.

To distinguish between these scenarios, we segmented and tracked placodal cells from time-lapse movies covering a -40 min to +70 min time interval of the invagination process, with $t = 0$ min defined as the first occurrence of tissue bending (see Figure 1-figure supplement 1 for time intervals of analysed movies and cell numbers analysed) and performed quantitative analyses (Fig. 1B, C). At the latest time point covered, the majority of secretory cells had completely internalised and formed the internal tube. As calculated previously (Sanchez-Corrales et al., 2018), we employ a radial coordinate system centred at the invagination point at $t = 0$ min. We specify the 'near to the pit' region as the region located between the invagination point and up to 33% of the distance between the invagination point and the far edge of the placode (with this region usually being within 13.7 +/- 3 µm of the pit), and with the rest of the cells defined as ‘far from the pit’.

Cells positioned near the pit at $t = 0$ min (Fig. 1B, pink) constricted their apices isotropically and rapidly and were internalised by about $t = +20$ min (Fig. 1B,C and dashed curve in E). Cells positioned far from the pit at $t = 0$ min (Fig. 1B, grey) and moving into a
position closer to the invagination point began constricting (Fig. 1B, D and solid curve in E; Video 1), suggesting that cells changed their behaviour depending on where they were positioned in the placode with respect to the invagination point. Cells near the pit displayed isotropic constriction and thus a negative cumulative apical area change from the start (Fig. 1E, pink highlighted), whereas cells initially positioned far from the pit did not change their apical area over the first ~20 min (Fig. 1C, grey highlighted), but then began to display isotropic apical constriction (Fig. 1E, blue highlighted). The cell behaviours and changes of cell behaviours were very apparent when we analysed individual cells: cells located in the near the pit region (Fig. 1F, yellow and green cells) constricted progressively, whereas cells located in the region far from the pit at t = 0 min (Fig. 1F, blue and magenta cells) retained their apical area initially and only began constricting from t > +20 min onwards. As described previously (Sanchez-Corrales et al., 2018), cells far from the pit initially showed predominantly a cell intercalation behaviour that constricts the tissue primordium circumferentially whilst expanding it radially, thereby moving cells closer to the invagination pit (Fig. 1G). As they approached the invagination pit, these cells also started to apically constrict (Fig. 1E solid line, highlighted in blue and 1F).

These data strongly suggest that an initially prepatterned behaviour, isotropic constriction at the pit and intercalation further away, is dynamically adjusted during tube budding from a flat epithelial sheet, so that cells within a similar distance to the invagination pit display the same behaviour.

**Sustained apical constriction near the pit is driven by sustained apical-medial myosin**

Early apical constriction at the pit is driven by a highly dynamic apical-medial actomyosin network (Booth et al., 2014; Sanchez-Corrales et al., 2018). We next sought to determine whether the apical-medial actomyosin dynamics would differ among regions in the placode. We analysed apical-medial actomyosin levels in cells across the placode, and firstly illustrate our findings in two snapshots: cells near the future invagination point at t = 0 min showed strong accumulation of apical-medial myosin (Fig. 2A, A”; C; see also Video 2), whereas cells far from the pit and close to the anterior placode boundary (Fig. 2A, A’) showed mainly junctional myosin II accumulation. At t = 30 min the cells now located right next to the invagination pit continued to display strong apical-medial myosin accumulation (Fig. 2B, B’), but in addition cells that were initially ‘far from the pit’, but were now in a closer proximity to the pit though still near the anterior boundary, now also started to display apical-medial actomyosin (Fig. 2B, B”). Thus, being moved into a position near the invagination pit appeared to determine levels of apical-medial myosin accumulation. Looking placode-wide over the whole time period analysed, the peak of the apical-medial myosin fluorescence intensity tracked in a near-fixed position to the edge of the pit over time (Fig. 2D, white}
dotted line). The location of the highest medial myosin intensity was also where cells showed the shortest cycle lengths of medial myosin II fluctuations (Fig. 2E, white dotted line). Interestingly, cells near the pit at t = 0 min showed an overall shorter myosin II cycle length (the time period elapsed between two peaks of highest myosin II intensity during the oscillation, see schematic in Fig. 2E) compared to cells located far from the pit (Fig. 2F). As longer cycle length correlates with unproductive myosin cycles (Booth et al., 2014), this was in line with cells further away from the pit showing less apical constriction. Furthermore, the strength of myosin oscillations can be expressed as the product of the amplitude and frequency of the oscillation (Booth et al., 2014). This myosin strength across the placode at t = 0 min was much higher in cells near the invagination point when plotted against the distance to the pit, with a gradual decrease in myosin strength towards the placode boundary (Fig. 2G). However, cells originally far from the pit at t = 0 min increased their myosin strength over time (and concomitantly decreased their cycle length; see Figure 2-figure supplement 1) whilst moving into a position closer to the pit, so that by t = 55 min their myosin strength had increased to levels previously shown by cells near the pit at t = 0 min (Fig. 2H).

Thus, our data show that the early asymmetric set-up of high apical-medial myosin II intensity and activity that was clustered near the pit was maintained during the continued invagination of the tube, in turn driving the continued apical constriction near the pit.

**hkb**<sup>−</sup> mutants show a delayed symmetrical apical constriction

In order to uncover the role of the initial asymmetric patterning of cell behaviours within the placode, we turned our attention to mutant situations: we already knew that the transcription factor Fkh was important for invagination, as *fkh*<sup>−/−</sup> mutants do not show localised apical constriction or any invagination (Myat and Andrew, 2000a; Sanchez-Corrales et al., 2018). A possibly more interesting situation is present in a previously published mutant of the transcription factor Huckebein (Hkb) that was reported to show a central invagination pit, combined with malformed invaginated salivary glands at later stages (Myat and Andrew, 2000b; 2002).

We collected and segmented time-lapse movies of *hkb*<sup>−/−</sup> mutant embryos over the same time period as the control wild-type embryos (Fig. 3 and Figure 3-figure supplement 1; Videos 3-6). Whereas in wild-type embryos, at t ~ +40 min, most secretory cells of the salivary gland placode have already invaginated (Fig. 3 A′′, B′′, C′′), in the *hkb*<sup>−/−</sup> mutants no invagination had occurred yet (Fig. 3 D′′, E′′, F′′). Eventually, these embryos invaginated a dilated tube through an enlarged pit (see Figure 3-figure supplement 2). We analysed the spatial distribution of apical area changes over time in wild-type and *hkb*<sup>−/−</sup> placodes (Fig. 3G, H). In the wild-type, the area of strongest apical area constriction was always located in a
position near the pit, similar to the distribution of medial myosin intensity and activity (Fig. 2D, E). In contrast, in hkb<sup>−/−</sup> mutant placodes cells began to constrict in an aberrant central position from about t = +20-30 min (Fig. 3G, H). Thus, hkb<sup>−/−</sup> mutants show a delayed central invagination pit compared to the eccentric pit observed in wild-type placodes (Fig. 3I, I'). For our further quantitative analyses, we now took this change to a central pit position in the mutants into account. We plotted cumulative apical area change for cells in hkb<sup>−/−</sup> mutant placodes, split into cells near the (now central) pit and cells far from the pit (at the placode periphery). This analysis showed that cells in hkb<sup>−/−</sup> mutant placodes located near the central pit started to constrict at a much slower rate than cells in wild-type placodes, and only from about t = +20 min on (Fig. 3J, wild-type data reproduced from Fig. 1E). In fact, the rate of the constriction of the central cells in hkb<sup>−/−</sup> mutants was very similar to that of the cells located at a distance to the pit in the wild-type (compare blue dashed and magenta solid curves), suggesting that the central cells in hkb<sup>−/−</sup> mutants continue to behave identical to the wild-type cells occupying the same position. Cells far from the central pit in hkb<sup>−/−</sup> mutants showed a slight apical area expansion up to t = +20 min followed by a slight constriction, but did not invaginate over the time interval shown in the plots. In agreement with the delayed central constriction of cells in hkb<sup>−/−</sup> mutant placodes, we found no accumulation of apical-medial actomyosin at the eccentric position where a pit forms in the wild-type (compare Fig. 3E, K to Fig. 2D). Instead, only at t ~ +40 min did a central group of cells in hkb<sup>−/−</sup> mutant placodes display increased accumulation of apical-medial myosin II (Fig. 3K), inducing a shallow tissue bending in this region at this point (see also Figure 3- figure supplement 2).

Thus, in comparison to wild-type placode with an eccentric invagination, in hkb<sup>−/−</sup> mutant placodes a central invagination formed with a significant delay in time.

**hkb<sup>−/−</sup> mutants display disrupted cell behaviours across the placode**

The above analyses strongly suggest that the initial asymmetrical set-up of cell behaviours in the placode might be disrupted in hkb<sup>−/−</sup> embryos. We therefore performed a strain (deformation) rate analysis of placodes in hkb<sup>−/−</sup> mutants in comparison to wild-type embryos to assess this quantitatively. The strain rate analysis is based on the assumption that any change in tissue shape can be accounted for by changes in cell shape and cell intercalations (Blanchard et al., 2009; Sanchez-Corrales et al., 2018). In many tissues cell division or cell death and delamination also contribute to strain rates, but these are absent in the salivary gland placode during invagination and can thus be neglected. The origin of the radial coordinate system used for this analysis was specified at t = 0 min and positioned at the centre of of the placode in the hkb<sup>−/−</sup> mutant embryos (where the central constriction eventually occurred) and in wild-type embryos at the dorsal-posterior corner of the placode (where the wild-type pit will form). In wild-type placodes, cells that were located near the
eccentric invagination pit (Fig. 4A; with the ‘near’ position defined at t = 0 min; see also Figure 4- figure supplement 1) constricted isotropically, with near equal change in strain at the tissue and cell shape level, and very little intercalation strain (Fig. 4A’, A’’, solid curves). These cells in wild-type placodes could only be tracked up to t = 20 min as at this point they had all invaginated into the embryo to form the initial part of the tube. In contrast, in the hkb<sup>-/-</sup> mutants cells near the forming central pit (Fig. 4A; defined to be in a ‘near’ position at t = 0 min) showed a much reduced tissue and cell shape strain rate, with near identical radial and circumferential contributions, and constriction only commenced at about half the rate of the wild-type placodes from t = +20 min onwards (Fig. 4A’, A’’, dashed curves; see also Suppl. Fig.S3_3). In wild-type placodes, cells far from the eccentric pit (Fig. 4B; defined to be ‘far’ at t = 0 min), showed cell intercalations leading to circumferential convergence and radial extension of the tissue over the first approximately 20 min after invagination commenced, as we also reported previously (Fig. 4B’, B”’, solid curves; tissue and intercalation strain rate showing expansion radially and contraction circumferentially; (Sanchez-Corrales et al., 2018)). Interestingly, we then observed a clear switch in cell behaviour of these cells to a second phase after t = +20 min. Once these cells were located closer to the pit position, they now also displayed apical contraction (beyond t = +20 min; Fig. 4 B’, B” solid green curves).

In the hkb<sup>-/-</sup> mutants, for cells at the placode periphery and hence far from the central constriction (Fig. 4B), the tissue expanded slightly in both radial and circumferential direction (Fig. 4B’, B”’, dashed grey curves), with cell shapes narrowing radially (dashed green curve in Fig. 4B’) and extending circumferentially (dashed green curve in Fig. 4 B”’). We could observe a similar rate of intercalation in these cells as in cells far from the pit in wild-type placodes, with some radial elongation and circumferential contraction (dashed orange curves in Fig. 4B’, B”’). As the tissue strain rate in the hkb<sup>-/-</sup> mutants showed no overall signature of directional changes with only a slight expansion both radially and circumferentially, we were curious to understand better what these tissue-scale signatures could represent at the individual cell level.

We therefore analysed individual events of neighbour gains across the tissue in wild-type and hkb<sup>-/-</sup> mutant embryos. We compared two types of intercalation events, those leading to circumferential neighbour gains that in wild-type placodes explain the observed intercalation strain rates, and those leading to radial neighbour gains, and thus potentially opposing the changes seen at the tissue and intercalation strain rate level (Fig. 4 C). In wild-type placodes, circumferential neighbour gains outweigh radial ones by a large margin throughout (Fig. 4D, dashed curves), leading to a steady increase in cumulative productive neighbour gains for cells across the placode (Fig. 4E, yellow curves). In contrast, in hkb<sup>-/-</sup> mutant embryos circumferential and radial neighbour gains across the placode were overall much reduced in number and occurred with equal frequency (Fig. 4D, solid curves).
Furthermore, cells near the delayed central pit in $\textit{hkb}^{-/}$ mutants showed no productive neighbour gains (defined as the difference between circumferential and radial neighbour gains, corrected for the proportion of interfaces that could successfully be tracked; Fig. 4E, purple dashed curve), whereas cells at the periphery away from the central pit showed productive neighbour gains (Fig. 4E, solid purple curve). Active neighbour gains in the placode and other morphogenetic events are initiated and driven by junctional actomyosin (Sanchez-Corrales et al., 2018; Tetley et al., 2016), prompting us to analyse junctional myosin polarisation in $\textit{hkb}^{-/}$ mutant embryos. We previously reported on the unipolar and bipolar enrichment of junctional myosin in the wild-type at early stages of placode morphogenesis (Sanchez-Corrales et al., 2018) and expand it here to the whole process of secretory cell invagination in comparison to $\textit{hkb}^{-/}$ mutants (Fig. 4 F, G). Whereas in control placodes junctional myosin is clearly enriched in circumferential junctions by both measures, up to the point of most secretory cells having internalised by $t = +40$ min (Fig. 4F, G; dashed green curve vs dashed purple curve), in $\textit{hkb}^{-/}$ mutant placodes myosin unipolarity and bipolarity are strongly reduced, even though some bipolarity remained (Fig. 4F, G; solid curves). Thus, the major driver of active intercalations appeared to be strongly diminished in the mutants, posing the question of what could drive the observed intercalations here. We also analysed cell elongation in cells far from the pit, with cells in wild-type placodes being initially elongated circumferentially at $t < +20$ min, while early intercalation events are initiated by circumferential junctional myosin (Fig. 4H, grey curve; (Sanchez-Corrales et al., 2018)). These cells then became increasingly elongated radially as they moved into a position nearer to the pit and were being actively funnelled towards the pit. In $\textit{hkb}^{-/}$ mutant embryos cells far from the pit at the periphery of the placode very quickly became circumferentially elongated and remained so throughout the time period analysed. This aberrant circumferential elongation together with the mild bipolarity of junctional myosin polarisation that we observed prompted us to analyse individual events of cell intercalation in time lapse movies (Fig. 4 I-L). In cells far from the central pit in $\textit{hkb}^{-/}$ mutants we could observe groups of cells clearly undergoing intercalations (Fig. 4I, J) with individual cells making circumferential neighbour gains and elongating (cells 1 and 2 in Fig. 4K), whilst the overall shape of the local group of cells undergoing the intercalation event was not changing (Fig. 4L). Thus, although cells far from the aberrant central pit in $\textit{hkb}^{-/}$ mutants still intercalated and gained circumferential neighbours, this behaviour lacked several key signatures of wild-type intercalations, and overall in the $\textit{hkb}^{-/}$ mutants intercalations did not lead to convergence and extension of the tissue towards the misplaced pit.

These analyses show that in the absence of Hkb, the initial radial patterning of cell behaviours across the placode is highly aberrant. Cells located at the position corresponding to where the eccentric pit would be located in the wild-type clearly failed to constrict apically,
suggesting that Hkb could be involved in the specification of this pit-behaviour observed in wild type.

The salivary gland placodal primordium is asymmetrically patterned by Hkb and Fkh prior to morphogenesis

The loss of correct patterning of cell behaviours in the early placode in hkb−/− mutant embryos suggested that Hkb was involved in establishing these in wild-type embryos. Furthermore, hkb mRNA expression has been reported to show a dynamic pattern by in situ hybridisation and β-gal reporter labelling (Myat and Andrew, 2000b). In order to be able to analyse Hkb protein levels dynamically and in comparison to cell shapes, we generated a Venus-tagged version of Hkb using CRISPR/Cas9 to tag the endogenous protein. Using this in vivo reporter, we detected Venus-Hkb at high levels concentrated in the dorsal-posterior region of the just forming placode, in the area where the invagination pit will form, already at t = -30 min prior to the first tissue bending (Fig. 5 A, A’, D, M and Figure 5- figure supplement 1 B-B”), and even as early as t = -63 min prior to invagination (Figure 5- figure supplement 1A).

The expression then expanded more broadly and anteriorly across the whole placode over time (Fig. 5B-C’, E, F, M and Video 7). At the moment of first tissue bending (t = 0 min) Venus-Hkb was found across the placode, with elevated levels still at the invagination pit position, and a second increase towards the anterior. At late stages (t >> 0 min) Venus-Hkb levels close to the invagination point were lower than further anterior (Fig. 5M).

As mentioned above, embryos mutant in fkh also show a lack of early apical constriction at the dorsal-posterior corner of the placode (Myat and Andrew, 2000a; Sanchez-Corrales et al., 2018). Similar to Hkb, analysis of fkh mRNA and protein levels in fixed embryos indicated an early enrichment near the forming invagination pit (Myat and Andrew, 2000a; Sanchez-Corrales et al., 2018). Using an in vivo genomic GFP-Fkh reporter (Spokoyny and White, 2013), we followed Fkh protein levels and patterning over the same time period whilst also assessing apical cell shapes (Fig. 5 G-L). Already at t = -60 min prior to tissue bending, Fkh-GFP was visible in the cells near the future invagination pit (Fig. 5 G, G’, J, N and Figure 5- figure supplement 1C-C”), from where it spread anteriorly and ventrally across the whole placode (Video 8). At t = 0 min, Fkh-GFP was already detected in all cells of the placode, though with levels still highest near the pit and decreasing towards the anterior edge of the placode (Fig. 5 H, H’, K, N). At t = +30 min and beyond, levels of Fkh-GFP were more uniform across the placode (Fig. 5 I, I’,L, N).

Thus, both Hkb and Fkh transcription factors showed a clear temporally and spatially graded pattern of expression within the early placode that developed dynamically. Changes were especially pronounced at the dorsal-posterior corner, with highest levels here already an hour before tissue bending commenced. Combined Hkb and Fkh action could thereby
define the future position of the invagination pit. We therefore compared Venus-Hkb as well as Fkh-GFP fluorescence intensity levels from time-lapse movies that in parallel allowed us to determine the corresponding apical area of cells (Fig. 5 O-P'). Venus-Hkb intensity levels peaked in cells located at the site of the forming eccentric invagination pit at about t~ -30 min (Fig. 5O), with an appearance of smaller apical areas in this position following on about 30-40 min later (Fig. 5O'). Similarly, Fkh-GFP intensity began to peak at about the same time, t~ -30 min, and then tracked at high level at a fixed distance to the pit over time (Fig. 5P).

Again also here, the increased reduction in apical area appeared with a delay of about 30-40 min (Fig. 5P'). Such a delay would be consistent with the estimated time required for the transcription of the fog locus, followed by translation, protein folding, secretion, binding to the receptor and signalling leading to myosin phosphorylation (Garcia et al., 2013; Shamir et al., 2016).

Thus, Hkb and Fkh dynamic expression at the future eccentric pit could prefigure the start of apical constriction at this site. The continuous high levels of Fkh tracking at a fixed distance to the pit suggest that Fkh is key to the continuous apical constriction of cells moving into a near pit position. These dynamic changes might trigger the graded and patterned expression of targets of both transcription factors, leading to the observed patterned cell behaviours we uncovered, as well as the switch between intercalation and constriction.

**Asymmetric Fog expression is controlled by Hkb and Fkh and is upstream of early differential behaviours in the placode**

With the transcription factors Fkh and Hkb both displaying intriguing protein expression patterns across the early placode pre-morphogenesis, targets of both factors are likely to play key roles in instructing cell behaviours such as wedging and cell intercalation.

Interestingly, an upstream activator of apical-medial actomyosin activity, the GPCR-ligand Folded gastrulation (Fog) was found to be dependent on Fkh (Chung et al., 2017; Dawes-Hoang et al., 2005) (Fig. 6) and suggested to be downstream of Hkb [(Myat and Andrew, 2000b); though in this publication as ‘data not shown’]. Fog is an apically secreted ligand that acts in an autocrine fashion, and thus the cells that switch on Fog expression will apically constrict (Dawes-Hoang et al., 2005). Previously published in situ hybridisation of fog mRNA suggested increased levels near the forming pit, similar to the early Hkb and Fkh enrichment (Nikolaidou and Barrett, 2004). Fog expression and signalling in particular could not only be upstream of the isotropic constriction of cells near the forming pit, but could also be involved in maintaining this behaviour in new coronas of cells moving into a position near the pit over time.
We therefore decided to carefully analyse Fog protein levels in comparison to apical area and apical-medial myosin intensity in salivary gland placodes from early to late invagination stages (Fig. 6A-I). Fog levels were low at mid stage 10 concomitant with placode specification (Fig. 6A, A', C). Just before tissue bending commenced at t = 0 min, Fog was already strongly enriched in cells near the forming invagination pit, and these cells were the ones already showing decreased apical area (Fig. 6 D-F). Fog protein levels remained enriched near the invagination point once the tube had started to internalise (Fig. 6G-I). In fact, Fog protein levels in cells were positively correlated with both smaller apical area (Fig. 6K) as well as higher average apical-medial myosin II intensity (Fig. 6L; Figure 6 – figure supplement 1).

hkβ−/− mutant embryos, as detailed above, showed a very clear disruption of the early asymmetric placodal patterning. At late stage 11, Fog protein levels in hkb−/− mutant embryos appeared to be similar to wild-type embryos and enriched in the placode compared to the surrounding epidermis, though the pattern of Fog enrichment was altered. Cells at the posterior corner, where the invagination pit would have formed in wild-type placodes, lacked high Fog protein levels, whereas only cells in the centre of the placode now showed high levels of Fog protein (Fig. 6 M-O). When quantitatively analysing many hkb−/− mutant embryos in comparison to wild-type, the peak intensity of Fog protein was clearly shifted to a central, much more symmetrical, position (Fig. 6 P). In contrast, although we could detect some Fog protein in fkh−/− embryos at late stage 11, it was no longer enriched at the position where the invagination pit forms in the wild-type. The levels also did not appear raised anywhere within the placode compared to the surrounding epidermis (Fig. 6Q-S and Figure 6 – figure supplement 1 D-D''), as the quantification of many placodes confirmed (Fig. 6T). In both mutants, altered levels and patterning of Fog protein still correlated with the now altered levels and patterns of residual apical-medial myosin (Fig. 6 M, Q).

In order to understand the cause of the central and delayed constriction in hkb−/− mutant embryos upstream of the also only centrally localised Fog, we examined Fkh protein localisation in hkb−/− mutants (Figure 6 – figure supplement 1E-J'). At late stage 10, when the Fkh protein expression pattern is just expanding from the eccentric pit position to the central part of the placode in the control, the pattern was indistinguishable in the hkb−/− mutants (Figure 6 – figure supplement 1 E-F' and H-I'). The pattern remained identical to wild-type placodes throughout stage 11 and beyond (Figure 6 – figure supplement 1 G,G', J, J'). Hence the natural expansion of Fkh protein expression to the cells in the centre of the placode that is also observed in the hkb−/− mutant placodes might be the cause of the delayed constriction of the cells in this region, whereas the earlier initial constriction at the site of the eccentric pit was absent in the mutant because it requires both Fkh and Hkb. Both transcription factors appeared in fact to be independently regulated, as also Hkb levels and...
protein expression pattern were unchanged in fkh'−/− mutant embryos (Figure 6 – figure supplement 1 K-N').

Thus, the asymmetrical set up of the salivary gland placode prior to initiation of tube budding relies on a correct spatial and temporal patterning of the key transcription factors, Hkb and Fkh. These transcription factors are in turn upstream of GPCR/Fog signalling that leads to patterned and sustained apical-medial myosin activity and hence contractile cell behaviours.

Changes to invagination pit size or position impact invagination and perturb final organ shape

Our data and previously published results show that the asymmetrical invagination point depends on patterning by transcription factors and downstream GPCRs/Fog signalling at the posterior corner, leading to a focussed and point-like invagination pit (Chung et al., 2017; Myat and Andrew, 2000a; b; 2002; Sanchez-Corrales et al., 2018). We next sought to test the importance of this asymmetric set-up for successful morphogenesis of a narrow-lumen and symmetrical tube. To do so, we overexpressed Fog in the salivary gland placode only, using UAS-Fog x fkhGal4 which led to placode-wide increased levels of Fog from early stages on (Fig. 7 and Figure 7 – figure supplement 1). Compared to the focused apical constriction near the forming invagination pit in wild-type placodes at early stage 11 (Fig. 7 A, A'), when Fog was overexpressed across the placode most secretory cells of the placode constricted simultaneously (Fig. 7 B, B', C, C'). At late stage 11, when in wild-type placodes cells had begun to invaginate and a short symmetrical narrow-lumen tube had formed (Fig. 7 D,D'), in UAS-Fog x fkhGal4 embryos the apically constricting cells had formed a large shallow depression across the whole dorsal part of the placode, with the beginning of a deeper invagination at the dorsal-posterior corner (Fig. 7 F, F'). Following on from this, at stage 12, in contrast to the narrow opening to the invagination in wild-type placodes (Fig. 7E), in UAS-Fog x fkhGal4 placodes this whole section usually formed a deepened very wide pit (Fig. 7 G). This widened tube in Fog-overexpressing embryos, at stage 15-16 when all cells had invaginated, displayed a significantly enlarged and flattened lumen compared to wild-type tubes (Fig. 7 H-J). Interestingly, overexpression of Fkh using UAS-Fkh x fkhGal4 led to a similarly enlarged area of apical constriction early on and invaginated tubes with aberrant and inflated lumens at late stages (Figure 7 – figure supplement 2).

We set out to explain the link between an enlarged or widened invagination pit in the placode early during tube formation and the expanded lumen tube observed at late stages by considering why the invagination pit forms in its eccentric position in wild-type placodes. We formulated a simplistic theoretical consideration that assumes that following the specification of an initial group of cells that apically constrict and form the initial invagination
(Fig. 7 K-M), neighbouring coronae of cells will follow and invaginate (Fig. 7 K’-M’). Using an idealised version of apices of a wild-type placode with an eccentric pit, the number of cells invaginating, going from corona to corona, remains very steady and low (Fig. 7K’ and green curve in N). In contrast, a centrally located pit (Fig. 7L) would lead to an ever-increasing number of cells per corona that would need to invaginate (Fig. 7L’ and magenta curve in N). An enlarged pit, a combination of the two previous scenarios and resembling aspects of the enlarged pit and invagination seen in UAS-Fog x fkhGal4 embryos (Fig. 7M, M’), would lead to the invagination of a large number of cells initially, followed by similarly large numbers in each corona (Fig. 7M’ and grey curve in N). In fact, when counting invaginating coronae of cells in frames of time-lapse movies of wild-type (Fig. 7O, green curve), hkb−/− (Fig. 7O, magenta curve) curve) and UAS-Fog x fkhGal4 (Fig. 7O, grey curve) embryos, the actual numbers observed were in very good agreement with the theoretical considerations outlined above (Fig. 7 K-N).

In the hkb−/− mutant embryos, the delayed central area of constriction turned into a widened invagination and the enlarged pit at late stages often collapsed into an enlarged crescent of subduction at the boundary (Figure 3 – figure supplement 2 and Videos 5 and 6), leading to malformed and enlarged lumen glands at the end of embryogenesis, as has been observed previously (Myat and Andrew, 2002).

Thus, the eccentrically placed invagination pit in the dorsal-posterior corner of the salivary gland placode is a key factor in ensuring the formation of a symmetrical narrow lumen tube during invagination, in part likely due to the control over the number of invaginating cells in each corona destined to internalise.
In this work, we investigated how the sustained morphogenesis of a tissue is achieved, in this case the continued invagination of epithelial cells forming a tubular organ. We previously identified cell behaviours that become established across the placode and drive the initial tissue bending (Sanchez-Corrales et al., 2018).

A number of mechanisms appear at play that in combination ensure that a symmetrical and narrow-lumen tube is formed from an initially flat epithelial primordium. A temporal and spatial component is provided by the dynamically changing pattern of the transcription factors Hkb and Fkh. These are expressed initially with the highest protein level at the point where the invagination pit will form, before expression expands further across the placode over time. This ensures that the cells in the dorsal-posterior corner are the first to experience high levels of activity of these transcription factors, and we show that one of the important targets, the GPCR-ligand Fog, is found at high levels in these cells in a Hkb- and Fkh-dependent manner with a slight temporal delay. A further spatial component is provided by the fact that, as cells continuously internalise to form the tube on the inside of the embryo, new coronas of cells are brought into a 'near the pit' position and adjust their behaviour accordingly. This position-specific behaviour of apical constriction is most likely triggered by the previous temporal expansion of Fkh and also Hkb expression to these cells, thereby priming them for their next step in the path to tube formation. Interestingly, though, the pattern of Fkh and Hkb expression from t = 0 min onwards, though generally expanding across the placode, is not identical. This could suggest that downstream targets begin to diverge at later stages. The hkb\textsuperscript{−/−} mutant analysis in particular suggests that the central cells in the placode that manage to constrict in the absence of Hkb might only require Fkh activity to initiate Fog expression and function. These cells therefore undergo their normal apical constriction once Fkh expression has reached the central position. As they now are the first cells to constrict and invaginate, the hkb\textsuperscript{−/−} mutants show a central and delayed constriction and invagination.

Our work strongly suggests that the combined early patterning of Hkb and Fkh expression defines the early invagination pit and its eccentric position. How though is Hkb and Fkh expression initially limited to this point? Both are expressed in the placode downstream of the homeotic factor Sex Combs Reduced (Scr; (Myat and Andrew, 2000b; Panzer et al., 1992)). They are also not the only factors expressed near exclusively at this corner first. At the transcription factor level in particular one of the parasegmentally repeated stripes of Wingless (Wg) expression overlays the posterior part of the placode, its expression domain defined by previous parasegmental patterning (Clark, 2017). And at the time point when Hkb and Fkh expression at the future pit location commences, the Wg expression within the placode region is also confined to this corner only, rather than
extending all the way to the ventral midline (data not shown). Future studies will show whether a combination of Scr and Wg transcription factor activity could activate early pit-defining expression of factors such as Fkh and Hkb. In addition to transcription factors, some potential downstream effectors also appear to be expressed near the future pit location first, these include Btk29/Tec29 (Chandrasekaran and Beckendorf, 2005), Crumbs (Myat and Andrew, 2002; Röper, 2012), 18-Wheeler (Kolesnikov and Beckendorf, 2007), and Klarsicht (Myat and Andrew, 2002). Cell-based genomics approaches such as single cell-RNAseq or ATAC-seq should in the future allow to precisely determine the genetic programme that underlies the regionalisation of the placode.

Activation of actomyosin contractility through GPCR signalling driven by the autocrine secretion of the ligand Fog is a module repeatedly used during tissue bending and invagination events in Drosophila embryogenesis. What differs are the upstream activating transcription factors: during mesoderm invagination Fog expression on the ventral side is controlled by Twist and Snail (Morize et al., 1998), whereas its expression during posterior midgut invagination is controlled by Tailless and Hkb (Weigel et al., 1990). The effect of Hkb on Fog expression is likely direct, as the Fog locus contains predicted Hkb-binding sites (using oPOSSUM, http://opossum.cisreg.ca/oPOSSUM3/). The identity of the GPCR that could mediate the Fog signal in the salivary gland placode is unclear thus far. Published and database (Flybase) in situ for the GPCRs previously reported to be involved in mesoderm invagination and germband extension, Mist and Smog (Kerridge et al., 2016; Manning et al., 2013), do not show an enriched expression in the placode. But with around 200 Drosophila GPCRs in existence, half of them orphan receptors (Hanlon and Andrew, 2015), the placode might well express yet another one, and we will focus on candidates previously identified in screens (Maybeck and Röper, 2009) and genomics approaches (our unpublished data) in the future.

The question of how cell behaviour is modulated over time after the initial transcriptional patterning that defines primordium identity is key to understand general mechanisms that allow continuous organ formation. Different solutions seem to have been adopted in different tissues. Recent work analysing mesoderm invagination in Drosophila has shown that the key morphogenetic effectors T48 and Fog show a gradient of expression due to differential timing of transcriptional activation, leading to different spatial accumulations of the transcripts (Lim et al., 2017) and proteins (Heer et al., 2017). Flattening the gradient experimentally (by expansion of the ventral domain) leads to a widened and perturbed invagination process. By contrast, the invagination of the posterior midgut or endoderm primordium in the fly embryo does not require sustained transcription, but instead a wave of apical constriction that is initiated by Fog expression in a subset of cells, appears to be propagated via mechanical feedback (Bailles et al., 2019). The salivary gland placode
seems to follow a model of a temporal expansion or wave of transcriptional activity
translating into positionally fixed behaviours. Nonetheless, our quantitative analysis of 3D
cell behaviours strongly indicated that mechanical feedback does also play a role (Sanchez-
Corrales et al., 2018). Which combination of mechanisms is ultimately at work to sustain a
morphogenetic process could depend on a number of factors including the developmental
stage, the speed of the process (consider ~15 min for mesoderm invagination versus ~30
min for endoderm invagination versus ~1.5 hours for secretory cell invagination in the
salivary gland placode), as well as the influence of other nearby concomitant morphogenetic
events.

The eccentric pit set-up of the placode we suggest is key to wild-type tube formation
with a narrow lumen, and we show that our simple geometrical considerations are in good
agreement with the actual in vivo data of number of cells per corona invaginating. Whether
the eccentricity only allows to control cell numbers of invaginating coronae, or whether such
set-up could also have further beneficial effects on for instance mechanical aspects of the
tissue will be something we will actively explore both in vivo and in silico in the future.
Curiously and maybe tellingly, the asymmetry of the placodal set-up is retained in another
tube formation process, the invagination of the posterior spiracles (Sidor et al., 2020; Simoes
et al., 2006). The posterior spiracle placode, like the salivary gland placode, is a flat
epithelium with an eccentric invagination point. In this case an initial tube is already present
due to the previous invagination of the most posterior tracheal placode. Whether the
asymmetrical set-up is thus a conserved feature of further tube budding processes during
development will be an exciting question to address. 3D mathematical modelling of tube
morphogenesis assuming an eccentric versus centrally positioned invagination point will
help to dissect the topological advantages of being asymmetric.

In summary, we have quantitatively unravelled a dynamic spatio-temporal patterning
of transcription factors and switches in cell behaviours leading to positionally fixed
behaviours during the morphogenesis of simple tubular organ. We foresee that such
mechanisms will be important for establishing general mechanisms and morphogenetic
modules at work during the morphogenesis of more complex tubular organs and potentially
also for engineered tissues in a dish.
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Declaration of Interests

The authors declare no competing interests.
### Materials & Methods

#### Key Resources Table

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Fly stocks and husbandry

The following transgenic fly lines were used: Armadillo-YFP (CPTI collection described in (Lye et al., 2014)), sqhAX3; sqh::sqhGFP42 (Royou et al., 2004) and fkhGal4 (Henderson and Andrew, 2000; Zhou et al., 2001) [kind gift of Debbie Andrew]; UAS-Fog (kind gift of
Thomas Lecuit); \( y^i w^i \) cv\(^{-i} \) sqh\(^{AX3} \); \( P[w^{\text{mC}}=\text{sqh-GFP}].RLC]C-42 \ M[w^{\text{mC}}=\text{Ubi-TagRFP-T-CAAX}].ZH-22A \) (Kyoto DGRC Number 109822, referred to as sqh\(^{AX3};\) sqhGFP; UbiRFP);

\[
\text{[sqhGFP42, UbiRFP, } fkh[6]/\text{TM3 Sb Twi-Gal4::UAS-GFP]} \ (fkh[6] \text{ allele from Bloomington}), [\text{hkb}^2, p^2, \text{Scribble-GFP}]; [\text{sqhGFP42, UbiRFP, } hkb^2, p^2 / \text{TM3 Sb Twi-Gal4::UAS-GFP}]. \]

\( hkb^2 \) allele from Bloomington BDSC: 5457), [UbiRFP, Venus-HKB] (this study), FkhGFP (Bloomington BDSC: 43951) and [FkhGFP, wgGal4, UAS-palmYFP] (generated from membrane Brainbow (Förster and Luschnig, 2012); (Hampel et al., 2011)).

See Table 1 for details of genotypes used for individual figure panels.

The CRISPR Venus-HKB was created using the following gRNA targets flanking the N-terminus of Hkb:

- ACAAGTGATTTAGTGTCGCGAGA
- CACCGCAACCTACTCGCGACTT

A linker sequence between the inserted fluorescent protein and the protein was also used:

- GGAGGCCAGGCTCGGGAGGCGAGGGCTCG.

**Embryo Immunofluorescence Labelling, Confocal, and Time-lapse**

Embryos were collected on apple juice-agar plates and processed for immunofluorescence using standard procedures. Briefly, embryos were dechorionated in 50% bleach, fixed in 4% formaldehyde, and stained with primary and secondary antibodies in PBT (PBS plus 0.5% bovine serum albumin and 0.3% Triton X-100). Anti-Hkb was a gift from Chris Doe (McDonald and Doe, 1997); anti-Fkh was a gift from Herbert Jäckle (Weigel et al., 1989) and anti-Fog was a gift from Naoyuki Fuse (Fuse et al., 2013), PY-20, (*P11120, Transduction laboratories); anti-CreB (DSHB). Secondary antibodies used were Alexa Fluor 488/Fluor 549/Fluor 649 coupled (Molecular Probes) and Cy3 coupled (Jackson ImmunoResearch Laboratories). Samples were embedded in Vectashield (Vectorlabs).

Images of fixed samples were acquired on an Olympus FluoView 1200 or a Zeiss 780 Confocal Laser scanning system as z-stacks to cover the whole apical surface of cells in the placode. Z-stack projections were assembled in ImageJ or Imaris (Bitplane), 3D rendering was performed in Imaris.

For live time-lapse experiments (see Table 1), embryos were dechorionated in 50% bleach and extensively rinsed in water. Stage 10 embryos were manually aligned and attached to heptane-glue coated coverslips and mounted on custom-made metal slides; embryos were covered using halocarbon oil 27 (Sigma) and viability after imaging after 24h was controlled prior to further data analysis. Time-lapse sequences were imaged under a 40x/1.3NA oil objective on an inverted Zeiss 780 Laser scanning system, acquiring z-stacks every 0.58-3 minutes with a typical voxel xyz size of 0.22 x 0.22 x 1 μm. Z-stack projections to generate movies in Supplementary Material were assembled in ImageJ or Imaris. The
absence of fluorescent **Twi-Gal4::UAS-GFP** was used to identify homozygous *hkb[2]* and
*fkh[6]* mutant embryos. The membrane channel images from time-lapse experiments were
denoised using *nd-safir* software (Boulanger et al., 2010).

**Cell segmentation and tracking**

Cell segmentation and tracking was performed using custom software written in (Blanchard
et al., 2009). First, the curved surface of the embryonic epithelium was located by draping a
'blanket' over all image volumes over time, where the pixel-detailed blanket was caught by,
and remained on top of binarised cortical fluorescence signal. Quasi-2D image layers were
then extracted from image volumes at specified depths from the surface blanket. This step
permitted to account for the curvature of the embryos in these quasi-2D projections. We took
maximum intensity projections of a small number of near surface image layers typically at 0-4 μm from membrane channels, for cell-apical analyses, apicominal myosin and anti-Fog
fluorescence channels and projections of image layers typically from 5-16μm encompassing
nuclear fluorescence for Venus-HKB and Fkh-GFP analyses. Membrane channels were
filtered with median, top-hat or high/low frequency filters as necessary to optimise
subsequent cell tracking.

Cells in membrane channels were segmented using an adaptive watershedding
algorithm as they were simultaneously linked in time. Manual correction of segmented cell
outlines was performed for all fixed and time-lapse data. The segmentation of all the movies
used in this study was manually corrected to ensure at least 90% tracking coverage of the
placode at all times. Tracked cells were subjected to various quality filters (lineage length,
area, aspect ratio, relative velocity) so that incorrectly tracked cells were eliminated prior to
further analysis. The number of embryos analysed and number of cells can be found in
Supplemental Figures 1 and 3.

**Mobile radial coordinate system for the salivary gland placode**

Wild-type movies were aligned in time using as t=0 min the frame just before the first sign of
invagination at the future tube pit was evident. *hkb*-*mutants were aligned using as a
reference of embryo development the level of invagination of the tracheal pits that are not
affected in the *hkb*-*mutant as well as other morphological markers such as appearance and
depth of segmental grooves in the embryo. Cells belonging to the salivary placode (without
the future duct cells that comprise the two most ventral rows of cells in the primordium) were
then manually outlined at t=0 min using the surrounding myosin II cable as a guide and
ramified forwards and backwards in time. Only cells of the salivary gland placode were
included in subsequent analyses.
At t= 0 min, the centre of the future tube pit was specified manually as the origin of a radial coordinate system, with radial distance (in µm) increasing away from the pit. Circumferential angle was set to zero towards Posterior, proceeding anti-clockwise for the placode on the left-hand side of the embryo, and clockwise for the placode on the right so that data collected from different sides could be overlaid.

The radial coordinate system was 'mobile', in the sense that its origin tracked the centre of the pit, forwards and backwards in time, as the placode translated within the field of view due to embryo movement or to on-going morphogenesis.

As calculated previously (Sanchez-Corrales et al., 2018), we employ a radial coordinate system centred at the invagination point at t = 0 min. We specify the 'near to the pit' region as the region located between the invagination point and up to 33% of the distance between the invagination point and the far edge of the placode (with this region usually being within 13.7 +/- 3 µm of the pit), and with the rest of the cells defined as ‘far from the pit’.

**Morphogenetic strain rate analysis**

Detailed spatial patterns of the rates of deformation across the placode and over time quantify the net outcome of active stresses, viscoelastic material properties and frictions both from within and outside the placode. We quantified strain (deformation) rates over small spatio-temporal domains composed of a focal cell and one corona of immediate neighbours over a ~3 min interval ((Blanchard et al., 2009) and reviewed in (Blanchard, 2017)). On such 2D domains, strain rates are captured elliptically, as the strain rate in the orientation of greatest absolute strain rate, with a second strain rate perpendicular to this.

For the early morphogenesis of the salivary gland placode, in which there is no cell division or gain/loss of cells from the epithelium, three types of strain rate can be calculated. First, total tissue strain rates are calculated for all local domains using the relative movements of cell centroids, extracted from automated cell tracking. This captures the net effect of cell shape changes and cell rearrangements within the tissue, but these can also be separated out. Second, domain cell shape strain rates are calculated by approximating each cell with its best-fit ellipse and then finding the best mapping of a cell’s elliptical shape to its shape in the subsequent time point, and averaging over the cells of the domain. Third, intercalation strain rates that capture the continuous process of cells in a domain sliding past each other in a particular orientation, is calculated as the difference between the total tissue strain rates and the cell shape strain rates of cells. Strain rates were calculated using custom software written in in L3Harris Geospatial IDL (code provided in (Blanchard et al., 2009) or by email from G.B.B.).
The three types of elliptical strain rate were projected onto our radial coordinate system (Sanchez-Corrales et al., 2018) using the eccentric pit location in wild-type (dorsal-posterior corner) or the centre of the misplaced invagination point in hkb−/− mutants. The radial coordinate system permitted us to analyse radial and circumferential contributions.

Strain rates in units of proportional size change per minute can easily be averaged across space or accumulated over time. We present instantaneous strain rates over time for spatial subsets of cells in the placode (Supplemental Figure 3_3), and cumulative strain ratios for the same regions over time. These plots were made from exported data using MATLAB R2014b. To test for differences in instantaneous strain rates of wt and hkb−/− time-lapse movies we used a mixed-effects model implemented in R (lmer4 package as in (Butler et al., 2009; Lye et al., 2015; Sanchez-Corrales et al., 2018)) with a significance threshold of p<0.05. The phenotype (wt or hkb−/−) was considered a fixed effect while the variation between embryos from the same phenotype was considered a random effect.

Cell shape elongation (Figure 4H) was calculated by fitting an ellipse into each cell. The two axes of each ellipse were projected into radial coordinates and the ratio of them was calculated.

**Neighbour exchange analysis**

We used changes in neighbour connectivity in our tracked cell data to identify neighbour exchange events (T1 processes). Neighbour exchange events were defined by the identity of the pair of cells that lost connectivity in t and the pair that gained connectivity at t+1. The orientation of gain we defined as the orientation of the centroid-centroid line of the gaining pair at t+1. We further classified gains as either radially or circumferentially oriented, depending on which the gain axis was most closely aligned to locally. We did not distinguish between solitary T1s and T1s involved in rosette-like structures.

From visual inspection, we knew that some T1s were subsequently reversed, so we characterised not only the total number of gains in each orientation but also the net gain in the circumferential axis, by subtracting the number of radial gains. Furthermore, when comparing embryos and genotypes, we controlled for differences in numbers of tracked cells by expressing the net circumferential gain per time step as a proportion of half of the total number of tracked cell-cell interfaces in that time step. We accumulated numbers of gains, net gains, and proportional rate of gain over time for WT and hkb embryos (Figure 4D,E).

Two sample Kolmogorov-Smirnov tests were used to determine significance at p<0.05 for data in Figure 3.

**Automated medial myosin II quantification and oscillatory behavior and polarity**

We extracted a quasi-2D layer image from the myosin II channel at a depth typically from 0-
3 μm that maximised the capture of apical-medial myosin. We background-subtracted the myosin images and quantified the average intensity of apical-medial myosin as the fluorescence inside the segmented cell, excluding the fluorescence along cell-cell interfaces encompassing the cell edge pixels plus 2 pixels in a perpendicular direction either side (considered junctional myosin).

The analysis of apical-medial fluorescence fluctuations was performed as described before in (Blanchard et al., 2010) and (Booth et al., 2014). Briefly, the apical-medial fluorescence values in the time series were detrended by subtracting a boxcar average with a window of 6 minutes (larger than the maximum expected fluctuation cycle length). All peaks and troughs were then identified in the fluorescence signal. Peaks or troughs that were associated with very small amplitude cycles were skipped. The remaining peaks and troughs were used to calculate the amplitude and the cycle length (peak to peak) and the amplitude (average peak to trough) of fluctuations.

The analysis of junctional myosin polarity was performed as described before in (Sanchez-Corrales et al., 2018; Tetley et al., 2016). Briefly, the average junctional fluorescence intensity is considered a periodic signal around the cell and decomposed into individual components (modes) using Fourier analysis. The strength of myosin bipolarity corresponds to the amplitude of the second mode while the strength of unipolarity corresponds to the amplitude of the first mode. Similarly, the phase of mode 1 and 2 (uni/bi-polarity) represents the orientation of cell polarity. The uni- and bipolarity amplitudes are expressed as a proportion of the mean cell perimeter fluorescence. Cells at the border of the placode that are part of the supra-cellular actomyosin cable at the boundary were excluded from the analysis.

**Hkb, Fkh and Fog fluorescence intensity quantifications**

Average fluorescence was calculated after background subtraction as the mean fluorescence inside a segmented cell excluding the cell edge pixels and 2 pixels in a perpendicular direction either side. Fixed samples of Venus-Hkb and FkhGFP embryos of mid to late stage 10 (t<< 0min), early stage 11 (t+ 0min) and late stage 11 (t>> 0 min) were pooled together with relevant time-points from live-imaging as shown in Table 1 and figure legends. Fluorescence was normalised in each image by dividing the average fluorescence of each cell by the 98th percentile value. Calculations were performed in R.

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References


**Figure 1. Patterned apical constriction remains fixed around the pit over time.**

**A** The tubes of the salivary glands form from a flat epithelial placode with a circular geometry. Cells invaginate through an asymmetrically positioned invagination pit at the dorsal-posterior corner, embryonic axes of anterior-posterior (AP) and dorso-ventral (DV) are indicated. In the placode at early stages cell behaviours are highly patterned, with cells near the pit predominately constricting isotropically and cells far from the pit predominantly intercalating (Sanchez-Corrales et al., 2018).

**A’** Apical constriction within the apical domain of an epithelial cell, a 2D change, equates to a behaviour of cell wedging within the 3D context of a whole cell, as shown in a cross-sectional view. **A”** Patterned cell behaviours are driven by distinct pools of apical actomyosin. Three pools of actomyosin can be distinguished in the placode: apical-medial actomyosin in cells near the invagination pit (dark orange), leading to isotropic apical constriction; polarised junctional actomyosin, driving the initiation of directed cell intercalation events in cells further away from the pit (light orange); a circumferential actomyosin cable at the boundary of the placode.

**B** Stills of a representative segmented time-lapse movie, colour-coded to indicate cell near (pink) and far (grey) from the invagination pit, with individual cell examples in both regions highlighted. As calculated previously (Sanchez-Corrales et al., 2018), we employ a radial coordinate system centred at the invagination point at t = 0 min. We specify the ‘near to the pit’ region as the region located between the invagination point and up to 33% of the distance between the invagination point and the far edge of the placode (with this region usually being within 13.7 ± 3 µm of the pit), and with the rest of the cells defined as ‘far from the pit’.

The cell outlines in the time-lapse movie are labelled using Armadillo-YFP. These and all confocal panels shown in the figures are always oriented with anterior to the left and dorsal up. See Video 1.

**C, D** Placodal cells visually split into near the pit (**C**) and far from the pit (**D**) cells and colour-coded for apical area size. The asterisks in B-D indicate the (future) position of the invagination pit, white dotted lines indicate the boundary of the placode in regions where cells are not highlighted.

**E** Analysis of apical area change expressed in proportion (pp) per minute, pooled from 7 time-lapse movies for cells that were specified at t= 0 min to be located near the invagination pit (dashed line) and cells that were specified at t= 0 min to be located far from the invagination pit (solid line). Wild-type movies were aligned in time using as t= 0 min the frame just before the first sign of invagination at the future tube pit was evident. Note that...
cells near the pit continuously constrict until internalised (pink shaded box), whereas cells far from pit do not constrict significantly until t= 20 min whilst moving closer to the pit (grey shaded box), but once they are in close proximity to the pit they also constrict until internalised (blue shaded box). Details of cell numbers and lengths of movies analysed are shown in Figure 1-figure supplement 1.

F Apical area size evolution of individual cell examples of cells near the pit and far from the pit as highlighted in colour in B.

G Example of a cell from the far from the pit region (blue cell in B and F) undergoing a cell intercalation process as part of a rosette of cells that contracts circumferentially and resolves elongating the tissue radially towards the invagination point.
**Figure 2. Sustained apical constriction near the pit is driven by apical-medial myosin.**

**A-B''** Still images of a time-lapse movie of embryos expressing SqhGFP to label myosin II at t= 0min (A-A'') and t= 30min (B-B''). White boxes indicate the corresponding higher magnifications shown below, with A'',B'' showing cells that are at these timepoints located near the pit, and A',B' showing cells near the boundary (small blue double arrows indicate similar closeness to the boundary). Magenta asterisk marks the invagination pit, blue dotted lines mark the boundary of the placode. **C** Schematic apical view of epithelial cells, illustrating junctional and apical-medial pools of actomyosin. Arrows in A'', B', B'' point to apical-medial actomyosin. See also Video 2.

**D** Spatial representation of the average medial myosin intensity from an exemplary time lapse movie, with radial location (collapsed into stripes) of cells specified at t= 0min. The dashed white line marks the peak of medial myosin intensity that is always adjacent to the invagination pit as cells flow into it over time. The schematic illustrates the evolution of the radial stripes analysed, with stripes initially close to the pit internalised first and hence a position close to the pit ‘moving’ across this type of plot diagonally in wild-type placodes.

**E** Spatial representation of the distribution of the myosin cycle length across the salivary gland placode, with radial location (collapsed into stripes) of cells specified at t= 0min. The dashed line marks the region of shortest cycle length that is always adjacent to the invagination pit as cells flow into it over time. The mean of 3 movies is shown. The schematic illustrates one cycle of a myosin pulsation defined as periodic increases and decreases in medial myosin II intensity.

**F** The distribution of myosin II cycle lengths between cells near the pit and far from the pit varies: the median of the cycle length for cells near the pit is 3.7 min (n =146) and SD+/- 2.08 min, while cells far from the pit have a median myosin II cycle length of 4.64 min (n=301) and SD+/- 2.72 min. Thus, the cells that are located far from the pit show longer cycle length (p < 0.0001 Mann Whitney test). Data is pooled from 3 movies at t=0.

**G** Spatial representation of the strength of myosin oscillations in radial stripes from the pit (0 µm) to the boundary of the placode (47µm) at t= 0min. Mean and standard error of the mean are shown. Data are pooled from 3 movies ranging from -7.5-56.25 min; -16.76 to 55.48 min and -17.42 to 67.26min. Regions corresponding to cells near the pit and far from the pit are indicated by coloured shading.

**H** Medial myosin strength, expressed as the product of amplitude x frequency of the oscillations, for all cells analysed from 3 movies, split into cells located near to the pit (n=148) compared to cell located far from the pit (n=302) at t=0 from 3 movies. Cells near the pit have significantly higher medial myosin strength than cells located far from the pit; statistical significance as determined (Mann Whitney test, with p<0.0001). Cells far from the
pit at later time points (t=55-60 min, n = 134) increased the medial myosin strength and were not significantly different from cells near the pit at t=0 (Mann Whitney test, p = 0.5690).

In all analyses, t= 0min is defined as the frame just before the first sign of invagination at the future pit was evident.

See also Figure 2- figure supplement 1 and Video 2.
Figure 3. hkb−/− mutants show a delayed symmetrical apical constriction.

A-F''' Stills of a representative wild-type (A-C''') and a hkb−/− mutant (D-F''') embryo time lapse movie at the indicated time points. A-A''' and D-D''' show the SqhGFP channel of the movies visualising myosin II, B-B''' and E-E''' show the average apical-medial myosin II intensity of segmented placodal cells, C-C''' and F-F''' show the corresponding apical cell areas. E-E''' hkb−/− mutants show apical-medial accumulation of myosin II at the centre at a delayed time point. Indicated colour scales correspond to the 5-95% range in each movie.

Magenta dotted lines indicate the boundary of the placode. F-F''' The delayed accumulation of myosin II in hkb−/− mutants is mirrored by the changed apical constriction pattern, with the wild-type constricting in the dorsal-posterior corner (C-C''') and hkb−/− mutants in the centre, but very delayed (F-F''').

G, H Rate of apical area change of cells in wild-type (G; data from 7 movies) and hkb−/− mutant (H; data from 7 movies) embryos shown over time and across radial distance of cells from the eccentric wild-type pit location (0 µm) at t = 0 min. 47 µm represents the boundary of the placode. The blue line in G indicates where cells have just disappeared into the pit in the wild-type. Greatest apical area change in the wild-type is always confined to the area in front of the pit (brackets in G), whereas in hkb−/− mutant embryos cells in a broader central region show a delayed apical constriction (blue brackets in H). See also Figure 3-supplement 1.

I, I' Schematic representation of a segmented wild-type and hkb−/− mutant placode, with cells near the eccentric wild-type pit and central hkb−/− mutant pit/area of constriction indicated in orange, and cells far from the pit for both in magenta.

J Comparative analysis of cumulative apical area change of hkb−/− mutant embryos expressed as proportion per minute (pp/min), pooled from 7 time-lapse movies for cells that were specified at t = 0 min to be located near the central constricting pit (dashed blue curve; see schematic on the right) and cells that were specified at t = 0 min to be located far from the central pit (solid blue line; see schematic on the right) versus wild-type placodal cells as previously shown in Fig. 1C.

K Spatial representation of the average medial myosin intensity from an exemplary time-lapse movie of a hkb−/− mutant embryo, with radial location (collapsed into stripes) of cells specified at t= 0min. The blue brackets mark the peak of medial myosin intensity that is located in the centre of the placode in hkb−/− mutant embryos rather than tracking near the forming eccentric pit as in the wild-type.

In all analyses of the wild-type, t= 0min is defined as the frame just before the first sign of invagination at the future pit was evident. hkb−/− mutants were aligned using as a reference of embryo development the level of invagination of the tracheal pits that are not affected in the
mutant as well as other morphological markers such as appearance and depth of segmental grooves in the embryo.

See also Videos 3-6.
**Figure 4.** *hkb*<sup>−</sup> mutants show aberrant cell behaviours during invagination.

A-B" Regional breakdown of time-resolved cumulative strain rates, with regions defined at t= 0min based on eccentric pit for the wild-type and a central pit for *hkb*<sup>−</sup> mutants. For cells near the pit in the wild-type (A), tissue constriction dominates (solid grey curves in A', A'""); and is due to isotropic cell constriction (solid green curves in A', A'"") whilst intercalation only plays a minor role in this region (solid orange curves in A', A'""). Cells in this region have completely internalised by about t= 20 min. By contrast, in *hkb*<sup>−</sup> mutant embryos, cells near the pit (A), show strongly reduced tissue (dashed grey curves in A', A'"") and cell strain rates (dashed green curves in A', A'"") and mildly reduced intercalation (dashed orange curves in A', A'""). For cells far from the pit in the wild-type (B), the tissue elongates towards the pit until t = 20 min (solid grey curve in B'), with a corresponding contraction circumferentially (solid grey curve in B'"), and this is predominantly due to cell intercalation (solid orange curves in B', B'""). Beyond t = 20 min, these cells have reached the invagination pit and also constrict isotropically, thereby leading the tissue change (solid grey curves in B', B'" >20 min) to mirror the cell shape change (solid green curves in B', B'" >20 min). By contrast, in *hkb*<sup>−</sup> mutant embryos, cells far from the (central) pit (B) show a slight tissue expansion both radially and circumferentially (dashed grey curves in B', B'"), paired with abnormal circumferential cell elongation (dashed green curves in B', B'"), and some reduced intercalation (dashed orange curves in B', B'"). The corresponding instantaneous strain rate plots can be found in Figure 4- figure supplement 1. Data from 9 wild type movies and 5 *hkb*<sup>−</sup> movies were analysed (see Figure 3- figure supplement 1).

C-E Quantification of neighbour gains as a measure of T1 and intercalation events.

Examples of a circumferential neighbour gain (leading to radial tissue expansion), and a radial neighbour gain (leading to circumferential tissue expansion) are shown in C.

D Circumferential neighbour gains dominate over radial neighbour gains in the wild-type (dashed curves), with the rate of neighbour exchanges dropping beyond 20 min. In contrast, in *hkb*<sup>−</sup> mutant embryos the amount of circumferential and radial gains is identical (solid curves). E Cumulative proportion of productive neighbour gains, defined as the amount of circumferential neighbour gains leading to radial tissue elongation and expressed as a proportion (pp) of cell-cell interfaces tracked at each time point, and split into cells near the pit (eccentric for wild-type and central for *hkb*<sup>−</sup> mutant) and far from the pit. Predicted productive neighbour gains are strongly reduced and near zero for cells near the pit in *hkb*<sup>−</sup> mutants compared to control (dashed curves), whereas cells far from the pit in *hkb*<sup>−</sup> mutant continue to intercalate similar to wild-type (solid curves).

F, G Myosin II junctional polarity was quantified from segmented and tracked time-lapse movies. Myosin enrichment at junctions can occur in two flavours: Myosin II unipolarity is defined as myosin II enrichment selectively on side of a cell (F, see schematic inset). Myosin
II bi-polarity is defined as myosin II enrichment at two parallel oriented junctions of a single cell, calculated as the magnitude of a vector pointing at the enrichment (G). Data from 6 wild-type movies and 5 hkb<sup>−/−</sup> movies, number of cells is shown in Figure 3- figure supplement 1. Plotted are the rates of change of the uni- and bipolarity amplitudes as a proportion per minute (pp/min) of the mean cell perimeter fluorescence.

**F** Circumferential myosin II uni-polar enrichment (i.e. the radial uni-polarity vector, red arrow in schematic, pointing at the myosin enrichment), increases and is high until ~40 min when it drops (G, green dashed curve). The circumferential uni-polar enrichment is always higher than the radial myosin II uni-polar enrichment (green solid curve in G). The myosin II uni-polar enrichment in hkb<sup>−/−</sup> mutants is overall strongly reduced compared to wild-type (solid curves in F).

**G** Circumferential myosin II bi-polar enrichment in the wild-type (i.e. the radial bi-polarity vector, red arrow in schematic, pointing at the myosin enrichment) is high until ~40 min when it drops (G, green dashed curve). Until this point, it is higher than the radial myosin II bi-polar enrichment (green solid curve in G). The myosin II bi-polar enrichment in hkb<sup>−/−</sup> mutants is strongly reduced compared to the wild-type (solid curves in G).

Statistical significance of p<0.05 (*), p<0.005 (**), p<0.0005 (***), using a mixed-effect model is indicated as shaded boxes at the top and bottom of the panels: comparing circumferential over radial enrichment for either the wild-type or hkb<sup>−/−</sup> mutants.

**H** Analysis of cell shape aspect ratio dynamics in cells far from the pit (eccentric pit for wild-type, central pit for hkb<sup>−/−</sup> mutant). In the wild-type, circumferential elongation as part of active circumferential neighbour gains (Sanchez-Corrales et al., 2018) persist until ~ t = +20 min, when cells start to become elongated radially (grey curve). In hkb<sup>−/−</sup> mutants cells become and remain circumferentially elongated (magenta curve). Data shown for 7 wild type and 5 hkb<sup>−/−</sup> mutant movies.

**I-L** Analysis of an exemplary cell intercalation event in cells far from the pit in a segmented and tracked time lapse movie of a hkb<sup>−/−</sup> mutant placode, stills at the beginning and end of the event shown are in I, and stills of the whole event in J. K Cells 1 and 2 gain a circumferential contact, mainly via cell elongation. L The cell cluster is already circumferentially elongated at t= +14:54 min (black outline) and remains near identically elongated at t= +26:49 min (orange outline).

Data for A-H pooled from 9 wild type and 5 hkb<sup>−/−</sup> mutant movies.

In all analyses of the wild-type, t= 0 min is defined as the frame just before the first sign of invagination at the future pit was evident. hkb<sup>−/−</sup> mutants were aligned using as a reference of embryo development the level of invagination of the tracheal pits that are not affected in the hkb<sup>−/−</sup> mutant as well as other morphological markers such as appearance and depth of...
segmental grooves in the embryo. Panels A, A', B, B', F, G are expressed as proportion per minute (pp/min).

See also Figure 3- figure supplements 1 and 2.
Figure 5. The salivary gland placodal primordium is asymmetrically patterned by Hkb and Fkh prior to morphogenesis.

A-F Stills of a time lapse movie of embryos with endogenously tagged Hkb, Venus-Hkb, at t = -38 min, +0 min and +38 min show the dynamic pattern of expression of Hkb. Expression starts at the posterior corner before tissue bending commences. Cell membranes are in magenta in A-C, Venus-Hkb is green in A-C and as a single channel in A’-C’. D-F show the quantification of Venus-Hkb fluorescence intensity at these time points.

G-L Stills of a time lapse movie of embryos with Fkh tagged by GFP under endogenous expression control, at t = -60 min, +0 min and +30 min show that as early as one hour before tissue bending, Fkh is already expressed at the posterior corner. Cell membranes are in magenta in G-I, Fkh-GFP is green in G-I and as a single channel in G’-I’. J-L show the quantification of Fkh-GFP fluorescence intensity at these time points.

M Venus-Hkb expression starts at the posterior corner in a region similar to ‘near the pit’ population (between pit location and dashed line). At the start of invagination (around t = 0min) the expression increases across the placode, in particular at the anterior edge. At late stages, the level of Venus-Hkb expression decreases in the pit region, but it remains high in the rest of the placode. Data is pooled from selected frames of time-lapse movies and corresponding fixed samples. Number of cells are as follows: t<<0 min (equivalent to stage 10), n = 777 cells from 8 embryos; t=0 min (equivalent to early stage 11), n = 1186 cells from 10 embryos and t>>0 min (equivalent to late stage 11/early stage 12), n = 885 cells from 10 embryos.

N Fkh-GFP expression initiates at the posterior corner and increases to cover the whole placode over time. Data is pooled from selected frames of time-lapse movies and corresponding fixed samples. Number of cells are as follows: t<<0 min (equivalent to stage 10), n = 365 cells from 4 embryos; t=0 min (equivalent to early stage 11), n = 1026 cells from 9 embryos and t>>0 min (equivalent to late stage 11/early stage 12), n = 659 cells from 9 embryos.

O, O’ Spatial representation of the Venus-Hkb intensity (O) and apical area (O’) from an exemplary time lapse movie of a wild-type embryo, with radial location (collapsed into stripes) of cells specified at t= 0min. The orange bracket in O marks the appearing peak of high Venus-Hkb intensity at the position of the eccentric pit, with the orange dotted line marking the highest point of intensity. The orange bracket in O’ marks the appearing apical constriction at the position of the eccentric pit, with the orange dotted line marking the midway bracket point. The blue dotted line in O’ marks where the smallest apical area begins to track in the near pit position (as shown in Fig. 3G).

P, P’ Spatial representation of the Fkh-GFP intensity (P) and apical area (P’) from an exemplary time lapse movie of a wild-type embryo, with radial location (collapsed into
stripes) of cells specified at t= 0min. The orange bracket in P marks the appearing peak of high Fkh-GFP intensity at the position of the eccentric pit, with the orange dotted line marking the highest point of intensity. The orange bracket in P' marks the appearing strongest apical constriction at the position of the eccentric pit, with the orange dotted line marking the midway bracket point. The blue dotted lines in P and P' mark where the Fkh-GFP intensity and the smallest apical area begin to track in the near pit position.

In all analyses, t= 0min is defined as the frame just before the first sign of invagination at the future pit was evident.

See also Figure 5- figure supplement 1.
Figure 6. Asymmetric Fog expression is controlled by Hkb and Fkh upstream of early differential behaviours in the placode.

A-I Wild-type embryos labelled for Fog protein (the ligand to the GPCR upstream of Rho-dependent myosin II activation, J) and myosin II, visualised by SqhGFP. Fog is magenta in A, D, G and as a single channel in A’, D’, G’; myosin II is green in A, D, G. Also shown are corresponding quantifications of apical cell area (B, E, H) and average medial Fog fluorescence intensity (C, F, I). Time points analysed were before any apical constriction and tissue bending commencing (t<<0 min; ~ stage 10), at t= 0 min (~early stage 11) and once invagination had commenced (t>>0 min; ~ late stage 11/early stage 12).

J Schematic of the GPCR pathway leading to myosin II activation.

K Average medial Fog fluorescence intensity negatively correlates with apical cell area. Pearson coefficient r = -0.436; n = 823 cells from 7 embryos.

L Average medial myosin II fluorescence intensity positively correlates with average medial Fog fluorescence intensity. Pearson coefficient r = 0.439; n = 823 cells from 7 embryos.

M-O In hkb\(^{-}\) mutant embryos at a time point where invagination would have been well advanced in wildtype embryos (t>>0 min), medial myosin II (M) and Fog fluorescence (M’ and quantified in O) are concentrated in cells in the centre of the placode, where cells also show constricted apices (N).

P Quantification of average medial Fog fluorescence intensity according to radial position in wild-type versus hkb\(^{-}\) mutant embryos. Whereas in the wild-type Fog intensity peaks at the posterior end of the placode where the invagination pit forms, in hkb\(^{-}\) mutant embryos Fog is dramatically reduced at the invagination pit. Number of cells: wild-type n=823 from 7 embryos; hkb\(^{-}\) n =765 cells from 5 embryos.

Q-S In fkh\(^{-}\) mutant embryos at a time point where invagination would have well advanced in wildtype embryos (t>>0min), medial myosin II (Q) and Fog fluorescence (Q’ and quantified in S) are very homogenous across the placode and no longer enriched within the placode compared to the surrounding epidermis. Apical area quantification shows there is only a mild central cell constriction and no invagination (R).

T Quantification of average medial Fog intensity in fkh\(^{-}\) mutant embryos. In contrast to wild-type embryos with Fog enrichment at the posterior pit, levels of Fog are more homogenous in the placode in fkh\(^{-}\) mutant embryos with an increase towards the anterior. Levels are comparable to the rest of the epidermis (see Supplemental Figure 5). The intensity curves shown for wild-type and fkh\(^{-}\) mutant embryos are comparable in their shape, though the absolute intensities shown are not directly comparable, as fluorescence intensity was normalised in each image by dividing the average fluorescence per cell by the 98\(^{\text{th}}\) percentile value to account for embryo to embryo variability in staining efficiency. Number of cells: wild type n = 823 from 7 embryos; fkh\(^{-}\) mutant n= 512 cells from 5 embryos.
In all analyses of the wild-type, $t = 0$ min is defined as the frame just before the first sign of invagination at the future pit was evident. $hkb^{-/-}$ mutants were aligned using as a reference of embryo development the level of invagination of the tracheal pits that are not affected in the $hkb^{-/-}$ mutant as well as other morphological markers such as appearance and depth of segmental grooves in the embryo. See also Figure 6- figure supplement 1.
Figure 7. Changes to asymmetric placode patterning and invagination pit shape lead to misshapen organs.

A-C' Whereas in wild-type placodes (A, A') apical constriction commences only at the dorsal-posterior corner prior to the start of pit invagination, in placodes where Fog is overexpressed (using UAS-Fog x fkhGal4; B, B') excess apical constriction occurs all across the dorsal side of the placode. A, B show cell apices labelled by anti-phospho-tyrosine labelling, A', B' show apical area size. Coloured scale for cell area as in Figure 1. C, C'

Widening the Fog expression domain leads to significantly more constricted apices: cell area distribution of UAS-Fog x fkhGal4 differs from wild type (Kolmogorov-Smirnov test, p-value < 0.0001). Number of cells: wild-type, n = 771 from 7 embryos; UAS-Fog x fkhGal4, n = 788 from 5 embryos.

D-G In contrast to the narrow symmetrical pit and early invagination in wild-type placodes (D-D'), in UAS-Fog x fkhGal4 placodes cells in the whole Fog-overexpression domain constrict and initially start to form a large shallow depression (F, F'). D-G are stills from time-lapse movies, with D' and F' showing surface view and xz/yz-cross sections, the arrow in F' points to the corner from where cells start to invaginate. In comparison to the small opening of the invagination observed in wild-type placodes at stage 12 (E), the initial large depression is still present in UAS-Fog x fkhGal4 placodes at stage 12 whilst cells invaginate through a large pit (G; magenta arrows).

H-J At late stage 15, early stage 16, when salivary gland invagination and morphogenesis has finished, the final shape, and in particular lumen shape, of salivary gland cells overexpressing Fog (I; UAS-Fog x fkhGal4) is altered compared to wild-type controls (H). Apical adherens junctions are marked by phospho-tyrosine labelling (white). Glands are shown in three orthogonal cross sections. J Quantification of lumen aspect ratio, allowing identification of altered tube shapes such as widened tubes (Mann-Whitney test, p<0.001; wild-type: n = 23 embryos, UAS-Fog x fkhGal4 n=20 embryos).

K-O Theoretical considerations and experimental test on how altering the shape and size of the original invagination pit will affect the geometry and shape of the invaginating and invaginated tube. K, K' In the wild-type, a focussed eccentric pit (red in K) leads to similar numbers (K') of cells invaginating within each corona even in the absence of any cell rearrangements (green curve in N). L, L' A central pit would lead to an increasing number of cells invaginating in each corona, leading to a widening tube (magenta curve in N). M, M' An enlarged initial pit would lead to an increased but steady number of cells (M') invaginating (grey curve in N), again leading to a tube with enlarged lumen. Numbers of cells per corona are indicated. O Experimental test of the number of cells per invaginating corona in wild-type placodes or when the pit is central (hkb−/− mutant embryos) or the pit is enlarged, covering eccentric and central position (UAS-Fog x fkhGal4 embryos). Shown are mean and SD of
n=30, n=11, and n=5 for wild-type, hkb \(^{\text{m}}\) mutant and UAS-Fog x fkhGal4 embryos, respectively. See also Figure 7- figure supplements 1 and 2.
Figure 1- figure supplement 1. Patterned apical constriction remains fixed around the pit over time.

A Illustration of time intervals covered by wild-type time-lapse movies analysed in Fig. 1G, and Fig. 3J.

B Number of cells in ‘near the pit’ region used in wild-type experiments per time interval to calculate cell area change shown in Fig.1G and 3J.

C Numbers of cells in ‘far from the pit’ region used in wild-type experiments per time interval to calculate cell area change shown in Fig.1G and 3J.

In all analyses, t= 0 min is defined as the frame just before the first sign of invagination at the future pit was evident.
Figure 2- figure supplement 1. Sustained apical constriction near the pit is driven by apical-medial myosin.

A Myosin cycle length for all cells analysed from 3 movies, split into cells located near the pit (n=148) compared to cell located far form the pit (n=302) at t=0. Cells near the pit have significantly higher shorter, myosin cycle length than cell located far from the pit; statistical significance as determined using Mann Whitney test, with p<0.0001. The myosin cycle length of cells far from the pit at later time points (t=55-60 min, n = 134) was not significantly different from cells near the pit at t=0 (p = 0.1101 using a Mann Whitney test). Related to Figure 2.

B Spatial representation of the distribution of the myosin amplitude across the salivary gland placode, with radial location (collapsed into stripes) of cells specified at t= 0min. The dashed line marks the region of highest amplitude that is always adjacent to the invagination pit as cells flow into it over time. The mean of 3 movies is shown.

C Spatial representation of the amplitude of myosin oscillations in radial stripes from the pit (0 µm) to the boundary of the placode (47µm) at t= 0min. Mean and standard error of the mean are shown. Data are pooled from 3 movies ranging from -7.5-56.25 min; -16.76 to 55.48 min and -17.42 to 67.26min. Regions corresponding to cells near the pit and far from the pit are indicated by coloured shading.

In all analyses, t= 0min is defined as the frame just before the first sign of invagination at the future pit was evident.
Figure 3- figure supplement 1. \( hkb^{+} \) mutants show a delayed symmetrical apical constriction.

A Number of cells used in \( hkb^{+} \) mutant experiments per time interval to calculate cell area change shown in Fig. 3H.

B Illustration of time intervals covered by the time-lapse movies for wild-type experiments used to calculate strain rates in Fig. 4 A-B' and T1 productive neighbour exchanges in Fig. 4D, E. For cell area change in the wild-type shown in Fig. 3G, the experiments ExpID356, ExpID0625, ExpID0692, ExpID0447, ExpID0707, ExpID0709, ExpID0712 were analysed (details of time interval for the last 4 experiments are shown in Suppl. Fig. 1).

C Illustration of time intervals covered by the time-lapse movies for \( hkb^{+} \) mutant experiments. All experiments shown were used to calculate the cell area change shown in Fig. 3H. Experiments ExpID0181, ExpID0190, ExpID0196, ExpID0689 and ExpID0678 were used to calculate strain rates in Fig. 3 I-J' and T1 neighbour exchanges in Fig. 4D, E.

D, E Number of cells in wild-type experiments (D) and \( hkb^{+} \) mutant experiments (E) used to calculate strain rates in Fig. 4A-B' and T1 exchanges in Fig. 4 D, E by time interval and region (near the pit and far from the pit, with eccentric pit for the wild-type and central pit for the \( hkb^{+} \) mutants).

F, G Total number of T1 exchanges detected in wild-type experiments (F) and \( hkb^{+} \) mutant experiments (G), divided into circumferential and radial T1s. The cumulative plots of T1 exchanges are shown in Fig. 4 D, E.

H Number of cells used for analysis of cell elongation ratio in Fig. 4H in wild-type and \( hkb^{+} \) mutant.
Figure 3- figure supplement 2. hkb\(^{-}\) mutants show a delayed symmetrical apical constriction followed by aberrant invagination.

A, A' The invagination pit in wild-type embryos at stage 11 and beyond is small, eccentric in its position in the placode (dorsal-posterior corner).

B-D' In hkb\(^{-}\) mutant embryos, the invagination pit that forms at a delayed stage is often centrally positioned within the placode and enlarged, leading to a very much widened invagination as visible in the cross-sections. These central pits often enlarge towards the boundary and sometimes collapse into a more crescent-shaped invagination at later stages.

Magenta crosshairs in A-D indicate the positions of the cross-sections shown. Labelling in these panels is for anti-phospho-tyrosine. In A'-D' the position and shape of the invagination pit, drawn from the images in A-D, is shown in magenta, and the boundary of the placode as well as the ventral midline, where visible, are in black.

E-H Still images from time lapse movie ExpID0678 shown in Supplemental Movie 5. The delayed central constriction is visible in F, the crescent-shaped enlarged invagination front is highlighted in magenta, the central constricting area in red in F, G, H. I-L Still images from time lapse movie ExpID0689 shown in Supplemental Movie 6. The delayed central constriction is visible in J, the enlarged invagination is highlighted in magenta in K, L.

Membrane channel and myosin II channel are shown as individual panels in E-L, placode boundaries are indicated by magenta dotted lines.

In all analyses of the wild-type, t= 0min is defined as the frame just before the first sign of invagination at the future pit was evident. hkb\(^{-}\) mutants were aligned using as a reference of embryo development the level of invagination of the tracheal pits that are not affected in the hkb\(^{-}\) mutant as well as other morphological markers such as appearance and depth of segmental grooves in the embryo.
**Figure 4- figure supplement 1.** *hkb<sup>−/−</sup> mutants show aberrant cell behaviours.*

A-D” Instantaneous strain rates relating to the cumulative strain rates shown in Figure 4 A-B’. Panels for cell near the pit and far from the pit are shown, with an eccentric pit for the wild-type and a central pit for the *hkb<sup>−/−</sup>* mutant. Instantaneous strain rates are split into tissue, cell shape and intercalation strain rate tensors, as well as the radial and circumferential contributions. Regions of statistically significant differences (p<0.05) are indicated by pink bars above the curves, and significance was determined using mixed-effects model.

In all analyses of the wild-type, t= 0min is defined as the frame just before the first sign of invagination at the future pit was evident. *hkb<sup>−/−</sup>* mutants were aligned using as a reference of embryo development the level of invagination of the tracheal pits that are not affected in the *hkb<sup>−/−</sup>* mutant as well as other morphological markers such as appearance and depth of segmental grooves in the embryo.
Figure 5- figure supplement 1. The salivary gland placodal primordium is asymmetrically patterned by Hkb and Fkh prior to morphogenesis.

A Still of Supplemental Movie 7 at t= -59.5min. Cell membranes are shown in magenta, Venus-Hkb is in green.

B-B’ Comparison of Venus-Hkb (green in B) and anti-Hkb antibody staining (red in B’) at late stage 10.

B-B’ Comparison of Fkh-GFP (green in B) and anti-Fkh antibody staining (red in B’) at late stage 10.
Asymmetric Fog expression is controlled by Hkb and Fkh and is upstream of early differential behaviours in the placode.

A-C Wild-type embryos expressing sqhGFP were analysed before, at initiation of and well beyond start of pit invagination and medial myosin II intensity quantified per cell.

D-D’’ anti-Fog antibody labeling (green in D and D’) in a fkh–/– mutant embryo, showing no increased Fog accumulation in the placode over the surrounding epidermis. Cell membranes are in magenta in D and in D’’.

E-J’ Analysis of Fkh levels and localisation pattern in control and hkb–/– mutant embryos. E-F’ show two examples of control Fkh pattern at late stage 10, G, G’ at stage 11. H-I’ show two examples of Fkh pattern in hkb–/– mutant embryos at late stage 10, J,J’ at stage 11. Anti-Fkh staining is in red, cell outlines are labelled by anti-phospho-tyrosine in blue and GFP from the balancer chromosome identifying control embryos is shown in green.

K-N’ Analysis of Hkb levels and localisation pattern in control and fkh–/– mutant embryos. K-L’ show two examples of control Fkh pattern at late stage 10 (H, H’) and early stage 11 (L, L’). M-N’ show two examples of Hkb levels and localisation pattern in fkh–/– mutant embryos at late stage 10 (M, M’) and early stage 11 (N, N’). Anti-Hkb staining is in red, cell outlines are labelled by anti-phospho-tyrosine in blue and GFP from the balancer chromosome identifying control embryos is shown in green.

The boundaries of placodes are indicated by dotted lines.
**Figure 7- figure supplement 1. Fog overexpression in the salivary gland placode.**

**A-A”** In wild-type placodes at early stage 11, when tissue bending has just commenced (visible in the cross-sections, apical side is indicated, the white bars indicate the positions of the cross-section panels) Fog protein is most concentrated in cells near the pit and located in vesicles near the apical side.

**B-C”** In UAS-Fog x fkhGal4 embryos overexpressing Fog, already at late stage 10 (i.e. earlier than in panels A-A”) Fog protein is found at very high levels and very homogeneously across the placode (with the exception of a few anterior cells where fkhGal4-driven expression usually increases slightly later). Cross-sections reveal that all cells show higher levels of Fog in vesicular structures, again mostly homogeneously across the placode in contrast to the gradient of Fog intensity observed in the wild-type.

**D-D”** At mid-stage 11 a very wide invagination has formed in UAS-Fog x fkhGal4 (as also shown in Fig. 7E, F) and Fog levels remain increased and homogeneous across the placode.

In **A-D”** cell outlines are in green using labeling of junctional phosphotyrosine (PY20), anti-Fog antibody labeling is in magenta. White dotted lines indicate the boundary of the placode, the asterisks indicate the position of the (future) invagination point.

**E** Quantification of medial myosin intensity in control embryos compared to embryos that overexpress Fog (UAS-Fog x fkhGal4). Shown is the ration of medial to junctional myosin for individual cells to account for embryo-to-embryo differences.
Figure 7- figure supplement 2. Overexpression of Fkh in the salivary gland placode.

A-B'' Embryos overexpressing Fkh across the placode, using UAS-Fkh x fkhGal4 UAS-srcGFP, show highly aberrant lumen shapes at stage 13 when all secretory cells have invaginated. A A stage 13 embryo with two glands with highly inflated lumens marked by apical-lateral srcGFP (green) and PY20, labelling phosphotyrosine at adherens junctions (magenta). B-B'' show a deep z-stack projection as well as two orthogonal views of one of the glands shown in A. C-D'' UAS-Fkh x fkhGal4 UAS-srcGFP glands at early stage 11, prior to any tissue bending, show an increased number of apically constricted cells compared to wild-type placodes, visible with both apical-junctional membrane markers, srcGFP (green) and PY20 (magenta). The dotted line marks the boundary of the placode.
List of Videos

Video 1, related to Figure 1. Continuous apical constriction near the pit and switch to apical constriction of cell approaching the pit.
Time-lapse movie of an embryo expressing Armadillo-YFP to label cell outlines. Placodal cells are labeled according to position, with grey cells located far from the pit and pink cells located near to the pit at t = 0 min (see Methods for definition of near and far). Four individual cells are highlighted and further analysed in Fig. 1 F and G.

Video 2, related to Figure 2. Continuous medial myosin oscillations near the pit.
Time-lapse movie of an exemplary embryo expressing SqhGFP to label myosin II. SqhGFP is shown in the left panel, and the calculated apical-medial myosin II intensity on the right.

Video 3, related to Figure 3. Extended wild-type embryo movie 1 for strain rate analysis.
Control embryo (sqh[AX3]; sqhGFP, UbiRFP) with cell membrane shown in the left panel and myosin II in the right panel. This is ExpID0618.

Video 4, related to Figure 3. Extended wild-type embryo movie 2 for strain rate analysis.
Control embryo (sqh[AX3]; sqhGFP, UbiRFP) with cell membrane shown in the left panel and myosin II in the right panel. This is ExpID0690.

Video 5, related to Figure 3. Extended hkb<sup>−/−</sup> mutant embryo movie 2 for strain rate analysis.
hkb<sup>−/−</sup> mutant embryo (sqhGFP42, UbiRFP; hkb[2]) with cell membrane shown in the left panel and myosin II in the right panel. This is ExpID0678.

Video 6, related to Figure 3. Extended hkb<sup>−/−</sup> mutant embryo movie 2 for strain rate analysis.
hkb<sup>−/−</sup> mutant embryo (sqhGFP42, UbiRFP; hkb[2]) with cell membrane shown in the left panel and myosin II in the right panel. This is ExpID0689.

Video 7, related to Figure 5. Hkb dynamics in the placode.
Embryo of the genotype UbiRFP, Venus-Hkb to allow dynamic analysis of Hkb protein levels.
Video 8, related to Figure 5. Fkh dynamics in the placode.
Embryo of the genotype UbiRFP, Fkh-GFP to allow dynamic analysis of Fkh protein levels.
Figure 2_Sanchez et al.
Figure 3_Sanchez et al.
Figure 5_Sanchez et al.
Figure 6_Sanchez et al.
**Figure 1_Supplement 1_Sanchez Corrales**

(A) Experiments and movie time intervals for panels 1G, 3J

(B) Number of cells panel 1G, 3J

- Number of cells
  - **Near the pit**: Orange bars
  - **Far from the pit**: Green bars

(B') Number of cells panel 1G, 3J

- Number of cells
  - **Near the pit**: Orange bars
  - **Far from the pit**: Green bars
near to the pit

far from the pit

Figure 4_Supplement 1_Sanchez Corrales
Figure 7_Supplement 1_Sanchez Corrales