Myosin-II-mediated cell shape changes and cell intercalation contribute to primitive streak formation

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Primitive streak formation in the chick embryo involves large-scale highly coordinated flows of more than 100,000 cells in the epiblast. These large-scale tissue flows and deformations can be correlated with specific anisotropic cell behaviours in the forming mesendoderm through a combination of light-sheet microscopy and computational analysis. Relevant behaviours include apical contraction, elongation along the apical–basal axis followed by ingression, and asynchronous directional cell intercalation of small groups of mesendoderm cells. Cell intercalation is associated with sequential, directional contraction of apical junctions, the onset, localization and direction of which correlate strongly with the appearance of active myosin II cables in aligned apical junctions in neighbouring cells. Use of class specific myosin inhibitors and gene-specific knockdown shows that apical contraction and intercalation are myosin II dependent and also reveal critical roles for myosin I and myosin V family members in the assembly of junctional myosin II cables.

Gastrulation is a key event in the development of higher organisms. In amniotes this process is characterized by the formation of the primitive streak, a structure through which the mesendoderm cells ingress to form the deeper layers of the embryo1,6. Before streak formation the embryo consists of a sheet of epithelial cells with a well-developed apical–basal polarity. Cells are connected by apical tight and adherens junctions, whereas at the basal side a developing basal membrane separates the cells from the forming hypoblast2–5. In chick embryos streak formation involves large-scale vortex-like tissue flows that transport the mesendoderm precursors located in the epiblast at the interface between the extra embryonic area opaca and the embryonic area pellucida into the central midline of the embryo6–9 (Fig. 1d). There has been considerable speculation about the cellular mechanisms driving these large-scale tissue flows10. Experiments so far have relied on labelling a small subset of cells and following their movements during streak formation7,9,11. Based on these observations, several hypotheses (oriented cell divisions, intercalation of cells in the streak region, chemotaxis of subpopulations of cells, movement of the extracellular matrix, and localized ingression of cells into the hypoblast) have been put forward to explain tissue flows during streak formation7,9,12–15. Progress has been impeded by the lack of a detailed description of the epiblast cell behaviours underlying streak formation, due to the absence of methods to investigate the behaviour of the >100,000 cells in the 4-mm-diameter epiblast disc at cellular resolution and good methods to identify all cells.

To address these problems we have developed a transgenic chick line in which the cell membranes of all cells in the embryonic and extra embryonic tissues are labelled with a green fluorescent protein tag (Myr-EGFP), allowing a detailed characterization of cell behaviours. We have furthermore built a dedicated light-sheet microscope (LSM) especially designed to image these large, fragile, flat, live tissue samples16–18. We also have developed new methods that allow us to culture the early chick embryos in liquid with the epiblast side up, conditions required to take advantage of the high-resolution long-working-distance immersion optics of the LSM. We have developed and implemented computational methods to characterize the large-scale tissue flows and automated segmentation and tracking methods to characterize cell behaviours during streak formation, allowing us to correlate tissue and cell behaviour on the scale of the embryo19,20.
RESULTS
LSM imaging of streak formation
To image most cells in the early chick embryo we have designed and constructed a LSM (Fig. 1a), and devised a dedicated liquid culture method (Fig. 1b) optimized for recording the early development of chick embryos at cellular resolution at 2–3 min time intervals (Fig. 1d,e,g,h). A key feature of the LSM is an automatic height adjustment that continuously calculates the position of the surface.
of the embryo and through dynamic feedback to the microscope stage keeps it at the focus of the light sheet during the scanning process, resulting in images with optimal resolution. The LSM enables us to see all of the cells in a 1.66 mm stripe across the embryo as a three-dimensional data stack (typically $\sim$8,000 $\times$ 2,560 $\times$ 500 voxels) at excellent cellular resolution (Fig. 1d and Supplementary Videos 1 and 2). Image series at full spatial and temporal resolution are available at DOI http://dx.doi.org/10.15132/00000100 to be viewed using an Omero dataviewer\textsuperscript{21}. A major challenge is to analyse these large data sets. We analyse tissue behaviour using particle image velocimetry\textsuperscript{7,11,22,23} (PIV) and automated segmentation and tracking algorithms to analyse detailed cell behaviours of epiblast cells. PIV provides information about the local velocity of small approximately cell-sized tissue regions ($10 \times 10 \mu$m) and allows calculation of detailed local spatiotemporal behaviours of these domains, such as movement and deformation over time.

We have used the LSM to obtain a detailed characterization of cell and tissue behaviours during primitive streak formation in Myr-GFP embryos. From the start of development, the behaviour of cells in the epiblast is highly dynamic and characterized by frequent cell divisions, cell shape changes and ingressions (Fig. 1e–h and Supplementary Videos 3 and 4). Cells at the area opaca boundary extend active lamellipodia and filopodia while actively moving outward over the vitelline membrane, keeping the growing embryo under tension (Fig. 1h). Tracking of epiblast cells over time confirms the existence of large-scale vortex cell flows during streak formation (Figs 1d and 2c)\textsuperscript{7,8}. We have used PIV to fate map the tissue regions that give rise to the streak, by marking the streak outline at the extended streak stage and following the tissue backward in time to the start of the experiment (Fig. 2a and Supplementary Video 5 and Supplementary Fig. 1A). This procedure identified a sickle-shaped area in the posterior area pellucida epiblast as the precursor region to the streak, a domain essentially congruent with known expression domains of mesoderm-specific genes\textsuperscript{25,26}. Tissue deformations during streak formation

To analyse the deformations of the mesendoderm tissue over time we have used the PIV-derived velocity fields to calculate the changes in shape of initially square tissue domains. Contracting domains are coloured blue and expanding domains are coloured red (Fig. 2b and Supplementary Fig. 1b and Supplementary Videos 6 and 7). Contrary to expectation the mesendoderm is strongly contracting (Fig. 2b and Supplementary Fig. 1b), while undergoing a characteristic biphasic deformation during streak formation. Domains located in front of the extending streak are also seen to contract, while elongating perpendicular to the direction of streak extension (Fig. 2b). Averaging of the deformations of 9 embryos shows the consistency of these deformation patterns (Supplementary Fig. 2 and Supplementary Video 8). The onset of tissue motion reveals that it starts in the central mesendoderm region and then rapidly spreads lateral, while the motion of the tissue (red arrows) is in the opposite direction, that is, towards the streak (Fig. 2d), implying that the mesendoderm tissue is pulled from the centre, rather than being pushed from the lateral sides (Fig. 2d).

To further characterize the mesendoderm deformation we have calculated the spatiotemporal distribution of the isotropic and anisotropic strain rate components from the PIV-derived velocity fields. The isotropic strain rate component describes the uniform tissue expansion or contraction rate; the anisotropic strain rate component describes the shearing rate. At the onset of the tissue motion the mesendoderm starts to contract (blue circles) and the shear strain rate in the direction of contraction increases in the direction towards the midline (blue lines). Both processes increase during development (Fig. 3a and Supplementary Fig. 1C and Supplementary Videos 7 and 9).

To analyse this behaviour in several embryos we calculated the average strain rate in 4 embryos in the direction perpendicular to the streak axis ($\partial V_x /\partial y$) and along the streak ($\partial V_y /\partial y$) as a function of time (Fig. 3b,c). The strain rate perpendicular to the streak (red curve) is always larger than the strain rate along the streak (green curve; Fig. 3d). Figure 3e explains how isotropic processes, such as uniform tissue contraction and uniform expansion, and anisotropic processes contribute to the overall deformation of the tissue\textsuperscript{20,27}. Separation of the strain rate into its isotropic and anisotropic components shows that the anisotropic strain rate increases continuously over time, while the isotropic strain rate increases only slowly at first, but then increases more rapidly at around $\sim$6 h of development, the onset of streak extension (Fig. 3f). The isotropic contraction processes accelerate the movement in the direction of the streak and counteract the expansion of the streak (Fig. 3b,d,f).

Cellular behaviours driving tissue deformations

To visualize local cell behaviours, the images are segmented and specific groups of cells are visualized in a moving frame of reference, allowing investigation of their behaviours for extended periods of time. Manual verification of the automatic segmentation and tracking data, in four selected regions in the embryo, has provided us with gold-standard data (Fig. 4a–c). The regions analysed include a region initially close to the centre of the mesendoderm (blue; Fig. 4b), a region in the lateral mesendoderm (black) moving towards the streak, a region in the posterior area opaca (red) and a region in front of the streak (green; Fig. 4c). To understand which cell behaviours contribute to the observed tissue flows we have analysed the following cell behaviours: apical contraction and ingression; division, intercalation; and alignment.

The apical cross-sectional area of cells in all areas decreased significantly over time (40–65% depending on position; Fig. 4b,d, Supplementary Fig. 3a); however measurement of the radii of the mitotically rounded cells showed that cell volumes changed only slightly (<10%) during streak formation (Supplementary Fig. 3b,c). This implies that mesendoderm cells elongate along their apical–basal axis, a process that continues until they start to ingress in the streak at around 6–7 h after the start of the experiment (Fig. 4e).

Cells divide on average every 6 h everywhere in the embryo (Supplementary Fig. 4a), but the cell division rates are not constant in time (Supplementary Fig. 4b). We note a rise in the number of cell divisions at the time of the initiation of streak elongation in all experiments, the significance of which is unknown (Supplementary Fig. 4a,b).

Cells in different domains showed marked differences in their local neighbourhood dynamics. Cells in the streak-forming region showed extensive directional rearrangements (Fig. 4b), whereas cells outside the mesendoderm did not (Fig. 4c). Within the sickle
region cells rearrange by directional sequential contraction of aligned apical junctions involving 2–6 cells (Fig. 4f and Supplementary Videos 10 and 11). These junctional contraction events result in local cell rearrangements and are aligned towards the midline, thereby contributing to the initial mesendoderm tissue contraction and expansion of the forming streak (Fig. 4g).

A marked alignment of mesendoderm cells in the direction of motion is already detectable before the onset of tissue motion. This alignment increases during the early stages of sickle contraction but then dissipates (Supplementary Fig. 5a,b). Cell divisions are also seen to align in the mesendoderm in the direction of motion especially in the early phases of streak formation (Supplementary Fig. 4c).

We have used recently developed statistical techniques to quantify the different cell behaviour changes over time\textsuperscript{20,28}. Calculation of the cell-based analogue of the isotropic and anisotropic tissue strain rate components (Fig. 4g, panels 1 and 3, Supplementary Video 12) shows them to be in excellent quantitative agreement with the PIV-based strain rates (Fig. 3a and Supplementary Video 9). These calculations also show that the onset of motion of the mesendoderm is accompanied by an increasing contraction rate of cells in the anterior mesendoderm (Fig. 4g, blue circles in the first and third panels, Supplementary Fig. 5b) as well as a strong increase in magnitude and alignment of the shear strain rate in the direction of contraction of the mesendoderm (Fig. 4g, blue bars in first and

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**Figure 2** Tissue dynamics using PIV analysis. (a) Three time points illustrating the formation of the primitive streak. Cells contained within the red and blue dotted boundary were marked at the streak stage (11.5 h) and tracked backwards to reveal their origin at 0 h. The white arrow indicates the direction of the anterior–posterior axis from posterior to anterior. (b) Three panels illustrating the contraction expansion fate map calculated using the velocity fields as described in Methods. (c) The cell flow pattern midway during streak formation. Red lines indicate cell tracks of 5% of randomly selected cells over a 150 min time interval with green dots indicating their final positions. The blue square indicates the location used to analyse the onset of motion. (d) Each panel is a maximum-intensity projection of 5 consecutive frames (10 min) 5 min apart highlighting actively moving cells. Red arrows indicate direction of the tissue flow. Red lines mark the area of moving cells at the current time, whereas the blue line shows the area moving cells at the previous time point. The area of actively moving cells expands from medial to lateral. The schematic drawing illustrates possible scenarios for cell motion and velocity propagation depending on the type of active force. In the case of a pulling force (upper schematic) the onset of motion propagates in a direction opposite to the direction of motion, whereas in the case of a pushing force (lower schematic) onset of cell motion and movement are oriented in the same direction. The white scale bars are 200 μm in length.
Figure 3 Analysis of strain rates during streak formation. (a) Evolution of the expansion/compression strain rate of the strain rate tensor and the shear strain rate of the strain rate tensor, calculated as described in Methods, during streak formation. The expansion/contraction rates of the strain tensor are shown as circles; blue indicates contraction, red expansion. The anisotropic part indicating the shear strain rate is shown as a blue line in the direction of contraction. (b) Velocity field at $t=600$ min. Green and red lines indicate the location of the velocity field vectors used for analysis. Black scale bar is 200$\mu$m. (c) Velocity components as a function of distance along the green and red lines in a. Red and green dots indicate velocity components from the red and green areas. Blue dots mark the fitting range used to determine the strain rate (spatial velocity gradients). Slopes of fitted magenta lines are tissue strain rates. (d) Mean tissue strain rates and standard errors as a function of developmental time of 4 experiments. The red and green lines indicate tissue strain rate perpendicular and parallel to the streak respectively. (e) Cellular events driving deformation of epithelial tissue. Contraction/ingression (blue square), cell intercalation (green) and cell growth (magenta). Arrows indicate the direction of the tissue flows generated by these processes. (f, g) Tissue strain rate (red lines) for: wild-type embryos (f), and an embryo treated with 50$\mu$M H1152 (g). The tissue strain rates are decomposed into the isotropic part (apical contraction/ingression and cell growth, blue lines) and the anisotropic part (intercalation, green lines). The white scale bar in a is a 200$\mu$m size marker; the red scale bar in a indicates a strain rate of $10^{-4}$ s$^{-1}$ and a tissue domain velocity of 4$\mu$m min$^{-1}$.

third panels). The anisotropic strain rate can be decomposed into the contributions made by the cell deformation rate (shape change) and the cell rearrangement rate (intercalation; Fig. 4g, panels 2 and 4, Supplementary Video 12). Before the onset of motion the cell shape changes (Fig. 4g green bars, second and fourth panels) and directional cell rearrangements (Fig. 4g blue bars, second and fourth panels) are of
similar magnitude and arranged in perpendicular orientation, thereby cancelling each other’s effects on local tissue rearrangement. At around 3.5 h the directional cell rearrangements become more prominent in the mesendoderm resulting in the onset of motion and contraction of the mesendoderm (Fig. 4g panels 2 and 4, Supplementary Video 12). From around 9 h, the time the streak starts to extend, it can be seen that the tissue in front of the streak shows increasing intercalation along the direction of streak extension (Fig. 4g panels 2 and 4, Supplementary Video 12) explaining the observed compression and lateral extension of the tissue in this area at this time of development (Fig. 2b).

The role of myosins in controlling cell behaviours
During *Drosophila* gastrulation apical junctional contraction and cell intercalation are mediated by myosin II (refs 29–32). This prompted us to investigate the patterns of myosin II activation through detection of the phosphorylation of the myosin light chain (Mlc). In the posterior embryo we detected active myosin II in apical cell junctions, organized in supercellular patterns of myosin cables spanning 2–8 cells (Fig. 5a,b and Supplementary Fig. 6a). These myosin cables appear at the time mesendoderm starts to contract and are aligned along the axis of contraction (Fig. 5b). Application of blebbistatin, a specific myosin II inhibitor (refs 7, 33), showed negligible effects
on streak formation at concentrations up to 50 μM and little effect on Mlc phosphorylation (Supplementary Fig. 6a,b). In *Drosophila*, the asymmetric apical accumulation of myosin II is dependent on the segment polarity genes and controlled by interactions with the PAR3 system in a RhoGef2- and Rho-kinase-dependent manner\(^\text{34-36}\). We found no detectable asymmetry in apical junctional localization of Par3 or the PDZ RhoGef (AHRGef11), the RhoGef2 homologue in the chick embryo (Supplementary Fig. 7a–c). The Rho kinase inhibitor H1152 (ref. 37) reduced Mlc phosphorylation in apical cell junctions and partially impaired tissue flows, resulting in embryos with short fat streaks (Supplementary Fig. 6a,b). Quantitative analysis showed that H1152 partially inhibited both the anisotropic and the isotropic strain rate (Fig. 3g). These results could suggest the involvement of other signalling pathways or other myosins. A global analysis of myosin expression in an RNA-seq experiment showed that several members of the myosin I, myosin II and myosin V families were expressed at detectable levels during early development (Table 1). To investigate the possible involvement of members of the myosin I, myosin II and myosin V families in streak formation we used two potent inhibitors, pentachloroepudilin (PCP) and pentabromopseudilin (PCB). PCB inhibits myosin V family members and to a lesser extent myosin II family members (half-maximum inhibitory concentration (IC$_{50}$) 1.2 μM and 28 μM respectively)\(^\text{38,39}\), whereas PCP very specifically inhibits members of the myosin I family (IC$_{50}$ 1–5 μM, IC$_{50}$ for class II and class 5 myosins >95 μM; ref. 40). PCB at 5 μM showed a very strong inhibitory effect on streak formation, and resulted in a significant decrease in myosin II light chain phosphorylation and cable formation (Fig. 6a). PCP showed an immediate inhibition of tissue motion as well as very strong inhibition of Mlc phosphorylation (Fig. 6a and Supplementary Video 13). It strongly inhibited the association of myosin Ib with apical junctions, but had only little effect on the association of myosin Va with apical junctions in the mesoderm (Supplementary Fig. 6c,d). PCP completely blocked apical contraction, intercalation and streak formation and resulted in a complete block of tissue flow (Fig. 6d,e and Supplementary Videos 13–15).

We downregulated the expressed myosins using a directed siRNA approach to independently verify their effects on streak formation (Fig. 7). Simultaneous downregulation of myosin Ia/Ib blocked streak formation and led to a strong reduction of Mlc phosphorylation in apical junctions in the mesoderm cells without a significant effect on myosin I localization (Fig. 7a). Myosin Ia and/or Ib are evidently key mediators of streak formation. Observation of cell behaviours in the LSM showed that 5–10 h after siRNA transfection the tissue flows stopped and the embryos started to contract (Supplementary Video 16). Simultaneous downregulation of myosin Ia/Ib also strongly inhibited streak formation and resulted in strong inhibition of Mlc phosphorylation (Fig. 7b). Knockdown of myosin Ia/Ib resulted in the formation of large actin-rich protrusions and hair-like structures especially at the cell boundaries (Fig. 7b). Knockdown of myosin Va/Vb was less effective as judged by myosin Va antibody staining, but still resulted in a significant loss of Mlc phosphorylation, whereas myosin Va/Vb knockdown has little effect on myosin Ib localization (Fig. 7c). Together the results of the knockdown of myosin Ia/Ib and myosin Va/Vb are in line with the observations obtained using their chemical inhibitors PCP and PCB respectively. The experiments highlight unexpected roles for unconventional myosins in controlling cell behaviours resulting in streak formation and suggest that these unconventional myosins most likely act through effects on Mlc phosphorylation.

**DISCUSSION**

The development of the LSM enabled us to visualize the large-scale tissue flows during streak formation in the chick embryo, while at the same time providing enough resolution to quantify individual cell behaviours underlying these flows. The streak forms in an essentially biphasic process; the mesoderm initially contracts in the direction of the midline and then extends along the anterior posterior axis
(Figs 2b and 4d,f and Supplementary Videos 1 and 5). The apical contraction of aligned asymmetric mesendoderm cells contributes to the directional shortening of the mesendoderm as does directional cell intercalation through shortening of aligned junctions (Fig. 8a–c). The alignment of asymmetrically shaped cells favours the formation of long junctional ‘cables’ spanning 2–8 cells, which favour asymmetric force propagation along these cables, contrary to cases where alignment does not occur (Fig. 8d–g). Therefore, the initial alignment of cells before the onset of motion (Supplementary Fig. 5) is probably a key factor in triggering the directional contraction of the mesendoderm (Figs 2a,b and 8a). The phase of streak extension is associated with increased ingression of cells in the streak and further strong directional cell intercalation perpendicular to the direction of streak elongation mediated by directional shortening of junctions (Figs 2a,b 3a and 4g and Supplementary Videos 1, 9 and 12). The apical contraction of ingressing cells and cell intercalations driven by myosin-II-mediated directional junctional contraction (Fig. 8ac). In several systems, cortical myosin II assembly has been shown to be tension sensitive\(^{33,34}\). Our working hypothesis is that a tension-dependent mechanism of assembly of active myosin in cell junctions underlies its supercellular alignment. Contraction of a given junction results in an increase in tension of neighbouring aligned junctions. The junctions rapidly assemble more myosin to counteract the experienced increase in tension. This myosin accumulation then in turn can result in contraction of those aligned junctions, leading to the observed sequential junctional contractions. The level of MLC phosphorylation is effectively a read-out of the tension on cell junctions. Surprisingly, inhibition of myosin V and myosin I activity by chemical inhibitors and knockdown strongly inhibits formation of active myosin II cables as well as streak formation (Figs 6c–e and 7b). Myosin I members have been shown to be able to act as tension sensors at the membrane–cytoskeleton interface\(^{35}\), possibly explaining their key role in apical myosin II cable formation.

In the mouse the role of cell intercalation seems to be less important and it has been proposed that the streak forms essentially by \textit{in situ} ingression of cells\(^{36}\). This difference could be due to the much larger size and faster development of the chick embryo requiring coordination of behaviours of around a hundred thousand cells by a robust highly self-organizing mechanism\(^{47}\).

The basic cellular mechanisms that drive gastrulation in the chick embryo seem very similar to those observed during \textit{Drosophila} gastrulation and germband extension, where epiblast cells move towards the cleavage furrow by pulling forces generated by apical contraction of ingressing cells and cell intercalations driven by myosin-II–mediated directional junctional contraction\(^{39,30,48}\), whereas the myosin V homologue Dachs has been shown to control junctional remodelling during epithelial morphogenesis in the dorsal thorax in \textit{Drosophila}\(^{20}\). The main difference is that in the chick embryo the junctional contractions seem to be sequential, whereas in \textit{Drosophila} they often seem to occur simultaneously giving rise to clear rosette structures\(^{30}\). In the chick embryo the rosette structures observed are

### Table 1

| Feature ID | Description | RKPM 0 h | RKPM 5 h | RKPM 10 h | RKPM 15 h | RKPM 20 h |
|------------|-------------|----------|----------|-----------|-----------|-----------|
| ENSGALT000000020472 | MYH9 (myosin IIa) | 98.21 | 256.26 | 294.09 | 143.99 | 202.84 |
| ENSGALT000000034774 | MYL9 (myosin light chain 2) | 28.31 | 156.29 | 110.16 | 26.43 | 67.04 |
| ENSGALT00000008744 | MYL3 (myosin light chain 1) | 37.48 | 86.09 | 118.94 | 11.54 | 29.19 |
| ENSGALT00000012496 | MYO1B | 36.61 | 67.18 | 51.03 | 35.19 | 37.8 |
| ENSGALT00000007538 | MYO1A | 9.69 | 31.77 | 45.52 | 3.98 | 7.59 |
| ENSGALT00000023397 | MYH10 (myosin IIb) | 9.82 | 20.91 | 17.52 | 21.13 | 16.61 |
| ENSGALT00000030933 | MyL12A (myosin light chain, smooth muscle isoform) | 4.46 | 10.07 | 4.77 | 1.93 | 2.09 |
| ENSGALT00000008228 | MYO1C | 2.36 | 3.64 | 1.92 | 3.5 | 2.51 |
| ENSGALT00000039066 | MYO5A | 1.9 | 2.98 | 2.44 | 2.46 | 1.82 |
| ENSGALT00000007393 | MYO5C | 0.89 | 0.89 | 1.1 | 0.29 | 0.36 |
| ENSGALT00000031656 | MYO6 | 0.03 | 0.15 | 0.12 | 0.05 | 0.03 |
| ENSGALT00000009051 | MYO1G | 0.11 | 0.13 | 0.24 | 0.07 | 0.08 |
| ENSGALT00000022380 | MYH7 | 0.04 | 0.06 | 0.05 | 0.04 | 0 |
| ENSGALT0000001184 | MYH13 | 0.02 | 0.06 | 0.07 | 0.02 | 0.05 |
| ENSGALT00000010535 | MYH11 | 0 | 0.02 | 0 | 0.02 | 0 |
| ENSGALT0000000816 | myosin light chain, embryonic (L23) | 0.24 | 0 | 0.11 | 0 | 0 |

The table shows the relative expression of all detected myosin heavy chain family members as well as myosin II light chains at various times of development in an RNA-seq experiment performed as detailed in Methods. 0 h represents freshly laid unincubated eggs, 5 h represents stage EGXIII, 10 h is stage HH2, 15 h is stage HH3, and 20 h is stage HH4. RKPM denotes the relative expression level (reads per kilobase per million reads).
Figure 6 Effect of inhibition of conventional and non-conventional myosins on streak formation. (a) Phosphorylated Mlc (green) and actin staining (red) for a control embryo at 8 h of development (left panel), an embryo developed for 6 h in EC culture followed by treatment with 5 μM PCB for 2 h showing partial inhibition of Mlc phosphorylation (middle panel), and an embryo developed for 6 h in EC culture followed by treatment with 5 μM PCP for 2 h showing complete inhibition of Mlc phosphorylation (right panel). White arrows indicate pMLC localization in contraction furrow of dividing cells in the PCP-treated embryo. The results shown are representative for 3–5 experiments with n > 20 embryos in control and treatment. All embryos were visually inspected by fluorescence microscopy and at least 1 control and 1 inhibitor-treated embryo was analysed in detail by confocal microscopy. Scale bars, 25 μm. (b) Effects of knockdown of different classes of myosins on streak formation. Results shown are means and standard deviations of myosin IIa/IIb (control n = 95 embryos/7 independent experiments, siRNA knockdown n = 111 embryos/10 independent experiments), myosin Ia/Ib (control n = 59 embryos/7 independent experiments), myosin Ia/Ib (control n = 90 embryos/10 independent experiments) and myosin Va/Vb (control n = 31 embryos/3 independent experiments, siRNA n = 41 embryos/3 independent experiments) on streak formation 18 h after transfection with the respective siRNAs. (c) Images of typical embryos 18 h after transfection with control or specific siRNAs. Knockdown of myosin IIa/IIb and myosin Ia/Ib results in very contracted embryos; knockdown of myosin Va/Vb results in embryos that expand but most do not develop streaks. Scale bars, 1 mm. (d) Contraction/expansion map of an embryo before and after treatment with 5 μM PCP. First panel: 5 h of development before treatment. Middle panel: 30 min after PCP addition. Right panel 5 h after PCP addition. Note the immediate relaxation of the epiblast after PCP addition followed by a later contraction of the embryo. Scale bars, 200 μm. (e) Analysis of the isotropic and anisotropic shear strain rate of the embryo shown in d. Analysis was performed as described for the embryo shown in Fig. 4g. Note the expansion of the tissue and the loss of shear strain after PCP addition, followed by contraction 5 h after addition of PCP. The white scale bars represent 200 μm; the red scale bars indicate a strain rate of 10^{-4} s^{-1} and an instantaneous tissue speed of 4 μm min^{-1}.

Methods

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.
Figure 7 Effects of siRNA-mediated myosin I, II and V downregulation. (a) Effect of simultaneous myosin IIa and myosin IIb knockdown. Upper panels show, from left to right: expression of myosin IIb in control embryos, and after myosin IIa/IIb knockdown; pMlc phosphorylation in control and after myosin IIa/IIb knockdown; myosin Ib expression in control and after myosin IIa/IIb knockdown. The lower panels show phalloidin staining for the same samples directly above. There is strong knockdown of myosin IIb and pMlc, but little effect on myosin Ib staining. Embryos were incubated for 3 h, put in EC culture and transfected with specific or control siRNAs. All samples were fixed 18 h after transfection. The controls had reached the primitive streak stages (HH3-4), while the transfected embryos did not develop into streaks. (b) Effect of simultaneous myosin Ia and myosin Ib knockdown. Upper panels show, from left to right: expression of myosin Ia in control and after myosin Ia/Ib double knockdown; myosin Ib expression in control and after myosin Ia/Ib double knockdown; pMlc expression in control and after myosin Ia/Ib knockdown. The red panels show phalloidin staining for the same samples positioned directly above. There is strong knockdown of myosin Ia, myosin Ib and pMlc; note the effect on the changes in actin distribution. Other conditions as in a. (c) Effects of simultaneous myosin Va and myosin Vb knockdown. Upper row, from left to right: myosin Va expression in control and after myosin Va/Vb knockdown; pMLC expression in a control embryo and after myosin Va/Vb double knockdown. The lower panels show phalloidin staining for the same samples directly above. There is a noticeable loss of myosin Va membrane staining in the knockdown samples and a great loss of pMlc expression, but little effect on myosin Ib expression. Other conditions as in a. The data shown in this figure are representative for outcomes of the siRNA knockdown experiments shown in Fig. 6b. All samples (control and siRNA of each experiment were stained with the relevant antibodies and inspected by fluorescence microscopy; at least 1 control embryo and 1 siRNA treated embryo of each experiment was investigated in detail by confocal microscopy). Scale bars in all panels, 25 μm.

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Figure 8 Model of the forces and cell behaviours controlling streak formation. (a) Diagrams depicting forces generating the tissue flows during streak formation. The active pulling forces, yellow arrows; the passive pushing forces, red arrows; the direction of tissue flows, green arrows. The sickle region is indicated in black; the area pellucida outline in blue. Light blue squares indicate scattered events of junctional contraction, whereas dark blue shapes indicate regions of ingression. (b) Schematic of sequential junctional contraction (in a region marked by the blue squares in a). The sequentially contracting junctions are indicated with different colours (red, grey, green, magenta). (c) Schematic of cells showing apical contraction (blue arrows), coupled to elongation (red arrows) along the apical axis followed by ingression (green arrow). (d,e) Propagation of the force generated by contracting/ingressing cells between symmetrically (d) and asymmetrically (e) shaped cells. In the case of symmetrical cells the magnitude of the force decreases strongly and symmetrically at every successive junction bifurcation. However, for asymmetric cells, the broken symmetry favours force transmission along the aligned junctions (red lines), while damping transmission in perpendicular directions (green lines). (f) Image of randomly oriented cells outside the sickle region, showing a lack of junctional alignment. (g) Image of cells inside the sickle, showing many aligned junctions in neighbouring cells forming long chains (red line). The alignment of asymmetrically shaped cells inside the sickle region (Supplementary Fig. 5) enables anisotropic force propagation by apical contraction and directional junctional shortening resulting in large-scale directed motion. Scale bar in f,g, 25 μm.
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METHODS
Production of the membrane-localized GFP transgenic chicken line. Gateway technology (Invitrogen) was used to generate a lentiviral expression vector for ubiquitous expression of Myr-EGFP. The Myr-Flag-pEGFP-N3 plasmid containing the membrane-targeting/anchor protein pentachloropseudilin (MCP) cDNA was digested using XhoI and NotI and the Myr-EGFP fragment was inserted into pENTR2B (Invitrogen), which had been linearized by NotI/Sall digestion. The CAG promoter/enhancer (CMV-IE enhancer fused to the chicken β-actin promoter/1st intron) was cloned between two EcoRI sites in the pENTR5'-TOPO vector (Invitrogen) from which the blastcidin resistance gene had been deleted. The three plasmids were recombined using LR Clonase II by screening for GFP fluorescence in newly hatched chicks. Nine G0 chicks were identified and analysis of genomic DNA by Southern blotting indicated that each chick had a single integrated copy of the Myr-EGFP expression vector and that all 9 chicks carried the same genomic site insertion of the lentiviral vector. A transgenic line was established from one of these chicks. Fertile eggs used in the experiments described here were hemizygous for the transgene. All experiments, animal breeding and care procedures were carried out under licence from the UK Home Office.

Culture conditions. Early-stage chicken embryos can develop in vitro up to the beating-heart stage in embryo culture (EC) and in New culture (NC) [16]. In the EC and NC culture methods, embryos younger than stage HH2 cannot be grown with their dorsal side up. Contact with a large volume of gel-based or liquid substrata impairs development, most likely because factors secreted by the embryo get diluted too much to sustain development. It is of great interest, however, to grow young stage embryos not only dorsal side up but also in a liquid environment to take advantage of high-NA immersion lenses as in light-sheet microscopy systems. To overcome these limitations we have designed special culture plates that allow the cultivation of unicubated chicken embryos dorsal side up and also in a liquid environment (Fig. 1b). Our method is an extension to the EC culture method [16]. The embryos were cultured on a specially designed plate 2 cm internal hole. The embryo is transferred on the culture plate with the side of the embryo o from the external environment. The silicon-oil filled well (volume of ~25 μl) and immersed in 10 ml pure albumin and imaged in 100 magnifications. The plates either contained no additives (control) or 5 μM pentachloropseudilin or 5 μM pentachloropseudilin prepared from 10 μM stock solutions in dimethylsulphoxide. The embryos were incubated overnight for live LSM microscopy or fixed after 2 h and 4 h for immunocytochemistry. Antibodies were fixed for 3 h with 4% paraformaldehyde/PBS on ice, and then washed three times in PBS, followed by antibody detection using published procedures [4]. The embryos were mounted and in investigated by confocal microscopy using a Leica TCS SP2 confocal microscope at ×40 and ×100 magnifications.

RNA-seq experiment. Total RNA was prepared from batches of 10–20 embryos of mixed sex incubated for 0, 5, 10, 15 and 20 h. The area pellucida was cut out with fine tungsten needles and collected in ice-cold PBS. RNA was isolated with a Qiagen RNA Easy kit according to the manufacturer’s instructions. The RNA sequencing libraries were prepared using the Illumina mRNA seq 8 sample prep kit, following the manufacturer’s protocol. Brieﬂy, high-quality total RNA (RIN > 7) is used as input to mRNA capture by Poly A. The mRNA is then fragmented and randomly primed for reverse transcription followed by second-strand synthesis to create double-stranded cDNA fragments. Ends are repaired to produce blunt ends and an ‘A’-base is added to the blunt ends followed by ligation to Illumina paired-end sequencing adapters. The library is enriched by a limited number of cycles of PCR ampliﬁcation. The libraries were assessed using Agilent Bioanalyzer DNA 100 chips and quantiﬁed by qPCR using the Kapa Biosystems Kapa Illumina Library quantiﬁcation kit. The concentration of the sequencing libraries was normalized to 10 nM before denaturation and dilution for loading at 4.75 pM concentration onto an Illumina paired-end flow cell using the Illumina paired-end cluster kit and cluster station instrument. The samples were loaded at one sample per lane. After the clustering the flow cell was immediately placed on the Illumina GAIIX sequencer, previously primed with Illumina SBS reagents sufﬁcient for 36-cycle paired-end sequencing, and the sequencing run initiated. Sequencing was carried out by Edinburgh Genomics, The University of Edinburgh. Sequence analysis was performed using CLC genomics work bench software.

siRNA knockdown experiments. For transfections, embryos were incubated for 3 h following by preparation in EC culture. Embryos were transfected using a dedicated electroporation apparatus. Embryos were placed epiblast apical side down on a 3-mm-diameter round electrode, covered with 20 μl of 0.6% saline epiblast side down. One microlitre of siRNA (1 μg ml⁻¹) dissolved in stabilization buffer was pipetted on the top, forming a small drop in which the second circular electrode was positioned 2 mm from the bottom electrode. The embryos were electroporated by 3 consecutive 50 ms pulses at a field strength of 17.5 V cm⁻¹. After electroporation the embryos were immediately transferred to EC culture and allowed to develop for 18 h after which they were fixed with 4% paraformaldehyde in PBS for 4 h and processed for immunofluorescence staining with a variety of antibodies using standard procedures [4]. Experiments were started with batches of 36 fertilized eggs. Typically 20–25 embryos were selected for transfection experiments, where half were controls and the other half were transfected with a mixture of myosin-class-specific siRNAs. All experiments were repeated at least three times with different batches of eggs. All fixed and stained embryos were investigated by epifluorescence microscopy and samples of 2–3 embryos for control and treatment were chosen for more detailed analysis and recording of results by confocal microscopy (Leica SP2) using standardized settings at ×100 magnification.

We used the following Dharmacon siRNA pairs for silencing of specific myosin genes. The ognucleotides were designed and checked for optimal specificity using BLAST searches against the chick genome database. MYH9: 5’-GAGGAAAGGCG GAAAGAUAUU-3’ and 5’-GGGAGAGGUGAGAAUAUU-3’; MYH6: 5’-GAGAACCUCGGAGGAAUAU-3’ and 5’-GGGAGAACGGGAGAAUAU-3’; MYO5A: 5’-UGUUGAGGGUGUGGAUAU-3’ and 5’-AGCUUGAAGCCCG AGAAUAU-3’; MYO5B: 5’-GGAGAUAUUGUGGAUAUAU-3’ and 5’-GG AUUGAGGGGCAUCAUUAU-3’; MYO5A: 5’-GGAGAUAUGGACAGAAUAU-3’ and 5’-GGGAGAAGGUGCAUGAAUAU-3’. Microscopy setup. To image chick embryo development on the scale of the whole organism and with cellular resolution we have designed and constructed a designated light-sheet ﬂuorescent microscope. Light-sheet microscopy offers high-speed high-resolution three dimensional time lapse imaging with very low levels

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of photobleaching and photo-toxicity. Our set-up is based on a standard scanned fluorescent light-sheet microscope design\(^2\); however, to image flat chick embryos we have placed imaging and illumination arms above the sample at 45° to the worktop surface. The system is equipped with a high-speed scientific CMOS camera (PCO.Edge), a GSI 2-axis galvo scanner and a Physik Instrumente 3-axis motorized stage combination, which allows for imaging at rates exceeding 50 frames per second. Illumination and imaging are performed using water-dipping \(\times 10\) Nikon objectives with a working distance of 3.5 mm and 0.3 numerical aperture. During the experiment the sample is kept in a heated chamber at 37°C. Imaging is performed by moving the sample through the light sheet along the \(x\) axis and collecting subsequent cross-sections, which after acquisition are transformed into ‘confocal’ stacks (Fig. 1a). At present, imaging of a 4-mm-diameter embryo at 1.88 \(\mu\)m steps takes 2 min. In addition, to accommodate for sample shape and its changes over time we have implemented an active surface contour detection algorithm, which corrects the sample position along the \(z\) axis for each scan to keep the top of the sample in the focal point of the light sheet, necessary for achieving high and consistent data quality. Typically, 3,000 images of 2,560 \(\times\) 400 pixels are collected per time point resulting in 1.5 TB of data per 15 h experiment.

### Data analysis

We have divided the data processing pipeline into a series of distinct steps, each of which not only generates input for the following ones but also provides information about embryo behaviour at the given level of analysis.

1. **First stage of data processing**
   - The embryo surface shape is determined and a flat \(z\) stack is constructed from sections conformal to the surface. The acquired three-dimensional stack is transformed into rectangular coordinates. Next, each \(z\) plane is divided into square interrogation areas (64 \(\times\) 64 pixels) for which an FFT power-spectrum is evaluated. Along the \(z\) axis the square that shows the highest number of frequencies above a certain threshold in the FFT spectrum is selected as the surface tile. This process is repeated for all squares in the stack. A new, flat three-dimensional stack is constructed by shifting tiles along the \(z\) axis so that the surface tiles are occupying the sample \(z\) plane. This method allows not only correcting for the large-scale height differences between different parts of the sample but also removes small surface undulations. The apical plane is selected for further steps of analysis.

2. **Tissue velocity calculation**
   - tissue velocity is calculated by image cross-correlation using standard particle image velocimetry (PIV) algorithm (PIVlab v1.32 for MATLAB\(^2\)). Results of this calculation are used to study local tissue deformation and tissue fate mapping, and as an input for the automatic cell tracking algorithm. We found that setting the interrogation window to 64 \(\times\) 64 pixels for the first pass and 32 \(\times\) 32 pixels for the second pass with 50% overlap at each pass gave the best results.

3. **To understand the way tissue deforms**
   - we have derived a new velocity field averaging scheme that focuses on tissue level dynamics of the developing chick embryo and helps to reduce noise by removing small fluctuations of vector fields. The embryo is divided into a square grid with a lattice constant of 125 pixels and 10 points per square side. Typically, the grid contains 60 \(\times\) 25 squares. Each lattice point is displaced in time according to the local velocity field. For points located in between the PIV grid points, their velocity vectors are linearly interpolated from the nearest points using the MATLAB built-in function interp2. Initial squares deform into polygons and their area is calculated at each time point using the MATLAB built-in function polyarea. The areas are colour coded on a dynamic grid as total change in area of the squares (Fig. 2b and Supplementary Fig. 1) to identify and select active tissue regions for further analysis of the cellular level.

   - **Averaging of velocity fields from different experiments**
     - To average the expansion contraction behaviour from several embryos we needed to align their developmental axis. This was achieved using points from the anterior and posterior end of the primitive streak, for each time point in each experiment. We also estimated the time of onset of motion from each experiment. The annotated points were used to translate the centre point of the primitive streak into the origin of a generalized coordinate system for each embryo and for each time point. The spatial alignment, rotation and temporal alignment were used to align their corresponding velocity fields, which were then averaged. The averaged velocity field over the nine experiments was then used to evaluate the grid deformation over the whole time sequence.

4. **Calculation and decomposition of tissue strain rate**
   - The strain rate describes how much the material (tissue) contracts or expands on the basis of the differences in velocity between neighbouring points/areas. To compare the contraction rate along the left-right axis (\(\frac{\partial v_y}{\partial x}\)) and the expansion rate (\(\frac{\partial v_x}{\partial y}\)) along the posterior–anterior axis we have calculated strain rates for those two directions as shown in Fig. 3b. To reliably estimate values we have taken 25 cuts for each direction at the resolution of PIV calculations and fitted a linear slope to linear parts of the curves (blue points, Fig. 3c). By analysing tissue flows generated by contraction/ingression, cell growth and intercalation (Fig. 3d), it is evident that intercalation generates anisotropic effects (expansion in one axis and contraction in the other), whereas growth and contraction lead to isotropic effects (expansion and contraction in both axes respectively). This allows us to decompose the tissue strain rate into the isotropic part (growth + apical contraction/ingression) and the anisotropic part (intercalation; Fig. 3e,f):

   - \(\begin{align*}
   \frac{\partial v_y}{\partial x} &= \text{anisotropic + isotropic} \\
   \frac{\partial v_x}{\partial y} &= \text{anisotropic - isotropic}
   \end{align*}\)

   Alternatively we have calculated the strain rate for the whole image. The velocity tensor \(L\) (ref. 67)

   \[L = \begin{pmatrix}
   \frac{\partial v_y}{\partial x} & \frac{\partial v_x}{\partial y} \\
   \frac{\partial v_x}{\partial x} & \frac{\partial v_y}{\partial y}
   \end{pmatrix}\]

   can be decomposed into a symmetric part, which defines the strain rate tensor \(\xi\) as follows \(\xi = (L+L^T)/2\).

   - We have plotted the isotropic contraction/expansion term as a circle, red for expanding and blue for contraction and the anisotropic shear strain rate tensor as a blue bar in the direction of contraction (the direction of the negative eigenvalue).

### Velocity field

- Velocity field gradients of the matrix \(L\) were estimated using central differences over intervals of 32 pixels. For visualization the computed strain rate fields were averaged over 10 time points (\(\sim 30\) min) and over 13 spatial points (\(\sim 200\) pixels) along both spatial dimensions.

- To smooth the images and equalize their intensities before segmentation the images are filtered with an FFT-based band-pass filter using standard ImageJ and MATLAB routines. A Watershed segmentation algorithm is used to find outlines of cells in membrane GFP images\(^4\). Local intensity minima are used as seed points for the algorithm. As a result of the segmentation each cell in an image is presented as a collection of labelled segments.

- During automatic tracking cells are tracked forward in time from the first time point onward. An initial segmentation is obtained using watershed segmentation\(^6\). To track cells between time points, the locations of cells in future time points are estimated from their previous locations using the tissue velocity fields calculated by PIV between these time points fields. These locations are used to constrain the seedpoints of the watershed algorithm. Ingression and division events are detected using the characteristic changes in cell size and shape of the segmented cells. The automatic tracking method results in the tracking of 70% of the >100,000 cells correctly for between >100 successive time points. For the cell-based statistical analysis described below it is only required to track cells confidently over ~20 successive time points. The segmentation routines are written in MATLAB.

- To improve the automatic tracking a manual curation option for smaller selected regions was introduced. The manual tracking scheme is similar to that used in the automatic tracking using algorithms coded in MATLAB. The initial image is segmented and checked by visual inspection. The locations of local minima in each image are adjusted manually if needed to achieve correct segmentation results. In addition, ingestions, divisions and emerging cells are marked manually if necessary.

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\(1\) T. Chen et al. Nature 527, 491–495 (2015)\n
\(2\) A. M. S. B. Thole et al. J. Phys. D: Appl. Phys. 46, 345101, 18pp (2013)\n
\(3\) M. G. M. Schwan et al. Biophys. J. 108, 2515–2523 (2015)\n
\(4\) S. A. E. Metz et al. Biophys. J. 111, 1078–1087 (2016)
To measure orientation of cells from segmented images, an ellipse is fitted into each segment using the normalized second-order central moments of pixels of the segment\(^\text{60}\). Orientation of a cell is taken to be the orientation of the major axis of the fitted ellipse. In this study we consider a cell to be polarized if the eccentricity of the fitted ellipse exceeds 0.87.

To calculate the isotropic and anisotropic tissue deformations from the segmented data we have used recently developed methods that are based on the spatio-temporal changes in the dynamics of the links connecting the centroids of neighbouring cells\(^\text{69}\). The tissue texture tensor \(M\) provides information on the size, shape and alignment of the cells at a given time point in a particular region\(^\text{70}\).

\[
M = \begin{pmatrix}
X^2 & XY \\
YX & Y^2
\end{pmatrix}
\]

where \((X, Y) = (x_i - x_j, y_i - y_j) = r_i - r_j = l\).

The links \(l\) are defined as vectors connecting the centroids of neighbouring cells. The length and orientation of the links are captured in the so-called link matrices that are averaged over the domain of interest. We calculate these averages based on the links between all neighbouring cells in circular domains of 65 \(\mu\)m radius that cover the embryo.

The dynamic changes in link length and orientation are used to calculate the statistical symmetrized velocity gradient \(V\), the discrete analogue of the strain rate tensor, as follows\(^\text{71}\):

\[
V = \frac{N_c}{2N_{\text{tot}}} \left( M^{-1}(t) \left( \frac{\partial M}{\partial t} \right) \frac{1}{\Delta t} + \left( \frac{\partial M}{\partial t} \right) \frac{1}{\Delta t} M^{-1}(t) \right)
\]

where

\[
\Delta t = t + \Delta t/2 - t - \Delta t/2.
\]

Here \(N_c\) is the number of links present at both end points of time interval \(\Delta t\) and \(N_{\text{tot}}\) is the number of all links of cells that have their centroid within the circular domain at the time \(t\). \(\otimes\) represents the tensor outer product. \(M\) is calculated as previously shown. The tensor variation was evaluated between successive time intervals, typically 2.5–3 min.

The variation of statistical internal strain rate tensor \(dU/dt\) quantifies how cell size and shape change contribute to the total tissue deformation. The tensor is defined as follows\(^\text{72}\):

\[
\frac{dU}{dt} = \frac{1}{2\Delta t} \left[ \log M(t + \Delta t/2) - \log M(t - \Delta t/2) \right]
\]

Here links are chosen from end points of the same time interval \(\Delta t\) as for evaluation of the tensor \(V\).

The statistical topological rearrangement rate tensor \(P\) measures the contribution of cell intercalation to the total tissue deformation. The tensor is defined as follows\(^\text{73}\):

\[
P = \frac{1}{2\Delta \alpha M_{\text{tot}}} \left( N_c(m_c) - N_c(m_a) \right) M^{-1}(t)
\]

where \(m_a\) are links that exist in the end of the time interval \(\Delta t\) but not in the beginning and \(m_c\) are links that exist in the beginning of the time interval but not in the end. \(N_c\) is the number of appearing links and \(N_c\) is the number of disappearing links. In the figures in the results we show the traceless parts of the \(dU/dt\) and \(P\) tensor in the direction of negative eigenvalues. These signify the rate of the change in cell shape and the rate for cell rearrangement respectively. For visualization, the computed tensor fields were shown as a moving average over 10 time points (~30 min).

**Code availability.** The code used for all data processing and analyses described above has been written in MATLAB. The scripts will be made available on request.

**Reproducibility of experiments.** For figure 1d–h the images presented are representative for over 150 successfully recorded embryos acquired over a 3-year period. For figure 2a the results shown were replicated in over 20 embryos; another example is shown in Supplementary Fig. 1. For figure 2c the results are representative for analyses in over 20 embryos. For figure 2d the results are representative for observations in 4 embryos. For figure 3a the detailed analysis was performed in 3 embryos. For figure 4a–e the analysis was performed in 2 embryos with comparable results. For figure 4g the analysis was performed in 3 embryos. For figure 6d,e the analysis was performed in 2 embryos out of 11 embryos recorded. For Supplementary Fig. 3 the analysis was performed in 4 embryos with similar results. For Supplementary Fig. 4 the analysis was performed in 4 embryos with similar results. For Supplementary Fig. 5 the analysis was performed in 3 embryos with similar results. For other results details of the statistical analyses and reproducibility are provided in the figure legends.

**Data access.** The RNA-seq data have been submitted to the European Nucleotide Archive (ENA) and can be accessed at [http://www.ebi.ac.uk/ena/data/view/PRJEB8414](http://www.ebi.ac.uk/ena/data/view/PRJEB8414).
Myosin-II-mediated cell shape changes and cell intercalation contribute to primitive streak formation

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Primitive streak formation in the chick embryo involves large-scale highly coordinated flows of more than 100,000 cells in the epiblast. These large-scale tissue flows and deformations can be correlated with specific anisotropic cell behaviours in the forming mesendoderm through a combination of light-sheet microscopy and computational analysis. Relevant behaviours include apical contraction, elongation along the apical–basal axis followed by ingression, and asynchronous directional cell intercalation of small groups of mesendoderm cells. Cell intercalation is associated with sequential, directional contraction of apical junctions, the onset, localization and direction of which correlate strongly with the appearance of active myosin II cables in aligned apical junctions in neighbouring cells. Use of class specific myosin inhibitors and gene-specific knockdown shows that apical contraction and intercalation are myosin II dependent and also reveal critical roles for myosin I and myosin V family members in the assembly of junctional myosin II cables.

Gastrulation is a key event in the development of higher organisms. In amniotes this process is characterized by the formation of the primitive streak, a structure through which the mesendoderm cells ingress to form the deeper layers of the embryo\textsuperscript{1}. Before streak formation the embryo consists of a sheet of epithelial cells with a well-developed apical–basal polarity. Cells are connected by apical tight and adherens junctions, whereas at the basal side a developing basal membrane separates the cells from the forming hypoblast\textsuperscript{2–7}. In chick embryos streak formation involves large-scale vortex-like tissue flows that transport the mesendoderm precursors located in the epiblast at the interface between the extra embryonic area opaca and the embryonic area pellucida into the central midline of the embryo\textsuperscript{6–9} (Fig. 1d). There has been considerable speculation about the cellular mechanisms driving these large-scale tissue flows\textsuperscript{10}. Experiments so far have relied on labelling a small subset of cells and following their movements during streak formation\textsuperscript{7,9,11}. Based on these observations, several hypotheses (oriented cell divisions, intercalation of cells in the streak region, chemotaxis of subpopulations of cells, movement of the extracellular matrix, and localized ingestion of cells into the hypoblast) have been put forward to explain tissue flows during streak formation\textsuperscript{7,9,12–15}. Progress has been impeded by the lack of a detailed description of the epiblast cell behaviours underlying streak formation, due to the absence of methods to investigate the behaviour of the >100,000 cells in the 4-mm-diameter epiblast disc at cellular resolution and good methods to identify all cells.

To address these problems we have developed a transgenic chick line in which the cell membranes of all cells in the embryonic and extra embryonic tissues are labelled with a green fluorescent protein tag (Myr-EGFP), allowing a detailed characterization of cell behaviours. We have furthermore built a dedicated light-sheet microscope (LSM) especially designed to image these large, fragile, flat, live tissue samples\textsuperscript{16–18}. We also have developed new methods that allow us to culture the early chick embryos in liquid with the epiblast side up, conditions required to take advantage of the high-resolution long-working-distance immersion optics of the LSM. We have developed and implemented computational methods to characterize the large-scale tissue flows and automated segmentation and tracking methods to characterize cell behaviours during streak formation, allowing us to correlate tissue and cell behaviour on the scale of the embryo\textsuperscript{19,20}.

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RESULTS

LSM imaging of streak formation

To image most cells in the early chick embryo we have designed and constructed a LSM (Fig. 1a), and devised a dedicated liquid culture method (Fig. 1b) optimized for recording the early development of chick embryos at cellular resolution at 2–3 min time intervals (Fig. 1d,e,g,h). A key feature of the LSM is an automatic height adjustment that continuously calculates the position of the surface. 

Figure 1 Light-sheet microscopy set-up to study gastrulation in chicken embryos. (a) Schematics of the LSM for large flat samples; the illumination and imaging objectives are positioned at 45° to the embryo’s surface. Successive 45° cross-sections are collected by moving the embryo through the light sheet. (b) Sample plate designed to keep the early embryo flat and isolated from the external environment. (c) Schematic representations of acquisition geometry (images marked with blue rectangles) and the transformed data for analysis. (d) Single z plane (red rectangle in c) overlaid with the cell tracks of 5% of all cells over a 180 min time interval shown as red lines with green dots indicating their final positions (7,950 × 2,560 pixels, 5.17 mm × 1.66 mm; Supplementary Video 1). (e) Four frames 5 min apart showing a dividing cell (red dots) and its local neighbourhood (blue and green dots) shown at full recorded resolution (Supplementary Video 4). (f) Cross-section through the dividing cell (red dots) seen in e. (g) Three frames 10 min apart showing an ingressing cell (red dot) and immediate neighbour cells (other coloured dots; Supplementary Video 3). (h) Three frames 20 min apart showing embryo expansion driven by area opaca boundary cells making active protrusions (red arrows). The AP arrow in d indicates the direction of the anterior–posterior axis. The white scale bar in d is 200 μm; the white scale bars in e–h are 25 μm in length.

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of the embryo and through dynamic feedback to the microscope stage keeps it at the focus of the light sheet during the scanning process, resulting in images with optimal resolution. The LSM enables us to see all of the cells in a 1.66 mm stripe across the embryo as a three-dimensional data stack (typically ~8,000 × 2,560 × 500 voxels) at excellent cellular resolution (Fig. 1d and Supplementary Videos 1 and 2). Image series at full spatial and temporal resolution are available at DOI http://dx.doi.org/10.15132/10000100 to be viewed using an Omero dataviewer21. A major challenge is to analyse these large data sets. We analyse tissue behaviour using particle image velocimetry (PIV) and automated segmentation and tracking algorithms to analyse detailed cell behaviours of epiblast cells. PIV provides information about the local velocity of small approximately cell-sized tissue regions (10 × 10 μm) and allows calculation of detailed local spatiotemporal behaviours of these domains, such as movement and deformation over time.

We have used the LSM to obtain a detailed characterization of cell and tissue behaviours during primitive streak formation in MyrGFP embryos. From the start of development, the behaviour of cells in the epiblast is highly dynamic and characterized by frequent cell divisions, cell shape changes and ingestions (Fig. 1e–h and Supplementary Videos 3 and 4). Cells at the area opaca boundary extend active lamellipodia and filopodia while actively moving outward over the vitelline membrane, keeping the growing embryo under tension (Fig. 1h). Tracking of epiblast cells over time confirms the existence of large-scale vortex cell flows during streak formation (Figs 1d and 2c). We have used PIV to fate map the tissue regions that give rise to the streak, by marking the streak outline at the extended streak stage and following the tissue backward in time to the start of the experiment (Fig. 2a and Supplementary Video 5 and Supplementary Fig. 1A). This procedure identified a sickle-shaped area in the posterior area pellucida epiblast as the precursor region to the streak, a domain essentially congruent with known expression domains of mesoderm-specific genes25,26.

Tissue deformations during streak formation

To analyse the deformations of the mesendoderm tissue over time we have used the PIV-derived velocity fields to calculate the changes in shape of initially square tissue domains. Contracting domains are coloured blue and expanding domains are coloured red (Fig. 2b and Supplementary Fig. 1b and Supplementary Videos 6 and 7). Contrary to expectation the mesendoderm is strongly contracting (Fig. 2b and Supplementary Fig. 1b), while undergoing a characteristic biphasic deformation during streak formation. Domains located in front of the extending streak are also seen to contract, while elongating perpendicular to the direction of streak extension (Fig. 2b). Averaging of the deformations of 9 embryos shows the consistency of these deformation patterns (Supplementary Fig. 2 and Supplementary Video 8). The onset of tissue motion reveals that it starts in the central mesendoderm region and then rapidly spreads lateral, while the motion of the tissue (red arrows) is in the opposite direction, that is, towards the streak (Fig. 2d), implying that the mesoderm tissue is pulled from the centre, rather than being pushed from the lateral sides (Fig. 2d).

To further characterize the mesendoderm deformation we have calculated the spatiotemporal distribution of the isotropic and anisotropic strain rate components from the PIV-derived velocity fields. The isotropic strain rate component describes the uniform tissue expansion or contraction rate; the anisotropic strain rate component describes the shearing rate. At the onset of the tissue motion the mesendoderm starts to contract (blue circles) and the shear strain rate in the direction of contraction increases in the direction towards the midline (blue lines). Both processes increase during development (Fig. 3a and Supplementary Fig. 1C and Supplementary Videos 7 and 9).

To analyse this behaviour in several embryos we calculated the average strain rate in 4 embryos in the direction perpendicular to the streak axis (∂Vx/∂x) and along the streak (∂Vy/∂y) as a function of time (Fig. 3b,c). The strain rate perpendicular to the streak (red curve) is always larger than the strain rate along the streak (green curve; Fig. 3d). Figure 3e explains how isotropic processes, such as uniform tissue contraction and uniform expansion, and anisotropic processes contribute to the overall deformation of the tissue20,27. Separation of the strain rate into its isotropic and anisotropic components shows that the anisotropic strain rate increases continuously over time, while the isotropic strain rate increases only slowly at first, but then increases more rapidly at around ~6 h of development, the onset of streak extension (Fig. 3f).

Cellular behaviours driving tissue deformations

To visualize local cell behaviours, the images are segmented and specific groups of cells are visualized in a moving frame of reference, allowing investigation of their behaviours for extended periods of time. Manual verification of the automatic segmentation and tracking data, in four selected regions in the embryo, has provided us with gold-standard data (Fig. 4a–c). The regions analysed include a region initially close to the centre of the mesendoderm (blue; Fig. 4b), a region in the lateral mesendoderm (black) moving towards the streak, a region in the posterior area opaca (red) and a region in front of the streak (green; Fig. 4c). To understand which cell behaviours contribute to the observed tissue flows we have analysed the following cell behaviours: apical contraction and ingestion; division, intercalation; and alignment.

The apical cross-sectional area of cells in all areas decreased significantly over time (40–65% depending on position; Fig. 4b,d, Supplementary Fig. 3a); however measurement of the radii of the mitotically rounded cells showed that cell volumes changed only slightly (<10%) during streak formation (Supplementary Fig. 3b,c). This implies that mesendoderm cells elongate along their apical–basal axis, a process that continues until they start to ingress in the streak at around 6–7 h after the start of the experiment (Fig. 4e).

Cells divide on average every 6 h everywhere in the embryo (Supplementary Fig. 4a), but the cell division rates are not constant in time (Supplementary Fig. 4b). We note a rise in the number of cell divisions at the time of the initiation of streak elongation in all experiments, the significance of which is unknown (Supplementary Fig. 4a,b).

Cells in different domains showed marked differences in their local neighbourhood dynamics. Cells in the streak-forming region showed extensive directional rearrangements (Fig. 4b), whereas cells outside the mesendoderm did not (Fig. 4c). Within the sickle
region cells rearrange by directional sequential contraction of aligned apical junctions involving 2–6 cells (Fig. 4f and Supplementary Videos 10 and 11). These junctional contraction events result in local cell rearrangements and are aligned towards the midline, thereby contributing to the initial mesendoderm tissue contraction and expansion of the forming streak (Fig. 4g).

A marked alignment of mesendoderm cells in the direction of motion is already detectable before the onset of tissue motion. This alignment increases during the early stages of sickle contraction but then dissipates (Supplementary Fig. 5a,b). Cell divisions are also seen to align in the mesendoderm in the direction of motion especially in the early phases of streak formation (Supplementary Fig. 4c).

We have used recently developed statistical techniques to quantify the different cell behaviour changes over time. Calculation of the cell-based analogue of the isotropic and anisotropic tissue strain rate components (Fig. 4g, panels 1 and 3, Supplementary Video 12) shows them to be in excellent quantitative agreement with the PIV-based strain rates (Fig. 3a and Supplementary Video 9). These calculations also show that the onset of motion of the mesendoderm is accompanied by an increasing contraction rate of cells in the anterior mesendoderm (Fig. 4g, blue circles in the first and third panels, Supplementary Fig. 5b) as well as a strong increase in magnitude and alignment of the shear strain rate in the direction of contraction of the mesendoderm (Fig. 4g, blue bars in first and second panels).
Figure 3 Analysis of strain rates during streak formation. (a) Evolution of the expansion/contraction strain rate of the strain rate tensor and the shear strain rate of the strain rate tensor, calculated as described in Methods, during streak formation. The expansion/contraction rates of the strain tensor are shown as circles; blue indicates contraction, red expansion. The anisotropic part indicating the shear strain rate is shown as a blue line in the direction of contraction. (b) Velocity field at t = 600 min. Green and red lines indicate the location of the velocity field vectors used for analysis. Black scale bar is 200 µm. (c) Velocity components as a function of distance along the green and red lines in a. Red and green dots indicate velocity components from the red and green areas. Blue dots mark the fitting range used to determine the strain rate (spatial velocity gradients). Slopes of fitted magenta lines are tissue strain rates. (d) Mean tissue strain rates and standard errors as a function of developmental time of 4 experiments. The red and green lines indicate tissue strain rate perpendicular and parallel to the streak respectively. (e) Cellular events driving deformation of epithelial tissue. Contraction/ingression (blue square), cell intercalation (green) and cell growth (magenta). Arrows indicate the direction of the tissue flows generated by these processes. (f, g) Tissue strain rate (red lines) for: wild-type embryos (f), and an embryo treated with 50 µM H1152 (g). The tissue strain rates are decomposed into the isotropic part (apical contraction/ingression and cell growth, blue lines) and the anisotropic part (intercalation, green lines). The white scale bar in a is a 200 µm size marker; the red scale bar in a indicates a strain rate of $10^{-4}$ s$^{-1}$ and a tissue domain velocity of 4 µm min$^{-1}$.

Third panels). The anisotropic strain rate can be decomposed into the contributions made by the cell deformation rate (shape change) and the cell rearrangement rate (intercalation; Fig. 4g, panels 2 and 4, Supplementary Video 12). Before the onset of motion the cell shape changes (Fig. 4g green bars, second and fourth panels) and directional cell rearrangements (Fig. 4g blue bars, second and fourth panels) are of
Figure 4 Analysis of cell behaviours controlling gastrulation.  
(a) Analysis of cell behaviour in four distinct regions marked with different coloured squares: the middle (blue) and lateral (black) sickle region, the area opaca (red) and a region in front of the sickle (green). The initial positions of the domains are shown as squares, the same-coloured irregular-shaped domains indicate their positions and shape after 600 min. Scale bar, 200 µm.  
(b) Outlines of cells manually tracked in the middle sickle domain (blue square) at the start, 0, and after 600 min respectively. Scale bars, 100 µm.  
(c) Cells tracked in the anterior domain (green) at 0 and 600 min respectively. Scale bars, 100 µm.
(d) Average cell size in the four domains (colours as in a).  
(e) Ingression of cells in the same four regions.  
(f) Example of a sequential junctional contraction event in the sickle region. The successive contractions generate a pulling force perpendicular to the streak axis and a pushing force along the AP axis as shown by the velocity field (green arrows, averaged over 60 min). Scale bar, 25 µm.  
(g) Quantitative analysis of the contraction expansion (red/blue circles) rate and shear rate (blue bar in the direction of contraction) of the total tissue strain rate at 3 and 8.5 h of development (first and third panel). The strain rates are calculated from the changes in the lengths and direction of the vectors linking the centroid positions of all cells with their immediate neighbours as described in Methods. The strain rate contribution of the rate of cell shape change (green bars) and the rate of cell rearrangement (blue bars) in the direction of contraction are shown in the second and fourth panel. The dotted yellow line indicates the outline of the deforming endoderm. The thin yellow lines indicate the instantaneous velocities of the tissue at the time the strain rate tensors were calculated. The white scale bar is a 200 µm size marker; the red scale bar indicates a strain rate of $10^{-4}$ s$^{-1}$ and a tissue velocity of $4$ µm min$^{-1}$.

The role of myosins in controlling cell behaviours
During Drosophila gastrulation apical junctional contraction and cell intercalation are mediated by myosin II (refs 29–32). This prompted us to investigate the patterns of myosin II activation through detection of the phosphorylation of the myosin light chain ($\text{Mlc}$).  
In the posterior embryo we detected active myosin II in apical cell junctions, organized in supercellular patterns of myosin cables spanning 2–8 cells (Fig. 5a,b and Supplementary Fig. 6a). These myosin cables appear at the time mesendoderm starts to contract and are aligned along the axis of contraction (Fig. 5b). Application of blebbistatin, a specific myosin II inhibitor$^{7,33}$, showed negligible effects

similar magnitude and arranged in perpendicular orientation, thereby cancelling each other’s effects on local tissue rearrangement. At around 3.5 h the directional cell rearrangements become more prominent in the mesendoderm resulting in the onset of motion and contraction of the mesendoderm (Fig. 4g panels 2 and 4, Supplementary Video 12). From around 9 h, the time the streak starts to extend, it can be seen that the tissue in front of the streak shows increasing intercalation along the direction of streak extension (Fig. 4g panels 2 and 4, Supplementary Video 12) explaining the observed compression and lateral extension of the tissue in this area at this time of development (Fig. 2b).
on streak formation at concentrations up to 50 μM and little effect on Mlc phosphorylation (Supplementary Fig. 6a,b). In *Drosophila*, the asymmetric apical accumulation of myosin II is dependent on the segment polarity genes and controlled by interactions with the PAR3 system in a RhoGef2- and Rho-kinase-dependent manner. We found no detectable asymmetry in apical junctional localization of Par3 or the PDZ RhoGef (AHRGef11), the RhoGef2 homologue in the chick embryo (Supplementary Fig. 7a–c). The Rho kinase inhibitor H1152 (ref. 37) reduced Mlc phosphorylation in apical cell junctions and partially impaired tissue flows, resulting in embryos with short fat streaks (Supplementary Fig. 6a,b). Quantitative analysis showed that H1152 partially inhibited both the anisotropic and the isotropic strain rate (Fig. 3g). These results could suggest the involvement of other signalling pathways or other myosins. A global analysis of myosin expression in an RNA-seq experiment showed that several members of the myosin I, myosin II and myosin V families were expressed at detectable levels during early development (Table 1). To investigate the possible involvement of members of the myosin I, myosin II and myosin V families in streak formation we used two potent inhibitors, pentachloropseudilin (PCP) and pentabromopseudilin (PCB). PCB inhibits myosin V family members and to a lesser extent myosin II family members (half-maximum inhibitory concentration ([IC₅₀]) 1.2 μM and 28 μM respectively) whereas PCP very specifically inhibits members of the myosin I family ([IC₅₀] 1–5 μM, [IC₅₀] for class II and class 5 myosins >95 μM; ref. 40). PCB at 5 μM showed a very strong inhibitory effect on streak formation, and resulted in a significant decrease in myosin II light chain phosphorylation and cable formation (Fig. 6a). PCP showed an immediate inhibition of tissue motion as well as very strong inhibition of Mlc phosphorylation (Fig. 6a and Supplementary Video 13). It strongly inhibited the association of myosin Ib with apical junctions, but had only little effect on the association of myosin Va with apical junctions in the mesendoderm (Supplementary Fig. 6c,d). PCP completely blocked apical contraction, intercalation and streak formation and resulted in a complete block of tissue flow (Fig. 6d,e and Supplementary Videos 13–15).

We downregulated the expressed myosins using a directed siRNA approach to independently verify their effects on streak formation (Fig. 7). Simultaneous downregulation of myosin Ia/Ib blocked streak formation and led to a strong reduction of Mlc phosphorylation in apical junctions in the mesoderm cells without a significant effect on myosin I localization (Fig. 7a). Myosin Ia and/or Ib are evidently key mediators of streak formation. Observation of cell behaviours in the LSM showed that 5–10 h after siRNA transfection the tissue flows stopped and the embryos started to contract (Supplementary Video 16). Simultaneous downregulation of myosin Ia/Ib also strongly inhibited streak formation and resulted in strong inhibition of Mlc phosphorylation (Fig. 7b). Knockdown of myosin Ia/Ib resulted in the formation of large actin-rich protrusions and hair-like structures especially at the cell boundaries (Fig. 7b). Knockdown of myosin Va/Vb was less effective as judged by myosin Va antibody staining, but still resulted in a significant loss of Mlc phosphorylation, whereas myosin Va/Vb knockdown has little effect on myosin Ib localization (Fig. 7c). Together the results of the knockdown of myosin Ia/Ib and myosin Va/Vb are in line with the observations obtained using their chemical inhibitors PCP and PCB respectively. The experiments highlight unexpected roles for unconventional myosins in controlling cell behaviours resulting in streak formation and suggest that these unconventional myosins most likely act through effects on Mlc phosphorylation.

**DISCUSSION**

The development of the LSM enabled us to visualize the large-scale tissue flows during streak formation in the chick embryo, while at the same time providing enough resolution to quantify individual cell behaviours underlying these flows. The streak forms in an essentially biphasic process; the mesendoderm initially contracts in the direction of the midline and then extends along the anterior posterior axis.
be squeezed out from the epiblast (Supplementary Videos 1, 9 and 12). These cells could either epiblast cells into the deeper parts of the embryo (Fig. 2a and 8b). The phase of streak extension is probably a key factor in triggering the directional contraction of the mesendoderm (Figs 2b and 4d,f and Supplementary Videos 1 and 5). The apical alignment does not occur (Fig. 8dg). Therefore, the initial alignment of asymmetrically shaped cells favours the formation of long junctional ‘cables’ spanning 2–8 cells, which favour asymmetric force propagation along these cables, contrary to cases where alignment does not occur (Fig. 8d–g). The alignment of cells before the onset of motion (Supplementary Fig. 5) is probably a key factor in triggering the directional contraction of the mesendoderm (Figs 2a,b and 8a). The phase of streak extension is associated with increased ingression of cells in the streak and further strong directional cell intercalation perpendicular to the direction of streak elongation mediated by directional shortening of junctions (Figs 2a,b 3a and 12). The alignment of asymmetrically shaped cells contributes to the directional shortening of the mesendoderm as does directional cell intercalation through shortening of aligned junctions (Fig. 8a–c). The alignment of asymmetrically shaped cells favours the formation of long functional ‘cables’ spanning 2–8 cells, which favour asymmetric force propagation along these cables, contrary to cases where alignment does not occur (Fig. 8d–g). Therefore, the initial alignment of cells before the onset of motion (Supplementary Fig. 5) is probably a key factor in triggering the directional contraction of the mesendoderm (Figs 2a,b and 8a). The phase of streak extension is associated with increased ingression of cells in the streak and further strong directional cell intercalation perpendicular to the direction of streak elongation mediated by directional shortening of junctions (Figs 2a,b 3a and 12). The alignment of cells helps to pull mesendoderm cells towards the streak, but counteracts its extension (Fig. 3e,f).

The deformation of tissue in the forming neural plate is probably caused by the pressure exerted by the extending streak. It results in directional cell intercalation in the direction of streak extension (Fig. 4g) and also coincides with ingestion of isolated epiblast cells into the deeper parts of the embryo (Fig. 2a and Supplementary Videos 1, 9 and 12). These cells could either be squeezed out from the epiblast or could ingress actively. The role of ingestion has been independently highlighted in a recently published study, in which it was argued that the ingestion of cells is cooperative and coordinated through a Nodal-dependent community effect. How Nodal signals mediate ingestion remains unresolved.

Junctional contraction is coincident with patterns of Mlc phosphorylation, organized in supercellular cable-like structures, spanning 2–8 cells that are aligned in the direction of motion towards the streak (Fig. 5). Inhibition of myosin II in aligned cell junctions and a strong inhibition of streak formation showing that myosin II is a key motor in junctional contraction (Fig. 6a–c).

Table 1 Analysis of RNA expression of myosin family members.

| Feature ID | Description | RKPM 0 h | RKPM 5 h | RKPM 10 h | RKPM 15 h | RKPM 20 h |
|-----------|-------------|---------|---------|----------|-----------|----------|
| ENSGALT000000020472 | MYH9 (myosin Ila) | 98.21 | 256.26 | 294.09 | 143.99 | 202.84 |
| ENSGALT000000034774 | MYL9 (myosin light chain 2) | 28.31 | 156.29 | 110.16 | 26.43 | 67.04 |
| ENSGALT00000008744 | MYL3 (myosin light chain 1) | 37.48 | 86.09 | 118.94 | 11.54 | 29.19 |
| ENSGALT00000012496 | MYO1B | 36.61 | 67.18 | 51.03 | 35.19 | 37.8 |
| ENSGALT00000007538 | MYO1A | 9.69 | 31.77 | 45.52 | 3.98 | 7.59 |
| ENSGALT00000023397 | MYH10 (myosin IIb) | 9.82 | 20.91 | 17.52 | 21.13 | 16.61 |
| ENSGALT00000030933 | MyL12A (myosin light chain, smooth muscle isoform) | 4.46 | 10.07 | 4.77 | 1.93 | 2.09 |
| ENSGALT00000008228 | MYO1C | 2.36 | 3.64 | 1.92 | 3.5 | 2.51 |
| ENSGALT00000039066 | MYO5A | 1.9 | 2.98 | 2.44 | 2.46 | 1.82 |
| ENSGALT000000077393 | MYO5C | 0.89 | 0.89 | 1.1 | 0.29 | 0.36 |
| ENSGALT000000031656 | MYO6 | 0.03 | 0.15 | 0.12 | 0.05 | 0.03 |
| ENSGALT00000009051 | MYO1G | 0.11 | 0.13 | 0.24 | 0.07 | 0.08 |
| ENSGALT00000022380 | MYH7 | 0.04 | 0.06 | 0.05 | 0.04 | 0 |
| ENSGALT0000001184 | MYH13 | 0.02 | 0.06 | 0.07 | 0.02 | 0.05 |
| ENSGALT00000010535 | MYH11 | 0 | 0.02 | 0 | 0.02 | 0 |
| ENSGALT00000000816 | myosin light chain, embryonic (L23) | 0.24 | 0 | 0.11 | 0 | 0 |

The table shows the relative expression of all detected myosin heavy chain family members as well as myosin II light chains at various times of development in an RNA-seq experiment performed as detailed in Methods. 0 h represents freshly laid unincubated eggs, 5 h represents stage EGXIII, 10 h is stage HH2, 15 h is stage HH3, and 20 h is stage HH4. RKPM denotes the relative expression level (reads per kilobase per million reads).
mainly caused by dividing or ingressing cells15,26,49. The mechanism of intercalation seems to be different from the sliding intercalation mechanism proposed to drive convergent extension of the mesoderm during gastrulation in the frog embryo50,51.

Another key question is why do myosin cables assemble preferentially in the forming mesendoderm? In Drosophila myosin assembly requires folded gastrulation (Fog) a ligand secreted by mesoderm cells52 which through a serpentine transmembrane receptor, a heterotrimeric G protein, a RhoGef, Rho and Rho kinase results in myosin II activation and apical junction contraction52–54.

Given the high mechanistic similarity at the cellular level it would appear likely that a similar signalling mechanism is required to initiate and coordinate cell behaviours during amniote apical contraction in a larger region during gastrulation.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.
Figure 7 Effects of siRNA-mediated myosin I, II and V downregulation.
(a) Effect of simultaneous myosin IIa and myosin IIb knockdown. Upper panels show, from left to right: expression of myosin IIb in control embryos, and after myosin IIa/IIb knockdown; pMlc phosphorylation in control and after myosin IIa/IIb knockdown; myosin Ib expression in control and after myosin IIa/IIb knockdown. The lower panels show phalloidin staining for the same samples directly above. There is strong knockdown of myosin IIb and pMlc, but little effect on myosin Ib staining. Embryos were incubated for 3 h, put in EC culture and transfected with specific or control siRNAs. All samples were fixed 18 h after transfection. The controls had reached the primitive streak stages (HH3-4), while the transfected embryos did not develop into streaks. (b) Effect of simultaneous myosin Ia and myosin Ib knockdown. Upper panels show, from left to right: expression of myosin Ia in control and after myosin Ia/Ib double knockdown; myosin Ib expression in control and after myosin Ia/Ib double knockdown; pMlc expression in control and after myosin Ia/Ib knockdown. The red panels show phalloidin staining for the same samples positioned directly above. There is strong knockdown of myosin Ia, myosin Ib and pMlc; note the effect on the changes in actin distribution. Other conditions as in a. (c) Effects of simultaneous myosin Va and myosin Vb knockdown. Upper row, from left to right: myosin Va expression in control and after myosin Va/Vb knockdown; pMLC expression in a control embryo and after myosin Va/Vb double knockdown. The lower panels show phalloidin staining for the same samples directly above. There is a noticeable loss of myosin Va membrane staining in the knockdown samples and a great loss of pMlc expression, but little effect on myosin Ib expression. Other conditions as in a. The data shown in this figure are representative for outcomes of the siRNA knockdown experiments shown in Fig. 6b. All samples (control and siRNA of each experiment were stained with the relevant antibodies and inspected by fluorescence microscopy; at least 1 control embryo and 1 siRNA treated embryo of each experiment was investigated in detail by confocal microscopy). Scale bars in all panels, 25 μm.

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Figure 8 Model of the forces and cell behaviours controlling streak formation. (a) Diagrams depicting forces generating the tissue flows during streak formation. The active pulling forces, yellow arrows; the passive pushing forces, red arrows; the direction of tissue flows, green arrows. The sickle region is indicated in black; the area pellucida outline in blue. Light blue squares indicate scattered events of junctional contraction, whereas dark blue shapes indicate regions of ingression. (b) Schematic of sequential junctional contraction (in a region marked by the blue squares in a). The sequentially contracting junctions are indicated with different colours (red, grey, green, magenta). (c) Schematic of cells showing apical contraction (blue arrows), coupled to elongation (red arrows) along the apical axis followed by ingression (green arrow). (d,e) Propagation of the force generated by contracting/ingressing cells between symmetrically (d) and asymmetrically (e) shaped cells. In the case of symmetrical cells the magnitude of the force decreases strongly and symmetrically at every successive junction bifurcation. However, for asymmetric cells, the broken symmetry favours force transmission along the aligned junctions (red lines), while damping transmission in perpendicular directions (green lines). (f) Image of randomly oriented cells outside the sickle region, showing a lack of junctional alignment. (g) Image of cells inside the sickle, showing many aligned junctions in neighbouring cells forming long chains (red line). The alignment of asymmetrically shaped cells inside the sickle region (Supplementary Fig. 5) enables anisotropic force propagation by apical contraction and directional junctional shortening resulting in large-scale directed motion. Scale bar in f,g, 25 μm.

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AUTHOR CONTRIBUTIONS
E.R. built the LSM hardware and software, performed experiments and PIV-based data analysis. M.C. performed the biological and myosin perturbation experiments. A.I.K. developed the cell-based image analysis software and analysed experiments. F.S. and H.M.S. developed the Myr-GFP embryos. R.M. and H-J.K. developed and produced the myosin inhibitors. C.J.W. and M.P.M. conceived the design and use of the LSM in the investigation of chick development.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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METHODS

Production of the membrane-localized GFP transgenic chicken line. Gateway technology (Invitrogen) was used to generate a lentiviral expression vector for ubiquitous expression of Myr-EGFP. The Myr-Flag-pEGFP-N3 plasmid containing the ampicillin/polyethylene glycol (PEG) cleavage sequence MGGCV was digested using XhoI and NotI and the Myr-EGFP fragment was inserted into pENTR2B (Invitrogen), which had been linearized by NotI/SalI digestion. The CAG promoter/enhancer (CMV-IE enhancer fused to the chicken β-actin promoter/1st intron) was cloned between two EcoRI sites in the pENTR5-TOPO vector (Invitrogen) from which the blastocidin resistance gene had been deleted. The three plasmids were recombined using LR Clonase enzyme mix to generate the Myr-EGFP lentiviral expression vector. HIV-GFP and the modified RNA regulatory element oPRE (ref. 56) were then inserted 5’ upstream of the CAG promoter and 3’ downstream of the Myr-EGFP gene respectively, to enhance viral titre and transgene expression.

Packaged viral vector stocks were generated by FuGENE6 (Roche) co-transfection of HEK 293T cells with the vector plasmid, gag/pol plasmid (psPAZX, Addgene) and VSV-G plasmid (pLP/VSV-G, Invitrogen). Viral particles were concentrated from cell culture medium by centrifugation and resuspended in a final volume of ~60–80 μL. The virus preparation had a titre of approximately 10^8 TU mL^(-1). A ~2 μL volume of concentrated virus was injected into the subgerminall cavity of newly laid eggs and the embryos were incubated in host shells to hatch. A total of 38 eggs were injected from which 23 (60.5%) chicks hatched. G1 birds were bred from one cockerel (MCG12-14), by screening for GFP fluorescence in newly hatched chicks. Nine G1 chicks were identified and analysis of genomic DNA by Southern blotting indicated that each chick had a single integrated copy of the Myr-GFP expression vector and that all 9 chicks carried the same genomic site insertion of the lentiviral vector. A transgenic line was established from one of these chicks. Fertile eggs used in the experiments described here were hemizygous for the transgene. All experiments, animal breeding and care procedures were carried out under licence from the UK Home Office.

Culture conditions. Early-stage chicken embryos can develop in vitro up to the beating-heart stage in embryo culture (EC) and in New culture conditions. In the EC and New culture methods, embryos younger than stage HH2 cannot be grown with their dorsal side up. Contact with a large volume of gel-based or liquid substrata impairs development, most likely because factors secreted by the embryo get diluted too much to sustain development. It is of great interest, however, to grow young stage embryos not only dorsal side up but also in a liquid environment to take advantage of high-NA immersion lenses as in light-sheet microscopy systems. To overcome these limitations we have designed special culture plates that allow the cultivation of uncubated chicken embryos dorsal side up and also in a liquid environment (Fig. 1b). Our method is an extension to the EC culture method79,80. The embryos were cultured on a specially designed plate 2 cm wide and 2.5 mm high as indicated in Fig. 1b. The central depression is 1 cm in diameter and 0.3 mm deep and 3 mm deep (volume of ~25 μL) filled with 30 μL of heavy silicone oil (Polydimethylsiloxane-co-methylphenylsiloxane, density 1.066 g ml^(-1), 125°C viscosity, Aldrich, 378488). Embryos are collected using circular 30 mm Whatman 1 filter paper carriers with pentachloropseudilin or 5 μM pentachlorobenzylidide for 3 h followed by preparation in EC culture. Embryos were transfected using a dedicated electroporation apparatus. Embryos were placed epiblast apical side down on a 3-mm-diameter round electrode, covered with 20 μL of 0.6% saline epiblast side down. One microlitre of siRNA (1 μg μL^(-1)) dissolved in stabilization buffer was pipetted on the top of the embryo. Another 20 μL of 0.6% NaCl was pipetted on the top, forming a small drop in which the second circular electrode was positioned 2 mm from the bottom electrode. The embryos were electroporated by 3 consecutive 50 ms pulses at a field strength of 17.5 V cm^(-1). After electroporation the embryos were immediately transferred to EC culture and allowed to develop for 18 h after which they were fixed with 4% paraformaldehyde in PBS for 4 h and processed for immunofluorescence staining with a variety of antibodies using standard procedures81. Experiments were started with batches of 36 fertilized eggs. Typically 20–25 embryos were selected for transfection experiments, where half were controls and the other half were transfected with a mixture of myosin-class-specific siRNAs. All experiments were repeated at least three times with different batches of eggs. All fixed and stained embryos were investigated by epifluorescence microscopy and samples of 2–3 embryos for control and treatment were chosen for more detailed analysis and recording of results by confocal microscopy (Leica SP2) using standardized settings at ×100 magnification.

We used the following Dharmaco siRNA pairs for silencing of specific myosin genes. The oligonucleotides were designed and checked for optimal specificity using BLAST searches against the chick genome database. MYH9: 5′-GAGAAAAGACGGAGAAAGAUUUU-3′ and 5′-GGGAGAAGGUGGAAGAAGAUUU-3′; MYH5: 5′-GAGAAAACCGGAGAAGAUUUU-3′ and 5′-GGGAGAAGGUGGAAGAAGAUUU-3′; MYO1A: 5′-UCAGAGAAGGGCAGGAGGAUUU-3′ and 5′-AGCAGUAGGCCGGGAGGAUUU-3′; MYO1B: 5′-GAAAGGCUUGGUUUGAGGAUUU-3′ and 5′-GGGAAUUGGGCACAUAGAUU-3′; MYO5A: 5′-AGAAGAAGCGGAGGAGGAUUU-3′ and 5′-GGGAAUUGGGCACAUAGAUU-3′; MYO5B: 5′-GAAAGAAGCGGAGGAGGAUUU-3′ and 5′-GGGAAUUGGGCACAUAGAUU-3′.

Microscopy setup. To image chick embryo development on the scale of the whole organism and with cellular resolution we have designed and constructed a designated light-sheet fluorescent microscope. Light-sheet microscopy offers high-speed high-resolution three dimensional time lapse imaging with very low levels of background.
we have placed imaging and illumination arms above the sample at 45° to the worktop surface. The system is equipped with a high-speed scientific CMOS camera (PCO.Edge), a GSI 2-axis galvo scanner and a Physik Instrumente 3-axis motorized stage combination, which allows for imaging at rates exceeding 50 frames per second. Illumination and imaging are performed using water-dipping ×10 Nikon objectives with a working distance of 3.5 mm and 0.3 numerical aperture. During the experiment the sample is kept in a heated chamber at 37°C. Imaging is performed by moving the sample through the light sheet along the x axis and collecting subsequent cross-sections, which after acquisition are transformed into ‘confocal’ stacks (Fig. 1a). At present, imaging of a 4-mm-diameter embryo at 1.88 μm steps takes 2 min. In addition, to accommodate for sample shape and its changes over time we have implemented an active surface contour detection algorithm, which corrects the sample position along the z axis for each scan to keep the top of the sample in the focal point of the light sheet, necessary for assuring high and consistent data quality. Typically, 3,000 images of 2,560 × 400 pixels are collected per time point resulting in 1.5 TB of data per 15 h experiment.

Data analysis. We have divided the data processing pipeline into a series of distinct steps, each of which not only generates input for the following ones but also provides information about embryo behaviour at the given level of analysis.

In the first stage of data processing the embryo surface shape is determined and a flat z stack is constructed from sections conformal to the surface. The acquired three-dimensional stack is transformed into rectangular coordinates. Next, each z plane is divided into square interrogation areas (64 × 64 pixels) for which an FFT power-spectrum is evaluated. Along the z axis the square that shows the highest number of frequencies above a certain threshold in the FFT spectrum is selected as the surface tile. This process is repeated for all squares in the stack. A new, flat three-dimensional stack is constructed by shifting tiles along the z axis so that the surface tiles are occupying the sample z plane. This method allows not only correcting for the large-scale height differences between different parts of the sample but also removes small surface undulations. The apical plane is selected for further steps of analysis.

Tissue velocity is calculated by image cross-correlation using standard particle image velocimetry (PIV) algorithm (PIVLab v1.32 for MATLAB®). Results of this calculation are used to study local tissue deformation and tissue fate mapping, and as an input for the automatic cell tracking algorithm. We found that setting the interrogation window to 64 × 64 pixels for the first pass and 32 × 32 pixels for the second pass with 50% overlap at each pass gave the best results.

To understand the way tissue deforms we have derived a new velocity field averaging scheme that focuses on tissue level dynamics of the developing chick embryo and helps to reduce noise by removing small fluctuations of vector fields. The embryo is divided into a square grid with a lattice constant of 125 pixels and 10 points per square side. Typically, the grid contains 60 × 25 squares. Each lattice point is displaced in time according to the local velocity field. For points located in between the PIV-boxes (a three-dimensional grid whose velocity vectors are linearly interpolated from the nearest points using the MATLAB built-in function interp2). Initial squares deform into polygons and their area is calculated at each time point using the MATLAB built-in function polyarea. The areas are colour coded on a dynamic grid as total change in area of the squares (Fig. 2b and Supplementary Fig. 1) to identify and select active tissue regions for further analysis at the cellular level.

Averaging of velocity fields from different experiments. To average the expansion contraction behaviour from several embryos we needed to align their developmental axis. This was achieved using points from the anterior and posterior end of the primitive streak, for each time point in each experiment. We also estimated the time of onset of motion from each experiment. The annotated points were used to translate the centre point of the primitive streak into the origin of a generalized coordinate system for each embryo and for each time point. The spatial alignment, rotation and temporal alignment were used to align their corresponding velocity fields, which were then averaged. The averaged velocity field over the nine experiments was then used to evaluate the grid deformation over the whole time sequence.

Calculation and decomposition of tissue strain rate. The strain rate describes how much the material (tissue) contracts or expands on the basis of the differences in velocity between neighbouring points/areas. To compare the contraction rate along the left–right axis (\(\partial v_x / \partial x\)) and the expansion rate (\(\partial v_y / \partial y\)) along the posterior–anterior axis we have calculated strain rates for those two directions as shown in Fig. 3b. To reliably estimate values we have taken 25 cuts for each direction at the resolution of PIV calculations and fitted a linear slope to linear parts of the curves (blue points, Fig. 3c). By analysing tissue flows generated by contraction/ingression, cell growth and intercalation (Fig. 3d), it is evident that intercalation generates anisotropic strain rates, whereas growth and contraction lead to isotropic effects (expansion and contraction in both axes respectively). This allows us to decompose the tissue strain rate into the isotropic part (growth + apical contraction/ingression) and the anisotropic part (intercalation; Fig. 3e,f):

\[
\begin{align*}
\begin{pmatrix}
\frac{\partial v_x}{\partial x} \\
\frac{\partial v_y}{\partial y}
\end{pmatrix}
&= \text{anisotropic + isotropic} \\
\begin{pmatrix}
\frac{\partial v_x}{\partial x} \\
\frac{\partial v_y}{\partial y}
\end{pmatrix}
&= \text{anisotropic – isotropic}
\end{align*}
\]

Alternatively we have calculated the strain rate for the whole image. The velocity tensor \(L\) (ref. 67)

\[
L = 
\begin{pmatrix}
\frac{\partial v_x}{\partial x} & \frac{\partial v_y}{\partial y} \\
\frac{\partial v_x}{\partial y} & \frac{\partial v_y}{\partial x}
\end{pmatrix}
\]

can be decomposed into a symmetric part, which defines the strain rate tensor \(\xi\) as follows \(\xi = (L+L^T)/2\). This can be written as

\[
\xi = \frac{1}{2} \left( \begin{array}{cc} \frac{\partial v_x}{\partial x} + \frac{\partial v_y}{\partial y} & 0 \\ 0 & \frac{\partial v_x}{\partial y} + \frac{\partial v_y}{\partial x} \end{array} \right) + \frac{1}{2} \left( \begin{array}{cc} \frac{\partial v_x}{\partial x} & \frac{\partial v_y}{\partial y} \\ \frac{\partial v_x}{\partial y} & \frac{\partial v_y}{\partial x} \end{array} \right)
\]

We have plotted the isotropic contraction/expansion term as a circle, red for expanding and blue for contraction and the anisotropic shear strain rate tensor as a blue bar in the direction of contraction (the direction of the negative eigenvalue). Velocity field gradients of the matrix \(L\) were estimated using central differences over intervals of 32 pixels. For visualization the computed strain rate fields were averaged over 10 time points (≈30 min) and over 13 spatial points (≈200 pixels) along both spatial dimensions.

To smooth the images and equalize their intensities before segmentation the images are filtered with an FFT-based band-pass filter using standard ImageJ and MATLAB routines. A Watershed segmentation algorithm is used to find outlines of cells in membrane GFP images. Local intensity minima are used as seed points for the algorithm. As a result of the segmentation each cell in an image is presented as a collection of labelled segments.

During automatic tracking cells are tracked forward in time from the first time point onward. An initial segmentation is obtained using watershed segmentation. To track cells between time points, the locations of cells in future time points are estimated from their previous locations using the tissue velocity fields calculated by PIV between these time points fields. These locations are used to constrain the seedpoints of the watershed algorithm. Ingression and division events are detected using the characteristic changes in cell size and shape of the segmented cells. The automatic tracking method resulted in the tracking of 70% of the >100,000 cells correctly for between >100 successive time points. For the cell-based statistical analysis described below it is only required to track cells confidently over ~20 successive time points. The segmentation routines are written in MATLAB.

To improve the automatic tracking a manual curation option for smaller selected regions was introduced. The manual tracking scheme is similar to that used in the automatic tracking using algorithms coded in MATLAB. The initial image is segmented and checked by visual inspection. The locations of local minima in each image are adjusted manually if needed to achieve correct segmentation results. In addition, ingestions, divisions and emerging cells are marked manually if necessary.

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To measure orientation of cells from segmented images, an ellipse is fitted into each segment using the normalized second-order central moments of pixels of the segment\(^6\). Orientation of a cell is taken to be the orientation of the major axis of the fitted ellipse. In this study we consider a cell to be polarized if the eccentricity of the fitted ellipse exceeds 0.87.

To calculate the isotropic and anisotropic tissue deformations from the segmented data we have used recently developed methods that are based on the spatio-temporal changes in the dynamics of the links connecting the centroids of neighbouring cells\(^7\). The tissue texture tensor \( M \) provides information on the size, shape and alignment of the cells at a given time point in a particular region\(^8\).

\[
M = \begin{pmatrix} X^2 & XY \\ XY & Y^2 \end{pmatrix}
\]

where \( (X, Y) = (x_i - x_j, y_j - y_i) = r_i - r_j = l \). The links \( l \) are defined as vectors connecting the centroids of neighbouring cells. The length and orientation of the links are captured in the so-called link matrices that are averaged over the domain of interest. We calculate these averages based on the links between all neighbouring cells in circular domains of 65 \( \mu \)m radius that cover the embryo.

The dynamic changes in link length and orientation are used to calculate the statistical symmetrized velocity gradient \( V \), the discrete analogue of the strain rate tensor, as follows\(^9\):

\[
V = \frac{N_c}{N_{active}} \left( M^{-1}(t) \left( I \otimes \frac{\Delta l}{\Delta t} \right) + \left( \frac{\Delta l}{\Delta t} \otimes I \right) M^{-1}(t) \right)
\]

where \( \Delta l = l(t + \Delta t/2) - l(t - \Delta t/2) \).

The variation of statistical internal strain rate tensor \( dU/dt \) quantifies how cell size and shape change contribute to the total tissue deformation. The tensor is defined as follows\(^10\):

\[
\frac{dU}{dt} = \frac{1}{2\Delta t} \left[ \log M(t + \Delta t/2) - \log M(t - \Delta t/2) \right]
\]

Here links are chosen from end points of the same time interval (\( \Delta t \)) as for evaluation of the tensor \( V \).

The statistical topological rearrangement rate tensor \( P \) measures the contribution of cell intercalation to the total tissue deformation. The tensor is defined as follows\(^11\):

\[
P = \frac{1}{2\Delta t N_{active}} (N_c(m_i) - N_c(m_i)) M^{-1}(t)
\]

where \( m_i \) are links that exist in the end of the time interval \( \Delta t \) but not in the beginning and \( m_o \) are links that exist in the beginning of the time interval but not in the end. \( N_c \) is the number of appearing links and \( N_d \) is the number of disappearing links. In the figures in the results we show the traceless parts of the \( dU/dt \) and \( P \) tensor in the direction of negative eigenvalues. These signify the rate of the change in cell shape and the rate for cell rearrangement respectively. For visualization, the computed tensor fields were shown as a moving average over 10 time points (~30 min).

Code availability. The code used for all data processing and analyses described above has been written in MATLAB. The scripts will be made available on request.

Reproducibility of experiments. For figure 1d–h the images presented are representative for over 150 successfully recorded embryos acquired over a 3-year period. For figure 2a the results shown were replicated in over 20 embryos; another example is shown in Supplementary Fig. 1. For figure 2c the results are representative for analyses in over 20 embryos. For figure 2d the results are representative for observations in 4 embryos. For figure 3a the detailed analysis was performed in 3 embryos. For figure 4a–e the analysis was performed in 2 embryos with comparable results. For figure 4g the analysis was performed in 3 embryos. For figure 6d,e the analysis was performed in 2 embryos out of 11 embryos recorded. For Supplementary Fig. 3 the analysis was performed in 4 embryos with similar results. For Supplementary Fig. 4 the analysis was performed in 4 embryos with similar results. For Supplementary Fig. 5 the analysis was performed in 3 embryos with similar results. For other results details of the statistical analyses and reproducibility are provided in the figure legends.

Data access. The RNA-seq data have been submitted to the European Nucleotide Archive (ENA) and can be accessed at http://www.ebi.ac.uk/ena/data/view/PRJEB8414.

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Supplementary Figure 1  Streak formation, expansion contraction dynamics and cell behaviour of a different sample embryo. **a-)** Images showing the formation of the streak on top images of the embryo as a function of time. **b-)** Time sequence of the expansion/contraction map. **c-)** Time sequence of the isotropic and anisotropic strain rates. Symbols and meaning are the same as for the embryo as shown in fig 3a. White scale bar indicates size in panels a, b and c is 200 µm. In panel c the white scale bar also indicates a strain rate of 10^{-4}/sec, as well as a domain displacement speed of 4 µm/min.
Supplementary Figure 2 Alignment of contraction expansion maps of 9 embryos. This result of averaging of the contraction expansion maps of nine embryos. The embryos were aligned and synchronised according to developmental time as described in detail in methods. The main features such as the initiation of contraction in the central mesendoderm followed by outward expansion of this contraction domain followed by streak formation and deformation of the area in front of the streak appear highly conserved. The black scale bar in the first image is 200 µm.
**Supplementary Figure 3** Changes in cell volume and cross section area during early development. 

a) Changes in cellular cross-sectional area over time. The average cell diameters were calculated from the segmented images in small domains and colour coded as indicated in the legend. Initially the average cross-sectional area of cells is smallest in the area pellucida. Over time the average cross-sectional area is decreasing in all the regions in the embryo. Size of each coloured square is initially 6600 µm². The white scale bar is 200 µm.

b) Relative average cell volume of rounded cells prior to cytokinesis (red line) and relative average apical cell diameter (green line) in the posterior domain (B) in the sickle region shown in a. It is seen that cell volume stays essentially constant, but that cross-sectional area decreases considerably.

c) Same volume and cross-sectional area measurements for the anterior domain (C) shown in panel a.
Supplementary Figure 4 Frequency of cell divisions and alignment of cell division axes. a) Number of cell divisions in the four domains shown in figure 4a as a function of time, showing that cell doubling times in all domains are similar (~6 h). b) Number of cell divisions as a function of time of the embryo shown in C. c) Histograms showing the distributions of the cell division axes occurring in domains in different parts of the embryo. Initially the cell divisions axes in the posterior parts of the embryo are aligned perpendicular to the midline of the embryo (0–4.3 h). From 4.4 hr of development onwards this polarity is gradually lost. All histograms contain the total number of divisions occurring over a 2.1 h period in the domain. Scale bar is 200 μm.
Supplementary Figure 5 Alignment of polarised cells in the embryo. 

a) Alignment of cells with a shape eccentricity of >0.87 in various domains before and during streak formation. The percentages show how many cells in each domain have eccentricity >0.87. It is evident that all ready at the beginning of development before the onset to motion (1 h) cells in the posterior Area Opaca are aligned perpendicular to the direction of the forming streak and that the number of polarised cells increase during the onset of motion (4.2 h) after which their fraction and orientation decreases again (7.3 h). Scale bar in each panel is 200 µm.

b) Tissue texture tensor (red bars). The tissue texture tensor is calculated from centres of mass of all cells in a given domain as described in detail in methods. The length and spatial orientation of the tensors shows that cells in the posterior embryo are initially aligned along their long axis. With the onset of streak formation the cells become smaller and the alignment becomes less pronounced supporting the data shown in fig S3a, S5a. White scale bar is 200 µm. The yellow scale bar corresponds to the tensor magnitude of 200 µm² and a domain speed 4 µm/min.
**Supplementary Figure 6** Effects of inhibitors on development and Myosin light chain phosphorylation. **a-)** Left panels phosphorylated Myosin light chain (green) and actin staining (red) for a control embryo incubated for 6 h in-ovo followed by 2 h in EC culture, middle panels, an embryo incubated for 6 hours in-ovo followed by 2 h treatment with 50µM of the Rho kinase inhibitor H1152 in EC culture, resulting in a partial inhibition of Myosin regulatory light chain phosphorylation, right panels an embryo incubated for 6 hours in-ovo followed by 2 h treatment with 50µM of the Blebbistatin in EC culture showing only very limited inhibition of Myosin light chain phosphorylation. **b-)** upper panel, embryo developed for 16 h in the presence of 50µM Blebbistatin showing a streak indistinguishable in shape from control embryos. Lower panel an embryo developed in the presence of 50µM, H1152 showing a characteristic short wide streak. **c-)** Myosin Ib (green) and actin (red) staining in the posterior epiblast in an embryo incubated for 6 h in ovo, followed by either 2 h in EC culture, or 2 h in EC culture containing 5uM PCP. **d-)** Myosin Va (green) and actin (red) staining in the posterior epiblast in an embryo incubated for 6 h in ovo, followed by either 2 h in EC culture, or 2 h in EC culture containing 5uM PCP. The results show are representative for several experiments Blebbistatin (control n=11 embryos/10 streaks/3 experiments, Blebbistatin 10 streaks/12 embryos/ 3 experiments), HH1152 experiments (control n=35 streaks/38 embryos/ 5 experiments, HH1152 n=28 streaks/33 embryos/ 5 experiments). White Scale bars in a,c,d, 25 µm. White scale bar in b 1 mm.
Supplementary Figure 7 Phospho Myosin light chain localisation in selective junctions is not reflected in Par3, PDZ RhoGef (AHRGef11) or phospho-Ezrin localisation. c-) Phospho-Myosin regulatory light chain shows only partial co-localisation with Myosin IIb, (similar results for IIa are not shown), suggesting that only a fraction of Myosin IIb is assembled into mini-filaments in apical junctions. b-) Phospho-Myosin light chain is only found in selective junctions, which are organised in a super cellular pattern in mesendoderm cells. c-) Par3, PDZRhoGef (AHRgef11) and phospho-Ezrin do not show an asymmetric cellular localisation in mesendoderm cells. The figures are representative of at least 2 experiments per antibody typically between 10 embryos /experiment. White scale bar 25 µm.
Supplementary Video legends

**Supplementary Video 1:** Development from EGXXII to HH3.
The image shown is a Z plane constructed from 2500 slices, 2560x400 voxels taken at 1.88um intervals, every 2.5 minutes. Small inserts show sequences at full resolution from the regions marked in colours in the main image. The data represent 286 time points of one Z plane through the middle of the embryo shown in Fig 1C, taken every 2.5 minutes. The data of this experiment as well as some of the results of the calculations can be seen at full resolution at DOI http://dx.doi.org/10.15132/10000100, they can be viewed at full resolution without downloading the primary data the Omero webbrowser. In the viewing option of the web browser the zoom can be selected as well as the data quality. By selecting the “Show ROIs” option in the bottom of the Viewing option panel the outline of the anterior middle and posterior streak can be seen as overlays over the data.

**Supplementary Video 2:** Cross section of images taken from the embryo shown in Video 1 through the developing primitive streak. The upper panel shows the full extent of the embryo, the lower panel shows a section around the streak tip at full resolution.

**Supplementary Video 3:** Example of cell ingression.
Cell ingression as described in text and shown in Fig. 1G.

**Supplementary Video 4:** Example of cell division.
Example of a cell division. Note the fact that after division the daughter cells are separated by some of the neighbour cells resulting effectively in an intercalation event, which is observed frequently.

**Supplementary Video 5 Fate mapping of streak forming area.**
Red dots indicate the outline of the streak, blue dots indicate the area pellucida of the experiment shown in Video S1.

**Supplementary Video 6:** Deformation visualised on a dynamic grid.
Colours indicate contraction (shades of blue) and expansion (shades of red).

**Supplementary Video 7:** Another example of streak formation, PIV based tissue dynamics and strain rate analysis
First and second panels illustrate primitive streak formation using the same notation as in figures 2A and 2B, respectively. The last panel illustrates strain rate tensor as in figure 3A. Each panel shows 10 hours on development.

**Supplementary Video 8:** Averaged contraction expansion maps for 9 embryos.
Video shows full data set of the embryos shown in figure S2.

**Supplementary Video 9:** Tissue strain rate tensor changes during streak formation.
Video illustrates the full temporal data set of the isotropic and anisotropic strain rate changes shown in figure 3A and calculated as described in methods.

**Supplementary Video 10:** Sequential contraction of apical junctions.
Sequential contraction of junctions coloured red bring two cells (initially 3 cells apart) indicated by red and blue dots together. The cells are taken from within the sickle area.

**Supplementary Video 11:** Another example of contracting apical junctions.
Conditions as described in legend to Video S10.

**Supplementary Video 12:** Cell based analysis of tissue deformation.
Left hand panel shows the isotropic (circles) and anisotropic strain rate tensor (blue bars). Right hand panel shows the combined cell deformation (green bars) and cell rearrangement tensor (blue bars) calculated as described in detail in methods. Video illustrates the full time course of data illustrated in figure 4G.

**Supplementary Video 13:** Development after addition of 5 µM pentachloropseudilin.
Embryos were allowed to develop for 2 h after which time 5 µM pentachloropseudilin was added. As can be seen this results in an initial relaxation followed by a loss of adhesion of the outer cells to the vitelline membrane and a contraction of the embryo.

**Supplementary Video 14:** Dynamics changes in the Expansion/Contraction map for Myosin I inhibitor treatment experiment.

**Supplementary Video 15:** Immediate inhibition of junctional contraction after addition of 5 µM pentachloropseudilin.

**Supplementary Video 16:** Streak formation is inhibited following Myh9/Myh10 (Myosin Ila/Ilb) siRNA transfection. Initially the embryo starts to develop normal vortex flows however from ~ 0 h after transfection the embryo starts to show severe aberrations in its development. The tissue flows stop and the surface of the embryo starts to buckle and show irregular contractions.