Different temporal requirements for tartan and wingless in the formation of contractile interfaces at compartmental boundaries

Thomas E. Sharrock, Jenny Evans, Guy B. Blanchard, Bénédicte Sanson*

Department of Physiology, Development and Neuroscience, Anatomy Building, Cambridge, CB2 3DY, University of Cambridge, UK

*Corresponding author: bs251@cam.ac.uk

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Abstract

Compartmental boundaries physically separate developing tissues into distinct regions, which is fundamental for the organization of the body plan in both insects and vertebrates. In many examples, this physical segregation is caused by a regulated increase in contractility of the actomyosin cortex at boundary cell-cell interfaces, a property important in developmental morphogenesis beyond compartmental boundary formation. We performed an unbiased screening approach to identify cell surface receptors required for actomyosin enrichment and polarisation at parasegmental boundaries (PSBs) in early Drosophila embryos, from the start of germband extension at gastrulation and throughout the germband extended stages (stages 6 to 11). First, we find that Tartan is required during germband extension for actomyosin enrichment at PSBs, confirming an earlier report. Next, by following in real time the dynamics of loss of boundary straightness in tartan mutant embryos compared to wildtype and ftz mutant embryos, we show that Tartan is required during
germband extension but not beyond. We identify candidate genes that could take over from Tartan at PSBs and confirm that at germband extended stages, actomyosin enrichment at PSBs requires Wingless signalling.

Introduction

The mechanisms underlying the partitioning of group of cells into immiscible compartments have fascinated scientists since the discovery of compartmental boundaries in Drosophila in the 1970s (Fagotto, 2020a). In many cases studied, this physical barrier is caused by a localised upregulation of actomyosin contractility at boundary cell-cell interfaces, found in both Drosophila and vertebrate models (Aliee et al., 2012; Calzolari et al., 2014; Canty et al., 2017; Landsberg et al., 2009; Monier et al., 2010). How this increase in cortex contractility is specified at boundary interfaces to create mechanical barriers remains only partially understood. Within homogeneous fields of epithelial cells, spatial regulation of transcription factors is key to initiate boundary formation (Dahmann et al., 2011; Monier et al., 2011). Downstream of these transcription factors, various cell surface receptors have been implicated in causing actomyosin enrichment at boundary interfaces. In vertebrates, for example at rhombomere boundaries in the hindbrain, the Ephrin/Eph receptors play a key role, but additional cell surface asymmetries have also been identified (Fagotto, 2020b; Pujades, 2020). In Drosophila, downstream receptors remained elusive for a long time, but recent work is starting to identify specific cell surface asymmetries required for the formation of mechanical boundaries (Sharrock and Sanson, 2020; Wang and Dahmann, 2020).

Beyond its role in compartmental cell sorting, increase in cortical contractility at epithelial cell-cell junctions underlies many cell and tissue behaviours (Amack and Manning, 2012; Bielmeier et al., 2016; Bosveld et al., 2016; Collinet and Lecuit, 2021). In particular, convergent extension, where cells intercalate to elongate a tissue, was shown in Drosophila to require planar polarized enrichment of the actomyosin cortex (Bertet et al., 2004; Collinet and Lecuit, 2021; Pare and Zallen, 2020; Zallen and Wieschaus, 2004). This planar
polarization is downstream of antero-posterior patterning, which generates the subdivision of the body axis by overlapping stripes of transcription factors encoded by the pair-rule genes (Bertet et al., 2004; Irvine and Wieschaus, 1994; Zallen and Wieschaus, 2004). In vertebrates, actomyosin planar polarization has now been linked to convergent extension in several examples, and in contrast to Drosophila axis extension, is generally thought to be regulated by the Planar Cell Polarity (PCP) pathway (Collinet and Lecuit, 2021; Nishimura et al., 2012; Pare and Zallen, 2020; Rozbicki et al., 2015; Shindo and Wallingford, 2014). It is not known, however, what upstream cues activate the PCP pathway to drive convergent extension in vertebrates and whether cell surface receptor asymmetries similar to those in Drosophila axis extension could contribute.

In this paper, we have searched for cell surface receptors driving actomyosin enrichment at compartmental boundaries, using an unbiased screening approach. We focused on parasegmental boundaries that subdivide the germband in early Drosophila embryos. Parasegmental boundaries (PSBs) form during convergent extension of the germband during gastrulation, a process called germband extension (GBE). We had shown previously that actomyosin enrichments form at PSBs in the course of GBE, progressively emerging from the tissue-wide actomyosin planar polarization that is initiated at gastrulation (Tetley et al., 2016). Tissue-wide planar polarization of actomyosin requires the LRR receptors Toll-2, 6 and 8, which are expressed in overlapping stripes downstream of the pair-rule transcriptional network (Pare et al., 2014). However, removal of all three receptors is not sufficient to abolish actomyosin enrichment at PSBs, suggesting that additional receptor(s) are required (Pare et al., 2019; Pare et al., 2014). In (Tetley et al., 2016), modeling cell-cell interactions during GBE, we proposed that an additional receptor expressed in a periodic, double-segment pattern, might be sufficient to confer polarization of actomyosin at PSBs. We undertook a systematic screen based on this hypothesis, which we present here. A second question concerned the role of surface receptors at different developmental stages. Once axis extension is completed, actomyosin enrichments are maintained at PSBs during the extended germband stages (Monier et al., 2010). Whereas actomyosin enrichments during
convergent extension (GBE, stages 7-8) require the pair-rule gene network, their maintenance at PSBs after completion of germband extension (stages 9-10-11) requires Wingless signalling (Monier et al., 2010; Scarpa et al., 2018; Urbano et al., 2018). No cell surface receptors had yet been identified downstream of Wingless signalling, so we also addressed this as part of our screening approach.

From our screen we find that Tartan, another LRR receptor, is required for actomyosin enrichment at PSBs throughout axis extension. This provides an independent confirmation of earlier findings by (Pare et al., 2019). By visualising transcription of parasegmental markers in combination with cell tracking in live embryos, we are able to follow how boundary straightness (a functional consequence of actomyosin enrichment at boundaries) evolves in the course of axis extension in wild-type and tartan mutant embryos and also in the pair-rule mutant ftz (we examined ftz because it is a known regulator of tartan’s expression (Chang et al., 1993)). This analysis showed that tartan is required for specifying contractile interfaces at PSB during early GBE, but not beyond. Our unbiased screen identifies candidate genes that could take over from Tartan to specify planar polarized mechanical interfaces at PSBs.

Results

A screen to find cell surface receptors expressed asymmetrically at parasegmental boundaries

In previous work, we predicted that the expression of a single surface molecule within either even or odd-numbered parasegments would constitute the minimal requirement for generating the missing molecular asymmetries at the parasegmental boundary during axis extension (Tetley et al., 2016). Based on this prediction, we performed an in silico screen to find genes meeting the following three criteria: i) they should be expressed in stripes along the anteroposterior (AP) axis ii) they should encode a protein that localizes to the cell surface and iii) they should be regulated by the pair-rule gene network. Mining publicly available data
for the 13,600 genes in the *Drosophila* genome, we identified 822 genes expressed in AP stripes, 5620 genes encoding proteins with a signal peptide and/or a predicted transmembrane domain and 3679 genes likely to be pair-rule regulated (Fig. 1A and Methods). Following standardization of the gene nomenclature using the unique Flybase Identifiers, we found 94 genes in common with these three datasets. Next, we applied manual quality controls to this initial list, whittling the number of candidates down to 31 genes (Fig. 1B and Methods). Excluded genes were those i) not showing an obvious striped pattern by eye in *in situ* libraries, ii) being likely to be expressed at very low levels in early embryos or iii) having a known localization in the literature different from the cell surface (e.g. transcription factors) (See Methods for details).

Next, we used *in situ* Hybridisation Chain Reaction (HCR) (Choi et al., 2018) to characterize the expression patterns of the 31 candidate genes during early embryogenesis relative to the parasegmental boundaries. HCR has several advantages over traditional *in situ* hybridization: i) because there is no enzymatic amplification, the signal is tightly localized within expressing cells, which helped identify the boundaries of expression with precision, ii) it enables the expression of several genes to be examined simultaneously and iii) it can be combined easily with antibody staining, which we used here to label the cell membranes to facilitate gene expression mapping relative to boundaries (see Fig. 2B,E). We focused on the period of embryogenesis from stage 5 (late cellularisation) to stage 10 (extended germband) (Fig. 1C). From 31 genes, we confirmed that 19 genes were expressed in AP stripes (Fig. S1), whereas 12 were not (Fig. S2) and were excluded from the candidate list. The 19 genes recovered included the three genes encoding the Toll-like receptors, Toll-2, Toll-6 and Toll-8, already identified by (Pare et al., 2014), thus validating our screening approach. The strength and type of striped patterns varied between the genes. Some were expressed in seven clear stripes at stages 5 to 7, indicating that they are likely under pair-rule gene network regulation: *ama, best1, comm, comm2, impl2, sca, toll-2, toll-6, toll-8* and *tartan* (Fig. 1C and Fig. S1). Some genes also show clear expression in every parasegment at later stages 9-10, when the germband is extended, either doubling their periodicity from an initial expression in seven
stripes (comm, comm2, impl2, toll-2) or initiating expression in every parasegment (dnt, drl, sli) (Fig. S1). For the genes with the clearest striped expression, it was possible to check the position of the stripes relative to the parasegmental boundary markers ftz and slp1. We found that best1, blot, comm, comm2, dnt, impl2, toll-6, toll-8 and tartan mRNA expression borders the parasegment boundaries at some point between stages 5 and 10 (Fig. S3). We decided to focus further investigations on comm, comm2 and tartan, as these genes were most clearly bordering the parasegmental boundaries and were also strongly expressed (Fig. 1C).

**A requirement for Tartan in actomyosin enrichment and boundary straightness at PSBs during germband extension**

Examining tartan expression patterns relative to parasegmental boundaries (PSBs) reveals that, from all the 19 candidates, tartan is the gene matching our initial prediction the closest (Tetley et al., 2016). Like the Toll-like genes, tartan encodes a LRR receptor that localizes at the plasma membrane (Chang et al., 1993). The protein localization pattern visualised by antibody labelling matches the mRNA expression patterns, validating the use of HCR to map boundaries (Fig. S4A). From stage 5 to stage 7, tartan is expressed throughout the even-numbered parasegments and borders even-numbered PSBs at the anterior and odd-numbered PSBs at the posterior of its domains (Fig. 2A,D and Fig. S3). Note that we summarise gene expression patterns in diagrams where representative parasegments are each divided into 4 domains (for example, see Fig. S3); while these 4 domains approximately correspond to the number of cells along AP at the beginning of GBE (3.7 cells in average, see Tetley et al 2016 (Tetley et al., 2016)), the cell number steadily increases with cell intercalation and then cell division (see Methods). tartan fulfils our prediction of a single receptor expressed in either even or odd parasegments and bordering every PSB. tartan’s expression however is not completely uniform across the even-numbered parasegments, being weaker towards the posterior of each stripe at stage 5 to 7. Towards the end of germband extension (stage 8), tartan’s posterior border of expression retracts away from the odd-numbered PSBs (Fig. S 3). This is similar to the dynamics of expression of the pair-rule
gene ftz, which is known to activate tartan expression (Chang et al., 1993). Note that from stage 8 onwards, tartan’s uniform expression starts breaking down along the DV axis and the PSBs become bordered only intermittently (Fig. S1).

Next, we quantified both Myosin II enrichment and boundary straightness in a tartan null mutant (trn^{28.4}), as a time course from stage 7 to 10. To measure Myosin II intensities, we used native fluorescence from Sqh-GFP^KI, a knock-in reporter for Myosin II Regulatory Light Chain (Proag et al., 2019). Quantifications were performed in fixed embryos by comparing the cell-cell interfaces at PSBs to control interfaces one cell diameter posteriorly (called +1 interfaces) (Example in Fig. 2B-C’ and Methods). The analysis shows a clear requirement for tartan for Myosin II enrichment at PSBs during germband extension, this requirement being strong at GBE onset and diminishing, with no contribution during extended germband stages (Fig. 2F). PSB straightness quantifications followed a similar trend (Fig. 2E). This matches remarkably the dynamics of expression of tartan mapped by HCR, suggesting that the specification of contractile cell-cell interfaces results from an immediate read-out of Tartan receptor asymmetries (Fig. S1 and 3). We conclude that our unbiased screening approach identifies a clear requirement for the LRR receptor Tartan to form actomyosin-enriched interfaces at PSBs, confirming a previous report (Pare et al., 2019). Our quantifications in fixed embryos further suggest that Tartan is required early during germband extension and that other cell surface receptors may be required later in development.

**Evaluating a possible requirement for Commissureless in boundary formation**

Next, we considered the candidate genes comm and comm2, as they are expressed in clear stripes bordering PSBs during GBE (Fig. 1C and 2A). comm and comm2 are duplicated genes located next to each other in the genome and exhibit identical expression patterns, which are markedly different from those of tartan (Fig. S1 and 3). At stage 5 and 6, their expression in seven stripes is not bordering any PSB and instead straddles the even-numbered PSBs. From stage 7 onwards, comm and comm2 expression doubles in periodicity and the expression becomes localized to the second half of every parasegment,
matching \textit{slp1} expression to border the anterior side of each PSB (Fig. 2A,G). \textit{comm} encodes a short transmembrane protein that does not localize to the cell surface but regulates the cell surface localization of the receptor Robo and possibly other receptors (Ing et al., 2007; Keleman et al., 2002). Comm protein localizes in puncta consistent with its known ER/Golgi localization and forms a striped pattern that matches the RNA expression (Fig. S4B). Comm2 has not been characterized but its amino-acid sequence presents homology with Comm in key domains (Justice et al., 2017). We quantified both Myosin II enrichment and boundary straightness in a \textit{comm} null mutant (\textit{comm}\textsuperscript{\textalpha e39}) (Fig. 2H,I). We find a small but significant difference in Myosin II enrichment and boundary straightness mid-germband extension (stage 8). This suggests that Comm, and perhaps its homologue Comm2, may have a role in boundary formation after Tartan.

\textbf{Evaluating a possible requirement for Toll-2 at PSBs during extended germband stages}

In addition to looking at germband extension, we also wanted to assess PSB function at extended germband stages (stages 9-11). At these stages, actomyosin enrichments initiated during GBE at PSBs are maintained by Wingless signalling (Monier et al., 2010). In \textit{wingless} mutants, both actomyosin enrichment and boundary straightness are lost at PSBs, as is the elevated tension along the cell-cell interfaces, shown by laser ablation experiments (Monier et al., 2010; Scarpa et al., 2018; Urbano et al., 2018). We reasoned that any cell surface receptor contributing to maintaining actomyosin enrichments at PSBs must be under Wingless signalling regulation. We therefore performed HCR in a \textit{wingless} null mutant (\textit{wg}\textsuperscript{\textalpha X4}), for the candidate genes expressed in stripes at extended germband stages (\textit{best1, comm, comm2, dnt, drl, sli}) as well as for \textit{tartan} and the \textit{toll-like} receptors (Fig. S5). Of these 11 genes, only \textit{toll-2} lost expression in \textit{wg}\textsuperscript{\textalpha X4} embryos compared to wildtype (Fig. 3A,B and Fig. S5). To confirm regulation by Wingless signalling, we examined \textit{toll-2} expression (alongside the other \textit{toll-like} receptors and \textit{tartan} as controls) in embryos ubiquitously
expressing Wingless (armGal4/UASwg). Again, toll-2 was the only gene responding robustly to Wingless signalling, its expression broadening towards the posterior to reach the anteriorly broadened expression of the slp1 domain (Fig. 3C and Fig. S5). The broadening of toll-2 expression is similar to the broadening in engrailed expression (a known target of Wg) in armGal4/UASwg embryos (Larsen et al., 2008; Scarpa et al., 2018; Urbano et al., 2018), confirming that Wg signalling regulates toll-2 expression.

Regulation of toll-2 by Wingless signalling could contribute to the maintenance of actomyosin enrichment at PSBs. toll-2 expression, however, does not border the PSBs at extended germband stages, but rather straddles it, similarly to its pattern during GBE (Fig. 3A and Fig. S3). To address a possible role of Toll-2 at PSBs, we quantified Myosin II enrichment and boundary straightness in a toll-2 null mutant (toll-2$^{\Delta 7-35}$) (Fig. 3D,E). Consistent with (Lavalou et al., 2021; Pare et al., 2019; Pare et al., 2014), we do not detect a loss of actomyosin enrichment or boundary straightness at PSBs in toll-2 mutants during germband extension (Fig. 3D,E). Despite the regulation of toll-2 by Wingless signalling, we also cannot detect a contribution of toll-2 at extended germband stages (Fig. 3D,E). As a positive control, we quantified actomyosin enrichment and boundary straightness in wg null mutants and found as expected a very significant decrease at extended germband stages (and not during GBE) (Fig. 3F,G). This confirms that our quantifications of Sqh-GFP native fluorescence are comparable to our prior quantifications using a P-Sqh antibody ((Monier et al., 2010; Urbano et al., 2018). Together, these results suggest that regulation of toll-2 by Wingless signalling does not explain the maintenance of actomyosin enrichments at PSBs at germband extended stages.

**Tools to monitor boundary activity during germband extension in live embryos**

Our analysis in fixed embryos suggests that Tartan’s requirement is limited to early convergent extension, matching closely its window of expression at PSBs. This is intriguing because it suggests that the specification of contractile interfaces is a rapid and short-lived response to Tartan’s asymmetric expression at boundaries. In order to analyse more
precisely the dynamics of requirement for *tartan*, we developed tools to monitor boundary mechanical properties during axis extension. In previous work, we had quantified Myosin II polarity in live embryos using Sqh-GFP (a reporter for Myosin II Regulatory Light Chain), while tracking cells with the cell membrane marker Gap43-Cherry (Tetley et al., 2016). Here we develop boundary straightness measurements in real time as a functional assay for boundary mechanical properties and a proxy for actomyosin enrichment.

To follow the dynamics of boundary straightness in live embryos, we took advantage of the MS2-MCP system implemented in *Drosophila* embryos (Garcia et al., 2013), to label in real time the transcription of the parasegmental boundary marker *engrailed* (Fig. S6A). The reason for using a transcriptional read-out rather than a protein reporter is that we found that tagged proteins of segmental markers, constructed by others or ourselves, do not give a fluorescent signal strong enough for tracking parasegments in live embryos. One exception is *eve*-YFP, which we used previously, but has the limitation of labeling only alternate parasegmental boundaries (Tetley et al., 2016). We fused a 2099bp region upstream of the *engrailed* promoter, the VT15159 enhancer, to a MS2 reporter containing 24 MS2 stem loops and *lacZ*, generating the construct EnVT15159-MS2 (Fig. S6B and Methods). We checked by HCR that *lacZ* expression from this construct recapitulates the endogenous pattern of *engrailed* expression during axis extension (Fig. S6C,D). The only difference is brighter Ftz-positive stripes (marking even-numbered parasegments), which might be a consequence of the known delay in transcription initiation of *engrailed* in odd-numbered parasegments compared to even-numbered ones (DiNardo et al., 1988).

Next, we visualised transcription from EnVT15159-MS2 by co-expressing MCP-GFP, which binds to the 24 MS2 stem loops in nascent transcripts and give rise to fluorescent dots corresponding to *engrailed* transcription in the nuclei of live embryos (Fig. 4B and Fig. S6A). A kymograph of the dots reveals that as for *lacZ* expression from the same reporter (Sup. Fig 6C,D), reported *engrailed* transcription is brighter in alternate parasegments (Fig. 4B’). This is useful as it gives us a means to distinguish even-numbered from odd-numbered parasegments. To associate *engrailed* transcriptional dots with given cells, we used Gap43-
mCherry as previously to label cell membranes and track cell positions (Fig. 4C) (Tetley et al., 2016). We developed additional computational methods to track the transcriptional dots in order to identify *engrailed* positive cells (Fig. 4C’) and thereby the cell-cell interfaces of the parasegmental boundaries (Fig. S7 and Methods). As shown below, we found that the parasegmental boundaries identified by these methods are, as expected, significantly straighter than control (non-boundary) interfaces throughout axis extension, thus validating the use of a reporter of *engrailed* transcription to identify parasegmental boundaries in live embryos.

**Mapping Tartan’s temporal requirement for parasegmental boundary straightness during axis extension**

To compare parasegmental boundary straightness, we analysed 3 movies each of wild-type and *tartan* mutant embryos carrying the transgenes *EnVT15159-MS2, MCP-GFP* and Gap43-mCherry. Movies were acquired as before (Tetley et al., 2016)(see field of view in Fig. 4A), cells were segmented automatically based on the Gap43-mCherry signal, then segmentation was corrected manually (Methods). Manual correction was important to recover enough cell-cell interfaces for the boundary straightness analysis. *engrailed* transcriptional dots from *EnVT15159-MS2/MCP-GFP* were tracked to locate the *engrailed* stripes and to find the parasegmental boundaries at the anterior border of each stripe (Fig. S7). We then compared the angle of cell-cell interfaces relative to the antero-posterior (AP) axis for parasegmental boundary interfaces and for control interfaces located one cell diameter posteriorly or anteriorly (+1 and -1 interfaces, respectively) (Fig. 5A-C). In wild-type embryos, cell-cell interfaces are quite straight at the beginning of axis extension, with 55 to 65% of PSB and control interfaces having an angle greater than 60 degrees relative to the AP axis (Fig. 5D). This initial interface straightness is caused by the invaginating mesoderm pulling on the ventral border of the ectoderm around the time axis extension starts (Butler et al., 2009; Lye et al., 2015). Once the mesoderm has invaginated, the control interfaces lose their alignment as cells intercalate during axis extension. In contrast, parasegmental
boundaries remain aligned throughout axis extension, with 60% of boundary interfaces having an angle greater than 60 degrees relative to the AP axis (Fig. 5D,D'). These trends are remarkably similar to our previous measurements for even-numbered PSBs identified using Eve-YFP (see fig2 K, L in (Tetley et al., 2016), validating our new method to identify boundaries, and confirming that parasegmental boundaries behave as mechanical boundaries during *Drosophila* germband extension.

In *tartan* mutants, the control interfaces anterior and posterior to the PSBs (-1 and +1) show the same behaviour as in wild-type embryos: they are initially aligned at the start of germband extension by the mesoderm pull, then lose their alignment as cells start intercalating. Strikingly, parasegmental boundaries in *tartan* mutants do not straighten at all at the start of tissue extension, losing their alignment even more quickly than control interfaces (Fig. 5E,E'). This suggests a complete absence of actomyosin enrichment at PSBs in *tartan* mutants at the start of axis extension. Moreover, direct comparison between *tartan* and wildtype demonstrate that PSBs in *tartan* null mutants are less straight throughout axis extension (Fig. 5F,F'). The straightness evolution over time in *tartan* mutants is consistent with our time-course of actomyosin enrichment and boundary straightness in fixed embryos (Fig. 2C,D). At 30 minutes into germband extension, the straightness of *tartan* and control interfaces are indistinguishable (Fig. 5E); from this point on, the PSB straightness starts recovering, diverging from control interfaces (Fig. 5E) and increasing towards wildtype (Fig. 5E). This suggests that other receptor systems take over then to promote actomyosin enrichment at PSBs. It also suggests that boundary straightness is an immediate read-out of the molecular asymmetries present at a given period of development.
Pair-rule regulation of tartan and its implication for boundary formation during germband extension

Our quantifications in fixed and live tartan embryos suggest that Tartan is the only patterned receptor required for actomyosin enrichment and straightness of PSBs at the start of GBE. This early requirement is consistent with the expression of tartan being controlled along the antero-posterior axis by pair-rule genes. Indeed, tartan has been reported to be regulated by Ftz (Chang et al., 1993). At stage 5, ftz is expressed throughout even-numbered parasegments, strongly at even-numbered PSBs and diminishing toward the odd-numbered PSBs. Because this tailing off appears to match tartan’s expression (Fig. S3), the simplest hypothesis is that Ftz is the sole regulator of tartan at both even and odd-numbered PSBs, which we went on to test.

We analysed boundary straightness in live ftz knock-down embryos, using the same methods as for tartan mutants. ftz dsRNA injection resulted in pair-rule cuticle phenotypes identical to those of ftz null mutants, showing that we have an efficient knockdown (Fig. S9D,E). We analysed 3 movies each of ftz dsRNA injected and control buffer injected embryos. engrailed transcriptional dots in buffer-injected embryos have brighter even-numbered stripes of engrailed dots, as in wildtype (Fig. 6 A,C). Straightness curves are similar to wildtype, with PSBs consistently straighter than control interfaces throughout axis extension (Fig. 6E, E'). In the ftz knockdown, we expect the even-numbered stripes to be lost, since Ftz is required to activate engrailed transcription in even-numbered parasegments (Florence et al., 1997; Howard and Ingham, 1986). Consistent with this, we find that alternate stripes of engrailed transcriptional dots are gone, with weak stripes remaining, which we infer are the odd-numbered stripes (Fig. 6B). Although weak, we were able to use those traces to track the odd-numbered PSBs in ftz knockdown embryos (Fig. 6D). If ftz is the sole regulator of tartan along the AP axis, the prediction is that odd-numbered PSBs should lose their straightness in ftz mutants, because tartan would be gone. This is not what we find: odd-numbered PSBs are clearly straighter than control interfaces in ftz RNAi embryos (Fig. 6F,F') and they have the same straightness as in buffer-injected embryos when compared side-by-
side (Fig. 6G,G’). We conclude that odd-numbered PSBs are fully functional in ftz mutant embryos.

Two possibilities could explain the above result: either there is enough tartan expression remaining at odd-numbered PSBs to support boundary formation, or other receptors such as toll-like 2, 6 or 8 rescue boundary formation via changes in their transcription patterns in absence of ftz. To address this, we performed HCR in ftz null mutants to map the expression patterns of these 4 genes (and also Best 1, comm, comm2, dnt, drl found in our screen) relative to both odd- and even-numbered PSBs (Fig. S8A,B). We find that while the expression of most of these genes change in the region where even-numbered PSBs have been lost, their expression is unchanged at odd-numbered PSBs. In particular, tartan is still expressed at odd-numbered PSBs in ftz mutants. Our quantification shows that while tartan’s expression bordering even-numbered PSBs is gone as expected, its expression bordering odd-numbered boundaries is unchanged (Fig. S8C-F). We conclude that the simplest explanation for why odd-numbered PSBs are still functioning normally in ftz mutants is that tartan’s expression is maintained there, presumably under other pair-rule regulatory inputs.

The above results suggest that tartan’s expression at even- and odd-numbered PSBs is regulated independently. We wondered whether this had a measurable impact on boundary function. To address this, we classified odd and even-numbered PSBs using the stronger signal for engrailed transcriptional dots in even-numbered PSBs. In both wildtype and buffer-injected controls, even-numbered PSBs are slightly straighter than odd-numbered ones throughout most of extension (Fig. S9B,C). This is consistent with an independent regulation of Tartan at both classes of PSBs. These differences, however, are subtle, and our statistical tests at a couple of timepoints are not significant (Fig. S9B’,C’). We also asked about Tartan’s requirement at either boundary and find that the loss of straightness at PSBs in tartan mutants is the same at odd versus even-numbered PSBs (Fig. S9A,A’). We conclude that tartan is required for actomyosin enrichment at every PSB during early extension.
Discussion

One of our motivations for taking a systematic approach was to evaluate how many cell surface receptors are required for compartmental boundary formation during axis extension. In (Tetley et al., 2016), we proposed that a single receptor expressed in either even- or odd-numbered stripes would be the minimal number. Tartan fits this single receptor hypothesis because it is expressed in even-numbered parasegments and is required for polarized contractility of interfaces at every parasegment boundary during axis extension (this study and (Pare et al., 2019). What we additionally show here, is that Tartan is required from the start of axis extension and that this requirement decreases as axis extension progresses, as shown both by our quantifications over time in fixed (Fig. 2E,F) and live embryos (Fig. 5E-F). So this suggests that other inputs take over to maintain actomyosin enrichment at PSBs during late axis extension and extended germband stages.

We have identified six other genes that encode cell surface receptors or regulators of cell surface receptors and that are differentially expressed at parasegment boundaries during axis extension: *Best1*, *blot*, *comm*, *comm2*, *dnt* and *ImpL2*. *comm* and *comm2* have the clearest differential expression at PSBs from stage 7 onwards, and the removal of *comm* on its own shows a modest contribution to interfacial contractility at PSBs (Fig. 2H,I), so this is a potential candidate for a role in late axis extension. *Comm* may act redundantly with *Comm2*, and together regulate *Robo* or another cell surface receptor. *toll-6* and *toll-8* are also expressed differentially at PSBs, at least some of the time (Fig. S3), but previous reports showed that these, along with *toll-2*, do not contribute significantly to interfacial contractility at PSBs during germband extension (Lavalou et al., 2021; Pare et al., 2019). During extended germband stages, *wingless* is required for maintaining interfacial contractility at PSBs (Monier et al., 2010; Scarpa et al., 2018; Urbano et al., 2018), and we confirm this property in this study, using a different quantification method (Fig. 3F,G). *toll-2* was the only gene to respond to Wg signalling, out of 11 candidates we tested (*best1*, *comm*, *comm2*, *dnt*, *drl*, *sli*, *toll-2*, *toll-6*, *toll-7*, *toll8*, *trn*). However, we cannot detect a contribution of *toll-2* on its
own at PSBs, so the regulation of Toll-2 by Wg signalling does not readily explain the requirement for Wg at PSBs in maintaining interfacial contractility.

Like \textit{toll-2}, \textit{toll-6} and \textit{toll-8}, \textit{tartan} is regulated transcriptionally by pair-rule genes at gastrulation. We confirm a previous report that \textit{ftz} is required for \textit{trn}'s expression (Chang et al., 1993), but found that although \textit{trn} expression is lost at even-numbered PSBs in \textit{ftz} mutants, it remains at odd-numbered PSBs. This might explain why odd-numbered PSBs in \textit{ftz} mutants behave as in WT in our boundary straightness analyses in live embryos. So distinct transcriptional inputs regulate \textit{trn} at even and odd-numbered PSBs, but despite this independent regulation, both boundaries behave similarly in WT embryos and both require \textit{trn} in early axis extension for boundary straightness (Fig. S9 A-C').

Our \textit{in silico} screen was based on the assumption that differential expression of receptors underlies interfacial contractility at PSBs. One limitation of our approach is that borders in mRNA expression detected by HCR do not necessarily equate with an asymmetry in protein localization, since post-translational regulation could modulate receptor localisation. However, this approach was sufficient to identify Tartan. Also, comparison of protein and mRNA expression patterns for \textit{tartan} and \textit{comm} suggests that these are comparable, with the main difference being that the mRNA pattern is ahead in time. For example, at the beginning of GBE \textit{tartan} mRNA expression retracts away from the odd-numbered PSBs (as does \textit{ftz}) while the protein pattern is still abutting the PSBs (Fig. S4). Recent reports have identified additional cell surface molecules that interact with LRR receptors at boundaries and become asymmetrically localised (Lavalou et al., 2021; Pare et al., 2019). Those are the cell surface molecules Ten-M (a teneurin) and Cirl (an adhesion GPCR), which become localized at boundary cell-cell interfaces via their interactions with Tartan and Toll-8, respectively (Lavalou et al., 2021; Pare et al., 2019). Ten-M and Cirl have a uniform RNA expression in embryos and thus were eliminated as candidates in our \textit{in silico} screen (Fig. S2). The planar polarization of those heterophilic receptor complexes is thought
to underlie the formation of contractile cell-cell interfaces, via pathways which are starting to be elucidated (Garcia De Las Bayonas et al., 2019; Tamada et al., 2021).

Our study shows that the measure of straightness is a sensitive and faithful readout for increased actomyosin contractility along a boundary and provides an alternative to Myosin II quantifications. Our analysis in live embryos also suggests that mechanical boundary formation responds in real time and with high sensitivity to molecular asymmetries, because the recovery of PSB straightness in tartan mutant parallels the loss of expression of tartan along PSBs. It is likely that mechanosensitive feedbacks contribute to this responsiveness; indeed, Myosin II-enriched cell interfaces connected to each other enrich more Myosin II and are under greater tension than isolated interfaces, both during germband extension (Fernandez-Gonzalez et al., 2009) and extended germband stages (Scarpa et al., 2018), suggesting the existence of a positive mechanosensitive feedback. Consistent with this notion, in both cases, decreasing tension at connected cell-cell interfaces using laser cuts also decreases Myosin II enrichment (Fernandez-Gonzalez et al., 2009; Scarpa et al., 2018). So it is possible that mechanosensitive feedback increases actomyosin enrichment along PSBs, contributing to the real-time responsiveness of boundary formation. This might also contribute to the robustness of boundary formation (Martin et al., 2021).

Acknowledgements

We would like to thank Erik Clark and Matt Benton for advice about in situ HCR; Julia Falo-Sanjuan for advice about the MS2-MCP system; Robert Zinzen, Nikolaos Karaiskos and Nick Brown for providing data for the in silico screen; Sarah Bray, Guy Tear and Shigeo Hayashi for reagents; Rob White and Erik Clark for critical reading of the manuscript; all present and past members of the Sanson lab for discussion.
Methods

In silico screen

To identify *Drosophila* genes expressed in anteroposterior (AP) stripes in the early embryo, the BDGP library (http://insitu.fruitfly.org/cgi-bin/ex/insitu.pl) was filtered using the descriptors “Pair-Rule” and/or “Segmentally Repeated”, the Fly-FISH library (http://flyfish.ccbr.utoronto.ca/) was filtered using the descriptors “Pair-Rule” and/or “Segment Polarity”, the Enhancer Library (http://enhancers.starklab.org/) was filtered using the descriptors “A-P Stripes” and/or “Pair-Rule”, and the vISH library (https://shiny.mdc-berlin.de/DVEX/) was manually interrogated for the expression pattern of 441 genes predicted to encode transmembrane adhesion protein in *Drosophila* (Hynes and Zhao, 2000). Manual clustering analysis of the vISH library raw data was also performed to identify the top 200 genes expressed in the same cells as those expressing *even-skipped* or *fushi-tarazu* in early embryos (personal correspondence from Nikos Karaiskos and Robert Zinzen, Max Delbrück Center for Molecular Medicine, Berlin).

To identify genes encoding proteins that localise to the cell surface, the UniProt data resource (http://www.uniprot.org) was filtered for the descriptors: annotation:(type:transmem) AND organism:"Drosophila melanogaster (Fruit fly)" and also annotation:(type:signal) AND organism:"Drosophila melanogaster (Fruit fly)".

To identify genes regulated by the pair-rule gene network, differentially expressed genes resulting from the knock-down of *even-skipped* and *runt* in early *Drosophila* embryos were obtained from (Pare et al., 2014). Further, the BDTNP database (http://bdtnp.lbl.gov/Fly-Net/) was queried to identify genes neighbouring *fushi-tarazu*, *sloppy paired1*, *paired* and *runt* DNA binding sites, while the DroID database (http://www.droidb.org/Index.jsp) was used to identify genes neighbouring *even-skipped*, *hairy* and *odd-skipped* DNA binding sites.
A custom R script was used to wrangle the downloaded filtered datasets into a standardised dataframe format and identified genes that fulfilled candidate criteria. The initial list of candidate genes was then trimmed as follow: i) Each candidate had their raw in situ hybridisation images, contained in each library, manually assessed and if a gene was found not to be expressed in AP stripes, the gene was excluded; ii) The ModEncode temporal expression data set (Roy et al., 2010) (annotated version kindly provided by Nick Brown, University of Cambridge) was used to exclude genes with less than 5% of their total embryonic expression (0-24hrs AEL) occurring between 0-6hrs AEL; iii) Finally, a manual investigation of protein localisation and described role was undertaken using Flybase (Gramates et al., 2022) and a search in the primary scientific literature, to eliminate genes which were unlikely to have a direct role at the cell surface, such as transcription factors.

Whole mount in situ HCR v3.0

Two to five hours old yw67 embryos were collected on apple juice agar plates at 25°C, fixed in 4% formaldehyde/heptane for 20 min, and stored at -20°C in methanol until required. In situ HCR v3.0 with split initiator probes were performed as in (Choi et al., 2018). The probes sets were designed by Molecular Instruments to target exons present within every gene isoform. Embryos for whole mount in-situ HCR were first post-fixed in 4% formaldehyde, then washed in PBT (PBS with 0.1% Tween-20), then 5XSSCT prior to hybridisation. Embryos were pre-hybridised in warm hybridisation buffer for 30 mins at 37°C. Embryos were incubated in the probe hybridisation solution (0.8pmol of each probe in 200uL) at 37°C overnight. Following overnight incubation, excess probes were removed by washing in wash buffer at 37°C, then in 5XSSCT at RT. The embryos were pre-amplified in buffer then final amplification solution was added (6pmol of each snap cooled fluorescently labelled hairpin added to 50-100μL of amplification buffer). Embryos were incubated in the amplification solution overnight then washed in 5XSSCT. If an antibody immunostaining was
to follow, embryos were washed in PBS-TX before being blocked in a PBS-TX-BSA solution as standard. Embryos were mounted in Vectashield (Vectorlabs) before imaging.

**Immunostaining and antibodies**

Embryos were fast fixed at the interface between 37% formaldehyde and 100% heptane for 8 minutes then washed thoroughly in PBS-TX. The vitelline membrane was either removed chemically using methanol or manually with a tungsten needle. Embryos were blocked in PBS-TX-BSA for 30mins at RT. Embryos were incubated with primary antibodies in blocking solution overnight at 4°C. Excess antibody was removed by washing embryos thoroughly in PBS-TX. Embryos were incubated with secondary antibodies in blocking solution for 1 hour at RT. Excess antibodies were removed by washing thoroughly in PBS-TX. Stained embryos were stored in Vectashield (Vectorlabs) until mounted.

Primary antibodies used were: mouse anti-phospho-Tyrosine (pTyr) (Cell signaling #9411; 1:1000), rat anti-DE-Cad (DSHB; 1:50), rabbit anti-Engrailed (Santa Cruz D300; 1:100), mouse anti-Wingless (DSHB; 1:25), chick anti-Beta-gal (Abcam ab9361; 1:500), rabbit anti-Tartan (1:2500)(Chang et al., 1993)(kind gift of Shigeo Hayashi), rabbit anti-Comm (1:50)(Tear et al., 1996)(kind gift of Guy Tear).

Secondary antibodies conjugated to fluorescent dyes were obtained from Jackson ImmunoResearch Laboratories, Invitrogen and Life Technologies. Streptavidin with Alexa Fluor 405 conjugate was from ThermoFisher Scientific.

**Confocal imaging of fixed embryos**

Embryos were mounted individually under a coverslip supported by a tape bridge on either side. This flattened the embryos sufficiently so that all cells were roughly in the same
z-plane. *In-situ* HCR stained embryos and immunostained embryos were imaged on an inverted SP8 Confocal Microscope (Leica Systems), with either a 20x 0.75NA air objective, 40x 1.3NA oil-immersion objective, or 63x 1.4NA oil-immersion objective. Either a PMT or HyD detector was used alongside a 405/488/546/594/647nm laser line. Image stacks of various Z separations were captured using the Leica Application Suite X Software.

**Embryo staging and mapping of expression patterns by HCR**

Fixed embryos were staged initially based on their morphology under a light microscope, before mounting in Vectashield. The staging was then refined when confocal imaging. Embryos with invaginated mesoderm but prior to the first mesectoderm cells dividing (identified by their dumbbell shapes) were classified as stage 7 or “early GBE”. Embryos past this stage and up to the time when the first neurectoderm cells divide were classified as stage 8 or “late GBE”. Embryos past this stage and up to the formation of tracheal pits are classified as “extended germband” and include stage 9 and 10 embryos. This staging was used in the quantifications presented in Fig. 2 and 3. Note that in our live embryos analyses, the first 30-40 minutes of GBE corresponds to stage 7/early GBE and the remainder, stage 8/late GBE (see for example Fig. 5D).

To classify embryos labelled by HCR, similar staging was used, with earlier embryos (stage 5 and 6) also included into the analysis. Candidate genes patterns were mapped relative to parasegmental boundaries using various markers by HCR (*ftz, slp1, en, wg*) and also membrane immunostaining (*pTyr* or DE-Cad, both labelling adherens junctions). This work is summarized in diagrams showing representative odd- and even-numbered parasegments separated by odd-numbered or even-numbered PSBs (see for example Fig. S3). For simplicity, each parasegment has been divided in 4 regions for all the developmental stages analysed, but it is important to note that cell numbers along AP slowly increase through polarized cell intercalation and then cell division. At the start of germband extension the 4 regions correspond approximately to the number of cells per parasegment, which we measured as 3.7 cells in average (Tetley et al., 2016). Germband cells undergo one round of
cell intercalation, bringing the parasegment width to 7.2 cells in average (Tetley et al., 2016). At the end of GBE, germband cells start dividing and the number of cells per parasegment increases further.

Fly genetics

We used yw^67 as control. Null mutant alleles were used for the following genes:

- **fushi-tarazu**: ftz^{11} (embryonic lethal, pair-rule cuticle phenotype, see Fig. S9E).
- **wingless**: wg^{CX4} (embryonic lethal, segment polarity cuticle phenotype).
- **tartan**: trn^{28.4} (Chang et al., 1993) for quantification s and trn^{S064117} for complementation tests (embryonic lethal).
- **commissureless**: comm^{Delta.e39} (https://flybase.org/reports/FBal0097023) (gift from Jimena Berni) for quantifications. Note that comm^{Delta.e39} is the same allele as comm^{E39} (https://flybase.org/reports/FBal0141222) (Guy Tear, personal communication), which is a deletion of the comm transcription unit (Georgiou and Tear, 2002). We used comm^{A990} (gift from Guy Tear) and Df(3L)BK10 for complementation tests (embryonic lethal).
- **toll-2**: toll-2^{Delta.7-35} (Eldon et al., 1994) for quantifications and toll-2^{K02701} (Yagi et al., 2010) and toll-2^{pTV} (Li et al., 2020) for complementation tests (embryonic semi-lethal, escapers have abnormal climbing behaviour (Li et al., 2020).

Transgenes were: Gap43mCherry (Martin et al., 2010) to label cell membranes, sqh^{EGFP.29B} (called sqhGFP here)(Proag et al., 2019) to label Myosin II, EnVT15159-peve-MS2-lacZ (this work), nos-MCP-eGFP on II (Garcia et al., 2013), armGal4 (Sanson et al., 1996) and UASwg (Lawrence et al., 1996).

Balancer chromosomes used for homozygous embryo selection were: CyO, eveGal4 or TM6C, twiGal4 (for fixed embryos) and TM3, twiGal4,UASGFP (TTG, for live embryos).
Quantification of Myosin II intensities and boundary straightness at PSBs

Quantifications were performed in fixed embryos using the following fly strains:

Fig. 2E,F: sqhGFP\textsuperscript{KI}; \textit{tmn}\textsuperscript{29,4}/TM6C, twilacZ
Fig. 2H,I: sqhGFP\textsuperscript{KI}; \textit{comm}\textsuperscript{\Delta e39}/TM6C, twilacZ
Fig. 3D,E: sqhGFP\textsuperscript{KI}; \textit{toll-2}\textsuperscript{\Delta e7-35}/CyO, evelacZ
Fig. 3F,G: sqhGFP\textsuperscript{KI}; \textit{wg}\textsuperscript{CX4}, enlacZ/CyO

Homozygous embryos were identified based on absence of immunostaining against LacZ except for \textit{wg} null mutant embryos, where loss of Engrailed immunostaining was used. The remainder of the embryos in the progeny were used as controls (WT). Using the plugin Simple Neurite Tracer in Fiji (https://imagej.net/plugins/snt/), lines 2 pixel in width were traced along the PSB and control +1 cell-cell interfaces, based on Engrailed or Wingless (or \textit{enlacZ} for \textit{wg} null mutants) and pTyr or DE-Cad immunostainings (see traces in Fig. 2B). Native fluorescence from sqhGFP\textsuperscript{KI} was then quantified in the corresponding traces. Normalisation was performed by removing background pixels using a threshold corresponding to 20% of total pixels. Ratios of PSB interface signal - background/ +1 interface signal- background were expressed in log10. Traces corresponding to PSBs were also used to calculate an index of straightness by dividing the length of the shortest path between the extremities of the trace and the length of the trace. Embryos of stage 7 to 10 were analysed. PSBs with boundary cells dividing were excluded from the analysis (see blue traces in Fig. 2B,C). As a consequence of the frequency of cell divisions, fewer PSBs of stage 9 were included in the analysis compared to stage 10 for extended germband stages.

dsRNA generation

Design of dsRNA was based upon the Heidelberg 2 (BKN) library (Horn and Boutros, 2013). First, to generate transcription templates for production of dsRNA, a PCR was undertaken on \textit{yw}\textsuperscript{67} fly gDNA using a Q5 polymerase master mix (NEB) and the following primer pair (preceded by the T7 promoter sequence: 5’-TAATACGACTCACTATAGGG-3’):
ssRNA was transcribed directly from the PCR amplicon product in a reverse transcription reaction using a HiScribe T7 polymerase (NEB). The DNA template was then removed through treatment with DNase1. ssRNA was annealed to form dsRNA through addition of 0.5M EDTA, 10% SDS and 3M NaCl, boiling the mixture then cooling to RT naturally. Annealed dsRNA was purified through a standard Phenol:Chloroform:IAA 25:24:1 extraction and precipitated from solution by adding of ethanol and ammonium acetate. The isolated dsRNA pellet was washed multiple times in 70% EtOH, air dried, and resuspended in injection buffer (0.1mM sodium phosphate buffer, 5mM KCl). The dsRNA was injected into pre-cellularised embryos at a concentration of 1.7μg/μL, as measured by nanodrop.

**Embryo injections**

Adult flies were kept at 25°C in a cage with an apple juice agar plate. Embryos were collected from the plate following a 30-minute laying period and were dechorionated. Approximately 20 embryos (for dsRNAi experiments) and 100 embryos (for transgenic injections) were aligned on a block of agar and transferred to a coverslip using a thin layer of heptane glue. If the injected embryos were for live imaging, embryos would be aligned with their ventral side facing the glue and coverslip. Embryos were desiccated in a jar of silica beads for 10 to 12 minutes before being covered with a thin layer of Voltalef (PCTFE H10S, Arkema). A brightfield microscope (Nikon), microinjection platform (Leica), and a pulled glass needle were used to inject the embryos through their posterior end. For RNA interference experiments, the expulsion of dsRNA was aimed for the centre of the embryo. For the generation of transgenic flies, plasmid solution was injected at the posterior end of the embryo (where the future pole cells form). Unfertilised, damaged, or old embryos were

<table>
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<th>Target</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Length</th>
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<tbody>
<tr>
<td>fushi-tarazu</td>
<td>5’-CCGCCACCTACTACGATAA-3’</td>
<td>5’-CAGCTGACGGATTCTCC-3’</td>
<td>577 bp</td>
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destroyed with forceps. Slides of injected embryos were placed in a 50mm petri dish at 18°C until the correct stage of development.

**Cuticle preparation**

Dechorionated embryos were transferred to a 50:50 mix of Hoyer’s medium and lactic acid and mounted under a 22x32mm coverslip. A permanent marker was used to draw black dots onto the surface of the coverslip to help locate embryos for microscopy. The slide was baked overnight at 62°C with a weight on top of the cuticle to prevent air bubbles forming. Cuticle preps were imaged using darkfield or phase-contrast microscopy.

**EnVT15159-MS2 generation**

The Stark Lab fly enhancer library was used to identify a small region of the *engrailed* enhancer that accurately recapitulates expression at germband extended stages of embryogenesis. Tile ID VT15159 contained a 2099bp region of DNA that neighbours the *engrailed* gene and drives *LacZ* in an *engrailed* pattern. The 2099bp region (EnVT15159) was PCR amplified from purified *yw* gDNA using Q5 DNA polymerase MasterMix (NEB) and the following primers:

<table>
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<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>EnVT15159</td>
<td>5’-GGG[AAGCTT]GGCGTTTG</td>
<td>5’-GGG[ACCGGT]TCTTAG</td>
</tr>
<tr>
<td></td>
<td>TGGGGATGTTTCAAGTTTG-3’</td>
<td>CCAGGCTTGTTAGCCGC-3’</td>
</tr>
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Square brackets indicate HindIII (Fwd primer) and AgeI (Rev primer) restriction enzyme cut sites. Primers were designed so the restriction cut site was preceded by 3 Guanine bases.
2μL of the PCR product was ran on a 1% agarose gel to confirm successful amplification of the EnVT15159 region. The PCR product was cleaned using the Qiaquick PCR clean-up kit (Qiagen). The EnVT15159 PCR product was then digested using HindIII and AgeI high fidelity restriction enzymes (NEB) in Cutsmart buffer (NEB).

The digested EnVT15159 region was cloned into *pattB-w+-pEve-24xMS2-LacZ* plasmid (kind gift of Julia Falo-Sanjuan and Sarah Bray) using the HindIII and *AgeI* sites. The plasmid was transformed into DH5-alpha library-efficiency competent cells (Invitrogen) through the standard heat shock protocol. Transformed colonies (displaying ampicillin resistance) were picked, grown into 50ml of culture and isolated via a MaxiPrep kit (Qiagen). Plasmid fingerprinting was undertaken using Bbs1 and EcoRV restriction enzymes (NEB) to confirm the EnVT15159 product had been inserted into the plasmid in the correct orientation. We injected the final plasmid construct *pattB-w+-EnVT15159-pEve-24xMS2-LacZ* into yw, M(eGFP, vas-int, dmRFP)ZH-2A;; M(attP)ZH-86Fb flies (sourced from Genetics Department Fly Facility, University of Cambridge). The construct was inserted, by phiC31 mediated integration into the attP-86Fb site on the third chromosome (86F8). F1 Transgenic flies were identified through the presence of w+. Crosses were undertaken to generate yw;;EnVT15159-MS2 flies that were homozygous viable and established as a stable stock.

**Live imaging**

Dechorionated embryos were mounted using an adapted hanging drop methodology (Reed et al., 2009). Briefly, a 22x64mm coverslip (#1) was attached to a rectangular metal microscope slide frame (Leica) using magictape (Scotch). Live embryos freely suspended in Voltalef (PCTFE - H10S - Arkema) were positioned with their ventral side towards the coverslip. The frame and coverslip were quickly inverted. The ventral side of the embryo remains in contact with the coverslip. Embryos were imaged under a 40x oil objective lens (NA of 1.3) on a Nikon Eclipse E1000 microscope with a Yokogawa CSU10 spinning disk head and a Hamamatsu EM-CCD camera. Embryos were illuminated using a Spectral
Applied Research LMM2 laser module (491 nm and 561 nm excitation). Images were captured using Volocity Acquisition Software (PerkinElmer) 32 Z-slices with a 1μm separation were obtained at each time point. Embryos were imaged every 30 s from late stage 5 for 100 min. Movies were recorded at 20.5 ± 1°C, measured with a high-resolution thermometer (Checktemp1). To check that embryos survived the imaging process to the end of embryogenesis, embryos were allowed to develop on the imaging insert to hatching in a humidified box. For mutants that are embryonic lethal, the cuticle of embryos was prepared using standard methods to check their phenotype. Occasional movies acquired for embryos that did not hatch or did not make a cuticle at the end of embryogenesis were discarded.

**Genetic tools for boundary straightness analysis in live embryos**

We used progeny embryos from females Gap43-mCherry/CyO; Nos-MCP-GFP/TM6B or Gap43-mCherry/CyO; Nos-MCP-GFP, trn^{28.4}/TTG crossed with males yw^{67}; ; EnVT15159-MS2 or yw^{67}; ; EnVT15159-MS2, trn^{28.4}/TTG. Note that there is sufficient maternal contribution of Gap43-mCherry to have a strong membrane signal in progeny embryos.

Fig. 5: For the comparison between WT and trn mutant embryos, trn homozygous embryos were identified by the lack of twiGal4,UASGFP fluorescence by mid-embryogenesis and WT embryos were the twiGal4,UASGFP positive embryos.

Fig. 6: Embryos are Gap43-mCherry/+; Nos-MCP-GFP/ EnVT15159-MS2 injected with either buffer (BI) or dsRNA against ftz (ftz KD).

**Cell tracking analysis**

Cell tracking based on the Gap43-mCherry membrane signal, spatiotemporal movie synchronization, domain strain rate calculations, cell selection criteria and contoured heat map generation were performed as in (Tetley et al., 2016).
Defining PSB interfaces and cell types

Tissue domains were defined in individual tracked movies by examining the position of EnVT15159-MS2 / MCP-GFP transcriptional dots. To assign dots to cells, cell tracking was undertaken using the custom oTracks software (Blanchard et al., 2009) on movies of embryos containing EnVT15159-MS2, MCP-GFP and Gap43-mCherry. First, the MS2-MCP signal was processed, and a pixel intensity threshold applied to identify dots in an automated manner. Next, the Gap43-mCherry signal was processed and a blanket correction applied to uncurve the 3D surface of the embryo. A few slices of the Gap43-mCherry signal, just under the apical surface of cells, were maximum intensity projected so cell outlines were clear and individual cells could be segmented. Segmented cells were tracked back and forth through time and each cell was marked with a unique identity. Fluorescent transcription dots (resulting from MS2-MCP binding) were also tracked back and forth through time and each dot was assigned to a corresponding cell. Based upon the assignment of dots, *engrailed* expressing cells were identified and cells could then be classified into parasegments meaning PSB interfaces could also be identified. Because cells were tracked over time, these classifications of parasegment identity could be automatically tracked through time to define the same groups of cells at all earlier and later time points.

Quantifying interface co-alignment

Interface orientations, relative to the embryonic axes, were calculated for PSB, -1 and +1 in all movies. All distributions of interface orientations (from 0, parallel to the AP embryonic axis, to 180°) were reflected around 90°, producing distributions from 0°, AP-aligned, to 90°, DV-aligned. As a measure of co-alignment, the proportion of interfaces oriented between 60 and 90° relative to the AP axis was plotted over time, from -10 to 50 min. Cumulative frequencies were calculated for each reflected distribution of interface orientations. Two-
sample Kolmogorov-Smirnov tests on the cumulative frequency distributions of interface orientation were used to compare treatments / genotypes.

Data availability

We are providing extended data for Figures S1, S2, S5 and S8, showing multi-channel images of the HCR experiments. The data has been deposited in Apollo, the institutional repository of the University of Cambridge, under the DOI: https://doi.org/10.17863/CAM.87959.

References


Fig. 1. *In silico* screen to identify cell surface factors responsible for actomyosin enrichment at PSBs. A) Venn diagram illustrating the three criteria used to identify candidate cell surface factors in an *in silico* screen and including the main datasets that were mined. B) Flow chart showing the successive steps taken to whittle down candidates. C) *In situ* HCR images for key candidate genes found in the screen (inverted images of data in Fig. S 1). *comm*, *comm2*, *tartan* and *toll-2* are all expressed in clear anteroposterior stripes in early embryos. We distinguish two developmental periods for actomyosin enrichment at parasegmental boundaries (PSBs), germband extension (GBE) and extended germband stages. At the onset of GBE, actomyosin becomes planar polarised at every AP cell-cell interface, including PSB interfaces (dashed green lines, stage 7). As GBE progresses and cells intercalate (stage 8), actomyosin enrichment become more prominent at PSBs (green lines). Weaker actomyosin enrichment is also detectable at two intraparasegmental boundaries (dashed lines)(Tetley et al., 2016). After completion of GBE, planar polarisation is lost except at PSBs (thick green lines, stages 9-10), where it is maintained throughout extended germband stages.
Fig. 2. Tartan is required for specifying contractile interfaces at PSB during GBE. A) Diagram based on in situ HCR images showing the expression patterns of comm, comm2, tartan, toll-2, toll-6 and toll-8 in relation to boundary markers ftz and slp1, at the beginning of GBE (see all the stages in Fig. S 3). Representative patterns are shown for even- and odd-numbered parasegments. Dark grey indicate high expression and light grey, lower
expression. B-C') Example of quantifications in fixed embryos at stage 10. In B, lines of interest are drawn along the PSB and control +1 interfaces using Engrailed and pTyr stainings, which label the PSBs and cell contours, respectively. Native fluorescence from shq-GFPKI shown in C, C' is then quantified along the lines. In this example, the orange and yellow lines have been used, whereas the blue lines could not be used because of the presence of cell divisions at the boundary. PSB lines are also used for measuring boundary straightness (see Methods). D) In situ HCR of tartan expression during GBE. tartan is expressed within each even-numbered parasegments and abuts both odd and even-numbered PSBs. E) Index of straightness measures in wildtype (WT) (n= 74 PSBs, from 24 embryos) and tartan mutant embryos (n=74 PSBs, from 22 embryos). The convention for P values for this graph and all subsequent graphs are: NS: p>0.05; *p<0.05; **p<0.01; ***p<0.001. D) Myosin II enrichment measures at PSBs in WT (n=67 PSBs and 24 embryos) and tartan mutant embryos (n=50 PSBs and 21 embryos). G) In situ HCR of comm expression during GBE. comm is expressed in a similar pattern to sloppy-paired during GBE and abuts both odd and even-numbered PSBs. H) Index of straightness measures in WT (n=83 PSBs, from 26 embryos) and comm mutant embryos (n=65 PSBs for 27 embryos). I) Myosin-II enrichment measures at PSBs in WT (n=83 PSBs, from 26 embryos) and comm mutant embryos (n=65 PSBs, from 27 embryos). “Early GBE” corresponds to stage 7 embryos; “late GBE”, stage 8; “Extended germband”, stages 9 and 10.
Fig. 3. Testing a requirement for *toll-2* at PSBs during extended germband stages. A) Expression patterns of *toll-2* and PSB marker *slp* in stages 8 to 10 WT embryos. Note that Toll-2 straddles the *slp* expression domain and thus the PSBs (merged signal in white). B) Expression patterns of *toll-2* and *slp* in *wingless* mutant embryos. C) Expression patterns of *toll-2* and *slp* in embryos overexpressing *wingless*. D) Index of straightness measures in WT (n=49 PSBs, from 18 embryos) and *toll-2* mutant embryos (n=34 PSBs, from 11 embryos). E) Myosin II enrichment measures at PSBs in WT (n= 49 PSBs, from 18 embryos) and *toll-2* mutant embryos (n=34 PSBs, from 11 embryos). Note that the ratio PSB/+1 increases significantly at late GBE in *toll-2* mutant, consistent with a loss of actomyosin enrichment at +1 interfaces in this mutant, as described by (Pare et al., 2019) and (Lavalou et al., 2021). F) Index of straightness measures in WT (n=83 PSBs, from 25 embryos) and *wg* mutant embryos (n=50 PSBs, from 14 embryos). G) Myosin II enrichment measures at PSBs in WT (n= 83 PSBs, from 25 embryos) and *wg* mutant embryos (n=50 PSBs, from 14 embryos). GBE corresponds to stage 8 embryos; extended germband, stages 9 and 10.
Fig. 4. Tracking parasegmental boundaries in live Drosophila embryos

A) Spinning disc confocal image of a live gastrulating Drosophila embryo expressing Gap43-mCherry. The cephalic furrow (CF) and ventral midline (VM) are annotated with dashed colored lines. The ventrolateral region shaded green is the approximate area imaged via spinning disc confocal microscopy. (Anterior = Left, Posterior = Right. 20x magnification).

B, C) Maximum intensity projection of a single time frame from an embryo expressing the transgenes EnVT15159-MS2, MCP-GFP and Gap43-mCherry. MCP-GFP binds the MS2 loops on nascent mRNA transcribed from the engrailed enhancer EnVT15159 and form spatially localized fluorescence within nuclei that resemble dots. B') Kymograph showing the distribution of transcriptional dots along the embryonic axis during GBE. Magenta asterix indicates stripes that coincide with odd-numbered PSBs, while yellow asterix indicates the brighter stripes that coincide with even-numbered PSBs. C) Gap43-mCherry protein localizes to cell membranes. C') Movie frame showing cell segmentation based on the Gap43-mCherry signal and boundary cell tracking based on the MCP-GFP dot signal. Assigning the dots to cells reveals the position of Engrailed-expressing cells.
Fig. 5. Live imaging reveals Tartan is required for PSB straightness throughout germband extension. A) In tracked movies, cell centroids colored by parasegment identity. PSBs, -1 and +1 interfaces are overlayed to show their position relative to one another. B) Diagram showing the relative position of -1, PSB, and +1 interfaces. -1 and +1 interfaces correspond to the column of AP interfaces one cell diameter to the anterior and to the posterior of PSBs, respectively. C) Diagram showing how the orientation of cell interfaces that make up each column is calculated. Theta represents the measured angle between the embryonic AP axis and each cell interface. Angles of each cell interface that make up a column are measured automatically in our tracking software. D, E) Plots showing the proportion of interfaces at PSB and control -1 and +1 interfaces that are greater than 60 degrees from the AP axis, in the course of GBE, for 3 wildtype (D) and 3 tartan mutant embryos (E). A loess curve (span 0.75) has been fitted to the data. D’, E’) Statistical comparison at time point 30 minutes with a Kolmogorov-Smirnov non-parametric test.
undertaken on the cumulative frequencies of interface angles. F) Subset of D and E curves to directly compare the PSB straightness in WT and *tartan* mutant embryos in the course of GBE, with statistics for timepoint 30 minutes in F'.
Fig. 6. Boundary tracking in live embryos reveals that odd-numbered PSBs in ftz mutant embryos retain their straightness during GBE. A,B) Kymographs showing the spatiotemporal distribution of transcriptional MCP-GFP dots marking the PSBs during GBE. Magenta asterix indicates stripes that coincide with odd-numbered PSBs, while blue asterix
indicates the brighter stripes that coincide with even-numbered PSBs. In A, buffer injected embryos show the same pattern as wildtype embryos (compare with Fig. 4B’), while in B, embryos injected with ftz dsRNA (ftz KD) have, as expected, lost the stronger stripes that coincide with even-numbered PSBs. C,D) Movie frames showing examples of boundary tracking in a buffer injected embryo (C) and a ftz KD embryo (D). Yellow arrows indicate the approximate position where even-numbered engrailed positive stripes are missing in ftz KB embryos. E, F) Straightness measurements in the course of GBE for 4 buffer injected (BL) and 4 ftz KD embryos. Plots show the proportion of interfaces that are greater than 60 degrees from the AP axis, for PSBs and +1 and -1 control interfaces. A loess curve (span 0.75) has been fitted to the data. Note that for ftz KD (F), only the odd-numbered PSBs and corresponding controls interfaces could be tracked. E’, F’) Statistical comparison for timepoint 30 minutes for plots E and F uses a Kolmogorov-Smirnov non-parametric test undertaken on the cumulative frequencies of interface angles. G) Subset of data from E and F curves to directly compare the straightness of odd-numbered PSBs in buffer injected and ftz KD mutant embryos in the course of GBE, with statistics for timepoint 30 minutes in G’.
Figure S1.
Fig. S1. **In situ HCR for candidate genes with antero-posterior striped expression.** HCR was performed for each gene in *yw*^{67} *Drosophila* embryos between stages 5 to 10 of embryogenesis. The 19 genes included in this figure were classified as having a striped expression pattern at some point during stages 5-10. For some genes, such as *tutl*, subtle stripes are only clearly visible at a subset of the stages surveyed (stages 9-10), whereas other genes, such as *trn*, have clear expression stripes visible at all stages. Scale bar = 100μm.
### Figure S2.

<table>
<thead>
<tr>
<th>Cellular blastoderm</th>
<th>Gastrulation</th>
<th>Germband extension</th>
<th>Extended germband</th>
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<td>Stage 6</td>
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- **Cellular blastoderm**: Images showing different stages of cellular blastoderm development.
- **Gastrulation**: Images depicting gastrulation stages.
- **Germband extension**: Images illustrating germband extension stages.
- **Extended germband**: Images showing extended germband stages.
Fig. S2. *In situ* HCR for candidate genes without AP striped expression pattern. HCR was performed for each gene in *yw* Drosophila embryos between stages 5 to 10 of embryogenesis and the 12 genes included here were found not to have any AP striped expression. Scale bar = 100μm.
Figure S3.
**Fig. S3. Map of expression patterns of candidate genes relative to the PSBs.** The maps are drawn based on gene expression patterns revealed by HCR for each candidate genes in combination with the parasegmental boundary markers ftz and slp1. HCR was performed in *yw*67 *Drosophila* embryos between stages 5 to 10 of embryogenesis. Representative expression for odd- and even-numbered parasegments are shown. Rather than trying to represent cell numbers, we have divided each parasegment into 4 regions, for simplicity of comparison across the different developmental stages (see Methods). Out of the 19 candidates with AP striped expression shown in Sup. Fig. 1, the 13 genes included here showed expression patterns that were clear enough to enable mapping relative to the PSBs.

*Note that for both tartan and toll-6, expression becomes discontinuous along DV.*
Figure S4.

A  Trn protein  
Stage 6

Stage 6

B  Comm protein  
Stage 7

Stage 7

trn mRNA

comm mRNA

.comm slp1 ftz

.comm slp1

Comm

20 µm

20 µm

Comm

20 µm

20 µm
Fig. S4. Comparison of mRNA and protein patterns for candidate genes tartan and comm. A) Left panel shows an immunostaining against the Tartan protein in combination with HCR for en and ftz. Right panel show HCR for tartan, in combination with slp1 and ftz. Both images show a stage 6 yw67 embryo. For the immunostain, maximum intensity projection are shown for the full z-depth of the engrailed and fushi-tarazu channels and for 2μm of Tartan signal, just below the apical surface of cells. Tartan protein localises to the membrane of cells located within even-numbered parasegments and is absent from cells within odd-numbered parasegments at stages 6-8 of embryogenesis. B) Left panel show immunostainings against Comm protein and right panel HCR for comm, in combination with slp1 and ftz markers. Images of stage 7 and 9 yw67 embryos show that comm patterns for both mRNA and protein undergo a doubling of periodicity (see also Sup. Fig. 3).
**Figure S5.**

A  

- Wildtype  
- 
- best1  
- comm2  
- dnt  
- drl  
- sl1  
- sli  
- wg  

B  

- Wildtype  
- 
- comm  
- toll-2  
- toll-5  
- toll-7  
- toll-8  
- tram  
- wg^{ca4}  
- arm-Gal4/UAS-wg
Fig. S5. In situ HCR to identify candidate genes regulated by Wingless signalling. A) HCR for a subset of candidate genes (best1, comm2, dnt, dnt, sli) in WT and wg^Cx4 mutant embryos, alongside wg and slp1 controls. B) HCR for an additional subset of candidate genes (comm, toll-2, toll-6, toll-7, toll-8, tm) in WT, wg^Cx4 mutant embryos and in armGal4/UASwg embryos expressing Wg ubiquitously. Out of all the genes tested, only toll-2 shows robust change in HCR, with loss of signal in wg^Cx4 mutant and increase in armGal4/UASwg embryos.
Figure S6.

A

Promoter

24x MS2 Loops

RNA Polymerase

RNA Polymerase

RNA Polymerase

LacZ

Enhancer

B

Chr2R

Engrailed

4207 bp

3722 bp

2099 bp

VT15159 Enhancer

C

D
**Fig. S6.** MS2-MCP system to image nascent mRNA transcripts from an *engrailed* enhancer in live embryos.  
A) Diagram showing the MS2-MCP system. The gene of interest’s enhancer region is cloned upstream of a strong promoter that initiates transcription. RNA polymerase transcribes the 24 MS2 stem loops followed by *lacZ*. The resulting mRNAs contains 24 copies of the MS2 loops that are bound by the MCP protein fused to GFP (box). The concentration of multiple MCP-GFP/MS2 loop complexes causes a spatially localised fluorescence that is detected at the locus as a fluorescent dot. Multiple transcripts are produced from a single locus simultaneously increasing the brightness of the fluorescent dot. *lacZ* transcription is accessory to the core system but prolongs the time the nascent mRNA stays within vicinity of the locus, making the fluorescent dot brighter. 
B) Gene diagram showing the position of the VT15159 enhancer region, which is located 3722bp upstream of the *engrailed* gene (adapted from the NCBI genome data viewer). 
C) HCR of *ftz* (blue), *engrailed* (red) and *lacZ* (green) in embryos transgenic for EnVT15159-MS2. *lacZ* expression from EnVT15159-MS2 coincides with endogenous *engrailed* expression demonstrating that EnVT15159 recapitulates endogenous *engrailed* expression during early embryogenesis. Note that brighter alternate *lacZ* stripes overlap with *ftz* expression, indicating differential patterning by the VT15159 enhancer between odd- and even-numbered parasegments, with the brighter stripes abutting even-numbered PSBs. (20x magnification. Scale bar = 100μm) 
D) Close-up to show the coincidence between endogenous *engrailed* expression and *lacZ* expression from EnVT15159. The brighter *lacZ* stripes overlap with *ftz* expression and abut even-numbered PSBs. (63x magnification and 2x zoom. Scale bar = 50μm)
Figure S7.

A Raw signal

EnVT15159-MS2 / MCP-GFP

C Raw signal

Gap43-mCherry

E Cell segmentation and tracking

G Assigning En positive cells

B Processed & dots identified

D Processed & blanketed cell outlines

F Dot tracking

H Parasegment classification
Fig. S7. Image processing and cell tracking pipeline to identify parasegmental boundaries in live embryos. A,B) Raw EnVT15159-MS2-MCP-GFP signal is processed in the custom software oTracks. A median background subtraction of 10 is applied. Fluorescent dots are identified in an automated manner by applying a pixel intensity threshold. C,D) Raw Gap43-mCherry signal is processed within oTracks. Median smoothing of 1 is applied and the corners of the image are brightened to correct for microscope artefacts. A blanketing operation is applied to correct for the 3D curvature of the embryonic volume. A 2μm z-plane is projected from the embryonic volume onto a flat 2D surface for image segmentation. E) oTracks segments each cell based on the Gap43-mCherry signal using a watershed algorithm. Each cell is tracked back and forth through frames and connected in time. F) Identified dots are tracked back and forth through time in oTracks. Dots are assigned a probability of belonging to each cell by determining the proximity of each dot to each cell during the lifetime of each dot. G) The probability of a cell being assigned to a dot can be displayed as a color (red = low probability, blue = high probability). Cells with a high probability of containing a dot form AP stripes throughout the embryo similar to the *engrailed* expression pattern. H) Based upon the color coding in G, parasegment identities can be assigned to each cell and the location of PSBs can be identified.
Fig. S8. Analysis of tartan expression at odd-numbered PSBs in ftz mutants. A, B) Diagrams summarising expression patterns by HCR of best1, comm, comm2, dnt, drl, toll-2, toll-6, toll-8 and tm, in relation to PSB markers ftz, wg and en, in wildtype and ftz mutant embryos. ftz mutant embryos are homozygous for the ftz\(^{11}\) null allele while wildtype embryos are the heterozygous embryos in the same progeny. ftz homozygous mutant embryos were identified based on the changes in en expression pattern. C-F) Quantification of tartan HCR at even- and odd-numbered PSBs in WT and ftz null mutants. The rectangles show the regions of interest where tartan and en HCR signal has been quantified. In WT, engrailed stripes mark both odd- and even-numbered PSBs, whereas in ftz mutants, only the engrailed stripes at odd-numbered parasegments remain. “Early GBE” corresponds to stage 7 embryos; “late GBE”, to stage 8. Scale bars =20 μm
Figure S9.

A  trn  10 mins  50 mins

B  WT  10 mins  50 mins

C  BI  10 mins  50 mins

A’  trn

B’  WT

C’  BI

D  fushi-tarazu

E  wild-type  ftz++  ftz KD
Fig. S9. Boundary straightness comparisons between even and odd-numbered PSBs and ftz dsRNA knockdowns A-C) Plots showing the proportion of odd- and even-numbered PSB boundary interfaces that are greater than 60 degrees from AP in 3 tartan mutant (A,A’), 3 wildtype (B,B’) and 4 buffer injected embryos (C, C’). Odd and even-numbered PSBs are distinguished by the differential expression of EnVT15159-MS2 transcriptional dots. A loess curve (span 0.75) has been fitted to the data. Statistical comparison is a Kolmogorov-Smirnov non-parametric test undertaken on the cumulative frequencies of interface angles at 10 and 50 minutes (shown in A’-C’). D) ftz gene model (adapted from NCBI genome viewer) showing the dsRNA target sequence in exon 1 of ftz mRNA. E) Dark field microscopy images of cuticle preps from wildtype, ftz11 null and ftz dsRNA knockdown embryos. The ftz KD phenocopies the ftz null mutant, displaying the expected pair-rule phenotype.