Odd-paired controls frequency doubling in *Drosophila* segmentation by altering the pair-rule gene regulatory network

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ABSTRACT

Drosophila segmentation is mediated by a group of periodically expressed transcription factors known as the pair-rule genes. These genes are expressed dynamically, with many transitioning from double segment periodicity to single segment periodicity at gastrulation. The myriad cross-regulatory interactions responsible for these expression changes have been studied for over 30 years, however a systems level understanding of pair-rule patterning is still lacking. We carefully analysed the spatiotemporal dynamics of pair-rule gene expression, and found that frequency-doubling is precipitated by multiple coordinated regulatory changes. We identify the broadly expressed but temporally patterned transcription factor, Odd-paired (Opa), as the cause of these changes, and propose a new model for the patterning of the even-numbered parasegment boundaries, which relies on Opa-dependent regulatory interactions. Our findings indicate that the pair-rule gene regulatory network has temporally-modulated topology and dynamics, permitting stage-specific patterning roles.

INTRODUCTION

Segmentation is a developmental process that subdivides an animal body axis into similar, repeating units (Hannibal & Patel 2013). Segmentation of the main body axis underlies the body plans of arthropods, annelids and vertebrates (Telford et al. 2008; Balavoine 2014; Graham et al. 2014). In arthropods, segmentation first involves setting up polarised compartment boundaries early in development to define "parasegments" (Martinez-Arias & Lawrence 1985). Parasegment boundaries are maintained by an elaborate and strongly-conserved signalling network of "segment-polarity" genes (Sanson 2001; Janssen & Budd 2013).

In all arthropods yet studied, the segmental stripes of segment-polarity genes are initially patterned by a group of transcription factors called the pair-rule genes (Green & Akam 2013; Peel et al. 2005; Damen et al. 2005) The pair-rule genes were originally identified in a screen for mutations affecting the segmental pattern of the *Drosophila melanogaster* larval cuticle (Nüsslein-Volhard & Wieschaus 1980). They appeared to be required for the patterning of alternate segment boundaries (hence "pair-rule"), and were subsequently found to be expressed in stripes of double-segment periodicity (Hafen et al. 1984; Akam 1987).

Early models of *Drosophila* segmentation speculated that the blastoderm might be progressively patterned into finer-scale units by some reaction-diffusion mechanism that exhibited iterative frequency-doubling (reviewed in Jaeger 2009). The discovery of a double-segment unit of organisation seemed to support these ideas, and pair-rule patterning was therefore thought to be an adaptation to the syncitial environment of the early *Drosophila* embryo, which allows diffusion of gene products between neighbouring nuclei. However, the transcripts of pair-rule genes are apically localised during cellularisation of the blastoderm, and thus pair-rule patterning occurs in an effectively cellular environment (Edgar et al. 1987; Davis & Ish-Horowicz 1991). Furthermore, double-segment periodicity of pair-rule gene expression is also found in sequentially segmenting ("short germ") insects (Patel et al. 1994), indicating that pair-rule patterning predates the evolution of simultaneous ("long germ") segmentation (Figure 1).

The next set of models for pair-rule patterning were motivated by genetic dissection of the early regulation of the segment-polarity gene *engrailed* (*en*). It was found that odd-numbered *en* stripes – and thus odd-numbered parasegment boundaries – require the pair-rule gene *paired* (*prd*), but not another pair-rule gene *fushi tarazu* (*ftz*), while the opposite was true for the even-numbered *en* stripes and parasegment boundaries (DiNardo & O'Farrell 1987). Differential patterning of alternate segment-polarity stripes, combined with the observation that the different pair-rule genes are expressed with different relative phasings along the anterior-posterior (AP) axis, led to models where static, partially-overlapping domains of pair-rule gene expression form a combinatorial regulatory code that patterns the blastoderm

with single cell resolution (DiNardo & O'Farrell 1987; Ingham & Gergen 1988; Weir et al. 1988; Morrissey et al. 1991).

However, pair-rule gene expression domains are not static. One reason for this is that their upstream regulators, the gap genes, are themselves dynamically expressed, exhibiting expression domains that shift anteriorly over time (Jaeger et al. 2004; El-Sherif & Levine 2016). Another major reason is that, in addition to directing the initial expression of the segment-polarity genes, pair-rule genes also cross-regulate each other. Pair-rule proteins and transcripts turn over extremely rapidly (Edgar et al. 1986; Nasiadka & Krause 1999), and therefore regulatory feedback between the different pair-rule genes mediates dynamic pattern changes throughout the period that they are expressed. Most strikingly, many of the pair-rule genes undergo a transition from double-segment periodicity to single-segment periodicity at the end of cellularisation. The significance of this frequency-doubling is not totally clear. In some cases, the late, segmental stripes are crucial for proper segmentation (Cadigan et al. 1994b), in others they appear to be dispensable (Coulter et al. 1990; Fujioka et al. 1995), and in other cases their function (if any) is not known (Klingler & Gergen 1993; Jaynes & Fujioka 2004).

More recent models of pair-rule patterning recognise that the pair-rule genes form a complex gene regulatory network that mediates dynamic patterns of expression (Edgar et al. 1989; Sánchez & Thieffry 2003; Jaynes & Fujioka 2004). However, whereas other stages of *Drosophila* segmentation have been extensively studied from a dynamical systems perspective (reviewed in Jaeger 2009; Grimm et al. 2010; Jaeger 2011), we do not yet have a good systems-level understanding of the pair-rule gene network (Jaeger 2009). This appears to be a missed opportunity: not only do the pair-rule genes exhibit fascinating transcriptional regulation, but their interactions are potentially very informative for comparative studies with other arthropod model organisms. These include the beetle *Tribolium castaneum*, in which the pair-rule genes form a segmentation oscillator (Sarrazin et al. 2012; Choe et al. 2006).

We wanted to understand exactly how pair-rule patterning works in *Drosophila*. We therefore carried out a careful analysis of pair-rule gene regulation during cellularisation and gastrulation, drawing on both the genetic literature and a newly-generated dataset of double-fluorescent *in situs*. Surprisingly, we found that the majority of regulatory interactions between pair-rule genes are not constant, but undergo dramatic changes just before the onset of gastrulation. These regulatory changes mediate the frequency-doubling phenomena observed in the embryo at this time.

We then realised that all of the regulatory interactions specific to the late pair-rule gene regulatory network seem to require the non-canonical pair-rule gene *odd-paired* (*opa*). *opa* was identified through the original *Drosophila* segmentation screen as being required for the patterning of even-numbered parasegment boundaries (Jürgens et al. 1984). However, rather than being expressed periodically like the rest of the pair-rule genes, *opa* is expressed ubiquitously throughout the trunk region (Benedyk et al. 1994). The reported appearance of Opa protein temporally correlates with the time we see regulatory changes in the embryo, indicating that it may be directly responsible for these changes. We propose that Opa provides a source of temporal information that acts combinatorially with the spatial information provided by the periodically-expressed pair-rule genes. Pair-rule patterning thus appears to be a two-stage process that relies on the interplay of spatial and temporal signals to permit a common set of patterning genes to carry out stage-specific regulatory functions.

RESULTS

High-resolution spatiotemporal characterisation of wild-type pair-rule gene expression

We carried out double fluorescent *in situ* hybridisation on fixed wild-type *Drosophila* embryos for all pairwise combinations of the pair-rule genes *hairy*, *even-skipped* (*eve*), *runt*, *fushi tarazu* (*ftz*), *odd-skipped* (*odd*), *paired* (*prd*), and *sloppy-paired* (*slp*). Because the expression patterns of these genes evolve dynamically but exhibit little embryo-to-embryo variability (Surkova et al. 2008; Little et al. 2013; Dubuis et al. 2013), we were able to order images of individual embryos by inferred developmental age. This allowed us to produce pseudo time-series that illustrate how pair-rule gene expression patterns change relative to one another during early development (Figure 2).

The expression profile of each individual pair-rule gene has been carefully described previously (Hafen et al. 1984; Ingham & Pinchin 1985; Macdonald et al. 1986; Kilchherr et al. 1986; Gergen & Butler 1988; Coulter et al. 1990;

Grossniklaus et al. 1992), and high quality relative expression data are available for pair-rule proteins (Pisarev et al. 2009). In addition, expression atlases facilitate the comparison of staged, averaged expression profiles of many different blastoderm patterning genes at once (Fowlkes et al. 2008). However, because the pair-rule genes are expressed extremely dynamically and in very precise patterns, useful extra information can be gleaned by directly examining relative expression patterns in individual embryos. In particular, we have found these data invaluable for understanding exactly how stripe phasings evolve over time, and for interrogating regulatory hypotheses. In addition, we have characterised pair-rule gene expression up until early germband extension, whereas blastoderm expression atlases stop at the end of cellularisation.

Our entire wild-type dataset (23 gene combinations, >500 individual embryos) is available from the authors upon request. We hope it proves useful to the *Drosophila* community.

Three main phases of pair-rule gene expression

We classify the striped expression of the pair-rule genes into three temporal phases (Figure 3A). Phase 1 (equivalent to phase 1 of Schroeder et al. 2011) corresponds to early cellularisation, before the blastoderm nuclei elongate. Phase 2 (spanning phases 2 and 3 of Schroeder et al. 2011) corresponds to mid cellularisation, during which the plasma membrane progressively invaginates between the elongated nuclei. Phase 3 (starting at phase 4 of Schroeder et al. 2011 but continuing beyond it) corresponds to late cellularisation and gastrulation. Our classification is a functional one, based on the times at which different classes of pair-rule gene regulatory elements (Figure 3B) have been found to be active in the embryo.

During phase 1, expression of specific stripes is established through compact enhancer elements mediating gap gene inputs (Pankratz & Jackle 1990). *hairy*, *eve* and *runt* all possess a full set of these "stripe-specific" elements, while *ftz* lacks an element for stripe 4, and *odd* lacks elements for stripes 2, 4 and 7 (Schroeder et al. 2011). These five genes are together classified as the "primary" pair-rule genes, because in all cases the majority of their initial stripe pattern is established *de novo* by non-periodic regulatory inputs. The regulation of various stripe-specific elements by gap proteins has been studied extensively (for example Small et al. 1992; Small et al. 1996).

Phase 2 is dominated by the expression of so-called "zebra" (or "7-stripe") elements (Hiromi et al. 1985; Dearolf et al. 1989; Butler et al. 1992). These elements, which tend to be relatively large (Gutjahr et al. 1994; Klingler et al. 1996; Schroeder et al. 2011), are regulated by pair-rule gene inputs and thus produce periodic output patterns. The stripes produced from these elements overlap with the stripes generated by stripe-specific elements, and often the two sets of stripes appear to be at least partially redundant. For example, *ftz* and *odd* lack a full complement of stripe-specific elements (see above), while the stripe-specific elements of *runt* are dispensable for segmentation (Butler et al. 1992). *hairy* and *eve* do not possess zebra elements, and thus their expression during phase 2 is driven entirely by their stripe-specific elements. We classify the "late" (or "autoregulatory") element of *eve* (Goto et al. 1989; Harding et al. 1989) as part of phase 3 rather than phase 2, since this element turns on considerably after other zebra elements (Schroeder et al. 2011).

In addition to the five primary pair-rule genes, there are two other pair-rule genes, *prd* and *slp*, that turn on after regular periodic patterns of the other genes have been established. These genes possess only a single, anterior stripe-specific element, and their trunk stripes are generated by a zebra element alone (Schroeder et al. 2011). Because (ignoring the head stripes) these genes are regulated only by other pair-rule genes, and not by gap genes, they are termed the "secondary" pair-rule genes.

The third, "late" phase of expression is the least understood. Around the time of gastrulation, all of the pair-rule genes except *hairy* and *ftz* undergo a transition from double-segmental stripes to single-segmental stripes. For *prd*, this happens by splitting of its early, broad pair-rule stripes. In contrast, *eve*, *odd*, *runt* and *slp* show intercalation of "secondary" stripes between their "primary" 7-stripe patterns, although in the case of *eve* these secondary stripes are very weak. In some cases, discrete enhancer elements have been found that mediate just the secondary stripes (Klingler et al. 1996), while in other cases all 14 segmental stripes are likely to be regulated coordinately (Fujioka et al. 1995). In certain cases, non-additive interactions between enhancers play a role in generating the segmental pattern (Prazak et al. 2010; Gutjahr et al. 1994). The functional significance of the late patterns is unclear, since they are usually not reflected

in pair-rule gene mutant cuticle phenotypes (Kilchherr et al. 1986; Coulter et al. 1990).

In the remainder of this paper, we investigate the nature and causes of the pattern transitions that occur between the end of phase 2 and the beginning of phase 3. A detailed analysis of the timing and dynamics of pair-rule gene expression during phase 2 will be covered elsewhere.

Frequency-doubling of different pair-rule gene expression patterns is almost simultaneous, and coincides with segment-polarity gene activation

As noted above, five of the seven pair-rule genes undergo a transition from double-segment periodicity to single-segment periodicity at the end of cellularisation (Figure 3). These striking pattern changes could be caused simply by feedback interactions within the pair-rule and segment-polarity gene networks. Alternatively, they could be precipitated by some extrinsic temporal signal (or signals).

Comparing between genes, we find that the pattern changes develop almost simultaneously (Figure 4; Figure 4–figure supplement 1), although there are slight differences in the times at which the first signs of frequency-doubling become detectable. (The splitting of the trunk *prd* stripes can be detected just before the *odd* secondary stripes start to appear and the *eve* stripes start to sharpen, which is just prior to the appearance of the secondary stripes of *slp* and *runt*). These events appear to be spatiotemporally modulated: there is a short but noticeable AP time lag, and also a DV pattern – frequency-doubling occurs first mid-laterally, and generally does not extend across the dorsal midline. In addition, the secondary stripes of *slp* are not expressed in the mesoderm, while the ventral expression of *odd* secondary stripes is only weak.

We also investigated the timing of the frequency-doubling events relative to the appearance of expression of the segment-polarity genes *en*, *gooseberry* (*gsb*) and wingless (*wg*) (Figure 4; Figure 4–figure supplement 2). We find that the spatiotemporal pattern of segment-polarity gene activation coincides closely with that of pair-rule frequency-doubling – starting at the beginning of phase 3, and rapidly progressing over the course of gastrulation. Only around 20 minutes separate a late stage 5 embryo (with double-segment periodicity of pair-rule gene expression and no segment-polarity gene expression) from a late stage 7 embryo (with regular segmental expression of both pair-rule genes and segment-polarity genes) (Campos-Ortega & Hartenstein 1985).

We can make three conclusions from the timing of these events. First, segment-polarity gene expression cannot be precipitating the frequency-doubling of pair-rule gene expression, because frequency-doubling occurs before segment-polarity proteins would have had time to be synthesised. Second, the late, segmental patterns of pair-rule gene expression do not play a role in regulating the initial expression of segment-polarity genes, because they are not reflected at the protein level until after segmental expression patterns of segment-polarity genes are observed. Third, the synchrony of pair-rule gene frequency-doubling and segment-polarity gene activation is consistent with co-regulation of these events by a single temporal signal.

The transition to single-segment periodicity is mediated by altered regulatory interactions

It is clear that a dramatic change overtakes pair-rule gene expression at gastrulation. For a given gene, an altered pattern of transcriptional output could result from an altered spatial pattern of regulatory inputs, or, alternatively, altered regulatory logic. Pair-rule proteins provide most of the spatial regulatory input for pair-rule gene expression at both phase 2 and phase 3. Therefore, the fact that the distributions of pair-rule proteins are very similar at the end of phase 2 and the beginning of phase 3 (Pisarev et al. 2009) suggests that it must be the "input-output functions" of pair-rule gene transcription that change to bring about the new expression patterns.

In this section we carefully examine pair-rule gene stripe phasings just before and just after the double-segment to single-segment transition. We find that these patterns do indeed indicate significant changes to the control logic of multiple pair-rule genes. Important conclusions from this section are summarised at the beginning of the next section.

paired (Figure 5)

Before frequency-doubling, the *prd* expression pattern is the additive result of broad stripes of medium intensity, and intense two-cell wide stripes at the posterior of each of the broad stripes ("P" stripes). The two sets of stripes are mediated by separate stretches of DNA (Gutjahr et al. 1994).

There is abundant experimental evidence that the splitting of the *prd* stripes is caused by direct repression by Odd protein. The primary stripes of *odd* lie within the broad *prd* stripes, and the secondary interstripes that form within these *prd* stripes at gastrulation correspond precisely to those cells that express *odd* (Figure 5). Furthermore, the *prd* stripes do not split in *odd* mutant embryos (Baumgartner & Noll 1990; Saulier-Le Dréan et al. 1998), and the broad *prd* stripes (although not the "P" stripes) are completely repressed by ectopically-expressed Odd protein (Saulier-Le Dréan et al. 1998; Goldstein et al. 2005).

However, prior to *prd* stripe splitting, *prd* and *odd* are co-expressed in the same cells, with no sign that *prd* is sensitive to repression by Odd (Figure 5). Because *prd* expression begins at a time when Odd protein is already present (Pisarev et al. 2009), this co-expression cannot be explained by protein synthesis delays. We therefore infer that Odd only becomes a repressor of *prd* at gastrulation, consistent with previous observations that aspects of Odd regulatory activity are temporally restricted (Saulier-Le Dréan et al. 1998). Other aspects of *prd* regulation will be discussed elsewhere (manuscript in preparation).

odd-skipped (Figure 6)

During phase 2, the primary stripes of *odd* have anterior boundaries defined by repression by Eve, and posterior boundaries defined by repression by Hairy (Manoukian & Krause 1992; Jiménez et al. 1996; and data not shown). These primary stripes narrow during phase 3, mainly from the posterior, and secondary stripes intercalate between them. It is not known whether all components of the single-segmental pattern observed at phase 3 are driven by a single enhancer, but we think it likely. The following analysis assumes that primary and secondary stripes of *odd* are governed by identical regulatory logic during phase 3.

The secondary stripes arise within cells expressing both Eve and Hairy, indicating that repression of *odd* by these proteins is restricted to phase 2. A loss of repression by Hairy during phase 3 is also supported by increased overlaps between *hairy* and the *odd* primary stripes. The posterior boundaries of the *odd* secondary stripes appear to be defined by repression by Runt. In wild-type embryos, these boundaries precisely abut the anterior boundaries of the *runt* primary stripes, whereas in *runt* mutant embryos they expand posteriorly (Jaynes & Fujioka 2004). However, *odd* is evidently not repressed by Runt during phase 2, because the *odd* primary stripes overlap with the posterior of the *runt* stripes.

Thus there appear to be multiple changes to the regulation of *odd* between phase 2 and phase 3: loss of repression by Eve and Hairy, and gain of repression by Runt. The lack of repression by Eve and Hairy does not compromise the late patterning of the primary *odd* stripes, because their patterning roles are taken over by new repressors. Slp protein appears at the end of cellularisation and takes over from Hairy at the posterior boundaries (Jaynes & Fujioka 2004). The new repression from Runt (and later, from En) seems to take over from Eve at the anterior boundaries (see below).

sloppy-paired (Figure 7)

The primary stripes of *slp* appear at the end of phase 2, while the secondary stripes appear shortly afterwards, at the beginning of phase 3. In contrast to the other pair-rule genes, *slp* stripes are static and stable, with dynamic pattern refinements restricted to the head region. The *slp* locus has a large, complex regulatory region, with many partially redundant enhancer elements (Fujioka & Jaynes 2012). A detailed study of two of these elements showed that the primary stripes are mediated by one element, while the secondary stripes require an additional enhancer that interacts non-additively with the first element (Prazak et al. 2010).

The primary stripes of *slp* are thought to be patterned by repression from Eve at their posteriors and repression by the

combination of Runt and Ftz at their anteriors (Swantek & Gergen 2004). There is plentiful evidence for repression of *slp* by Eve throughout segmentation (Fujioka et al. 1995; Riechmann et al. 1997; Jaynes & Fujioka 2004; Swantek & Gergen 2004; Prazak et al. 2010). However, while the posterior boundaries of the Runt primary stripes do appear to define the anterior boundaries of the *slp* primary stripes, we are not convinced that Runt and Ftz act combinatorially to repress *slp* (Figure 8–figure supplement 1).

We find that in *ftz* mutant embryos, the *slp* primary stripes form fairly normally during phase 2, with their anterior boundaries still seemingly defined by Runt, rather than expanding anteriorly to overlap the (Eve-negative) posterior halves of the *runt* stripes. Ectopic *slp* expression does not appear until phase 3. This indicates that Runt is able to repress *slp* in the absence of Ftz, at least temporarily. We therefore propose that during phase 2, *slp* is repressed by both Eve and Runt, regardless of whether Ftz is present, and that the anterior boundaries of the *slp* primary stripes are initially patterned by Runt alone.

During phase 3, the *slp* secondary stripes appear in the anterior halves of the *runt* stripes. There are competing models for how they are regulated. One model proposes that they are activated by Runt, but repressed by the combination of Runt and Ftz, so that their anterior boundary is defined by Runt and their posterior boundary is defined by Ftz (Swantek & Gergen 2004; Prazak et al. 2010). A different model proposes that their anterior boundaries are defined by repression by Eve, while their posterior boundaries are defined by repression by Odd (Jaynes & Fujioka 2004).

The posterior borders of the *eve* primary stripes abut the anterior borders of the *runt* primary stripes during phase 3. Mutual repression between Eve and Runt (Ingham & Gergen 1988; Manoukian & Krause 1992; Manoukian & Krause 1993; Klingler & Gergen 1993) stabilises these expression boundaries, which also correspond to the anterior boundaries of the *slp* secondary stripes. Because of the regulatory feedback between Eve and Runt, the distinct regulatory hypotheses of repression by Eve *versus* activation by Runt actually predict identical effects on the expression of *slp* in a variety of genetic backgrounds. Therefore, much of the experimental evidence cited in favour of each of these models does not really discriminate between them.

When we look carefully at the early expression of the *slp* secondary stripes, we occasionally find *slp* expression in a *runt*-negative cell, but we never observe cells expressing both *eve* and *slp*. This indicates that Eve directly patterns the anterior boundaries of the *slp* secondary stripes, while the regulatory role of Runt is indirect. Consistent with this hypothesis, a reporter study found that Runt did not appear to directly regulate a *slp* enhancer that drives 14 stripes at phase 3 (Sen et al. 2010; Fujioka & Jaynes 2012).

While *ftz* and *odd* are subject to similar regulation during phase 2 and consequently have similar expression domains, the slightly broader Ftz stripes appear to define the posterior boundary of *slp* secondary stripe expression. This does not rule out Odd as a repressor of *slp*, however. Indeed, experimental evidence supports direct repression of *slp* by Odd (Saulier-Le Dréan et al. 1998) as well as by Ftz (Nasiadka & Krause 1999; Swantek & Gergen 2004; Prazak et al. 2010).

We see no compelling evidence that the repressive activity of Ftz on *slp* is mediated by Runt. It is clear that the presence or absence of Runt has dramatic effects on the expression pattern of *slp*, and that this is modified by the presence or absence of Ftz (Swantek & Gergen 2004; Prazak et al. 2010). However, we think that these effects are likely to be explained either by indirect interactions or by the repressive role of Runt during phase 2 (see above).

We thus conclude that regulation of *slp* undergoes several changes at phase 3. Repression by Runt is lost, while repression by Ftz and Odd is gained. Our proposed repressive role of Runt is in contrast to previous reports that Runt activates *slp*. Also in contrast to previous reports, we do not find evidence for a combinatorial interaction between Ftz and Runt. Instead, we think that their roles are temporally separate, with Runt acting at phase 2 and Ftz acting at phase 3.

runt (Figure 8)

During phase 2, the primary stripes of *runt* are broadly out of phase with those of *hairy*. There is clear evidence for repression of *runt* by Hairy (Ingham & Gergen 1988; Klingler & Gergen 1993; Jiménez et al. 1996), and it is commonly

thought that Hairy defines both the anterior and posterior boundaries of *runt* expression (e.g. Edgar et al. 1989; Schroeder et al. 2011). However, we find clear gaps between the posterior boundaries of *runt* expression and the anterior boundaries of *hairy* expression, indicating that some other pair-rule gene must be repressing *runt* from the posterior. We propose that the posterior boundaries of the *runt* primary stripes are defined by repression from Odd. This hypothesis is strongly supported by the observations that the *runt* stripes widen slightly in *odd* mutant embryos and are directly repressed by ectopic Odd (Saulier-Le Dréan et al. 1998).

During phase 3, new *runt* expression appears to the posterior of the primary stripes, and gradually intensifies to form the secondary stripes. At the same time, the primary stripes narrow from the posterior, producing a "splitting" of the broadened *runt* domains (Klingler & Gergen 1993). The two sets of stripes are initially driven by different enhancers, although each of the two enhancers later drive 14 segmental stripes during germband extension (Klingler et al. 1996). This indicates that the primary and secondary *runt* stripes are subject to different regulatory logic during phase 3

During cellularisation, the anterior of each *runt* stripe overlaps with *eve* expression, and accordingly Eve does not appear to repress *runt* during this stage (Manoukian & Krause 1992). However, Eve starts to repress *runt* at phase 3 (Manoukian & Krause 1992; Klingler & Gergen 1993). Eve appears to act on both sets of *runt* stripes, defining the posterior boundaries of the secondary stripes as well as the anterior boundaries of the primary stripes.

It has been hypothesised that the narrowing of the *runt* primary stripes is caused by direct repression by Ftz (Klingler & Gergen 1993; Wolff et al. 1999). However, this is not supported by Ftz misexpression (Nasiadka & Krause 1999). Indeed, we find that the posteriors of the *runt* primary stripes continue to overlap with the anteriors of the *ftz* stripes for a considerable period during phase 3 ruling out direct repression by Ftz. Instead, the posteriors of the *runt* primary stripes appear to be repressed by the even-numbered En stripes, which are activated by Ftz (Klingler & Gergen 1993; DiNardo & O'Farrell 1987). Before the appearance of En protein, the posterior boundaries continue to be defined by repression from Odd.

We have not investigated whether Hairy continues to repress the regulatory element driving the *runt* primary stripes during phase 3, although it is possible it does not. However, it is clear that Hairy does not repress the element driving the *runt* secondary stripes, because they are located within domains of *hairy* expression. The secondary stripes also overlap with Odd expression, indicating that, unlike the primary stripes, they are not sensitive to repression by Odd.

It is not clear what defines the anterior boundaries of the *runt* secondary stripes. The locations of these stripes correlate very closely with those of the *slp* primary stripes, in both wild-type and *ftz* mutant embryos. However, because *runt* expression is not noticeably affected in *slp* mutant embryos (Klingler & Gergen 1993), this must result from shared regulation rather than a patterning role for Slp itself. Indeed, Eve defines the posterior boundaries of both the *slp* primary stripes and the *runt* secondary stripes (see above). The anterior boundaries of the *slp* primary stripes are defined by repression by the Runt primary stripes, raising the possibility that the *runt* secondary stripes are regulated in the same way. If true, this would be the first example of direct autorepression by a pair-rule gene during segmentation.

Finally, Prd is required for the expression of the secondary stripes (Klingler & Gergen 1993). Prd appears to provide general activatory input to the element driving the stripes, but is unlikely to convey specific positional information, because the expression boundaries of the Prd stripes do not correspond to those of the *runt* secondary stripes. Prd is also unlikely to provide temporal information to the element: the expression of the *runt* secondary stripes is delayed relative to the appearance of Prd protein (Pisarev et al. 2009), suggesting that Prd alone is not sufficient for their activation.

In summary, there is one important change to the regulation of the *runt* zebra element at phase 3. Repression by Eve is gained, and may potentially replace repression by Hairy. In addition, a separate element driving the secondary stripes begins to be expressed at phase 3. This element appears to be repressed by Eve and perhaps Runt, and activated by Prd.

even-skipped

eve does not possess a zebra element active during phase 2, and therefore its regulation does not come under control of the pair-rule network until its "late" element turns on at phase 3. This element generates strong expression in the anterior halves of the pre-existing early *eve* stripes. The posterior boundaries of the late stripes are defined by repression

by Runt, while the anterior boundaries are defined by repression by Slp (Jaynes & Fujioka 2004). Odd also represses late *eve* (Saulier-Le Dréan et al. 1998), and will temporarily compensate for the lack of repression by Slp in *slp* mutant embryos (Jaynes & Fujioka 2004). The late *eve* stripes do not persist long after gastrulation, largely owing to the appearance of En protein, another repressor of *eve* (Harding et al. 1986).

In addition to the strong "major" stripes at the anteriors of the odd-numbered parasegments, faint "minor" stripes of *eve* expression appear during gastrulation in the anteriors of the even-numbered parasegments (Macdonald et al. 1986; Frasch et al. 1987). These stripes are also driven by the late element (Fujioka et al. 1995), and are therefore likely to share the same regulatory logic as the major stripes. They do not appear to play any role in patterning, since deletions of the *eve* late element do not affect the patterning of the even-numbered parasegment boundaries (Fujioka et al. 1995; Fujioka et al. 2002).

Other pair-rule genes

In contrast to the other pair-rule genes, *hairy* and *ftz* do not show signs of significantly altered spatial regulation at gastrulation. The *hairy* stripes, which are regulated by stripe-specific elements, begin to fade away. During phase 2, the anterior boundaries of the *ftz* stripes are defined by repression by Eve, while the posterior boundaries are defined by repression by Hairy (Ish-Horowicz & Pinchin 1987; Carroll et al. 1988; Frasch et al. 1988; Ingham & Gergen 1988; Vavra & Carroll 1989; Manoukian & Krause 1992; Jiménez et al. 1996). The *ftz* stripes narrow from the posterior at phase 3, but this appears to be simply due to the new appearance of Slp protein, which also represses *ftz* (Cadigan et al. 1994b), rather than evidence for altered regulatory logic.

A candidate temporal signal: Odd-paired

To summarise the results of the previous section, a number of regulatory interactions seem to disappear at the beginning of phase 3: repression of *odd* by Hairy, repression of *odd* by Eve, and repression of *slp* by Runt. These regulatory interactions are replaced by a number of new interactions: repression of *prd* by Odd, repression of *odd* by Runt, repression of *runt* by Eve, and repression of *slp* by Ftz. At the same time that these regulatory changes are observed, new elements for *eve* and *runt* turn on and various segment-polarity genes start to be expressed.

Having identified all of these regulatory changes, we wanted to know how they are made to happen in the embryo. Because they all occur within a very short time window (Figure 4), they could potentially all be regulated by a single temporal signal that would instruct a regulatory switch. We reasoned that if this hypothetical signal were absent, the regulatory changes would not happen. This would result in a mutant phenotype in which frequency-doubling events do not occur, and segment-polarity expression is delayed.

We then realised that this hypothetical phenotype was consistent with descriptions of segmentation gene expression in mutants of the non-canonical "pair-rule" gene, *opa* (Benedyk et al. 1994). This gene is required for the splitting of the *prd* stripes and the appearance of the secondary stripes of *odd* and *slp* (Baumgartner & Noll 1990; Benedyk et al. 1994; Swantek & Gergen 2004). It is also required for the late expression of *runt* (Klingler & Gergen 1993), and for the timely expression of *en* and *wg* (Benedyk et al. 1994). In contrast, *ftz*, which does not exhibit altered regulation at gastrulation, is expressed normally in *opa* mutant embryos (Benedyk et al. 1994).

The *opa* locus was originally isolated on account of its cuticle phenotype, in which odd-numbered segment boundaries (corresponding to even-numbered parasegment boundaries) are lost (Jürgens et al. 1984). For many years afterwards, *opa* was assumed to be expressed in a periodic pattern of double-segment periodicity similar to the other seven pair-rule genes (for example, Coulter & Wieschaus 1988; Ingham et al. 1988; Weir et al. 1988; Baumgartner & Noll 1990; Lacalli 1990). When *opa*, which codes for a zinc finger transcription factor, was finally cloned, it was found - surprisingly - to be expressed uniformly throughout the trunk (Benedyk et al. 1994). Presumed to be therefore uninstructive for spatial patterning, it has received little interest in the context of segmentation since. However, we realised that Opa could still be playing an important role in spatial patterning. By providing temporal information that would act combinatorially with the spatial information carried by the canonical pair-rule genes, Opa might permit individual pair-rule genes to carry out different patterning roles at different points in time.

Expression of *opa* spatiotemporally correlates with patterning events

We examined *opa* expression relative to other segmentation genes, and found an interesting correlation with the spatiotemporal pattern of segmentation (Figure 9). As previously reported, the earliest expression of *opa* is in a band at the anterior of the trunk, which we find corresponds quite closely with the head stripe of *prd* (data not shown). Expression in the rest of the trunk quickly follows, and is stronger ventrally than dorsally. *opa* begins to be transcribed throughout the trunk during phase 1, before regular patterns of pair-rule gene expression emerge. The sharp posterior border of the *opa* domain at first lies just anterior to *odd* stripe 7, but gradually shifts posteriorly over the course of gastrulation to encompass it. Notably, *odd* stripe 7 is the last of the primary pair-rule gene stripes to appear, and segmentation of this posterior region of the embryo appears to be significantly delayed relative to the rest of the trunk (Kuhn et al. 2000).

The timing of *opa* transcription has been shown to rely on nuclear / cytoplasmic ratio (Lu et al. 2009), and begins relatively early during cellularisation. However, it takes a while for the *opa* expression domain to reach full intensity. Unlike the periodically-expressed pair-rule genes, which have compact transcription units (all <3.5 kb, FlyBase) and are therefore rapidly synthesised, the *opa* transcription unit is large (~17 kb, FlyBase), owing mainly to a large intron. Accordingly, during most of cellularisation we observe a punctate distribution of *opa*, suggestive of nascent transcripts located within nuclei (Figure 9–figure supplement 1). Unfortunately, the available polyclonal antibody against Opa (Benedyk et al. 1994) did not work well in our hands, so we have not been able to determine precisely what time Opa protein first appears in blastoderm nuclei. However, Opa protein levels have been reported to peak at late cellularisation and into gastrulation (Benedyk et al. 1994), corresponding to the time at which we observe regulatory changes in the embryo, and consistent with our hypothesised role of Opa as a temporal signal.

opa mutant embryos do not transition to single-segment periodicity at gastrulation

If our hypothesised role for Opa is correct, patterning of the pair-rule genes should progress normally in *opa* mutant embryos up until the beginning of phase 3, but not undergo the dramatic pattern changes observed at this time in wild-type. Instead, we would expect that the double-segmental stripes would persist unaltered, at least while the activators of phase 2 expression remain present. The pair-rule gene expression patterns that have been previously described in *opa* mutant embryos (those of *prd*, *slp*, *odd*, *runt* and *ftz*, see above) seem consistent with this prediction, however we wanted to characterise the *opa* mutant phenotype in more detail in order to be sure.

During cellularisation, we find that pair-rule gene expression is relatively normal in *opa* mutant embryos (Figure 10A), consistent with our hypothesis that this phase of expression is not regulated by Opa. The one exception is that the appearance of the *slp* primary stripes may be slightly delayed compared to wild-type. These stripes normally appear towards the end of cellularisation, only shortly before the secondary stripes appear at phase 3.

In contrast, pair-rule gene expression becomes dramatically different from wild-type at gastrulation (Figure 10B). Most notably, the transition from double-segment to single-segment periodicity is not observed for any pair-rule gene. As previously reported (Benedyk et al. 1994; Swantek & Gergen 2004), the secondary stripes of *odd* and *slp* do not appear. The *prd* stripes do not split (Baumgartner & Noll 1990), although we note that cells in the centres of the stripes do exhibit markedly less intense expression than those at the anterior and posterior edges. The *ftz* stripes persist as normal (Benedyk et al. 1994), although they seem a little wider than wild-type, perhaps owing to the delayed expression of the *slp* primary stripes. *hairy* expression fades away as normal (data not shown). *eve* expression in *opa* mutant embryos has not to our knowledge been previously described. We find that *eve* expression fades away at gastrulation, with no sign of the sharpened “late” expression normally activated in the anteriors of the early stripes. Finally, as previously reported (Klingler & Gergen 1993), *runt* expression is much reduced; only primary stripes 6 and 7 continue to be expressed strongly, while the secondary stripes appear but are irregular and weak.

In summary, *odd*, *slp*, *prd* and *ftz* remain expressed strongly in stripes of double-segment periodicity, similar to their expression at the end of phase 2, while expression of *hairy*, *eve* and *runt* is largely lost.

Opa accounts for the regulatory changes observed at gastrulation

Many of the altered expression patterns in *opa* mutant embryos (Figure 10B) appear to directly reflect an absence of the regulatory changes normally observed in wild-type at gastrulation. The altered *prd* expression in Opa mutants is consistent with Odd continuing not to repress *prd*, indicating that Odd only acts as a repressor of *prd* in combination with Opa. Similarly, the absence of the secondary stripes of *odd* and *slp* suggest that Eve continues to repress *odd* in the absence of Opa and Runt continues to repress *slp*.

Whereas the expression of *prd*, *slp* and *odd* persists strongly in *opa* mutant embryos, albeit in abnormal patterns, the late expression of *eve* and *runt* is either absent or strongly reduced. This indicates first that the activators that drive expression of these genes during phase 2 do not persist in the embryo after the end of cellularisation, and second that the expression of these genes during phase 3 is directly activated by the new appearance of Opa. This is not too surprising for *eve*, which has phase 2 expression driven by stripe-specific elements and phase 3 expression driven by a separate element. Expression of stripe-specific elements is known to fade away at gastrulation, as seen for the entire *hairy* pattern (Ingham et al. 1985), or for stripe-specific reporter elements (Bothma et al. 2014). However, a single stretch of DNA drives *runt* primary stripe expression at both phase 2 and phase 3 (Klingler et al. 1996). This suggests that the organisation and regulatory logic of this element may be complex, as it is evidently activated by different factors at different times.

We have not investigated whether Hairy still represses its targets after gastrulation in *opa* mutant embryos. However, all of the other phase-specific regulatory interactions we detected in wild-type appear to be modulated by Opa, and thus explained by the onset of Opa regulatory activity at gastrulation. Therefore, the presence or absence of Opa significantly affects the topology of the pair-rule gene regulatory network.

opa mutant embryos fail to pattern the even-numbered parasegment boundaries because they lack Slp secondary stripes

We also examined pair-rule gene expression in *opa* mutant embryos during early germband extension (Figure 10–figure supplement 1). Interestingly, some recovery of the normal pattern of *odd*, *prd* and *runt* is observed. The *prd* stripes eventually split, while weak secondary stripes of *odd* appear in some segments. Having faded away at gastrulation, *runt* expression becomes re-established. *runt* appears to be expressed everywhere except the presumptive odd-numbered parasegment anteriors, and is expressed particularly strongly in the presumptive even-numbered parasegment posteriors, which would normally express *runt* in wild-type. In addition, it has previously been shown that segment-polarity gene expression partially recovers during germband extension (Benedyk et al. 1994; Ingham & Martinez-Arias 1986), suggesting that segmentation gene expression becomes largely Opa-independent at this stage.

We do also observe abnormalities of certain stripe widths and spacings, for example the *ftz* stripes appear wider than wild-type, and "pairing" of the segmental *prd* stripes is observed, with the gaps corresponding to the initial interstripes appearing wider than those formed by the splitting of pair-rule stripes. However, since cell intercalation occurs during germband extension and is indirectly controlled by pair-rule gene expression patterns (Irvine & Wieschaus 1994; Paré et al. 2014), it is not immediately clear whether these specific abnormalities derive from altered gene regulation or altered morphogenesis.

Why exactly do *opa* mutants fail to pattern the even-numbered parasegment boundaries? The most significant difference in the final pattern of pair-rule gene expression between wild-type and *opa* mutant embryos appears to be the absence of the secondary stripes of *slp* (Figure 10–figure supplement 2). *opa* mutant embryos also never express odd-numbered *wg* stripes (Benedyk et al. 1994). These *wg* stripes are normally located within the *slp* secondary stripes, and Slp is known to be required for the expression of *wg* during germband extension (Cadigan et al. 1994b). The absence of the odd-parasegment *wg* stripes in *opa* mutant embryos leads to the eventual loss of the adjacent even-parasegment *en* stripes, because persistent *en* expression must be maintained by Wingless signaling from neighbouring cells (Benedyk et al. 1994; DiNardo et al. 1988; Vincent & Lawrence 1994). We therefore surmise that the failure to activate the secondary stripes of *slp* is the root cause of the pair-rule phenotype of *opa* mutants.

Opa appears to activate the *eve* late element

The element driving "late" *eve* expression is sometimes referred to as the *eve* "autoregulatory" element, because expression from it is lost in *eve* mutant embryos (Harding et al. 1989; Jiang et al. 1991). However, the observed "autoregulation" appears to be indirect (Goto et al. 1989; Manoukian & Krause 1992; Fujioka et al. 1995; Sackerson et al. 1999). Instead of being directly activated by Eve, the element mediates regulatory inputs from repressors such as Runt and Slp, which are ectopically expressed in *eve* mutant embryos (Vavra & Carroll 1989; Klingler & Gergen 1993; Riechmann et al. 1997; Jaynes & Fujioka 2004). The element is thought to be activated by Prd, and functional *prd* binding sites have been demonstrated within the element (Fujioka et al. 1996). However, while Prd protein appears at roughly the right time to activate the *eve* late element (Pisarev et al. 2009), we do not think that activation by Prd is an adequate explanation for the expression generated from this element, because much of the early expression from this element occurs in cells that do not express *prd* (Figure 11–figure supplement 1).

Instead, we suggest that the *eve* late element may be directly activated by Opa. In *opa* mutant embryos, the strong, sharply-defined expression that normally appears in the anteriors of the *eve* stripes at phase 3 is not observed (except for stripe 1), leaving only the weaker and broader stripe domains generated by the stripe specific elements (Figure 11). This is similar to what is observed in embryos in which the late element has been deleted (Fujioka et al. 1995; Fujioka et al. 2002). We think that the lack of late *eve* expression in *opa* mutant embryos results from a failure to activate the late element, rather than the ectopic expression of repressive inputs, since none of *runt*, *odd* or *slp* are ectopically expressed in the domains where *eve* late element expression would normally be seen (Figure 10).

A new model for the patterning of the even-numbered *engrailed* stripes

One particularly intriguing feature of *opa* mutant embryos is that the offset between the anterior boundaries of the *ftz* and *odd* stripes is largely absent (Benedyk et al. 1994; Figure 12). In wild-type embryos, the anterior boundaries of the *odd* primary stripes are shifted posteriorly relative to those of the *ftz* stripes by about one cell row. This relative phasing is responsible for patterning the even-numbered *en* stripes, which are activated by Ftz but repressed by Odd (Coulter et al. 1990; Manoukian & Krause 1992; Mullen & DiNardo 1995).

The offsets between the anterior boundaries of *ftz* and *odd* require the presence of the early Eve stripes (Fujioka et al. 1995). It is thought that the posterior halves of these stripes act as morphogen gradients that repress *odd* at lower concentrations of Eve than required to repress *ftz*, and thus differentially position the expression domains of the two genes (Fujioka et al. 1995; Manoukian & Krause 1992). We find this explanation unsatisfactory, for two reasons.

First, a careful analysis of wild-type gene expression calls into question the hypothesis that the early Eve stripes are functioning in this manner. Both *ftz* and *odd* lack a stripe-specific element for stripe 4, and so the expression seen in these stripes is a true reflection of regulatory control by pair-rule proteins, whereas inferences from the remaining stripes are complicated by gap protein-regulated contributions to the overall expression pattern. When the zebra element-driven expression of *ftz* and *odd* kicks in and stripe 4 appears, clear one cell wide offsets are seen at the anterior borders of most of the stripes, but are absent from stripe 4 (Figure 12). This suggests that Eve is not differentially regulating the two genes, and that the offsets that are seen in the other stripes are instead generated by bespoke positioning of individual stripes by stripe-specific elements.

Secondly, maintenance of the offsets between *ftz* and *odd* expression seems to require Opa function (Figure 12). In wild-type embryos, offsets are observed at gastrulation for all stripes, including stripe 4, indicating that *ftz* and *odd* must be differentially regulated by pair-rule proteins at this later stage. In *opa* mutant embryos, we find that the relative phasing of *ftz* and *odd* appears normal at cellularisation (with offsets present for most stripes, but absent for stripe 4). By gastrulation, however, the anterior boundaries of the two sets of stripes often coincide. We therefore do not think that the early Eve stripes can be directly patterning the offsets, because early *eve* expression is normal in *opa* mutant embryos. Late *eve* expression is lost in *opa* mutant embryos (see above), but this phase of expression cannot be regulating the pattern either, because *eve* rescue constructs lacking the *eve* late element still produce the offsets (Fujioka et al. 1995). Therefore, the offsets must be patterned by a pair-rule protein other than Eve, by way of an Opa-dependent regulatory interaction.

Coincident anterior boundaries of *ftz* and *odd* could be produced by a posterior retraction of *ftz* expression, or alternatively by an anterior expansion of *odd* expression. We interpret the patterns in *opa* mutant embryos as representing the latter scenario. The *odd* stripes still share posterior boundaries with the *ftz* stripes (defined by repression from the Slp primary stripes), but appear wider than in wild-type embryos, consistent with de-repression at the anterior. Furthermore, when we compare phasings of the *odd* stripes with those of *eve*, the domains of *odd* expression appear significantly anteriorly expanded in *opa* mutant embryos compared to wild-type (Figure 12–figure supplement 1)

Following from this reasoning, it appears that the *ftz*/*odd* offsets observed at gastrulation in wild-type embryos must be caused by anterior repression of *odd* (and not *ftz*) by an appropriately-located pair-rule protein in combination with Opa. We suggest that this protein is Runt. Above, we hypothesised that in wild-type embryos, Runt starts to repress *odd* at gastrulation, thus defining the anterior boundaries of the *odd* primary stripes (Figure 6). We then identified Opa as being required for Runt to exert this regulatory activity. In the absence of Opa we would therefore expect that Runt would fail to repress *odd*, and the anterior boundaries of *odd* expression would presumably continue to be defined by the posterior boundaries of the Eve stripes, which also define the anterior boundaries of the *ftz* stripes, resulting in the loss of the *ftz*/*odd* offsets, (Figure 12–figure supplement 2). This new model appears to explain the experimental observations, and suggests that the even-numbered *en* stripes are patterned by late-acting regulatory interactions.

Opa spatially patterns *odd* stripe 7

We noticed that in *opa* mutant embryos, *odd* stripe 7 is expressed across the ventral midline, whereas in wild-type embryos it is only expressed laterally (Figure 13). *odd* stripe 7 is both spatially and temporally unusual: in addition to its unique dorsoventral restriction, it first appears considerably after the other six *odd* stripes have been established. In fact, it is the only primary pair-rule stripe to appear after the trunk stripes of the secondary pair-rule gene *prd* are established (Figure 13–figure supplement 1).

We have described above how the anterior boundaries of the *odd* stripes are defined first by repression by Eve, and subsequently by repression by Runt, which requires the presence of Opa. When *odd* stripe 7 first appears, its anterior boundary correlates well with the posterior boundary of *eve* expression, and is likely be patterned by repression by Eve. The posterior boundary of *eve* stripe 7 then markedly shifts anteriorly, while *odd* stripe 7 remains static, suggesting that its anterior boundary is maintained by repression from some other protein (Figure 13–figure supplement 2). However, the seventh stripe of *runt* is abnormally broad and completely encompasses the domain of *odd* expression. Consequently, Runt cannot be providing spatial information to *odd* in this region of the embryo. It is therefore not clear which protein spatially delimits the anterior boundary of *odd* stripe 7 at gastrulation.

We suggest that it is actually Opa that patterns the anterior boundary of *odd* stripe 7. *odd* is repressed by the combination of Runt and Opa, but not by either gene alone. Theoretically, it makes no difference which protein provides the spatial information to pattern an expression domain of *odd*, as long as the repressive activity of the co-expressed proteins is appropriately localised. For *odd* stripes 2-6, Opa is expressed ubiquitously, while Runt is patterned. For *odd* stripe 7, we find that the position of its anterior boundary is prefigured by the posterior boundary of the broad *opa* expression domain (Figure 9). Therefore, in the posterior of the embryo the situation seems to be the other way around: Runt is expressed ubiquitously, while Opa provides the necessary spatial information (Figure 13–figure supplement 3).

Because *odd* stripe 7 is so delayed relative to the other primary pair-rule stripes, there is only a short time between its appearance and the first signs of Opa regulatory activity in the embryo. Therefore, while the early expression of *odd* stripe 7 is likely to be patterned by Eve, repression by Runt + Opa would soon take over, explaining why *odd* stripe 7 remains static rather than shifting anteriorly in concert with *eve*. Accordingly, we observe that in *opa* mutant embryos, where the *odd* anterior boundaries are presumably defined by Eve at all times, *odd* stripe 7 expands both anteriorly and ventrally over time, correlating well with the shifting posterior boundary of *eve* stripe 7. Indeed, in *opa* mutant embryos the anterior boundary of *odd* 7 is located at a similar position to the anterior boundary of *prd* stripe 8 (also likely to be defined by repression by Eve), whereas in wild-type it is offset from it posteriorly.

The distinctive shape of *odd* stripe 7 can therefore be explained by the curvature of the *opa* posterior boundary. Thus,

expression of Opa appears to convey both temporal and spatial information to the segmentation process in *Drosophila*.

DISCUSSION

Opa alters the pair-rule gene regulatory network

We have found that many regulatory interactions between the pair-rule genes are not constant over the course of *Drosophila* segmentation, but instead undergo coordinated changes at the end of cellularisation. We are not the first to notice that certain regulatory interactions do not apply to all stages of pair-rule gene expression (Baumgartner & Noll 1990; Manoukian & Krause 1992; Manoukian & Krause 1993; Fujioka et al. 1995; Saulier-Le Dréan et al. 1998). However, cataloguing and analysing these changes for the whole pair-rule system led us to the realisation that they are almost simultaneous and mediate the transition from double-segment to single-segment periodicity. We propose that the pair-rule system should not be thought of as a static gene regulatory network, but rather two temporally and topologically distinct networks, each with their own dynamical behaviour and consequent developmental patterning role.

Having recognised that the pair-rule gene regulatory network changes at gastrulation, we hypothesised that the non-canonical pair-rule gene Opa might act as a temporal signal and mediate the changes. We found that the spatiotemporal expression and mutant phenotype of Opa were consistent with this hypothesis. In *opa* mutant embryos, the regulatory changes do not occur and as a consequence the even-numbered parasegment boundaries are not patterned. Therefore, rather than being an uninteresting protein required but not instructive for gene expression, it appears that Opa actually plays a crucial and fascinating role in segmentation, by orchestrating a fundamental patterning transition.

What is the mechanism of Opa regulatory activity?

opa is the *Drosophila* ortholog of *zinc finger of the cerebellum* (*zic*) (Aruga et al. 1994). *zic* genes code for zinc finger transcription factors closely related to Gli proteins and have many important developmental roles.

In the *Drosophila* embryo, Opa is involved in the formation of visceral mesoderm (Cimbora & Sakonju 1995; Schaub & Frasch 2013), in addition to its role in segmentation. Opa is later highly expressed in the larval and adult brain (FlyAtlas – Chintapalli et al. 2007), and is likely to be involved in neuronal differentiation (Eroglu et al. 2014). It is also involved in the regulation of adult head development (Lee et al. 2007).

This neuronal function is likely to reflect the ancestral role of Zic, as involvement of Zic genes in nervous system development and neuronal differentiation is pervasive throughout metazoans (Layden et al. 2010). Lineage-specific duplications have resulted in five *zic* genes in most vertebrate taxa, and seven in teleosts (Aruga et al. 2006; Merzdorf 2007). While partial redundancy between these paralogs complicates the interpretation of mutant phenotypes, it is clear that Zic proteins play crucial roles in early embryonic patterning, neurogenesis, left-right asymmetry, neural crest formation, somite development, and cell proliferation (reviewed in Merzdorf 2007; Houtmeyers et al. 2013).

Zic proteins have been shown to act both as classical DNA-binding transcription factors, and as cofactors that modulate the regulatory activity of other transcription factors via protein-protein interactions (reviewed in Ali et al. 2012; Winata et al. 2015). They show context-dependent activity and can both activate and repress transcription (Yang et al. 2000; Salero et al. 2001). In particular, they appear to be directly involved in the modulation and interpretation of Wnt and Hedgehog signalling (Murgan et al. 2015; Pourebrahim et al. 2011; Fujimi et al. 2012; Koyabu et al. 2001; Chan et al. 2011; Quinn et al. 2012). Finally, they may play a direct role in chromatin regulation (Luo et al. 2015).

The roles that Opa plays in the *Drosophila* segmentation network appear to be consistent with the mechanisms of Zic regulatory activity that have been characterised in vertebrates. Opa appears to transcriptionally activate a number of pair-rule gene enhancers, including those driving late expression of *eve* and *slp*. In the case of the *slp* enhancer, this has been verified experimentally (Sen et al. 2010). In other cases, the role of Opa is likely to be restricted to modulating the effect of other regulatory inputs, such as mediating the repressive effect of Odd on *prd* expression. Finally, Opa seems often to provide a function that is intermediate between these activatory and modulatory roles, as when it (presumably)

cooperates with Prd to activate segment-polarity gene expression (Benedyk et al. 1994; Morrissey et al. 1991; Copeland et al. 1996). It will be interesting to investigate the enhancers mediating late pair-rule gene expression and determine how Opa interacts with them to bring about these varied effects.

Is Opa sufficient for the regulatory changes we observe at gastrulation?

Our data seem consistent with Opa being "the" temporal signal that precipitates the 7 stripe to 14 stripe transition. However, it remains possible that Opa acts in conjunction with some other, as yet unidentified, temporally patterned factor, or has activity that is overridden during cellularisation by some maternal or zygotic factor that disappears at gastrulation. Indeed, combinatorial interactions with DV factors do seem likely to be playing a role in restricting the effects of Opa: despite the *opa* expression domain encircling the embryo, many Opa-dependent patterning events do not extend into the mesoderm or across the dorsal midline. Identification of these factors should yield interesting insights into cross-talk between the AP and DV patterning systems of the *Drosophila* blastoderm.

The activity of Opa has previously been suggested to be concentration-dependent (Swantek & Gergen 2004). Supposing that Opa protein concentration increases progressively at the end of cellularisation, differential sensitivity to Opa activity might underlie the slightly different times at which we observe particular Opa-dependent expression changes in the embryo. For example, the splitting of the *prd* stripes moderately precedes the appearance of the secondary stripes of *odd* and *slp*. The effect on *prd* temporally coincides with the appearance of the *slp* primary stripes, which are slightly delayed in *opa* mutant embryos. These two events seem to reflect the earliest regulatory effects of Opa.

We note that while Opa may contribute to their timely activation, the *slp* primary stripes do not strictly require Opa activity. This is not surprising, since the *slp* locus has been shown to possess multiple partially redundant regulatory elements driving spatially and temporally overlapping expression patterns (Fujioka & Jaynes 2012). From our own observations, we have found several cases where mutation of a particular gene causes the *slp* primary stripes to be reduced in intensity, but not abolished (data not shown), suggesting that regulatory control of these expression domains is redundant at the *trans* level as well as at the *cis* level. Partially redundant enhancers that drive similar patterns, but are not necessarily subject to the same regulatory logic, appear to be very common for developmental transcription factors (Cannavò et al. 2015; Perry et al. 2011; Staller et al. 2015; Wunderlich et al. 2015).

Opa-dependent regulatory interactions pattern the even-numbered parasegment boundaries

Future parasegment boundaries are positioned essentially by painting a stripe of *en* expression just posterior to an abutting stripe of *slp* expression (Cadigan et al. 1994b). In the extending germband, instances of this pattern are separated by stripes of *odd* expression, which prevent the formation of ectopic compartment boundaries with reverse polarity (Mullen & DiNardo 1995; Jaynes & Fujioka 2004; Meinhardt 1986).

The odd-numbered parasegment boundaries are pre-patterned by the combination of the "P" stripes of *prd* and the primary stripes of *slp*, neither of which are Opa-dependent. Current models for the patterning of the even-numbered parasegment boundaries implicate an early role for the Eve stripes. However, we have shown that the effect of Eve is likely indirect. Instead, we propose a model whereby the patterning of the even-numbered parasegment boundaries occurs later, and relies upon Opa-dependent regulatory interactions.

It therefore seems that pair-rule patterning is a two stage process. The first stage, characterised by the absence of Opa, patterns one set of parasegment boundaries. The second stage, characterised by the presence of Opa and a consequently different regulatory network, patterns the other set of parasegment boundaries. Each stage uses the same source of positional information (the primary stripes of the pair-rule genes), but uses different sets of regulatory logic to exploit this information in different ways.

The pair-rule network exhibits general regulatory principles

By carefully analysing pair-rule gene expression patterns in the light of the experimental literature, we have clarified

our understanding of the regulatory logic responsible for them. In particular, we propose significantly revised models for the patterning of *odd*, *slp* and *runt*. Because the structure of a regulatory network determines its dynamics, and its structure is determined by the control logic of its individual components, these subtleties are not merely developmental genetic stamp-collecting. Our reappraisal of the pair-rule gene network allows us to re-evaluate some long-held views about *Drosphila* blastoderm patterning.

Firstly, pair-rule gene interactions are combinatorially regulated by an extrinsic source of temporal information, something not accounted for by textbook models of the *Drosophila* segmentation cascade. We have characterised the role of Opa during the 7 stripe to 14 stripe transition, but there may well be other such signals acting earlier or later. Indeed, context-dependent transcription factor activity appears to be very common (Stampfel et al. 2015).

Secondly, our updated model of the pair-rule network is in many ways simpler than previously thought. While we do introduce the complication of an Opa-dependent network topology, this effectively streamlines the sub-networks that operate early (phase 2) and late (phase 3). At any one time, each pair-rule gene is only regulated by two or three other pair-rule genes. We do not see strong evidence for combinatorial interactions between these inputs (DiNardo & O'Farrell 1987; Baumgartner & Noll 1990; Swantek & Gergen 2004). Instead, pair-rule gene regulatory logic seems invariably to consist of permissive activation by a broadly expressed factor (or factors) that is overridden by precisely-positioned repressors (Edgar et al. 1986; Weir et al. 1988). This kind of regulation appears to typify other complex patterning systems, such as the vertebrate neural tube (Briscoe & Small 2015).

Finally, pair-rule gene cross-regulation has traditionally been thought of as a mechanism to stabilise and refine stripe boundaries (e.g. Edgar et al. 1989; Schroeder et al. 2011). Consistent with this function, as well as with the observed digitisation of gene expression observed at gastrulation (Baumgartner & Noll 1990; Pisarev et al. 2009), we find that the late network contains a number of mutually repressive interactions (Eve/Runt, Eve/Slp, Ftz/Slp, Odd/Runt, Odd/Slp). However, these switch-like interactions do not appear to characterise the early network. Interestingly, pair-rule gene expression during cellularisation has been observed to be unexpectedly dynamic (Keränen et al. 2006; Surkova et al. 2008), something that is notable given the oscillatory expression of pair-rule gene orthologs in short-germ arthropods (Sarrazin et al. 2012; El-Sherif et al. 2012; Brena & Akam 2013).

Opa activates the earliest phase of segment-polarity gene expression

Genetic dissection of *en* regulation suggests that there are several phases of segment-polarity gene regulation, each responding to distinct sets of regulatory inputs. Early segment-polarity gene expression is spatially patterned by pair-rule genes, whereas later expression is maintained by positive feedback loops within the segment-polarity network that rely on an appropriate prepattern being present (DiNardo et al. 1988; von Dassow et al. 2000). Finally, *en* expression becomes independent of signalling and is instead dependent upon polycomb repression (Moazed & O'Farrell 1992).

The absence of segment-polarity gene expression in *opa* mutant embryos indicates that Opa acts as an explicit temporal signal regulating the onset of the first phase of expression. Therefore, activation of segment-polarity gene expression is not merely determined by the emergence of an appropriate pattern of pair-rule proteins, as in simple models of hierarchical gene regulation. The necessity for an additional signal had been surmised previously, based on the delayed appearance of odd-numbered *en* stripes in cells already expressing Eve and Prd (Manoukian & Krause 1993).

Temporally regulating segment-polarity activation makes good sense from a patterning perspective. Correct segmentation depends upon the initial expression of segment-polarity genes being precisely positioned, therefore it is imperative that a regular pair-rule pattern is present before the segment-polarity genes first turn on. Notably, another temporal signal is deployed to prevent precocious pair-rule gene expression while gap gene expression is being established. In this case, a ubiquitously-expressed maternal protein, Tramtrack, represses pair-rule gene expression during early embryogenesis (Harrison & Travers 1990; Read et al. 1992; Brown & Wu 1993). Thus it appears that both activators and repressors provide extrinsic temporal information to the *Drosophila* segmentation cascade.

Why do pair-rule genes show a segmental phase of expression?

prd, *odd*, *slp* and *runt* are expressed in regular segmental stripes after gastrulation. However, mutation of these genes causes pair-rule defects rather than segment-polarity phenotypes. In the case of *slp*, this has been shown to be due to redundancy with a paralog, *slp2* (Grossniklaus et al. 1992; Cadigan et al. 1994a). *prd* and *odd* also have paralogs expressed in segment-polarity patterns (Baumgartner et al. 1987; Hart et al. 1996). The *prd* paralog, *gsb*, gives a segment-polarity phenotype if mutated, but Prd and Gsb are able to substitute for each other if expressed under the control of the other gene's regulatory region (Li & Noll 1993; Li & Noll 1994; Xue & Noll 1996). This indicates that the same protein can fulfil both pair-rule and segment-polarity functions, and that the two roles require different regulation.

We have shown that the transition to single-segment periodicity is mediated by substantial re-wiring of pair-rule gene regulatory interactions. Furthermore, we have shown that this rewiring is controlled by the same signal that activates segment-polarity gene expression. We propose that Opa's main role is to usher in a "segment-polarity phase" of expression. In several cases, the presence of Opa induces pair-rule genes to effectively become segment-polarity genes, and these genes then work in concert with other segment-polarity genes that do not have an earlier, non-segment-polarity function. For example, En protein is involved in patterning the late expression of *eve*, *odd*, *runt* and *slp* (Harding et al. 1986; Mullen & DiNardo 1995; Klingler & Gergen 1993; Fujioka et al. 2012), while Slp is a critical component of the segment-polarity network (Cadigan et al. 1994b).

We envisage that ancestrally, certain genes would have sequentially fulfilled both pair-rule and segment-polarity functions, employing different regulatory logic in each case. Serendipitous gene duplications would later allow these roles to be divided between different paralogs, leaving the transient segmental pattern of the earlier expressed gene as an evolutionary relic. Consistent with this hypothesis, the roles of *prd* and *gsb* seem to be fulfilled by a single co-ortholog, *pairberry1*, in grasshoppers, with a second gene, *pairberry2*, expressed redundantly (Davis et al. 2001).

Is the role of Opa conserved?

In light of our data, it will be interesting to characterise the role of Opa in other arthropod model organisms. The best studied short-germ insect is the beetle *Tribolium castaneum*, which also exhibits pair-rule patterning. An RNAi screen of pair-rule gene orthologs reported no segmentation phenotype for *opa* knock-down, and concluded that *opa* does not function as a pair-rule gene in *Tribolium* (Choe et al. 2006). However, the authors also state that *opa* knock-down caused high levels of lethality and most embryos did not complete development, indicating that this conclusion may be premature. In contrast to this study, iBeetle-Base (Dönitz et al. 2015) reports a segmentation phenotype for *opa* knock-down. The affected cuticles show a reduced number of segments including the loss of the mesothorax (T2). This could indicate a pair-rule phenotype in which the even-numbered parasegment boundaries are lost, similar to the situation in *Drosophila*. If true, this suggests that at least some aspects of the role of Opa are conserved between long-germ and short-germ segmentation.

MATERIAL AND METHODS

Drosophila mutants and genetics

Wild-type embryos were Oregon-R. The pair-rule gene mutations used were opa^{8} (Bloomington stock no. 5335) and ftz^{11} (gift of Bénédicte Sanson). These mutations were balanced over *TM6C Sb Tb twi::lacZ* (Bloomington stock no. 7251) to allow homozygous mutant embryos to be easily distinguished. Embryos were collected and fixed according to standard procedures.

Double fluorescent *in situ* hybridisation

Digoxigenin (DIG) and fluorescein (FITC) labelled riboprobes were generated using full-length pair-rule gene cDNAs from the *Drosophila* gene collection (Stapleton et al. 2002). The *lacZ* cDNA was a gift from Nan Hu. *In situ* hybridization was carried out essentially as described in Tautz & Pfeifle 1989. Embryos were simultaneously hybridised with DIG and FITC probes to different pair-rule genes. Embryos from mutant crosses were additionally hybridised with a DIG probe to *lacZ*. After hybridisation, embryos were incubated in 1:4000 peroxidase-conjugated sheep anti-FITC

and 1:4000 alkaline phosphatase(AP)-conjugated sheep anti-DIG antibodies (Roche, Basel, Switzerland). TSA biotin amplification (Perkin Elmer, Waltham, MA) followed by incubation in streptavidin Alexa Fluor 488 conjugate (ThermoFisher Scientific, Waltham, MA) was used to visualise the peroxidase signal. A Fast Red reaction (Fast Red tablets, Kem-En-Tec Diagnostics, Taastrup, Denmark) was subsequently used to visualise the AP signal. Embryos were mounted in Prolong Gold (ThermoFisher Scientific) before imaging.

Microscopy and image analysis

Embryos were imaged on a Leica SP5 Upright confocal microscope, using a 20x objective. Minor brightness and contrast adjustments were carried out using Fiji (Schindelin et al. 2012; Schneider et al. 2012). Thresholded images were produced using the "Make Binary" option in Fiji.

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COMPETING INTERESTS

The authors declare that no competing interests exist.

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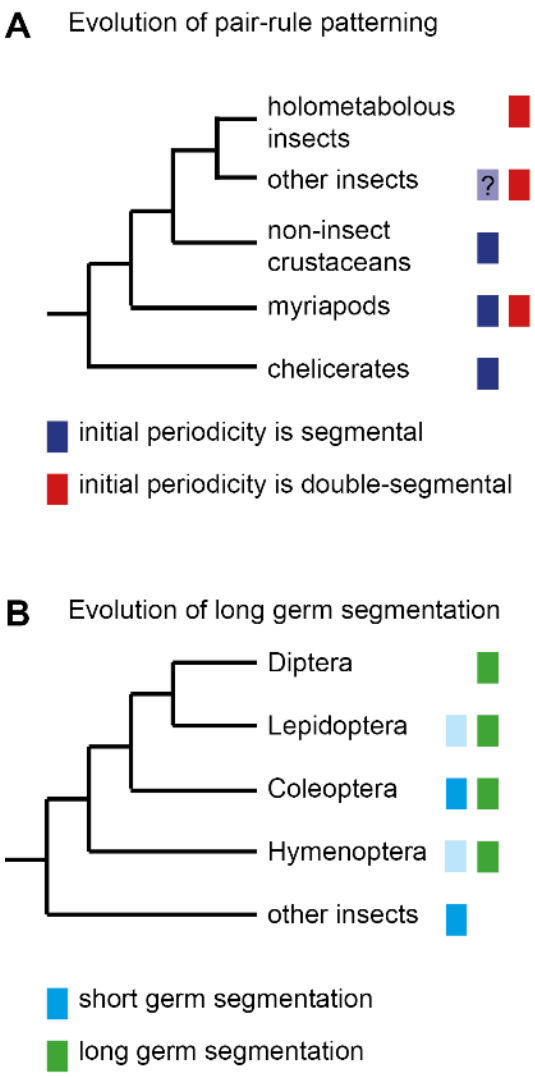

Figure 1.
The evolution of pair-rule patterning pre-dates the evolution of long germ segmentation.

(A) Single segment periodicity is ancestral in arthropod segmentation, being found in spiders, millipedes, crustaceans, and probably some insects. "Pair-rule" patterning, involving an initial double segment periodicity of pair-rule gene expression, appears to have evolved independently at least twice. It is found in certain centipedes and also in most insects. (**B**) Long germ segmentation is likely to have independently evolved multiple times within holometabolous insects, from an ancestral short germ state.

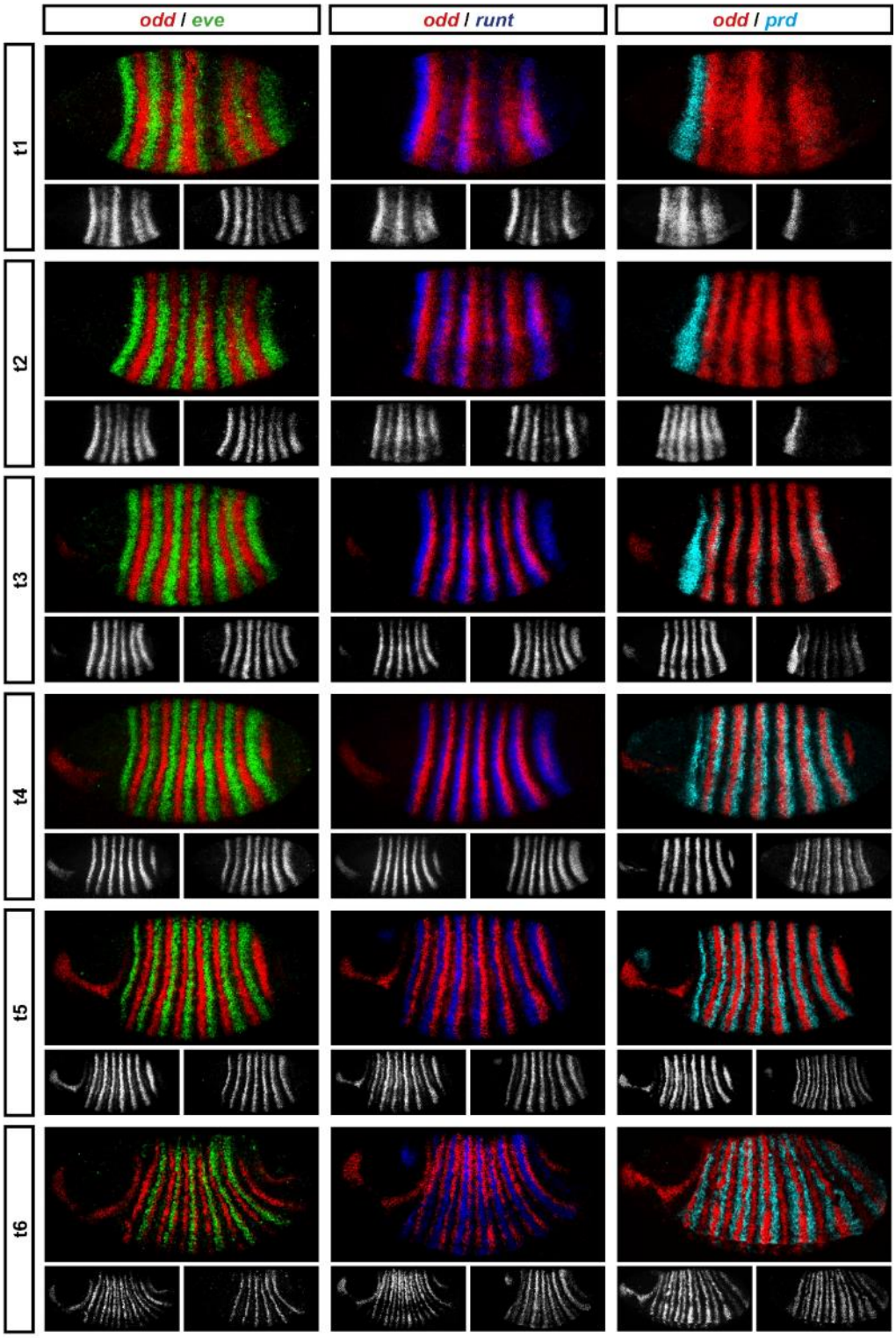

Figure 2.
Representative double fluorescent *in situ* hybridisation data for three combinations of pair-rule genes.

This figure shows a small subset of our wild-type dataset. Each column represents a different pairwise combination of *in situ* probes, while each row shows similarly-staged embryos of increasing developmental age. Channels are false-coloured for ease of comparison, and individual channels are shown in grayscale below each double-channel image. Time classes are arbitrary, meant only to illustrate the progressive stages of pattern maturation between early cellularisation (t1) and late gastrulation (t6). Note that the evolving pattern of *odd* expression in the head provides a distinctive and reliable indicator of embryo age.

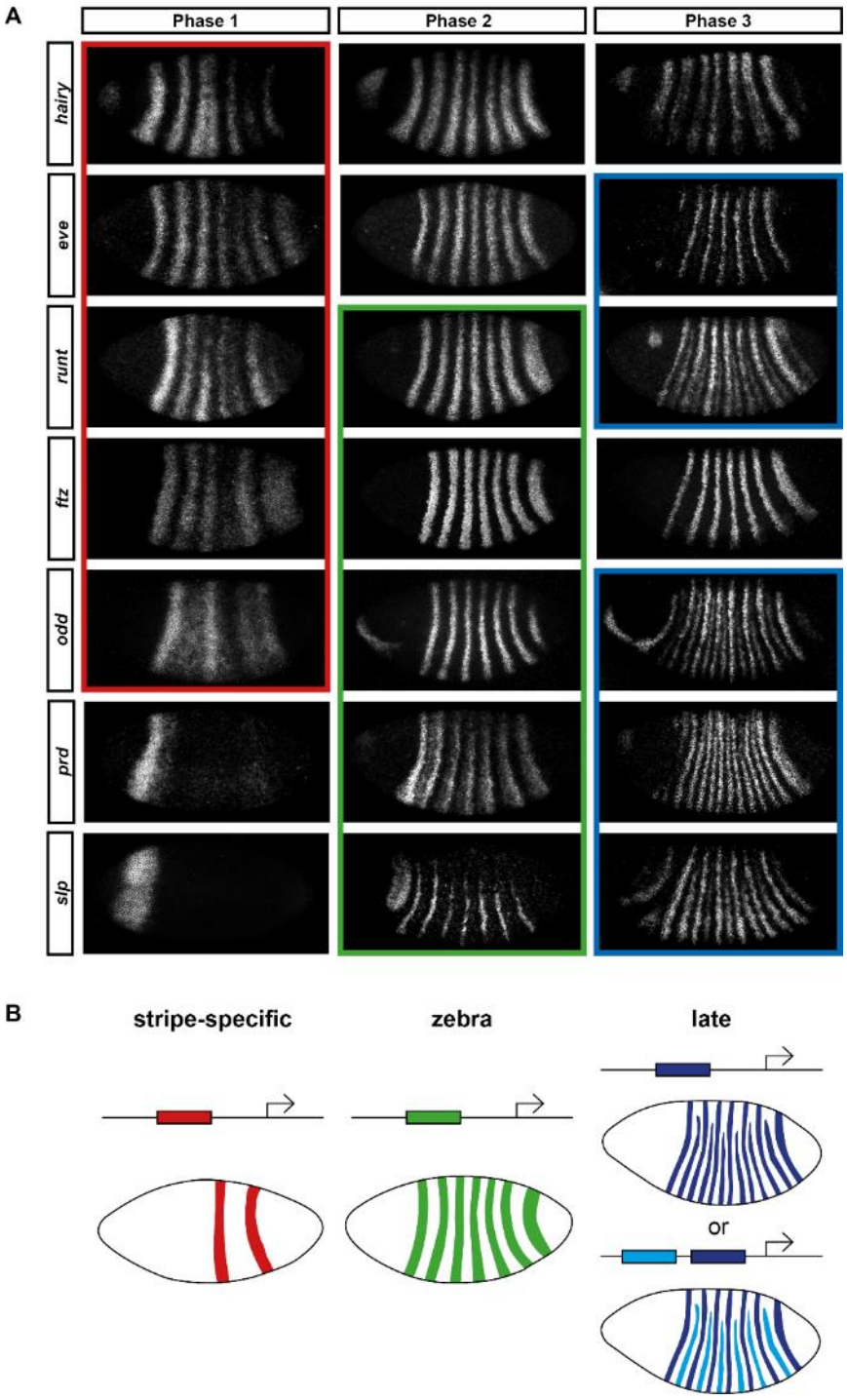

Figure 3.
Three phases of pair-rule gene expression, usually mediated by different classes of regulatory element.

(**A**) Representative expression patterns of each of the seven pair-rule genes at phase 1 (early cellularisation), phase 2 (mid cellularisation), and phase 3 (late cellularisation and gastrulation). Pair-rule genes are classified as "primary" or "secondary" based on their expression during phase 1. (**B**) Diagrams of the different kinds of regulatory elements mediating pair-rule gene expression. Stripe-specific elements are regulated by gap genes and give rise to either one or two stripes each. Zebra elements are regulated by pair-rule genes and give rise to seven stripes. Late expression patterns may be generated either by a single element generating segmental stripes, or by a combination of two elements each generating a distinct pair-rule pattern. The coloured outlines around the panels in (**A**) correspond to the colours of the different classes of regulatory elements in (**B**), and indicate how each phase of expression is regulated for each pair-rule gene.

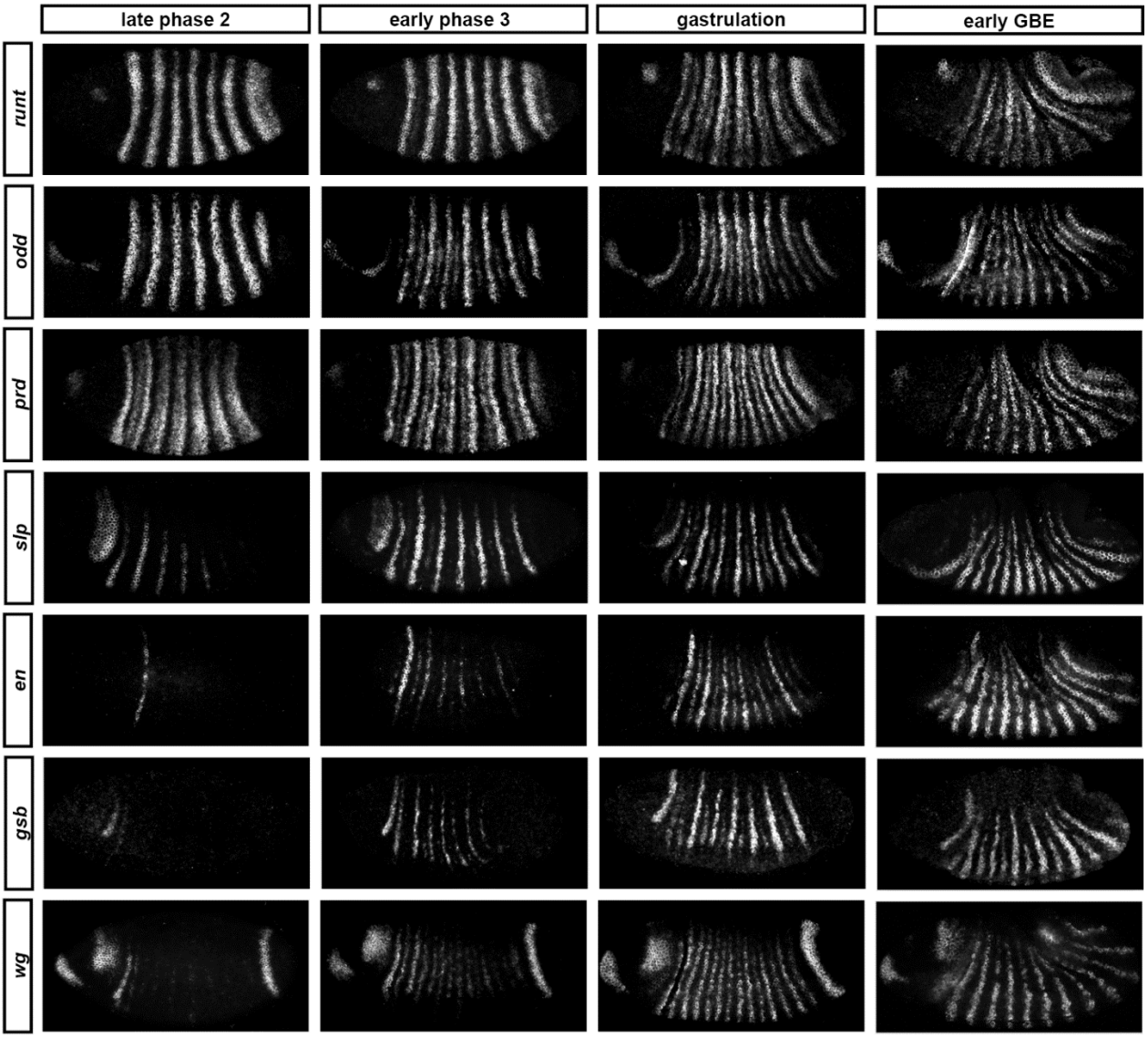

Figure 4
Frequency-doubling of pair-rule gene expression patterns is almost simultaneous, and coincides with the first expression of the segment-polarity genes.

Each row shows the expression of a particular pair-rule gene or segment-polarity gene, while each column represents a particular developmental stage (late phase 2 and early phase 3 = late stage 5, gastrulation = stage 6, early germband extension = stage 7). GBE = germband extension.

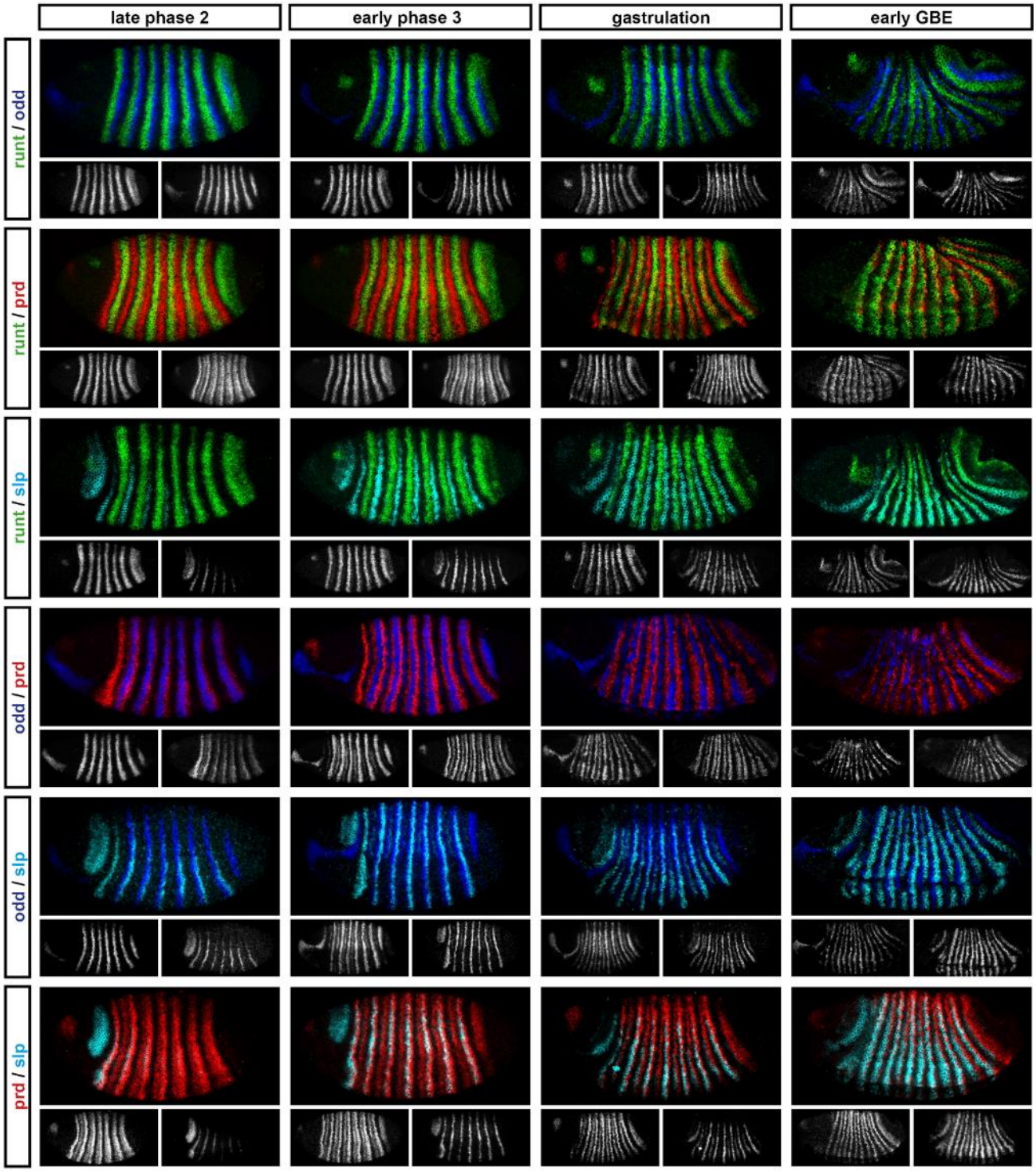

Figure 4–figure supplement 1
Relative expression of pair-rule genes during frequency-doubling.

Each row shows the relative expression of two pair-rule genes, while each column represents a particular developmental stage (late phase 2 and early phase 3 = late stage 5, gastrulation = stage 6, early germband extension = stage 7). Single channel images are shown in greyscale below each false-coloured double channel image (the channel listed first in the row label is always on the left). GBE = germband extension.

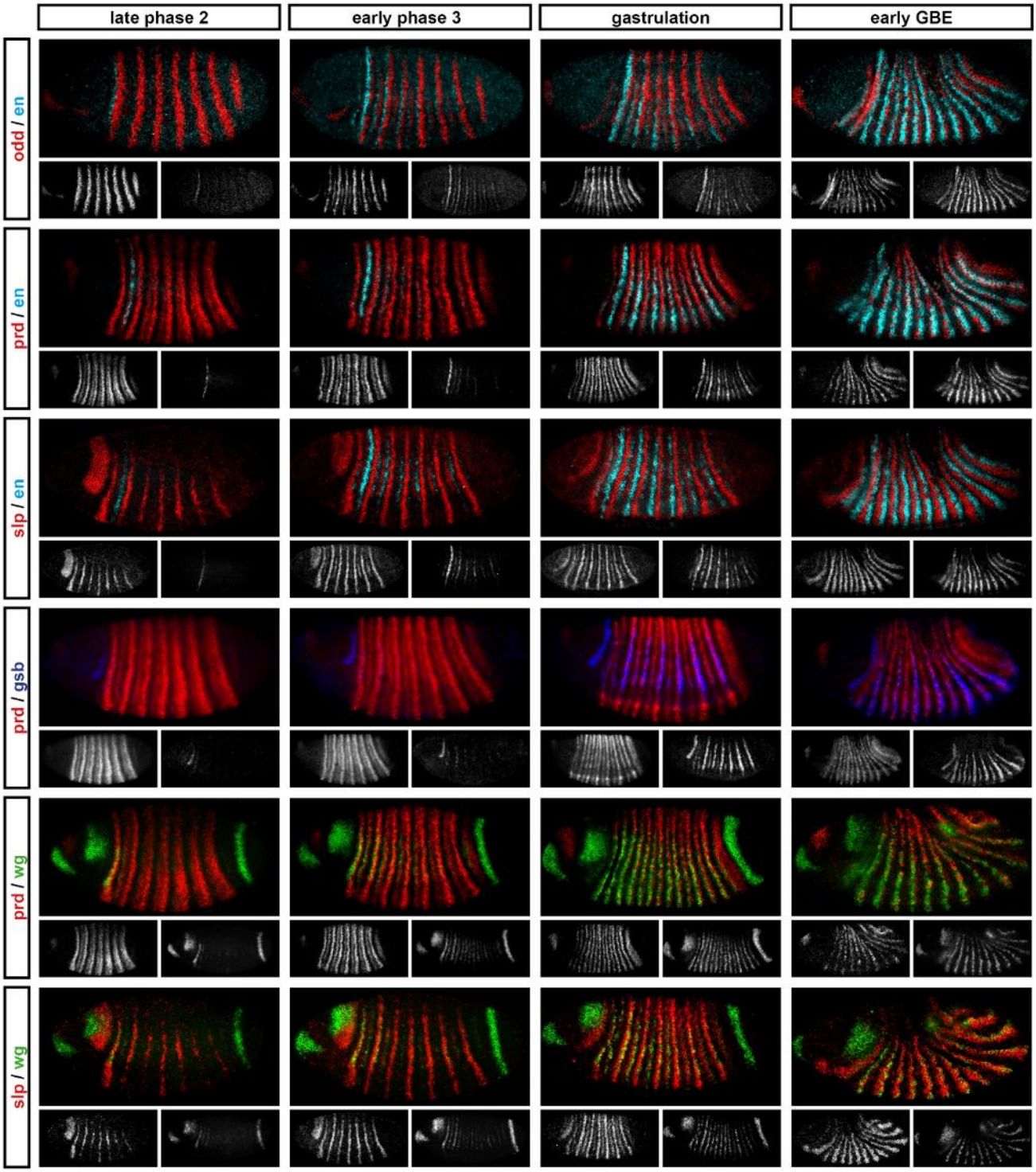

Figure 4–figure supplement 2
Relative expression of segment-polarity genes and pair-rule genes during frequency-doubling.

Each row shows the relative expression of a particular pair-rule gene and segment-polarity gene combination, while each column represents a particular developmental stage (late phase 2 and early phase 3 = late stage 5, gastrulation = stage 6, early germband extension = stage 7). Single channel images are shown in greyscale below each false-coloured double channel image (the channel listed first in the row label is always on the left). GBE = germband extension.

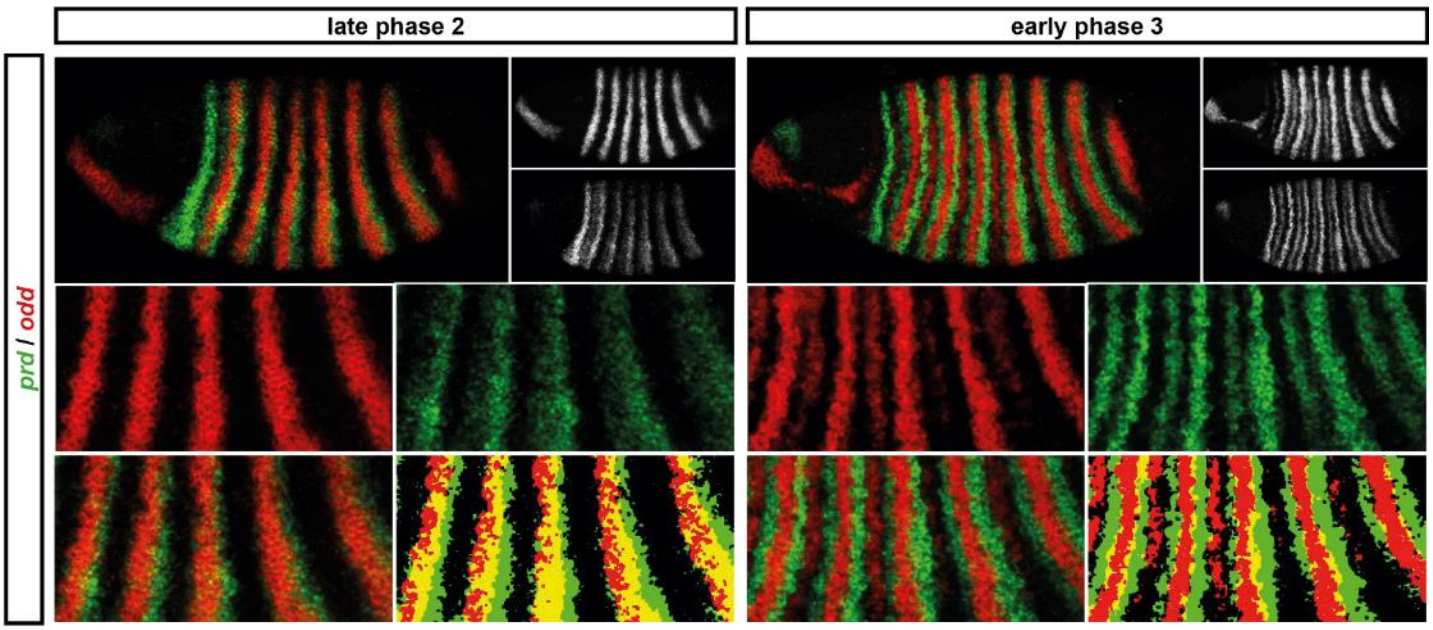

Figure 5
Regulation of *prd* transcription at phase 2 *versus* phase 3.

Relative expression of *prd* and *odd* is shown in a late phase 2 embryo (just prior to frequency doubling) and an early phase 3 embryo (showing the first signs of frequency doubling). For each phase, double and single channel images of the whole embryo are shown (single channel images are in the same vertical order as the panel label). The remaining images show close ups of expression in stripes 2-6. The double channel image (bottom left) and individual channels (middle row) are shown. A processed version of the double channel image (bottom right) highlight regions of overlapping expression (yellow pixels). Considerable overlap between *prd* and *odd* expression is observed at phase 2 but not at phase 3.

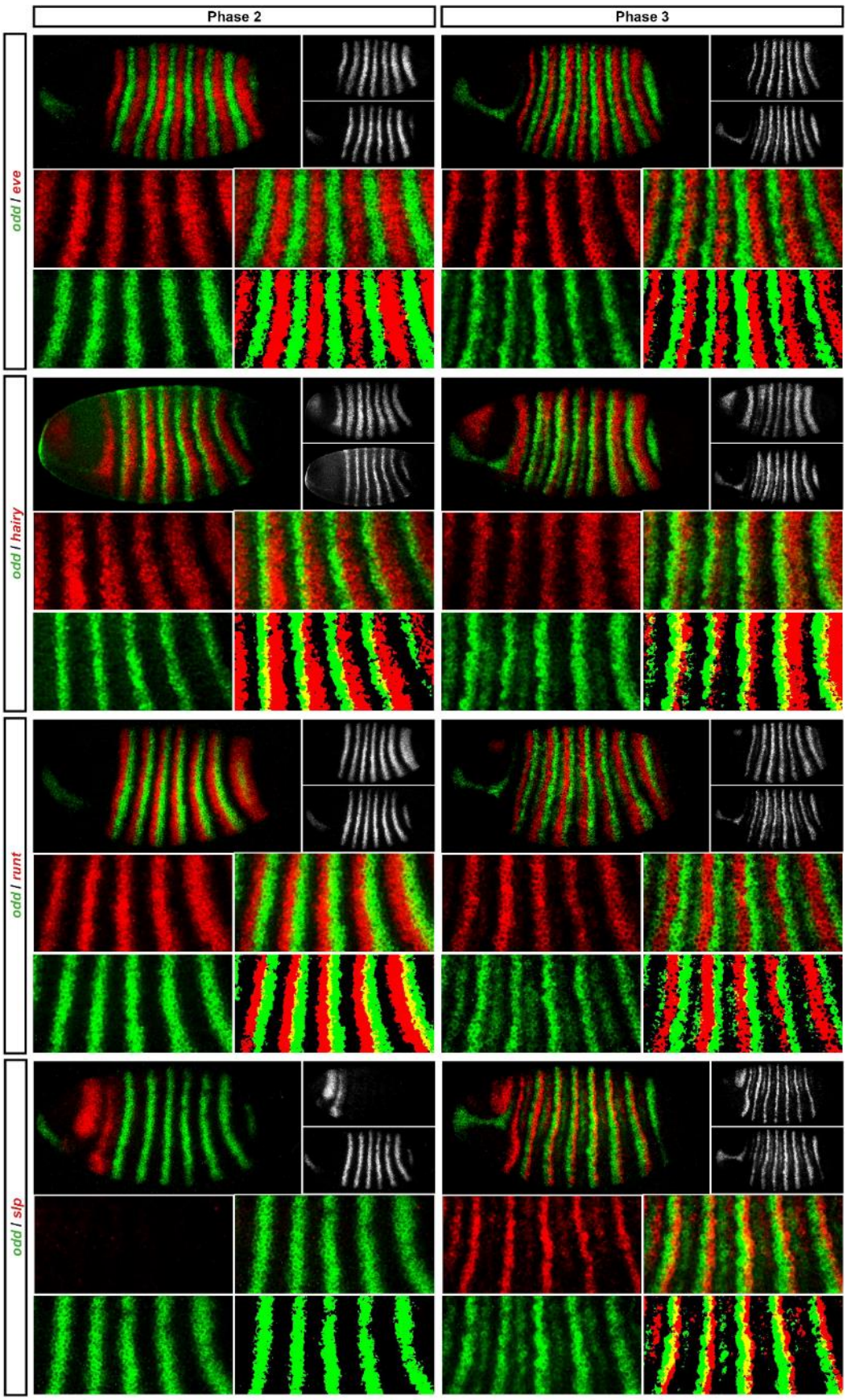

Figure 6
Regulation of *odd* transcription at phase 2 vs phase 3.

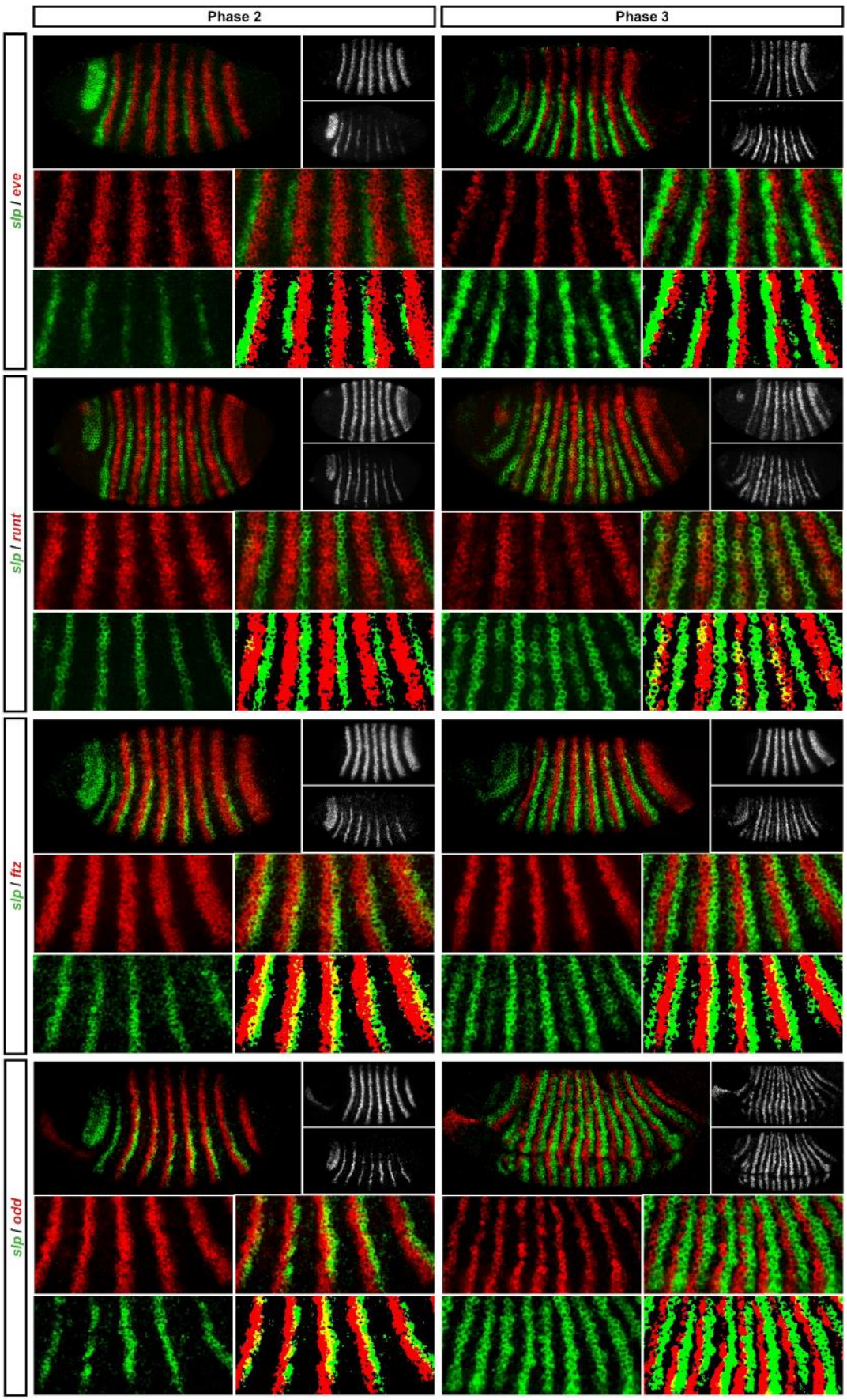

Figure 7
Regulation of s*lp* transcription at phase 2 vs phase 3.

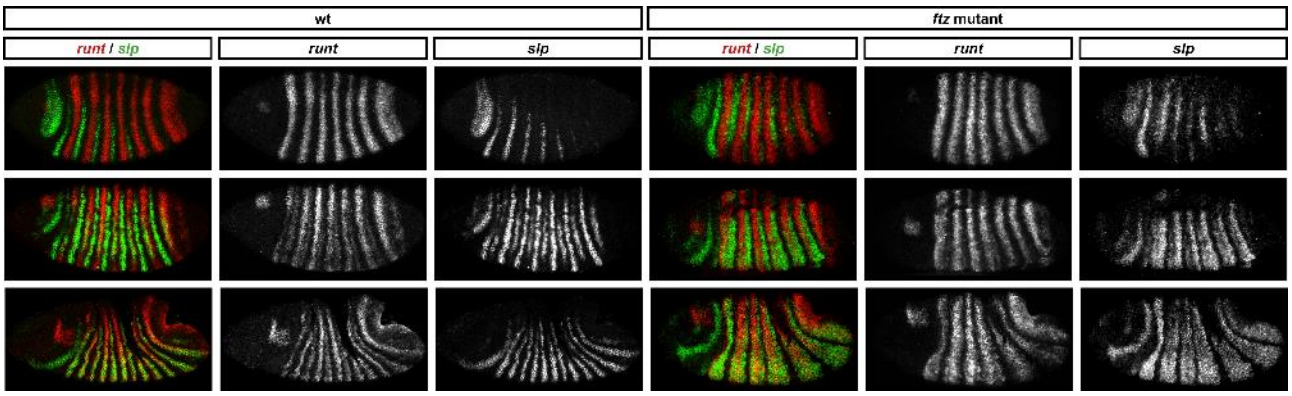

Figure 7–figure supplement 1
Runt represses *slp* during phase 2 even in *ftz* mutant embryos

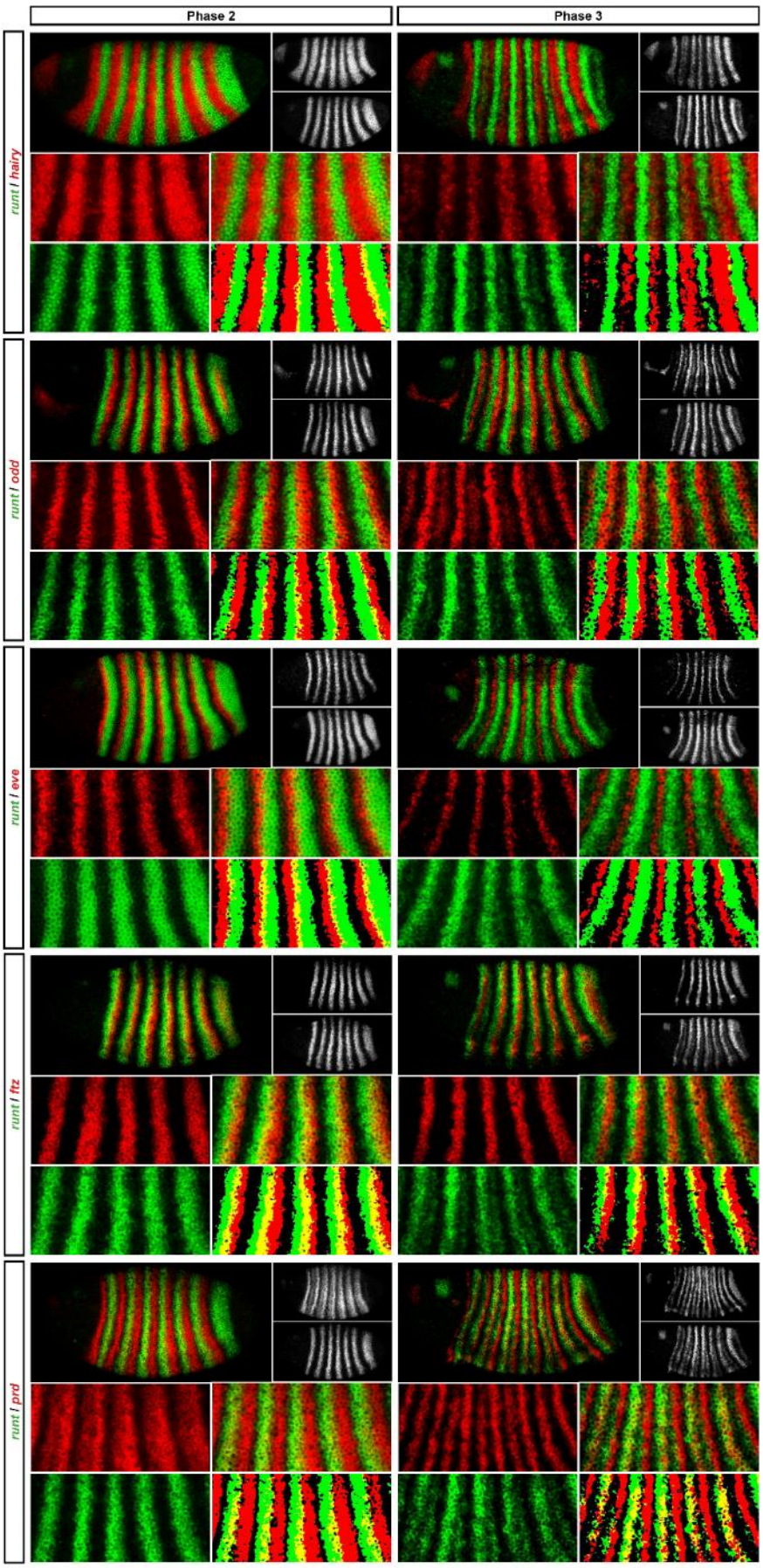

Figure 8
Regulation of *runt* transcription at phase 2 vs phase 3.

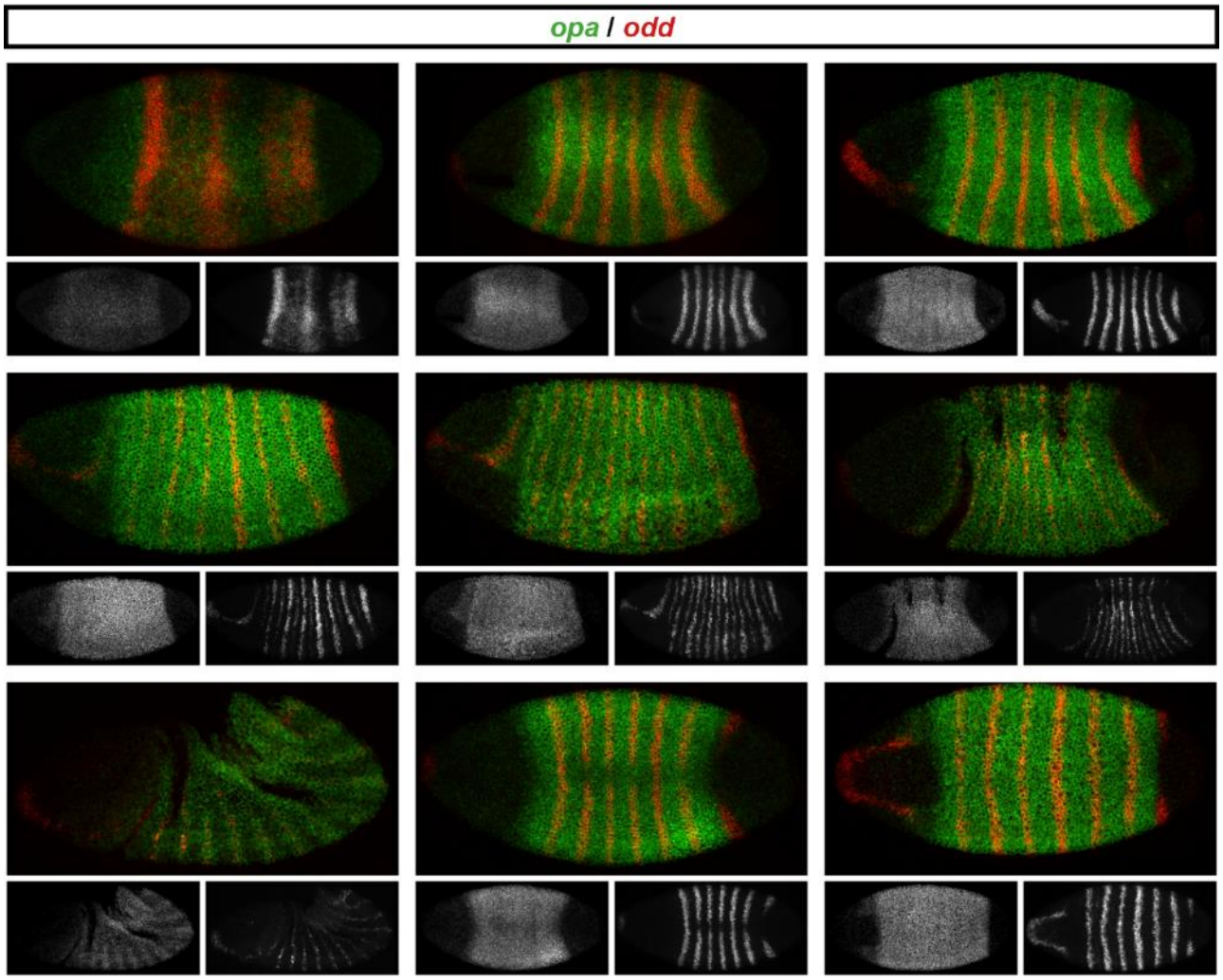

Figure 9
Spatiotemporal expression of *opa* relative to *odd.*

Expression of *opa* relative to *odd* from early cellularisation until mid germband extension. Single channel images are shown in greyscale below each double channel image (*opa* on the left). The last two panels show dorsal and ventral views respectively of late phase 2 embryos.

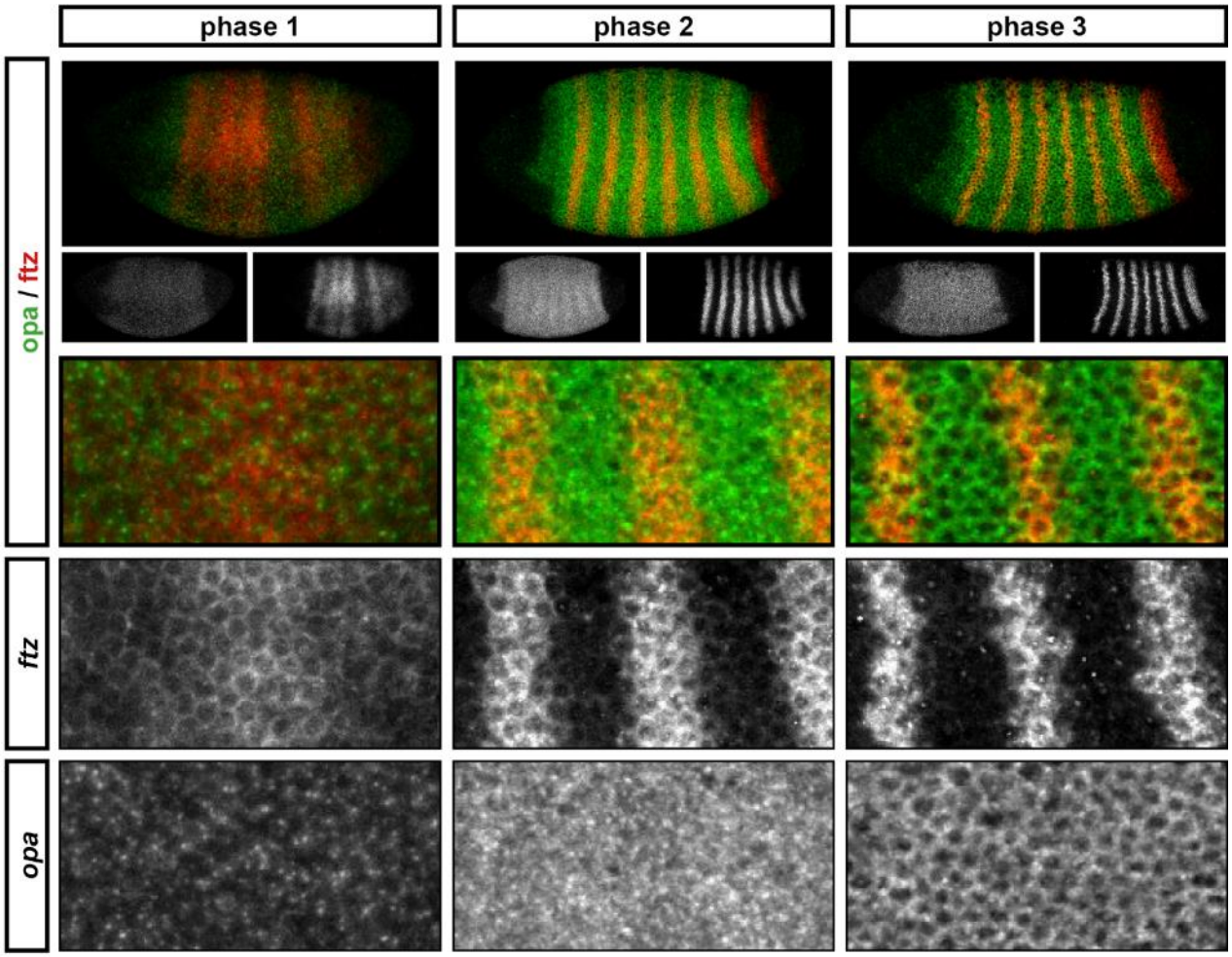

Figure 9–figure supplement 1
The cellular localisation of *opa* transcripts changes over the course of segmentation

Relative expression of *opa* and *ftz* is shown in embryos at phase 1, phase 2 and phase 3. Single channel images are shown in greyscale below each double channel whole embryo image (*opa* on the left). Lower panels show double and single channel close up images of these embryos.

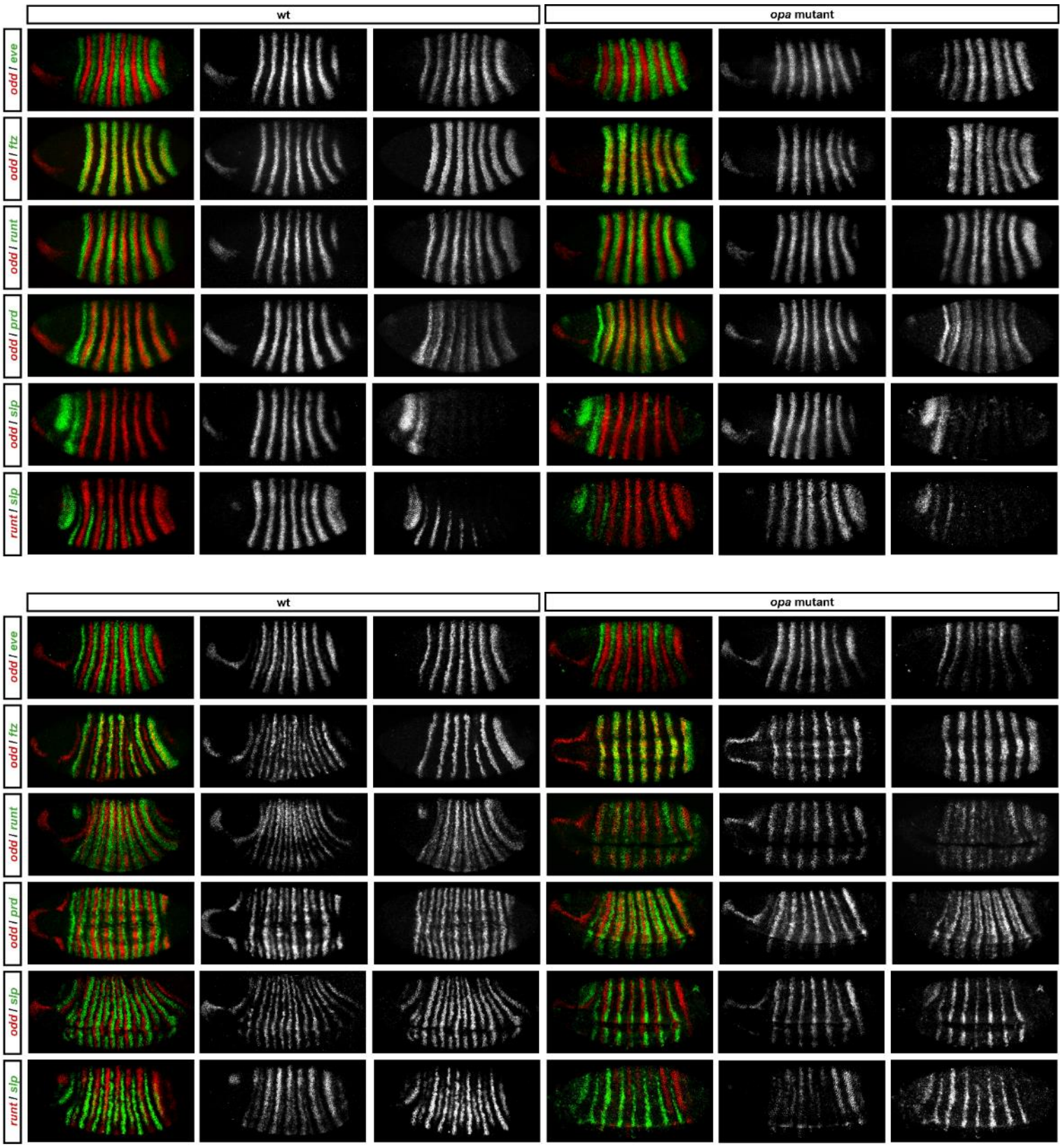

Figure 10
Pair-rule gene expression is relatively normal during cellularisation in *opa* mutant embryos, but becomes peturbed at gastrulation.

(**A**) Pair-rule gene expression in wild-type and *opa* mutant embryos at late phase 2. (**B**) Pair-rule gene expression in wild-type and *opa* mutant embryos towards the end of gastrulation (late stage 3). Single channel images are shown in the same order left-to-right as they are listed in the row label.

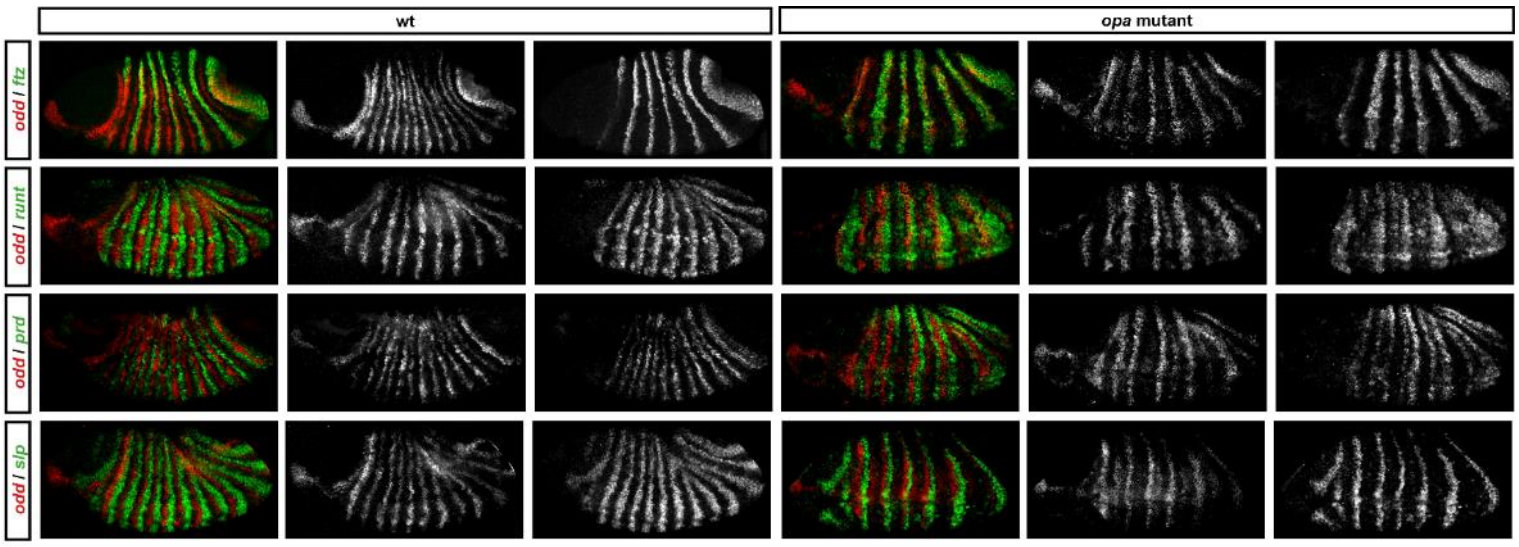

Figure 10–figure supplement 1
Pair-rule gene expression at early germband extension in *opa* mutant embryos.

Pair-rule gene expression in wild-type and *opa* mutant embryos during early germband extension. Single channel images are shown in the same order left-to-right as they are listed in the row label.

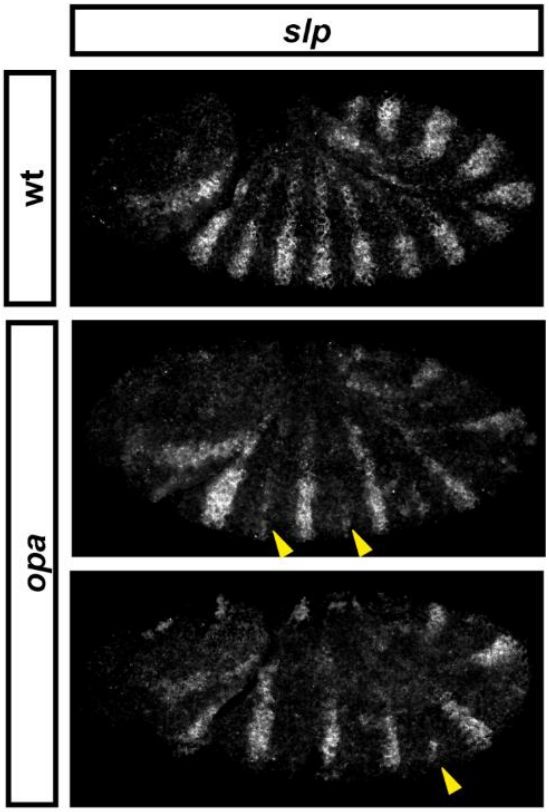

Figure 10–figure supplement 2
The *slp* secondary stripes do not recover in *opa* mutant embryos.

Single channel images of *slp* expression in wild-type and *opa* mutant embryos at late germband extension. Arrowheads point to weak or partial secondary stripes that are sometimes observed.

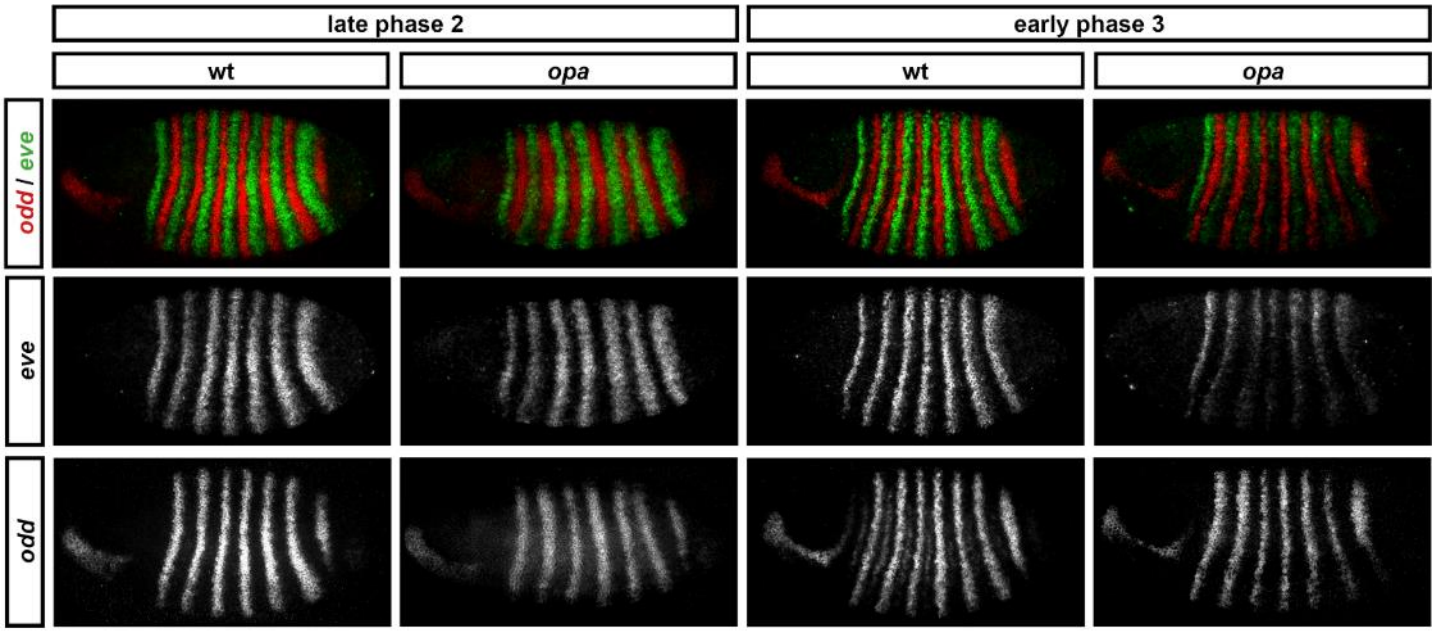

Figure 11
Opa activates the *eve* "late" element.

eve and *odd* expression in wild-type and *opa* mutant embryos at late phase 2 and early phase 3. *eve* expression largely fades away at phase 3 in *opa* mutant embryos, in contrast to wild-type embryos, where the "late" element generates strong, sharp expression in the anterior halves of the early stripes. The pattern of *odd* expression in the head was used for embryo staging.

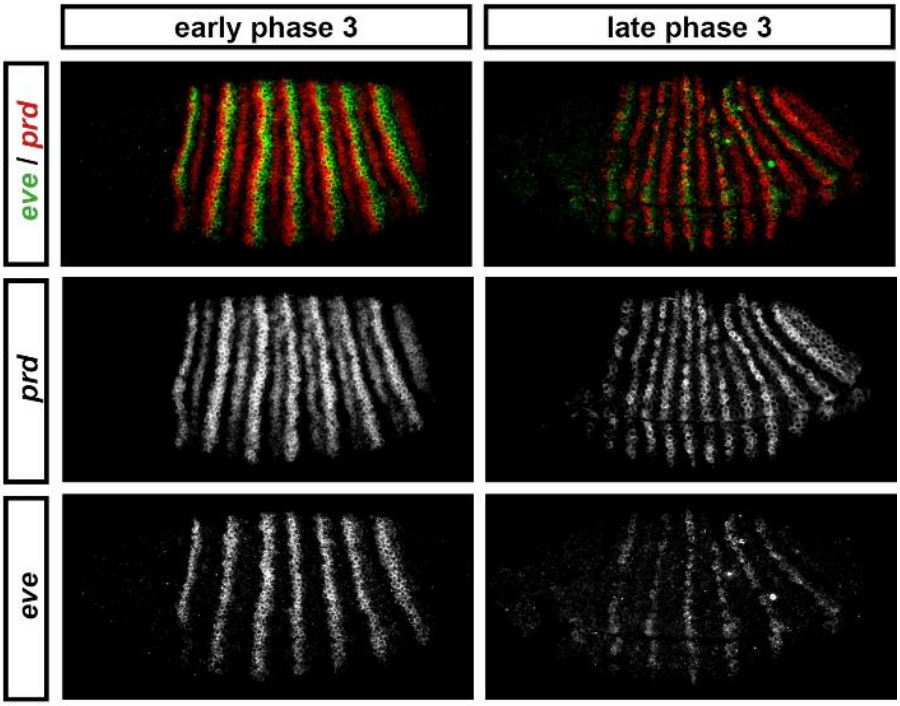

Figure 11–figure supplement 1
"late" *eve* expression is observed in cells that do not express *prd*

eve and *prd* expression in wild-type embryos during phase 3. During early phase 3, eve is strongly expressed in stripes ~2 cells wide. Only the anterior halves of these stripes overlap with the "P" stripes of *prd* expression, meaning that the *eve* "late" element is expressed in many cells that have never expressed *prd*. *eve* expression is largely lost from non-*prd* expressing cells by the end of gastrulation, indicating that *prd* may nevertheless be required for the maintenance of *eve* late element expression. [ADD ARROWHEADS].

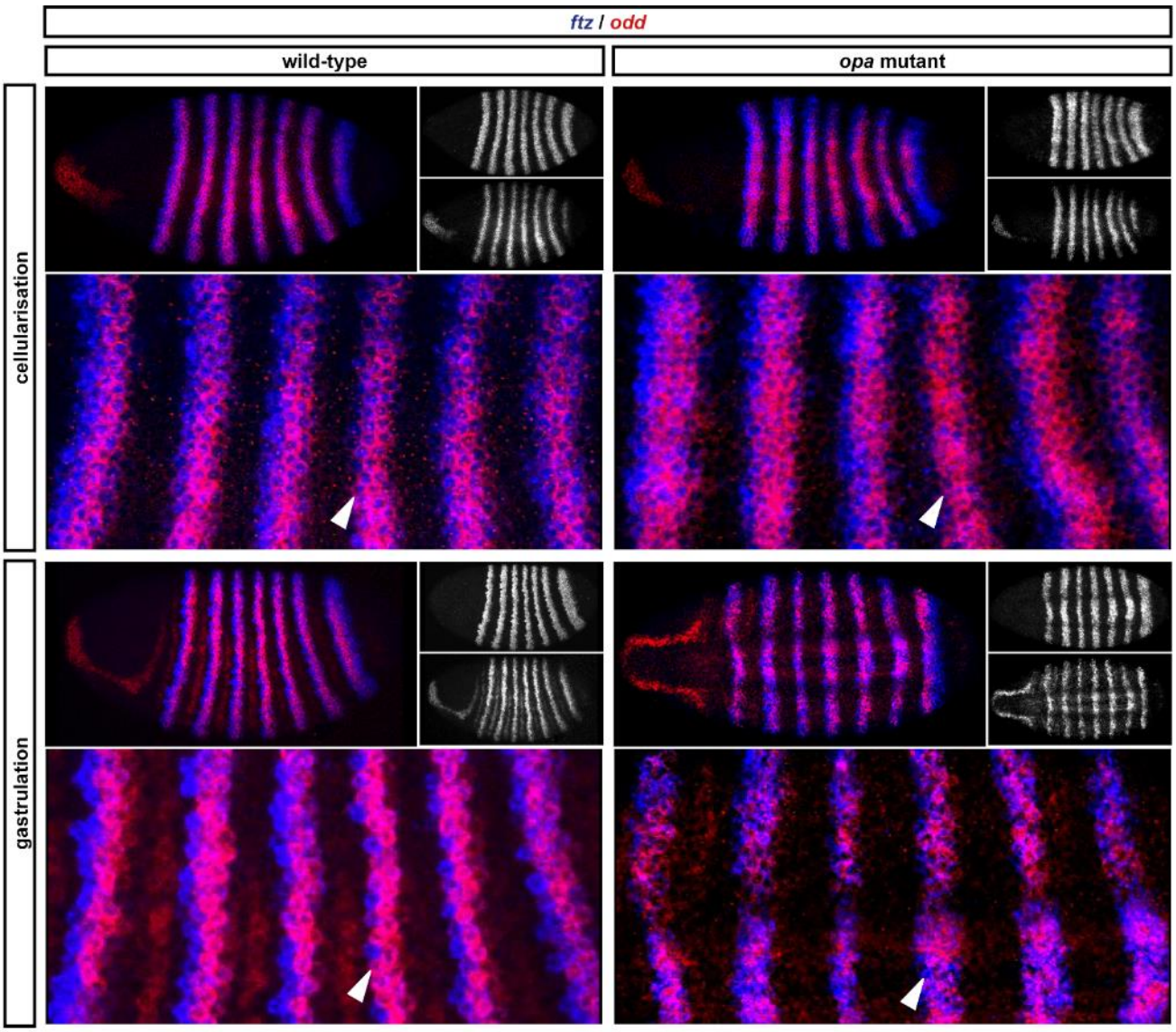

Figure 12:
The *ftz/odd* anterior boundary offsets are lost in *opa* mutant embryos at gastrulation.

At cellularisation, off sets are present in most stripes, but absent in stripe 4, in both wild type and *opa* mutant embryos. At gastrulation, offsets are present for all stripes in wild-type embryos, but lost for all stripes in *opa* mutant embryos. Arrowheads point to stripe 4. In single channel images, *ftz* expression is shown top.

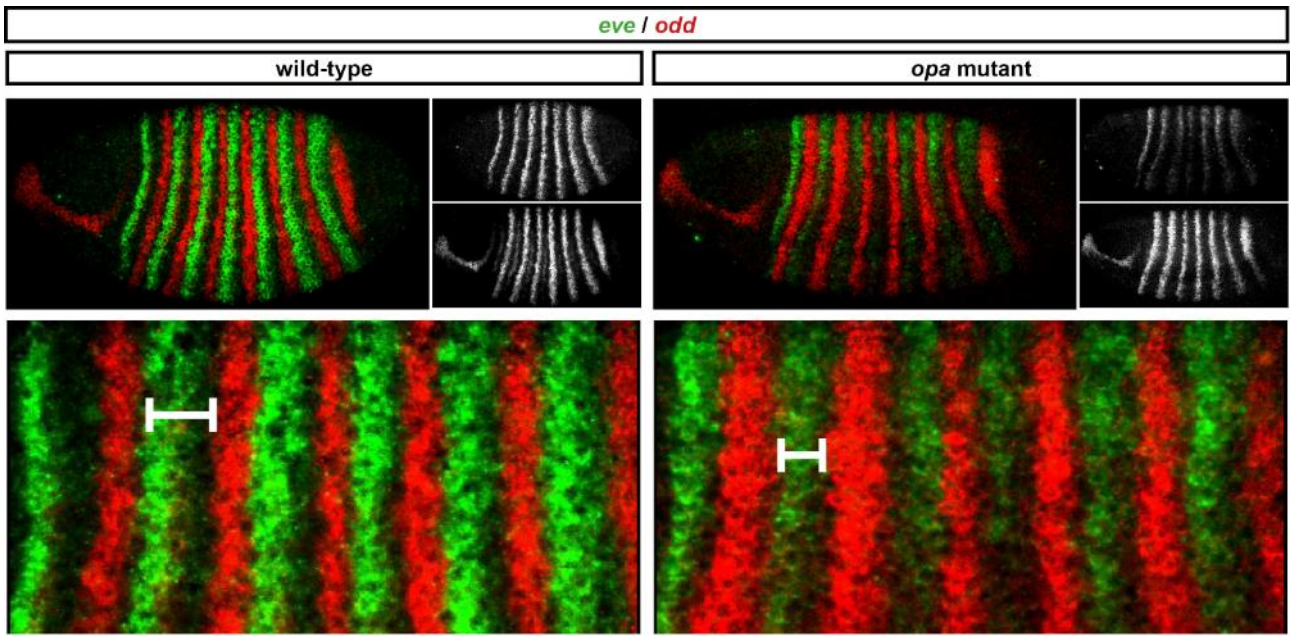

Figure 12–figure supplement 1
The *odd* primary stripes expand anteriorly in *opa* mutant embryos.

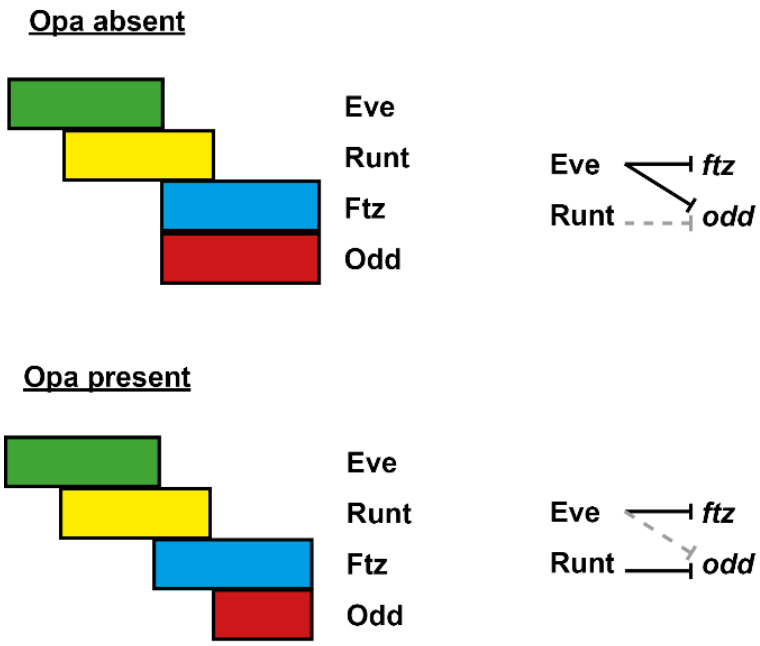

Figure 12–figure supplement 2
Model for the patterning of the anterior boundaries of *ftz* and *odd*.

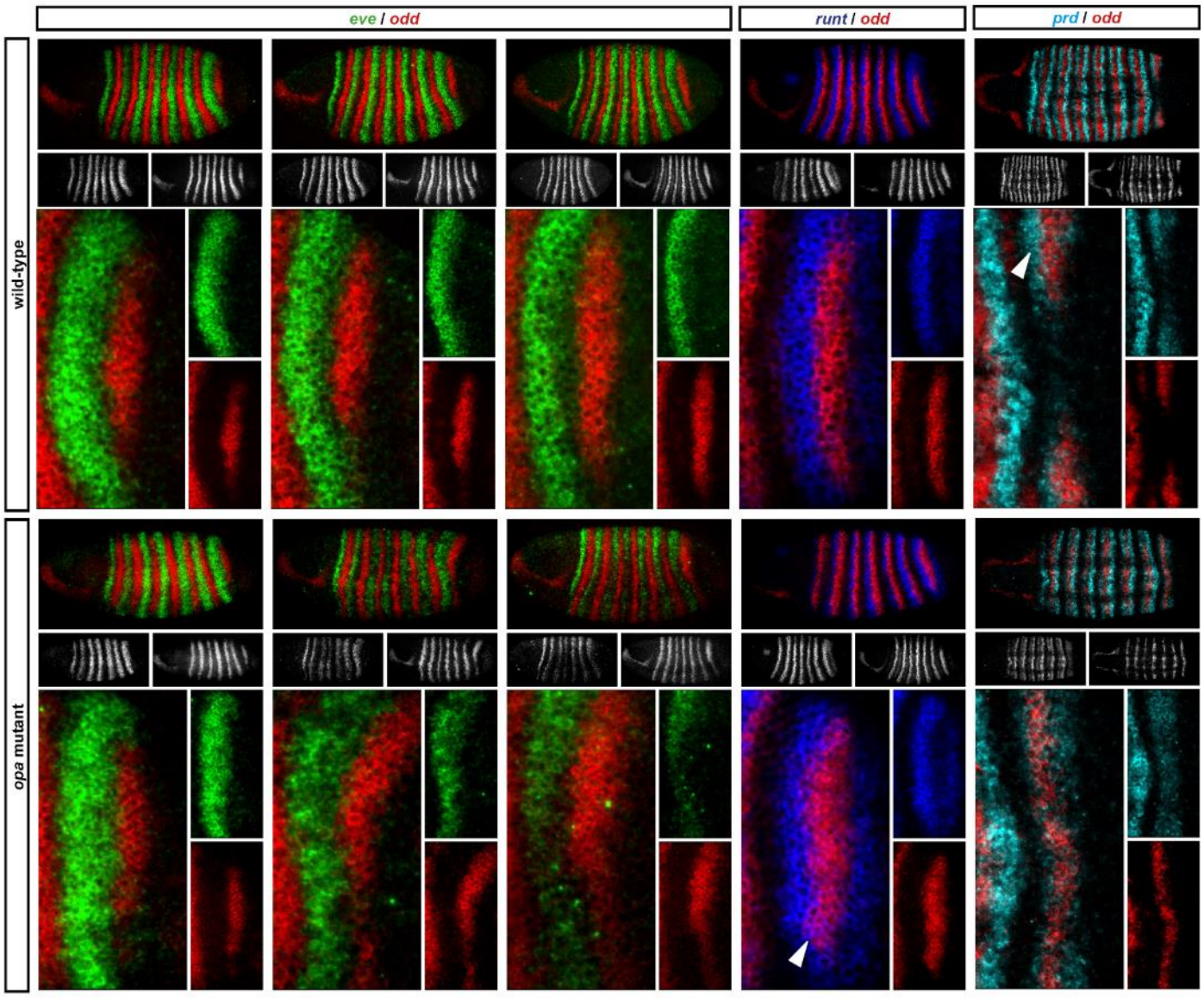

Figure 13
***odd* stripe 7 expands anteriorly and ventrally in *opa* mutants.**

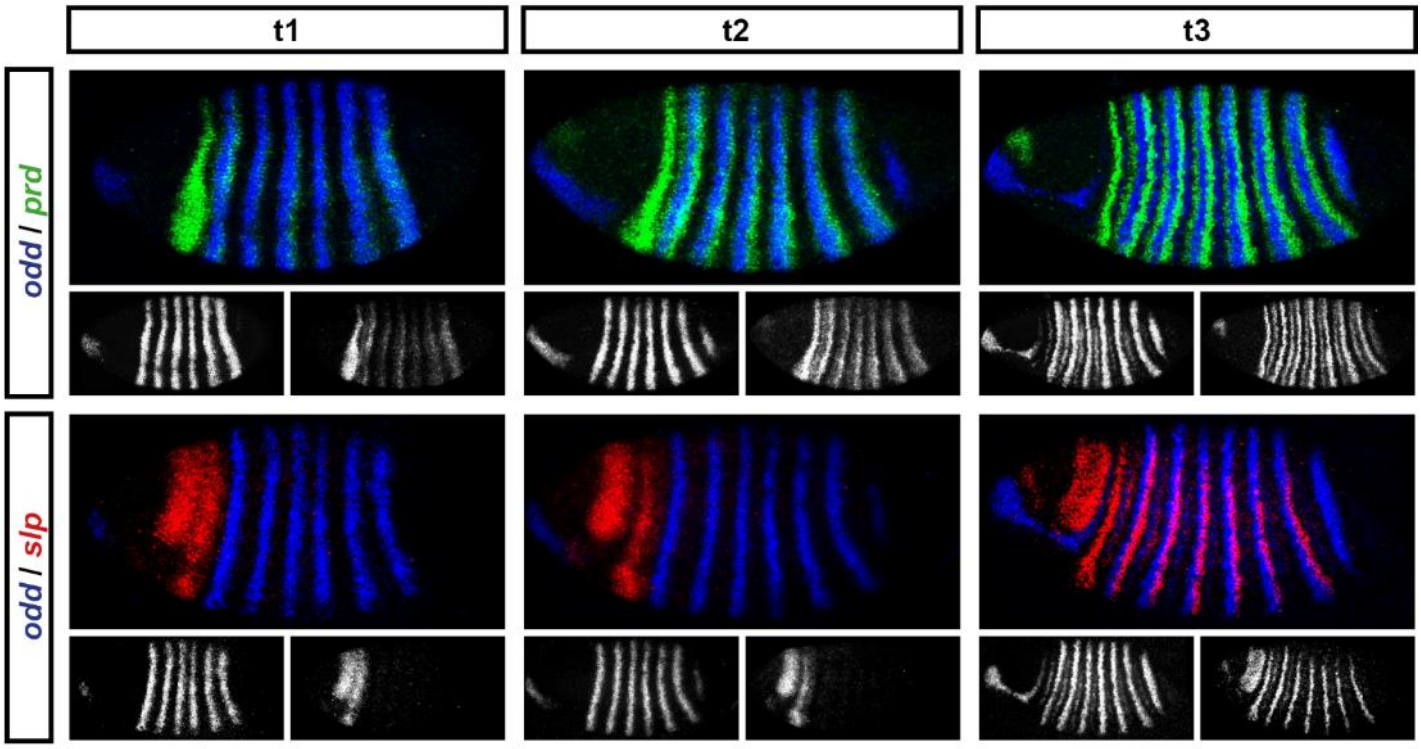

Figure 13–figure supplement 1
***odd* stripe 7 appears after the primary stripes of *prd*, but before the primary stripes of *slp*.**

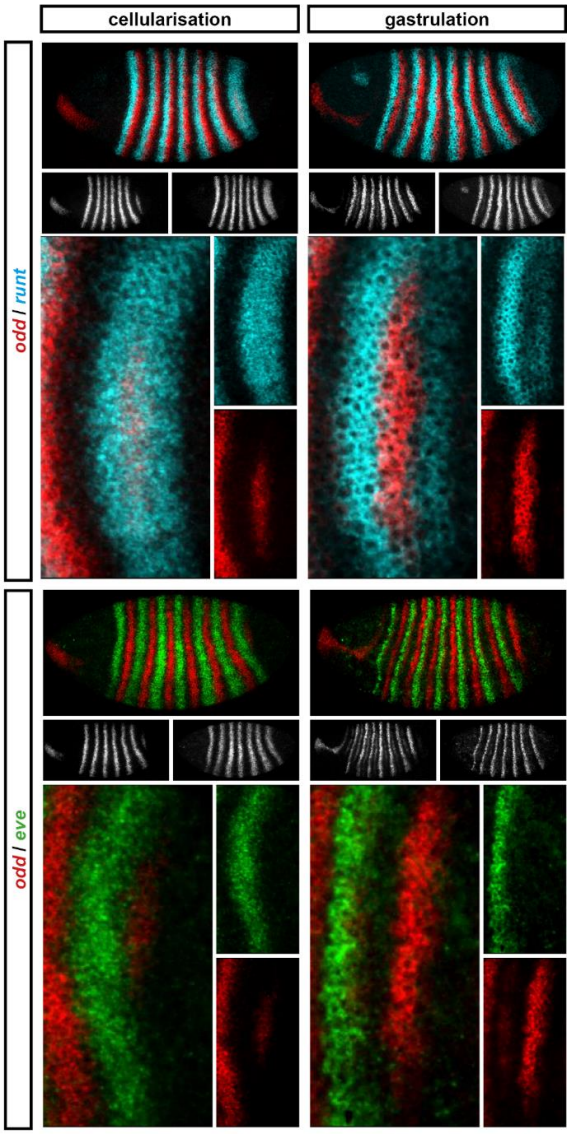

Figure 13–figure supplement 2
***eve* stripe 7 shifts anteriorly over time, but *odd* stripe 7 does not.**

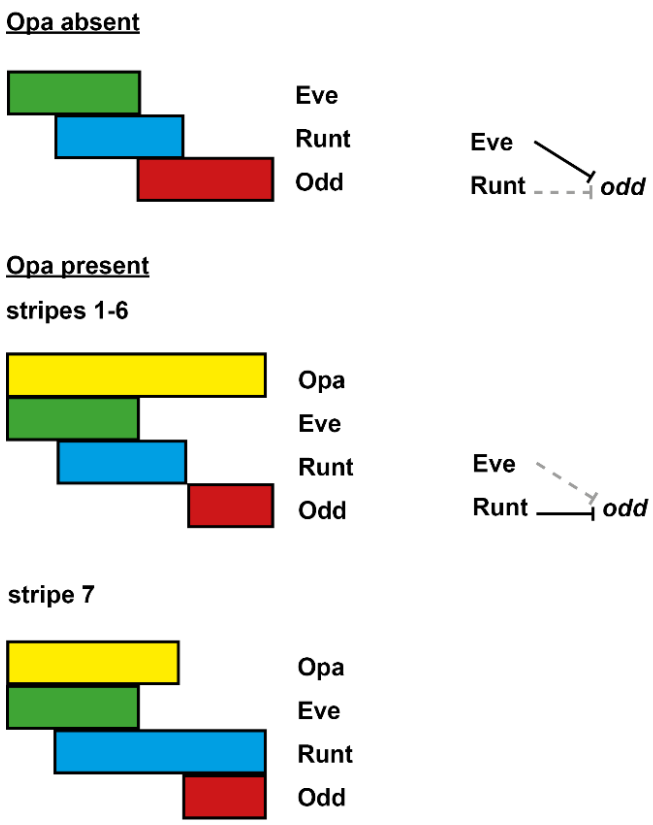

Figure 13–figure supplement 3
Model for the patterning of the anterior boundaries of *odd* primary stripes