

# **Evidence for the temporal regulation of insect segmentation by a conserved set of developmental transcription factors**

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## **ABSTRACT**

Long-germ insects, such as the fruit fly *Drosophila melanogaster*, pattern their segments simultaneously, whereas short germ insects, such as the beetle *Tribolium castaneum*, pattern their segments sequentially, from anterior to posterior. While the two modes of segmentation at first appear to be very different, many details of segmentation gene expression are surprisingly similar between long-germ and short-germ species. Collectively, these observations hint that insect segmentation may involve fairly conserved patterning mechanisms, which occur within an evolutionarily malleable spatiotemporal framework. Based on genetic and comparative evidence, we now propose that, in both *Drosophila* and *Tribolium* embryos, the temporal progression of the segmentation process is regulated by a temporal sequence of Caudal, Dichaete, and Odd-paired expression. These three transcription factors are broadly expressed in segmenting tissues, providing spatiotemporal information that intersects with the information provided by periodically-expressed segmentation genes such as the pair-rule factors. However, they are deployed differently in long-germ versus short-germ insects, acting as simple timers in *Drosophila*, but as smooth, retracting wavefronts in *Tribolium*, compatible with either gap gene-based or oscillator-based generation of periodicity, respectively.

## **INTRODUCTION**

An ancestral, conserved and defining feature of arthropods is their possession of a modular body plan composed of distinct segments serially arrayed along the anterior-posterior (AP) body axis. Over the last few decades, comparative developmental studies have revealed that

33 phylogenetically diverse arthropod species share a conserved network of “segment-polarity”  
34 genes such as *engrailed* (*en*) and *wingless* (*wg*) (Patel 1994; Damen 2002; Farzana & Brown  
35 2008; Janssen & Budd 2013). These genes, which are expressed in a segmentally-reiterated  
36 pattern of stripes, encode transcription factors and signalling molecules that together function  
37 to organise and maintain the boundaries between segments, as well as the polarity of  
38 anatomical features within them (DiNardo et al. 1994; Perrimon 1994; Sanson 2001). However,  
39 these comparative studies have also revealed that, across the phylum, quite disparate  
40 developmental strategies are used to generate this conserved segmental pattern (reviewed in  
41 Peel et al. 2005; Lynch et al. 2012; Williams & Nagy 2016).

42 The contrast is especially striking for insect segmentation. Although insects exhibit a highly  
43 conserved body plan compared to other arthropod clades (the number and identities of  
44 segments being largely invariant), the early stages of insect development vary dramatically  
45 between species. The distinction between “long-germ” and “short-germ” embryos is  
46 particularly notable (Krause 1939; Sander 1976; Davis & Patel 2002; Liu & Kaufman 2005;  
47 Rosenberg et al. 2009).

48 In long-germ embryos, e.g. those of the fruit fly *Drosophila melanogaster*, almost all segments  
49 are patterned during the blastoderm stage (Akam 1987; Nasiadka et al. 2002). (The term  
50 “long-germ” derives from the fact that, in this “simultaneous” mode of segment formation, the  
51 germ rudiment fills much of the volume of the egg from an early stage of embryogenesis.) We  
52 now have a detailed understanding of how the *Drosophila* embryo uses a bespoke set of  
53 “stripe-specific” enhancer elements, regulated by maternal and “gap” factors, to rapidly  
54 establish a periodic pattern of “pair-rule” gene transcription factor expression (Pankratz &  
55 Jäckle 1990; Small & Levine 1991; Schroeder et al. 2011). The pair-rule genes are so-called  
56 because their expression, each in a pattern of seven regularly-spaced stripes, reflects a  
57 transient double-segment periodicity within the *Drosophila* embryo (Nüsslein-Volhard &  
58 Wieschaus 1980; Hafen et al. 1984). The positional information in this pattern is then used, at  
59 gastrulation, to establish precise patterns of segment-polarity gene expression (14 stripes  
60 each). At the same time as the segment-polarity genes begin to be expressed, several of the  
61 pair-rule genes also begin to be expressed segmentally, owing to a rewiring of the pair-rule  
62 gene network which results in frequency doubling (Clark & Akam 2016a).

63 In contrast, short-germ embryos, e.g. those of the beetle *Tribolium castaneum*, have retained  
64 the ancestral arthropod condition of patterning their segments in a pronounced sequential  
65 sequence from anterior to posterior over the course of embryogenesis (Patel et al. 1994; Patel

66 1994; Choe et al. 2006; Choe & Brown 2007). (The term “short-germ” derives from the fact  
67 that these embryos pattern only their anterior segments at the blastoderm stage, from what is  
68 usually a shorter germ rudiment restricted to a posterior-ventral region of the early egg.) The  
69 more posterior segments are patterned after gastrulation, in a process that is often coupled to  
70 embryo growth.

71 In *Tribolium*, as well as a species of myriapod, the segmentation process has been shown to  
72 involve periodic oscillations of pair-rule gene expression in the posterior of the embryo  
73 (Sarrazin et al. 2012; El-Sherif et al. 2012; Brena & Akam 2013). Similar dynamic patterns of  
74 pair-rule gene expression have also been found in spiders, crustaceans, and other short-germ  
75 insects (for example, Schönauer et al. 2016; Eriksson et al. 2013; Mito et al. 2007). These  
76 findings have drawn parallels with vertebrate somitogenesis, which is thought to occur via a  
77 “clock and wavefront” mechanism (Cooke & Zeeman 1976; Palmeirim et al. 1997; Oates et al.  
78 2012). Indeed, pair-rule gene orthologs in short-germ arthropods appear to be either  
79 components of, or entrained by, a segmentation clock (Stollewerk et al. 2003; Choe et al.  
80 2006; Pueyo et al. 2008). Their striped expression patterns are thus generated by sustained  
81 oscillatory dynamics rather than through precise positioning by gap gene orthologs.

82 The terms “short-germ” and “long-germ” on their own do not adequately describe the true  
83 diversity of developmental modes observed within the insect clade, and are perhaps better  
84 considered opposite ends of an embryological and/or evolutionary spectrum (Davis & Patel  
85 2002). Individual insect species may fall anywhere on a continuum from extreme “short-germ”  
86 to extreme “long-germ” development, depending partly upon the number of segments  
87 patterned before versus after gastrulation. Although long-germ development is only found  
88 within holometabolous insects, each order within Holometabola contains both shorter-germ  
89 and longer-germ species, suggesting that long-germ segmentation has evolved from a short-  
90 germ ancestral state several times independently (Davis & Patel 2002; Jaeger 2011). There is  
91 also at least one documented case of long-germ segmentation reverting to the short-germ  
92 state (Sucena et al. 2014).

93 These observations argue that evolutionary transitions between the sequential/short-germ and  
94 simultaneous/long-germ modes of segment patterning must be relatively straightforward. At  
95 first sight, this inference is hard to square with the contrasting patterning strategies involved.  
96 However, close inspection of orthologous segmentation gene expression between long-germ  
97 and short-germ embryos (Figure 1) reveals striking commonalities, suggesting that these overt  
98 differences might mask an underlying mechanistic conservation (Patel et al. 1994; Davis et al.

99 2001; Damen et al. 2005; Davis et al. 2005; Choe et al. 2006; Janssen, Budd & Damen 2011;  
100 Green & Akam 2013). First, as alluded to above, all arthropods use periodically expressed pair-  
101 rule gene orthologs to initialise the segment pattern. Second, across all arthropods there is a  
102 temporal distinction between the earlier-expressed “primary” pair-rule genes (i.e. *hairy*, *even-*  
103 *skipped*, *runt*, *odd-skipped*, and, in some species, *ftz*) and the later-expressed “secondary” pair-  
104 rule genes (i.e. *paired* and *sloppy-paired*). Third, the primary pair-rule genes tend to be initially  
105 expressed dynamically, while the secondary pair-rule genes are expressed in stable stripes that  
106 prefigure segment-polarity gene expression and static segmental boundaries. Finally, judging  
107 by relative expression patterns, the way that the secondary pair-rule genes regulate the  
108 segment-polarity genes is likely strongly conserved (Figure 1C, right).

109 The unexpected similarities between long-germ and short-germ segmentation detailed above  
110 are much easier to rationalise in the light of a revised model of *Drosophila* segment  
111 patterning, which posits that dynamic shifts of pair-rule stripes are essential for the correct  
112 patterning of segment-polarity genes (Clark 2017). With this model, the *Drosophila* pair-rule  
113 gene network can easily be modified into a clock and wavefront-type system; depending on  
114 initial conditions, the simulated network can broadly recapitulate the patterning dynamics  
115 seen in either long-germ or short-germ insects, in both cases giving rise to the same final  
116 output pattern of segment-polarity gene expression. The model therefore suggests that long-  
117 germ and short-germ modes of segmentation represent alternate tissue-level behaviours of a  
118 fundamentally conserved transcriptional network, with similar cellular-level expression  
119 dynamics observed in each case. Specifically, the model proposes that the choice between  
120 these alternate macroscopic behaviours (simultaneous or sequential segment patterning) is  
121 determined by the particular spatiotemporal expression patterns of extrinsic inputs that  
122 control the timing of state transitions within the pair-rule network.

123 The insights from the modelling study rest on two key assumptions/predictions, which can be  
124 experimentally examined and tested in short-germ and long-germ insect models. First, there  
125 should exist broadly expressed factors that, via their influences on the segmentation network,  
126 control the temporal progression of the segmentation process. Two such factors have already  
127 been identified: in *Drosophila*, the transcriptional cofactor Odd-paired (Benedyk et al. 1994),  
128 which precipitates the transition from double-segment to single-segment periodicity (Clark &  
129 Akam 2016a), and, in *Tribolium*, the homeodomain transcription factor Caudal (Schulz et al.  
130 1998; Macdonald & Struhl 1986), which is thought to quantitatively tune the dynamics of the  
131 segmentation clock (El-Sherif et al. 2014). Second, evolutionary transitions between  
132 segmentation modes should be effected in part by altered expression profiles of these extrinsic

133 “timing factors”, which then drive altered downstream expression of the segmentation genes.  
134 Consequently, the spatiotemporal expression of Odd-paired, Caudal, and other timing factors  
135 should remain tightly correlated with specific phases of segmentation gene expression across  
136 all insect embryos, regardless of whether they exhibit a long-germ or short-germ mode of  
137 development.

138 In this manuscript, we first identify Caudal and Dichaete as additional factors playing  
139 important temporal roles in *Drosophila* segmentation. We chose to study these two genes  
140 because earlier studies have shown that mutations in these genes affect the segment pattern  
141 (Macdonald & Struhl 1986; Russell et al. 1996; Nambu & Nambu 1996), but the precise reasons  
142 why have never been clear. We then show that, as predicted, *Tribolium* orthologs of *caudal*,  
143 *Dichaete*, and *odd-paired* are expressed in the same temporal order, and preserve the same  
144 correlations with segmentation gene expression as are observed in *Drosophila*. However, while  
145 in *Drosophila* these factors are expressed ubiquitously throughout the trunk and thus act as  
146 simple timers, in *Tribolium* they provide retracting or advancing wavefronts, and thus could  
147 represent the primary source of spatial information within the short-germ segmentation  
148 process. Finally, we discuss the significance of these findings for the evolution of  
149 segmentation.

150

## 151 **RESULTS**

### 152 *caudal*, *Dichaete*, and *odd-paired* are expressed sequentially during *Drosophila* segmentation

153 Figure 2 shows the expression of *caudal* (*cad*), *Dichaete* (*D*), and *odd-paired* (*opa*) transcripts  
154 over the course of *Drosophila* segmentation, relative to the expression of the pair-rule gene  
155 *odd-skipped* (*odd*), which is here used as a marker to show the progressive refinement of  
156 segment patterning. We find that all three of these candidate timer genes are widely expressed  
157 throughout the trunk at some point during segmentation, consistent with previous  
158 descriptions of their expression patterns (Macdonald & Struhl 1986; Russell et al. 1996; Nambu  
159 & Nambu 1996; Benedyk et al. 1994). However, the phase of widespread expression spans a  
160 different developmental time period in each case, meaning that the segmentation process  
161 occurs against a changing background of these transcription factors’ expression.

162 Following a staging scheme introduced in a recent study of pair-rule gene regulation (Clark &  
163 Akam 2016a), pair-rule gene expression can be divided into three broad phases, spanning from  
164 early cellularisation to early germband extension (Figure 2A). During phase I (early

165 cellularisation), individual stripes of primary pair-rule gene expression are established in an ad  
166 hoc manner by stripe-specific enhancer elements, resulting in irregular/incomplete periodic  
167 patterns. During phase 2 (mid- to late-cellularisation), pair-rule factors cross-regulate each  
168 other through “zebra” elements, resulting in regular stripes of double-segment periodicity, and  
169 the secondary pair-rule genes turn on in the trunk. During phase 3 (gastrulation onwards), the  
170 regulatory network changes and the expression patterns of some of the pair-rule genes  
171 (including *odd*) transition to single-segment periodicity.

172 *cad* and *Dichaete* are both expressed prior to cellularisation and continue to be expressed  
173 during phase 1, meaning that the periodic patterns of the primary pair-rule genes emerge in  
174 the context of Cad and Dichaete activity. (Indeed, both Cad and Dichaete have been found to  
175 regulate stripe-specific elements: La Rosée et al. 1997; Häder et al. 1998; Ma et al. 1998; Fujioka  
176 et al. 1999). *cad* expression retracts to a narrow posterior domain early in phase 2, while  
177 *Dichaete* expression persists but gradually fades, passing through a transient phase of periodic  
178 modulation. Since the dynamics of Cad and Dichaete protein expression appear to closely  
179 reflect their respective transcript patterns (Macdonald & Struhl 1986; Ma et al. 1998), most of  
180 phase 2 is likely to be characterised by Dichaete activity alone. (*opa* expression builds up  
181 during this time, but owing to the length of the *opa* transcription unit, Opa protein levels are  
182 likely to be absent or low (Benedyk et al. 1994).) Finally, phase 3 is characterised by strong *opa*  
183 expression, while *Dichaete* becomes expressed in a narrow posterior domain and throughout  
184 the neuroectoderm. The widespread changes in pair-rule gene expression that occur during  
185 this time are now known to be triggered by Opa activity (Clark & Akam 2016a).

186

187 *caudal*, *Dichaete*, and *odd-paired* expression spatially correlates with segmentation timing  
188 along the anteroposterior axis

189 In addition to this general temporal progression within the trunk, there are also interesting  
190 spatial correlations with the segmentation process (Figure 2B-D). Although *Drosophila*  
191 segmentation is generally described as “simultaneous”, the AP axis can actually be divided into  
192 three distinct regions, which undergo segment patterning at slightly different times (Figure  
193 2C; Surkova et al. 2008). In region 1 (near the head-trunk boundary, encompassing *odd* stripe  
194 1 and *paired* (*prd*) stripes 1 and 2), both primary and secondary pair-rule genes are expressed  
195 very early, and head-specific factors play a large role in directing gene expression (Schroeder  
196 et al. 2011; Andrioli et al. 2004; Chen et al. 2012). In region 2 (the main trunk, encompassing  
197 *odd* stripes 2-6 and *prd* stripes 3-7), primary pair-rule genes turn on at phase 1, secondary pair-

198 rules turn on during phase 2, and segment-polarity genes turn on at gastrulation. Finally, in  
199 region 3 (the tail, encompassing *odd* stripe 7 and *prd* stripe 8), the expression of certain pair-  
200 rule genes is delayed, while segment-polarity gene expression emerges only during germband  
201 extension (Kuhn et al. 2000). This region of the fate map eventually gives rise to the “cryptic”  
202 terminal segments A9-All (Campos-Ortega & Hartenstein 1985; Kuhn et al. 1995).

203 Suggestively, the boundaries of these three regions correspond to expression boundaries of  
204 our candidate timing factors at mid-cellularisation (Figure 2B, left). Region 1 never expresses  
205 *cad* or *Dichaete*, but does express *opa*, whose expression domain extends further anterior than  
206 those of the other two genes. Region 2 corresponds to the early broad domain of *Dichaete*  
207 expression, which extends from just behind *odd* stripe 1 to just behind *odd* stripe 6 (the *opa*  
208 expression domain shares the same posterior boundary). Finally, region 3 is characterised by  
209 *cad* expression, which extends further posterior than the other two genes, and persists in this  
210 posterior region. The expression profiles of *cad*, *Dichaete*, and *opa* thus correlate with the  
211 current state of the segmentation process across space, as well as over time.

212 Region 3 is particularly intriguing, because as *odd* stripe 7 begins to be expressed, the cells also  
213 start to express *Dichaete* (Figure 2B), and as gastrulation proceeds, the cells begin to express  
214 *opa* (Figure 2D), thus recapitulating the same temporal sequence that occurred earlier in  
215 region 2. Interestingly, we noticed that the expression boundaries of *cad*, *Dichaete*, and *opa* all  
216 shift posteriorly relative to *odd* stripe 7 over time (Figure 2E; for evidence that these are “true”  
217 kinematic shifts, see Figure SI).

218 Given 1) that *Opa* activity triggers many of the pair-rule gene expression changes observed at  
219 gastrulation, 2) that proper segment patterning is also dependent on *Cad* and *Dichaete*  
220 activity, and 3) that the temporal progression of *cad/Dichaete/opa* expression is correlated  
221 with the temporal progression of the segmentation process, it is possible that all three genes  
222 provide important temporal regulatory information to the pair-rule system. We therefore  
223 investigated whether *Cad* and *Dichaete*, like *Opa*, show regulatory effects on the *Drosophila*  
224 pair-rule gene network.

225

### 226 Caudal controls the onset of *paired* expression

227 The clearance of *cad* transcript during cellularisation occurs in a distinctive spatiotemporal  
228 pattern: expression is lost from anterior regions before posterior regions, and from ventral  
229 regions before dorsal regions. A similar spatiotemporal pattern is seen at the protein level

230 (Macdonald & Struhl 1986), suggesting that Cad protein turns over rapidly, and that *cad*  
231 transcript patterns therefore provide a reasonable proxy for Cad protein dynamics during this  
232 period.

233 Figure 3 shows that this retraction of *cad* expression is tightly spatiotemporally associated  
234 with the emergence of the *prd* stripes. *prd* stripe 1 is established during early cellularisation  
235 under the control of a stripe-specific element (Schroeder et al. 2011) and is located just  
236 anterior to the broad *cad* domain in the trunk. *prd* stripe 2 follows and then stripes 3-7 emerge  
237 during mid-cellularisation, appearing with a marked anterior-to-posterior and ventral-to-  
238 dorsal progression that is the inverse of the retracting *cad* domain. Finally, *prd* stripe 8  
239 (asterisk in Figure 3) emerges towards the end of cellularisation, as the *cad* domain in the tail  
240 retracts posteriorly (see Figure S1).

241 Setting aside the periodic nature of the *prd* output, *cad* and *prd* thus exhibit strikingly  
242 complementary patterns of expression throughout the entire segmentation process,  
243 suggesting that Cad might repress *prd* and thus control the time of onset of its expression. To  
244 test this, we examined the emergence of the *prd* stripes in *cad* zygotic mutant embryos (Figure  
245 4). Note that we chose to analyse *cad* zygotic mutants, which retain maternal *cad* expression  
246 and undergo largely normal segment patterning, rather than complete *cad* nulls, because Cad  
247 has pleiotropic effects on the segmentation process, and we wanted to be able to distinguish a  
248 possible direct effect on *prd* expression from expected indirect effects stemming from  
249 perturbations to early gap gene expression (reviewed in Jaeger 2011).

250 Consistent with the proposal that Cad represses *prd* expression, we found that *prd* expression  
251 is precocious and more intense in *cad* zygotic mutants. In addition, the stripes turn on  
252 simultaneously along the dorsoventral axis rather than in a ventral to dorsal progression,  
253 correlating with the absence of the spatially-patterned zygotic Cad domain. (Note, however,  
254 that the *prd* stripes remain less intense in dorsal regions of the embryo than elsewhere,  
255 indicating that some of the remaining regulators of *prd* expression must be dorsoventrally  
256 modulated.)

257 In contrast to the marked effects on early *prd* expression, other aspects of segmentation gene  
258 expression are largely unperturbed in *cad* zygotic mutant embryos, aside from a modest  
259 posterior expansion of the fate map (asterisks in Figure S2B). For example, there are only very  
260 subtle spatiotemporal changes to the *odd*, *slp*, and *en* stripes (Figure 4 and Figure S2), and late  
261 *prd* expression is also fairly normal, with stripe splitting (an effect of Opa activity) occurring at  
262 the same time as in wild-type (Figure 4). These observations indicate that the temporal

263 regulatory information provided by *Cad* is at least partially decoupled from the general  
264 progression of the segmentation process in *Drosophila*.

265

### 266 Dichaete influences the topology of the early pair-rule network

267 The cuticles of *Dichaete* null larvae exhibit segmentation defects of varying severity, often  
268 involving several segment fusions and/or deletions (Russell et al. 1996; Nambu & Nambu  
269 1996). These defects are not randomly distributed, but tend to affect particular segments (e.g.  
270 loss of the A2 and A8 denticle belts, narrowing of the A3 belt, and fusions between A4-A5 and  
271 A6-A7). Since gap gene expression is normal in *Dichaete* mutants but pair-rule gene  
272 expression is perturbed (Russell et al. 1996), *Dichaete* is thought to directly regulate pair-rule  
273 gene expression, consistent with high levels of *Dichaete* binding around pair-rule gene loci  
274 (Macarthur et al. 2009; Aleksic et al. 2013). However, the specific aetiology of the *Dichaete*  
275 mutant phenotype and the reason for its variable severity are not known.

276 At least some of *Dichaete*'s regulatory effects are mediated by stripe-specific elements (Ma et  
277 al. 1998; Fujioka et al. 1999). However, *Dichaete* might also act on pair-rule gene “zebra”  
278 elements (which take inputs from other pair-rule genes), and thereby influence pair-rule gene  
279 cross-regulation. To investigate this possibility, we examined whether any of the interactions  
280 within the early (phase 2) pair-rule gene network (Clark & Akam 2016a; Clark 2017) seemed to  
281 be perturbed in *Dichaete* mutants.

282 Five of the pair-rule genes are patterned by other pair-rule genes during phase 2: *runt*, *ftz*, *odd*,  
283 *prd*, and *slp*. Based on double in situ data, the regulatory logic governing *ftz*, *odd*, and *slp*  
284 expression appears to be normal in *Dichaete* mutant embryos: despite the irregularity of the  
285 expression patterns, sharp expression boundaries between repressors and their targets are still  
286 observed at mid-cellularisation (Figure S3A). (In other words, even though both the regulatory  
287 inputs and transcriptional outputs of each gene are clearly perturbed, normal input-output  
288 relationships appear to be preserved.) Note that, in contrast to previous descriptions of  
289 *Dichaete* mutants (Russell et al. 1996), we find that pair-rule gene expression patterns are  
290 fairly consistent across different embryos, as long as the embryos are similarly-staged.

291 Surprisingly, we noticed that *odd* is expressed almost ubiquitously throughout the trunk  
292 during early cellularisation (phase 1), even though it resolves into a fairly normal pair-rule  
293 pattern as cellularisation proceeds (Figure S3B). This ectopic expression looks very similar to  
294 the expression pattern of *odd* in *hairy* mutant embryos (Figure S3B), indicating that the effect

295 may be mediated by a functional interaction between *Dichaete* and *Hairy*. However, any such  
296 interaction would have to be both temporary and target-specific: temporary because *Hairy* is  
297 evidently still able to repress *odd* normally during phase 2, and target-specific because no  
298 similar ectopic expression is seen for *ftz*, which also requires repression from *Hairy* during  
299 phase 1 (Figure S3C).

300 In contrast to *ftz*, *odd*, and *slp*, the transcriptional outputs of *runt* and *prd* in *Dichaete* mutants  
301 are perturbed in ways that indicate significant deviations from their wild-type regulatory logic  
302 (Figure 5; Figure 6). The expression patterns of these two genes markedly overlap with those  
303 of genes that would normally repress them, demonstrating that these particular regulatory  
304 interactions are crucially dependent upon *Dichaete* activity.

305 *prd* shows the most dramatic expression changes (Figure 5). The early *prd* stripes are normally  
306 patterned by direct repression from *Eve* – they broaden somewhat in *eve* heterozygotes, and  
307 fuse into a largely aperiodic expression domain in *eve* mutant embryos (Figure 5B;  
308 Baumgartner & Noll 1990; Clark 2017). Although the *eve* stripes are still present in *Dichaete*  
309 mutant embryos (Figure 5A), early *prd* expression shows almost as great a loss of periodicity as  
310 observed in *eve* mutant embryos, suggesting that the repression of *prd* by *Eve* requires  
311 *Dichaete* activity. This apparent cofactor role for *Dichaete* must be target-specific, since other  
312 *Eve* targets are repressed normally in *Dichaete* mutant embryos (see above). Note that the  
313 repression of *prd* by *Eve* is restricted to earlier phases of segmentation in wild-type embryos,  
314 and that its temporary nature is likely to be important for segment patterning (Fujioka et al.  
315 1995; Clark 2017). Our results now suggest a mechanistic explanation for the temporal  
316 modulation of this regulatory interaction: the loss of *Dichaete* expression over the course of  
317 cellularisation.

318 Unlike *prd*, *runt* is still expressed in seven stripes in *Dichaete* mutants, but these stripes are  
319 very irregular (Figure 6). Stripes 3 and 7 are broadened compared to wild-type, while stripes 2,  
320 5, and 6 are narrow and fairly weak. In wild-type embryos, the regular stripes of *runt* seen at  
321 mid-cellularisation (phase 2) are defined by repression from *Hairy* at their anteriors, and  
322 repression from *Odd* at their posteriors (Figure 6C; Klingler & Gergen 1993; Saulier-Le Dréan  
323 et al. 1998; Clark & Akam 2016a), resulting in sharply abutting expression domains (Figure  
324 6A,B left). However, *runt* expression in *Dichaete* mutant embryos tends to overlap with the  
325 stripes of both *hairy* and *odd* (Figure 6A,B right), indicating that this cross-repression is either  
326 ineffective or absent. Given the weakened, although still periodic, expression of *runt* in  
327 *Dichaete* mutants, combined with the apparent failure to respond to pair-rule inputs, we think

328 that the phase 2 expression we see may derive mainly from *runt*'s stripe-specific elements,  
329 rather than mainly from *runt*'s zebra element as in wild-type (Figure 6D). This inference  
330 implicates Dichaete as an activator of the zebra element (Figure 6C).

331 Later aspects of both *runt* and *prd* expression then appear fairly normal in *Dichaete* mutant  
332 embryos – as might be expected since Dichaete activity would be largely absent from the trunk  
333 during these stages even in wild-type embryos. The *runt* stripes sharpen, and become  
334 complementary with those of both *odd* and *hairy*, events that are presumably triggered by  
335 known, Opa-dependent regulatory interactions taking effect (Figure 6C; Clark & Akam 2016a).  
336 *prd* expression first becomes spatially modulated, and then transitions to a segmental pattern  
337 as normal. The initial expression changes are probably regulated by Runt (compare Figure 6),  
338 accounting for their irregularity, while stripe splitting is another Opa-dependent effect  
339 (Baumgartner & Noll 1990).

340 However, although pair-rule gene regulatory logic thus appears completely wild-type during  
341 these later stages, the resulting expression patterns are of course abnormal, owing to the  
342 previous misregulation of key genes. Figure S4 shows how these early patterning irregularities  
343 are carried forward, eventually resulting in segment boundary defects that prefigure the  
344 cuticle defects observed at the end of development. (Specifically, irregular *runt* stripes lead to  
345 abnormal widths and spacing of the *slp* stripes, something which then has knock-on effects for  
346 the final pattern of segment-polarity fates.) We have therefore shown that the *Dichaete*  
347 segmentation phenotype follows in a predictable manner from abnormal patterns of pair-rule  
348 gene expression laid down at the blastoderm stage, illustrating the importance of precise early  
349 stripe phasing for producing a viable embryo.

350

351 *Tc-caudal*, *Tc-Dichaete*, and *Tc-odd-paired* provide staggered wavefronts that likely regulate  
352 *Tribolium* segmentation

353 We have shown above that Cad, Dichaete, and Odd-paired all have important, and temporally  
354 distinct, influences on the *Drosophila* pair-rule gene network. Given that pair-rule gene  
355 orthologs carry out segment patterning in both long-germ and short-germ insects, we  
356 wondered whether the roles of these “timing factors” might also be broadly conserved. We  
357 therefore examined the expression of their orthologs, *Tc-cad*, *Tc-Dichaete*, and *Tc-opa*, in the  
358 short-germ beetle *Tribolium castaneum*. The expression patterns of *Tc-cad* and *Tc-Dichaete*  
359 have been described previously (Schulz et al. 1998; Oberhofer et al. 2014), but only for a small

360 number of stages, affording only a limited view into their spatiotemporal dynamics over the  
361 course of segmentation. *opa* expression has been previously looked at in spiders and  
362 myriapods (Damen et al. 2005; Janssen, Budd, Prpic, et al. 2011), but to our knowledge has not  
363 been examined in non-dipteran insects.

364 Figure 7 shows staged expression of *Tc-cad*, *Tc-Dichaete*, and *Tc-opa*, all relative to a common  
365 marker, *Tc-wg*, over the course of germband extension. A more extensive set of stages is  
366 shown in Figure S5, and direct comparisons between *Tc-cad/Tc-Dichaete*, *Tc-cad/Tc-opa*, and  
367 *Tc-Dichaete/Tc-opa* are shown in Figure S6.

368 *Tc-cad* is continuously expressed in the growth zone, resulting in a persistent posterior  
369 domain that gradually shrinks over time. *Tc-cad* therefore turns off in presegmental tissues  
370 just as they emerge from the anterior of the growth zone, and well before the *Tc-wg* stripes  
371 turn on.

372 *Tc-Dichaete* is also broadly expressed within the growth zone, but is excluded from the most  
373 posterior tissue, turning on slightly anterior to the terminal *Tc-wg* domain. The growth zone  
374 expression of *Tc-Dichaete* extends slightly further to the anterior than that of *Tc-cad*, turning  
375 off just before the *Tc-wg* stripes turn on. (*Tc-Dichaete* expression anterior to the *Tc-cad*  
376 domain tends to be at lower levels and/or periodically modulated.) In a subset of embryos we  
377 observe a separate stripe of *Tc-Dichaete* expression, anterior to growth zone domain, which  
378 clearly encompasses lateral ectodermal cells (Figure 8; Figure S6, A-L). *Tc-Dichaete* expression  
379 later transitions into persistent expression within the neuroectoderm, with expression now  
380 absent from the more lateral ectodermal regions.

381 Finally, *Tc-opa* is absent from the posterior half of the growth zone, but is expressed in a broad  
382 posterior domain starting in the anterior growth zone and extending anteriorly far enough to  
383 overlap nascent *Tc-wg* stripes. The intensity of expression in this domain is periodically-  
384 modulated, with *Tc-opa* expression transitioning more anteriorly into relatively persistent  
385 segmental stripes. The segmental stripes cover the central third of each segment, lying  
386 posterior to each *Tc-en* stripe (Figure 8R).

387 The most striking aspect of these expression patterns is that while they are obviously dynamic,  
388 retracting posteriorly as the embryo elongates, they appear remarkably consistent from one  
389 stage to the next: the posterior expression domains of each of the three genes retain the same  
390 relationship to the gross morphology of the embryo, and to each other, at each timepoint. This  
391 means that each cell that starts off within the growth zone will at some point experience a

392 temporal progression through the three transcription factors, similar to that experienced by  
393 cells within the *Drosophila* trunk over the course of cellularisation and gastrulation (compare  
394 Figure 2). For most of the cells that contribute to the *Tribolium* trunk, this sequence is likely  
395 to start with Cad+Dichaete, transit through Dichaete+Opa, and end with Opa alone (compare  
396 Figure 7 against the *Tribolium* lineage tracing experiments from Nakamoto et al. 2015).

397 We also compared the expression domains of *Tc-cad*, *Tc-Dichaete*, and *Tc-opa* to the  
398 expression patterns of a number of *Tribolium* segmentation genes (Figure 8; and more  
399 extensive developmental series in Figures S7-S10). Strikingly, we found that the temporal  
400 associations of each factor with specific phases of segmentation gene expression are very  
401 similar to the temporal associations observed in *Drosophila*. In *Drosophila*, we found that the  
402 onset of *prd* expression correlated with the retraction of *cad* expression (Figure 3), and that  
403 the early, pair-rule phase of *prd* expression involved regulation by Dichaete (Figure 5); in  
404 *Tribolium*, *Tc-prd* turns on at the anterior limit of the *Tc-cad* domain (Figure 8A-C; Figure S7),  
405 with the pair-rule phase of *Tc-prd* expression falling within the *Tc-Dichaete* domain, and stripe  
406 splitting occurring anterior to it (Figure 8D-F; Figure S8). In *Drosophila*, we found that the  
407 phase 2 (zebra element mediated) expression of *runt* seemed to be activated by Dichaete  
408 (Figure 6); in *Tribolium*, the *Tc-runt* pair-rule stripes turn on at the very posterior of the *Tc-*  
409 *Dichaete* domain (Figure 8G-I; Figure S9). Finally, in *Drosophila*, Opa is required for the  
410 frequency doubling of pair-rule gene expression and the activation of segment-polarity gene  
411 expression (Clark & Akam 2016a; Benedyk et al. 1994); in *Tribolium*, frequency doubling of all  
412 the pair-rule genes examined occurs within the *Tc-opa* domain, and the stripes of the  
413 segment-polarity genes *Tc-en* and *Tc-wg* emerge within it, as well (Figure 8j-R; Figure S10).

414 The overall temporal progression of the segmentation process thus seems to be remarkably  
415 similar in both species: primary pair-rule genes are expressed dynamically in the context of  
416 Cad and Dichaete expression, secondary pair-rule genes turn on as Cad turns off, and  
417 frequency doubling and segment-polarity activation occur in the context of Opa expression.  
418 Although we have not yet tested these relationships functionally in *Tribolium*, close  
419 spatiotemporal associations between segmentation gene expression and our candidate timing  
420 factors are evident throughout development, strongly suggesting that patterns of *Tc-Cad*, *Tc-*  
421 *Dichaete*, and *Tc-Opa* activity coordinate the segmentation process, as they do in *Drosophila*.  
422 Furthermore, the similarity of the correlations with segmentation gene expression to those in  
423 *Drosophila* make it likely that orthologs of Caudal, Dichaete, and Odd-paired temporally  
424 regulate the segmentation process in similar (although not necessarily identical) ways in the  
425 two different insects.

426 However, while the regulatory functions of these transcription factors might be broadly  
427 conserved between *Drosophila* and *Tribolium*, the spatiotemporal deployment of those  
428 functions is obviously divergent. In *Drosophila*, the factors are expressed ubiquitously  
429 throughout the trunk and each turns on or off all at once (Figure 2), consistent with  
430 controlling the temporal progression of a simultaneous segmentation process. In *Tribolium*,  
431 their expression domains are staggered in space, with anterior regions always subjected to a  
432 “later” regulatory signature than more posterior tissue (Figure 7). Furthermore, these  
433 expression domains retract over the course of germband extension, effectively generating  
434 smooth wavefronts that sweep posteriorly across the tissue, consistent with controlling the  
435 temporal progression of a sequential segmentation process built around a segmentation clock.

436

## 437 DISCUSSION

### 438 Caudal, Dichaete, and Odd-paired play temporal regulatory roles in *Drosophila* segmentation

439 Our dissection of the temporal regulatory roles of Cad and Dichaete builds on other recent  
440 work on the *Drosophila* pair-rule gene network (Clark & Akam 2016a; Clark 2017), and  
441 represents an important step towards a full understanding of this paradigmatic developmental  
442 patterning system. Our findings highlight the overall complexity of pair-rule patterning, which  
443 consists of several distinct phases of gene expression, each requiring specific regulatory logic.  
444 However, our results also demonstrate how the whole process is orchestrated using just a  
445 small number of key regulators expressed sequentially over time. (Although note that there  
446 may well be other important timing factors involved in regulating the segmentation process,  
447 whose roles are yet to be discovered.) In particular, by rewiring the regulatory connections  
448 between other genes, factors like Dichaete and Opa allow a small set of pair-rule factors to  
449 carry out multiple different roles. This kind of control logic makes for a flexible and fairly  
450 modular regulatory network, and may therefore turn out to be a hallmark of other complex  
451 patterning systems.

452 Further work will be required in order to dissect how this combinatorial regulation is  
453 implemented at the molecular level. Dichaete is known to act both as a repressive cofactor  
454 (Zhao & Skeath 2002; Zhao et al. 2007) and as a transcriptional activator (Aleksic et al. 2013),  
455 therefore a number of different mechanisms are plausible. The Odd-paired protein is also  
456 likely to possess both these kinds of regulatory activities (Ali et al. 2012). It should prove  
457 particularly interesting to analyse the enhancer regions of genes such as *runt* and *prd*, which

458 are regulated by both Dichaete and Opa, to find out how exactly their complex expression  
459 profiles are generated.

460

461 The segmentation roles of Caudal, Dichaete, and Odd-paired are likely to be broadly  
462 conserved between long-germ and short-germ insects

463 In the second half of this paper, we revealed that Cad, Dichaete, and Opa are likely to play a  
464 similar set of roles in *Tribolium* and *Drosophila* segmentation. Given the phylogenetic distance  
465 between beetles and flies (separated by at least 300 million years, Wolfe et al. 2016), we  
466 suggest that the similarities seen between *Drosophila* and *Tribolium* segmentation are likely to  
467 hold true for other insects, and perhaps for many non-insect arthropods as well. These  
468 predictions can be tested by comparative studies in various arthropod model organisms. In  
469 particular, we anticipate that the expression of *cad*, *Dichaete*, and *opa* orthologs should retain  
470 broadly the same relationship to segmentation gene expression across a wide diversity of  
471 developmental modes, and that manipulating the expression of these factors should alter  
472 segmentation gene expression accordingly. Note that such manipulations would have to be  
473 designed carefully in order to be informative, since *cad* knockdowns in short-germ arthropods  
474 cause severe axis truncations (Copf et al. 2004), while Dichaete might exhibit redundancy  
475 with other Sox factors, such as SoxN (Overton et al. 2002).

476 Assuming the temporal regulatory roles of these factors are conserved, they would represent a  
477 common spatiotemporal framework for insect segmentation that unifies long-germ and short-  
478 germ modes of development. Our findings therefore strengthen recent proposals of regulatory  
479 homology between the *Drosophila* early pair-rule gene network and the “segmentation clock”  
480 of short germ insects (Clark 2017). This framework could also make sense of other differences  
481 between long-germ and short-germ development: for example, if our timing factors turn out  
482 to temporally regulate Toll gene expression, the various locations of their expression domains  
483 would likely explain the different spatiotemporal distributions of intercalation-based  
484 convergent extension observed in different insect species (Paré et al. 2014; Benton et al. 2016)

485

486 The evolution of arthropod segmentation

487 Finally, we think that the regulatory roles we have uncovered for Cad, Dichaete, and Odd-  
488 paired may be highly significant regarding the evolutionary origins and subsequent  
489 modification of arthropod segmentation. The patterns of *Tc-cad*, *Tc-Dichaete*, and *Tc-opa*

490 expression that we have characterised are striking, because essentially similar spatiotemporal  
491 expression patterns are observed for these genes in other bilaterian clades, including  
492 vertebrates. Cdx genes are expressed in the posterior of vertebrate embryos, where they play  
493 crucial roles in axial extension and Hox gene regulation (van Rooijen et al. 2012; Neijts et al.  
494 2016). Sox2 (a Dichaete ortholog) has conserved expression in the nervous system, but is also  
495 expressed in a posterior domain, where it is a key determinant of neuromesodermal  
496 progenitor (posterior stem cell) fate (Wood & Episkopou 1999; Wymeersch et al. 2016).  
497 Finally, Zic2 and Zic3 (Opa orthologs) are expressed in presomitic mesoderm and nascent  
498 somites, and have been functionally implicated in somitogenesis (Inoue et al. 2007). All three  
499 factors thus have important functions in posterior growth, roles which may well be conserved  
500 across Bilateria (Copf et al. 2004).

501 In *Tribolium*, we think it likely that all three factors are integrated into an ancient gene  
502 regulatory network downstream of Wnt signalling, which generates their sequential  
503 expression and helps regulate posterior proliferation and/or differentiation (McGregor et al.  
504 2009; Oberhofer et al. 2014; Williams & Nagy 2016). For example, it is suggestive that we  
505 observe mutually exclusive patterns of *Tc-wg* and *Tc-Dichaete* in the growth zone: Wnt  
506 signalling and Sox gene expression are known to interact in many developmental contexts  
507 (Kormish et al. 2010), and these interactions may form parts of temporal cascades  
508 (Agathocleous et al. 2009) .

509 We therefore suggest the following outline as a plausible scenario for the evolution of  
510 arthropod segmentation. 1) In non-segmented bilaterian ancestors of the arthropods, Cad,  
511 Dichaete and Opa were expressed broadly similarly to how they are expressed in *Tribolium*  
512 today, owing to conserved roles in posterior elongation. 2) At some point, segmentation genes  
513 started to be expressed under the regulatory control of these factors, which represented a pre-  
514 existing source of spatiotemporal information. Pair-rule genes started oscillating in the  
515 posterior, perhaps under the control of Cad (El-Sherif et al. 2014; Schönauer et al. 2016), while  
516 the retracting expression boundaries of the timing factors provided wavefronts that effectively  
517 translated these oscillations into a periodic patterning of the AP axis, analogous to the roles of  
518 the opposing retinoic acid and FGF gradients in vertebrate somitogenesis (Oates et al. 2012).  
519 3) Much later, in certain lineages of holometabolous insects, the transition to long-germ  
520 segmentation occurred. This would have involved two main modifications of the short-germ  
521 segmentation process: A) changes to the expression of the timing factors, away from the  
522 situation seen in *Tribolium*, and towards the situation seen in *Drosophila* (thus causing a  
523 major heterochronic shift in the deployment of the segmentation machinery, plus a

524 coordinated shift in all the other processes downstream of the timing factors), and B)  
525 recruitment of gap genes to pattern pair-rule stripes, via the ad hoc evolution of stripe-specific  
526 elements. This transition process would likely have occurred gradually along the body axis,  
527 from anterior to posterior (Peel & Akam 2003; Rosenberg et al. 2014).

528 In other words, this model involves first creating new regulatory interactions between existing  
529 domains of expression and the downstream genes that are to be patterned, and later changing  
530 these upstream expression domains, without altering the existing downstream regulatory  
531 network. The recruitment/reuse of existing developmental features at each stage reduces the  
532 number of regulatory changes that would have to evolve de novo, facilitating the evolutionary  
533 process. Note that under this hypothesis arthropod segmentation would not be homologous to  
534 segmentation in other phyla, but would probably have been fashioned from common parts  
535 (Chipman 2010; Graham et al. 2014).

536

## 537 **METHODS**

### 538 *Drosophila melanogaster*

539 Embryo collections were carried out at 25° C. The *Drosophila* mutants used were *cad*<sup>2</sup> (gift of  
540 Helen Skaer), *D*<sup>r72</sup> (gift of Steve Russell), *eve*<sup>3</sup> (gift of Bénédicte Sanson), and *h*<sup>22</sup> (Bloomington  
541 stock no. 5529). Wild-type flies were Oregon-R. In order to distinguish homozygous mutant  
542 embryos, mutant alleles were balanced over *CyO hb::lacZ* (Bloomington stock no. 6650) or  
543 *TM6c Sb Tb twi::lacZ* (Bloomington stock no. 7251). DIG-labelled and FITC-labelled riboprobes  
544 were generated using full-length cDNAs from the *Drosophila* gene collection (Stapleton et al.  
545 2002), obtained from the *Drosophila* Genomics Resources Centre. The clones used were  
546 LD29596 (*cad*), LD16125 (*opa*), RE40955 (*hairy*), MIP30861 (*eve*), IP01266 (*runt*), GH22686  
547 (*ftz*), RE48009 (*odd*), GH04704 (*prd*), LD30441 (*slp*), and F107617 (*en*). The cDNA for  
548 *Dichaete* was a gift from Steve Russell, and the cDNA for *lacZ* was a gift from Nan Hu.

549 Double fluorescent in situ hybridisation was carried out as described previously (Clark &  
550 Akam 2016a). Images were acquired using a Leica SP5 confocal microscope. Contrast and  
551 brightness adjustments of images were carried out using Fiji (Schindelin et al. 2012; Schneider  
552 et al. 2012). Some of the wild-type images were taken from a previously published dataset  
553 (Clark & Akam 2016b).

554

555 *Tribolium castaneum*

556 *Tribolium castaneum* eggs were collected on organic plain white flour (Doves Farm Foods Ltd,  
557 Hungerford, U.K.) at 30°C over a period of 48 hours. Alkaline phosphatase in situ  
558 hybridization on whole mount embryos were carried out as previously described (Schinko et  
559 al. 2009). RNA probes were DIG-labelled (all genes) and in most cases also FITC-labelled (*Tc-*  
560 *Dichaete*, *Tc-opa*, *Tc-prd*, *Tc-wg* & *Tc-en*) and prepared according to Kosman et al. (2004),  
561 using gene fragments amplified from *Tribolium castaneum* genomic DNA (for *Tc-cad*, *Tc-eve*,  
562 *Tc-odd* & *Tc-run*) or cDNA (for *Tc-Dichaete*, *Tc-opa*, *Tc-prd*, *Tc-wg* & *Tc-en*) and cloned into  
563 the pGEM-T Easy Vector (Promega, Madison, WI). Clones, and their details, are available on  
564 request.

565 The generation of DIG-labelled probes against *Tc-cad* has been previously described in Peel &  
566 Averof (2010), and the generation of DIG-labelled probes against *Tc-eve* and *Tc-odd* has been  
567 previously described in (Sarrazin et al. 2012). The remaining gene fragments were amplified  
568 using the following primers: *Tc-run*: 5'-CAACAAGAGCCTGCCCATC-3' & 5'-  
569 TACGGCCTCCACACTTT-3' (amplifies 3,158bp fragment). *Tc-Dichaete* (TC0B163): 5'-  
570 TAACAACCGACACCCAACAG-3' & 5'-TTGACGACCACAGCGATAATAA-3' (921bp fragment).  
571 *Tc-opa* (TC0I0234): 5'-CCCAAGAATGGCCTACTGC-3' & 5'-TTGAAGGGCCTCCCGTT-3'  
572 (710bp 5' fragment), 5'-GCGAGAAGCCGTTCAAAT-3' & 5'-  
573 TCTCTTTATAACAATTGTGGTCTAC-3' (705bp 3' fragment); two probes made separately and  
574 combined. *Tc-prd*: 5'-GAATACGGCCCTGTGTTATCT-3' & 5'-ACCCATAGTACGGCTGATGT-3'  
575 (1179bp fragment). *Tc-wg*: 5'-CAACGCCAGAAGCAAGAAC-3' & 5'-  
576 ACGACTTCCTGGGTACGATA-3' (1095bp fragment). *Tc-en*: 5'-TGCAAGTGGCTGAGTGT-3' &  
577 5'-GCAACTACGAGATTTGCCTTC-3' (1001bp fragment).

578 In the double in situ hybridizations where *Tc-cad* mRNA is detected in red, the primary  
579 antibodies were switched such that anti-DIG-AP was used second (after anti-FITC-AP) to  
580 detect *Tc-cad* DIG probe and signal developed using INT/BCIP (see Schinko et al. (2009) for  
581 more details). Embryos were imaged on a Leica MI65FC Fluorescence Stereo Microscope with  
582 a Q Imaging Retiga EXI colour cooled fluorescence camera and Q Capture Pro 7 software.

583

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595

#### 596 **COMPETING INTERESTS**

597 The authors declare that no competing interests exist.

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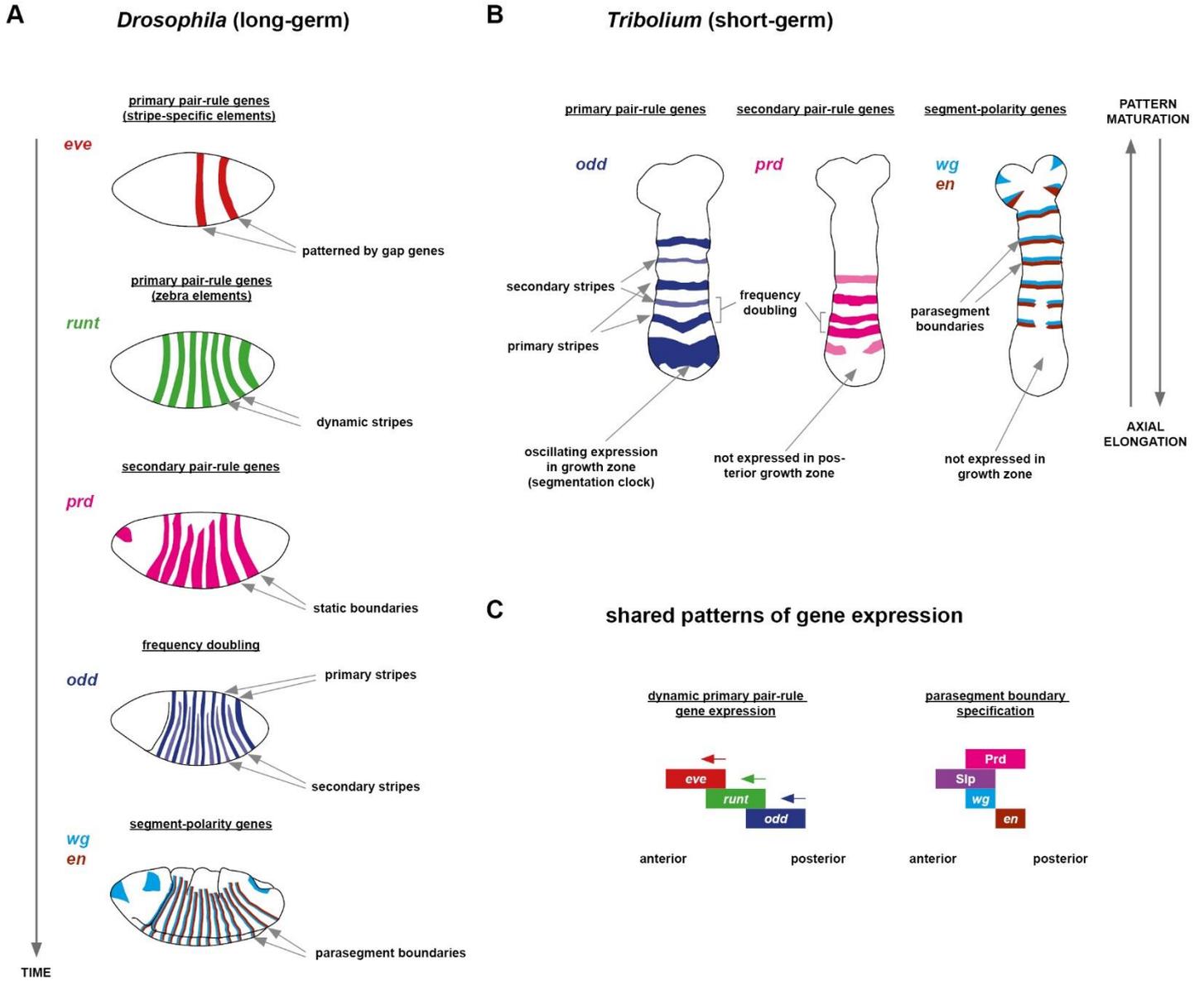
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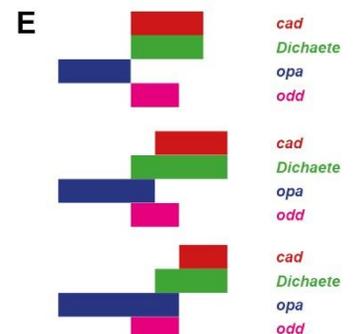
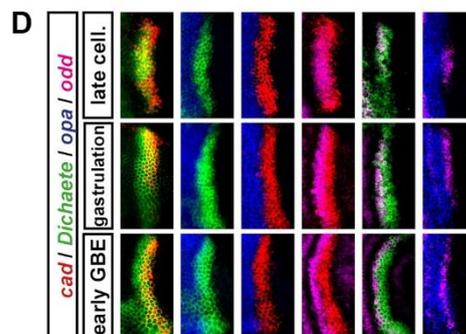
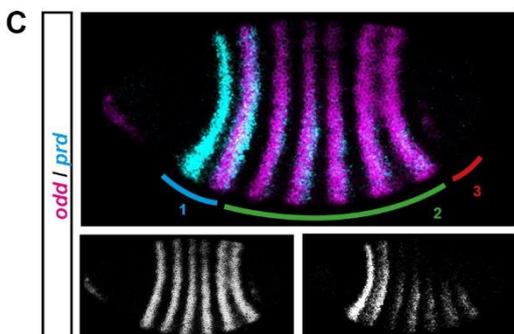
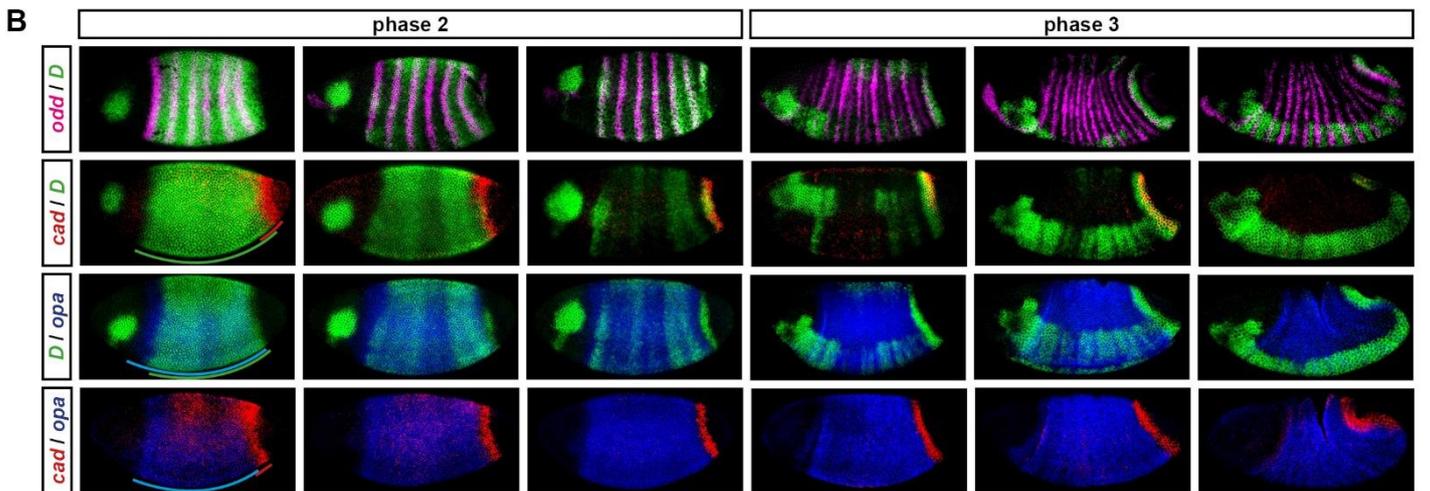
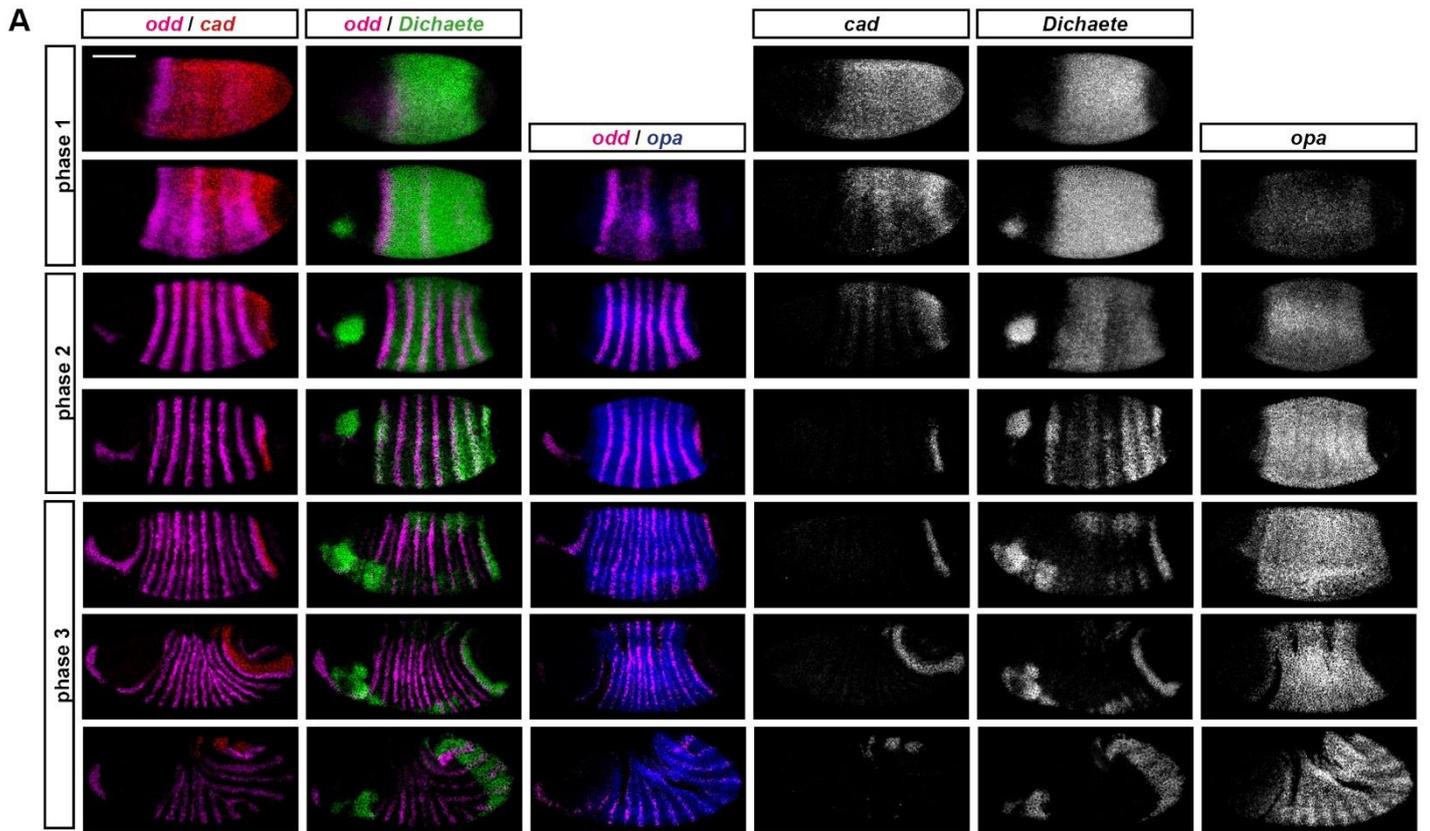


**Fig. 1: Schematic overview of segment patterning in *Drosophila* versus *Tribolium*.**

**A:** Overview of pair-rule patterning in *Drosophila*. Top row depicts expression from the *eve* 4+6 reporter element, other rows depict endogenous expression patterns. Time axis runs from early cellularisation (top) to early germband extension (bottom).

**B:** Overview of segmentation in *Tribolium*. Embryos are depicted at mid germband extension, but gene expression patterns are essentially similar throughout axial elongation.

**C:** Similar relative patterns of gene expression are seen in both long-germ and short-germ species. Left: a dynamic sequence of *eve*, *runt*, and *odd* expression is seen both *Drosophila* blastoderm and *Tribolium* growth zone (Clark 2017; Choe et al. 2006). Right: the same pattern of secondary pair-rule gene expression is seen upstream of parasegment boundary specification in all arthropods yet studied, including *Drosophila* and *Tribolium* (Green & Akam 2013). Morphological boundaries in the embryo form later, located between the abutting *wg* and *en* domains.



**Fig. 2: *cad*, *Dichaete*, and *opa* are expressed dynamically over the course of *Drosophila* segmentation.**

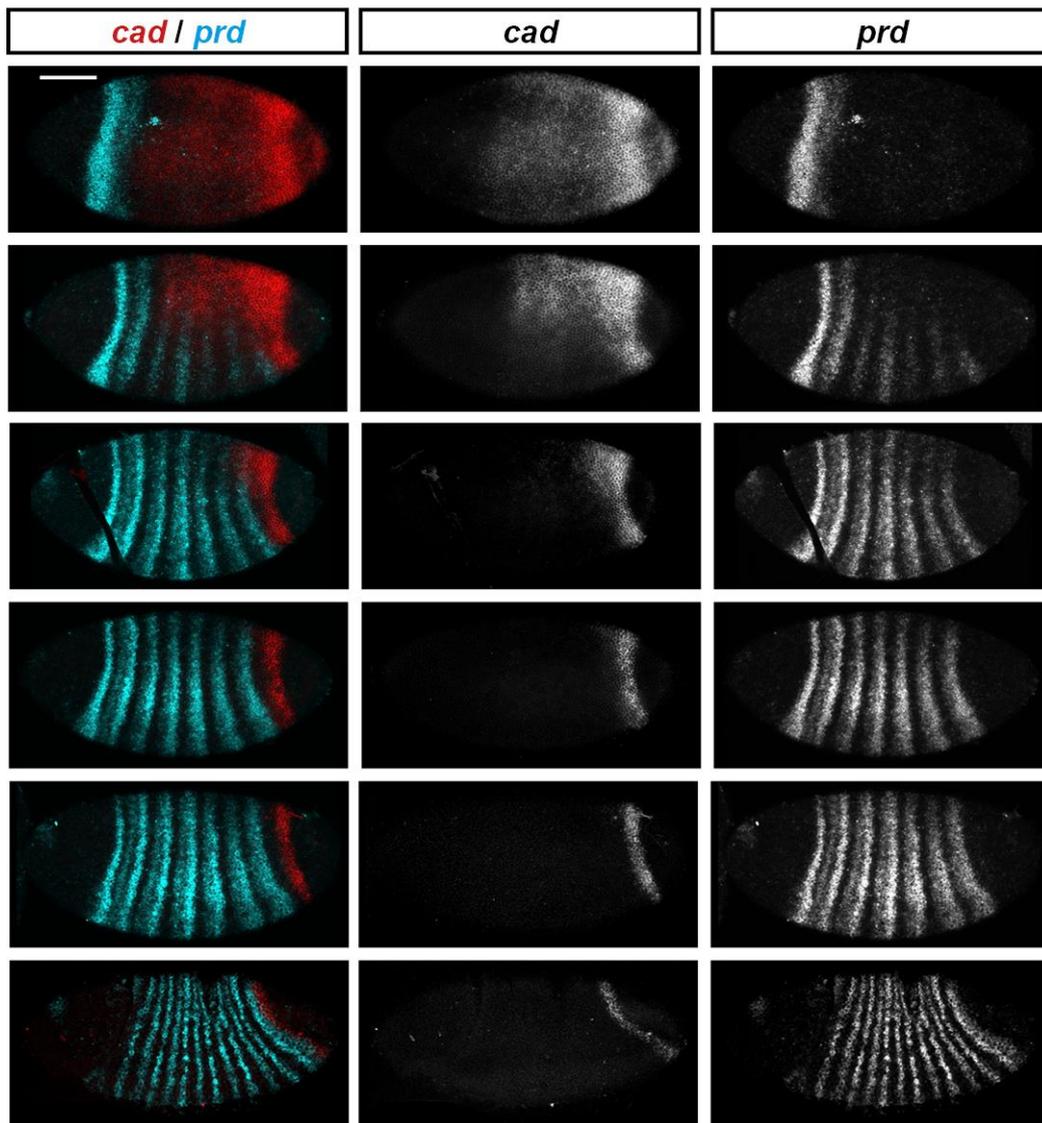
**A:** Spatiotemporal expression of *cad*, *Dichaete*, and *opa*, relative to the pair-rule gene *odd*. Embryo age increases from top to bottom. “Phase 1” = early cellularisation; “phase 2” = mid-late cellularisation; “phase 3” = gastrulation and early germband extension. Individual channels for *cad*, *Dichaete*, and *opa* are shown on the right of the false-coloured dual-channel images. All *Drosophila* embryos here and elsewhere are aligned anterior left, dorsal top. Scale bar = 100  $\mu\text{m}$ . The *opa* / *odd* in situs are from Clark and Akam (2016a).

**B:** Relative locations of the *cad*, *Dichaete*, and *opa* expression domains, from mid-cellularisation (early phase 2) onwards. Equivalently-staged *Dichaete/odd* in situs are also shown (top row), for easy cross-referencing with **A**. Embryo age increases from left to right. Annotations in left-most column highlight the relative extent of each domain at mid-cellularisation.

**C:** Three distinct regions of pair-rule gene expression timing along the AP axis. The embryo shown is just slightly older than those in the left-most column of **B**. Individual channels for *odd* (left) and *prd* (right) are shown below the false-coloured dual-channel image.

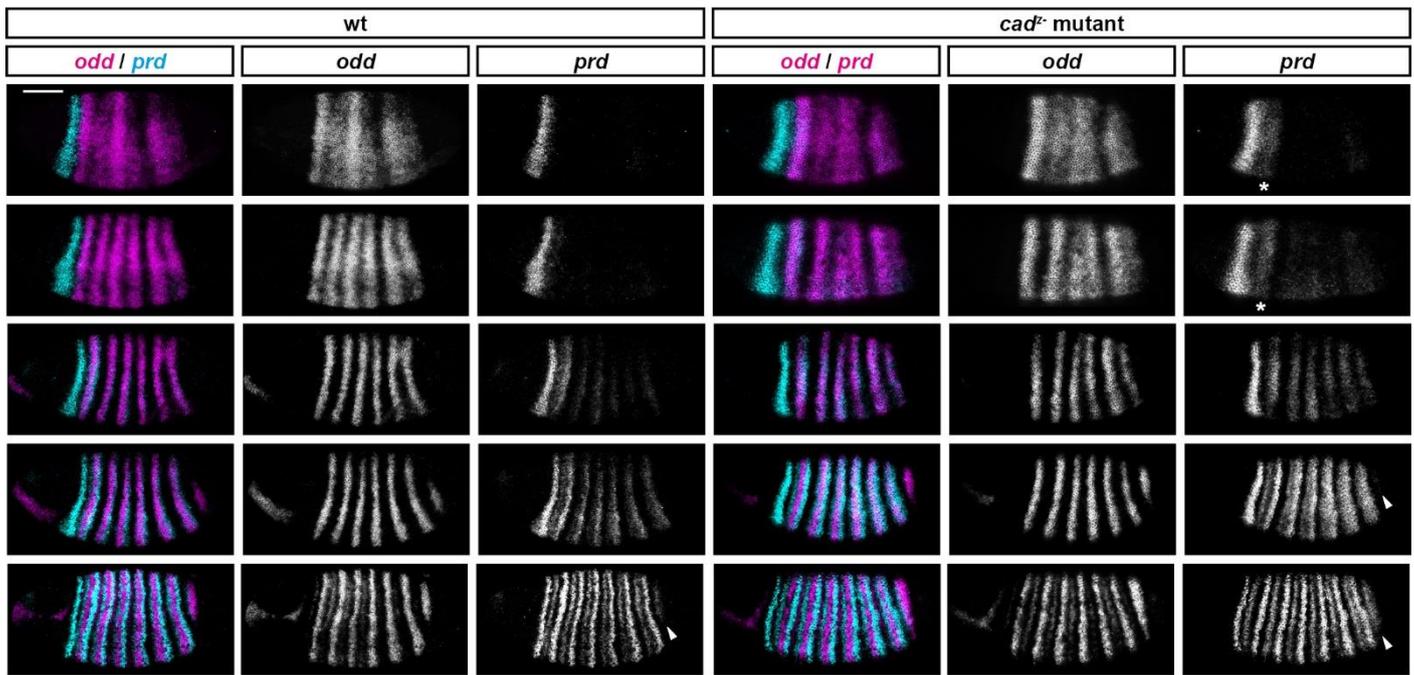
**D:** Dynamic gene expression within the tail (region 3). Cropped and rotated enlargements of the *odd* stripe 7 region are shown for three timepoints (equivalent to rows 4-6 in **A** and columns 3-5 in **B**).

**E:** Schematic diagram of shifting expression domains within the tail, based on the data in **D**. The anterior boundary of *odd* stripe 7 is assumed to be static (see Figure SI legend).



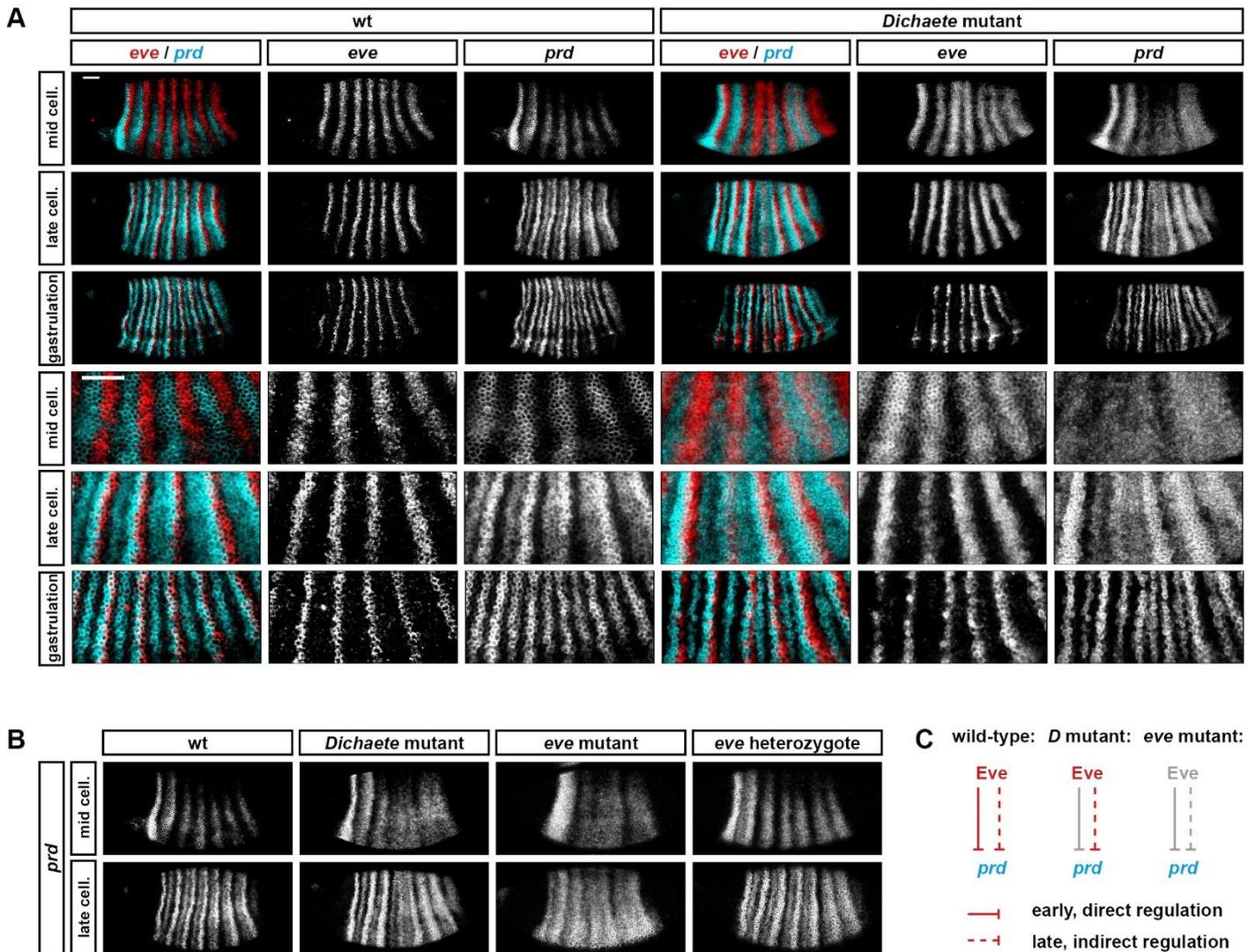
**Fig. 3: *cad* and *prd* exhibit anti-correlated spatiotemporal dynamics during cellularisation.**

Embryo age increases from top (early cellularisation) to bottom (gastrulation). Arrowheads mark the posterior border of *prd* stripe 7; asterisk marks *prd* stripe 8. Scale bar = 100  $\mu$ m.



**Fig. 4: The spatiotemporal onset of *prd* expression is regulated by Cad.**

*prd* expression is shown in wild-type embryos and *cad* zygotic mutants, using *odd* as a staging marker. (Note the characteristic development of *odd* expression within the head, which is necessarily independent of Cad levels.) Mutant embryos are conservatively staged: note that embryos in rows 2 and 3 are slightly younger than their wild-type counterparts. Asterisks indicate precocious *prd* stripe 2 expression; arrowheads point to emerging *prd* stripe 8 expression. Note the ectopic *prd* expression throughout the trunk in the second mutant embryo down, and the even intensity of the *prd* stripes along the DV axis in the third and fourth mutant embryos down. Scale bar = 100  $\mu$ m.

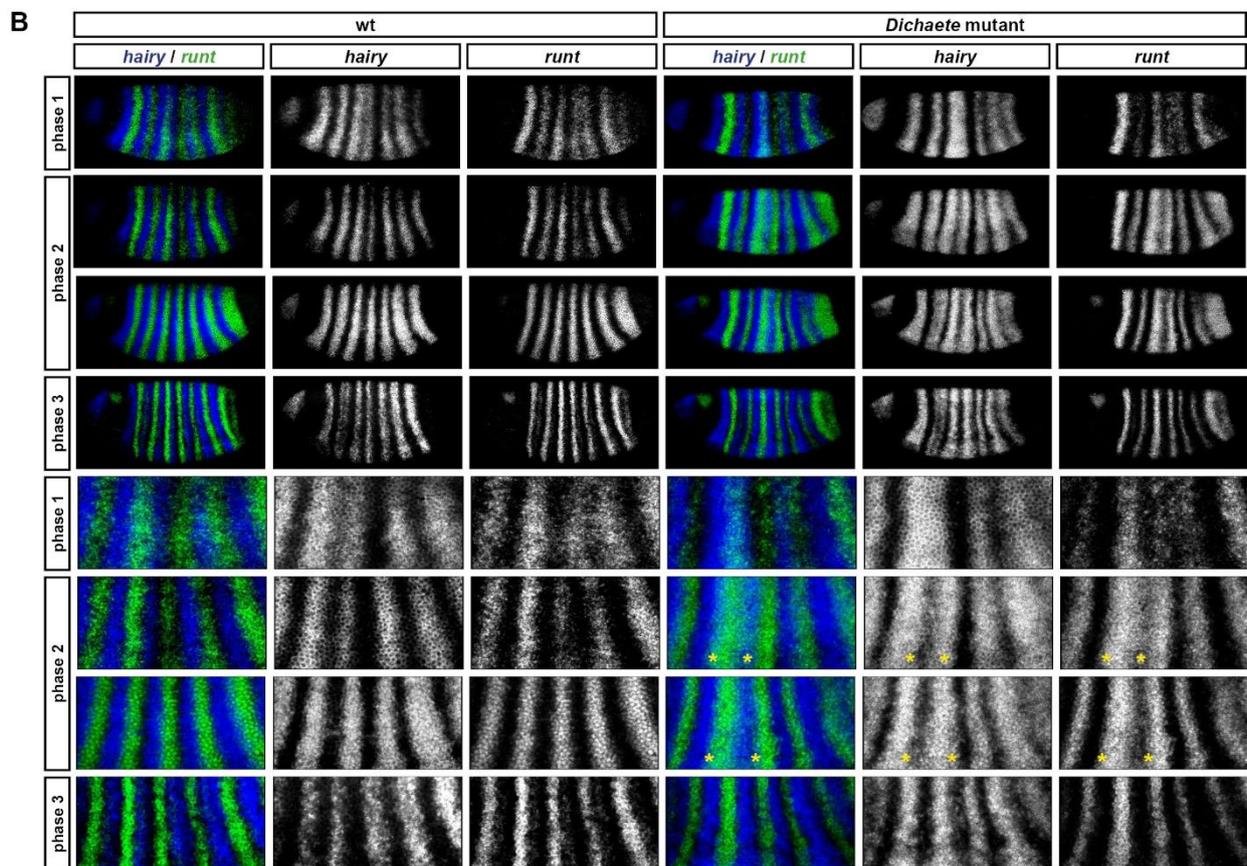
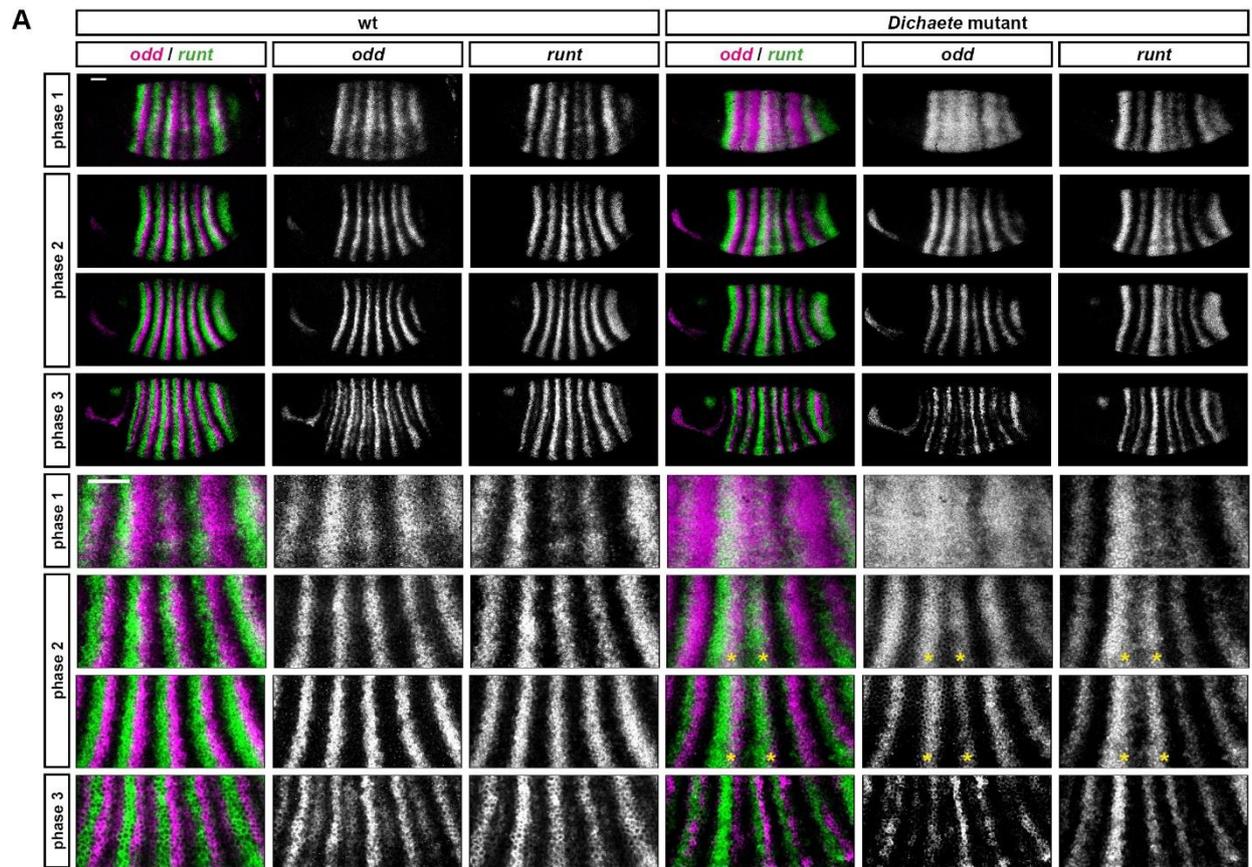


**Fig. 5: Eve requires Dichaete in order to repress *prd*.**

**A:** Expression of *prd* relative to *eve* in wild-type and *Dichaete* mutant embryos, at mid-cellularisation, late cellularisation, and gastrulation. Single channels are shown to the right of each false-coloured dual-channel image, and enlarged views of the trunk region are shown below the whole embryo views. Periodicity of early *prd* expression is lost in the *Dichaete* mutants, but the timing and DV patterning of expression remain normal. Note that *prd* and *eve* stripes overlap at later stages in both wild-type and mutant embryos. Scale bars = 50  $\mu$ m.

**B:** *prd* expression at mid-cellularisation and late-cellularisation, in a variety of genetic backgrounds. A similar loss of *prd* periodicity within the trunk is seen in *Dichaete* and *eve* mutant embryos at mid-cellularisation, but patterning subsequently diverges.

**C:** Interpretation of *prd* regulation in **B**.

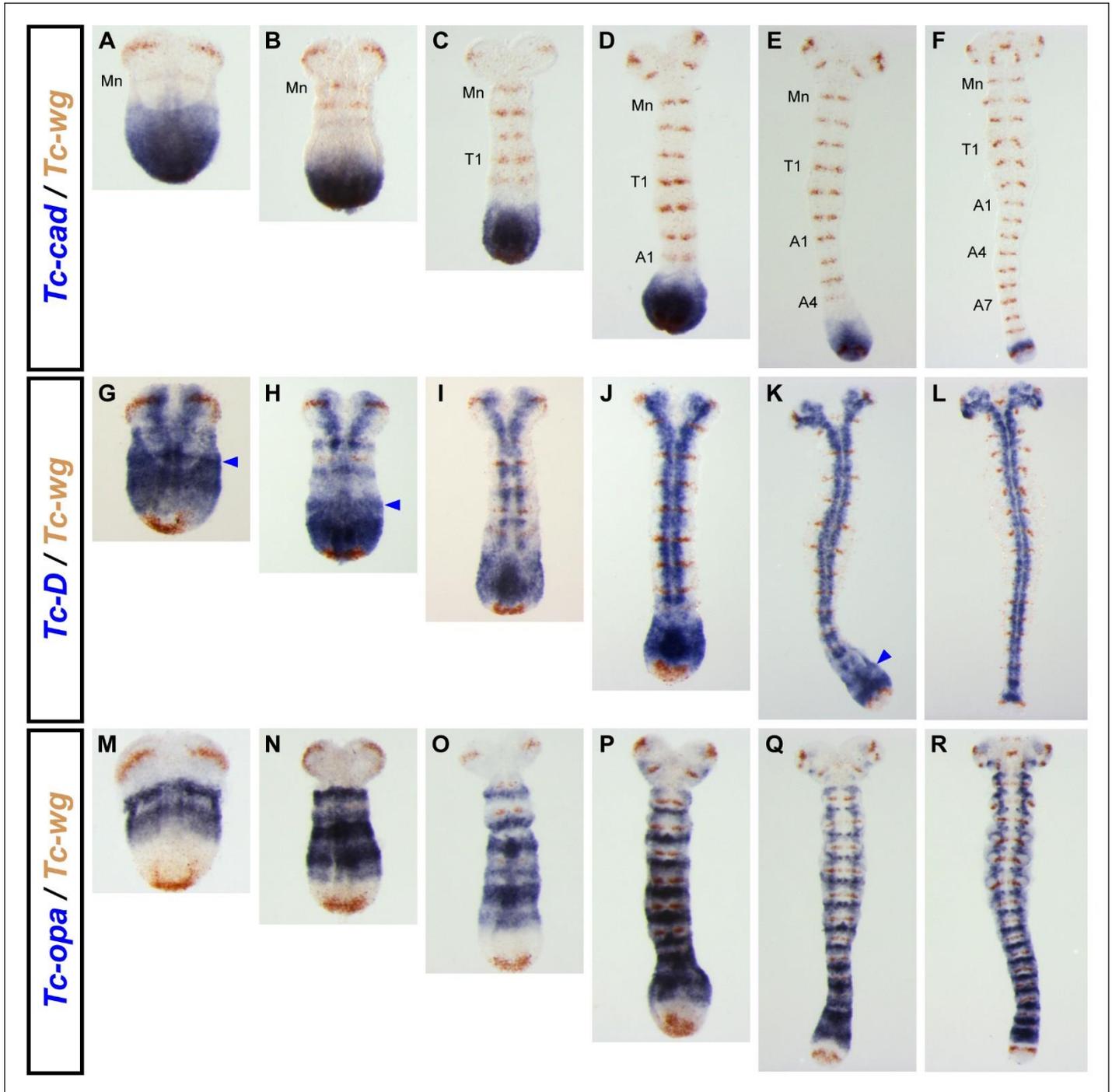


**Fig. 6: *runt* is not patterned by pair-rule inputs in *Dichaete* mutant embryos**

**A, B:** Expression of *runt* relative to *odd* (**A**) and *hairy* (**B**) in wild-type and *Dichaete* mutant embryos, over the course of cellularisation. Single channels are shown to the right of each false-coloured dual-channel image, and enlarged views of the trunk region are shown below the whole embryo views. *runt* expression continues to overlap with *odd* and *hairy* expression throughout phase 2 in *Dichaete* mutants, but not wild-type. Asterisks indicate extensive regions of coexpression associated with *runt* stripes 3 and 4. (Note that narrow strips of overlapping expression in wild-type embryos are caused by the dynamic nature of the pair-rule stripes, which shift anteriorly across the tissue until the beginning of phase 3, causing protein domains to lag slightly behind transcript domains.) Scale bars = 50  $\mu$ m.

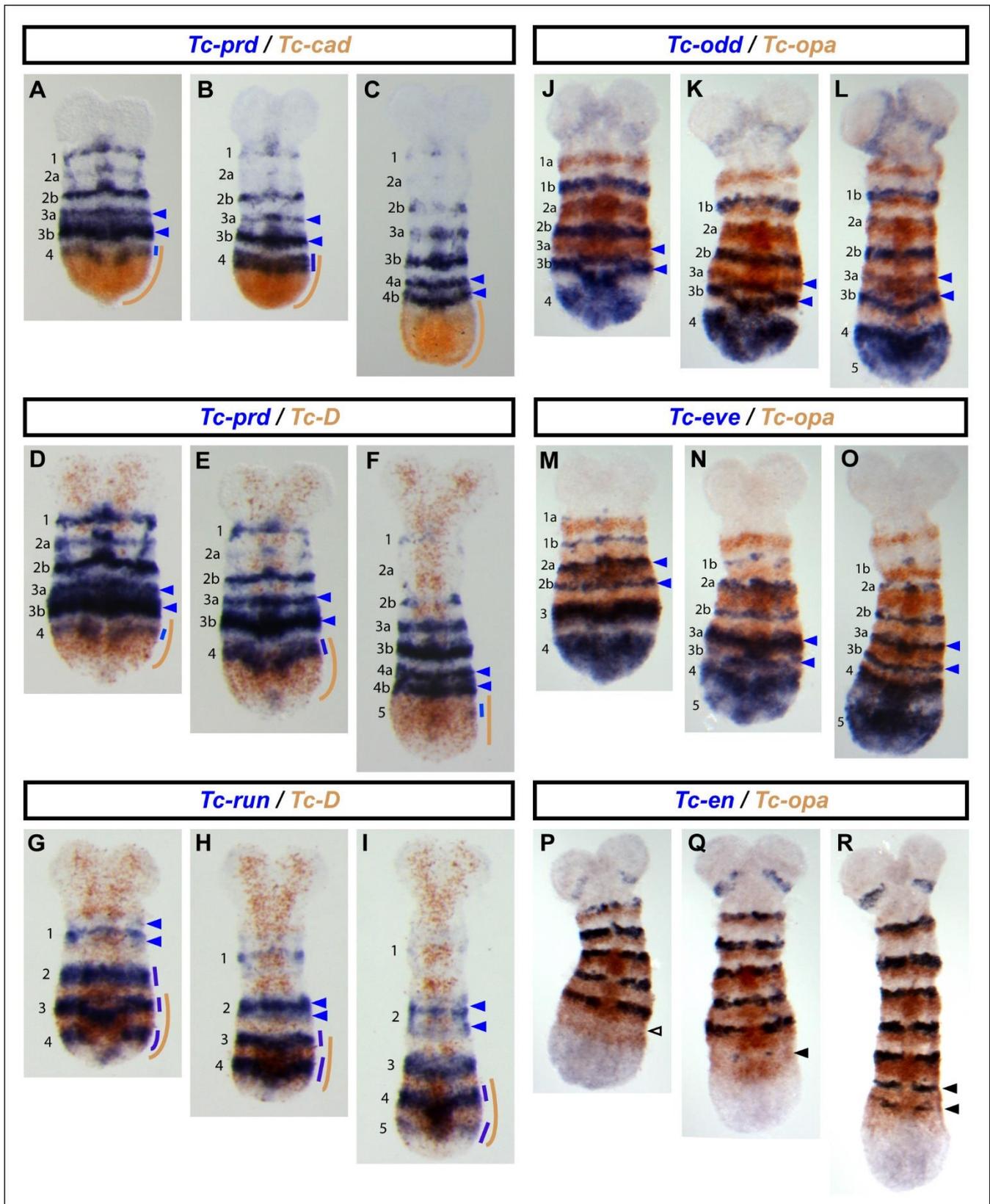
**C:** Schematic model of *runt* regulation over the course of segmentation. Only the cross-regulatory interactions between *hairy*, *odd*, and *runt* are included.

**D:** Interpretation of *runt* regulation in **A** and **B**.



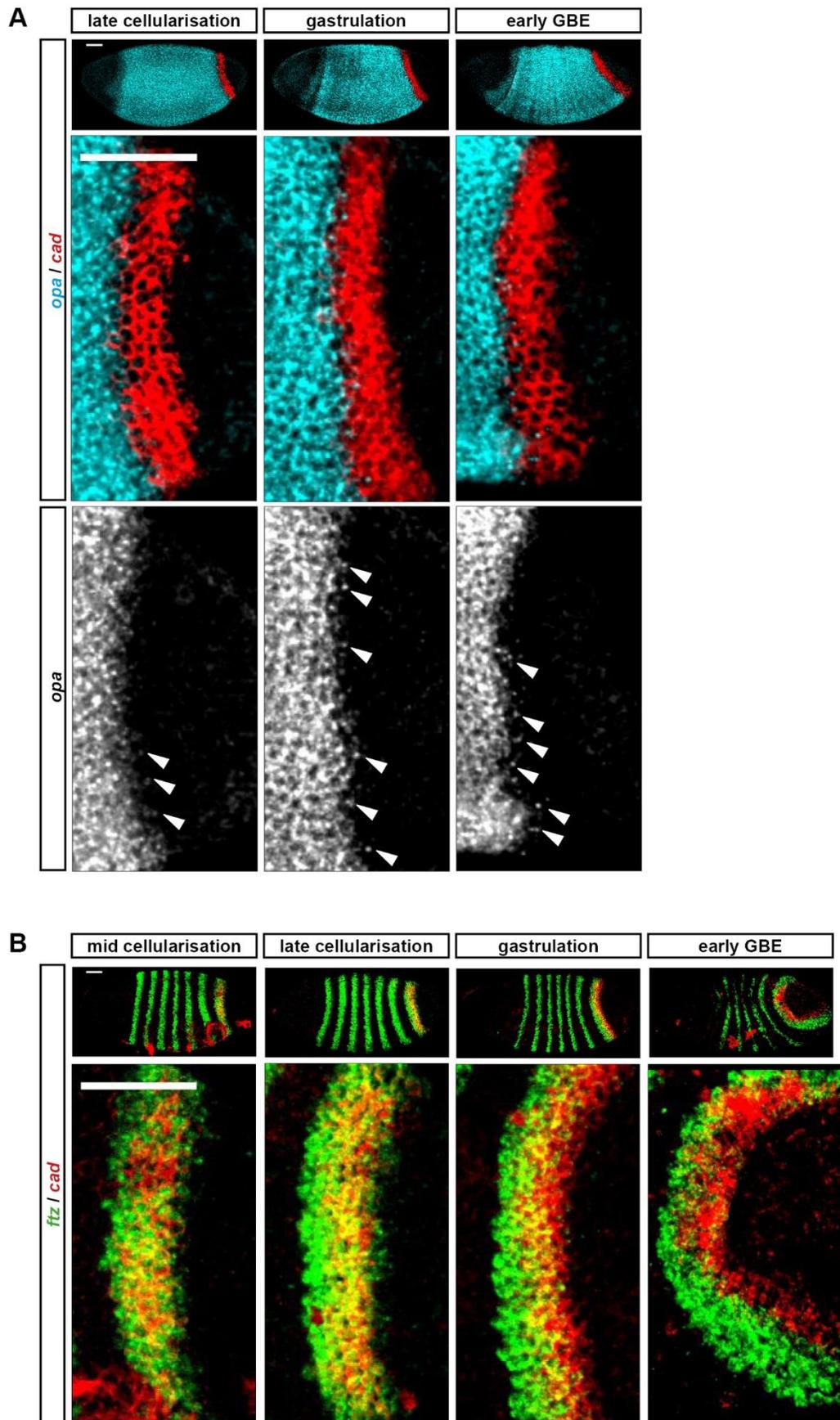
**Fig. 7. Expression of *Tc-cad*, *Tc-Dichaete* & *Tc-opa* relative to a common segment marker, *Tc-wg*, in *Tribolium castaneum* germband stage embryos.**

(A-F). Double in situ hybridization for *Tc-cad* (blue) and *Tc-wg* (brown) in embryos of increasing age from left (A) to right (F). The mandibular (Mn), prothoracic (T1), 1<sup>st</sup> abdominal (A1), 4<sup>th</sup> abdominal (A4) & 7<sup>th</sup> abdominal (A7) stripes of *Tc-wg* expression have been labeled. (G-L). As for panels A-F, but this time double in situ hybridization for *Tc-Dichaete* (blue) and *Tc-wg* (brown). Blue arrowheads point to a stripe of *Tc-Dichaete* expression that has formed, or is forming, anterior to the strong posterior expression domain (see text for further details). (M-R). As for A-F, but this time double in situ hybridization for *Tc-opa* (blue) and *Tc-wg* (brown). Panels in the same column have been stage matched using *Tc-wg* expression patterns. Note how the *Tc-cad*, *Tc-Dichaete*, and *Tc-opa* expression patterns shift posteriorly relative to *Tc-wg* expression as embryos elongate, and the remarkable consistency of these posterior expression domains during the process of germband elongation. Consult Fig. S5 for a more extensive range of stages, which are presented in a manner that aids comparison between *Tc-cad*, *Tc-Dichaete* & *Tc-opa* expression patterns, rather than variation across developmental stages.



**Fig. 8. Expression of *Tc-cad*, *Tc-Dichaete* & *Tc-opa* relative to selected segmentation genes in *Tribolium castaneum* germband stage embryos.**

(A-C). Double in situ hybridization for *Tc-prd* (blue) and *Tc-cad* (brown) on three embryos that span the formation and splitting of the 4<sup>th</sup> *Tc-prd* primary pair-rule stripe. (D-F). As for panels A-C, but this time double in situ hybridization for *Tc-prd* (blue) and *Tc-Dichaete* (brown). Note how the primary pair-rule stripe of *Tc-prd* expression forms at the anterior of the *Tc-cad* domain (A, B) and the anterior of the posterior-most *Tc-Dichaete* domain (D, E), but only splits to form segmental stripes (labeled 4a & 4b) anterior to these domains (C, F). (G-I). Double in situ hybridization for *Tc-run* (blue) and *Tc-Dichaete* (brown) on three embryos that span the formation of the 4<sup>th</sup> and 5<sup>th</sup> *Tc-run* primary pair-rule stripes, plus splitting of the 2<sup>nd</sup> *Tc-run* primary pair-rule stripe. Note how *Tc-run* stripe splitting occurs anterior to the posterior-most *Tc-Dichaete* domain. (J-L). Double in situ hybridization for *Tc-odd* (blue) and *Tc-opa* (brown) on three embryos that span the formation of the 4<sup>th</sup> and 5<sup>th</sup> *Tc-odd* primary pair-rule stripes and the resolution of the 3<sup>rd</sup> *Tc-odd* primary pair-rule stripe into two segmental stripes (labeled 3a & 3b). (M-O). As for J-L, but this time double *in situ* hybridization for *Tc-eve* (blue) and *Tc-opa* (brown). Note how segmented stripes of *Tc-eve* and *Tc-odd* expression resolve within the *Tc-opa* domain (K, L, N, O). In A-O, blue arrowheads mark *Tc-prd*, *Tc-run*, *Tc-odd* & *Tc-eve* segmental stripes that have recently resolved, or are in the process of resolving. (P-R). Double in situ hybridization for *Tc-en* (blue) and *Tc-opa* (brown) on three embryos that span the formation of the T3 and A1 *Tc-en* segmental stripes. Note how the forming *Tc-en* stripes (solid black arrowheads in Q, R) appear within the *Tc-opa* domain, but in a stripe-shaped region where *Tc-opa* expression is already clearing (empty black arrowhead in P). Colour-coded lines on the right-hand side of the embryos indicate our interpretations of the expression patterns in A-I. For a more detailed description of these expression patterns, consult the main text and supplementary figures 7-10.



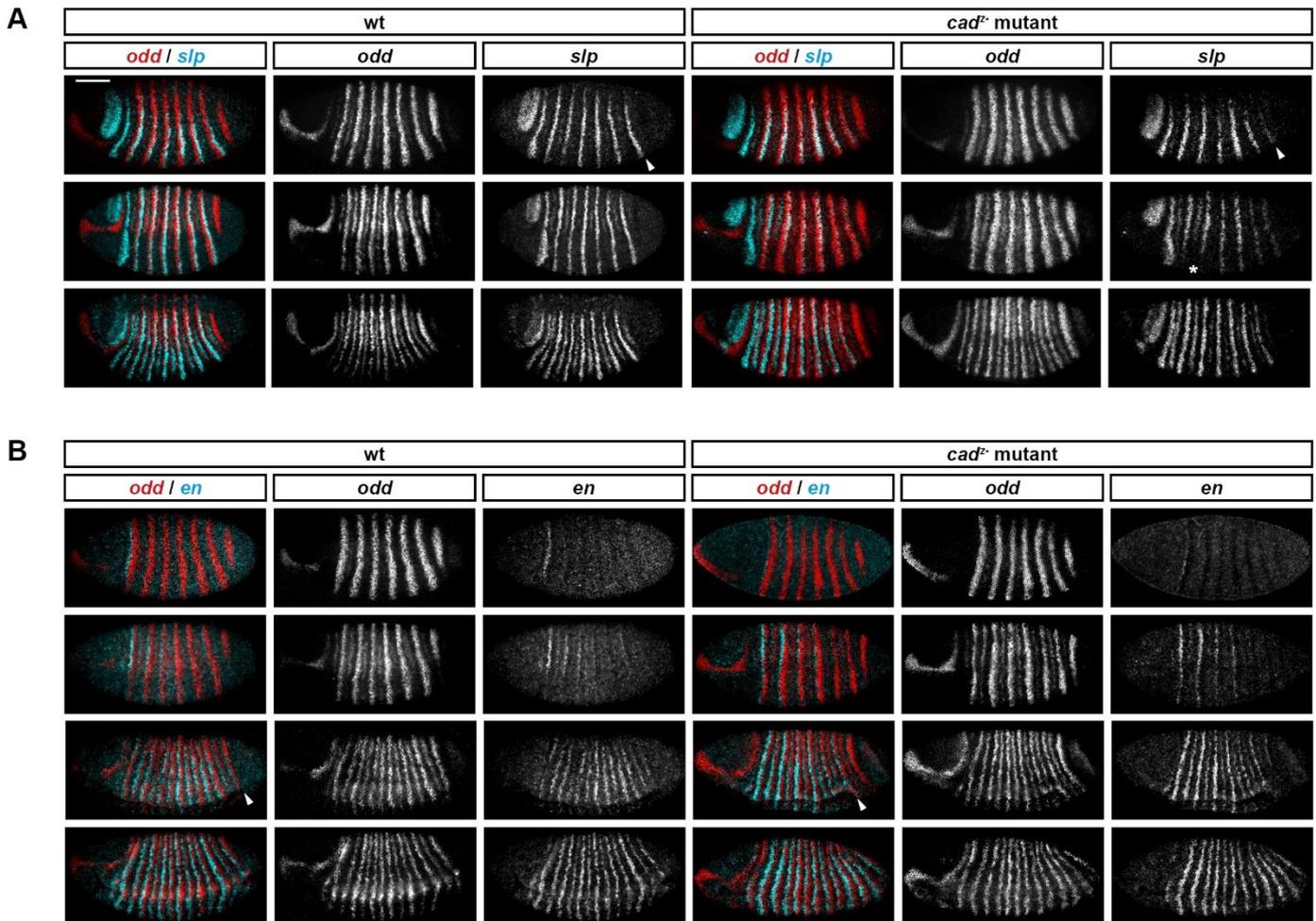
### **Fig. S1: Shifting boundaries in the posterior of the embryo**

**A:** Nascent transcription (nuclear dots, marked by arrowheads) of *opa* expression are observed within the *cad* domain, indicative that the *opa* expression domain is expanding posteriorly.

Scale bars = 50  $\mu\text{m}$ .

**B:** Marked posterior shifting of the *cad* posterior domain relative to stripe 7 of *ftz*. Cropped and rotated enlargements of the stripe 7 region are shown below the whole embryo views. (The bright red regions of staining outside the *cad* posterior domain are artefacts caused by bits of debris stuck to the embryos.) Scale bars = 50  $\mu\text{m}$ .

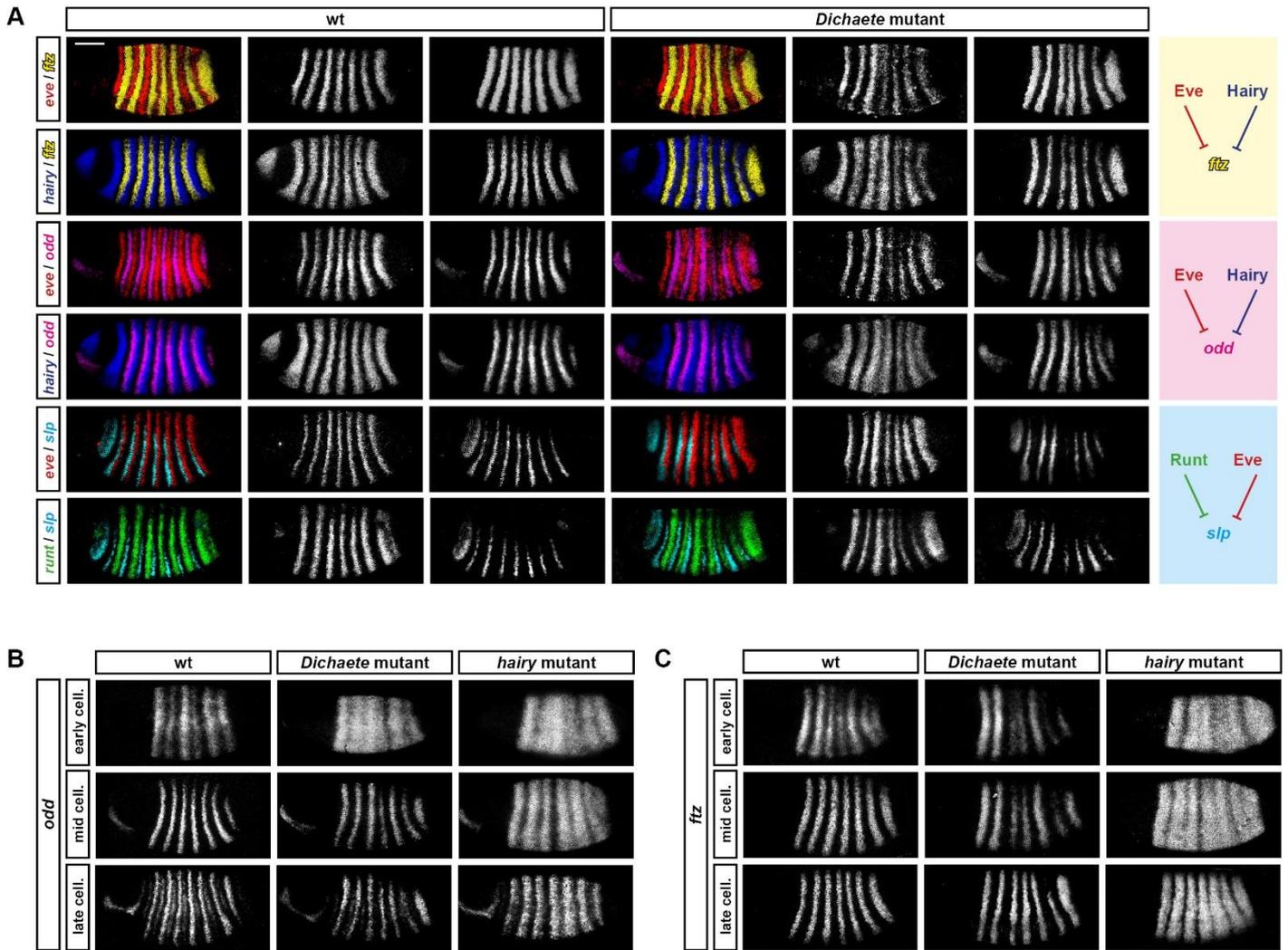
Note that without live reporters, it is hard to determine unambiguously which of the *cad*, *Dichaete*, *opa*, and *odd* expression domains are moving relative to cells, and which are static (see Figure 2D,E). Nevertheless, we are reasonably sure that the *cad*, *Dichaete*, and *opa* domains are dynamic, for three reasons. First, quantitative measurements in populations of fixed embryos indicate that, in contrast to other primary pair-rule stripes, neither *odd* stripe 7 nor *ftz* stripe 7 (**B**) shift significantly within the embryo (Surkova et al. 2008). Second, *odd* stripe 7 is repressed anteriorly by *Opa* expression, since it emerges within Runt stripe 7, and Runt+*Opa* combined repress *odd* (Clark & Akam 2016a). Third, at the same time as the domains are shifting relative to one another, the *opa* domain appears to be expanding posteriorly (**A**). These anterior-to-posterior shifts of *cad*, *Dichaete*, and *opa* expression are a notable counterpoint to the posterior-to-anterior shifts observed for gap and pair-rule gene expression (Jaeger et al. 2004; Surkova et al. 2008), and it will be interesting to find out how they are generated.



**Fig. S2: Normal temporal patterning of *slp*, *odd*, and *en* in *cad* zygotic mutants.**

**A:** *slp* expression emerges at the normal time in *cad* zygotic mutants, although there are some changes to the intensity of the stripes, particularly in anterior ventral regions (asterisk). DV patterning of the *slp* stripes is normal. Arrowheads mark the emergence of *slp* stripe 7, which is considerably weakened in *cad* zygotic mutants, likely owing to the posterior expansion of the fate map (by about 10% AP length, based on measurements of posterior stripes). In the mutant, this stripe probably experiences a different background of activators and repressors than the more anterior stripes, which are located within the main trunk. Scale bar = 100  $\mu$ m.

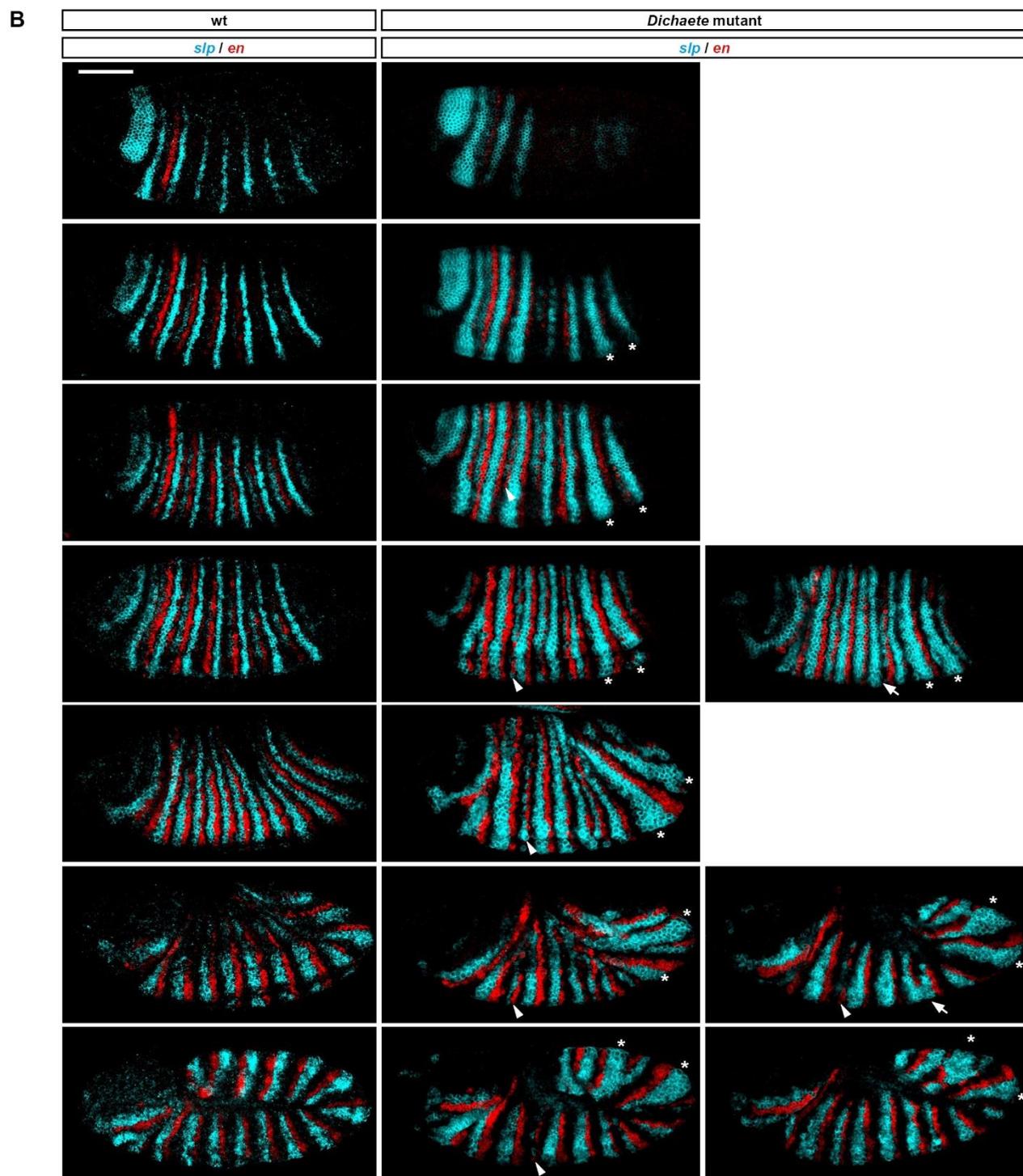
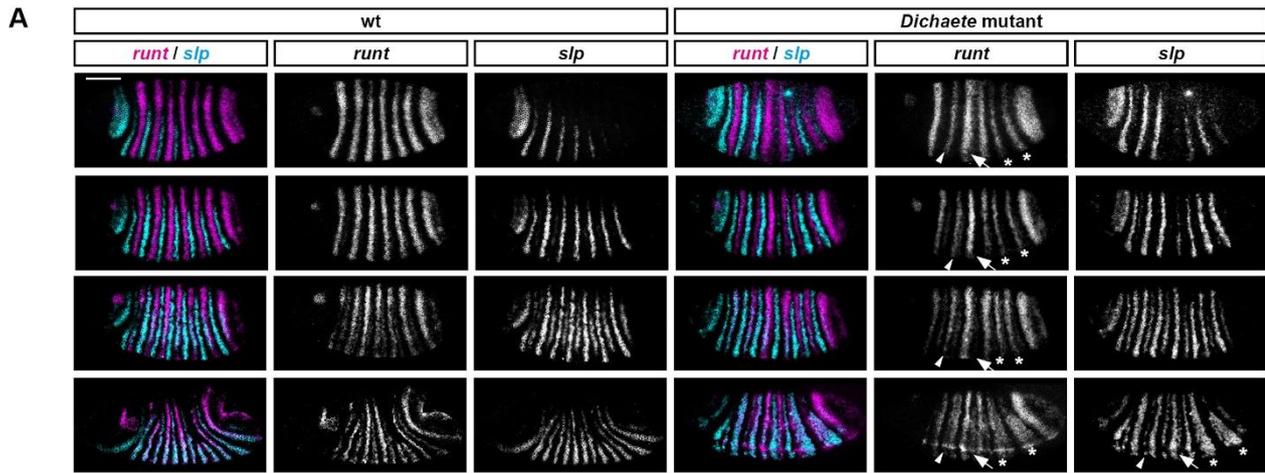
**B:** *en* expression emerges at the correct time in *cad* zygotic mutants, although the order of stripe emergence and the DV patterning of the stripes is altered. Arrowheads mark the posterior extent of the ventral furrow, which aligns with the posterior extent of segmentation gene expression in wild-type embryos, but not *cad* zygotic mutants.



**Fig. S3: *ftz*, *odd*, and *slp* are cross-regulated normally in *Dichaete* mutant embryos**

**A:** Expression of *ftz*, *odd*, and *slp*, relative to their pair-rule repressors, at mid-cellularisation (phase 2). Single channel images are shown to the right of each false-coloured dual-channel image (*ftz*, *odd*, and *slp* are always on the right). Right: arrow diagrams describing *ftz*, *odd*, and *slp* regulation during phase 2. Hammerhead arrows represent repression. Activators (likely to be ubiquitously-expressed factors) are not included. Scale bar = 100  $\mu$ m.

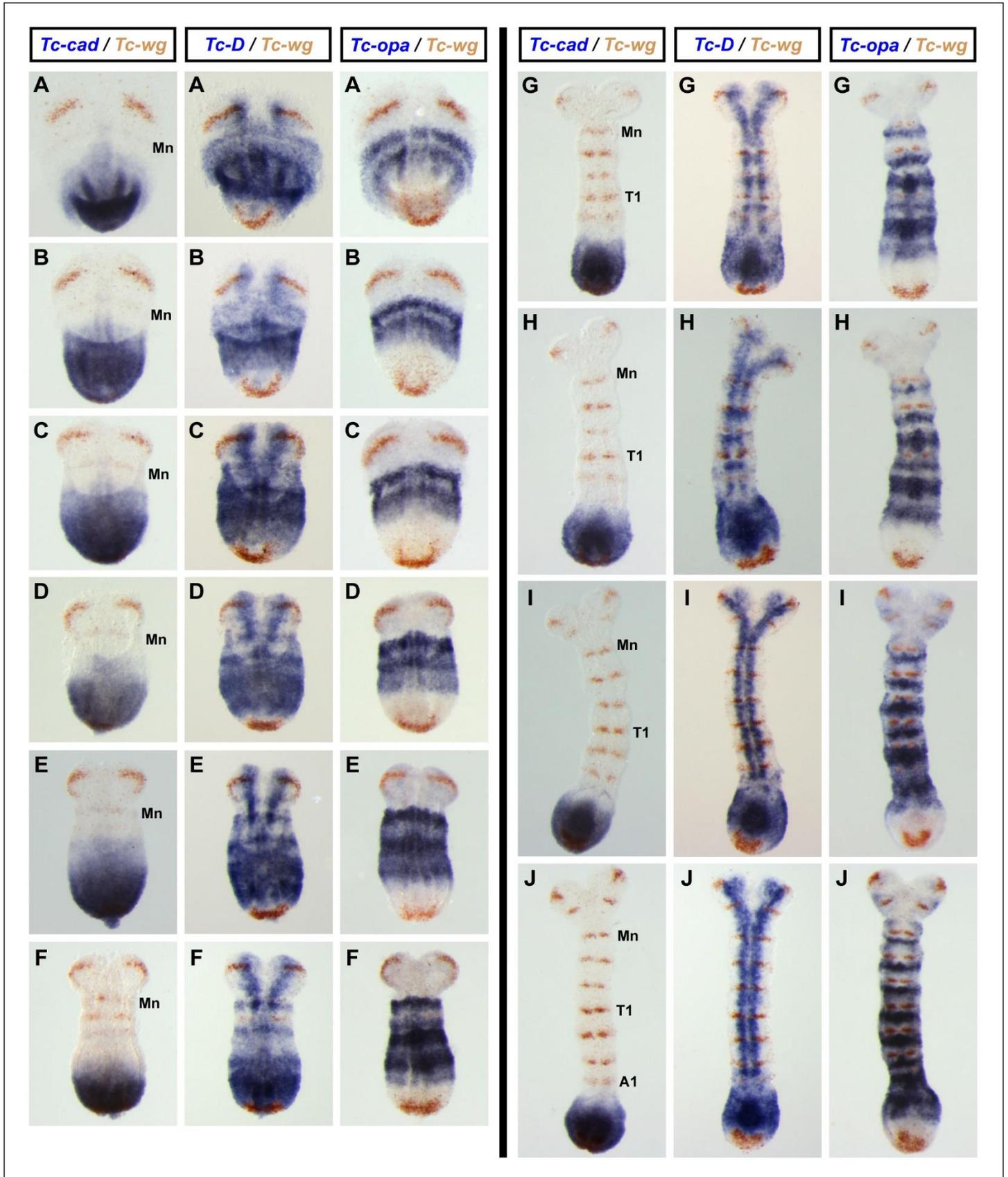
**B, C:** Expression of *odd* (**B**) or *ftz* (**C**) in wild-type, *Dichaete* mutant, and *hairy* mutant embryos, over the course of cellularisation. Note the ectopic *odd* expression seen at early cellularisation in *Dichaete* mutants.

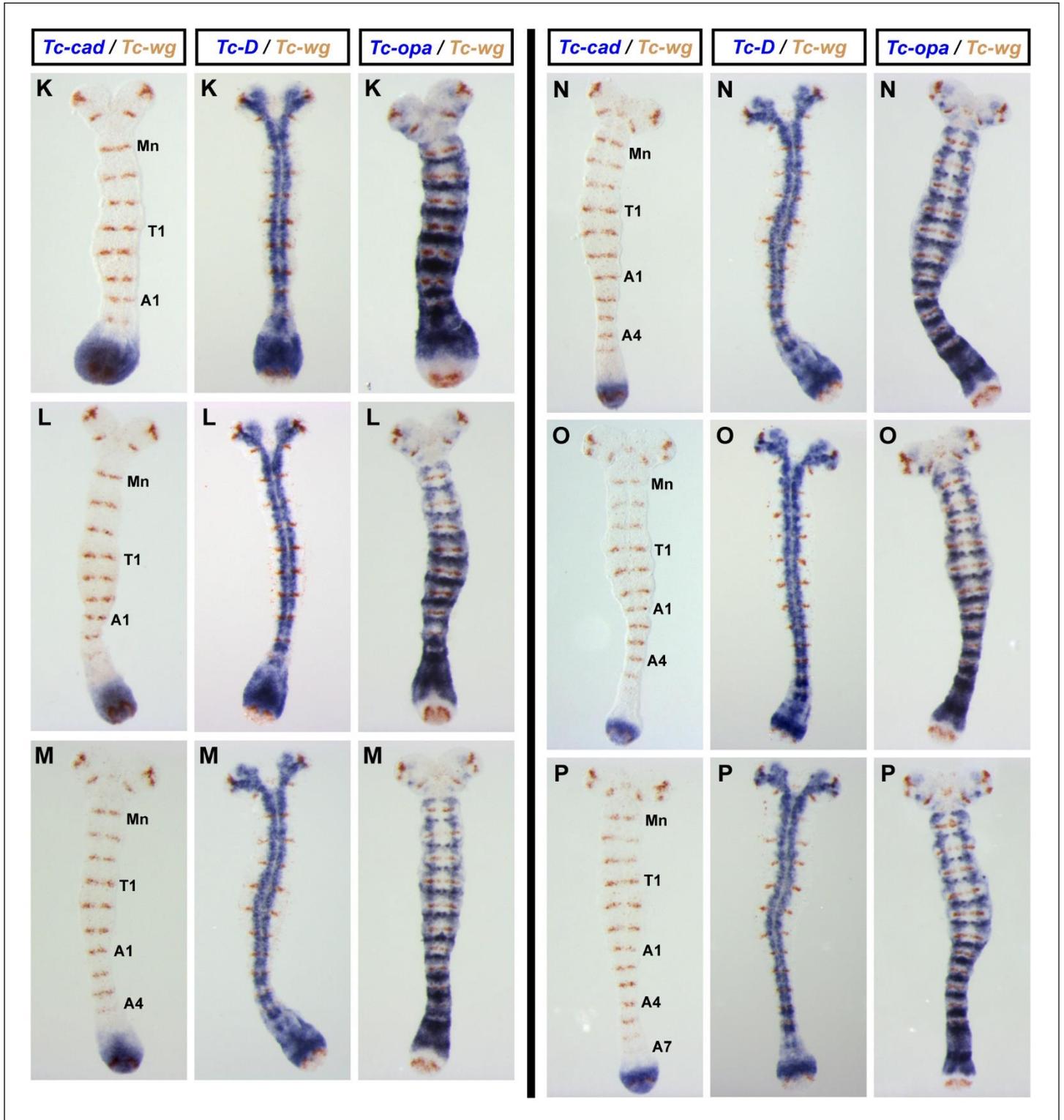


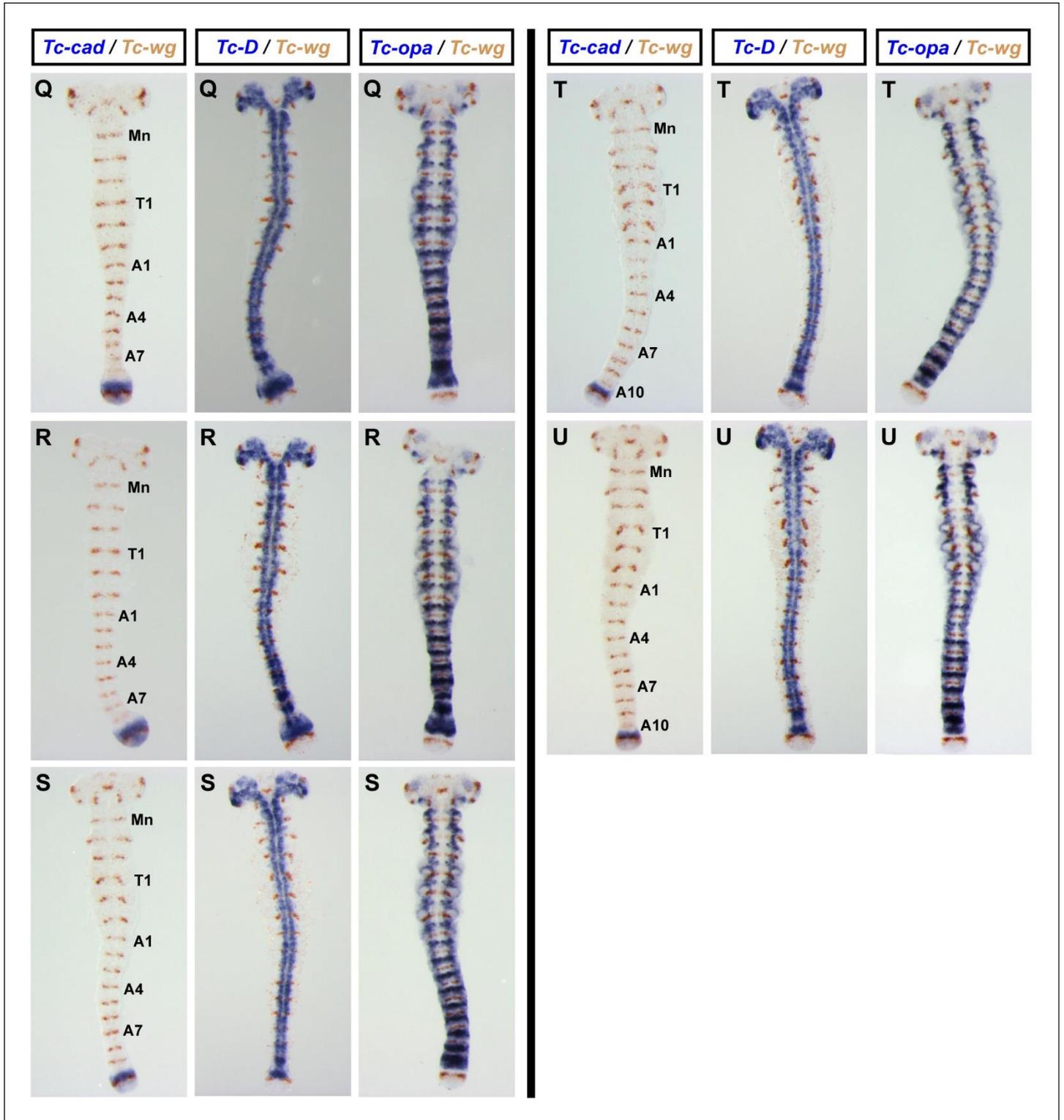
**Fig. S4: Late pair-rule gene expression in *Dichaete* mutants reveals the aetiology of the *Dichaete* cuticle phenotype.**

**A:** Irregular *runt* expression in *Dichaete* mutants at cellularisation (top) results in an irregular pattern of *slp* expression at the end of gastrulation (bottom). Embryo age increases from top to bottom. Asterisks indicate weakened *runt* stripes 5 and 6 in the mutant embryos. These stripes are responsible for patterning the anterior boundaries of *slp* stripes 6 and 7, which tend to be expanded anteriorly in the mutants. (Note that the third embryo down is unusually mildly affected, compare equivalently-stage *slp* expression in **B**.) These anterior expansions often result in fusions with the fifth and sixth secondary *slp* stripes (asterisks in bottom row, right). Late *runt* is patterned in the same way as *slp*, meaning that the fifth and sixth *runt* secondary stripes form contiguously with the posteriors of the primary stripes (asterisks in bottom row, left). Arrowheads in the mutant point to weakened *runt* stripe 2, which leads to an expanded *slp* stripe 3, and eventually a narrow and incomplete second *slp* secondary stripe (arrowhead in bottom row, right). Finally, arrows in the mutant point to the expanded *runt* stripe 3, which represses *slp* stripe 4 at early stages, and can lead to patterning irregularities later on (arrow, bottom row, right). Scale bar = 100  $\mu\text{m}$ .

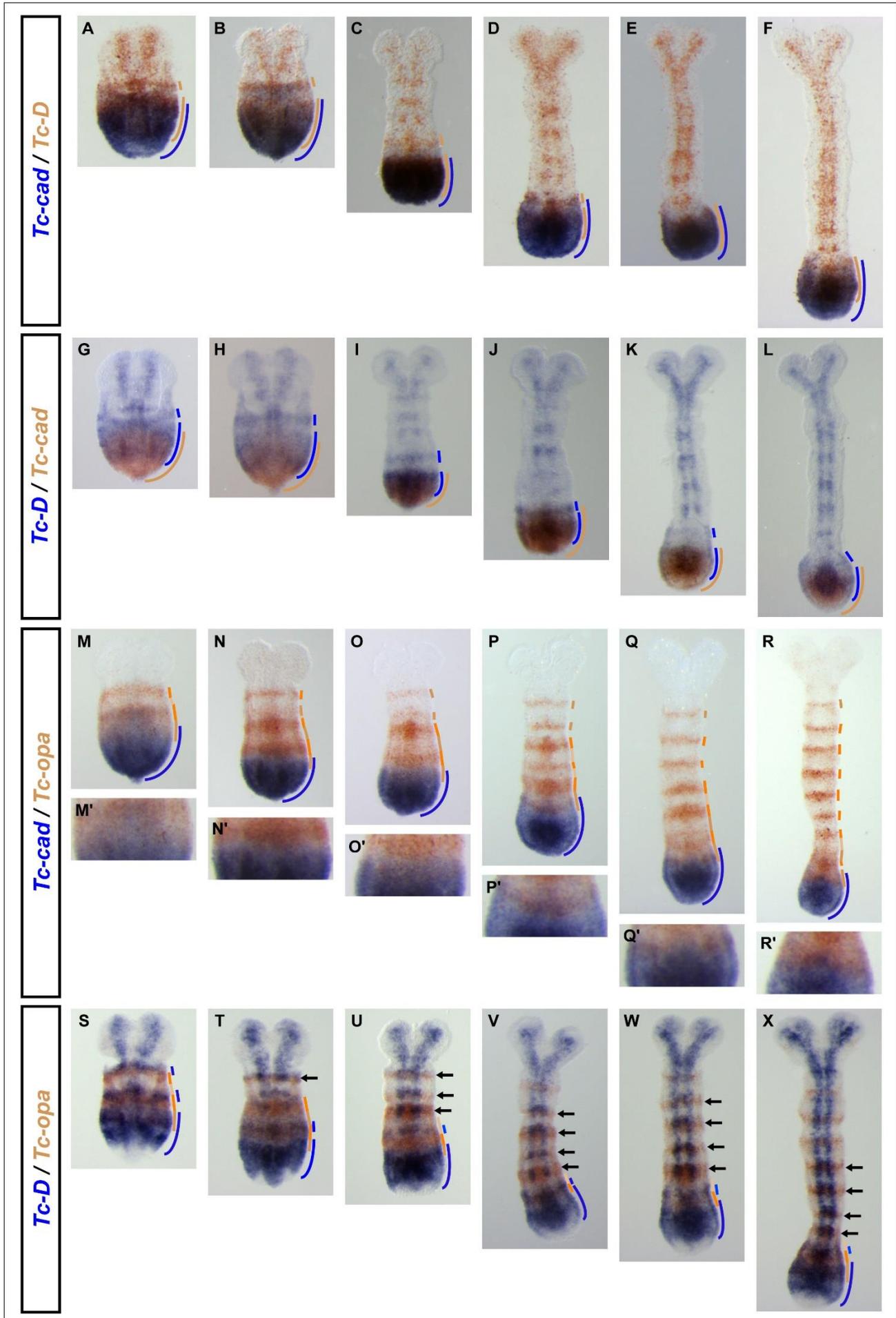
**B:** Irregular boundary specification in *Dichaete* mutant embryos. Parasegment boundaries are specified in wild-type embryos (left) by abutting stripes of *slp* and *en* expression, separated by clear buffer zones (correspond to segmental stripes of *odd* expression), which allow the boundaries to maintain polarity. The irregular patterns of *slp* expression, resulting ultimately from irregular *runt* expression (see **A**) lead to various boundary specification defects: fusions with more anterior boundaries (asterisks); narrow/incomplete boundaries (arrowheads); and fusions with more posterior boundaries (arrow). Scale bar = 100  $\mu\text{m}$ .





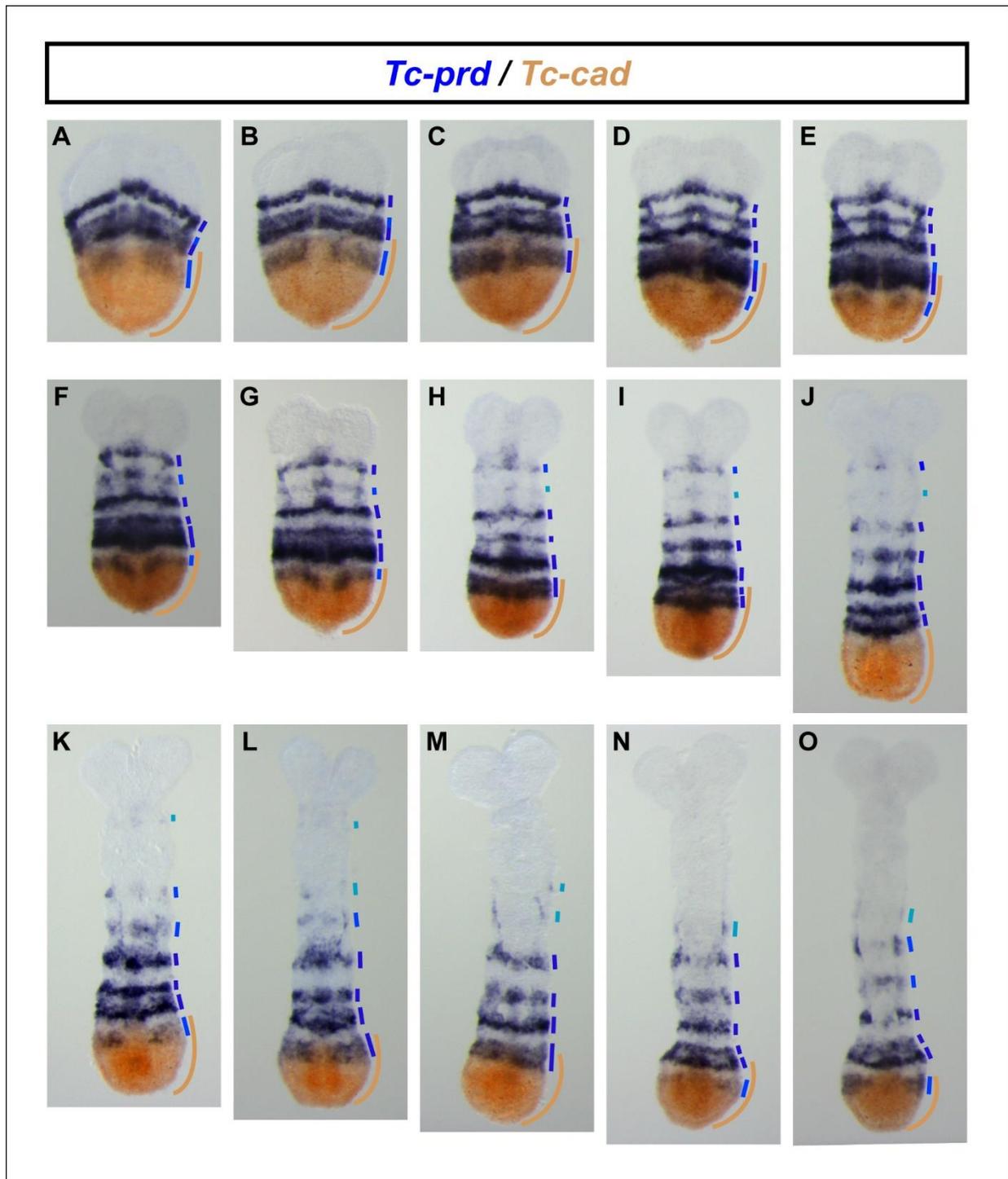


**Fig. S5. Expression of *Tc-cad*, *Tc-Dichaete* & *Tc-opa* relative to a common segment marker, *Tc-wg*, in *Tribolium castaneum* germband stage embryos. (A-U).** Sets of three *Tribolium castaneum* germband stage embryos that have been stage matched using *Tc-wg* expression patterns (*Tc-wg* expression brown in all panels). Stage matched germband embryos increase in age from **A** to **U**. In each set of embryos, the left-hand embryo is also stained for *Tc-cad* expression, the middle embryo is stained for *Tc-Dichaete* expression and the right-hand embryo is stained for *Tc-opa* expression (all blue stains). In the double in situ hybridizations for *Tc-cad* & *Tc-wg* (left-hand embryos) the mandibular (Mn), prothoracic (T1), 1<sup>st</sup> abdominal (A1), 4<sup>th</sup> abdominal (A4), 7<sup>th</sup> abdominal (A7) and/or 10<sup>th</sup> abdominal (A10) stripes of *Tc-wg* expression have been labeled. Note how the relative position of the expression domains of these three genes is remarkably conserved across progressive germband elongation stages. Consult Fig. 7 for a clear comparison across different developmental stages, rather than between *Tc-cad*, *Tc-Dichaete* & *Tc-opa* expression patterns.



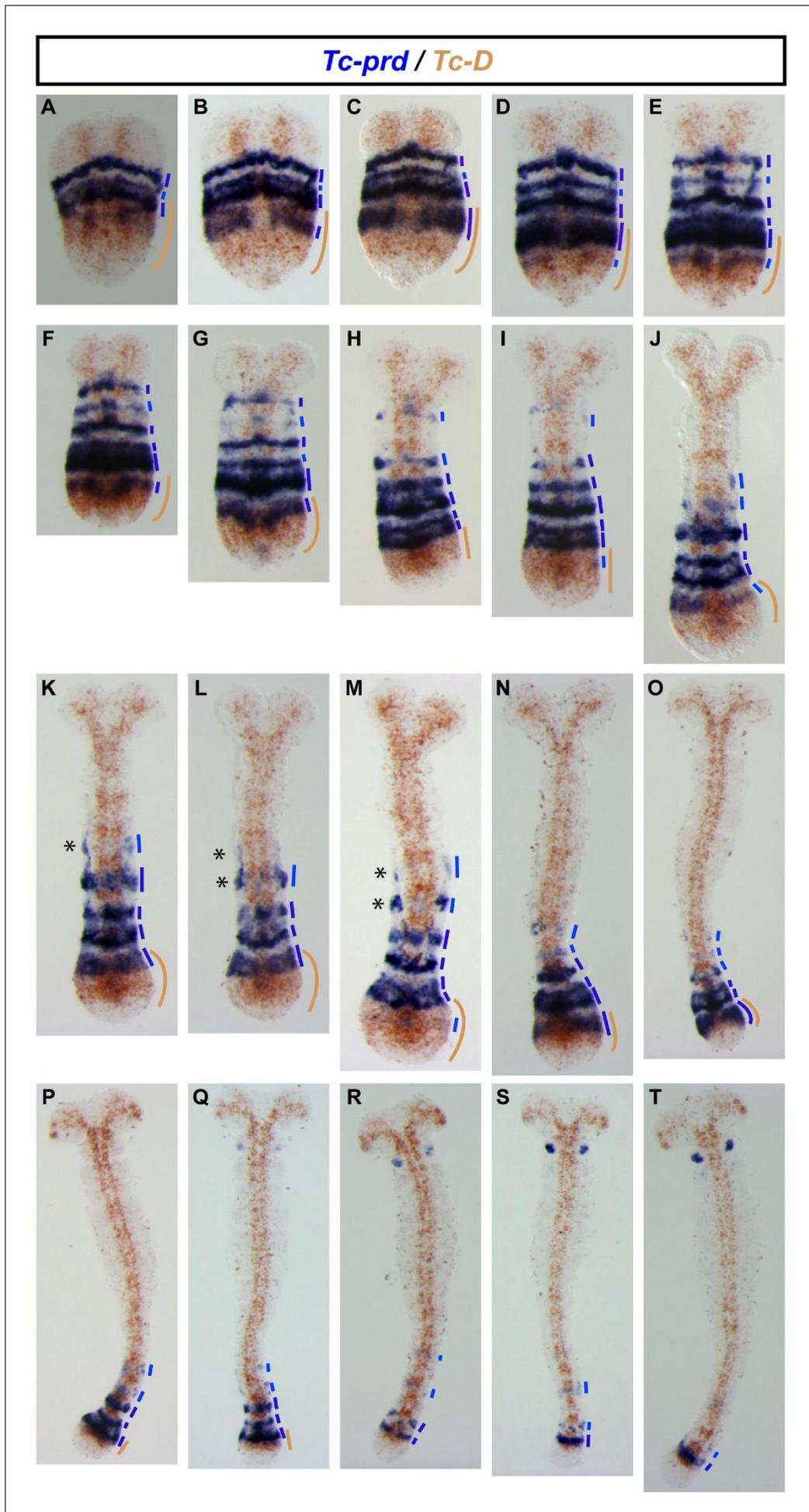
**Fig. S6. Expression patterns of *Tc-cad*, *Tc-Dichaete* & *Tc-opa* in relation to each other in *Tribolium castaneum* germband stage embryos.**

(A-F). Double in situ hybridization for *Tc-cad* (blue) and *Tc-Dichaete* (brown) in embryos of increasing age from left (A) to right (F). (G-L). As for panels A-F, but this time *Tc-Dichaete* DIG and *Tc-cad* FITC RNA probes were used instead of *Tc-cad* DIG and *Tc-Dichaete* FITC RNA probes such that the colours are reversed. Note the stripe of *Tc-Dichaete* expression that is observed anterior to the *Tc-cad* domain in some, but not all, embryos (see text for further details). (M-R). Double in situ hybridization for *Tc-cad* (blue) and *Tc-opa* (brown) in embryos of increasing age from left (M) to right (N). Panels M'-R' show higher magnification images of the regions in M-R where *Tc-cad* and *Tc-opa* expression overlaps. These data suggest that as posterior germband cells move anteriorly relative to the posterior tip of the elongating embryo due to convergent extension cell movements, they experience a drop as *Tc-cad* expression levels as *Tc-opa* expression levels increase. (S-X). Double in situ hybridization for *Tc-Dichaete* (blue) and *Tc-opa* (brown) in embryos of increasing age from left (S) to right (X). Black arrows point to late *Tc-opa* segmental stripes that overlap strong, segmentally-reiterated *Tc-Dichaete* expression domains that are limited to the medially positioned neuroectoderm. Colour-coded lines on the right-hand side of the embryos indicate our interpretations of the expression patterns in A-X.



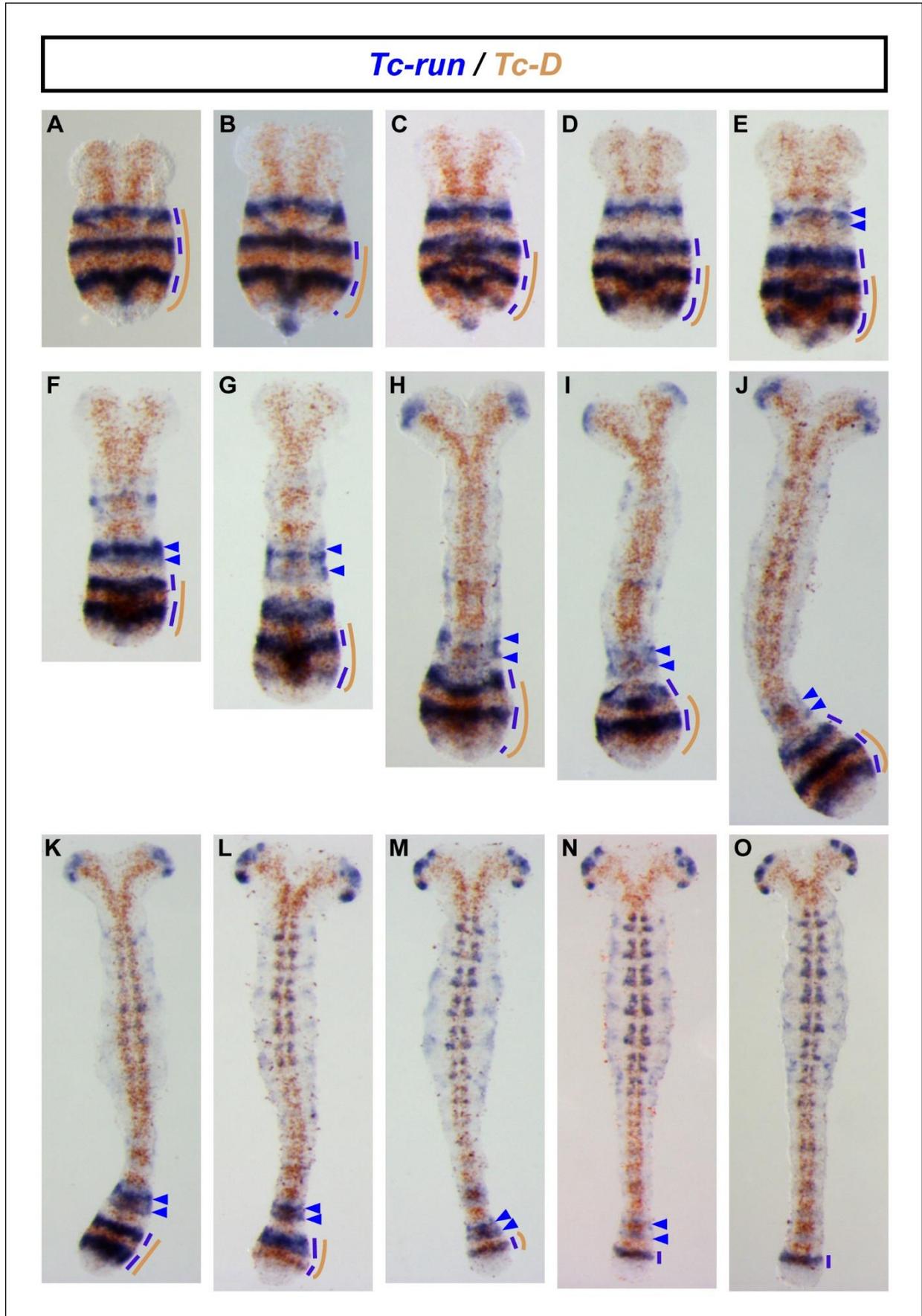
**Fig. S7. Expression of *Tc-prd* relative to *Tc-cad* in *Tribolium castaneum* germband stage embryos.**

(A-O). Double in situ hybridization for *Tc-prd* (blue) and *Tc-cad* (brown) in embryos of increasing age from youngest (A) to oldest (O). Colour-coded lines on the right-hand side of the embryos indicate our interpretations of the expression patterns in A-O. Note how the primary pair-rule stripes of *Tc-prd* first appear and form within the anterior half of the posterior *Tc-cad* domain (see where blue lines overlap brown lines). In contrast, segmental stripes of *Tc-prd* expression resolve by splitting just anterior to the *Tc-cad* domain (see where blue lines lie anterior to the brown line).



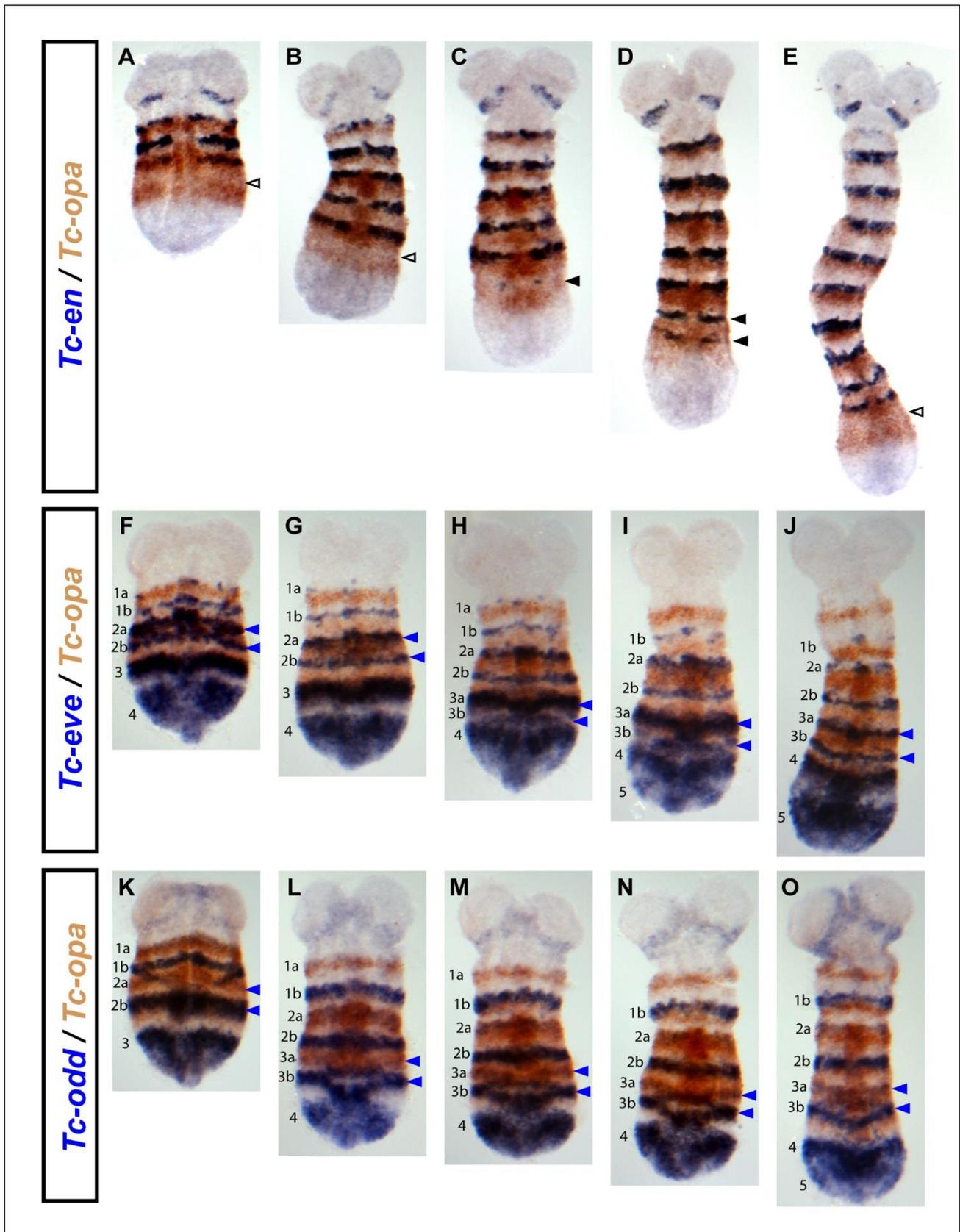
**Fig. S8. Expression of *Tc-prd* relative to *Tc-Dichaete* in *Tribolium castaneum* germband stage embryos.**

(A-T). Double in situ hybridization for *Tc-prd* (blue) and *Tc-Dichaete* (brown) in embryos of increasing age from youngest (A) to oldest (T). Colour-coded lines on the right-hand side of the embryos indicate our interpretations of the expression patterns in A-T. Note how the primary pair-rule stripes of *Tc-prd* first appear and form within the posterior-most *Tc-Dichaete* domain (see where blue lines overlap brown lines). In contrast, segmental stripes of *Tc-prd* expression resolve by splitting anterior to this domain (see where blue lines lie anterior to the brown line). While dissecting and cleaning the embryos we noted that *Tc-prd* expression remains on stronger and longer in the overlying amnion compared to the underlying ectoderm; this is particularly apparent in panels K to M where the *Tc-prd* stained amnion has been ripped away while cleaning the embryo of yolk to reveal ectoderm free from *Tc-prd* expression (asterisks). Amnion-related expression can be seen down the lateral margins of many of the embryos where some amniotic cells survived dissection and cleaning.



**Fig. S9. Expression of *Tc-run* relative to *Tc-Dichaete* in *Tribolium castaneum* germband stage embryos.**

(A-O). Double in situ hybridization for *Tc-run* (blue) and *Tc-Dichaete* (brown) in embryos of increasing age from youngest (A) to oldest (O). Colour-coded lines on the right-hand side of the embryos indicate our interpretations of the expression patterns in A-O. In A-O blue arrowheads mark the primary pair-rule stripes that have most recently resolved – or are in the process of resolving – to a segmental periodicity. In some younger embryos (A-H), more than two stripes are apparent due to differences in the timing and/or positioning of this process between the amnion and ectoderm cell layers. Note how *Tc-run* stripe splitting occurs anterior to the posterior-most *Tc-Dichaete* domain (as judged by brown line by side of embryo). Older embryos show additional domains of *Tc-run* expression in the head lobes (H-O) and neuroectoderm (J-O) that were used to help stage the embryos.



**Fig. S10. Expression of *Tc-opa* relative to *Tc-en*, *Tc-eve* and *Tc-odd* in *Tribolium castaneum* germband stage embryos.**

(A-E). Double in situ hybridization for *Tc-en* (blue) and *Tc-opa* (brown) in embryos of increasing age from left (A) to right (E). Note how the *Tc-en* stripes (solid black arrowheads in C, D) form within the *Tc-opa* domain, but in a stripe-shaped region that is already clearing of *Tc-opa* expression (empty black arrowheads in A, B, E). (F-J). As for A-E, but this time double in situ hybridization for *Tc-eve* (blue) and *Tc-opa* (brown). (K-O). As for A-E, but this time double in situ hybridization for *Tc-odd* (blue) and *Tc-opa* (brown). In F-O, note how the segmental stripes of *Tc-odd* and *Tc-eve* (labeled a & b) resolve within the *Tc-opa* domain. In F-O, blue arrowheads mark *Tc-odd* & *Tc-eve* segmental stripes that have most recently resolved, or are in the process of resolving.