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Dynamic expression of *Drosophila* segmental cell surface-encoding genes and their pair-rule regulators

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Abstract

Drosophila segmentation is regulated by a complex network of transcription factors that include products of the pair-rule genes (PRGs). PRGs are expressed in early embryos in the primorida of alternate segmental units, establishing the repeated, segmental body plan of the fly. Despite detailed analysis of the regulatory logic among segmentation genes, the relationship between these genes and the morphological formation of segments is still poorly understood, since regulation of transcription factor expression is not sufficient to explain how segments actually form and are maintained. Cell surface proteins containing Leucine rich repeats (LRR) play a variety of roles in development, and those expressed in segmental patterns likely impact segment morphogenesis. Here we explore the relationships between the PRG network and segmentally expressed LRR-encoding (sLRR) genes. We examined expression of *Toll2*, *Toll6*, *Toll7*, *Toll8* and *tartan* (*trn*) in wild type or PRG mutant embryos. Expression of each sLRR-encoding gene is dynamic, but each has a unique register along the anterior-posterior axis. The registers for different sLRRs are off-set from one another resulting in a continually changing set of overlapping expression patterns among the sLRR-encoding genes themselves and between the sLRR-encoding genes and the PRGs. Accordingly, each sLRR-encoding gene is regulated by a unique combination of PRGs. These findings suggest that one role of the PRG network is to promote segmentation by establishing a cell surface code: each row of cells in the two-segment-wide primordia expresses a unique combination of sLRRs, thereby translating regulatory information from the PRGs to direct segment morphogenesis.

Keywords

Toll family; *tartan*; pair rule genes; segmentation

Introduction

Segmentation can be broken down into three steps: segment specification, differentiation or morphogenesis of segments, and maintenance of segments once they are formed. The

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process of segmentation has been extensively studied in *Drosophila melanogaster* where a cascade of regulatory genes specifies formation of embryonic segments (Lawrence and Johnston, 1989; Nusslein-Volhard and Wieschaus, 1980). This hierarchy is initiated by a set of maternal genes that define the anterior-posterior (AP) axis. Gap genes then define broad regions within the embryo, and, together with maternal genes, control expression of the pair rule genes (PRGs). PRGs are expressed in the primordia of alternating segment-wide units, typically in a pattern of 7 stripes at the cellular blastoderm stage (PR-stripes). The PRGs are essential for formation of segments, evidenced by the finding that in embryos homozygous for mutations in any of the nine PRGs, alternate segmental units are missing. The last set of segmentation genes, the segment polarity genes (SPGs), define anterior and posterior compartments within each segment and are typically expressed in 14-stripe patterns (segmental stripes). Definition of segments is completed very early, by the end of the cellular blastoderm stage of development, when PR-stripes peak (Chan and Gehring, 1971; Lohs-Schardin et al., 1979). The PRGs and SPGs encode regulatory proteins, primarily transcription factors, whose cross-regulatory interactions have been well-studied. Although these regulatory interactions have been documented in some detail, regulatory proteins themselves cannot direct segment formation. Rather, these regulators must control downstream target genes that encode products more directly involved in segment morphogenesis.

Genes that encode cell surface proteins are good candidates for playing direct roles in segment morphogenesis. Subsequent to segment establishment, the embryo undergoes two dramatic morphogenetic movements, germband extension and germband retraction. During these movements, cells specified to be part of a particular segment remain associated and retain their segmental identity (Chan and Gehring, 1971; Gergen et al., 1986; Gilbert, 2010; Grosshans and Wieschaus, 2000; Irvine and Wieschaus, 1994; Lohs-Schardin et al., 1979; Wieschaus et al., 1991). Several groups previously showed that genes encoding cell surface proteins belonging to the Toll family are expressed in patterns suggestive of PR-regulation. Specifically, *Drosophila Toll-2*, *Toll-6*, *Toll-7* and *Toll-8* were shown to be expressed in early embryos in PR-like stripes (Eldon et al., 1994; Kambris et al., 2002). The *Toll-2* stripes lie posterior to those of *even-skipped* (*eve*), and *Toll-8* stripes overlap those of *eve* (Kambris et al., 2002). By germband extension, *Toll-2* and *Toll-8* stripes have doubled in the trunk, with *Toll-2* overlapping and possibly extending posterior to *wingless* (*wg*) and *Toll-8* lying posterior to *wg* stripes and potentially partially overlapping *Toll-2* (Eldon et al., 1994; Kambris et al., 2002). *Toll-6* is expressed in stripes late in cellular blastoderm, with secondary stripes evident by early germband extension (Kambris et al., 2002). *Toll-7* is expressed during germband extension in 14 stripes that overlap those of *engrailed* (*en*) (Kambris et al., 2002).

Tartan (Trn), like the segmentally expressed Tolls, contains leucine rich repeats (LRR) in its extracellular domain (Figure 1A), is apically localized, and has been shown to function as an adhesion molecule in a variety of contexts (Artero et al., 2003; Mao et al., 2008; Milan et al., 2005; Milán et al., 2001). Thus, Trn may act with the segmentally expressed Toll family members to translate the spatial information provided by the PRGs into actions that produce or maintain actual segments. *trn* is expressed in a PR-like pattern of eight stripes, seven of which overlap with the PRG *fushi tarazu* (*ftz*). These seven stripes were lost in a *ftz* mutant

background (Chang et al., 1993; Field et al., 2016). Regulation of *trn* by Ftz, and its partner Ftz-F1, appears to be direct, as a *trn*-enhancer was identified that directs reporter gene expression in a *ftz*-dependent seven-stripe pattern (Field et al., 2016).

Functional roles for these cell surface proteins remained elusive for some time, because deletion of any single *Toll* family gene failed to cause segmentation defects (Yagi et al., 2010, W.R.A. and L.P., unpublished). However, RNAi-mediated knock-down indicated that *Toll-2*, *Toll-6* and *Toll-8* have roles in germband extension (Pare et al., 2014). While knockdown of any single gene did not affect elongation, double and triple gene knock-downs reduced axis elongation. Similarly, in the flour beetle *Tribolium castaneum*, *Toll-7* and *Toll-10* are expressed in striped patterns, and simultaneous knock-down of both genes reduced mediolateral intercalation during germband extension (Benton et al., 2016). The defects in germband extension seen for knockdown of *Drosophila Tolls* are reminiscent of defects seen in *eve* and *runt* (*run*) mutants, consistent with these genes regulating *Toll* gene expression (Butler et al., 2009; Irvine and Wieschaus, 1994; Pare et al., 2014).

In this paper, we determined the phylogenetic relationships between *trn* and the segmentally expressed *Toll* genes, referred to here collectively as ‘segmentally expressed Leucine-Rich Repeat (sLRR)’-encoding genes. We examined the expression patterns of these sLRR-encoding genes in wild type and segmentation gene mutant backgrounds throughout early embryogenesis and developed a model for the dynamic positioning of these genes relative to one another and the PRGs during early embryogenesis. Expression of each sLRR-encoding gene was affected by multiple PRGs. However, regulatory interactions are complex: the complement of PRG regulators, and the direction of regulation by PRGs were unique for each sLRR, suggesting that each is regulated by different elements of the PRG network.

Materials and Methods

Drosophila genetics

Drosophila mutant strains used in this study: ftz^{9h39}/*TM3Ser*, *actin-GFP*; h^{22}/*TM3Ser*, *actin-GFP*; run^{2}/*FM7*, *actin-GFP*; prd^{12}/*CyO*, *actin-GFP*; odd^{5}/*CyO*, *actin-GFP*; eve^{1}/*CyO*, *actin-GFP*; $slp^{\Delta 34}$/*CyO*, *actin-GFP*; and en^{54}/*CyO*, *actin-GFP*. Each allele has been characterized as a null or a strong hypomorphic allele, except for eve^{1}. Null alleles of *eve* produce a denticle lawn phenotype rather than a PR phenotype. At 25°C eve^{1} produces a PR phenotype (Frasch et al., 1988). Initially, we used *actin-GFP* balancers to identify homozygous mutant embryos, expecting that the GFP marker would be visible throughout embryogenesis, such that homozygous mutant embryos would be identified by the lack of expression. However, we found that reliable identification of the mutant embryos was difficult. Thereafter, we examined expression of genes of interest in embryos at cellular blastoderm, gastrulation and germband extension stages for each genotype, without double staining for a marker gene. Because both parents were heterozygous for each PRG mutation, 25% of the embryos would be expected to be homozygous mutant. A PRG mutation was considered to have a strong effect on a particular sLRR when expression differed from wild type in at least 20% of the embryos. If expression was altered in fewer than 10% of the embryos, the PRG was considered to have no effect. Those PRG mutations that affected expression in 10 - 15% of

embryos are described as having a weak effect; between 15 and 20% affected are described as having a moderate effect.

Phylogenetic analysis

Orthologs were identified by blasting the protein sequence (blastp) of the *Drosophila* gene of interest (Trn, Cap, Chp, Con, Slit, Toll1, Toll2/18w, Toll3/MstProx, Toll4, Toll5/Tehao, Toll6, Toll7, Toll8/Tollo, or Toll9) against the non-redundant protein sequences (nr) collection of Caenorhabditis, Chelicerata, Myriapoda, Crustacea, Collembola, Orthoptera, Isoptera, Hemiptera, Hymenoptera, Coleoptera, Lepidoptera, Siphonaptera, and Mecoptera in NCBI. The protein sequence of the top 3-4 "hits" (excluding sequences of low-quality proteins, partial sequences and additional isoforms of a single gene) was blasted back against the *Drosophila melanogaster* non-redundant protein sequences (nr) collection in NCBI. If the reciprocal blast identified the original *Drosophila* gene as the first hit, the putative ortholog was retained for use in the phylogenetic analysis. Sequences for all orthologs of a particular gene were aligned in Clustal Omega (Li et al., 2015), then imported into Aliview (Larsson, 2014) for evaluation. Sequences that were at least 100 amino acids shorter than the majority of the orthologs were presumed to be partial sequences and were discarded from the pool. A master set of sequences for all orthologs of all genes was generated by combining the subsets of individual gene sequences. The master set was aligned in Clustal Omega, then imported into Topali (Milne et al., 2009) for phylogenetic analysis using the Maximum Likelihood algorithm RaxML, WAG model with 100 bootstraps (Stamatakis and Swiss Federal Institute of Technology Lausanne, 2018). Additional formatting of trees was performed in MEGA7 (Tamura et al., 2013) or Dendroscope3 (Huson and Scornavacca, 2012).

Drosophila gene expression analysis

Drosophila embryo *in situ* hybridization with digoxygenin-, biotin- or fluorescein-labeled probes was carried out using modifications of the protocols in Tautz and Pfeifle 1989 (Tautz and Pfeifle, 1989) and Kosman et al. 2004 (Kosman et al., 2004). Details are provided in supplemental material. Reagents for TSA detection were: Sigma goat anti-biotin HRP conjugated (1:1000 dilution), Roche mouse anti-digoxygenin (1:250 dilution), Life Technologies goat anti-mouse IgG HRP conjugated (1:100 dilution), ThermoFisher AlexaFlour 488 tyramide reagent, and ThermoFisher AlexaFluor 555 tyramide reagent. Reagents used for colorometric detection were: Roche anti-digoxygenin AP conjugated Fab fragments (1:2000 dilution), Roche anti-fluorescein AP conjugated Fab fragments (1:200 dilution), MP Biomedicals mouse anti-beta-galactosidase biotin conjugated (1/1000), NBT (Roche 11383213001) -BCIP (Roche 11383221001) -INT -BCIP (Roche 11681460001), ABC (vector labs Vectastain Elite ABC-HRP Kit) and DAB (SigmaFAST 3,3-Diaminobensidine tablets).

Results

As mentioned above, cell surface proteins likely play direct roles in morphogenesis in the *Drosophila* embryo. The *Toll* family genes have been examined in this light (Pare et al. 2014), but Trn encodes another cell surface protein expressed in PR-like stripes, which could

function similarly to or together with Tolls. Trn shares LRR and transmembrane domains with the Toll family but lacks a Toll/Il-1 Receptor (TIR) domain (Fig. 1A). To assess whether Trn is related to the Tolls, we constructed a phylogenetic tree comparing *Toll1-9*, *trn*, and *capricious (caps)* which is closely related to *trn* (Shishido et al., 1998). We included three additional divergent LRR protein-encoding genes that mediate cell adhesive interactions in *Drosophila*: Choaptin (Chp) and Connectin (Con) are LRR proteins that are membrane associated via a phosphatidylinositol link rather than a transmembrane domain (Krantz and Zipursky, 1990; Nose et al., 1992; Van Vactor et al., 1988), while Slit is a secreted LRR protein (Rothberg et al., 1988; Rothberg et al., 1990). Orthologs for each gene were identified from up to three species within the following groups: Caenorhabditis, Chelicerata, Myriapoda, Crustacea, Collembola, Orthoptera, Isoptera, Hemiptera, Hymenoptera, Coleoptera, Lepidoptera, Siphonaptera, Mecoptera. We used *Drosophila* protein sequences as queries for blastp searches of the non-redundant protein sequences database for each order. The protein sequences of candidate orthologs were then used for reciprocal blastp searches of the *Drosophila* non-redundant protein sequences database. Only candidates that returned the original *Drosophila* gene were considered as orthologs.

As shown in Figure 1B, and in more detail in Supplemental Figure 1, the orthologs of each gene form well defined clades with high confidence levels. *capricious* and *tartan* orthologs are so closely related that they cannot be separated into individual clades. The sLRR-encoding genes most closely related to *trn* and *caps* are *slit* and *connectin*. All the *Tolls* and *chaoptin* form a clade separate from that containing *trn*, *caps*, *slit* and *con*.

The *Tolls* that are expressed in segmental patterns (*Toll2*, *6*, *7* and *8*) form a clade of their own separate from other *Tolls* and *chaoptin*. Within this clade, *Toll8* orthologs show the most divergence from one another. The *Toll6* orthologs all fall within one clade, and most potential orthologs of *Dmel-Toll2* return *Dmel-Toll7* when blasted against *Drosophila* genes. Since *Toll2* and *Toll7* are closely linked on chromosome 2, this suggests they are the results of a recent duplication in lineages leading to *Drosophila*.

trn and *Toll2*, *Toll6*, *Toll7* and *Toll8* are expressed in non-overlapping segmental patterns

The expression of the sLRR-encoding genes was compared to that of segmentation genes expressed in early embryos by double in situ hybridization (Fig. 2). Expression of each sLRR-encoding gene overlapped exactly with that of one segmentation gene. Specifically, at the cellular blastoderm stage, the seven anterior *Toll2* stripes overlapped with those of the PRG *run* (Fig. 2A-C). The anterior 7 *Toll8* stripes at cellular blastoderm overlapped with the seven stripes of the PRG *hairy* (*h*) (Fig. 2D-F). *trn* was detected in eight stripes at the cellular blastoderm stage, with stripes 2-8 overlapping the seven *ftz* stripes (Fig. 2G-I). Two sLRR-encoding genes were not detectable at early cellular blastoderm. *Toll6* expression was first reliably detectable at late cellular blastoderm. The number of *Toll6* stripes changes rapidly, but *Toll6* stripes overlapped *sloppy paired (slp1)* stripes (Fig. 2J-L). Finally, *Toll7* was detected during germband extension in a pattern of 14 stripes that overlapped ectodermal *en* stripes (Fig. 2M-O). Thus, each sLRR-encoding gene was expressed in a distinct spatio-temporal domain, overlapping the pattern of one segmentation gene.

sLRR genes are expressed dynamically during *Drosophila* embryogenesis

Although a static pattern of sLRR expression is implied by single snapshot-views of expression such as that shown in Figure 2, the sLRR-encoding genes are expressed in dynamic patterns that change rapidly during early embryogenesis. We and others (Chang et al., 1993; Eldon et al., 1994; Kambris et al., 2002) monitored expression of the sLRR-encoding genes through cellular blastoderm, gastrulation and germband extension stages (Fig. 3, Supp. Fig. 2). *Toll2* expression was detected in a pattern of 8 stripes at the cellular blastoderm stage with an additional anterior stripe that does not reach the dorsal side and a region of expression near the anterior end of the embryo (Fig. 3A). The cephalic furrow formed between the partial stripe and the first full stripe. The 5 most anterior full stripes remained a constant 2-3 cells wide from the cellular blastoderm stage through germband extension, while the two most posterior stripes were initially 4-5 cells wide, but narrowed to 2-3 cells before germband extension. Secondary stripes began to appear during gastrulation or early germband extension (Fig. 3B). Initially 1 cell wide, they quickly widened to 2-3 cells like the primary stripes. During germband extension (Figure 3C), 14 *Toll2* stripes were detected in a segmentally repeating pattern.

Toll8 expression first appeared at cellular blastoderm (Fig. 3D). Near the anterior of the embryo, there was a wide stripe (4-5 cells), then a gap of about 12 cells. Posterior to the gap, there was a set of 8 stripes, with the first stripe being slightly wider (4-5 cells) than the others (3-4 cells). The most anterior stripe faded during gastrulation while the set of 8 stripes was maintained, and the first stripe in the set narrowed to 3-4 cells (Fig. 3E). The cephalic furrow formed just posterior to first stripe in this set of 8 stripes. Weak secondary *Toll8* stripes appeared between the primary stripes during mid to late germband extension. By the end of germband extension, 14 *Toll8* stripes were apparent in the trunk, each approximately 2 cells wide (Fig. 3F), along with patches of stain in the head.

As described previously, *trn* was initially detected as a set of 8 stripes approximately 3 cells wide at the cellular blastoderm stage (Fig. 3G). These stripes were maintained during gastrulation (Fig.3H) and early germband extension. The cephalic furrow formed between the first and second stripe. Late in germband extension, epidermal *trn* was very briefly and weakly expressed in a segmental pattern of stripes 1-2 cells wide, with additional expression in neuronal tissue (Fig. 3I).

The first set of *Toll6* stripes consists of 7 full stripes posterior to a single partial stripe that does not reach the ventral side (Fig. 3J). The stripes were 2-3 cells wide and appeared late in cellular blastoderm or early in gastrulation with the most anterior stripe appearing first. At gastrulation, the cephalic furrow formed on top of the most anterior stripe, and slightly narrower secondary stripes were detected between the primary stripes (Fig. 3K). When the germband was fully extended, a set of 14 *Toll6* stripes that had narrowed to single cell width were detected (Fig. 3L). In addition, there were patches of expression in the head region throughout this period of development.

Toll7 expression was first detected at early germband extension, when a set of 14 ‘messy’ stripes appear. As the germband extended, the stripes became more refined until they were a single cell wide (Fig. 3M, N). In addition, there were patches of expression in the head

region. In a variable percent of embryos (0-30% depending upon the sample), a broad band of expression was observed at the internal edge of the *Toll7* stripes, which is partially visible in the embryos shown in Fig. 3N, 6J.

In sum, *Toll 2*, *6*, and *8* were expressed in striped patterns from cellular blastoderm through germband extension which shifted from double to single segment periodicity. The secondary stripes appeared during gastrulation for *Toll2* and *Toll6*. *Toll8* secondary stripes appeared in the middle of germband extension, *trn* was expressed in alternate segment primordia from cellular blastoderm to mid germband extension and shifted to single segment periodicity for a very short period at the end of germband extension. *Toll7* was expressed only during germband extension and with single segment periodicity.

Unique expression for each sLRR-encoding gene

To examine how relative expression of the sLRR-encoding genes changes over the course of development, we first examined expression of *Toll2* relative to each of the others (Figure 4; see Figure 7 for summary). At cellular blastoderm, *Toll6* stripe 1 was evident as a full stripe but other stripes were just beginning to emerge, allowing us to determine stripe register. The 9 *Toll2* stripes were positioned adjacent and posterior to *Toll6* stripes (Fig. 4 A, B; Supp. Fig. 3 K, L). During gastrulation, *Toll2* secondary stripes formed between the *Toll6* stripes (Figure 4 C, D; Supp. Fig. 3 M-P). As the germband elongated, the *Toll6* stripes narrowed from the original width of 2-3 cells to 1-2 cells (Fig. 4 E, F; Supp. Fig. 3 Q, R).

Toll2 and *Toll8* stripes never overlapped (Fig. 4 G-L). Initially they alternated with small or no gaps (Fig. 4 G, H), but during germband elongation, the *Toll8* stripes narrowed, generating gaps between the anterior edge of the *Toll2* stripes and the posterior edge of the *Toll8* stripes (Fig. 4 K, L). The relationship between *Toll7* and *Toll2* remained stable, with *Toll2* stripes overlapping and extending anterior to the *Toll7* stripes (Fig. 4 M, N, Supp. Fig. 3A, B). Finally, *Toll2* stripes were positioned adjacent and anterior to *trn* stripes through cellular blastoderm and gastrulation stages of development (Fig. 4 O-R). Colorimetric double in situs are shown in Supplemental Figure 3 with better resolution for the *Toll2-Toll6* and *Toll2-Toll7* double *in situ* hybridizations and additional combinations of sLRR-encoding genes. In sum, the sLRR-encoding genes are expressed in evolving patterns. While each is expressed in a unique domain, expression is partially overlapping for several (Fig. 7A), similar to the situation for the PRGs.

Mutations in segmentation genes have specific effects on the expression sLRR-encoding genes

The sLRR-encoding genes are expressed in overlapping segmental patterns that closely match those of segmentation genes. To determine whether mutations in these genes affect expression of the sLRR-encoding genes, we examined expression of each sLRR-encoding gene in PRG or *en* mutants. All sLRR-encoding genes showed altered expression in multiple PRG mutant backgrounds suggesting that the network of sLRR-encoding genes responds to the PRG network. Each sLRR-encoding gene was affected by a different set of PRGs, but *run*, *eve*, *ftz*, and *paired* (*prd*) had strong effects on all of the sLRR-encoding genes during at least one stage of embryogenesis.

Changes in expression at gastrulation were observed in some PRG mutant backgrounds. *Toll2* expression was altered in *ftz*, *h* and a small fraction of *odd skipped* (*odd*) mutant embryos (Fig. 5B-D), with changes evident earliest at gastrulation. In all three mutant backgrounds, fusion and/or uneven spacing of the stripes, resulting in doublets of stripes with a wider gap between the doublets, was observed. Changes in *Toll8* expression were not observed at gastrulation in *h*, *ftz*, *odd* or *prd* mutant backgrounds, but *Toll8* stripes were fused or expanded in *run* mutant embryos (Fig. 5F). The only effects seen at cellular blastoderm were on *trn*. At cellular blastoderm, *trn* stripes 2-8 were lost in *ftz* mutant embryos (Fig. 5H) and all *trn* stripes expanded throughout the trunk region in *h* mutants (Fig. 5I). No effect of PRG mutant background was seen for *Toll6* or *Toll7* at these developmental stages.

Changes in sLRR expression patterns were more common in PRG mutant embryos during germband extension (Figure 6). All sLRR-encoding genes are expressed in a 13-14-stripe pattern in wild type embryos at this stage (Fig. 6 A, F, J, P; Fig. 3I for *trn*). Alternate *Toll2* stripes were absent in *run* and, *prd*, mutant embryos (Fig. 6B, C). The primary stripes were missing in *run* mutant embryos, while the secondary stripes were missing in *prd* mutant embryos. Embryos mutant for *eve* and *ftz* had fused *Toll2* stripes (Fig. 6D, E). In a moderate fraction of *odd* mutant embryos, *Toll2* stripes were unevenly spaced, as they were in younger *odd* mutants (data not shown). Although *Toll2* stripes were unevenly spaced in many *h* mutant embryos at gastrulation, only a small fraction of the *h* mutant embryos were affected at germband extension. Those that were affected were missing alternate stripes (data not shown).

The *Toll6* pattern was altered in most *ftz*, *prd* and *h* mutant embryos. *Toll6* stripes appeared to be fused in embryos mutant for *ftz* (Fig 6G), *prd* (Fig. 6H), or *h* (Fig. 6I). The *Toll7* pattern was altered in a moderate fraction of *run*, *ftz*, *prd*, and *eve* mutant embryos, with even numbered stripes missing in *ftz* and *run* mutant embryos and odd numbered stripes missing in *prd* mutant backgrounds (Fig. 6K-M). *eve* mutant embryos had unevenly spaced *Toll7* stripes (Fig. 6N) while in a moderate number of *en* mutant embryos, all stripes were missing (Fig. 6O).

Secondary *Toll8* stripes were missing in *ftz* mutants (Fig. 6Q). Since it was somewhat difficult to determine which stripes were missing in the *prd* mutants (6R), *prd* mutant embryos and their heterozygous siblings were double stained for En and *Toll8*. During early germband extension, all the En stripes were present, but only the primary *Toll8* stripes were clearly visible. The secondary stripes were very weak or absent. In *prd* mutant embryos at a similar stage, the remaining En stripes also showed strong *Toll8* staining (data not shown). This suggests that the remaining *Toll8* stripes are the primary stripes and that the *Toll8* stripes lost in *prd* mutants are the secondary stripes. In *eve* mutants, *Toll8* stripes were unevenly spaced (Fig. 6S), while in *run* mutants, *Toll8* stripes were fused, as they were at earlier stages (Fig. 5F compared to Fig. 6T). In *h* mutant embryos, *Toll8* stripes appear expanded or fused (Fig. 6U). Since there is only fleeting expression of *trn* in stripes during germband extension, it was difficult to discern alterations in the *trn* pattern at this stage in PRG mutant embryos.

Discussion

Genes regulating segmentation have been studied intensively over many years, and a complex network of regulatory interactions among them has been described in *Drosophila* (Reviewed in (Jaynes and Fujioka, 2004; Peel et al., 2005; Schroeder et al., 2011; Wieschaus and Nüsslein-Volhard, 2016). However, the downstream targets of this network responsible for the mechanical implementation of PRG network directives are not well understood. As cell surface proteins capable of forming heterodimers, the sLRRs are good candidates for these functional PRG-target genes involved in defining and/or maintaining cellular interactions within and between segments. Although PRG expression patterns are often described in relatively simple terms (e.g., *ftz* is expressed in seven stripes at blastoderm), they actually change rapidly and continuously (Clark and Akam, 2016). As shown in Figure 7A, expression of both the PRGs and the sLRR-encoding genes changes significantly and quickly during the early stages of *Drosophila* embryogenesis, and spatial relationships between different pairs of genes shift over time. Because expression of each sLRR-encoding gene overlaps expression of more than one PRG at various points during development, each sLRR-encoding gene has the potential to be controlled by more than one PRG. Consistent with this, expression of each sLRR-encoding gene was altered in embryos carrying mutations in different PRGs (Figures 5 and 6).

Considering our observations here, along with data from the extensive literature documenting expression patterns of and regulatory interactions among PRGs (i.e., PR-gene cross-regulation) summarized in Table S1, we propose a model for regulatory interactions between PRGs and the sLRRs (Fig. 7B). Note that the experiments presented in this manuscript do not indicate whether interactions between regulators and their targets are direct or indirect; however, they provide a framework for testing such interactions in the future. The following logic was used to establish this model: If a set of sLRR stripes is lost in a particular PRG mutant background, and the PRG expression pattern overlaps with the sLRR stripes that are lost, the simplest explanation is that the PRG activates that sLRR. This scenario is observed in the following cases: *run* and *Toll2* primary stripes (Fig. 6B); *prd* and *Toll2* secondary stripes (Fig. 6C); *en* and *Toll7* (Fig. 6O); *run* and *Toll7* even stripes (Fig. 6K); *ftz* and *Toll8* secondary stripes (Fig. 6Q); *ftz* and *trn* stripes 2-8 (Fig. 5H).

If we observed that a PRG was expressed in the regions between sLRR stripes, and loss of the PRG caused expansion of the sLRR stripes, the simplest explanation would be that the PRG acts as a repressor of that particular *Toll* gene. This pattern fits the expansion of *Toll8* in *run* mutant embryos (Fig. 5F, 6T); *Toll2* in *h* and *odd* mutant embryos (Fig. 5B, C and 6E); and *trn* in *h* mutant embryos (Fig. 5I). In the case of the fusion or doublets of *Toll2*, *7* and *8* in an *eve* mutant background (Fig. 6D, N and S), it seems likely that this is due to the effects of the *eve* mutation on the width of the odd-numbered parasegments. As clearly described in the model of Jaynes and Fujioka (2004), partial loss of Eve causes the odd-numbered parasegments to narrow (Coulter and Wieschause, 1988; DiNardo and O'Farrell, 1987; Fujioka et al., 1995; Jaynes and Fujioka, 2004), which would bring the *Toll* stripes closer together, explaining the doublets we observed.

If we observed an effect on an sLRR from a PRG that only partially or transiently overlapped the sLRR, we checked to see if activation or repression during that period would be reasonable in light of the observed phenotype. For example, although *h*, *ftz* and *prd* partially overlap the *Toll6* primary stripes, loss of any of these PRG causes fusion/doublets of *Toll6* (Fig. 6G-I) suggesting loss of repression. One possibility is that these PRGs act as repressors but expression of these PRGs is normally low enough in the overlap region to allow expression of *Toll6*. When the PRG is missing, *Toll6* spreads from the normal region into adjacent cells. Similarly, *prd* expression overlaps part of the regions covered by the *Toll8* primary and secondary stripes (Fig. 7A). Since the secondary stripes of *Toll8* are missing in *prd* mutant embryos (Figure 6R and data not shown) *prd* probably acts with *ftz* to activate the secondary stripes. In this model, the *Toll8* primary stripes lack an activator. It may be that *Toll8* is activated by a gene that is expressed throughout the early embryo (eg. *opa* or *ftz-f1*), and the stripes are produced by repression of *Toll8* by *runt* (Fig. 7A). Alternatively, there may be a specific activator that was not included in this study.

For some regulatory interactions, alternative hypotheses can be proposed by considering known relationships among PRGs. First, since *ftz* activates *trn*, it is likely that the expansion of *trn* expression in *h* mutant embryos (Figure 5I) is due to the expansion of *ftz* in *h* mutants (Ingham and Gergen, 1988). Second, the expansion of *Toll2* in *h* mutant embryos (Fig. 5C) would be due to the expansion of *runt* which occurs in *h* mutant embryos (Ingham and Gergen, 1988; Jiménez et al., 1996). Third, the loss of *Toll2* in *runt* mutant embryos would be explained by persistence of the proposed *Toll2* repressor *eve*, since *runt* would normally repress *eve* in the posterior of odd-numbered parasegments (Ingham and Gergen, 1988; Jiménez et al., 1996; Manoukian and Krause, 1993).

Three cases fit none of the categories mentioned above. The expansion of *Toll2* in *ftz* mutant embryos (Fig. 5B, 6E) suggests that *ftz* acts as a repressor of *Toll2*. However, *ftz* expression overlaps the posterior of the even *Toll2* stripes through much of early embryogenesis, making this simple explanation unlikely. The expansion of *Toll2* in *ftz* mutant embryos is probably due to loss of *odd* in *ftz* mutants, (Jaynes and Fujioka, 2004; Nasiadka and Krause, 1999), with *odd* acting as a *Toll2* repressor. *ftz* and *prd* regulate alternate *en* stripes (DiNardo and O'Farrell, 1987; Fujioka et al., 1996; Howard and Ingham, 1986; Ish-Horowicz et al., 1989), and all three genes impact *Toll7* expression (Fig. 6L, M and O), suggesting that *ftz* and *prd* could exert their effects on *Toll7* via *en*. However, we propose that the effects of *ftz* and *prd* on *Toll7* are only partially indirect, since mutations in both *ftz* and *prd* have more consistent, strong effects on even and odd *Toll7* stripes, respectively, than does *en* (Fig. 6L, M, O). It should also be noted that the *Toll7* and *en* expression patterns do not completely overlap since *en* expression extends beyond the ventral ectoderm (Kornberg, 1981a, b) while *Toll7* expression does not (Kambris et al., 2002), Fig. 2N, 4M). Thus, it seems likely that although *en* likely regulates part of the *Toll7* expression pattern there are additional factors regulating *Toll7*. The effect of *h* on *Toll8* (Fig 6U) remains difficult to interpret.

These results extend those of Pare et al. (2014), who examined the effect of *eve* and *run* on three *Toll* family genes. Our study made use of strong hypomorphs of *eve* and *run* (Frasch et al., 1987; Lifschytz and Falk, 1968; Torres and Sánchez, 1992) while Pare et al. (2014) used *eve* and *run* null mutants (Duffy and Gergen, 1991; Nusslein-Volhard and Wieschaus, 1980).

This led to some differences in expression patterns observed but these differences are consistent with the use of these different types of alleles. For example, we observed *Toll2* doublets in *eve* mutants (Fig. 5f, 6t), while Pare et al. observed a broad expansion of *Toll2* stripes in an *eve* mutant background. In one case, our results were inconsistent with Pare et al. (2014): they reported complete loss of *Toll8* stripes in *eve* mutants while we found doublets. One possible explanation is that, given that *eve* represses *run* (Fujioka et al., 1995; Manoukian and Krause, 1992) a null mutation in *eve* would allow the *run* domain to expand more widely than a weaker *eve* allele causing complete repression of the *Toll8* stripes.

Overall, our data suggest that regulatory information from the PRGs establishes a code of cell surface proteins that mark cells with unique identities. Specifically, the repeating units comprised of one odd- and one even-numbered parasegment primoridia are generally thought to consist of 8 rows of cells at the blastoderm stage. Combinatorial action of the PRGs directs expression of the sLRRs such that each row expresses a unique combination of sLRR genes at gastrulation (Supp. Fig. 4). Specifically, in these repeating units, cell 1 is marked by the expression of *Toll2,7* and *8*; cell2 expresses only *Toll8*; cell 3 expressed *Toll6*; cell 4, *Toll2* and *6*; cell 5, *Toll 2,7,8,* and *trn*; cell 6, *Toll 6,8, trn*; cell 7, *Toll 6, trn*; cell 8, only *Toll 2*. By the end of germband extension, a segmentally repeating matching pattern emerges that matches that seen in the odd PS at gastrulation (Supp. Fig. 4). Thus, the alternate segment periodicity and combinatorial action of the PRGs directs the establishment of sets of cells along the anterior-posterior axis in double-width segmental units, each with a unique cellular identity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Tartan and related Toll proteins form a subset of the **L**eucine **R**ich **R**epeat family of cell surface proteins that is distinguished by expression in **segmentally** repeating pattern during *Drosophila* development (sLRR proteins).
- sLRR-encoding genes have dynamic expression patterns, with each expressed in a unique register along the anterior-posterior axis, marking each cell in the double segment primorida with a unique cell surface code.
- The sLRR-encoding gene are regulated by the pair-rule gene network, linking determination to differentiation during segmentation.

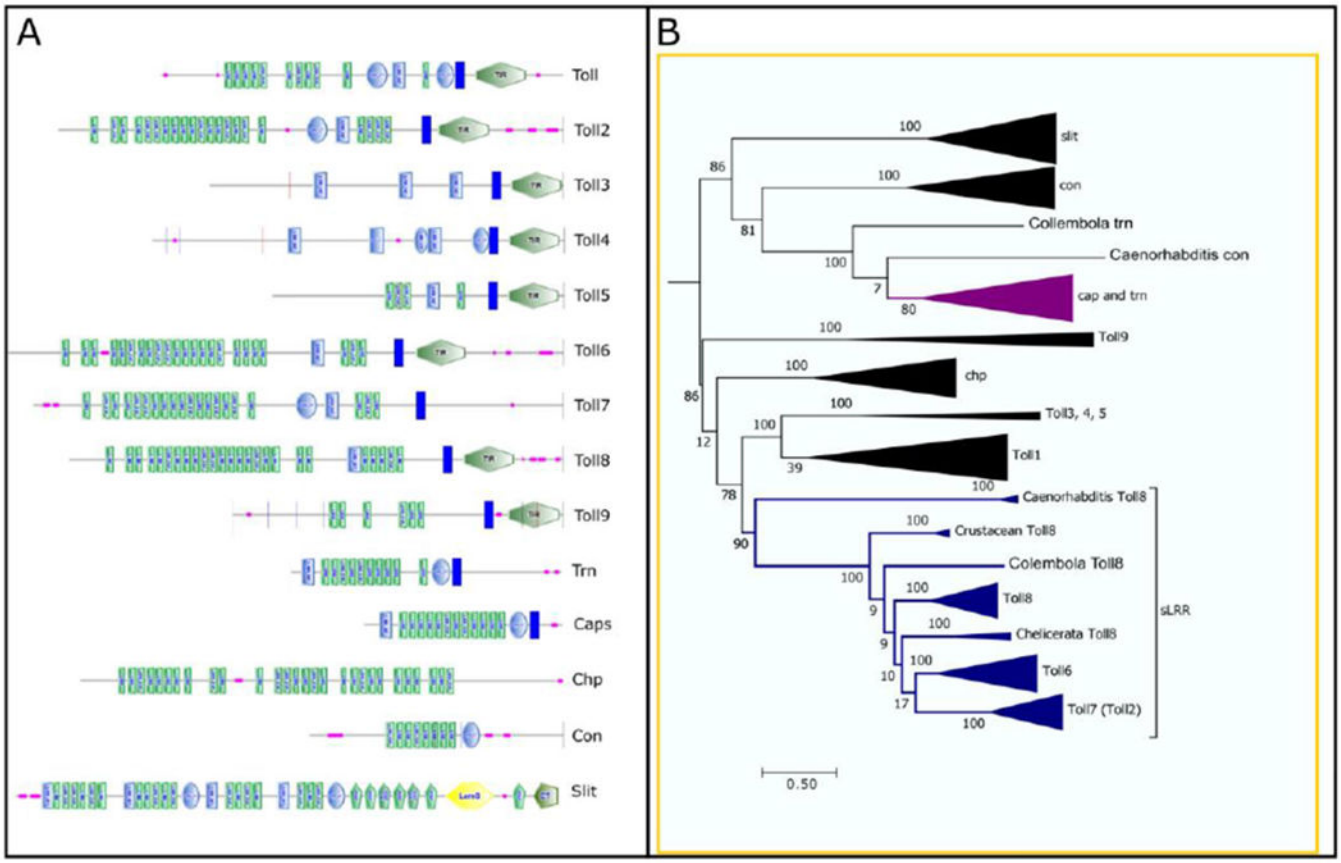

Figure 1.

Trn shares LRR repeats with the Toll family proteins. A) Diagrams of *Drosophila* sLRR proteins domains. Diagrams modified from those produced by the SMART program. Most Tolls have large Leucine Rich Repeat regions (LRR, green rectangles), C- (blue ovals) or N- (light blue rectangles) type cysteine rich flanking regions, a transmembrane domain (TM, dark blue rectangles) and an internal Toll/Interleukin-1 Receptor (TIR, green hexagons) domain. Pink squares represent regions of low complexity. Tartan (Trn) and Capricious (Caps) share two of these features, the LRR region and the TM domain, but lack the TIR domain. Chaoptin (Chp), Connectin (Con) and Slit all contain LRR domains and are involved in cell adhesion, but lack the TM and TIR domains. Slit also contains a laminin G domain (LamG, yellow hexagon), an EGF domain (GFP, vertical green pentagons), and a cystein knot-like domain (CT, horizontal green pentagon). B) Simplified phylogenetic tree showing the relationship between Trn and Toll family proteins. For the full tree see Supplemental Figure 1. Orthologs were identified by reciprocal blast of protein sequences, aligned with Clustal Omega and placed in a midpoint rooted RaxML phylogenetic tree using Topali. The clade containing Trn is purple. The clades containing the Tolls that are expressed in a segmental pattern are blue. Numbers at the junctions are bootstrap values from 100 iterations which provide statistical support for the nodes. The size of the triangle is proportional to the number of orthologs within the clade.

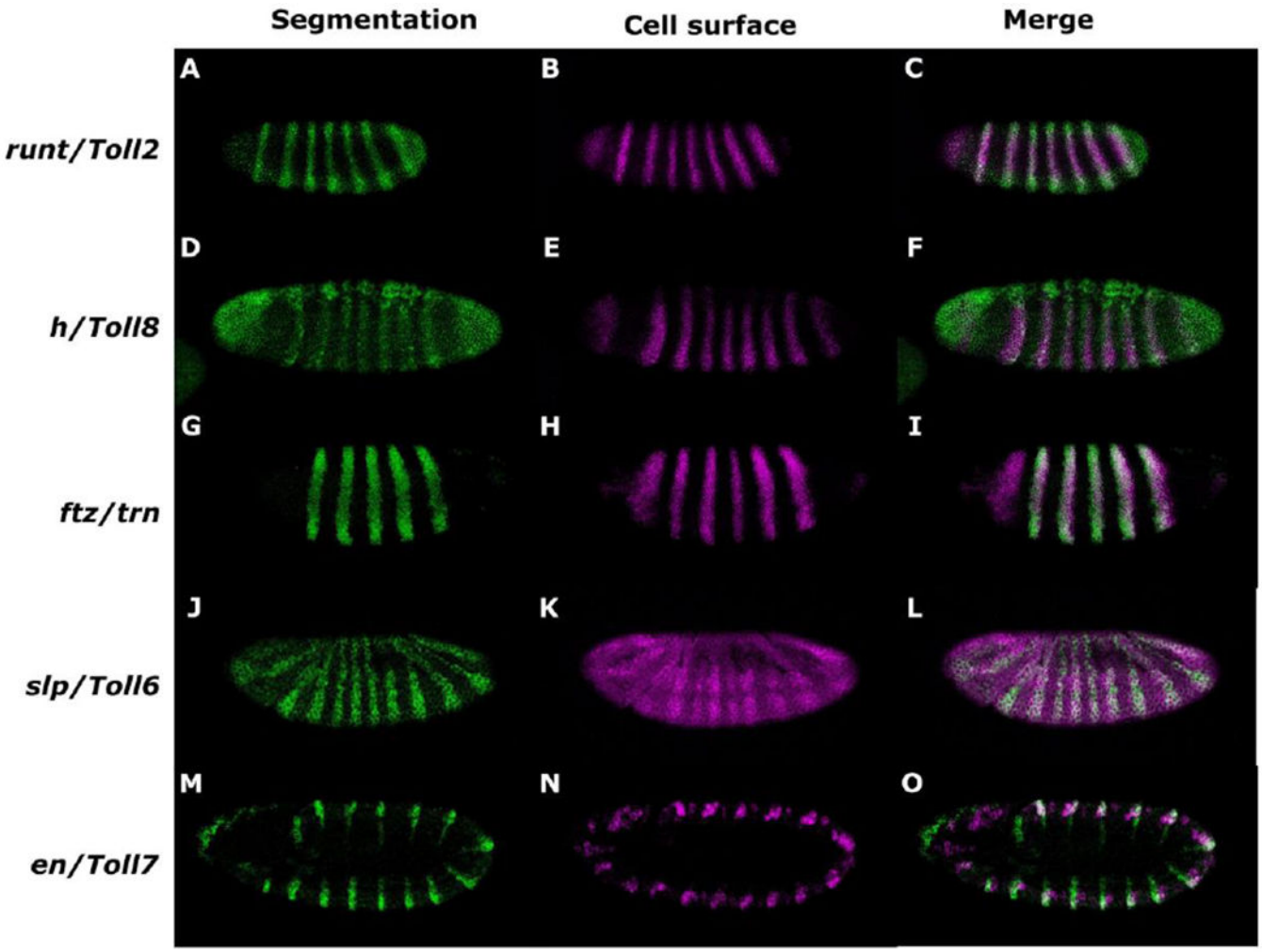

Figure 2.

Expression of each sLRR encoding gene overlaps with a segmentation gene in early *Drosophila* embryos. Expression patterns were monitored in w^{1118} embryos by *in situ* hybridization using digoxigenin- or fluorescein-labeled probes, as indicated. In each row, the first panel shows the pattern of the segmentation gene, the second shows expression of the sLRR-encoding gene and the final shows the merge. (A, D, G, J, M) segmentation gene expression. (B, E, H, K, N) sLRR-encoding gene expression. (C, F, I, L, O) merged images. Expression of *run* (A, C) overlaps with *Toll2* (B, C). *h* (D, F) overlaps *Toll8* (E, F). *ftz* (G, I) overlaps *trn* (H, I). *slp* (J, L) overlaps *Toll6* (K, L), and *en* (M, O) overlaps *Toll7* (N, O). Embryos are oriented anterior, left; dorsal, top.

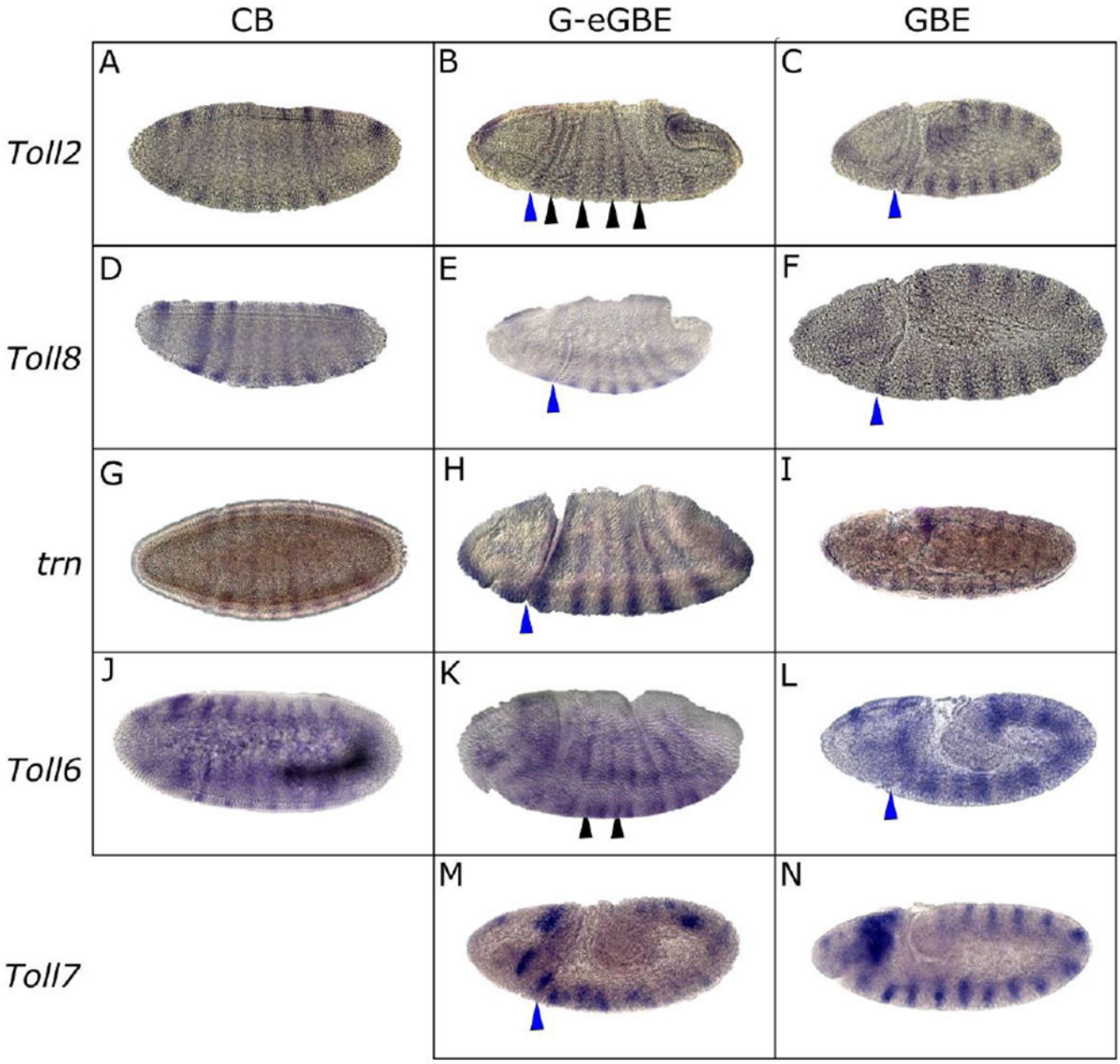

Figure 3.

sLRR-encoding gene expression evolves continuously throughout early embryogenesis. Expression patterns were monitored in w^{1118} embryos by *in situ* hybridization using digoxigenin labeled probes. *Toll2* (A-C); *Toll8* (D-F); *trn* (G-I); *Toll6* (J-L); *Toll7* (M-N). In each row, embryos are ordered in increasing age from cellular blastoderm (CB), through gastrulation (G) or early germband extension (eGBE), to fully extended germband (GBE) stages. Embryos oriented anterior, left; dorsal, top. Black arrowheads indicate secondary stripes. Blue arrowheads indicate the cephalic furrow.

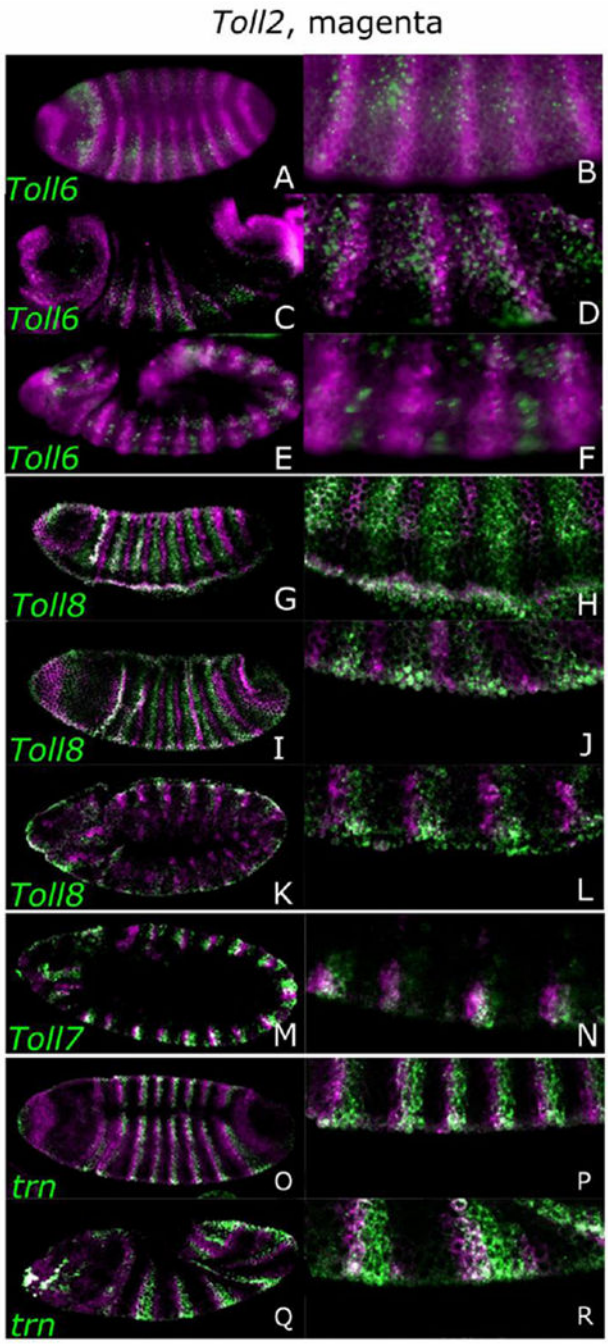

Figure 4.

sLRR relationships change during development. Expression patterns were monitored in w^{1118} embryos by *in situ* hybridization using digoxigenin- or biotin-labeled probes, as indicated. Embryos are oriented anterior, left; dorsal, top. Images to the right of each panel are a higher magnification view of the embryo to the left. *Toll2*, expression (magenta) is compared to that of a second sLRR-encoding gene (green), as indicated in the lower left corner, at different stages of embryogenesis: *Toll6* at cellular blastoderm (A, B), gastrulation (C, D) and germband extension (E, F), *Toll8* at cellular blastoderm (G, H), gastrulation (I, J)

and germband extension (K, L), *Toll7* at germband extension (M, N) and *trn* at CB (O, P), early germband extension (Q, R).

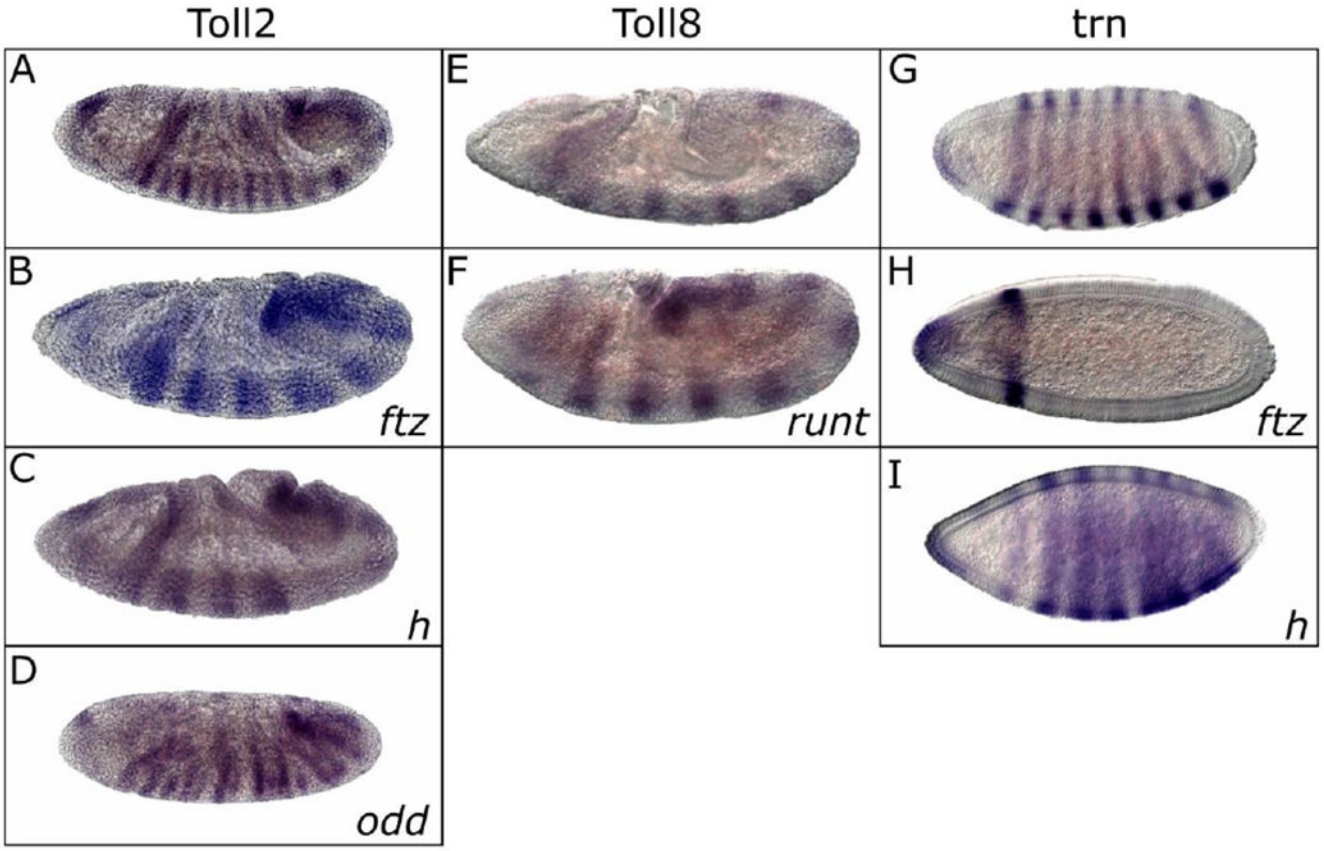

Figure 5.

A few PRG mutations alter early expression of *Toll2*, *Toll8* and *trn*. Expression patterns were monitored during the cellular blastoderm or gastrulation stage of development in PRG mutant embryos by *in situ* hybridization using digoxigenin labeled probes. sLRR-encoding gene indicated at the top of each column; PRG mutation indicated in the lower right corner of each panel. The top panel (A, E, G) in each column shows the wild type pattern for comparison. (A-D) *Toll2* in *ftz* (B), *h* (C) or *odd* (D) (E-F) *Toll8* in *run* (F) (G-I) *trn* in *ftz* (H) and *h* (I). Embryos are oriented anterior, left; dorsal, top.

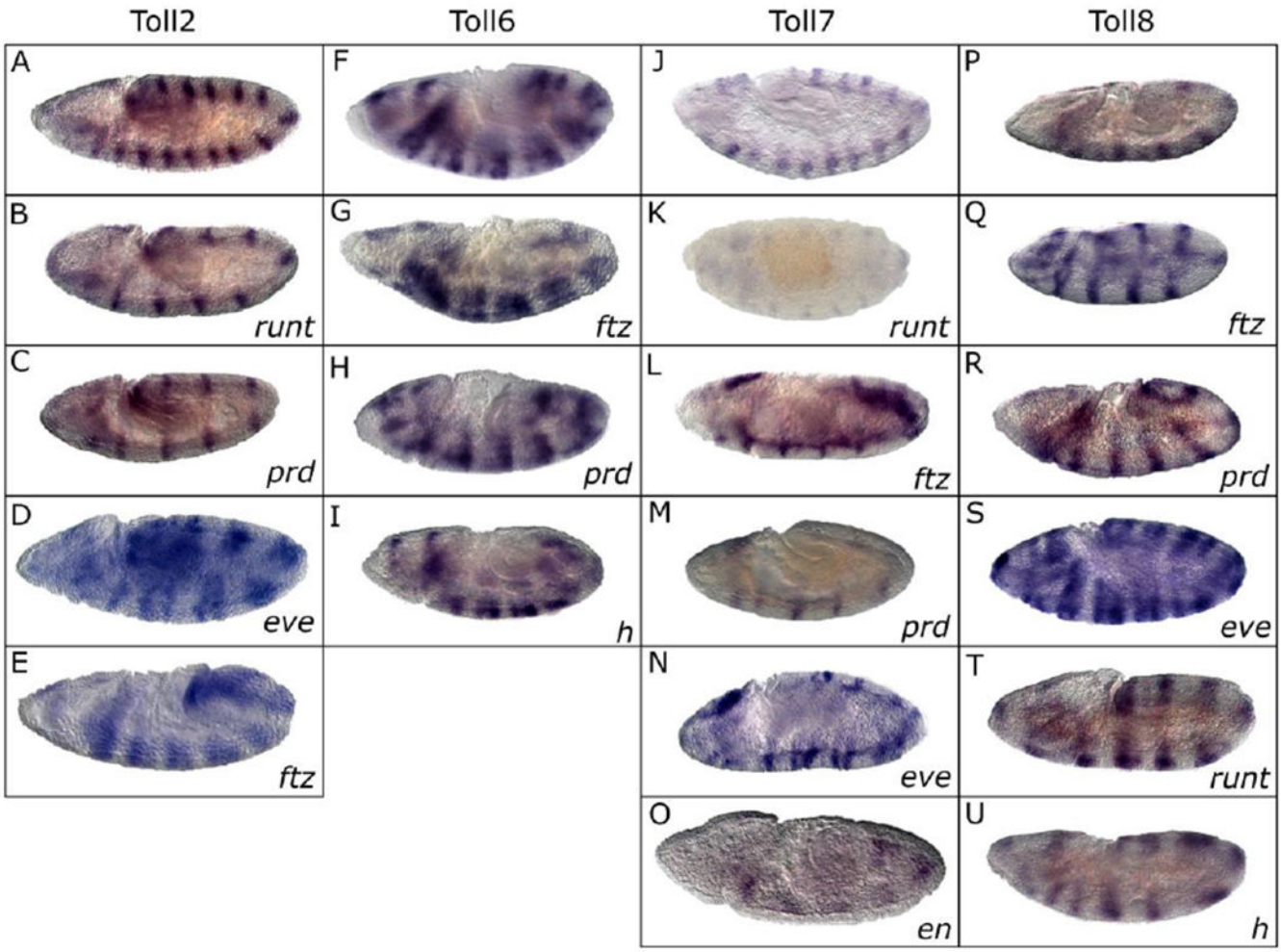

Figure 6.

Several PRG mutations alter expression of each sLRR at the germband extended stage. Expression patterns were monitored by *in situ* hybridization using digoxigenin labeled probes to the *sLRR* encoding gene listed at the top of each column on embryos homozygous for a PRG mutation as indicated in the lower right corner of each panel. The top panel in each column (A,F,J, P) shows the wild-type pattern for comparison. (A-E) *Toll2* in *run* (B), *prd* (C), *eve* (D) and *ftz* (E); (F-I) *Toll6* in *ftz* (G), *prd* (H) and *h* (I); (J-O) *Toll7* in *run* (K), *ftz* (L), *prd* (M), *eve* (N) and *en* (O); (P-U) *Toll8* in *ftz* (Q), *prd* (R), *eve* (S) *run* (T) and *h* (U). Embryos are oriented anterior, left; dorsal, top.

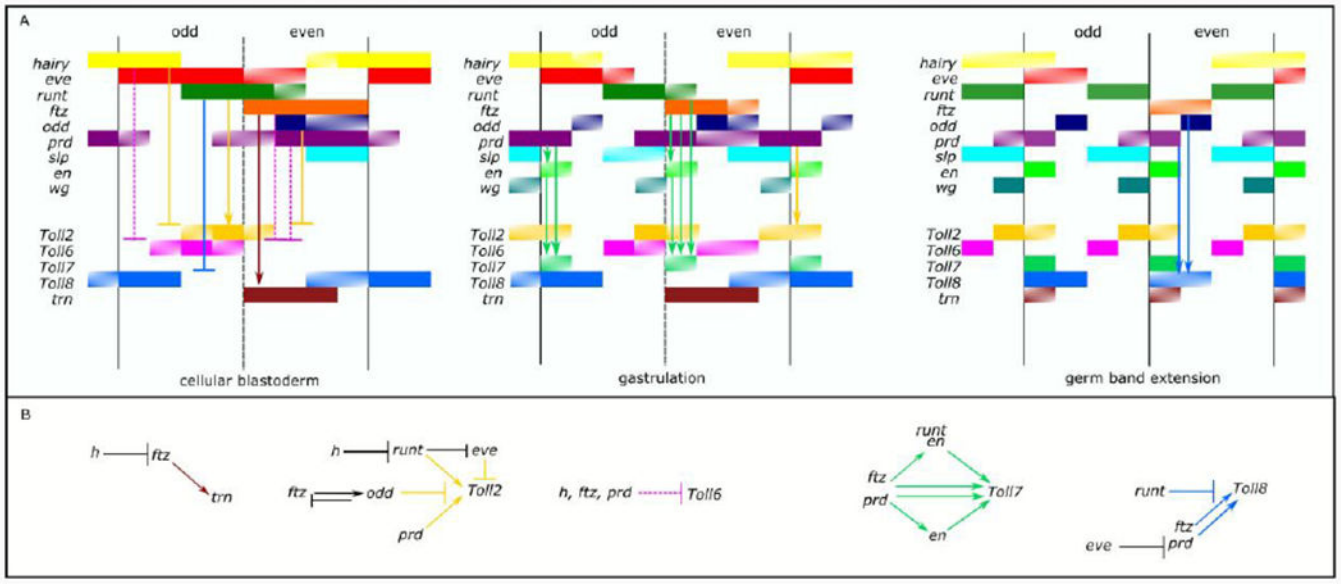

Figure 7.

The relationships between the PRGs and the sLRR genes are complex and suggest that multiple PRGs regulate each sLRR gene. A) Schematic representation of PRG and sLRR-encoding gene expression. The diagrams, using the format of Clark and Akam (Clark and Akam, 2016), represent snapshots of a continuously evolving pattern at cellular blastoderm, gastrulation and germ-band extension. Gene expression is indicated by a colored bar to the right of the gene name: *hairy* (yellow), *eve* (red), *run* (dark green), *ftz* (orange), *odd-skipped* (dark blue), *paired* (purple), *sloppy-paired* (light blue), *engrailed* (light green), *wingless* (blue-green), *Toll2* (gold), *Toll6* (pink), *Toll7* (medium green), *Toll8* (medium blue), *trn* (brown). Patterned bars represent rapidly changing expression. The black vertical lines represent the eventual position of the parasegment boundaries. Arrows or T-bars represent possible activation or repression respectively. Solid arrows or T-bars indicate possible direct regulations. Interactions affecting the primary *Toll* stripes are shown in the cellular blastoderm panel while those affecting the secondary *Toll* stripes are diagramed in the gastrulation or germband extended panel. Arrow color matches that of the *Toll* gene affected. B) Regulatory relationships between PRGs and sLRR genes. The diagrams show proposed regulatory interactions between PRGs and sLRR-encoding genes. PRGs that activate primary sLRR stripes are located above the sLRRs. PRGs that activate secondary sLRR stripes are located below the sLRRs. PRGs that repress sLRR stripes are on the same level as the sLRR. Previously described regulatory relationships are shown as black arrows or bars.