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[REVIEW]

Proximodistal Development of the Leg in *Drosophila* –Creation of New Developmental Fields by Gene Regulatory Interaction and Tissue Growth–*

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Abstract

The leg development in *Drosophila melanogaster* has been studied extensively at a molecular level during past dicades and giving many important insights into development and evolution of appendages. In this review, recent advances that greatly improve our understanding of the molecular mechanism of leg development are briefly described.

Introduction

Arthropods have legs consisting of several segments connected by flexible joints along the proximodistal (PD) axis, as the phylum name stands for. The number and arrangement of segments are basically conserved within a subphylum and similar even between subphyla, in spite of the fact that the overall morphology is sometimes highly diverse between species (Snodgrass, 1935). This makes arthropod legs have attracted a considerable attention from a viewpoint of evolutionary biology. Furthermore, legs are also a good model system in the view of developmental biology, since interplay between tissue growth and patterning, which is a fundamental process of tissue development, can be illustrated.

Generally, it is thought that a tissue is patterned, or subdivided into several distinct regions, by region-specific expression of patterning genes according to the positional information determined by morphogens (Lawrence and Struhl, 1996). However, the tissue pattering merely occurs at once but rather, new regions are added progressively with growth of the tissue. In addition, expression pattern of patterning genes is changed dramatically as development proceeds. Thus, it is important to know how pattering and tissue growth are integrated, in other words, how patterning gene expression influences tissue growth and how tissue growth regulates patterning gene expression.

The leg development has been studied extensively at a molecular level using a well-established model insect, *Drosophila melanogaster*, and a substantial amount of knowledge has been accumulated during past decades (previously reviewed in detail by Kojima, 2004 and Estella *et al.*, 2012). Especially, several recent researches clearly demonstrate that although morphogens are indeed important to set the initial state of patterning gene expression, addition of new regions occurs *de novo* through changes in the patterning gene expression by the regulatory interaction between them and the interplay between patterning gene expression and tissue growth. In this review, these new findings are briefly described.

Overview of the *Drosophila* leg development

The *Drosophila* leg has six segments, which are, the coxa, trochanter, femur, tibia, tarsus and pretarsus, in a proximal to distal direction, as with other insect species (Fig. 1, lower panel). These segments are called "true" segments and their number and arrangement are generally conserved in almost all insect species. Unlike the true segments, the tarsus is often subdivided further into several tarsal segments. The number of tarsal segments is diverse from species to species, with the number generally ranging from one to five (Snodgrass, 1935). The *Drosophila* leg has five tarsal segments, which are designated as tarsal segment 1 (ta1) to tarsal segment 5 (ta5) (Fig. 1, lower panel).

Drosophila is a complete holometabolous insect and adult structures, such as antennae, wings and legs, are derived from primordial tissues called imaginal discs (eye-antennal discs, wing discs and leg discs, respectively). Cells for an imaginal disc are determined in the epithelium of the embryo during embryogenesis and invaginate from the

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epidermis to form a sac-like structure composed of a sheet of mono-layered epithelial cells. Imaginal discs proliferate within a larva during three larval stages without contributing to larval structures and terminally differentiate into adult structures during the pupal stage (Cohen, 1993; Fristrom and Fristrom, 1993).

By the genetic analysis, the leg disc is thought to consist of only about 10–30 cells at its birth. Subsequently, cells in the leg disc proliferate to contain over 10,000 cells by the late third instar stage (Cohen, 1993). Morphologically, two types of cells are recognized in the leg disc: large, flat and squamous cells of the peripodial membrane that contributes to the adult body wall and thick, columnar cells of the disc epithelium that differentiate into the leg proper (Fig. 1, upper panel; Fristrom and Fristrom, 1993). The disc epithelium is morphologically a "flat" sheet by the beginning of the third instar stage, but subsequently folded over concentrically several times and becomes no more "flat" in its appearance, although it remains to be a mono-layered sheet. By the late third instar stage, three major foldings and folds are recognized (in this review, the "fold" refers to a protruding-, or mountain-, part of the disc). The pretarsus and ta5–ta3 come from the most central fold, ta2–distal part of the tibia from the second one (mid fold), the proximal tibia and the femur from the third fold (peripheral fold), and the more proximal parts are derived from the more peripheral parts (Fig. 1; Kojima *et al*., 2000). After the puparium formation, the disc epithelium begins to telescope out from its center and elongates by cell rearrangement during the pupal stage to form the adult leg (Fig. 1; Condic *et al*., 1991; Fristrom and Fristrom, 1993). Since distal segments of the adult leg are derived from central portions of the leg disc and more proximal segments from more peripheral portions, each leg segment is determined concentrically by the concentric expression of patterning genes on the leg disc. Specification of regions corresponding to each segment is completed by the late third instar stage and each leg segment becomes visible morphologically by the puparium formation.

Determination of precursor cells for the adult leg during embryogenesis

It has been suggested that arthropod legs are primarily subdivided along the PD axis to the coxopodite, the proximal portion that is a simple outgrowth of the body wall, and the telopodite, the distal portion that are movable (Snodgrass, 1935; reviewed by Boxshall, 2004). In insects, the coxopodite includes the coxa and the telopodite corresponds to all the more distal leg segments. Studies on *Drosophila* legs and appendages in other arthropod species indicate that the telopodite is specified by a homeobox gene, *Distal-less* (*Dll*), and proper formation of the coxopodite requires two homeobox genes, *homothorax* (*hth*) and *extradenticle* (*exd*), and two genes encoding zinc-finger-containing transcription factors, *escargot* (*esg*) and *teashsirt* (*tsh*) (Cohen *et al*., 1989; Cohen and Jürgens, 1989; Cohen, 1990; Fasano, *et al.*, 1991; Whiteley *et al*., 1992; Rauskolb *et al*., 1993, 1995; González-Crespo and Morata, 1995, 1996; Gorfinkiel *et al*., 1997; Goto and Hayashi, 1997, 1999; Panganiban *et al*., 1997; Reickhof *et al*., 1997; Campbell and Tomlinson, 1998; González-Crespo *et al*., 1998; Azpiazu and Morata, 2002; reviewed by Pnaganiban and Rubenstein, 2002).

The first sign of the leg development is the expression of *Dll* in ventrolateral regions at each side of thoracic segments at the mid embryonic stage, starge 11 (Fig. 2, upper-left panel; Cohen, 1990; Vachon *et al*., 1992; González-Crespo *et al*., 1998). Genetic evidence indicated that at this stage, *Dll* expression domain contains precursor cells for the wing or haltere disc and for the larval sensory organ called the Keilin's organ, in addition to those for the leg disc, and thus, the initial *Dll* domain is called the limb primordium (Wieschaus and Gehring, 1976; Cohen *et al*. 1993; Kubota *et al*. 2000). By stage 14, cells dorsally situated in the initial *Dll* domain migrate dorsally, cease *Dll* expression and eventually form the wing or haltere disc (Cohen *et al*., 1993; Fuse *et al*., 1996; Kim *et al*., 1996). Before the appearance of the initial *Dll* expression, *hth* is expressed in almost all cells in the thoracic segments (González-Crespo *et al*., 1998). After that, the central region of *Dll* domain gradually ceases *hth* expression, leaving cells with *Dll* but no *hth* expression, by stage 14 (Kubota *et al*., 2003; González-Cresop *et al*., 1998). Since *Dll* and *hth* specify the telopodite and coxopodite, respectively, it was thought previously that this central, *Dll*-only domain corresponds to the telopodite and surrounding *hth*-expressing cells, which also express *esg*, to the coxopodite. This view was revised by recent findings, however, from analyses of regulatory regions of *Dll* and the cell lineage tracing experiments (Estella *et al*., 2008; McKey *et al*., 2009; Galindo, *et al*., 2011).

Extensive studies on regulatory regions of *Dll* has revealed several enhancers existing upstream and downstream of its coding region, which drive leg-related *Dll* expression (Vachon *et al*., 1992; Estella *et al*., 2008; Galindo *et al*., 2011). These enhancers are activated differentially and sequentially (Fig. 2). First to be activated is an enhancer called *Dll304* (Fig. 2, left panel), which regulates *Dll* expression in the limb primordia (Vachon *et al*., 1992). This enhancer is controlled by a combination of Wingless (Wg), Decapentaplegic (Dpp) and epidermal growth factor receptor (EGFR) signalings. Wg is a Wnt family member and Dpp is a TGF-beta family member (for review of these signalins, see Barolo and Posakony 2002). At the mid embryonic stage, *Dll304* is activated by Wg but repressed dorsally by Dpp signaling and ventrally by EGFR signaling (Cohen, 1990; Goto and Hayashi, 1997). Thus, *Dll304* activity is restricted to the ventrolateral region. In addition, *Hox* genes determining identities of abdominal segments, *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*), repress *Dll304* and thus, its activity is restricted to thoracic segments (Vachon *et al*., 1992; Castelli-Gair and Akam, 1995; Gebelein *et al*., 2002, 2004). *Dll304* activity is transient and with its disappearance, an enhancer called *DllLP* (Leg Primordium) is activated in the precursor cells for the Keilin's organ and leg disc through

activation by all of the Wg, Dpp and EGFR signalings (Fig. 2, middle pannel; Galindo *et al*., 2011). Since *DllLP* is not activated in precursor cells for the wing and haltere discs, they lose *Dll* expression with the disappearance of *Dll304* activity. Shortly after the *DllLP* activation, two other enhancers called *DllLT* (Leg Trigger) and *DKO* (Distal-less Keilin's Organ) becomes active in cells with *DllLP* activity (Fig. 2, right panel; Estella *et al*., 2008; Galindo *et al.*, 2011). *DKO* is activated in the central cells destined to the Keilin's organ and *DllLT* in cells surrounding it (Mckay *et al.*, 2009). *DllLT* is activated by positive inputs from Dll protein itself and high levels of Wg and Dpp signalings (Estella *et al*., 2008). Although *DllLP* activity declines during first to second instar stages, *DllLT* activity persists and with autoregulatory modules called *DllM* (Maintenane) and *DllLL* (Leg Late), regulates all aspects of *Dll* expression during all remaining stages (Estella *et al*., 2008; Galindo *et al*., 2011).

Cell lineage analysis of cells with *Dll304*, *DllLT* or *DKO* activity showed a fine-scale fate map of the limb primordium (Fig. 2; McKay *et al*., 2009). Cells in which *Dll304* is activated at mid embryonic stage (stage 11) actually contributed to all descendants of the limb primordium, that is, the wing or haltere disc, the Keilin's organ as well as the proximal and distal portions of the leg disc (Fig. 2, left panel). *DKO* is activated in the *Dll*-only domain and these cells indeed differentiated into the Keilin's organ (Fig. 2, right panel), consistent with the finding from the detailed analysis of the Keilin's organ development (Bolinger and Boekhoff-Falk, 2005). Most strikingly, *DllLT* is first activated in cells surrounding the *Dll*-only domain and cells with *DllLT* activity contributed to the entire telopodite region of the leg disc (Fig. 2, right panel). Initially, these cells also express *hth* and *esg* but lose their expression later and proliferate to form the telopodite region without their expression. In addition, the coxopodite region of the leg disc was demonstrated to derive from cells in the vicinity of the *Dll*-only domain but without *DllLT* activity (only expressing *hth* and *esg* but not *Dll*) (Fig. 2, right panel; McKay *et al*., 2009).

These results provide an important implication that although *Dll* is continuously expressed in precursor cells for the telopodite during embryogenesis, enhancers regulating its expression change from moment to moment. In other words, the precursor cells for the telopodite are determined through several changes in their state.

Subdivision of the telopodite along the PD axis during larval stages

As described above, the leg disc consists of only two regions at the initial stage of the leg disc determination: the *Dll*-expressing telopodite and the *hth*-expressing coxopodite. After that, however, a new region expressing *dachshund* (*dac*) but neither *Dll* nor *hth* emerges between *Dll* and *hth* domains by the beginning of the third instar stage (Abu-Shaar and Mann, 1998). *dac* encodes a transcription factor required for the formation of the medial portion of the leg (Mardon *et al*., 1994). Subsequently, *Dll* and *dac* expression becomes overlapping with each other and an additional new region expressing both *Dll* and *dac* is formed (Lecuit and Cohen, 1997; Abu-Shaar and Mann, 1998). Consequently, the telopodite region of the leg disc is now subdivided into *dac*-only domain, *Dll* + *dac* domain and *Dll*-only domain, in a proximal (peripheral) to distal (central) direction (Fig. 3).

To achieve this subdivision, Wg and Dpp have been thought to act cooperatively as morphogens (Diaz-Benjumea *et al*., 1994; Lecuit and Cohen, 1997). Since Wg and Dpp is expressed in the ventral and dorsal sides of the leg disc, respectively (Fig. 3, left panel), it seems that the central portion, near the contact point between their expression domains, receives high levels of Wg + Dpp signaling and progressively more peripheral regions are exposed to lower levels of Wg + Dpp signaling. It has been thought that high levels of Wg + Dpp signaling induce *Dll* expression but repress *dac* expression and intermediate levels activate *dac* but not *Dll* expression, whereas lower levels activate neither gene and allows expression of the coxopodite genes, such as *hth* and *esg* (Lecuit and Cohen, 1997; Abu-Shaar and Mann, 1998; González-Crespo *et al*., 1998; Wu and Cohen, 1999). Recently, however, this model has been revised remarkably.

The analysis on an enhancer called *dacRE* (Ring Enhancer), which regulates *dac* expression in the leg disc, indicated that although high levels of Wg + Dpp signaling activity repress *dac* expression as has been thought, *dac* expression is not activated by intermediate levels of Wg + Dpp signaling. Rather, *Dll* activates *dac* expression by directly binding to *dacRE*. In addition, *dacRE* is repressed also by transcription factors expressed more distally than *dac*, such as *BarH1* and *BarH2* (collectively referred to as *Bar* hereafter; see below) (Giorgianni and Mann 2011). According to these results and those from the analyses of enhancers for *Dll* expression, the following scenario is proposed (Fig. 3; Giorgianni and Mann, 2011; Estella *et al*., 2012):

By the early second instar stage, *Dll* is expressed through the activation of *DllLT* by high levels of Wg + Dpp signaling, which counteracts the activation of *dacRE* by *Dll* and remains *dac* unexpressed (Fig. 3, left panel). Towards the late second instar stage, growth of the disc drives some *Dll*-expressing cells outside the region with high levels of Wg + Dpp signaling. This leads to the activation of *dacRE* by *Dll* and the initiation of *dac* expression. Since *dac* has a repressive activity on *Dll* expression (Abu-Shaar and Mann, 1998), these cells lose *Dll* expression (Fig. 3, middle panel). After the fading of *Dll* expression, dac expression may be maintained by the autoregulation and thus, the *dac*-only domain is formed, maintained and enlarged with the growth of the disc (Fig. 3, right panel). At the early third instar onwards, further growth of the disc makes more cells move out of the central region. In these cells, levels of Wg + Dpp signaling are not high enough and *DllLT* loses its activity. Instead, however, autoregulatory elements, *DllM* and *DllLL*, maintain *Dll* expression in these cells. Since *Dll* activates *dac* expression through the activation

of *dacRE*, this leads to the stable formation of the *Dll* + *dac* domain (Fig. 3 right panel). The distal extent of the *Dll* + *dac* domain is determined through the repression of *dacRE* by distally expressed transcription factors, such as *Bar* (Fig. 3, right panel).

As described above, medial regions in the telopodite is formed mainly by the regulatory interaction between patterning genes and tissue growth but not by intermediate levels of morphogen signaling. This means that a major role of morphogens is to set the initial state of the regulatory interaction cascade and once the cascade is triggered, new regions are formed *de novo* by interplay between patterning genes themselves and tissue growth.

Formation and determination of five tarsal segments and pretarsus

During the third instar stage, the *Dll*-only domain is further subdivided into five tarsal segments and the pretarsus. Considerable numbers of patterning genes are known to be expressed within this domain in a region-specific manner (Fig. 4B). At the late third instar stage, the future pretarsal region expresses *aristaless* (*al*) and *clawless* (*cll*; also known as *C15*), encoding homeodomain transcription factors, as well as *Lim1*, encoding a LIM-homeodomain transcription factor (Campbell *et al*., 1993; Schneitz *et al*., 1993; Lilly *et al*., 1999; Pueyo *et al*., 2000; Tsuji *et al*., 2000). Just proximal to the pretarsal region, another genes encoding homeodomain transcription factors, *Bar*, is expressed strongly in the future ta5 region and weakly in the ta4 region (Kojima *et al*., 1991; Higashijima *et al*., 1992; Kojima *et al*., 2000). The ta5 region also expresses *nubbin* (*nub*), which encodes a POU-homeodomain transcription factor, while the ta4 region expresses *apterous* (*ap*), a LIM-homeodomain transcription factor (Billin *et al*., 1991; Cohen *et al*., 1992; Rauskolb and Irvine, 1999; Kojima *et al*., 2000). *trachealess* (*trh*), encoding a bHLH-PAS type transcription factor, is expressed in both pretarsal and ta5 regions (Isaac and Andrew, 1996; Wilk *et al*., 1996; Tajiri *et al*., 2007). Furthermore, *dac* is expressed strongly in the ta1 region and weakly in the ta2 region (Mardon *et al*., 1994; Lecuit and Cohen, 1997; Natori *et al*., 2012). In addition, *bric à brac 1* (*bab1*) and *bric à brac 2* (*bab2*), which encodes BTB/POZ domain containing transcription factors, are expressed from the distal region of ta1 to the ta4 region with their expression stronger towards ta2 and ta3 regions (Godt *et al*., 1993; Couderc *et al*., 2002). The process of tarsal segment and pretarsus formation has been illustrated in great detail in relation to the regulation and function of these patterning genes.

Morphogen signaling for the tarsal and pretarsal development

In the process of tarsal and pretarsal development, EGFR signaling is acting as a morphogen regulating patterning gene expression (Fig. 4A). Ligands for EGFR are produced by cells at the most central region of the leg disc according to high levels of Wg + Dpp signalings. They emanate from the most central region and set a concentric gradient of EGFR signaling activity in a central (distal) to peripheral (proximal) direction (Campbell, 2002; Galindo *et al*., 2002; Galindo *et al*., 2005).

Precise establishment of the pretarsal region

At the early third instar stage, when *al*, *cll* and *Bar* expression initiates, there is an overlap between their expression domains. But later, by the mid third instar stage, the overlap is resolved and a sharp boundary is formed between them (Kojima *et al*., 2000). During this refinement process, *al* and *cll* repress *Bar* expression cooperatively in the pretarsal region, whereas their expression is repressed by *Bar* in the ta5 region (Fig. 4B; Campbell, 2005; Kojima *et al*., 2005). The expression of *Lim1*, which is required for the sufficient levels of *al* and *cll* expression, is activated by the cooperative function of *al* and *cll*, while it is repressed by *Bar* (Tsuji *et al*., 2000). The expression of *Bar* in the ta5 is strengthened by autoactivation (Kojima *et al*., 2000). In addition, *trh* potentiates the repressive activity of *al* and *cll* on *Bar* expression in the pretarsal region, while it also facilitates the autoactivation of *Bar* expression in the ta5 region (Tajiri *et al*., 2007). In this manner, the pretarsal region is precisely determined through the induction of patterning genes in an approximate region by the morphogen signaling, followed by the refinement of their expression domains through regulatory interaction between them (Fig. 4B). Interestingly, it was reported that *al* expression in the pretarsal region and *Bar* expression in the ta5 region is unchanged by the loss of EGFR signaling after the mid third instar stage (Campbell, 2002). This suggests that their expression no more requires the input from the morphogen signaling. Therefore, the morphogen only determines initial, rough expression domains of patterning genes and once rough domains are set, the regulatory interaction between them establishes and maintains their precise expression domains.

Formation of five tarsal segments

After the puparium formation, the leg disc is partially elongated and each tarsal segment becomes morphologically visible (Fig. 1 middle panel). These five segments are prefigured by the expression of several patterning genes by the late third instar stage (Fig. 4B). As described below, however, the expression of the patterning genes changes dramatically during the third instar stage (Fig. 5). For example, *Bar* is strongly expressed in the ta5 region and weakly in the ta4 region, while *dac* is expressed weakly in the ta2 region and strongly in the ta1 region. Neither gene is expressed in the ta3 region (Mardon *et al*., 1994; Lecuit and Cohen, 1997; Kojima *et al*., 2000; Natori *et al*., 2012). At the early third instar stage, however, their expression is homogeneous within each domain and abuts each other (Fig. 5, upper-left panel). Then, a gap region devoid of both *Bar* and *dac* expression emerges between their domains (Fig. 5, upper-middle panel). After the

appearance of the gap, the central folding, or the central fold formation, occurs just outside the *Bar* domain (Kojima *et al*., 2000; Natori *et al*., 2012). As the central folding deepens, or the central and second folds (*i.e*. the tarsal region) grow, the gap region continues to expand into the proximal part of the central fold due to the progressive repression of *Bar* expression (Fig. 5, upper-right panel). Around the mid third instar stage, *Bar* expression in the ta5 region is strengthened (Fig. 5, lower-left panel; Kojima *et al*., 2000) and at very late third instar stage, weak *dac* expression appears in the ta2 region (Fig. 5, lower-right panel; Natori *et al*., 2012). Eventually, the tarsal region is subdivided into five regions corresponding to each tarsal segment. Therefore, at most only two regions exist in the tarsal region initially and subdivision into five segments occurs *de novo* afterward. The complex but ingenious regulatory interaction between

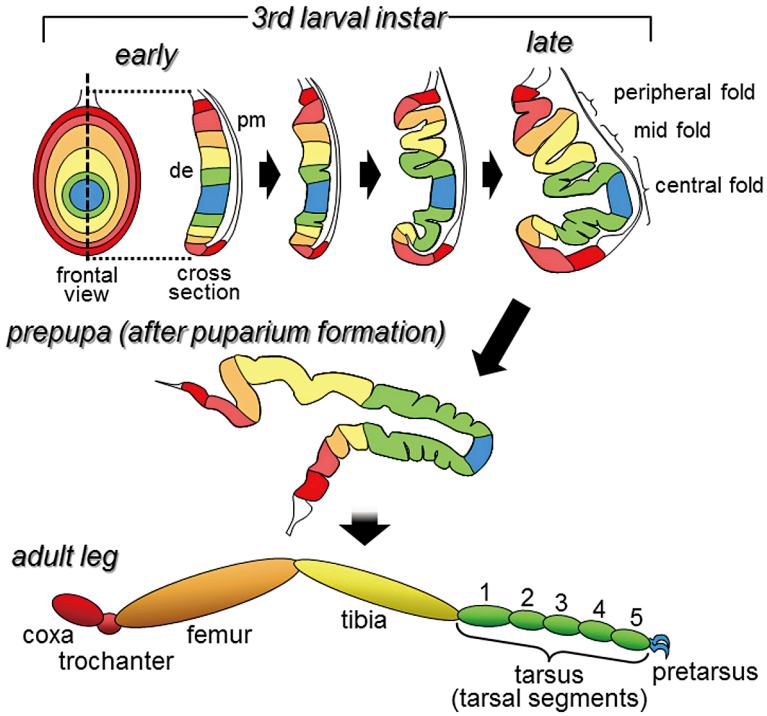

Fig. 1 Schematic representation of the adult leg and leg disc development. Dorsal is to the top. Distal is towards right except for the left-most figure in the top panel. Regional relationships are represented by colors.
de: disc epithelium, pm: periodial membrane.

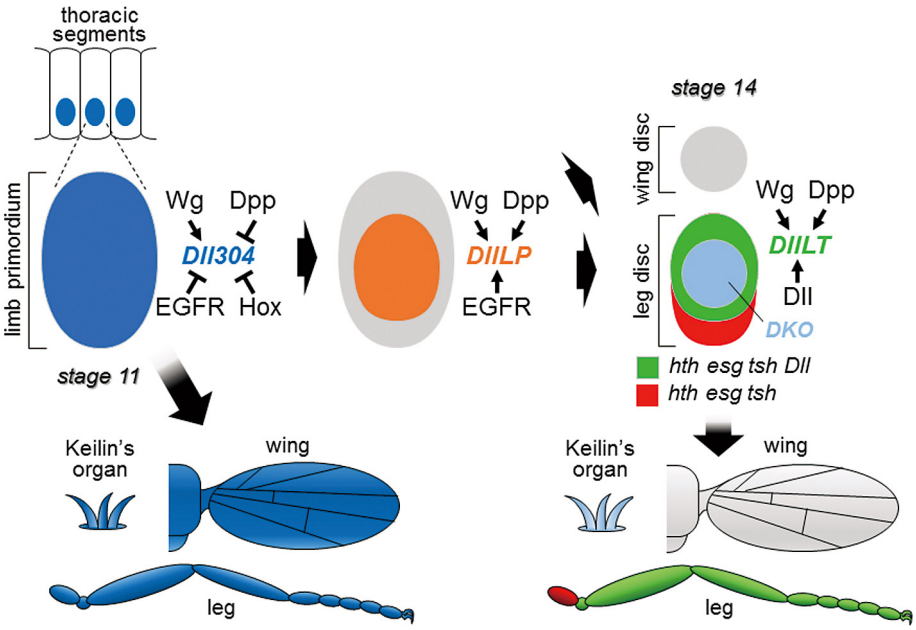

Fig. 2 Formation of the leg disc during embryogenesis. Upper panel represents changes in the usage of *Dll* enhancers and their regulation. Thin arrows and T-bars indicate activation and repression, respectively. Genes expressed in the telopodaite and coxopodite region are shown below the leg disc. Progenitors of each *Dll*-expressing cells are shown in lower panel. Lineage relationships are indicated by colors. Dorsal is to the top except for the Keilin's organs and wings.

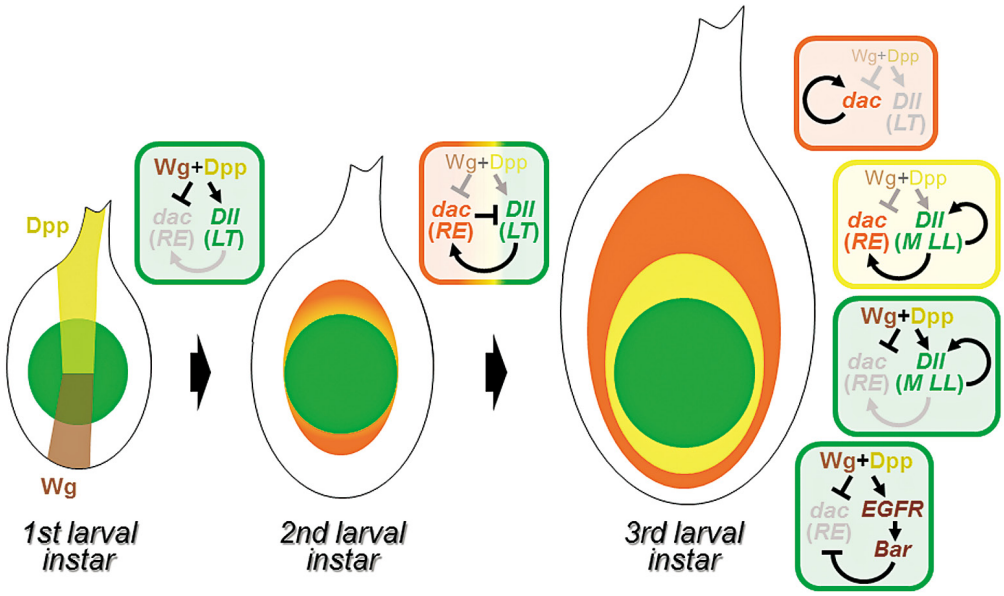

Fig. 3 Telopodite subdivision along the PD axis during larval stages. Arrows and T-bars indicate positive and negative regulation, respectively. Gray indicates inactive state. Size and color depth of letters represent a combined activity of Wg and Dpp signalings. Dorsal is to the top.

patterning genes and growth of the tarsal region again play important roles in this process (Figs. 4B and 5).

In addition to the patterning genes described above, genes transiently expressed in the medial tarsal region, such as *rotund* (*rn*), *spineless* (*ss*) and *tarsal-less* (*tal*; also known as *polished rice*, *pri*) play key roles. *rn* and *ss* encode a zinc-finger transcription factor and a bHLH-PAS transcription factor, respectively, while *tal* encodes four short peptides known to act cell-non-autonomously (Duncan *et al*., 1998; St Pierre *et al*., 2002; Kozu *et al*., 2006; Galindo *et al*., 2007; Kondo *et al*., 2007; Pueyo and Couso, 2008). *Bar* activates *tal* expression cell-non-autonomously, whereas attenuates it cell-autonomously (Pueyo and Couso, 2008). Through this regulation, *tal* expression starts weakly in nearly the same domain as *Bar* at early stages but becomes strongly expressed around the *Bar* domain later (Fig. 5; Pueyo and Couso, 2008; Natori *et al*., 2012). Since *ss* expression is activated by *tal* cell-non-autonomously, *ss* is expressed in the same timing as, but in a larger domain than, *tal* (Fig. 5; Pueyo and Couso, 2008; Natori *et al*., 2012). *rn* expression is activated by *tal* directly and also indirectly through the activation of *ss* expression (Pueyo and Couso, 2008; Natori *et al*., 2012). By the late third instar stage, *ss* and *rn* expression fades with the cessation of *tal* expression (Fig. 5, lower panels; Duncan *et al*., 1998; St Pierre *et al*., 2002; Pueyo and Couso, 2008).

With the initiation of its expression, *Bar* represses *dac* expression to limit the distal extent of the dac domain (Kojima *et al*., 2000; Giorgianni and Mann, 2011). Conversely, the proximal extent of the *Bar* domain is determined by the concerted action of *dac* and *tal* (Pueyo and Couso, 2008). Thus, *Bar* and *dac* domains come to abut each other (Fig 5, upper-left panel). Since *tal* and *ss* are already expressed at this early stage (Pueyo and Couso, 2008; Natori *et al*., 2012), *rn* is ready to be expressed. However, *rn* expression is blocked by *nub*, which is expressed by EGFR signaling in a broad domain that spans all the tarsal and pretarsal regions (Fig. 5, upper-left panel; Natori *et al*., 2012). Then, the growth of the tarsal region possively drives some cells outside the levels of EGFR signaling required for the activation of *nub* expression, leading to an emersion of a region not expressing *nub*. This results in the initiation of *rn* expression in the region devoid of *nub* expression (Fig. 5, upper-middle panel; Natori *et al*., 2012). Afterwards, by the late third instar stage, the region without *nub* expression expands continuously with the growth of the tarsal and pretarsal regions, restricting *nub* expression distally to the ta5 region and proximally to the distal tip of the tibia (Figs. 4B and 5, lower panel; Rauskolb and Irvine, 1999; Natori *et al*., 2012). In association with this, *rn* expression domain also expands. Since *rn* has an activity of repressing *Bar*, the expansion of the *rn* domain into the proximal part of the central fold results in the repression of *Bar* expression there, leading to the formation of the ta3 region (Fig. 5, upper-right panel; Natori *et al*., 2012).

From the mid third instar stage onward, the *Bar* domain starts overlapping with the expanding *rn* expression. This is due to the initiation of *ap* expression in cells at the proximal part of the *Bar* domain (Fig. 5, lower-left panel). *ap* expression is activated by the concerted action of the cell-autonomous function of *Bar* and cell-non-autonomous function of *tal*, while repressed in the distal region of the *Bar* domain by Notch signaling (Campbell, 2005; Pueyo and Couso, 2008; Natori *et al*., 2012). *ap* renders *Bar* refractory to the repression by *rn*, so that *Bar* expression is no more repressed. This leads to the formation of the ta4 region, in which weak *Bar* expression and *ap* expression is observed at the late third instar stage (Fig. 5, lower panels; Natori *et al*., 2012).

The strong expression of *Bar* in the ta5 region at the late third instar stage is regulated by a dedicated enhancer called

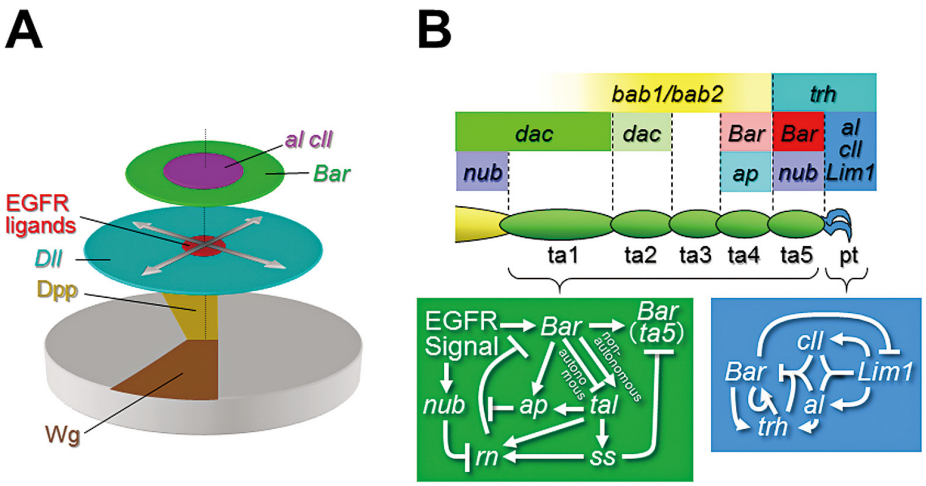

Fig. 4 Subdivision of the *Dll*-only domain into the pretarsus and five tarsal segments during the third instar stage. A. From the onset of the third instar stage, ligands for EGFR signalins are produced and secreted at the most centar of the disc by Wg and Dpp signalings, set a center to peripheral gradient of EGFR signaling activity, and induce the region-specific expression of patterning genes, such as al, *cll*, *Bar*. B. Relationship between adult leg segments and expression domains of several patterning genes at the late third instar stage (upper panel) and regulatory interaction between them (lower panel). Arrows and T-bars indicate positive and negative regulation, respectively. Distal is to the right.

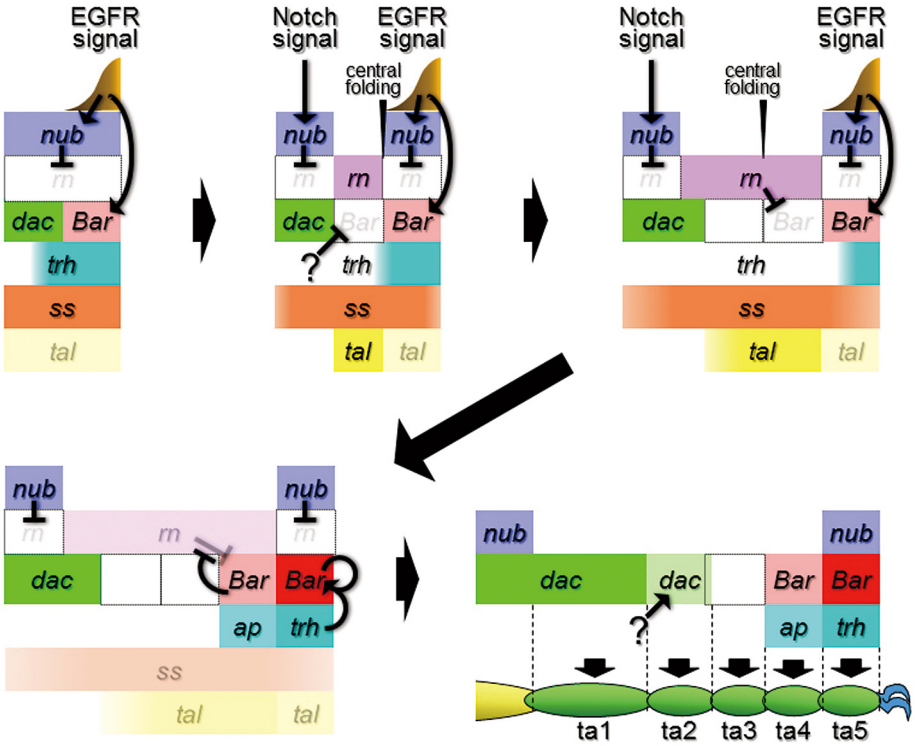

Fig. 5 Expression changes in and some key regulatory interactions betwee patterning genes during formation of five tarsal segments during the third instar stage. Thin arrows and T-bars indicate positive and negative interaction, respectively. Depth of colors represents expression levels and light gray indictes a repressed state. Distal to the right.

ta5-enhancer (Kojima *et al*., 2000; Kozu *et al*., 2006). Although ta5-enhancer is activated by the function of *Bar* itself and *trh* (Tajiri *et al*., 2007), and they are already expressed at the early third instar stage, the activity of ta5-enhancer is repressed directly by *ss* at this early stages. The cessation of *ss* expression by the late third instar stage releases the activity of ta5-enhancer. This leads to the strong expression of *Bar* in the distal part of its domain and thus, the specification of the ta5 region (Fig. 5, lower panels; Kozu *et al*., 2006).

It has been suggested that the ta2 region is derived from cells in the initial gap between *Bar* and *dac* domains before the central fold formation and cells in the most proximal part of the early central fold (Kojima *et al*., 2000; Natori *et al*., 2012). The initial gap appears to be formed mainly by the cessation of *Bar* expression. Although *rn* is already expressed in the initial gap, however, *rn* is dispensable for its formation (Natori *et al*., 2012). At present, the mechanism of *Bar* repression here is unknown as well as the mechanism of weak *dac* expression in

the ta2 region at the late third instar stage (Fig. 5, upper-middle and lower-right panels).

Other than the patterning genes described above, *lines* (*lin*) and *odd-skipped* family genes, *odd-skipped* (*odd*), *brother of odd with entrails limited* (*bowl*), *sister of odd and bowl* (*sob*) and *dramstick* (*drm*), are also implicated in the subdivision and specification of the tarsal region (de Celis Ibeas and Bray 2003; Hao *et al*., 2003; Greenberg and Hatini, 2009). *odd* family genes encode zinc-finger transcription factors and *lin* encodes a protein that degrades Bowl protein (Green *et al*., 2002; Hatini *et al*., 2005; Greenberg and Hatini, 2009; Del Signore *et al*., 2012). The activity of Lin is in turn inhibited by Odd, Sob and Drm. Lin protein gradually accumulated in the tarsal region by the late third instar stage and represses strong *dac* expression, while promote *ap* and weak *Bar* expression, in the medial tarsal region through the degradation of Bowl protein (Greenberg and Hatini, 2009). A detailed investigation of the regulatory and functional relationship between this system and those described above will provide a more perfect picture of tarsal development.

As described above, the dynamic regulatory interaction between patterning genes and growth of the tarsal region play key roles in the subdivision of the tarsal region into each tarsal segment. This also has an important implication for leg or appendage evolution and diversity. Even if functions and regulatory relationship between patterning genes are unchanged, small alterations in the timing of patterning gene expression and in the growth rate of the tissue could lead to changes in the number and proportion of leg segments. For example, it is easily imagined that the number and proportion of each tarsal segment can be altered by changes in the timing of the initiation of *ap* expression and the disappearance of *ss* and *nub* expression. Especially, the alteration in the timing of *nub* disappearance might occur by changes in the tissue growth rate and/or the sensitivity of *nub* to EGFR signaling, that is, a balance between tissue growth and EGFR signaling strength. This might be one of mechanisms underlying the diversity in tarsal segmentation among different insect species.

Concluding remarks

As described in this review, the remarkable progress has been made recently in understanding the molecular mechanism of leg development in *Drosophila*. One thing becoming clear is the importance of temporally dynamic changes in patterning gene expression by regulatory interaction between them and their relation to growth of the tissue. In addition to its importance in developmental biology, it also gives an important insight into the evolution of legs or appendages. Close investigations of temporal changes in expression and function of patterning genes and their relationship with tissue growth in other insect or arthropod species based on knowledge in *Drosophila* described here will greatly help us understand the evolutionary mechanism of divergence and evolution in legs or appendages. Recent advances in techniques of gene disruption or genome editing, such as RNAi, TALEN and CRISPER (reviewed by Wei *et al*., 2013), allows us to analyze not only gene expression but also gene function in non-model organisms. So, we have a good opportunity for tackling this issue now.

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