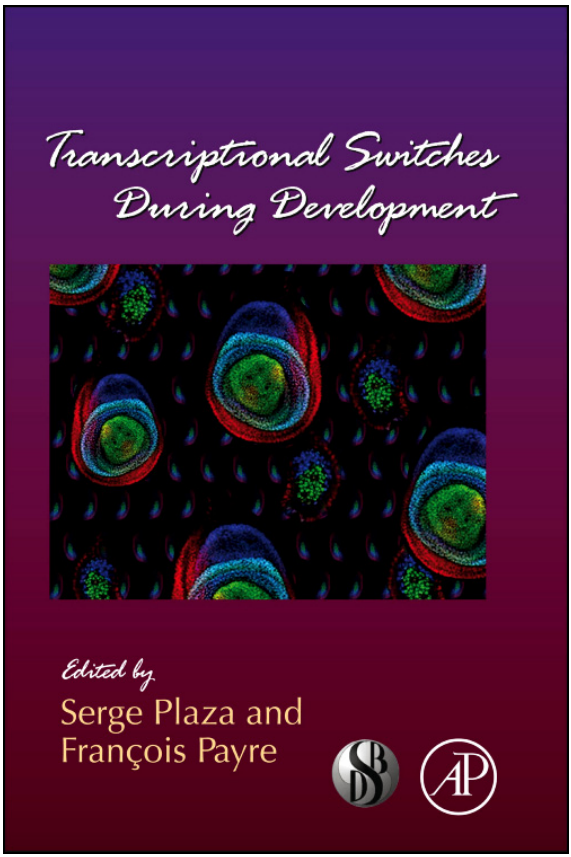

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CHAPTER SEVEN

A Dynamic Network of Morphogens and Transcription Factors Patterns the Fly Leg

Carlos Estella,*,[1] Roumen Voutev,* *and* Richard S. Mann*

Contents

Abstract

Animal appendages require a proximodistal (PD) axis, which forms orthogonally from the two main body axes, anteroposterior and dorsoventral. In this review, we discuss recent advances that begin to provide insights into the molecular mechanisms controlling PD axis formation in the *Drosophila* leg. In this case, two morphogens, Wingless (Wg) and Decapentaplegic (Dpp), initiate a genetic

* Department of Biochemistry and Molecular Biophysics, Columbia University, New York, New York, USA
[1] Current Address: Fundación Instituto Valenciano de Infertilidad (FIVI), Valencia University, and Instituto Universitario IVI/INCLIVA, Valencia, Spain

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cascade that, together with growth of the leg imaginal disc, establishes the PD axis. The analysis of *cis*-regulatory modules (CRMs) that control the expression of genes at different positions along the PD axis has been particularly valuable in dissecting this complex process. From these experiments, it appears that only one concentration of Wg and Dpp are required to initiate PD axis formation by inducing the expression of *Distal-less* (*Dll*), a homeodomain-encoding gene that is required for leg development. Once *Dll* is turned on, it activates the medially expressed gene *dachshund* (*dac*). Cross-regulation between *Dll* and *dac*, together with cell proliferation in the growing leg imaginal disc, results in the formation of a rudimentary PD axis. Wg and Dpp also initiate the expression of ligands for the EGFR pathway, which in turn induces the expression of a series of target genes that pattern the distal-most portion of the leg.

1. Introduction

Animal appendages are external projections from the body wall that are used for very diverse functions including locomotion, grooming, and feeding. In the thorax of diptera, such as the fruit fly *Drosophila melanogaster*, there are dorsal appendages required for flight—a pair of wings in the second thoracic (T2) segment and a pair of halteres in T3—and three pairs of legs used for walking and grooming. The fly leg, the subject of this review, is composed of 10 morphologically unique segments: coxa, trochanter, femur, tibia, tarsal segments 1–5, and the claw. Together, these segments comprise the proximodistal (PD) axis, in which the proximal coxa is closest to the body and the claw is furthest from the body (Fig. 7.1).

Unlike the two other primary body axes (anteroposterior, AP; dorsoventral, DV), for each appendage, the PD axis is established during embryogenesis *de novo*. In contrast, at all stages of development, even in the unfertilized egg, rudimentary AP and DV axes exist. Thus, in this respect, the PD axis is unique among the main body axes. This topic, how so-called

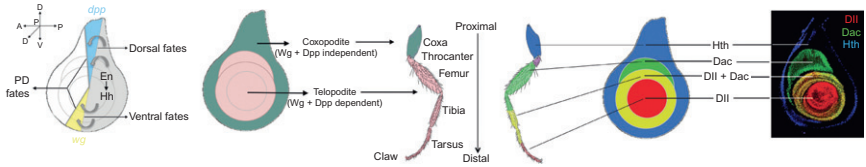

Figure 7.1 Overview of fly leg development. On the left shows the relationship between En, Hh, *wg*, and *dpp* and the definition of the telopodite (Hh, Wg, and Dpp-dependent domain) and the coxopodite (Hh, Wg, and Dpp-independent domain). On the right shows the relationship between the three primary PD gene expression domains established by Hth, Dac, and Dll.

secondary developmental fields are established from preexisting developmental information, has been debated for decades both from theoretical perspectives and by classical developmental biologists (reviewed by Baker, 2011). Data generated over the past several years have provided novel mechanistic and molecular insights that build upon these earlier studies, providing interesting connections between cell division, secreted morphogens, and the use of dedicated *cis*-regulatory modules (CRMs) for transcriptional regulation of genes expressed along the PD axis. It is the goal of this review to summarize our current understanding of the intimate interplay between these components, orchestrated over developmental time, which establishes, elaborates, and fine-tunes the leg's PD axis.

2. The Molecular Players in PD Axis Formation

As for much of the adult fly, fly legs are derived from imaginal discs, elliptical sheets of epithelia that are highly folded by the end of larval development. The fate map of the leg disc is such that cells at its center will give rise to distal-most structures, while cells further away from the center generate more proximal structures (Fig. 7.1). Imaginal discs do not only give rise to appendages: cells at the periphery of the leg disc, for example, generate the ventral portion of the adult body wall, the ventral and sterno pleura. Thus, in the leg imaginal disc, the PD axis—from distal claw to proximal body wall—is displayed as concentric rings within these elliptical epithelia (Fig. 7.1).

Many genes are expressed in rings or subdomains in the leg disc that mark distinct positions along the PD axis (Abu-Shaar and Mann, 1998; Campbell and Tomlinson, 1998; Diaz-Benjumea *et al.*, 1994; Duncan *et al.*, 1998; Emmons *et al.*, 1999; Erkner *et al.*, 1999; previously reviewed by Kojima, 2004; Kojima *et al.*, 2000; Mardon *et al.*, 1994). From this perspective, the problem of how the PD axis is established can be reformulated by asking the simpler question: how are these gene expression domains established? As will be discussed more below, much attention has been focussed on dissecting the regulation of two genes that are broadly expressed in distal and medial domains of the leg disc, *Distal-less* (*Dll*) and *dachshund* (*dac*), respectively (Fig. 7.1). *Dll*, in particular, is a critical player in leg development as it is one of the earliest known markers for the appendage, not just in flies, but also throughout the animal kingdom (Cohen, 1990; Cohen *et al.*, 1989; Panganiban *et al.*, 1997; reviewed in Panganiban and Rubenstein, 2002). Moreover, in flies both *Dll* and *dac* are required for the development of their respective distal and medial domains of the leg (Cohen and Jurgens, 1989; Mardon *et al.*, 1994). *homothorax* (*hth*), encoding a homeodomain transcription factor, and *teashirt* (*tsh*), encoding a zinc-finger transcription factor, are coexpressed in an even more proximal domain (Abu-Shaar and

Mann, 1998; Erkner *et al.*, 1999; Rieckhof *et al.*, 1997; Wu and Cohen, 1999). The domains resulting from the expression of *hth/tsh* (proximal), *dac* (medial), and *Dll* (distal), together with regions that have overlapping expression of these factors, broadly define the PD axis (Fig. 7.1). Other genes, also expressed at specific positions along the PD axis, for example, those expressed in the tarsal segments, are activated later in development in response to EGFR signaling and are required for forming the joints that separate each of the leg segments (see below).

Theoretical modeling and classical limb grafting experiments both led to the idea that the juxtaposition of three different cell types—in particular, posterior, anterior dorsal, and anterior ventral—leads to the induction of new PD axes (reviewed in Baker, 2011; Fig. 7.2). We now have a molecular understanding of this phenomenon, namely, that the juxtaposition of cells expressing Decapentaplegic (Dpp) next to cells expressing Wingless (Wg), two secreted morphogens used widely in animal development, is sufficient within the context of leg development to generate a new PD axis (Campbell *et al.*, 1993; Diaz-Benjumea *et al.*, 1994; Lecuit and Cohen, 1997). In the wild-type leg imaginal disc, *dpp* and *wg* are expressed along the AP compartment boundary in dorsal and ventral cells, respectively, both in response to Hedgehog (Hh) emanating from the posterior compartment (Basler and Struhl, 1994; Fig. 7.2). Accordingly, in the wild-type leg disc, cells expressing Wg and Dpp are adjacent to each other only at the center of the disc, which will give rise to the distal-most portion of the appendage. Thus, the expression patterns of Hh (posterior), Dpp (dorsal-anterior), and Wg (ventral-anterior) account for the three cell types initially postulated by the grafting and theoretical studies (Fig. 7.2).

Significant effort over the past several years has attempted to connect these two sets of molecular players in PD axis formation. At the top of the hierarchy are Wg and Dpp, which together are sufficient for initiating a PD axis in the leg, inducing the correct expression domains of *Dll*, *dac*, and

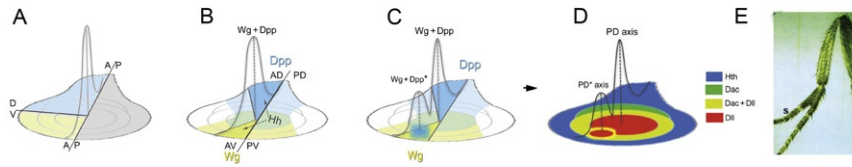

Figure 7.2 Wg+Dpp initiate the PD axis. (A) Meinhardt's "three-sector" model for induction of the PD axis. (B) Hh, from the P compartment, induces Wg (yellow) in the anterior ventral (AV) domain and Dpp (blue) in the anterior dorsal (AD) domain; note that Wg- and Dpp-expressing cells are only adjacent in the center of the wild-type disc. (C–E) An ectopic source of Dpp (C) in the ventral domain induces an ectopic PD axis visualized in the disc (D) and in the adult appendage (E; from Campbell *et al.*, 1993).

other genes expressed along this axis. How do these morphogens activate these PD genes? Is the activation direct or indirect? What is the relationship between the leg primordia and the dorsal appendage (e.g., wing) primordia? What genes provide the ventral identity of the appendage? Here, we provide an update on efforts to answer these questions and provide an initial view of the molecular architecture underlying PD axis specification.

3. The Initial Establishment of the PD Axis is Encoded in the *cis*-Regulatory Architecture of *Dll*

A common principle that has emerged from studying transcriptional regulatory mechanisms in many developmental genes is that dedicated CRMs drive small subsets of complex expression patterns (reviewed in Maeda and Karch, 2011). Dissection of the *Dll* locus revealed a similar level of CRM dedication, but with the additional finding that distinct *Dll* CRMs control *Dll* expression in cells that have different degrees of developmental potential (Fig. 7.3). The discovery of these CRMs has allowed

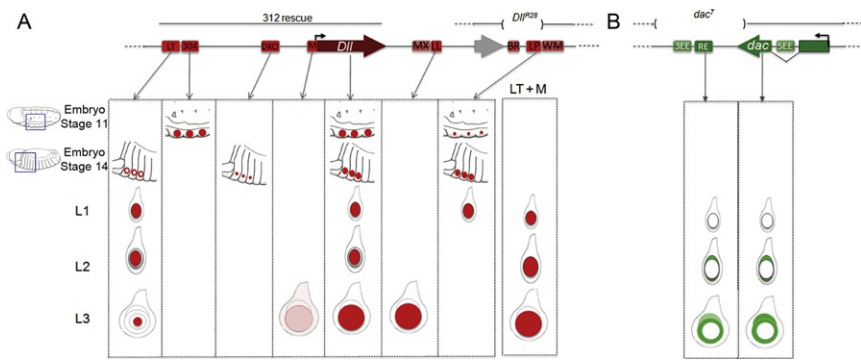

Figure 7.3 *Dll* and *dac* CRMs. (A,B) Schematic of the *Dll* (A) and *dac* (B) genomic regions showing the positions of identified CRMs (colored boxes) and transcription units (large arrows). The expression patterns driven by individual CRMs is indicated and compared to the intact genes. All CRMs are mentioned in the text except for *DllMX*, *DllWM*, *dac3EE*, and *dac5EE*, which are not active during leg development, or *DllBR*, which is active very late in leg development (Galindo *et al.*, 2011; Pappu *et al.*, 2005). A *Dll* rescue transgene ("312 rescue") and a small *Dll* deficiency (Dll^{R28}) both result in a nearly complete PD axis, with defects primarily in the tarsal segments. dac^{7}, which removes *dacRE*, is a deficiency that eliminates both *dac* expression and the medial Dac domain in the leg.

lineage tracing experiments to be carried out, which helped redefine the fate map of the early appendage primordia. Below, we discuss this *cis*-regulatory architecture and its implications for leg development.

3.1. *Dll304*

The first sign of appendage formation in the *Drosophila* embryo is the activation of *Dll* at ~6 h after egg laying (AEL; stage 11) in circular domains comprising ~20-30 cells per thoracic hemisegment (Cohen, 1990). At this stage, an early *Dll* regulatory element situated 11 kb 5′ of the start of *Dll* transcription, called the *Dll* early enhancer or *Dll304*, is able to drive a pattern similar to *Dll* (Vachon *et al.*, 1992). Genetically, *Dll* and *Dll304* activity at this early stage depend on a positive input from Wg, but not from Dpp (Cohen, 1990; Cohen *et al.*, 1993). In fact, *Dll304* is repressed dorsally and ventrally by the Dpp and EGFR pathways, respectively (Goto and Hayashi, 1997; Kubota *et al.*, 2000). Although a molecular dissection of the Wg, Dpp, or EGFR inputs into this enhancer has not yet been described, direct repressive inputs from the abdominal Hox proteins Ultrabithorax (Ubx) and Abdominal-A (AbdA) have been identified (Castelli-Gair and Akam, 1995; Gebelein *et al.*, 2002, 2004; Mann, 1994; Vachon *et al.*, 1992). By directly repressing *Dll304*, these Hox inputs block *Dll* expression and consequently limb development in the abdominal segments.

In the 1970s, Wieschaus and Gehring used X-ray somatic recombination and gynandromorphs to genetically follow marked cells by lineage analysis into the adult (Gehring *et al.*, 1976; Wieschaus and Gehring, 1976). When induced at the blastoderm stage or earlier, marked clones often included both the T2 leg and wing, suggesting a common embryonic origin to both appendages. This conclusion has been corroborated by using the *Dll304* element to carry out lineage tracing experiments, at first by following β-galactosidase perdurance from *Dll304-lacZ* reporter genes and later by genetic methods (Cohen *et al.*, 1993; McKay *et al.*, 2009). The results show that the cells that express *Dll304* give rise not only to the entire ventral appendage (the entire leg) but also to all parts of the two dorsal appendages (the wing in T2 and haltere in T3; Fig. 7.4). In fact, together the ~20-30 early *Dll304*-expressing cells in each thoracic hemisegment have the potential to give rise to the entire adult thorax.

3.2. *DllLT*

The activity of *Dll304* decays within a few hours, but *Dll* expression is maintained in a similarly positioned group of cells in each thoracic hemisegment, suggesting that other regulatory elements must assume control of *Dll* expression. One such element is the late-acting enhancer or "**L**eg **T**rigger" (*DllLT*), situated adjacent to *Dll304* (Cohen *et al.*, 1993; Estella *et al.*, 2008; Vachon *et al.*, 1992; Fig. 7.3). *DllLT* begins to be active at ~8 h and is robustly

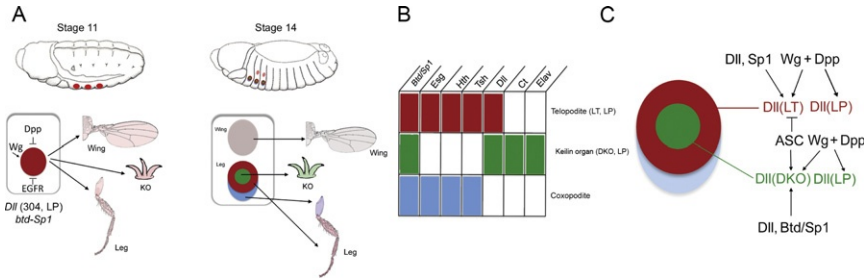

Figure 7.4 Embryonic appendage fate map. (A) Cells expressing *Dll* at stage 11 via the 304 CRM can give rise to the entire adult thorax, while those expressing *Dll* at stage 14 give rise to either the KO or telopodite, depending whether DKO or LT is driving expression, respectively. (B) Genes expressed in the progenitors to the telopodite, KO, and coxopodite. (C) Fate map and regulatory network defining the activity of *Dll* CRMs. The blue cells, expressing *esg* but not *Dll*, are fated to become coxopodite.

expressed by ~10h AEL. This element is not active in all *Dll*-expressing cells but just in ~15 cells at the periphery of the *Dll* expression domain in each hemisegment (Cohen *et al.*, 1993; McKay *et al.*, 2009; Fig. 7.4). *DllLT*, in contrast to *Dll304*, requires positive inputs from both Wg and Dpp. Moreover, the Wg and Dpp input into *DllLT* is direct, mediated by several binding sites for their respective downstream transcription factors Pangolin (Pan) and Mothers against Dpp (Mad; Estella *et al.*, 2008). Interestingly, *DllLT* also requires *Dll* for its activation, presumably derived from the earlier acting *Dll304* element in the early primordia (Castelli-Gair and Akam, 1995; Estella and Mann, 2010; McKay *et al.*, 2009).

Not only is there a temporal hand off from one *Dll* CRM to another, lineage tracing studies reveal that *DllLT*-expressing cells are dramatically more limited in developmental potential compared to *Dll304*-expressing cells. Specifically, the ~15 *DllLT*-expressing cells per hemisegment only give rise to the mature *Dll* and *dac* expression domains of the leg disc, but not to more proximal regions of the leg nor to any part of the dorsal appendages (McKay *et al.*, 2009; Fig. 7.4). This restricted lineage is interesting for two reasons. For one, the combined Dll+Dac domain coincides with the so-called "telopodite" or "true leg" that was originally described by the American entomologist Robert Snodgrass (Snodgrass, 1935; Fig. 7.1). According to Snodgrass, the telopodite is evolutionarily distinct from the more proximal coxopodite, which evolved as a primitive and unsegmented extension from the body wall. Second, as shown more recently, the telopodite, but not the coxopodite, depends on Hh, Wg, and Dpp signaling (Diaz-Benjumea *et al.*, 1994; Gonzalez-Crespo and Morata, 1996), and *DllLT* directly integrates the Wg and Dpp pathways for its

activation (Estella *et al.*, 2008). Thus, the telopodite/coxopodite subdivision concept gained significant molecular support by the observation that a single *Dll* CRM, *DllLT*, is dependent on Wg+Dpp signaling and is active in cells that give rise to the entire telopodite, and only the telopodite.

The telopodite/coxopodite subdivision idea is also supported by genetic analysis of the *Dll* gene, which encodes a homeodomain transcription factor: *Dll* null mutants lack the telopodite but retain the coxopodite (Cohen *et al.*, 1993). There are two interesting follow-up points to be made here. First, even though *Dll* (via the *Dll304* CRM) is expressed earlier, in the progenitors of the entire leg and wing, *Dll* function is only required for the development of the telopodite (Campbell *et al.*, 1993; Cohen *et al.*, 1993). Second, these genetic results imply that *Dll* is required for establishing the Dac domain, even though it is not expressed in part of the Dac domain by the end of larval development. As discussed below, these observations have recently been supported by a molecular analysis of a *dac* CRM, which requires direct Dll input for its activity (Giorgianni and Mann, 2011).

3.3. *DllDKO*

DllLT is active in only ~15 of the *Dll*-expressing cells in each thoracic hemisegment of stage 14 embryos. The remaining *Dll*-expressing cells have a neural identity as revealed by the expression of genes such as *achaete* (*ac*) and *cut* (*ct*; Bolinger and Boekhoff-Falk, 2005; Cohen and Jurgens, 1989; McKay *et al.*, 2009). A third *Dll* CRM, named *DllDKO* (for **D**istal-less **K**eilin's **O**rgan) was identified ~3kb 5′ from the start of *Dll* transcription and is specifically activated in these *Dll*-expressing, *DllLT*-negative cells (Figs. 7.3 and 7.4). *DllDKO* receives positive input from members of the *achaete–scute* complex (ASC) and *Dll*, thus restricting its activity to the neurogenic cells in the limb primordia (McKay *et al.*, 2009; Fig. 7.4).

At this point in embryogenesis, cells in the leg and wing primordia express the zinc-finger transcription factor *escargot* (*esg*), which is required to maintain diploidy and therefore an imaginal disc fate (Fuse *et al.*, 1996; Hayashi *et al.*, 1993). Notably, there is a gap in *esg* expression in the leg primordia that is filled by the expression of the neuronal genes *ct* and *ac*, suggesting that these cells do not contribute to the leg imaginal disc but instead are fated to form the Keilin's Organ (KO), a larval sensory organ thought to be a vestige of larva legs present in more primitive insects (Bolinger and Boekhoff-Falk, 2005; Keilin, 1915). Lineage tracing experiments confirm that *DllDKO*-expressing cells do not give rise to any adult structures, consistent with the idea that they are dedicated to forming the KO (McKay *et al.*, 2009; Fig. 7.4).

3.4. Other *Dll* CRMs

Dll304, *DllLT*, and *DllDKO* are all located within a 14-kb region 5′ to the start of *Dll* transcription (Fig. 7.3). Recently, additional *Dll* CRMs 3′ to the *Dll* transcription unit that are also able to produce some aspects of the *Dll* expression pattern in the leg imaginal disc have been identified (Galindo *et al.*, 2011; Fig. 7.3). One element, called *DllLP* for **L**eg **P**rimordium, is active in a subset of *Dll*-expressing cells in stage 10 embryos and remains active until the end of first larval instar when its activity decays. *DllLP*, like *DllLT*, is activated by Wg and Dpp in the embryo, although it is not known if this activation is direct. A second 3′ element, called *DllLL* for **L**eg **L**ate, is only active in mid-third instar larvae and depends, like *DllLT*, on *Dll* for its activity. Although specific deletions do not exist to assess the necessity (or sufficiency) of each individual CRM, the available data suggest that both the 5′ and 3′ enhancers are important for wild-type *Dll* expression and function. While *Dll* null mutants lack the entire telopodite, a *Dll* minigene that includes the 5′ CRMs (including *Dll304* and *DllLT*) but not the 3′ CRMs significantly rescues telopodite development with the exception of the tarsal segments (Galindo *et al.*, 2011; Vachon *et al.*, 1992). This partial rescue, which could be due to inaccurate timing or levels of expression, is very similar to a *Dll* deletion allele that removes the *DllLP* element but leaves the 5′ elements intact (Galindo *et al.*, 2011; Fig. 7.3). Given the complexity of *Dll* regulation during development, it is not surprising that it is governed by multiple CRMs with partially overlapping activities.

The lineage tracing experiments cited above using individual *Dll* CRMs, combined with additional gene expression studies, resulted in a revised fate map of the ventral appendage primordia (Bolinger and Boekhoff-Falk, 2005; McKay *et al.*, 2009; Fig. 7.4). At stage 14, this fate map comprises three domains that correspond to a rudimentary PD axis: (1) an *esg* on, *hth* on, *tsh* on, and *Dll* off domain that is fated to form the coxopodite and the body wall, (2) an *esg* on, *hth* on, *tsh* on, and *Dll* on (*DllLT* on) domain will give rise to the entire telopodite, and (3) an *esg* off, *hth* off, *tsh* off, and *Dll* on (*DllDKO* on) domain fated to generate the KO (Fig. 7.4). This revised fate map is distinct from an earlier version in which the overlap between *hth* and *Dll* was not recognized, likely based on an analogy with third instar discs where the expression of these factors is largely nonoverlapping (Fig. 7.1; Gonzalez-Crespo and Morata, 1996; Gonzalez-Crespo *et al.*, 1998). The overlapping expression of *Dll*, *hth*, and *tsh* in the embryonic telopodite precursors is surprising and is no longer observed by the second instar stage, when these cells begin to divide. Interestingly, the coxopodite precursors (*hth* on, *tsh* on, *Dll* off) begin to proliferate slightly earlier than the telopodite precursors (Bryant and Schneiderman, 1969; McKay *et al.*, 2009). The different timing in proliferation between these two domains may be a consequence of the coexpression of *hth* and *Dll* in the telopodite precursors. Consistently, the

forced expression of *hth* in *Dll*-expressing cells blocks cell proliferation and telopodite formation (Azpiazu and Morata, 2002; McKay *et al.*, 2009). Why this temporal asynchrony in the start of proliferation exists between these two domains of the leg is unknown.

4. The Role of *Sp1* in Distinguishing Ventral Appendage from Dorsal Appendage Fates

Although the above focus on *Dll* regulation reveals how the initial PD axis and leg fate map are established, several questions remain concerning this early stage of leg development. Some of these questions are answered by two paralogous genes, *buttonhead* (*btd*) and *Sp1* (Estella and Mann, 2010; Estella *et al.*, 2003; Wimmer *et al.*, 1996). Both genes encode Sp family zinc-finger transcription factors that share a similar expression pattern throughout development. Despite their similar expression patterns, the lack of *Sp1* function (but not *btd*) completely abolishes leg formation. Unlike *Dll*, which is required only for telopodite development, *Sp1* is required for the development of both the coxopodite and telopodite. Also noteworthy is that the closest vertebrate homolog of *Sp1* is *Sp8*, which is required for limb development in the mouse, suggesting an evolutionarily conserved role for these transcription factors (Bell *et al.*, 2003; Kawakami *et al.*, 2004; Treichel *et al.*, 2003).

The *Sp1* expression pattern is also consistent with an early role for this transcription factor in leg development. In parallel to *Dll304*, *Sp1* is first activated at stage 10 in the initial appendage primordia. *Sp1* activation requires Wg and is repressed dorsally by Dpp and in the abdomen by Ubx. However, in contrast to *Dll*, *btd* and *Sp1* are both expressed throughout the entire leg primordia, coinciding with *esg* expression (Estella *et al.*, 2003). In embryos without *btd* and *Sp1* function, *Dll* is activated normally but decays as the embryo develops. This is likely because *Dll304* does not require *Sp1/btd* function, but *DllLT* does (Estella and Mann, 2010). Thus, *Sp1* is initially activated in parallel to *Dll* but is required for the maintenance of *Dll* expression and is required for both telopodite and coxopodite fates. *disconnected* (*disco*) has also been proposed to play a role in the maintenance of *Dll* expression (Dey *et al.*, 2009).

In addition to being required for the entire ventral appendage, *Sp1* also appears to be required for suppressing dorsal appendage fates. When clonal analysis was used to generate ventral appendages devoid of both *Sp1* and *btd*, in some cases dramatic homeotic transformations from leg to wing were observed in the adult (Estella and Mann, 2010). Together, these experiments suggest that *Sp1* is a ventral appendage selector gene, and that in its absence dorsal appendage fates become derepressed.

Although many questions remain concerning this early aspect of appendage fate specification, there are several other relevant and interesting observations in the literature. First, as noted above, the dorsal appendage primordia (wing and haltere) and ventral appendage primordia (leg) are derived from the same set of *Dll304*-expressing cells. As the embryo develops, a small number of cells leave this early ventral (*Dll304*-expressing) primordia, migrate dorsally, stop expressing *Dll*, and contribute to the establishment of the dorsal appendage primordia (Cohen *et al.*, 1993; McKay *et al.*, 2009). Second, the balance between the sizes of the ventral and dorsal appendage primordia is sensitive to two opposing signaling pathways: Dpp and EGFR. High levels of Dpp signaling promote dorsal appendage and coxopodite fates, while high levels of EGFR signaling repress dorsal appendage fates and promote ventral appendage fates (Goto and Hayashi, 1997; Kubota *et al.*, 2000). Finally, the T-box transcription factor-encoding genes *Dorsocross1* (*Doc1*), *Doc2*, and *Doc3* are required for establishing the dorsal, but not the ventral, primordia (Hamaguchi *et al.*, 2004; Reim *et al.*, 2003). Although the relationships between these various inputs (*Sp1*, Dpp, EGFR, and *Doc*) are not understood, these observations provide tantalizing hints at a complex process that establishes the fates of these two appendage primordia from a common group of cells.

5. Elaboration of the PD Axis: The Role of *brk*

By the end of embryogenesis, a rudimentary PD axis of the leg is apparent in the expression patterns of *Dll* (via *DllLT*, *DllDKO*, and *DllLP)*, *hth*, and *tsh*. How are these initial patterns elaborated upon to create the mature PD axis present in the third larval instar stage? The separation between these two time points is huge both in terms of time (96h) and tissue growth (from ~60 cells to ~10,000 cells). Also, at the end of embryogenesis, *dac* has not yet been activated but begins to be expressed in a circular medial domain in the second instar. Nevertheless, despite these dramatic changes, we are beginning to understand how the late embryonic patterns of gene expression evolve and are eventually stabilized during this phase of development.

As discussed above, many observations in the literature support the idea that the juxtaposition of Wg- and Dpp-expressing cells in the center of the leg disc leads to the formation of the PD axis, including the proper domains of *Dll* and *dac* expression. The activation of these genes by Wg and Dpp does not rely on a third signal, arguing that these two signals are both necessary and sufficient to induce the leg's PD axis (Lecuit and Cohen, 1997). How can the obligate synergy between Wg and Dpp be explained at the molecular level? One answer is that, in the leg disc as in other places during fly development, Dpp functions mainly by repressing the transcriptional repressor, *brinker* (*brk*; Campbell and Tomlinson, 1999; Jazwinska

et al., 1999; Minami *et al.*, 1999). In contrast, Wg is an obligate activator of *Dll* and *dac* (Lecuit and Cohen, 1997). In the absence of Dpp, *brk* expands throughout the leg disc and *Dll* and *dac* fail to be activated. Importantly, however, in *brk* and *dpp* double mutants, *Dll* and *dac* expression domains are rescued, in patterns reminiscent to those in a wild-type leg (Estella and Mann, 2008). Thus, in the absence of *brk*, Dpp is not required for either *Dll* or *dac* activation. Further, by uncovering a role for Brk repression in *dac* and *Dll* regulation, they provided an explanation why both Wg and Dpp signals are required for PD axis formation: in the early disc, *DllLT* and *Dll* are only expressed in cells that (1) do not have Brk and (2) are exposed to high Wg levels. It was also suggested that different ratios of Wg (a positive input) and Brk (a negative input) determined whether *dac* or *Dll* would be activated (Estella and Mann, 2008). According to this idea, *Dll* is activated by high levels of Wg and very low or no Brk. In contrast, *dac* is activated by lower levels of Wg and is less sensitive to Brk repression than *Dll*.

6. Elaboration of the PD Axis: The Role of a Transcription Factor Cascade and Cross-regulation

In addition to positing that Wg and Dpp are critical for initiating the PD axis, Lecuit and Cohen proposed a gradient model to account for the PD axis expression patterns of *dac* and *Dll*. According to this model, the expression of *dac* and *Dll* along the PD axis depends on the levels of Wg and Dpp a cell perceives: high concentrations of both Wg and Dpp activate *Dll* and repress *dac* in the center of the leg disc; intermediate levels activate *dac* but not *Dll* in medial regions of the disc; and low levels of these morphogens fail to activate either gene (Fig. 7.5A). Two main observations supported this model (Lecuit and Cohen, 1997): (1) ectopic expression of *wg* in the dorsal half of the leg (where Dpp levels are high) recapitulated the wild-type nested pattern of PD gene expression, with *Dll* expressed closest to the Wg source and *dac* further away and (2) mutant clones for *mad* or *disheveled* (*dsh*; essential components of the Dpp and Wg pathways, respectively) in the center of the leg disc derepressed *dac*. In addition, based on their expression patterns, the highest levels of Wg and Dpp would be located in the center of the leg disc where the expression of these two morphogens meet, and the levels would gradually diminish in cells closer to the periphery of the disc (Fig. 7.2).

Although very attractive, the gradient model is difficult to envision at the molecular level, where the inputs from the Wg and Dpp signaling pathways must converge onto the CRMs of *Dll* and *dac* (Fig. 7.5A). The model is also difficult to reconcile with the observation that *brk dpp* mutant discs,

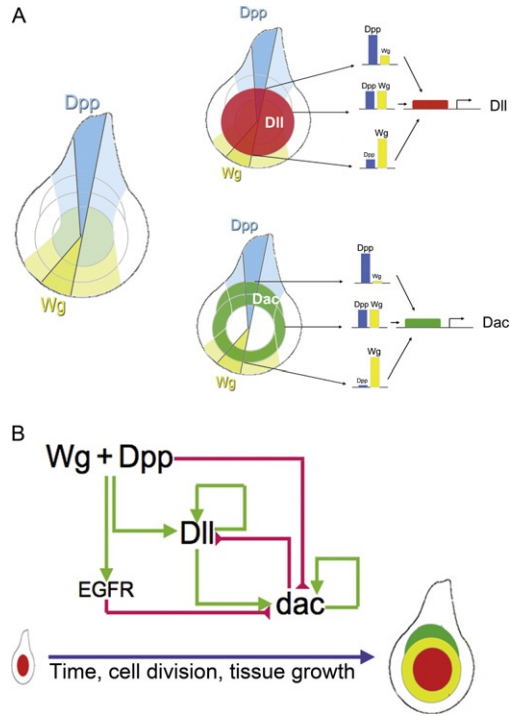

Figure 7.5 Gradient versus cascade models. (A) The gradient model, highlighting that, depending on a cell's position in the disc, *Dll* and *dac* CRMs must interpret very different ratios of Dpp:Wg signaling. (B) The cascade model, in which Wg+Dpp are only required to initiate PD axis formation by activating *Dll* and ligands for the EGFR pathway. Dll in turn activates *dac*, and both *Dll* and *dac* maintain their expression in a Wg+Dpp-independent manner. EGFR activity maintains *dac* repression, while Wg+Dpp repress *dac* in the center of the leg disc early in leg development.

which do not have a Dpp gradient, have a PD axis (Estella and Mann, 2008). The dissection of *Dll* and *dac* CRMs has provided insights into how these genes respond to Wg and Dpp signaling. As described above, *DllLT* is first activated in the progenitors of the telopodite cells in late embryogenesis. Although *DllLT* remains active during larval development, in third instar discs, it is restricted to cells at the center of the disc, close to where the Wg and Dpp domains touch. This is a small subset of the overall *Dll* domain at this time (Fig. 7.3). Moreover, in contrast to *Dll*, *DllLT* continuously depends on Wg and Dpp signals and it integrates these inputs directly by the binding of the transcription factors Mad, Brk, and Pan (Estella *et al.*, 2008). Although Wg and Dpp meet in other tissues such as the wing disc,

DllLT activity is restricted to the leg imaginal disc by *Sp1* and *Dll*, although it is not yet known if this regulation is direct (Estella and Mann, 2010; McKay *et al.*, 2009).

As noted above, the dependency of *Dll* on Wg and Dpp inputs is only transient: by the second instar, these signals are no longer required for *Dll* expression in the leg disc. It has been suggested that the Wg- and Dpp-independent expression phase or "maintenance" is achieved in combination with another *Dll cis*-regulatory element that includes the *Dll* transcription start site. On its own, this element, named *DllM* for **M**aintenance, is weakly active in *Dll*-expressing cells. But when placed in *cis* with *DllLT*, it produces an accurate and robust *Dll* expression pattern in the leg disc (Fig. 7.3; Estella *et al.*, 2008). Moreover, *DllLT+M*, like *Dll*, is able to maintain its expression in the absence of Wg and Dpp inputs, in part by a positive autoregulatory feedback loop. The *M* element contains Dll binding sites that are required for maintenance. Interestingly, the *M* element is also able to produce an accurate Dll pattern when placed close to other *Dll* CRMs that, on their own, do not drive a Dll-like expression pattern (Estella *et al.*, 2008). Although it is very likely that additional CRMs are involved (Estella *et al.*, 2008; Galindo *et al.*, 2011; Vachon *et al.*, 1992), the "trigger-maintenance" mechanism provides a molecular explanation for how Wg and Dpp activate *Dll* and how *Dll* expression is maintained as the disc grows, despite widely varying levels of Wg+Dpp signaling in Dll domain. Importantly, this mechanism does not require *Dll* CRMs to integrate gradients of Dpp and Wg inputs.

The molecular dissection of a *dac* CRM (*dacRE* for **R**ing **E**nhancer), which recapitulates most of the medial expression pattern of this gene (Fig. 7.3B), suggests that *dac* also does not need to interpret Wg and Dpp gradients for its activation in the medial leg domain (Giorgianni and Mann, 2011). Eliminating most of the putative Pan- and Mad-binding sites has no or very little effect on *dacRE* activity in third instar leg discs, suggesting that this element is not integrating intermediate levels of Wg and Dpp. Instead of being activated by Wg and Dpp, *dacRE* is directly activated by Dll, consistent with lineage tracing experiments showing that the Dac domain is derived from *DllLT*-expressing cells (McKay *et al.*, 2009). Moreover, *dacRE* repression in the distal tip of the leg by Wg and Dpp is transient and is maintained by other transcription factors expressed later in development (see below). In summary, instead of using a gradient mechanism, these results suggest that this phase of PD axis formation depends on a genetic cascade, in which Wg+Dpp activate *Dll*, and Dll activates *dac* (Fig. 7.5B).

If gradients of Wg and Dpp signaling are not required for *Dll* and *dac* activation, how are these two genes differentially expressed along the PD axis of the leg? One plausible scenario is as follows (Fig. 7.6): During the first and second instar leg disc, high levels of Wg and Dpp activate *Dll* expression, in part via *DllLT*, and repress *dac* via the *dacRE* element. As the disc grows, *dac* is activated by Dll in cells where the levels of Wg and Dpp

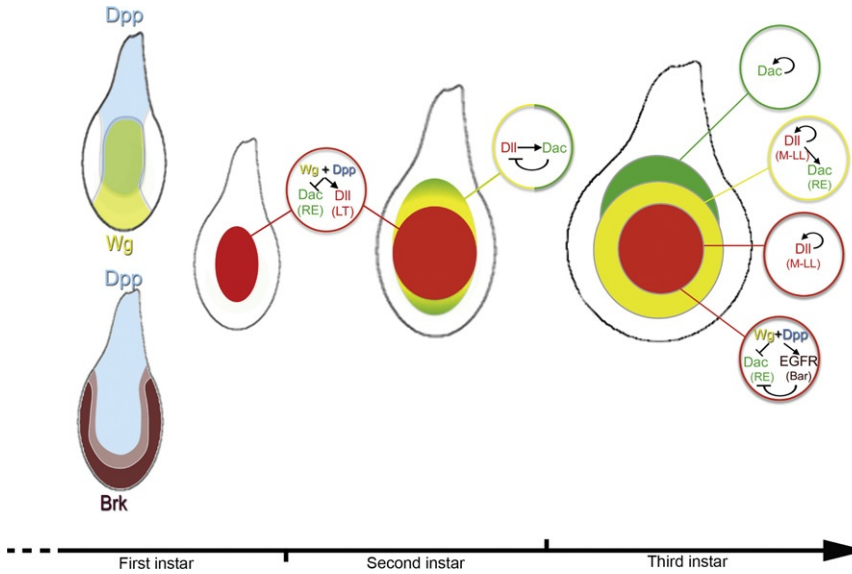

Figure 7.6 Summary of the dynamic network establishing the PD axis. The relationship between Dpp, Wg, and Brk triggers the expression of *Dll* and represses *dac* in the center of the disc. As the disc grows, Dll activates *dac* in cells that escape repression by Wg+Dpp. These initial domains are likely maintained by a combination of autoregulation, cross-regulation, and transcriptional memory systems.

signaling are low enough to escape repression. As cells continue to divide, some *Dll*- and *dac*-expressing cells will end up removed from peak Wg and Dpp levels and lose *Dll* expression, generating the Dac-only domain. In this manner, the three primary PD domains of gene expression, Dll only, Dll+ Dac, and Dac only, have been specified. At about the same time, the expression of these genes starts to become independent of the activating signals and may be maintained, in part, by an autoregulatory mechanism (via the *M* element for *Dll*) or by a transcriptional memory mechanism (Kim *et al.*, 2008). One key aspect to this model is that Wg+Dpp are only required to initiate the PD cascade: by initially activating *Dll* (and repressing *dac*) in the center of the imaginal disc, Wg+Dpp trigger the cascade but are no longer essential after this step (Figs. 7.5B and 7.6). Although there are many aspects to this model that need confirmation, such a mechanism, coupled to tissue growth, is sufficient to account for the generation of stable domains of gene expression along the PD axis.

Finally, we highlight the role of cross-regulation and additional repressive interactions for the maintenance of PD domains. As noted above, the coxopodite domain is defined by the expression of *hth* and *tsh* in the body wall and coxa (Abu-Shaar and Mann, 1998; Erkner *et al.*, 1999; Gonzalez-Crespo

and Morata, 1996; Rieckhof *et al.*, 1997; Wu and Cohen, 1999, 2000). There is evidence that the combined input from Wg and Dpp signaling represses *hth* and *tsh* expression in the telopodite cells via activation of *Dll* and *dac* (Abu-Shaar and Mann, 1998; Gonzalez-Crespo *et al.*, 1998) and by a *Dll*-independent mechanism (Wu and Cohen, 1999). This *Dll*-independent repression mechanism may be mediated via the *elbow–no ocelli* gene complex, which depends on Wg and Dpp signaling for expression and delimits the appendage field in the leg imaginal disc (Weihe *et al.*, 2004). In addition, repression of *dac* by the homeodomain proteins Bar (BarH1/BarH2) is one way in which *dac* repression is maintained in distal leg cells (Giorgianni and Mann, 2011; Kojima *et al.*, 2000). Additional cross-regulation between PD genes is also observed among other transcription factors expressed in the tarsal segments, downstream of EGFR signaling (see below).

7. Patterning the DV Axis

If gradients of Wg and Dpp are not used to establish the PD axis, what purpose might they serve? The posterior expression of the homeodomain transcription factors *engrailed* (*en*) and *invected* (*inv*) divides the leg into anterior and posterior compartments, which have distinct cell lineages (Morata and Lawrence, 1975). In contrast to strict lineage restrictions along the AP axis, the distinction between dorsal and ventral fates is controlled by the secreted molecules Wg and Dpp in a nonlineage-dependent manner. As noted above, in the leg imaginal disc, Hh signals from the posterior compartment to anterior compartment cells to activate the expression of *wg* and *dpp* in the ventral and dorsal halves of the disc, respectively (Basler and Struhl, 1994). The anterior dorsal and anterior ventral expression of these two genes in the leg imaginal disc is maintained by a mutual antagonism that prevents the expression and/or signaling of these pathways in the other half of the disc. Dpp specifies dorsal fates and represses ventral ones, whereas Wg specifies ventral fates and represses dorsal ones (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Morimura *et al.*, 1996; Penton and Hoffmann, 1996; Struhl and Basler, 1993; Theisen *et al.*, 1996; Wilder and Perrimon, 1995). Hypomorphic mutations in *wg* result in the mirror image duplication of the dorsal leg pattern at the expense of the ventral pattern. Analogously, mutations in *dpp* have the opposite effects (Brook and Cohen, 1996; Jiang and Struhl, 1996; Theisen *et al.*, 1996). Thus, although gradients of Wg and Dpp signaling may not be required for specifying distinct PD axis fates, they appear to play a critical role in establishing positional information along the DV axis.

In the tip of the leg, all cells are likely to perceive high levels of Wg and Dpp, so understanding how a cell is able to discriminate between these

signals to promote dorsal or ventral fates is an important question. One possibility is that there are dorsal and/or ventral selector genes activated downstream of these signals. Recently, Svendsen *et al.* (2009) characterized the expression and function of two redundant Tbx20 transcription factors, *H15* and *midline* (*mid*), two genes that can fulfil this role (Svendsen *et al.*, 2009). These genes are expressed in the ventral half of the leg disc, in a broader domain than *wg* that coincides with the domain deleted in *wg* mutants. In *H15* and *mid* mutants, ventral leg structures are transformed to the corresponding dorsal ones, without affecting *wg* or *dpp* expression, probably due to the low levels of Dpp signaling found in the ventral domain (Blackman *et al.*, 1991). Moreover, ectopic *mid* and *H15* expression is able to induce ventral fates in dorsal cells. Although Dpp-dependent dorsal fates have been suggested to be mediated by the dorsal-specific T-box gene *optomotor-blind* (*omb*; Grimm and Pflugfelder, 1996; Maves and Schubiger, 1998), *omb* is not derepressed in *mid* mutant clones (Svendsen *et al.*, 2009). One possibility is that the DV "ground state" is dorsal, but this is argued against by the observation that lateral structures are formed in legs with reduced expression of both *wg* and *dpp* (Held *et al.*, 1994). Thus, other factors in addition to *omb* are probably required to define dorsal fates.

8. EGFR Signaling Patterns the Tarsus

While Wg and Dpp play an important role in initiating the PD axis, by the early third instar, Wg and Dpp are no longer required for the PD axis and the role of further elaborating this axis is handed off to the EGFR signaling pathway (Campbell, 2002; Galindo *et al.*, 2002; Fig. 7.7). Shifts of the temperature-sensitive mutant $Egfr^{[ts]}$ ($Egfr^{[tsla]}/Egfr^{[null]}$) to the restrictive temperature in the beginning of the third larval stage lead to development of legs without pretarsus and one or more tarsal segments depending on the

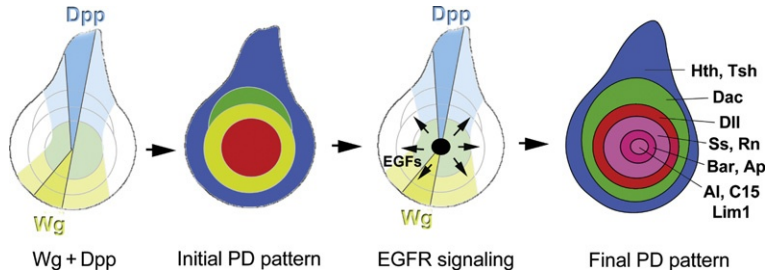

Figure 7.7 EGFR signaling patterns the tarsal segments. After the initial PD domains are established, EGFR ligands are produced at the center of the disc and activate a series of secondary PD targets in the progenitors to the tarsal segments.

severity of the restrictive temperature. Similar results were obtained when dominant-negative forms or inhibitors of EGFR signaling components were ectopically expressed in leg discs (Campbell, 2002; Galindo *et al.*, 2002, 2005). Genetic experiments suggest that initiation of EGFR signaling in the center of leg discs is dependent on *wg*, *dpp*, and *Dll* (Galindo *et al.*, 2002, 2005). Although it is not known how this occurs at a mechanistic level, it is plausible that in this case graded EGFR activity may be important for setting up distinct PD fates in the tarsus.

EGFR signaling occurs in waves, by the consecutive activation of ligands, proteases, and inhibitors (reviewed in Shilo, 2005). In leg imaginal discs, EGFR signaling is initiated in early third instar larvae by expression of the secreted ligand Vein (Vn) in the central region of leg disc (Campbell, 2002; Galindo *et al.*, 2002), which we refer to as the EGF Organizing Center (EOC). Shortly after *vein* is activated, another component of the EGFR signaling cascade is expressed: the protease Rhomboid (Rho; Campbell, 2002; Galindo *et al.*, 2005). Rho is required for processing of the membrane-bound ligands Spitz (Spi), Keren (Krn), and Gurken (Grk); without Rho these ligands are not secreted (reviewed in Urban, 2006). Although none of the membrane-bound ligands have been detected in the center of leg discs, Vn cannot be the only ligand that plays a role in the EOC because *vn* single mutant leg discs do not phenocopy *Egfr* mutants (Campbell, 2002; Galindo *et al.*, 2005). Only the triple mutant *ru rho vn* produces strong phenotypes that resemble medium $Egfr^{[ts]}$ leg mutants (*rougHoid* (*ru*) encodes a paralog of *rho*). Small regions of wild-type tissue in the center of otherwise *ru rho vn* mutant leg discs can rescue tarsal formation (Campbell, 2002), providing additional evidence that the EOC serves as a source of secreted EGFR ligands that pattern the tarsus.

Drosophila EGFR signaling is a typical Ras–Raf–MAPK (Map kinase) signaling pathway. In the leg imaginal disc, phosphorylated MAPK is detected shortly after expression of *vn* in the EOC (Campbell, 2002). Moreover, the Ets transcription factor PointedP2 (PntP2), a common downstream effector of receptor tyrosine kinase (RTK) signaling in *Drosophila* (Brunner *et al.*, 1994; O'Neill *et al.*, 1994; Scholz *et al.*, 1997), is also expressed in the EOC, and misexpression of a dominant-negative form of *pntP2* ($pntP2^{[DN]}$) abolishes tarsal segments 4 and 5 and pretarsus (Galindo *et al.*, 2005). Although the truncation phenotype in this experiment is not as strong as that of $Egfr^{[ts]}$, it is possible that $PntP2^{[DN]}$ cannot completely block PntP2 activity or that additional EGFR downstream effectors participate in this process. Interestingly, gene repression by general repressors such as Capicua and Groucho was recently shown to be relieved by EGFR signaling in other developmental contexts (Ajuria *et al.*, 2011; Cinnamon *et al.*, 2008). Similar mechanisms might be involved in leg disc patterning by EGFR.

The expression of several transcription factors required for PD patterning is dramatically changed in *Egfr* mutants, *Egfr* mosaic clones, or ectopic EGFR activation. The results of several studies (Campbell, 2002, 2005;

Galindo *et al.*, 2002, 2005) show that high levels of EGFR signaling are required for activation of *aristaless* (*al*), *C15* (*clawless*), and *Lim1* in small overlapping circular domains in the very center of the leg discs (Fig. 7.7). These homeodomain proteins are required for the specification of the pretarsus (Campbell, 2005; Campbell and Tomlinson, 1998; Kojima *et al.*, 2005; Pueyo and Couso, 2004; Pueyo *et al.*, 2000; Tsuji *et al.*, 2000). Lower levels of EGFR signaling are required for expression of another set of homeodomain-encoding genes, *BarH1/BarH2* and *apterous* (*ap*), in rings surrounding the *al/C15/Lim1* domain. Apterous and BarH1/BarH2 specify tarsal segments 4 and 5 (Kojima *et al.*, 2000; Pueyo and Couso, 2004; Pueyo *et al.*, 2000). *tarsal-less* (*tal*), *spineless* (*ss*), *rotund* (*rn*), and *bric-a-brac* (*bab*) are expressed in concentric rings just proximal to the rings of *BarH1/BarH2* and *ap*. These genes are required for the proper development of tarsal segments 1–5 (Couderc *et al.*, 2002; Duncan *et al.*, 1998; Galindo *et al.*, 2007; Godt *et al.*, 1993; St Pierre *et al.*, 2002). *tal*, *ss*, *rn*, and *bab* are repressed directly or indirectly by EGFR signaling in the EOC since mild loss of EGFR signaling abolishes *al*, *C15*, and *Lim1* expression, while *Bar*, *ap*, *ss*, *rn*, and *bab* expression shifts toward the center of leg discs. Strong loss of EGFR signaling abolishes expression of most tarsal patterning genes while at the same time allows for *dac* expression in the center of leg discs (Campbell, 2002; Galindo *et al.*, 2002). This latter observation is consistent with mutagenesis studies of the *dacRE* element, which suggested that Bar is a direct repressor of *dac* in third instar leg discs (Giorgianni and Mann, 2011). Spineless and the zinc-finger transcription factor Rotund are also involved in delimiting the distal margin of the *dac* expression domain in mid-third instar—*ss; rn* double mutant leg discs show expansion of the *dac* domain distally and the *BarH1/H2* domain proximally until they juxtapose each other (Pueyo and Couso, 2008).

In addition to being targets of EGFR signaling, the tarsal PD patterning genes show complex interactions with each other. For example, Al and C15 form a protein complex and are required for the expression of *Lim1*, while Lim1 together with its cofactor Chip maintains the expression domain of *al* and *C15* by repressing *BarH1/H2* in the very center of leg discs (Campbell, 2005; Kojima *et al.*, 2005; Pueyo and Couso, 2004). BarH1/H2, in turn, keeps *dac* off in the EOC (Giorgianni and Mann, 2011; Kojima *et al.*, 2000) and also activates the polycistronic gene *tal* in a ring between its own expression domain and the *dac* domain (Pueyo and Couso, 2008). Interestingly, all of the above-mentioned EGFR targets in the tarsus encode transcription factors except for *tal*, which encodes four short peptides (Galindo *et al.*, 2007). During embryogenesis and later during leg joint formation, these peptides act posttranslationally to cleave and modulate the activity (repressor vs. activator) of the zinc-finger transcription factor Shavenbaby (Svb; Kondo *et al.*, 2010; Pueyo and Couso, 2011). As a result of Tal function, a cell-nonautonomous signal is released that triggers the expression of *spineless* and *rotund* in cells in between the domains of Dac and

BarH1/H2 (Pueyo and Couso, 2008). *bab* (BTB/POZ pair of genes *bab1/bab2*) is expressed in a similar domain as *rn* and *ss* (Godt *et al.*, 1993), but its early regulation seems to be independent of Tal and Ss. However, *bab1/2* later expression is aberrant in *ss* or *tal* mutants suggesting that it is at least partially regulated by Ss (Chu *et al.*, 2002; Pueyo and Couso, 2008).

9. Leg Segmentation and Growth

The process of leg segmentation, or forming the joints that separate each of the leg segments, is one critical downstream consequence of PD gene expression (Rauskolb, 2001). Several genes and pathways required for forming the joints have been defined (Bishop *et al.*, 1999; Chu *et al.*, 2002; Ciechanska *et al.*, 2007; de Celis Ibeas and Bray, 2003; de Celis *et al.*, 1998; Galindo *et al.*, 2005; Greenberg and Hatini, 2009, 2011; Hao *et al.*, 2003; Kerber *et al.*, 2001; Mishra *et al.*, 2001; Pueyo and Couso, 2011; Rauskolb and Irvine, 1999; Shirai *et al.*, 2007). A key step in leg segmentation is the induction of the Notch ligands Delta and Serrate (Bishop *et al.*, 1999; de Celis *et al.*, 1998; Rauskolb and Irvine, 1999), which activate the Notch signal transduction pathway (reviewed by Greenwald, 1998; Kimble and Simpson, 1997). Notch activation at the presumptive borders between leg segments, in turn, induces the expression of several downstream genes, including *d-AP2* and the *odd-skipped* family members, *drumstick* (*drm*), *odd-skipped* (*odd*), *brother of odd with entrails limited* (*bowl*), and *sister of odd and bowl* (*sob*; de Celis Ibeas and Bray, 2003; Hao *et al.*, 2003; Kerber *et al.*, 2001; Rauskolb and Irvine, 1999). Together with the nuclear protein Lines, which antagonizes Bowl, the activity of these genes establishes a feedback mechanism that stabilizes these borders, allowing joint morphogenesis to ensue (Greenberg and Hatini, 2009). In addition, EGFR signaling is induced in the interjoint regions. These waves of EGFR signaling prevent formation of supernumerary joints by antagonizing some of the Notch effector genes (Galindo *et al.*, 2005; Shirai *et al.*, 2007). Other Notch downstream genes, such as *nubbin* and *d-AP2*, play a role in leg growth via mechanisms that have not yet been established (Kerber *et al.*, 2001; Rauskolb and Irvine, 1999).

10. Concluding Remarks

The above review reveals that a molecular framework of PD axis formation is now emerging. Yet, many questions remain. For one, the initial stages of dorsal and ventral primordia establishment are not well understood. How, for example, does *Sp1* block dorsal appendage fates? Second, although

graded levels of Wg and Dpp activities may not be relevant to elaborating the PD axis, it remains an open question whether a gradient of EGFR signaling is used to turn on its targets in the tarsus. Third, it is not clear how large a role transcriptional memory mechanisms play in maintaining PD gene expression domains once they are initially established, and how the transition from establishment to maintenance occurs. However, now that there are working hypotheses and a molecular framework for how the initial PD pattern is formed, it is likely that these and other questions will soon be answered.

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