The origins of the Drosophila leg revealed by the cis-regulatory architecture of the Distalless gene

Daniel J. McKay*, Carlos Estella* and Richard S. Mann†

Limb development requires the elaboration of a proximodistal (PD) axis, which forms orthogonally to previously defined dorsoventral (DV) and anteroposterior (AP) axes. In arthropods, the PD axis of the adult leg is subdivided into two broad domains, a proximal coxopodite and a distal telopodite. We show that the progressive subdivision of the PD axis into these two domains occurs during embryogenesis and is reflected in the cis-regulatory architecture of the Distalless (Dll) gene. EarlyDll expression, governed by theDll304 enhancer, is in cells that can give rise to both domains of the leg as well as to the entire dorsal (wing) appendage. A few hours afterDll304 is activated, the activity of this enhancer fades, and two later-acting enhancers assume control overDll expression. TheLt enhancer is expressed in cells that will give rise to the entire telopodite, and only the telopodite. By contrast, cells that activate theDKO enhancer will give rise to a leg-associated larval sensory structure known as the Keilin’s organ (KO). Cells that activate neitherLt norDKO, but had activatedDll304, will give rise to the coxopodite. In addition, we describe the trans-acting signals controlling theLt andDKO enhancers, and show, surprisingly, that the coxopodite progenitors begin to proliferate ~24 hours earlier than the telopodite progenitors. Together, these findings provide a complete and high-resolution fate map of the Drosophila appendage primordia, linking the primary domains to specific cis-regulatory elements inDll.

KEY WORDS: Distalless, Drosophila melanogaster, Leg, Limb primordium

INTRODUCTION

Evolutionary and genetic studies in arthropods suggest that the proximodistal (PD) axis of the leg is initially established by defining two primary domains (Snodgrass, 1935) (reviewed byBoxshall, 2004). The coxopodite, which includes the coxa, the most proximal leg segment, is thought to have been derived as an outgrowth of the body wall and may have been the ancestral, unsegmented appendage. The telopodite, or leg proper, includes all of the more distal leg segments, and is thought to have evolved subsequently, to allow more sophisticated leg movements by bendable joints that separate each leg segment. However, despite the existence of this primary subdivision, and its importance in arthropod evolution, a molecular understanding of this process is lacking.

Most of the molecular dissection of arthropod leg development has come from studying the leg imaginal discs of the fruit fly, Drosophila melanogaster. These studies suggest that the formation of the telopodite is under the control of the Hedgehog (Hh) signaling pathway, whereas the coxopodite forms independently of this pathway (Gonzalez-Crespo et al., 1998; Gonzalez-Crespo andMorata, 1996). For the telopodite to form, Hh induces the expression of two downstream signals, Wingless (Wg), ventrally, and Decapentaplegic (Dpp), dorsally (Basler andStruhl, 1994). The combinatorial action of Wg plus Dpp creates the PD axis of the leg by activating target genes such as Distalless (Dll) and dachshund (dac) (Campbell et al., 1993; Diaz-Benjumea et al., 1994; Estella andMann, 2008; Lecuit andCohen, 1997). Based on these studies, the sum of theDll and dac expression domains in a mature leg imaginal disc may correspond to the telopodite, a conclusion that is supported by studies in other arthropods (Abzhanov andKaufman, 2000). By contrast, there is no clear molecular marker for the coxopodite. Initially, the presence of nuclear Extradenticle (nExd), a homeodomain protein that requires the co-expression of homothorax (hth) for nuclear localization, was proposed to be a marker for the coxopodite in the leg imaginal disc (Gonzalez-Crespo andMorata, 1996; Rieckhoff et al., 1997). However, a true coxopodite gene should not be expressed distal to the coxa, and Hth-nExd are also expressed in the next-most distal leg segment, the trochanter (Abu-Shaar andMann, 1998). The molecular definition of these two domains is also complicated by the observation that the relative expression patterns ofDll and Hth-nExd change over time. WhenDll, the earliest marker of the leg primordium, is first activated in embryogenesis, allDll-expressing cells co-expressHth-nExd in circular domains comprising ~20 cells per thoracic hemisegment (Gonzalez-Crespo et al., 1998). Slightly later Hth-nExd are no longer expressed in a central subset of theDll domain, but the three proteins remain co-expressed in the remaining cells (Bolinger andBoekhoff-Falk, 2005; Mann andAbu-Shaar, 1996). Eventually, in the third instar leg imaginal disc, the expression domains of Hth-nExd andDll are mutually exclusive except for a thin ring of cells that co-express these genes and gives rise to the trochanter (Abu-Shaar andMann, 1998; Gonzalez-Crespo andMorata, 1996).

We reasoned that insights into how the telopodite and coxopodite are specified might come from characterizing the cis-regulatory elements that regulateDll in the embryonic leg primordia.Dll is initially activated at ~6 hours of embryonic development under the control of an early-acting enhancer calledDll304 (Vachon et al., 1992). Wg provides the anteroposterior (AP) positional cue that activatesDll304 (Cohen et al., 1993). Two other signaling pathways, Dpp and Epidermal growth factor receptor (Egfr) signaling, limit the leg progenitor domain dorsally and ventrally, respectively (Goto andHayashi, 1997; Kubota et al., 2000). Furthermore, although the Wg, Dpp and EGFR signals are deployed similarly in all embryonic trunk segments,Dll expression...
is limited to the thoracic segments by the abdominal Hox genes that directly repress Dll304 activity in the abdomen (Gebelein et al., 2002; Vachon et al., 1992).

Although Dll304 is activated by Wg and repressed by Dpp, Dll expression in the imaginal disc is activated by both signals (Campbell et al., 1993; Diaz-Benjumea et al., 1994), implying that additional Dll regulatory elements must exist. Recently, such a leg disc regulatory element, termed LT for ‘leg-trigger’, has been described (Estella et al., 2008). Unlike Dll304, LT continuously requires Wg and Dpp input for its activity in the leg disc. Although LT (also called Dll215) has been reported to be active in late stage embryos (Castelli-Gair and Akam, 1995; Cohen et al., 1993), its spatial relationship compared to Dll304 and its regulation by Wg and Dpp during embryogenesis has not been described. In addition, the lineages that Dll304- and LT-expressing cells give rise to have not been examined and may help inform how the coxopodite and telopodite are specified.

Another important unresolved set of questions concerns the relationship between the development of the adult and larval legs. As a holometabolous insect, Drosophila undergoes complete metamorphosis, meaning that the tissues that give rise to the adult structures, the imaginal discs, grow within the larva but do not contribute to the larval body plan. Nevertheless, Drosophila has rudimentary larval appendages called Keilin’s organs (KOs) that serve as thoracic-specific sensory organs. KOs are intimately associated with the developing leg imaginal disc (Lakes-Harlan et al., 1991; Madhavan and Schneiderman, 1977) and, like the adult telopodite, require Dll to form (Cohen and Jurgens, 1989). Although a group of cells within the Dll-expressing leg primordia express neural markers and is therefore thought to give rise to the KOs (Bolinger and Boekhoff-Falk, 2005; Cohen, 1993), its relationship to other Dll-dependent lineages has not been clearly defined.

Here we compare the spatial relationships, subsequent lineages and genetic inputs that regulate three Dll cis-regulatory elements, Dll304, LT, and a newly defined element, DKO, dedicated to the formation of the KOs. We show that when the leg primordia are first allocated, coincident with the activity of Dll304, this domain is multipotent and has the potential to give rise not only to the entire telopodite, coxopodite and KO, but also to dorsal (e.g. wing) appendage fates. A few hours later, Dll304 activity fades, and LT and DKO are activated in mutually exclusive subsets of the Dll304-expressing domain. In contrast to the multipotency of the Dll304 expression domain, LT-expressing cells give rise to the entire telopodite and only the telopodite, while DKO-expressing cells give rise to the KO. As in the leg imaginal discs, LT requires both Wg and Dpp to be activated during embryogenesis. In addition, we show that the telopodite fate is repressed by the KO fate, suggesting that these two sets of progenitor cells are mutually antagonistic. Surprisingly, we also find that the onset of coxopodite growth is advanced relative to the telopodite fate is repressed by the KO fate, suggesting that these two sets of progenitor cells are mutually antagonistic. Surprisingly, we also find that the onset of coxopodite growth is advanced relative to the telopodite. As in the leg imaginal discs, LT requires both Wg and Dpp input for its activity in the leg disc.

**RESULTS**

**The LT and DKO enhancers are active in mutually exclusive subsets of the limb primordia**

In light of the identification of the LT enhancer as a direct integrator of Wg and Dpp during leg imaginal disc development (Estella et al., 2008), we examined the activity of this enhancer relative to Dll and Dll304 during embryonic stages. Dll protein in the thorax was first detected during embryonic stage 11 (Fig. 1B), and continued to be visualized in this region until the end of embryogenesis (Fig. 1C; data not shown). Although a Dll304-lacZ transgene recapitulated the initial pattern of Dll expression (Fig. 1D), the activity of this enhancer decayed within a few hours (Cohen et al., 1993). By contrast, an LT-lacZ reporter gene became active in Dll-expressing cells of the thorax after germ-band retraction, with robust expression by stage 14 (Fig. 1E). Importantly, the LT enhancer was not active in all of the Dll-expressing cells of the thorax. LT-lacZ was expressed in the outermost ring of ~15 cells of the Dll clusters (Fig. 1G). At this stage, LT-lacZ-expressing cells also expressed lth, esg and teashirt (tsb; Fig. 1H, I, and data not shown). Because esg is required for the maintenance of diploidy, it has been suggested that...
esg-expressing cells give rise to the imaginal discs (Hayashi et al., 1993). By contrast, the Dll-expressing LT-lacZ-nonexpressing cells within the LT ring did not express esg. Instead, these cells expressed cut (ct), which encodes a transcription factor required for the development of external sensory organs (Bodmer et al., 1987). Accordingly, these cells may give rise to the KO (Bolinger and Boekhoff-Falk, 2005; Cohen et al., 1993).

Because the ct-expressing cells expressed Dll but not LT-lacZ, there must be additional cis-regulatory elements controlling Dll expression in these cells. To identify these elements, we cloned a conserved region of the Dll gene located approximately 3 kb 5’ to the start of transcription to create a transgenic reporter gene which we named DKO-lacZ (Fig. 1A). By stage 14 DKO was active in the Dll-expressing cells that also express ct (Fig. 1J; see Fig. S1B,C in the supplementary material). Unlike LT-lacZ, DKO-lacZ was not expressed in third instar leg discs (data not shown). However, DKO-lacZ was expressed in other cells of the embryonic peripheral nervous system that do not express Dll. The ectopic expression of DKO-lacZ indicates that this element lacks repressor input that normally limits its activity to the limb primordia. Within the leg primordia, however, the LT and DKO expression domains are mutually exclusive in stage 14 embryos, such that the sum of the LT and DKO expression domains accounts for all Dll expression (Fig. 1J). These data suggest that by stage 14 the fates of the Dll-expressing cells of the thoracic limb primordia have been determined, and they can be subdivided into two distinct populations of cells in which different Dll cis-regulatory elements are active.

Existence of different cell fates in the ventral limb primordia

To determine whether the two cell types defined above give rise to different fates, we performed lineage-tracing experiments with a panel of Gal4 drivers, including drivers made with these enhancer elements. In general, lineages were analyzed by using these Gal4 drivers to express the yeast recombinase Flp, which deleted a transcriptional stop cassette from an actin-lacZ transgene (actin>stop>lacZ; see Materials and methods for details).
Lineage tracing using Dll304-Gal4 labeled cells in both the dorsal and ventral appendages (Fig. 2A), indicating that this enhancer was active prior to the separation of leg and wing fates, consistent with previous cell-lineage analyses (Wieschaus and Gehring, 1976). Within the leg, cells were labeled in both the coxopodite and telopodite, unlike the distal-only pattern of Dll immunostaining in mature third instar leg discs (Fig. 2A). This pattern matches that generated by Dll-Gal4, an enhancer trap into the Dll locus (line MD 23, see Material and methods; see Fig. S2A in the supplementary material) (Campbell and Tomlinson, 1998).

By contrast, lineage-tracing using LT-Gal4 demonstrated that LT was not active in any cells that give rise to the wing or other dorsal appendages. In both the leg disc and adult leg, the LT lineage coincided with the Dll- and dac-expressing telopodite and did not contribute to the peripodial epithelium (Fig. 2B; see Fig. S2B in the supplementary material). In the coxopodite, the LT lineage analysis consistently labeled a small group of cells in the dorsal-most stalk region (Fig. 2B). The few labeled cells in the coxopodite may be the result of the imperfection of the LT enhancer when out of its normal genomic context. Importantly, the entire telopodite was also labeled when LT-Gal4 activity was limited exclusively to embryogenesis by using a tub-Gal80 ts transgene to suppress Gal4 activity during larval stages (see Material and methods for experimental details). These data suggest that the ~15 LT-expressing cells of the embryonic limb primordia give rise to the entire telopodite. This is surprising given that, in the embryo, these cells also express hh and tsh (Fig. 1H), which are genes that are expressed only in the proximal domain of the third instar leg disc and have therefore been considered to be coxopodite markers.

Fig. 2. Lineage analyses of genes active in the ventral limb primordia. All discs except for those in F were stained for the lineage marker (red), Dll (green, subset of telopodite), and Hth (blue, coxopodite); see Materials and methods for details. (A) The progeny of cells in which Dll304 was active contribute to both dorsal (wings and halteres) and ventral (legs, both coxopodite and telopodite) thoracic limbs. Although individual wing discs show labeling in only a subset of the disc, labeled cells can contribute to any part of the disc. (B) The progeny of cells in which LT was active generate the telopodite of the leg. Expression in the dorsal coxopodite (arrow) may be due to the imperfection of the LT-Gal4 driver. The arrowhead marks a clone in the trochanter region. (C,D) The progeny of cells that expressed tsh become more restricted over time. Restricting tsh-Gal4 activity to the beginning of second instar (48-72 hours AEL) results in the labeling of both the coxopodite and telopodite (C). Allowing tsh-Gal4 to be active beginning at third instar (72-96 hours AEL) results in the labeling of only the coxopodite (D). The asterisk in D indicates lacZ-positive adepithelial cells that are not part of the disc epithelium. (E) The progeny of esg-expressing cells adopt both wing and leg (coxopodite and telopodite) fates. (F) The progeny of the cells in which DKO was active (red) occasionally contribute to larval neurons that co-express Elav (blue, arrow). All lacZ-positive cells express elav but not all elav cells are lacZ positive (see inset).
To confirm that the telopodite progenitor cells also express hth and tsh, we performed lineage-tracing experiments using tsh-Gal4 and hth-Gal4. Because the entire thoracic ectoderm expresses tsh and hth prior to the initiation of Dll expression, the tub-Gal80ts transgene was used to control the activity of these Gal4 drivers. Raising the animals at the temperature where Gal80ts was active for all of development (the permissive temperature) resulted in no lacZ expression (data not shown), confirming the efficacy of the Gal80ts protein. Switching the animals to the nonpermissive temperature at the beginning of the second larval instar (~48 hours) resulted in lacZ expression throughout the entire leg disc (Fig. 2C), indicating that tsh was active in both coxopodite and telopodite progenitors long after LT activation in the embryo. By contrast, switching the animals to the nonpermissive temperature at the beginning of the third larval instar (~72 hours) consistently labeled the coxopodite, but rarely labeled the telopodite (Fig. 2D). Similar results were obtained using hth-Gal4 instead of tsh-Gal4 (data not shown).

Although these experiments identify the progenitors of the telopodite, they open the question of which embryonic cells give rise to the coxopodite. Because the product of the esg gene is required for all imaginal disc fates, we reasoned that Esg-positive, LT-nonexpressing cells would be the progenitors of the coxopodite. Such a population of cells exists just ventral to the LT-expressing ring (Fig. 1I). Consistently, all leg and wing disc cells were labeled when a lineage analysis was performed using esg-Gal4 (Fig. 2E). Thus, we conclude that the coxopodite is derived from the esg-expressing cells that are present just ventral to the LT-positive cells of the leg primordia (Fig. 1I).

Because the Dll- and DKO-lacZ-expressing cells also express ct and elav, but not esg (Fig. 1G-J; see Fig. S1A in the supplementary material), these cells were predicted to be the progenitors of the larval KO. To test this, we carried out lineage tracing using a DKO-Gal4 transgene. One-third (n=40) of these third instar leg discs had no lacZ expression, demonstrating that DKO-expressing cells did not contribute to imaginal disc fates. Approximately one third of the discs contained small numbers of lacZ-positive cells that co-expressed the neural marker Elav (Fig. 2F, see inset). These neurons may be the same as previously described, embryonically born neurons that persist until larval stages (Tix et al., 1989). The cell bodies of these neurons reside in the leg imaginal disc and project dendrites to the KO in the larval epidermis (Tix et al., 1989). Finally, approximately one-third of the discs had lacZ-expressing clones present in the disc epithelia. Because the DKO element is expressed in Dll-negative cells (see above), these clones probably result from the spurious activity of this enhancer. Altogether, these data are consistent with an earlier report (Bolinger and Boekhoff-Falk, 2005) and support the conclusion that the Dll-positive, Ct-positive cells in the center of the leg primordia, previously considered to be the progenitors of the telopodite, are the progenitors of the Keilin’s organ and do not contribute to the imaginal disc. These conclusions were further confirmed by using these Gal4 drivers to express the proapoptotic gene hid (Zhou et al., 1997) to induce cell death (see Fig. S2C in the supplementary material).

In summary, when Dll304 is first activated, Dll-positive cells have the potential to give rise to all regions of the dorsal and ventral appendages. A few hours later three cell types are defined: the KO progenitors [Dll(DKO)-positive, esg-, hth- and tsh-negative], the

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*Fig. 3. Regulation of LT by Wg and Dpp. (A) Cells that activate LT (red) at stage 14 in the limb primordia are close to cells expressing high levels of Wg (blue) and Dpp (visualized by pMad staining, green). (B) A wgts stage 14 embryo raised at the permissive temperature stained for Ct (blue), Dll (green) and LT activity (red). (B') An enlargement of the leg primordium boxed in B. LT activity in the head segments (arrows) is not affected in the wgts embryos. (C) A wgts stage 14 embryo shifted to the restrictive temperature at 10-14 hours, after the initial activation of Dll and Dll304 (see methods), stained for Dll (green), Ct (blue), and LT activity (red). Dll expression is still observed, probably due to the activity of Dll304, but LT activity and Ct are not observed. (C') An enlargement of the leg primordium boxed in C. (D-F) Dpp and Wg activate LT. prd-Gal4 is expressed in T2 but not T1 and T3. LT (red) and Dll (green) are activated dorsally by prd-arm* in T2 (arrow; D). LT activity (red) and Dll levels are reduced via prd-Dad (arrow; E). LT activity (red) and Dll (green) are expanded ventrally via prd-Tkv65D (arrow; F). Insets show single channels for Dll and LT activity.*
telopodite progenitors [Dll(LT)-positive, esg-, hth- and tsh-positive], and the coxopodite progenitors (Dll-negative, esg-, hth- and tsh-positive). Together, these cells comprise the entire thoracic ventral limb primordia (Fig. 1F).

**Regulation of LT and DKO activity by Wg and Dpp**

To determine how each of these cell fates in the limb primordia is specified, we carried out genetic experiments to identify the regulators of the LT and DKO enhancers. Consistent with LT’s dependency on wg and dpp for leg disc expression (Estella et al., 2008), LT is activated in the embryo in cells that receive both inputs, as monitored by anti-Wg and anti-PMad staining (Fig. 3A). To determine whether wg is required for LT activity, we used a temperature-sensitive allele of wg to allow earlier Dll activation (Cohen et al., 1993). Switching the embryos to the restrictive temperature at stage 11 resulted in the absence of LT activity, despite the presence of Dll protein (probably derived from Dll304 activity; Fig. 3C). In addition, ectopic activation of the wg pathway [using an activated form of armadillo (arm*)] resulted in more LT-lacZ-expressing cells (Fig. 3D).

Like wg, the dpp pathway is necessary for LT-lacZ expression in leg discs. Paradoxically, dpp signaling represses Dll in the embryo because dpp mutants show an expansion in Dll304-lacZ expression (data not shown) (Goto and Hayashi, 1997). By contrast, LT-lacZ is not expressed in dpp null embryos (data not shown). LT-lacZ, but not Dll protein, was also repressed by two dpp pathway repressors, Dad and brk (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999; Tsuneizumi et al., 1997) (Fig. 3E; and data not shown). Conversely, stimulation of the dpp pathway [using an activated form of the Dpp receptor (TkVDSL)] resulted in ectopic activation of LT ventrally (Fig. 3F).

Taken together, these data demonstrate that LT is activated by Wg and Dpp in the embryonic limb primordia, just as it (and Dll) is in the leg disc. Similarly, DKO activity also requires Wg and Dpp input (see Fig. S3D,E in the supplementary material).

**Dll and btd confer ventral thoracic-specificity to LT expression**

Although LT is activated by wg and dpp in the leg primordia, these signals are also present in each abdominal segment. Consequently, there must be additional factors that restrict LT activity to the thorax. One possibility is that LT is repressed by the abdominal Hox factors, such as Dll304 (Gebelein et al., 2002; Vachon et al., 1992). Alternatively, LT might be regulated by Dll, itself (Castelli-Gair and Akam, 1995). We found that in Dll null embryos LT-lacZ was initially expressed in a stripe of cells instead of a ring, but then...
expression decayed (Fig. 4C). Ectopic expression of Dll resulted in weak ectopic expression of LT-lacZ in the thorax and abdomen (see Fig. S3A in the supplementary material). These data suggest that LT activity is restricted to the thorax in part because of the earlier restriction of Dll304 activity to the thorax.

The related zinc-finger transcription factors encoded by buttonhead (btd) and Sp1 are also expressed in the limb primordia and are also required for ventral appendage specification (Estella et al., 2003). In strong btd hypomorphs, the activity of LT was still detected but the number of cells expressing LT-lacZ was decreased and its pattern was disrupted (see Fig. S3C in the supplementary material). LT-lacZ expression was completely eliminated in animals bearing a large deficiency that removes both btd and Sp1 (Fig. 4B). By contrast, Dll304 was activated normally in these animals (data not shown). Importantly, LT-lacZ expression was rescued by expressing bid in these deficiency embryos (Fig. 4B). By contrast, expressing Dll, tkbD, or arm did not rescue LT expression in these deficiency embryos (data not shown). Ectopic expression of btd resulted in weak ectopic activation of LT-lacZ in cells of the thorax and abdomen (see Fig. S3B in the supplementary material). Strikingly, the simultaneous expression of Dll and bid resulted in robust ectopic expression of LT-lacZ in abdominal segments in the equivalent ventrolateral position as the thoracic limb primordia (Fig. 4D). bid and Dll were not sufficient to activate LT in wg null embryos (data not shown). These data indicate that the thoracic-specific expression of the LT enhancer is controlled by the combined activities of bid and/or Sp1, Dll and the wg and dpp pathways.

Proneural genes activate DKO and repress LT

Although the above data suggest that LT is activated by a combination of Wg, Dpp, Btd and Dll, these activators are also present in the precursors of the KO, which activate DKO instead of LT. Because the KO is a sensory structure, we tested the role of members of the achaete-scute complex (ASC) that are expressed in these cells (Bolinger and Boekhoff-Falk, 2005). In embryos hemizygous for a deficiency that removes the achaete-scute complex (ASC) that are expressed in the limb primordia (asense) repressed LT and increased the number of Ct-expressing cells (Fig. 4F). These data suggest that there is a mutual antagonism between the progenitors of the telopodite and those of the KO. We also found that DKO-lacZ expression in the leg primordia was lost in Dll or bid null embryos, consistent with the loss of KOs in these mutants (Cohen and Jurgens, 1989; Estella et al., 2003) (data not shown). DKO activity was also lost from the limb primordia in embryos deficient for the ASC (Fig. 4H). These results indicate that DKO is activated by the same genes that promote LT expression but, in addition, requires proneural input from the ASC.

Distinct cell proliferation dynamics in the coxopodite and telopodite

To follow the development of the telopodite, we performed a time-course experiment to visualize LT-expressing cells throughout larval development. esg-lacZ was used to identify all imaginal disc cells and LT-Gal4, UAS-GFP was used to mark the progenitors of the telopodite. We estimate that there are ~15 progenitor cells each for the telopodite and coxopodite in stage 14 embryos (Fig. 5A). Previous clonal analyses suggested that the cells of the leg primordia stop dividing during embryogenesis and resume proliferation at approximately 48 hours (Bryant and Schneiderman, 1969). Consistent with these studies, the number of Esg-positive cells began increasing at the beginning of the second larval instar, about 48 hours AEL (Fig. 5A). Surprisingly, at ~60 hours, the coxopodite progenitors (Esg-positive, LT- and Dll-negative) far outnumbered the telopodite progenitors (LT- and Dll-positive). In addition, the nuclei of the telopodite progenitor cells were larger than those of the coxopodite progenitor cells in these early leg discs. The telopodite progenitors also appear to be tightly associated with the larval epidermis, and they continued to express hth and tsh, in addition to Dll and esg (Fig. 5A,B). By the end of the second larval instar stage, between 60 and 72 hours AEL, the entire leg disc invaginated from the larval cuticle, and the number of LT-positive cells was dramatically increased (Fig. 5B). At this stage, these cells no longer expressed tsh or hth (Fig. 5B; and data not shown).

These data suggest that there is a difference in the time when the progenitor cells of the coxopodite and the telopodite begin to proliferate. By direct observation, we estimate that the coxopodite progenitors begin to divide between 12 and 24 hours earlier than those of the telopodite. To rule out that LT-positive cells start to proliferate at the same time, and LT is rapidly shut off in some progeny, we repeated the LT-Gal4 lineage analysis, comparing at early time points the number of cells in which LT had been active with the number that continued to express LT. Fig. 5C shows that these two cell populations are identical, suggesting that the progenitors of the telopodite rarely proliferate prior to this time.

We confirmed the delay in the onset of telopodite proliferation by inducing marked clones in both domains between 12 and 24 hours AEL, and quantifying the location and number of cells 36 and 48 hours later. For these experiments, we defined the coxopodite as being Hth- or Tsh-positive and LT-GFP-negative. Conversely, the telopodite progenitors were defined as being LT-GFP-positive. At the 36-hour time point, the average number of cells in telopodite clones was 1.3 (n=31; Fig. 5D; see Fig. S4A in the supplementary material). By contrast, the average number of cells in coxopodite clones was 3.2 (n=51; Fig. 5D; see Fig. S4A in the supplementary material). All KO clones (LT-GFP-negative and Hth/Tsh-negative) remained as single cells (n=11). When measured 48 hours after clone induction, the average number of cells in telopodite clones was 4.6 (n=9) while the average number of cells located in coxopodite clones was 5.6 (n=20; Fig. 5D; see Fig. S4B in the supplementary material). These data suggest that the progenitors of the coxopodite resume proliferating approximately one to two cell divisions earlier than the progenitors of the telopodite.

Interestingly, we found that telopodite and coxopodite clones stayed within their respective domains (LT-GFP expressing or non-expressing, respectively) (see Fig. S4A,B in the supplementary material). When clones (n=25) induced between 12 and 24 hours were allowed to grow to the third instar, their progeny continued to demonstrate a restriction in lineage (Fig. 5E). However, both sets of clones could enter the trochanter, which expresses both hth and Dll (see Fig. S5A,B in the supplementary material). By contrast, clones induced prior to stage 14 (5.5-7 hours AEL) occasionally spanned both the coxopodite and telopodite (19%, n=32; see Fig. S4C in the supplementary material). These data suggest that there is a lineage restriction along the PD axis of the developing leg that forms at stage 14, about the same time that LT is activated in the limb primordia. This lineage restriction does not constitute a compartment boundary, however, because when cells were given a growth advantage using the Minute technique (Morata and Ripoll, 1975) we observed clones that did not respect this boundary (data not shown). Moreover, this restriction is not a discreet border, but is instead defined by a region (the trochanter), that expresses both telopodite (Dll) and coxopodite (Hth) markers.
DISCUSSION
A cascade of Dll cis-regulatory elements prefigures cell fates in the appendage primordia
One of the most interesting findings from this work is that the temporal control of Dll expression in the limb primordia by three cis-regulatory elements is linked to cell-type specification (Fig. 6). The earliest acting element, Dll304, is active throughout the appendage primordia. At the time Dll304 is active, the cells are multipotent and can give rise to any part of the dorsal or ventral appendages, or KO. A few hours later, Dll304 activity fades, and two alternative cis-regulatory elements become active. Together, these two elements allow for the uninterrupted and uniform expression of Dll within the appendage primordia. However, their activation correlates with a higher degree of refinement in cell fate potential: LT, active in only the outer ring of the appendage primordia, is only expressed in the progenitors of the telopodite. By contrast, DKO,
active in the cells within the LT ring, is only expressed in the progenitors of the KO. Thus, although the pattern of Dll protein appears unchanged, the control over Dll expression has shifted from singular control by Dll304 to dual control by LT and DKO. Moreover, not only is there a molecular handoff from Dll304 to LT and DKO, the two later enhancers both require the earlier expression of Dll. Thus, the logic of ventral primordia refinement depends on a cascade of Dll regulatory elements in which the later ones depend on the activity of an earlier one.

The high-resolution view of the embryonic limb primordia provided here allows us to clarify some contradictions that currently exist in the literature. Initial expression of Dll in the thorax overlaps entirely with Hth-nExd. Subsequently, hth expression is lost from most, but not all, of the Dll-expressing cells of the leg primordia. The first reports describing these changes failed to recognize the persistent overlap between Dll and Hth-nExd in some cells (Gonzalez-Crespo et al., 1998; Gonzalez-Crespo and Morata, 1996). As a result, and partly because of the analogy with the third instar leg disc, the predominant view of this fate map became that the Dll-positive, Hth-nExd-negative cells of the embryonic primordia gave rise to the telopodite, while the surrounding Hth-positive cells gave rise to the coxopodite (reviewed by Morata, 2001). The expression pattern of esg, a gene required for the maintenance of diploidy, was also misinterpreted as being a marker exclusively of proximal leg fates (Goto and Hayashi, 1997; Kubota et al., 2000; Kubota et al., 2003). Counter to these earlier studies, our experiments unambiguously show that the Dll-positive, Hth-nExd-negative cells in the center of the primordia give rise to the KO, the ring of Dll-positive, Esg-positive, Hth-nExd-positive cells gives rise to the telopodite, and the remaining Esg-positive, Dll-negative cells give rise to the coxopodite (Fig. 6).

The spurious expression of DKO-lacZ in Dll-non-expressing cells outside the leg primordia complicates the interpretation of several experiments. Attempts to refine DKO activity by changing the size of the cloned fragment proved unsuccessful. Nevertheless, our evidence supports the idea that DKO-positive, Dll-positive cells of the leg primordia give rise to the Keilin’s organ, and not the adult appendage.

**Regulation of proliferation along the PD axis of the developing leg**

The progenitors of the coxopodite begin to proliferate at approximately 48 hours of development, consistent with previous measurements of leg imaginal disc growth, whereas the progenitors of the telopodite do not resume proliferating for an additional 12 to 24 hours. According to estimates of the cell cycle time in leg discs
(Postlethwait, 1978), this difference in the onset of proliferation results in one to two additional cell divisions in the coxopodite, consistent with images of late second instar leg discs presented here. Why might the telopodite and coxopodite begin proliferation at different times? One possibility is that the cells of the coxopodite give rise to the peripodial epithelium that covers the leg imaginal disc, and therefore require additional cell divisions relative to the telopodite. It is also possible that the telopodite is delayed because the neurons of the Keilin’s organ serve a pathfinding role for larval-born neurons that innervate the adult limb (Jan et al., 1985). Perhaps this pathfinding function requires that the KO and telopodite remain associated with each other through the second instar. Consistently, the leg is the only imaginal disc that has not invaginated as a sac-like structure in newly hatched first instar larvae (Madhavan and Schneiderman, 1977).

A possible explanation for the delay in the onset of telopodite proliferation is the persistent co-expression of hth andDll in these cells; hth and tsh expression is turned off in these cells at about the same time they begin to proliferate. Consistent with this idea, maintaining the expression of hth throughout the primordia blocks the proliferation of the telopodite (see Fig. S5C in the supplementary material) (Azpiazu and Morata, 2002). Also noteworthy is the finding that the genes no ocelli and elbow have been shown to mediate the ability of Wg and Dpp to repress coxopodite fates (Weihe et al., 2004). Together with our findings, it is possible that the activation of these two genes in the LT-expressing progenitors is the trigger that turns off hth and tsh in these cells.

Restriction of cell lineage between coxopodite and telopodite

Our experiments suggest that once LT is activated, and under normal growth conditions, there is a lineage restriction between the telopodite and coxopodite. By contrast, previous lineage-tracing experiments using tsh-Gal4 concluded that the progeny of proximal cells could adopt more distal leg fates (Weigmann and Cohen, 1999). However, these authors were unaware that tsh is still expressed in the telopodite progenitors far into the second instar, providing an explanation for their results. In contrast to this early restriction, there is no evidence for a later lineage restriction within the telopodite. For example, the progeny of a Dll-positive cell can lose Dll expression and contribute to the dac-only domain (Gorfinkiel et al., 1997).

Interestingly, the lineage restriction between coxopodite and telopodite is not defined by the presence or absence of Hth-nExd or Tsh because both progenitor populations express hth and tsh after their fates have been specified. By contrast, when these two domains are specified, the telopodite expresses Dll, while the coxopodite does not, suggesting that Dll may be important for the lineage restriction. However, later in development, some cells in the telopodite lose Dll expression and express dac, but continue to respect the coxopodite-telopodite boundary. Thus, either Dll expression in the telopodite is somehow remembered or the telopodite-coxopodite boundary can be maintained by dac, which is expressed in place of Dll immediately adjacent to the telopodite-coxopodite boundary. Also noteworthy is our finding that clones originating in the coxopodite can contribute to the trochanter, the segment inbetween the proximal and distal components of the adult leg that expresses both Dll and hth in third instar imaginal discs (Abu-Shaar and Mann, 1998). However, the progeny of such clones do not contribute to fates more distal than the trochanter. Likewise, a clone originating in the telopodite can also contribute to the trochanter, but will not grow more proximally into the coxa (see Fig. S5A,B in the supplementary material). Thus, the lineage restriction uncovered here seems to be determined by distinct combinations of transcription factors expressed in the coxopodite and telopodite progenitors at stage 14. The progeny of cells that express Dll, tsh and hth can populate the telopodite or trochanter, whereas the progeny of cells that express tsh and hth, but not Dll, can populate the coxopodite or trochanter. In light of the Minute-positive results, however, the lineage restriction between coxopodite and telopodite does not satisfy the classical definition of a compartment boundary. A similar non-compartment lineage restriction has also been documented along the PD axis of the developing Drosophila wing (Zirin and Mann, 2007).

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/1/61/DC1

References


Drosophila embryonic limb primordium


