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

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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. Early *Dll* expression, governed by the *Dll304* 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 after *Dll304* is activated, the activity of this enhancer fades, and two later-acting enhancers assume control over *Dll* expression. The *LT* enhancer is expressed in cells that will give rise to the entire telopodite, and only the telopodite. By contrast, cells that activate the *DKO* enhancer will give rise to a leg-associated larval sensory structure known as the Keilin's organ (KO). Cells that activate neither *LT* nor *DKO*, but had activated *Dll304*, will give rise to the coxopodite. In addition, we describe the trans-acting signals controlling the *LT* and *DKO* 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 in *Dll*.

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 by Boxshall, 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 and Morata, 1996). For the telopodite to form, Hh induces the expression of two downstream signals, Wingless (Wg), ventrally, and Decapentaplegic (Dpp), dorsally (Basler and Struhl, 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 and Mann, 2008; Lecuit and Cohen, 1997). Based on these studies, the sum of the *Dll* 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 and Kaufman, 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 and Morata, 1996; Rieckhof 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 and Mann, 1998). The molecular definition of these two domains is also complicated by the observation that the relative expression patterns of Dll and Hth-nExd change over time. When *Dll*, the earliest marker of the leg primordium, is first activated in embryogenesis, all *Dll*-expressing cells co-express Hth-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 the Dll domain, but the three proteins remain co-expressed in the remaining cells (Bolinger and Boekhoff-Falk, 2005; Mann and Abu-Shaar, 1996). Eventually, in the third instar leg imaginal disc, the expression domains of Hth-nExd and Dll are mutually exclusive except for a thin ring of cells that co-express these genes and gives rise to the trochanter (Abu-Shaar and Mann, 1998; Gonzalez-Crespo and Morata, 1996).

We reasoned that insights into how the telopodite and coxopodite are specified might come from characterizing the cis-regulatory elements that regulate *Dll* in the embryonic leg primordia. *Dll* is initially activated at ~6 hours of embryonic development under the control of an early-acting enhancer called *Dll304* (Vachon et al., 1992). Wg provides the anteroposterior (AP) positional cue that activates *Dll304* (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 and Hayashi, 1997; Kubota et al., 2000). Furthermore, although the Wg, Dpp and EGFR signals are deployed similarly in all embryonic trunk segments, *Dll* expression

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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 onset of telopodite growth, which begins only after Hth-nExd are turned off in these cells at ~60 hours of development. These experiments thus provide a complete description of all the cell types within the *Drosophila* leg primordia, their temporal development, and the subsequent structures they generate.

MATERIALS AND METHODS

Transgenes

LT-lacZ and *LT-Gal4* have been previously described (Estella et al., 2008). The DKO fragment was selected based on sequence conservation to other *Drosophilids* (Vista Genome Browser), and cloned by PCR (details are available upon request) into the *hs43-nuc-lacZ* vector (Estella et al., 2008). The DKO and 304 enhancers were also cloned in pChs-Gal4.

Immunostaining

Discs and embryos were stained using standard procedures. The primary antibodies used were, rabbit and mouse anti-β-Gal (Cappell and Promega), mouse anti-Wg, mouse anti-Cut, mouse anti-En, mouse anti-Dac and mouse anti-Elav (Developmental Studies Hybridoma Bank, University of Iowa). Guinea pig anti-P-Mad (a gift from E. Laufer and T. Jessell), guinea pig anti-Dll (generated against the full-length protein), rabbit anti-Hth generated against the full-length protein, mouse anti-Ubx (Crickmore and Mann, 2006), guinea pig anti-Tsh (a gift from G. Struhl).

Drosophila stocks and mutant analysis

lacZ lines, Gal4 lines and lineage analysis

esg-lacZ (Hayashi et al., 1993), *esg*-Gal4 (Goto and Hayashi, 1999), *Dll*-Gal4 (line MD 23) (Calleja et al., 1996), *Dll*-Gal4 (line 212) (Gorfinkiel et al., 1997), *tsh*-Gal4 (Wu and Cohen, 2000), *hth*-Gal4 (GETDB-Gal4 Enhancer Trap Insertion Data Base), *prd*-Gal4 (Gebelein et al., 2004). Two *Dll*-Gal4 lines were used: MD 23 recapitulates all *Dll* expression whereas em 212 does not capture early (*Dll304*) expression.

Lineage analyses and neutral clones used *act5C>stop>lacZ; UAS-flp* (Struhl and Basler, 1993). In the *tsh-Gal4* and *hth-Gal4* lineage experiments flies of the genotype *tub-Gal80^{ts}; tsh-Gal4; tub-Gal80^{ts}* (Zirin and Mann, 2007) were crossed to *act5C>stoP>lacZ; UAS-flp* at 18°C. At various time points, 12-hour collections were transferred to 29°C. Female larvae were dissected at the third instar. Developmental stage at time of transfer to the restrictive temperature was determined based upon the amount of time spent at 29°C before dissection. *tub-Gal80^{ts}* was also used to show that limiting *LT-Gal4* activity to the embryo is sufficient to label the entire telopodite. For the *Minute* experiment we generated wild-type clones in a *Minute* heterozygous background in telopodite precursor cells using the *LT-Gal4*; UAS-*flp*.

Mutant lines

Dll^{SA1} (Vachon et al., 1992), *Df(1)sc-B57* (Dominguez and Campuzano, 1993), *btd^{XG81}* and *Df(1)C52* (Cohen, 1990) were used. The *wg* temperature-sensitive experiments (*Wg^{IL114}*) were performed as previously described (Cohen et al., 1993) with modifications. Embryos were kept at the permissive temperature (18°C) until *Dll* expression is initially activated (~10-14 hours), then transferred to the restrictive temperature (29°C) until stage 14.

UAS lines

UAS-*hid* (Zhou et al., 1997), UAS-*ase* (Brand and Dormand, 1995), UAS-*arm* (delta N) (Chan et al., 1997), UAS-*tkv^{QD}* (Abu-Shaar and Mann, 1998), UAS-*dad* (Tsuneizumi et al., 1997), UAS-*brk* (Jazwinska et al., 1999), UAS-*Dll* (Gorfinkiel et al., 1997), UAS-*btd* (Schock et al., 1999) and UAS-*p35* (Bloomington Center) were used.

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 *hth*, *esg* and *teashirt* (*tsh*; Fig. 1H,I, and data not shown). Because *esg* is required for the maintenance of diploidy, it has been suggested that

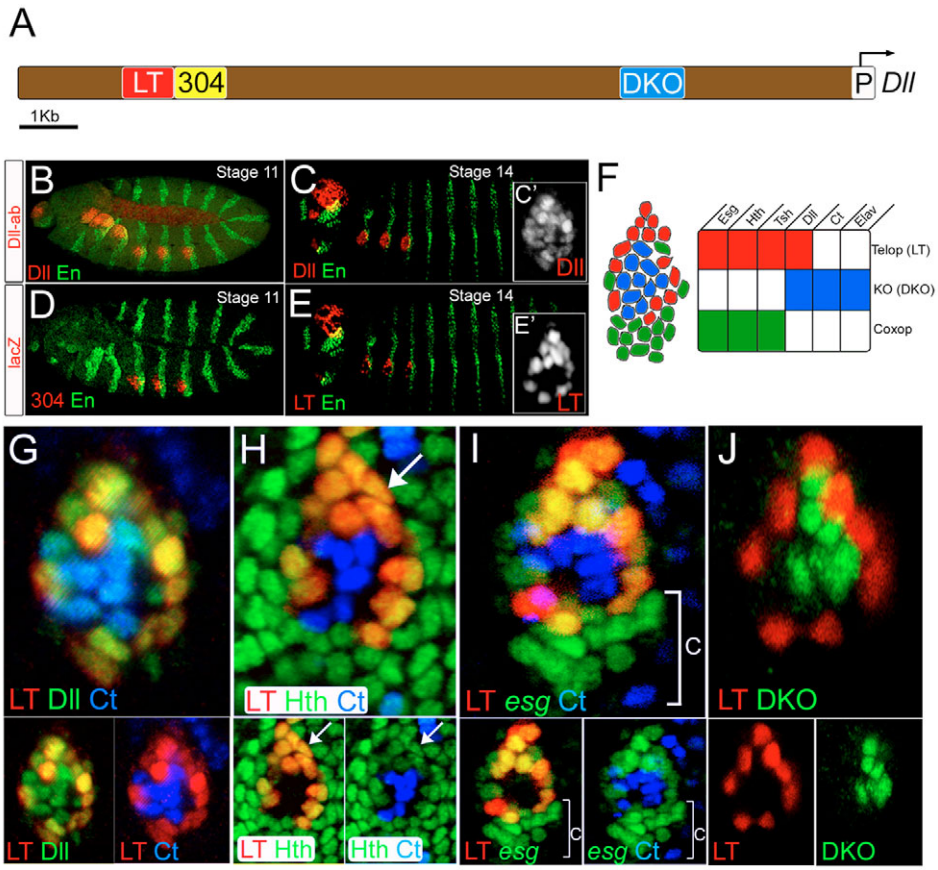

Fig. 1. *LT* and *DKO* are active in mutually exclusive subsets of the limb primordia. Embryos are oriented anterior to the left and dorsal up. (**A**) The *Dll* 5′ cis-regulatory region. *LT* is in red, *Dll304* in yellow, *DKO* in blue, and the *Dll* promoter in white. (**B**,**C**) Stage 11 (B) and stage 14 (C) embryos stained for Dll (red) and En (green). (**C′**) A magnification of the Dll-positive cells at stage 14. (**D**) A stage 11 embryo stained for *Dll304* activity (red) and En (green). (**E**) A stage 14 embryo stained for *LT* activity (red) and En (green). (**E′**) A magnification of *LT*-positive cells at stage 14. LT is active in a subset of *Dll*-expressing cells (compare with C′). (**F**) Schematic representation of a leg primordium at stage 14. (**G**) Dll (green), Ct (blue) and *LT* activity (red) in a leg primordium at stage 14. *LT* activity is mutually exclusive with Ct expression. *LT*-positive and Ct-positive cells are subsets of the *Dll* expression domain. (**H**) Hth (green), Ct (blue) and *LT* activity (red) in a leg primordium at stage 14. *LT*-positive cells also express Hth (arrow). *ct* and *hth* are expressed in mutually exclusive domains. (**I**) *esg-lacZ* (green), Ct (blue) and *LT* activity (red) in a leg primordium at stage 14. *esg* and Ct are mutually exclusive and *LT* activity overlaps with a subset of the *esg*-expressing cells. The ventral *esg*-expressing, *LT*-negative cells (bracket) are the coxopodite progenitor cells. (**J**) *LT* (red) and *DKO* (green) activities are mutually exclusive within the *Dll*-positive cells of the leg primordium at stage 14.

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 *hth* 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.

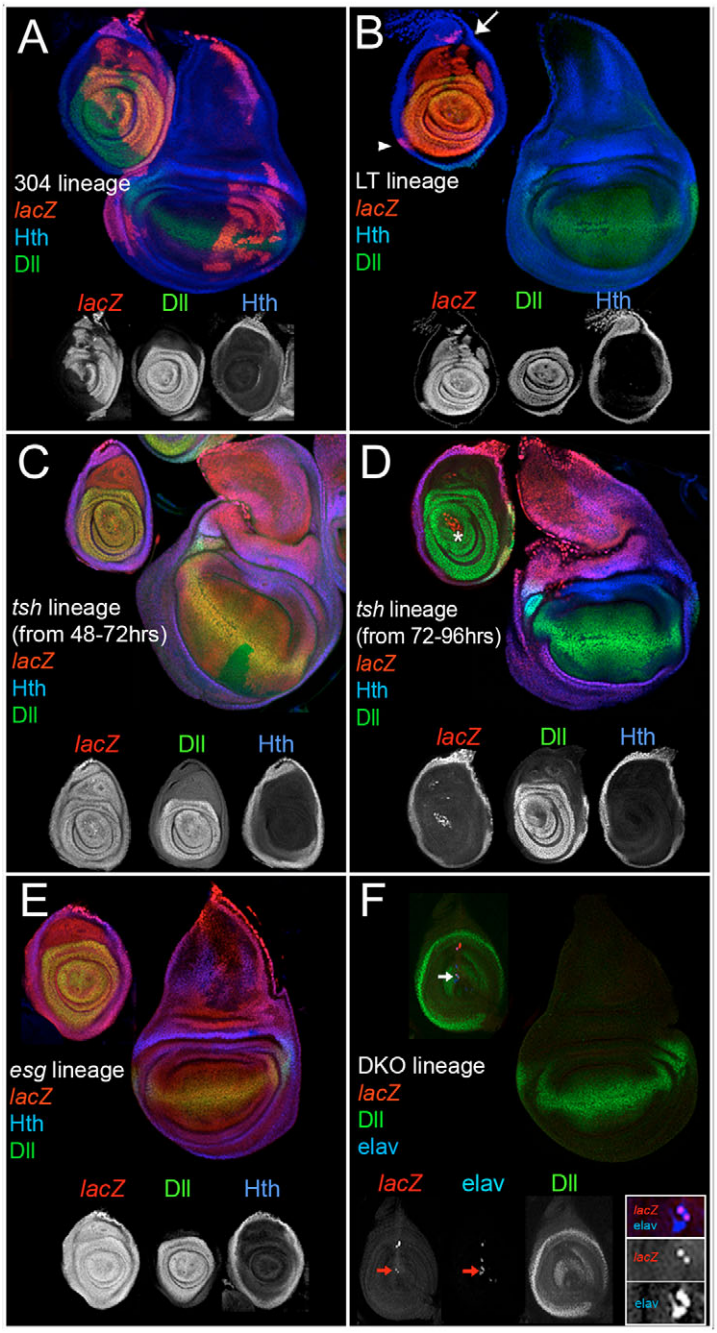

Fig. 2. Lineage analyses of genes active in the ventral limb primordia. All discs except for those in F were stained for the lineage marker (red), Dll (green, subset of telopodite), and Hth (blue, coxopodite); see Materials and methods for details. (**A**) The progeny of cells in which *Dll304* was active 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-Gal80^{ts}* transgene was used to control the activity of these *Gal4* drivers. Raising the animals at the temperature where Gal80^{ts} was active for all of development (the permissive temperature) resulted in no *lacZ* expression (data not shown), confirming the efficacy of the Gal80^{ts} 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 leave 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

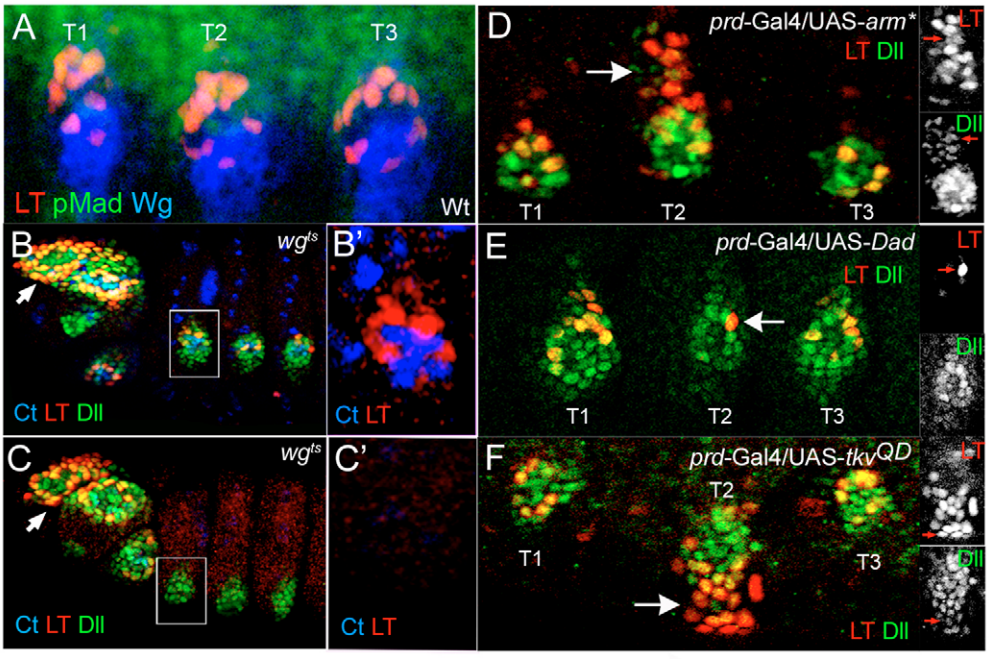

Fig. 3. Regulation of LT by Wg and Dpp. (**A**) Cells that activate *LT* (red) at stage 14 in the limb primordia are close to cells expressing high levels of Wg (blue) and Dpp (visualized by pMad staining, green). (**B**) A *wg^{ts}* stage 14 embryo raised at the permissive temperature stained for Ct (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 *wg^{ts}* embryos. (**C**) A *wg^{ts}* 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*>Tkv^{QD} (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 (Tkv^{QD})] 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

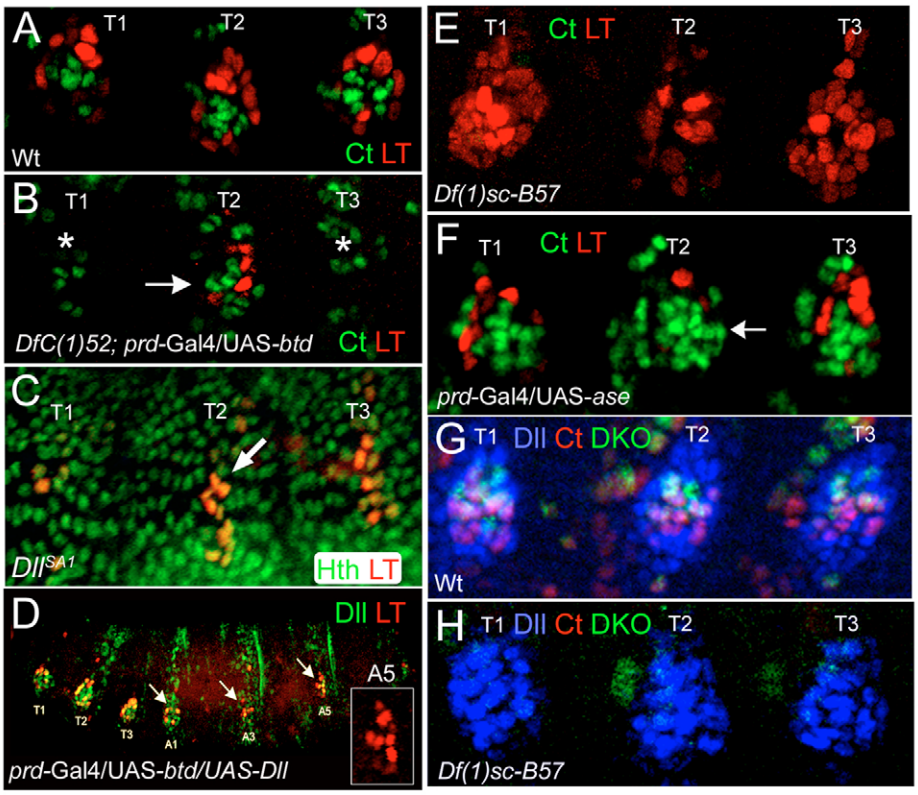

Fig. 4. Regulation of *DKO* and *LT*. (**A**) Wild-type stage 14 limb primordia stained for *LT* activity (red) and Ct (green). *LT* activity and Ct are present in mutually exclusive domains. (**B**) A stage 14 *prd>btd; DfC(1)52* embryo stained for *LT* (red) and *ct* (green). In the absence of *btd* and *Sp1* both LT and *ct* are not activated (in T1 and T2; asterisks). Resupplying Btd in T2 rescues *LT* and *ct* activity (arrow). (**C**) In a stage 14 Dll^{SA1} mutant embryo *LT* activity (red) initiates, but decays over time. Unlike in wild type (A), the *LT* expression domain is a stripe. (**D**) Ectopic expression of Dll (green) and Btd using *prd-Gal4* activates *LT* (red) in the abdomen (arrows). The inset shows ectopic activation of *LT* in abdominal segment 5. (**E**) A *Df(1)sc-B57* mutant embryo, deleted for the ASC, stained for *LT* activity (red) and Ct (green). *LT* activity is expanded at the expense of the *ct*-expressing cells, which are lost. (**F**) Ectopic expression of the proneural gene *asense* (*ase*) in T2 represses *LT* activity (red) and expands the number of cells that express *ct* (green; arrow). (**G**) Wild-type limb primordia stained for Dll (blue), *DKO* activity (green) and Ct (red). *DKO* activity and Ct expression overlap within the Dll-positive domain. (**H**) *Df(1)sc-B57* mutant embryo stained for *DKO* activity (green), Ct (red) and Dll (blue). *DKO* activity and Ct staining are absent in the leg primordia, but the number of cells expressing *Dll* is not changed.

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 *btd* in these deficiency embryos (Fig. 4B). By contrast, expressing *Dll*, tkv^{QD}, 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 *btd* resulted in robust ectopic expression of *LT-lacZ* in abdominal segments in the equivalent ventrolateral position as the thoracic limb primordia (Fig. 4D). *btd* 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 *btd* 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, *LT-lacZ* expression was expanded at the expense of the Ct-expressing cells (Fig. 4E). Consistently, ectopic expression of the ASC gene *asense* (*ase*) 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 *btd* 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 cells) 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, arguing 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.

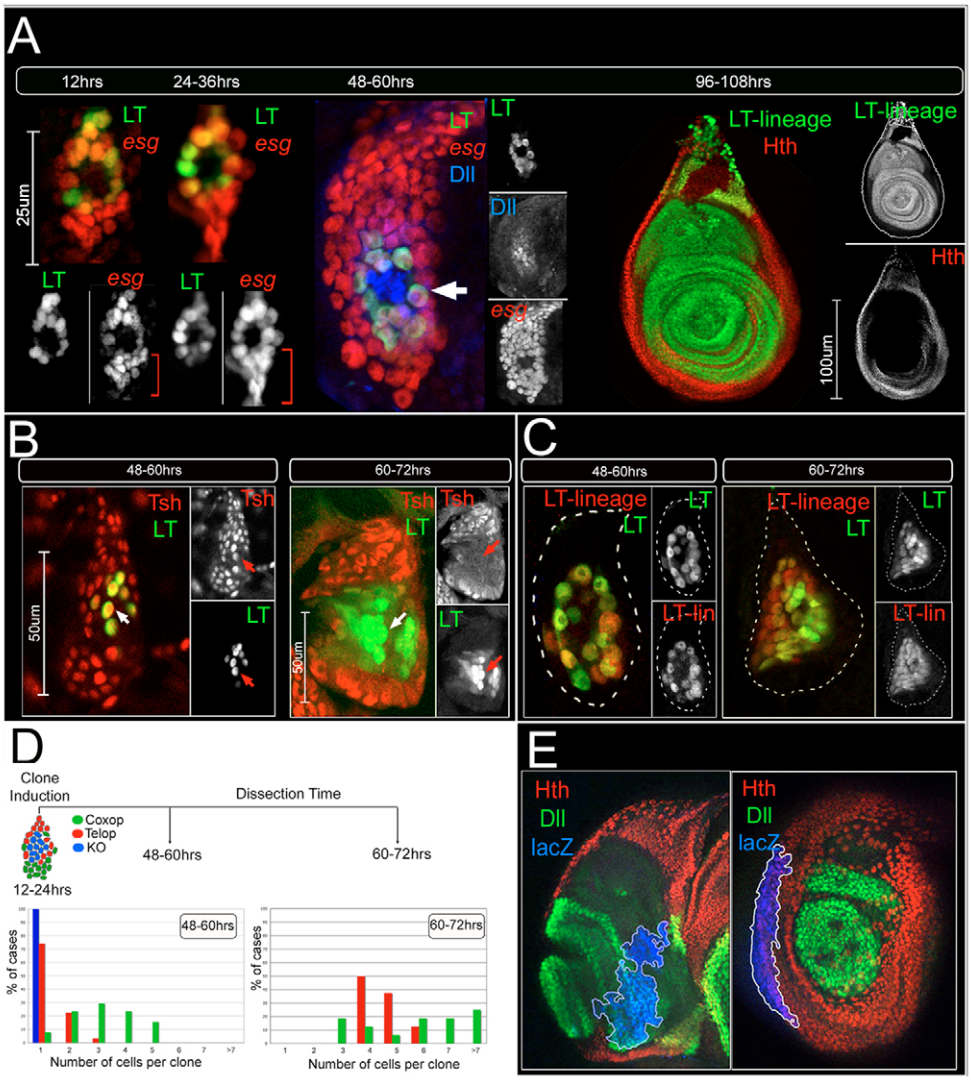

Fig. 5. Distinct cell proliferation dynamics in the coxopodite and telopodite. (**A**) Time course of limb primordia development, staining for *LT* activity (green) and *esg-lacZ* (red). At 12 hours AEL (stage 14) the number of *LT*-positive telopodite progenitors is approximately equal to the number of *esg*-positive, *LT*-negative coxopodite progenitors (~15 cells, each). At 24-36 hours AEL (early first instar) the number of *LT*-positive cells and *esg*-positive, *LT*-negative cells remain about the same. At 48-60 hours AEL (early second instar) the number of *esg*-positive, *LT*-negative cells is far greater than the number of *LT*-positive cells. *LT*-positive (arrow) nuclei are noticeably larger than *esg*-positive, *LT*-negative nuclei. By 96-108 hours AEL (third instar) the progeny of the *LT*-expressing cells now populate the entire telopodite. (**B**) *LT* activity (green) compared to *tsh* expression (red) during development. At early second instar (48-60 hours AEL) the number of cells that are *tsh*-positive but *LT*-negative is greater than the number of cells where *LT* is active. At this time the *LT*-positive cells also express *tsh* (arrow). At the end of the second instar (60-72 hours AEL) the number *LT*-positive cells has increased. At this time the *LT*-positive cells no longer express *tsh* (arrow). (**C**) At 48-60 hours AEL, the ~15 cells in which *LT* is active (green) exist as a ring that is coincident with the *LT* lineage labeling. At 60-72 hours AEL, the *LT* lineage-labeled cells, although greater in number, all actively express *LT*. Discs have been outlined with a dotted line. (**D**) Randomly generated neutral clones induced at 12-24 hours of development were quantified for their position and cell number 36 and 48 hours after clone induction. Examples for each of these experiments are shown in Fig. S4 in the supplementary material. (**E**) Third instar discs containing neutral clones stained for Hth (red), Dll (green) and β-gal (blue, the clone marker). The subset of randomly marked neutral clones induced between 12 and 24 hours of development that reach the border do not cross between the coxopodite and the telopodite (*n*=25).

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*,

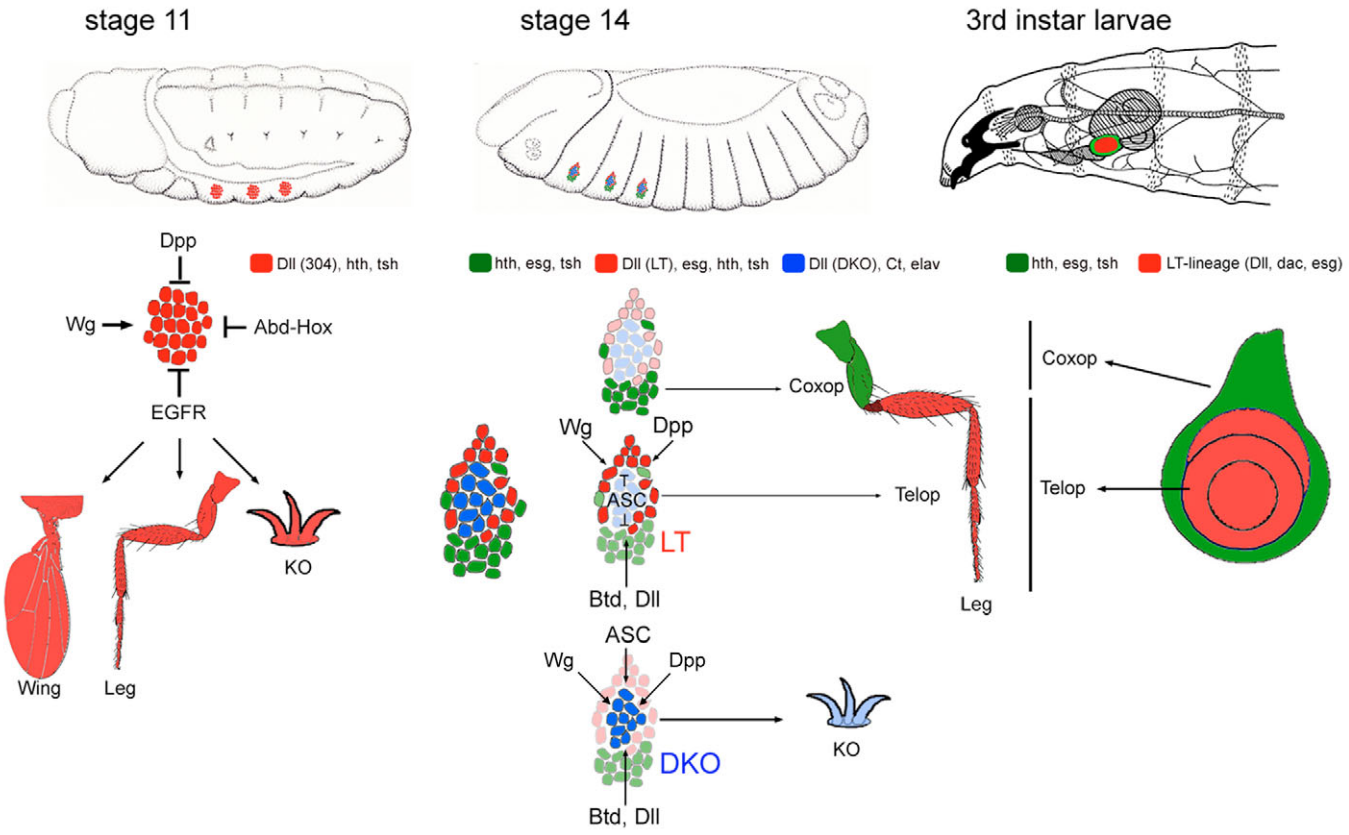

Fig. 6. The fate map of the thoracic limb primordia. At stage 11 the cells of the limb primordia are multipotent and can contribute to the dorsal appendage (e.g. wing), coxopodite, telopodite, and Keilin's Organ (KO). Also shown are the genetic inputs that control the early *Dll304* enhancer at this stage. Approximately 4 hours later (stage 14) the cells in the limb primordia are restricted in their potential. Cells that activate *LT* (red) give rise only to the telopodite. *LT* activation requires Wg and Dpp and is restricted to the thorax by Btd and Dll. *LT* is also repressed by the proneural genes of the ASC in the KO primordia. Cells that activate *DKO* (blue) are fated to form the KO. The *DKO* enhancer is controlled by Wg, Dpp, ASC, Btd and Dll. By stage 14 the coxopodite precursor cells (green) do not express *Dll* and are primarily located ventral to the *Dll*-positive cells. At the end of larval development the *LT* lineage gives rise to the entire telopodite (Dll and Dac, in red), while the coxopodite is marked by the restricted expression of *tsh* and *hth* (in green).

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 primorida 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* and *Dll* 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

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