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Dissecting insect leg regeneration through RNA interference

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Abstract. Nymphs of hemimetabolous insects such as cockroaches and crickets exhibit a remarkable capacity for regenerating complex structures from damaged legs. Until recent years, however, approaches to elucidate the molecular mechanisms underlying the leg regeneration process have been lacking. Taking the cricket *Gryllus bimaculatus* as a model, we found that a phenotype related to regeneration frequently appears during leg regeneration, even though no phenotype is induced by RNA interference (RNAi) in the cricket nymph, designated as regeneration-dependent RNAi. Since then, we have investigated the functions of various genes encoding signaling factors and cellular adhesion proteins like Fat and Dachsous during leg regeneration. In this review, we summarize the classical knowledge about insect leg regeneration and introduce recent advances concerning the signaling cascades required for regenerating a leg. Our results provide clues to the mechanisms of regeneration which are relevant to vertebrate systems. (Part of a Multi-author Review)

Keywords. *Gryllus bimaculatus*, legs, regeneration, positional information, Wnt signaling, Fat signaling.

Introduction

Nymphs of hemimetabolous insects such as cockroaches and crickets have three pairs of legs consisting of six segments, arranged along the proximodistal (P/D) axis in the following order: coxa, trochanter, femur, tibia, tarsus, and pretarsus (Fig. 1a). Damaged legs show a remarkable capacity for regenerating complex structures from a blastema (Fig. 1b). Since those nymphal legs are relatively large, they are easy to manipulate surgically. Numerous grafting experiments have been done whose results provide clues to elucidate mechanisms of pattern formation and also offer relevant information on how tissues or organs regenerate, including vertebrate systems. Such experiments show that each leg segment has a similar set of P/D and circumferential positional values; furthermore, when positional values are missing in amputated or grafted legs, the legs can intercalate the missing values [1–3]. In this intercalary regeneration, a rule, designated as the shortest intercalation rule, was discovered, which states that the confrontation of cells with different positional values would result in intercalation of intermediate values via the shortest route [1–3]. Although the nymphal leg system was used extensively by scientists interested in the mechanisms of appendage regeneration until 1986, very few groups are currently working on this system. This was mainly due to the paucity of molecular data and the lack of tools for functional analyses.

However, the situation gradually changed as sequencing techniques improved and RNA interference (RNAi) proved to work efficiently in cricket nymphs [4]. When double-stranded RNA (dsRNA) for a given gene is injected in a cricket nymph, as shown in Figure 1c, in some cases a phenotype appears after molting and lasts to the adult stage (nymphal RNAi, nyRNAi). Interestingly, we found that a phenotype related to regeneration frequently appears during leg

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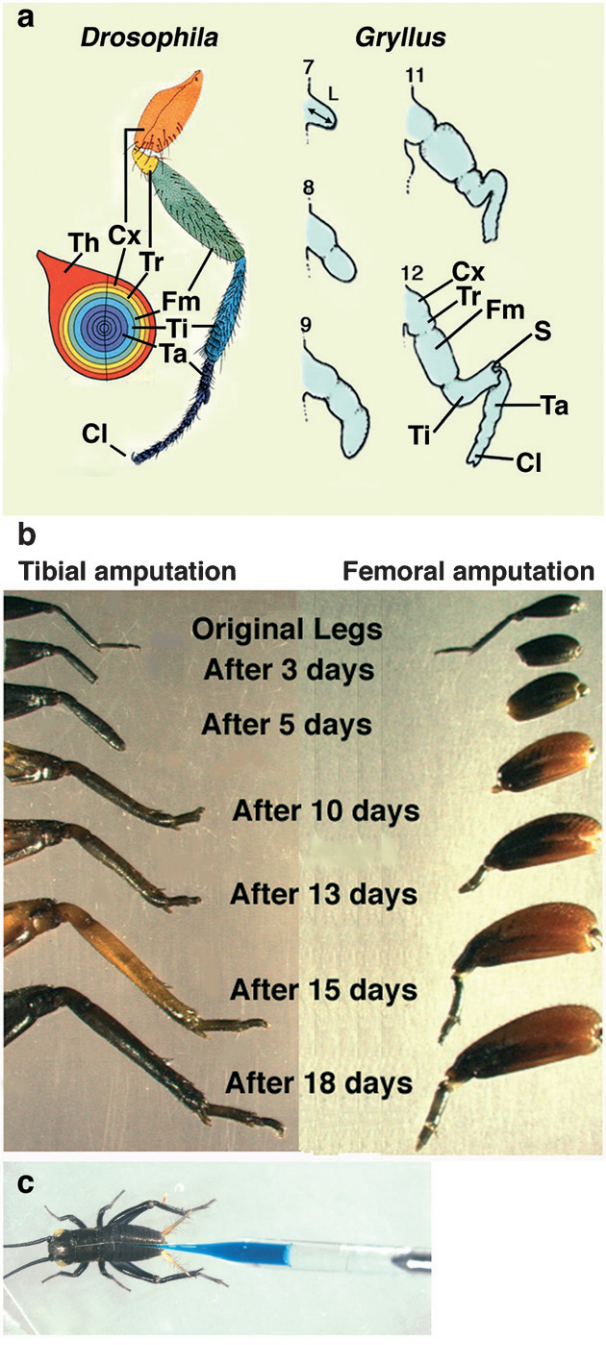

Figure 1. Leg development and regeneration in the cricket *Gryllus bimaculatus*. (*a*) In *Drosophila*, the adult leg forms from the larval leg imaginal disc [32], whereas in the cricket, the adult leg forms from the embryonic limb bud. (*b*) Regeneration process of the amputated leg of the cricket nymph. When amputated at the distal tibia of the 3rd instar nymph, it takes about two weeks to complete regeneration (left panel). When amputated at the femur, although regeneration occurs, it takes longer to complete regeneration (right panel). (*c*) Injection of double-stranded RNA solution into the body cavity of the cricket nymph. Th, thorax; Cx, coxa; Tr, trochanter; Fm, femur; Ti, tibia; Ta, tarsus; Cl, claw; S, spur.

regeneration, even though no phenotype is induced by nyRNAi, designated as regeneration-dependent RNAi (rdRNAi) [4]. Observed RNAi phenotypes could be sorted into five types according to their timing and the intensity (Table 1). In Type I, neither nyRNAi nor rdRNAi effect is detected; in Type II, nyRNAi effects are observed without rdRNAi; in Type III, RNAi effects can be detected only after amputation (rdRNAi only); in Type IV, nyRNAi effects are observed concomitantly with rdRNAi; finally, in Type V, nyRNAi makes nymphs lethal. Genes analyzed so far are classified into the five types as listed in Table 1. For example, since the *chico* nyRNAi phenotype is a small body [unpublished data], while *Laccase 2* is a white body, both without phenotype during leg regeneration, they belong to Type II. As shown later, *armadillo*, *hedgehog*, *engrailed*, *Distal-less*, *fat*, and *dachsous* belong to Type III because their RNAi effects are observed only after amputation. The *Epidermal growth factor receptor* (Egfr) gene belongs to Type IV because the body size becomes small as an effect of nyRNAi, whereas long cercal hairs are not formed. In addition, although no phenotype was observed in the leg before amputation, the distal structures are not restored after amputation as an effect of rdRNAi. We identified many genes belonging to this type. Although we do not know the mechanisms supporting the specificity of the RNAi, genes belonging to Type III provide a unique entry point for dissecting, through loss-of-function assays, the mechanisms underlying leg regeneration in cricket nymphs.

The regeneration process likely relies on a dynamic web of gene regulatory networks which creates a robust self-organizing system. The last 20 years have provided a wealth of genetic information on *Drosophila* leg development [5]. However in *Drosophila*, the presumptive legs develop from imaginal discs, which indeed display regenerative abilities but grow inside the larval body, making the analysis of RNAi phenotypes very difficult. Therefore, the cricket leg provides two major advantages: on the one hand, as an arthropod, the genetic regulatory networks regulating leg development are supposedly close to those active in *Drosophila* and therefore benefit from the genetic advances of the *Drosophila* model system; on the other hand, the cricket leg provides an easily amenable model system for screening genes involved in regeneration through RNAi. This approach should help elucidate the gene regulatory networks supporting regeneration in insects. Here we will first summarize the classical knowledge about insect leg regeneration and then explore the putative gene regulatory networks involved in cricket leg regeneration. We will focus especially on the molecular clues that might determine positional information in regenerating leg segments.

Table 1. Classification of *Gryllus* genes into five types, according to the RNA interference (RNAi) phenotypes induced at nymphal stages.

	RNAi-induced phenotypes	*Gryllus* genes
Type I	none	*four-jointed*, *wingless*, *decapentaplegic*
Type II	nymphal only (nyRNAi)	*chico*, *Laccase 2*
Type III	regeneration-dependent only (rdRNAi)	*armadillo*, *hedgehog*, *engrailed*, *Distal-less*, *dachshund*, *fat*, *dachsous*
Type IV	nymphal and regeneration-dependent (nyRNAi + rdRNAi)	*Egfr*
Type V	lethal at nymphal stages	*Notch*, *Delta*, *extradenticle*, *homothorax*

Establishment of the proximodistal axis in the developing insect leg

Appendage regeneration is divided into three phases: wound healing, blastema formation, and pattern formation and growth [6]. In this review, we will focus on the latter two. It is generally considered that the third phase actually recapitulates the leg developmental processes, especially the establishment of the P/D (proximodistal) axis, which is assumed to follow a common process both during development and regeneration in the insect leg. Thus, we will first summarize the molecular mechanisms supporting the development of the insect leg. Those have been extensively investigated in *Drosophila* [5], but not in hemimetabolous insects such as crickets [7]. In contrast to the *Drosophila* leg, which develops from a specific imaginal disc (Fig. 1a, left), the cricket leg develops from a limb bud (Fig. 1a, right), as in the vertebrate limb, in the absence of any leg imaginal disc. Then nymphal legs develop, looking like small adult legs. As shown later, although the leg developmental processes considerably differ in cricket and *Drosophila*, the signaling molecules involved in development and regeneration are essentially similar. This implies that the genetic information gathered about leg development in *Drosophila* can be used as a starting point to understand the molecular mechanisms supporting leg development in hemimetabolous insect.

Based on *Drosophila* experimental data, Meinhardt proposed the boundary model [2] to explain the formation of the P/D axis of the leg in 1983. The leg is divided into three compartments, posterior, dorsal/anterior, and ventral/anterior (Fig. 2a). The site where the boundaries between these compartments intersect defines the presumptive distal tip of the leg and is the source of a diffusible morphogen that induces outgrowth and specifies cell fate along the P/D axis. Ten years later, three reports demonstrated that *Drosophila* limbs are indeed subdivided into three domains, which derive from adjacent cell populations founded early in development (Fig. 2a) [8–10]. Posterior cells organize growth and cell patterning in the three domains by secreting the Hedgehog (Hh) protein, which acts indirectly by inducing neighboring anterior cells to secrete the Decapentaplegic (Dpp) or Wingless (Wg) protein at the dorsal anteroposterior and ventral anteroposterior boundaries respectively [8–10]. A P/D axis is initiated at the site where *dpp*-expressing cells come into close association with those expressing *wg* [8]. In 2002, Campbell showed that the distal region is actually patterned by a distal-to-proximal gradient of receptor tyrosine kinase (RTK) activity [11]. This graded activity is established, depending on the concentration of epidermal growth factor (EGF)-related ligands. Its source is located at the presumptive tip – the most distal region that requires the highest level of EGFR activity – whereas more proximal regions require progressively less [11]. This indicated that EGF corresponds to the predicted morphogen produced by the distal organizer and that the tarsus may be patterned by a distal-to-proximal gradient of EGFR activity [12]. These findings thus provided the molecular basis of the boundary model, termed as the molecular boundary model (Fig. 2a).

We next asked whether the molecular boundary model could be applied to the developing hemimetabolous insect legs. We observed the expression patterns of the *hh*, *wg*, and *dpp* homologs in the cricket *Gryllus bimaculatus* (named *Gb'hh*, *Gb'wg*, and *Gb'dpp* respectively) and found no striking differences between *Drosophila* and *Gryllus* expression patterns [7]. In fact, those genes were all expressed in the *Gryllus* limb bud (Fig. 2b), even though the *Gb'dpp* transcripts were detected as a spot in the dorsal tip of the limb bud, whereas *dpp* transcripts form a stripe along the dorsal side of the anteroposterior boundary in the *Drosophila* leg imaginal disc. The morphogenetic significance of this difference is currently unknown. However, these results allowed us to conclude that the molecular boundary model is a fundamental and universal mode for forming the P/D axis in the insect leg.

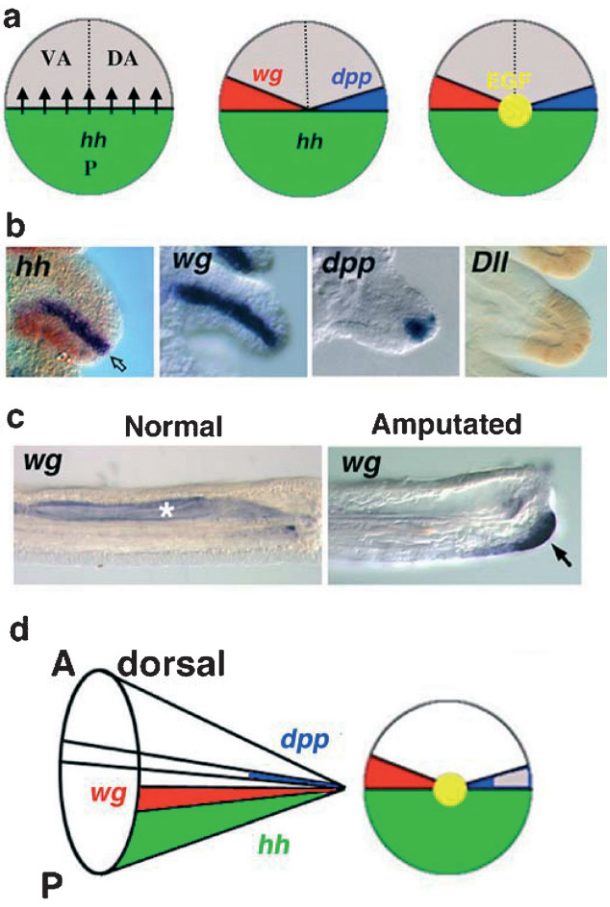

Figure 2. Establishment of the P/D axis of the cricket leg during development and regeneration. (*a*) The molecular boundary model supports the establishment of the proximodistal (P/D) axis in the *Drosophila* leg. The *Drosophila* leg disc is divided into three cell populations: posterior, which expresses *hh* (green), and dorsal anterior (DA) and ventral anterior (VA). Hh induces expression of *wg* (red) along the ventral side of the anteroposterior boundary and *dpp* (blue) along the dorsal side. The interaction between *wg* and *dpp* is involved in the P/D axis formation of the leg: the only site at which *dpp*-expressing cells abut *wg* cells may become a P/D axis organizer center expressing EGF-related ligands (yellow). (*b*) Expression of *Gb'hh*, *Gb'wg*, *Gb'dpp*, and *Gb'Dll* cricket genes during limb development. The left most panel shows double staining for *Gb'hh* (red) and *Gb'wg* (dark blue, open arrow). *Gb'hh* is expressed in the posterior compartment of the early leg bud, whereas *Gb'wg* is expressed along the ventral side of the anteroposterior boundary. *Gb'dpp* is expressed at the dorsal side of the anteroposterior boundary as a spot, which is different from the *dpp* expression pattern in *Drosophila*. *Gb'Dll* is expressed at the distal side of the leg bud (the right most panel shows anti-*Gb'*Dll immunostaining). (*c*) *Gb'wg expression* in the nymphal and regenerating (2 days post-amputation dpa) tibia. *Gb'wg* exhibits no significant expression in the nymphal tibia, but is strongly expressed at the ventral side of the regeneration blastema (arrow). The asterisk indicates nonspecific staining in the trachea. (*d*) The molecular boundary model can be applied to the developing and regenerating cricket leg.

Establishment of the P/D axis in the regenerating hemimetabolous insect leg

The classical approach to understand the nature of appendage regeneration relied on transplantation experiments, initially performed on cockroaches and newts. One interesting phenomenon was the formation of supernumerary legs after grafting the distal part of a leg onto the contralateral leg stump, a way to bring into contact at the amputation plane two structures with inverted anteroposterior axes [1–3]. The formation of the supernumerary legs in that context meant that additional P/D axes were induced upon grafting. In 1976, the polar coordinate model was proposed to interpret the regeneration phenomena [13]. The model has two rules. First, the rule of shortest intercalation states that the confrontation of cells with different positional values would result in the intercalation of intermediate values via the shortest route; second, the full circle rule stated that leg outgrowth and ultimately a P/D axis would be generated at the site where there was a full complement of circumferential positional values [14]. This model can account for the formation of supernumerary legs following grafting experiments (see Fig. 3), because a full circumference of positional values will be intercalated at the two positions of maximal circumferential misalignment [13]. The model, based on local interactions, provides a simple and unified interpretation for a wide range of experimentally produced and naturally occurring regenerative phenomena such as limb regeneration in insects, crustaceans, and amphibians, duplication of structures, and formation of complete, tapering, or branching supernumeraries. In 1983, Meinhardt interpreted the formation of the supernumerary legs with the boundary model [2]. In 1995, the molecular boundary model was proposed to explain the formation of the supernumerary legs and may thus provide a molecular basis for the polar coordinate model [3].

To verify that the molecular boundary model would apply during regeneration, we observed the expression patterns *of Gb'hh*, *Gb'wg*, and *Gb'dpp* in regenerating legs (Fig. 2c, d) [6] and silenced those genes through RNAi. In nymphs silenced for *Gb'hh*, regeneration was abnormal, with formation of a supernumerary leg as shown in Figure 3a, b, whereas no phenotype was observed in the absence of amputation. In nymphs silenced for *Gb'dpp* and *Gb'wg*, no phenotype was found either during development or during regeneration. However, when expression of *Gb'armadillo* (the beta-catenin ortholog) was knocked down through RNAi, no regeneration was observed, as shown in Figure 3c, indicating that the canonical Wnt pathway may be involved in initiation of regeneration [4].

Concerning *Gb'Egfr*, during leg regeneration *Gb'Egfr* is expressed in the blastema (Fig. 4b), as observed in the limb bud. In the nymphs exposed to *Egfr* dsRNAs, although the leg was formed normally without amputation, the distal structures of the tarsus and claw were not normal after tibial amputation (Fig. 3d [unpublished data]). Furthermore, during formation of the supernumerary legs upon grafting, the genes encoding the three main signaling factors involved in leg development, Hh, Dpp, and Wg, were expressed as expected from the molecular boundary model (Fig. 3e) [6]. However, the *Egfr*-RNAi nymphs were unable to form the distal structures of the supernumerary legs (Fig. 3f,g), indicating that EGFR is indeed involved in the regeneration of distal structures. This is consistent with the role of EGF signaling during *Drosophila* leg development [11]. Thus, these data indicate that the molecular boundary model is the most plausible one to explain the various phenomena observed during the formation of the P/D axis of both the developing and regenerating insect leg. Furthermore, the model may be applicable to the regenerating vertebrate limb, as supernumerary legs can be formed by grafting in a way similar to that described above [15].

Segment identity in the developing hemimetabolous insect leg

Nymphs of hemimetabolous insects have six leg segments, arranged along the P/D axis in the following order: coxa, trochanter, femur, tibia, tarsus, and pretarsus. These leg segments are formed not simultaneously but sequentially from the presumptive trochanter/femur, femur/tibia, tibia/tarsus boundaries, the initial structure consisting of the presumptive coxa/pretarsus [16]. During formation of the leg segments, a proximal leg segment appears to be intercalated from the proximal side between the most proximal and the most distal structures. Segment identity may be determined by the *homothorax* (*hth*), *dachshund* (*dac*), and *Distal-less* (*Dll*) genes in the cricket limb bud (Fig. 4a) [17], at least as revealed in the fly imaginal disc (Fig. 4a) [5, 18]. Their expression patterns are likely to be regulated by Hh, Wg, and Dpp, which are expressed in the limb bud [5]. Since recent findings show that, in *Drosophila* legs, the sharp discontinuity of Dpp signaling is required for forming the leg joint, expression patterns of *Gb'dpp* in the cricket leg bud may be also involved in the formation of the leg joint [19]. Thereafter, intrasegmental pattern formation, which is characterized by spines and hairs, follows the determination of segment identity. So far we do not

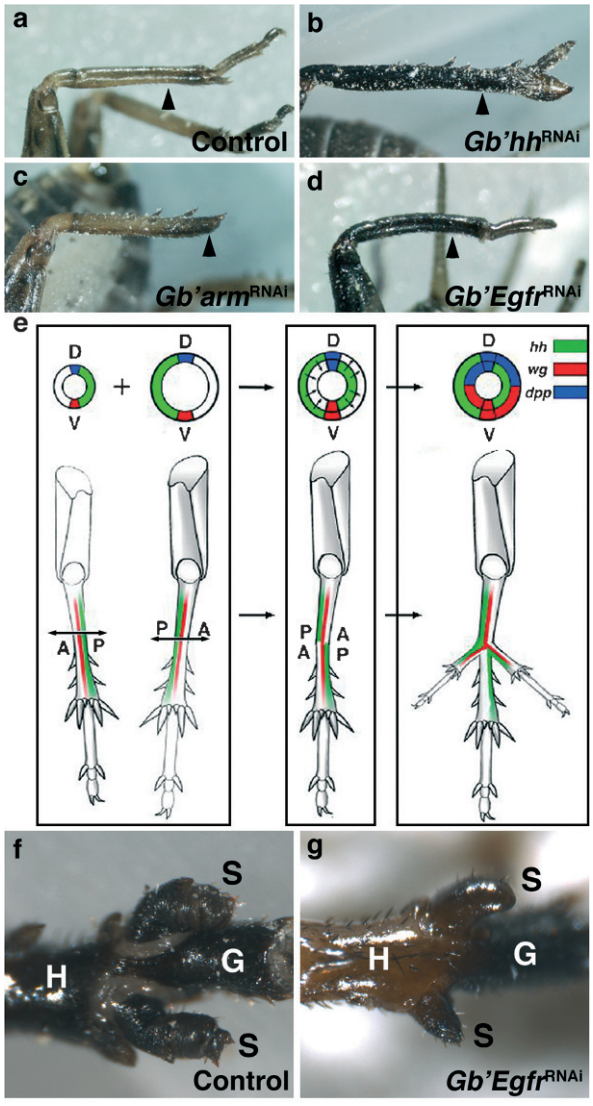

Figure 3. P/D patterning during leg regeneration in the *Gryllus* nymph. (a–d) Leg regeneration in nymphs amputated at the distal tibia level and observed after the second molt. Arrowheads indicate amputated positions. (*a*) Leg fully regenerated at 11 dpa. (*b*) *Gb'hh* RNAi results in altered P/D patterning and supernumerary axis formation. (*c*) *Gb'arm* RNAi nymphs show no significant regeneration after the second molt, indicating that *Gb'arm* is essential for regeneration. (*d*) *Gb'Egfr* RNAi results in the loss of the pretarsus and distal tarsus. (*e*) Formation of supernumerary legs upon grafting the controlateral tibia onto the amputated tibia. The anteroposterior axis of the graft is rotated by 180° with respect to the host. The *hh* (green) and *wg* (red) expression patterns are drawn based on the molecular boundary model. In the upper series, the two first circles represent cross-sections of the graft and the host, respectively, where the *hh* (green), *wg* (red), and *dpp* (blue) expression domains are depicted. When the anteroposterior polarity is reversed, the two Hh domains of the host and graft legs induce the expressions of *wg* and *dpp* in the ventral side and dorsal side of their adjacent anterior compartment (double circles) [6]. The sites where the *wg*-expressing cells abut the *dpp*-expressing cells become organizers that induce formation of the P/D axis. A, anterior; P, posterior; D, dorsal; V, ventral. (*f*) Supernumerary legs (S) formed as a result of the grafting operation shown in (*e*). H, host; G; graft. (*g*) *Gb'Egfr* RNAi results in the loss of the distal structures of the supernumerary legs.

know the mechanisms driving intrasegmental pattern formation, but information about the regulation of the pattern within a segment has been obtained from intercalary regeneration experiments in hemimetabolous insects [2].

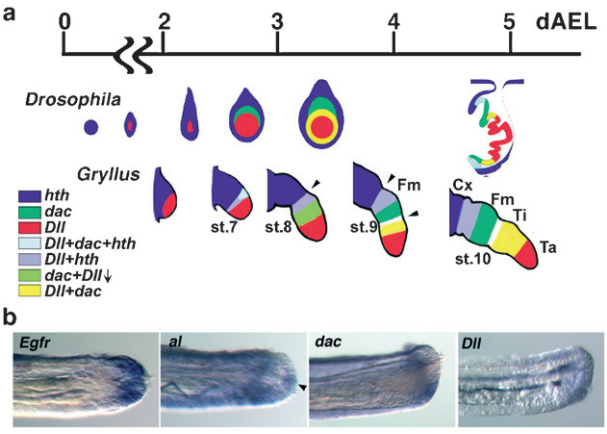

Figure 4. Expression patterns of the genes involved in the P/D patterning of the insect leg. (*a*) Summary of the observed expression patterns in the developing leg of *Drosophila* and *Gryllus*. In the *Gryllus* leg bud, *Gb'hth* and *Gb'Dll* are first expressed in adjacent but nonoverlapping domains, then subsequently overlap along their common border (purple). The proximal region corresponds to the trochanter/femur boundary. Thereafter *Gb'dac*-expressing cells intercalate between *Gb'Dll* and *Gb'hth*, overlapping with *Gb'Dll* (light green). This expression pattern is not observed in the *Drosophila* imaginal disc. Subsequently the *Gb'dac (green)* and *Gb'dac/Gb'Dll* expression domains (yellow) become distinct. These patterns may be related to distal segmentation. The latest patterns detected in the fully segmented legs of *Drosophila* and *Gryllus* are similar to each other. (*b*) Expression patterns in the regenerating blastema. In the early blastema (2 dpa) an intense expression of *Gb'Egfr* is induced in epithelial cells of the distal blastema. *Gb'al* is expressed as a spot at the ventral side of the distal tip of the blastema (epidermis, arrowhead) and broadly in a more proximal region of the blastema. *Gb'dac* is expressed in the whole blastema except its distal tip and its most proximal region. *Gb'Dll* is expressed in the entire blastema.

Segment identity in the regenerating hemimetabolous insect leg

Complete regeneration of the P/D pattern of the leg results from the superposition of at least two systems, as pointed out by Meinhardt [2]: (1) the first is the determination of leg segment identity and joint formation: (2) the second correspond to intrasegmental pattern formation. Concerning the former process, the *Gb'hth*, *Gb'dac*, and *Gb'Dll* genes are expressed during regeneration of the cricket leg in a fashion similar to that observed in the developing limb buds (Fig. 4b). Moreover, when *Gb'Dll* expression was knocked down by nymphal RNAi, the regenerated leg segments appeared to be abnormal [unpublished data]. These results indicate that determination of segment identity during regeneration may take place in the same manner as during leg development.

The latter process of the intrasegmental pattern formation is closely related to the phenomenon of intercalary regeneration, which may differ from the former process. Several examples of intercalary regeneration are depicted in Figure 5, where the normal tibia segment was numbered according to Meinhardt [2], from 1 to 9 (123…9, Fig. 5a), 1 corresponding to the most proximal value. In the first example, a host tibia of the metathoracic leg (T3) amputated between positional values 3 and 4 was grafted with a distal tibia of the mesothoracic leg (T2) amputated between positions 7 and 8, resulting in a proximal stump of positional value 3 put into contact with a distal graft of position 8 (Fig. 5c). Structures with positional values 4567 intercalated between those two surfaces (Fig. 5d). In a second example, a large proximal tibia stump (12345678) was grafted with a large distal fragment (456789), resulting in a proximal stump of positional value 8 put into contact with a distal tissue of position 4 (Fig. 5e). As anticipated from the polar coordinate model [13], regenerated structures with reversed polarity (765) intercalated (Fig. 5f). Meinhardt pointed out that this polarity reversal is difficult to explain by a morphogen gradient generated by a source at one segment border and a sink at the other, since this would lead to a monotonic gradient but never to the observed polarity reversal [2]. In order to explain the intercalary regeneration, Meinhardt and Gierer [20] proposed a theoretical model, designated here as the 'discrete cell model': According to this model, pattern formation within a segment and its intercalary regeneration can be explained by assuming that the pattern consists of a sequence of discrete cell states which mutually activate each other over a longer range but exclude each other locally.

In their discrete cell model, they assumed that (1) genes (or more general feedback loops) must exist that have a positive feedback on their own activation; (2) these activities are locally exclusive – only one of the alternative genes can be active in a given cell; (3) long-ranging molecules provide mutual activation of those cell states that eventually become neighbors. Each cell state in a given cell depends on the help from different cell states in neighboring regions (see details at http://www.eb.tuebingen.mpg.de/departments/former-departments/h-meinhardt/segments.html).

Since intersegmental graft at the similar position, i.e. the graft of two distinct segments amputated at the same position, here between 7 and 8, did not result in intercalary regeneration (Fig. 5 g), one can deduce that each leg segment has a similar set of P/D positional values. So far, however, molecules predicted by this model have not been identified.

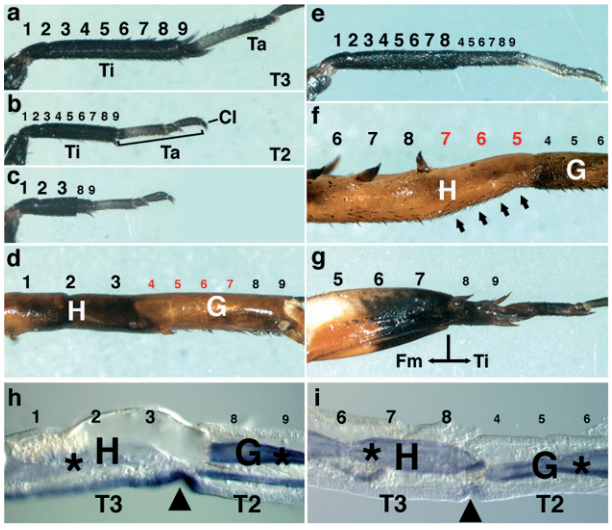

Figure 5. Induction of intercalary regeneration in the nymphal tibia of the cricket leg. A distally amputated mesothoracic leg was grafted onto a proximally amputated metathoracic leg at the 3rd instar stage. To describe the experimental results, the P/D sequence of the tibial structures is numbered from 1 to 9, 1 corresponding to the most proximal value, 9 to the most distal one according to Meinhardt [2]. (*a, b*) Normal metathoracic (T3) and mesothoracic (T2) legs in a 3rd instar cricket. Ti, tibia; Ta, tarsus; Cl, claw. (*c, d*) When an amputated T2 tibia (89) is grafted to an amputated T3 tibia (123, *c*), intercalary structures of the tibia (4567, *d*), originating from the distal graft (G), are observed at 28 days after grafting. (*e, f*) When an amputated T2 tibia (456789) is grafted onto an amputated T3 tibia (12345678), intercalary structures of the tibia (765, *f*), derived from the proximal host (H) are observed at 28 days after grafting. (*g*) The confrontation of different segments amputated at the same internal level does not lead to intercalary regeneration. Here a T2 tibia sequence (89) was grafted onto a T3 femur (Fm) sequence (567), and the result was observed at 28 days after grafting. (*h*) *Gb'wg expression* at 2 days after grafting a distal T2 tibia (89, G) to a proximal T3 tibia (123, H). An arrowhead indicates the host-graft junction, asterisks the nonspecific staining detected in the trachea. (*i*) *Gb'wg* expression at 2 days after grafting a proximal T2 tibia (456789) to a distal T3 tibia (12345678).

To identify the molecules possibly involved in intercalary regeneration, we investigated the *Gb'wg* and *Gb'hh* expression patterns during regeneration [4]. We found *Gb'hh* and *Gb'wg* induced in the host, proximally to the amputation plane, but not in the structure grafted distally to the amputation plane (Fig. 5 h) [4]. This directional induction occurs even in the reversed intercalation (Fig. 5i). Because these results are consistent with a distal-to-proximal respecification of the regenerate, *Gb'wg* may be involved in the re-establishment of the positional values in the regenerate. Furthermore, we found that no regeneration occurs when *Gb'armadillo* was knocked down by RNAi [4]. These results indicate that the canonical Wnt/Wingless signaling pathway is involved in the process of leg regeneration and determination of positional information in the leg segment.

The segments of insect legs have an internal polarity, frequently visible by overt structures such as hairs or spines. As indicated by the reversed orientation of the hairs, the surplus structures are intercalated with a reversed polarity [3, 4]. Thus, the polarity of the pattern within a segment results from the sequence of elements. Recently, a set of 'tissue polarity' genes that serve to coordinate planar cell polarity (PCP) in the developing wings, legs, and eyes were identified by genetic analysis in *Drosophila* [21–23]. Fat (Ft) and Dachsous (Ds) are large atypical cadherins, whereas the third one, Four-jointed (Fj), is a type II membrane protein. Mutations in each of these three genes give rise to flies with defects in the ratio of P/D to anteroposterior growth of the wing imaginal disc [21], indicating that they are involved as a cassette in regulating these modulations in growth. In *Drosophila* wing imaginal disc, these genes are nonuniformly expressed along the P/D axis: *ds* expression is intense in the proximal region of the wing disc, whereas *fj* is most highly expressed distally [21–23] and Ft protein is present more uniformly throughout. It was suggested that the Fat/Dachsous/Four-jointed cassette acted upstream of Frizzled to control planar polarization in the wing [24]. In fact, ectopic gradients of Ft and Ds can reorientate polarity in the wing [25]. In the *Drosophila* eye, during the development of PCP, cells respond to long-range directional signals that specify the axis of polarization. It has been proposed that a crucial step in this process is the establishment of graded expression of Ds and Fj [26].

From these data, we speculated that the Fat/Dachsous/Four-jointed cassette may be involved in the establishment of PCP in the cricket leg. In the cricket limb buds, the three genes are expressed in all leg segments with a pattern rather reverse to that observed in the *Drosophila* wing disc, i.e. *Gb'ds* is expressed distally, whereas *Gb'ft* and *Gb'fj* are detected proximally in each leg segment (Fig. 6). RNAi silencing revealed that Ft is involved in determination of the positional value [unpublished data], supporting a possible role for the Fat/Dachsous/Four-jointed cassette not only in the regulation of growth but also in the determination of positional information in each leg segment. This may be closely related to the Salvador-Warts-Hippo pathway, which may regulate the size of developing organs [27, 28]. Recently, Smith et al. [29] showed that the chick *Fat-1* gene is expressed in limb buds, and exhibits remarkably dynamic expression patterns throughout tissue differentiation and limb maturation. These results suggest some evolutionarily conserved mechanisms in leg pattern formation from protostomes to deuterostomes.

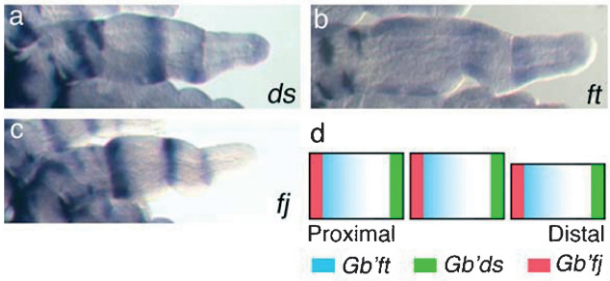

Figure 6. Expression patterns of *Gb'fat*, *Gb'dachsous,* and *Gb'four-jointed* in the elongating leg bud. (*a*) *Gb'dachsous (Gb'ds)* is expressed in the distal region of each leg segment. (*b*) *Gb'fat (Gb'ft)* is expressed in each leg segment with a gradient from the proximal to mid-region. (*c*) *Gb'four-jointed (Gb'fj)* is expressed in the proximal region of each leg segment. (*d*) Schematic diagram of the *Gb'ds* (green), *Gb'ft* (blue), and *Gb'fj* (red) expression domains. Distal is to the right.

Concluding remarks

The results described here indicate that during leg regeneration, cricket nymphs use the same molecules as those required for developing legs from imaginal discs in *Drosophila.* Two directions in future work on the cricket leg should provide original contributions to the general field of regeneration. First, this robust model system will help identify new molecules whose function in regeneration can only be discovered through powerful genome-wide RNAi screens. Second, the experimental versatility of the cricket leg makes possible a precise dissection of the molecular mechanisms underlying intercalary regeneration. Ultimately, this system will tell us which signaling pathways and what genetic circuitry define positional information in regenerating structures. However, one tool is missing for studying regeneration in the cricket leg, which is the possibility to use transgenesis. Although we succeeded in introducing genes in the cricket genome through *piggyBac* [30] and *Minos* [31] transposon elements, germline transmission of exogenous sequences has not yet been observed. Nevertheless, the cricket system remains easily amenable to functional genetic experimentation and should provide valuable information to elucidate the mechanisms underlying regeneration in both invertebrates and vertebrates.

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