Involvement of *dachshund* and *Distal-less* in distal pattern formation of the cricket leg during regeneration

Yoshiyasu Ishimaru¹, Taro Nakamura¹*, Tetsuya Bando², Yuji Matsuoka¹, Hideyo Ohuchi², Sumihare Noji³ & Taro Mito¹

¹Department of Life Systems, Institute of Technology and Science, The University of Tokushima Graduate School, 2-1 Minami-Jyosanjima-cho, Tokushima City, Tokushima, 770-8506, Japan, ²Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-cho, Kita-ku, Okayama City, Okayama, 700-8530, Japan, ³Center for Collaboration among Agriculture, Industry and Commerce, The University of Tokushima, 2-24 Shinkura-cho, Tokushima City, Tokushima 770-8501, Japan.

Cricket nymphs have the remarkable ability to regenerate a functional leg following amputation, indicating that the regenerating blastemal cells contain information for leg morphology. However, the molecular mechanisms that underlie regeneration of leg patterns remain poorly understood. Here, we analyzed phenotypes of the tibia and tarsus (three tarsomeres) obtained by knockdown with regeneration-dependent RNA interference (rdRNAi) against *Gryllus dachshund* (*Gb’dac*) and *Distal-less* (*Gb’Dll*). We found that depletion of *Gb’Dll* mRNA results in loss of the tarsal segments, while rdRNAi against *Gb’dac* shortens the tibia at the two most distal tarsomeres. These results indicate that *Gb’Dll* expression is indispensable for formation of the tarsus, while *Gb’dac* expression is necessary for elongation of the tibia and formation of the most proximal tarsomere. These findings demonstrate that mutual transcriptional regulation between the two is indispensable for formation of the tarsomeres, whereas *Gb’dac* is involved in determination of tibial size through interaction with *Gb’ds/Gb’ft*.

The principal differences between leg development and leg regeneration are in their initiation. In the amputated leg, the starting point of regeneration consists of multiple differentiated tissues such as muscle, epidermis, peripheral nerve components, and various cells. Thus, leg regeneration relies on cell dedifferentiation in invertebrates as well as in vertebrates. Following leg amputation, a blastema consisting of the dedifferentiated cells is formed at the amputated surface. How the blastema can redifferentiate to form the lost portion of the leg is a long-standing problem, and how regenerating cells have positional identity in the leg remains unknown.

We used the cricket *Gryllus bimaculatus* to elucidate the molecular mechanisms underlying development and regeneration of the leg. A cricket leg consists of six basic segments along the proximodistal (PD) axis: the coxa, trochanter, femur, tibia, tarsus, and claw. The cricket tarsus is subdivided into three tarsomeres, which are not true segments as they lack the individual musculature seen in the other segments. We observed that when the tibia of the cricket third-instar nymph is amputated at any level of the PD axis, the leg regenerates and recovers its allometric size and proper shape in the sixth instar, and the amputated leg is subsequently restored to almost normal adult leg size and shape. Our previous work has shown that *Gryllus wingless* (*Gb’wg*) and *decapentaplegic* (*Gb’dpp*) are expressed in the ventral and dorsal sides of blastemal cells, respectively, while *Gryllus hedgehog* (*Gb’hh*) is expressed in the posterior side of the blastema, similar to that observed in the leg bud and in the *Drosophila* leg imaginal disc.

In *Drosophila*, genes involved in establishing the PD axis of the leg have been identified and include *Distal-less* (*Dll*), which encodes a homeodomain transcription factor and is indispensable for the development of distal leg parts, *dachshund* (*dac*), which encodes a transcriptional co-repressor and is required for the development of medial leg parts, and *extradenticle* (*exd*) and *homothorax* (*hth*), which encode homeodomain transcription factors and together instruct proximal leg fates. Rauskolb designated these four genes as the “leg gap genes” by analogy with embryonic segmentation, because the absence of *Dll, dac, and hth* functions results in deletion of distal, intermediate, and proximal leg segments, respectively. The expression of these genes roughly
corresponds to the regions of the leg affected by their absence and is related to the initial crude positional values along the PD axis of the holometabolous fly legs.

To examine whether Dll, dac, exd and hth function as gap genes in the legs of other species, we observed their expression patterns in the developing hemimetabolous cricket leg bud and found that these patterns are essentially the same as those observed in the fly leg. These findings imply that the functions of the leg gap genes are conserved in the insect leg.

Gryllus Dll (Gb’Dll) is expressed in the distal domain that corresponds to the three tarsomes, which are designated here as tarsal segment 1,2, and 3 (Ta1,2,3), and in the distal tibia; dac (Gb’dac) is expressed in the proximal domain of Ta1 and in the tibial spurs25. Our previous studies demonstrated that Gb’Dll and Gb’dac are expressed in regenerating blastema after tibial amputation in cricket nymphs, similar to that observed in the cricket leg bud22,23.

To determine the functions of the leg gap genes in the cricket leg, we performed loss-of-function analyses using regeneration-dependent RNAi (rdRNAi) that occurs specifically in the amputated leg of cricket nymphs injected with double-strand RNA (dsRNA) for a target gene24. The functions of the leg gap genes are generally indispensable in many organs besides the leg, and therefore their knock-out mutants tend to be lethal. To overcome this, we used an rdRNAi knockdown approach, the apparent effect of which could be restricted to the regenerating leg. Thus, we could examine gene functions during leg development in a leg-regeneration system.

Recently, we successfully applied rdRNAi to elucidate the functions of the Dachsous (Gb’Ds)/Fat (Gb’Ft) signaling pathway. We demonstrated that tibial size and shape along the PD axis in the regenerating cricket leg are regulated through the Gb’Ds/Gb’Ft signaling pathway25-27. Furthermore, classical transplantation experiments showed that, when two leg stumps with discontinuous positional values were grafted, intercalary regeneration restored the missing positional values28,29. Interestingly, intercalary regeneration was not observed in Gb’Ds/Gb’Ft rdRNAi legs, indicating that Gb’Ds/Gb’Ft signaling is also essential for specification of positional identity in regenerating tibia25. These findings clearly show that rdRNAi is useful for analyzing gene functions during leg regeneration.

Here, we analyzed the functions of Gb’Dll and Gb’dac in regeneration of the tarsus and tibia. We demonstrated that mutual transcriptional regulation exists between Gb’Dll and Gb’dac in the tarsal segments, leading to proper pattern formation of these segments. The short-tibia phenotypes obtained by rdRNAi against Gb’dac closely resemble those obtained by rdRNAi against Gb’dac/Gb’Ft. In addition, intercalary regeneration did not occur in legs treated with rdRNAi against Gb’dac, as observed in legs treated with rdRNAi against Gb’Ds/Gb’Ft. These results indicated that cell proliferation along the PD axis in regenerating tibia depends on the expression of Gb’dac through interaction with Gb’Ds/Gb’Ft. Based on our results, we proposed molecular cascades functioning in leg regeneration.

Results
RNAi against Gryllus Distal-less and dachshund inhibits regeneration of the leg along the proximodistal axis. We first examined whether rdRNAi occurs against Gb’dac or Gb’Dll during regeneration of the leg amputated at the tibia. We confirmed that dsRNA for Gb’dac or Gb’Dll injected into nymphs at the third instar without leg amputation (nymphal RNAi [nyRNAi]) had no significant effect on legs (treated nymphs vs. adults, data not shown), suggesting that nyRNAi against Gb’dac or Gb’Dll does not affect normal leg growth. Next, we also confirmed that when a DsRed2 dsRNA-injected control leg was amputated at the tibia (red arrowhead in Fig. 1a, b), the normal-looking shapes of the tarsus and claw were observed in a regenerating leg of a control fifth-instar nymph at 7 days post-amputation (dpa) (Fig. 1c, b), although these structures were slightly shorter than those of the contralateral leg. The regenerating tibia also recovered its length with the tibial spurs at the tibial end (Fig. 1b, c). Three tarsal segments with the tarsal spurs and claw were fully restored in the adult regenerated leg (Fig. 1d; n = 64/64). In contrast, when the right metathoracic legs of third-instar nymphs were amputated at the tibia immediately after injection of dsRNA for Gb’dac or Gb’Dll, the length of regenerated legs of treated adults was changed (Fig. 1e, i, m) relative to controls (Fig. 1a).

In the Gb’dac rdRNAi nymphs at 7 dpa, the regenerating leg became short and showed obvious defects in the tarsus and tibia (Fig. 1f). Ta1 failed to form, but no significant defects were observed in Ta2 and 3 or in claws (Fig. 1g). Furthermore, the regenerated tibia was shorter than the control tibia, and the tibial spurs appeared in the distal side (Fig. 1g). In the Gb’Dll rdRNAi adult, Ta3 and claws were restored, whereas Ta1 was remarkably shortened (n = 8/64) or deleted (n = 56/64) (Figs. 1h and 2a). In addition to this structural change, the tibial spurs formed but tibia size along the PD axis was not fully restored (Fig. 1h; n = 61/64). These results indicate that the loss of Gb’dac function leads to defects in Ta1 and the tibia but does not affect the formation of Ta2 and 3 and the tibial decorations.

In Gb’Dll rdRNAi nymphs and adults, we found two distinct phenotypes in rdRNAi tarsi: either a short (Fig. 1j, l) or long (Fig. 1n, p) Ta1, depending on the dsRNA concentration of Gb’Dll. The regenerated tarsus of nymphs injected with dsRNA against Gb’Dll at a concentration of 20 μM became shorter (Fig. 1j), while at a lower concentration (0.2 μM), the regenerated tarsus became much longer than the normal tarsal segment (Fig. 1n). Interestingly, the claws and tarsal spurs were not clearly observed in either rdRNAi leg (Fig. 1k, o). In the Gb’Dll rdRNAi (20 μM) adult legs, Ta1 became short (Fig. 1l; n = 26/38), but the longer Ta1 was also observed in 31% of rdRNAi legs (Fig. 2a; n = 12/38). By contrast, approximately 91% of Gb’Dll rdRNAi (0.2 μM) legs showed the long-segment phenotype (Figs. 1p and 2a; n = 42/46). Ta2 and 3 and claws were entirely absent in Gb’Dll 20 and 0.2 μM rdRNAi legs. The regenerated tibiae of Gb’Dll rdRNAi legs achieved almost normal size (see Fig. 2), whereas distal decorations, including tibial spurs, did not appear in Gb’Dll 20 μM rdRNAi legs (Fig. 1l). In Gb’Dll 0.2 μM rdRNAi legs, loss of tibial and tarsal spurs was an unstable phenotype (Fig. 1p). These results suggest that: (1) normal Gb’Dll expression is essential for formation of the tarsal segments; and (2) specification of the tarsal segments and formation of tibial spurs depend on the expression level of Gb’Dll.

To examine whether the leg phenotypes obtained by rdRNAi against Gb’dac or Gb’Dll depend on amputation position in the tibia, we measured the length of the regenerated adult tarsus and tibia after amputation at the proximal, middle, or distal level (Fig. 2b). We compared the length of Ta1 obtained by distal amputation (n = 11) with that obtained by middle (n = 8) or proximal (n = 12) amputation. Middle and proximal amputation reduced the length of Ta1 to approximately 86% (t-test; P < 0.05, n = 8) and 87% (t-test; P < 0.05, n = 12), respectively (Fig. 2c). The length of the regenerated tibia after middle and proximal amputation became short, to approximately 88% (t-test; P < 0.01) and 85% (t-test; P < 0.01) respectively, compared with that after distal amputation (Fig. 2d). These results indicated that amputation itself could reduce the size of the regenerated leg segment to 85% of adult length.

We then investigated the effects of amputation position on knock-down leg phenotype by rdRNAi against Gb’dac or Gb’Dll (dsRNA: 20 or 0.2 μM). In the case of Gb’Dll rdRNAi (20 μM), the relative ratios of length of Ta1 to the control were reduced to 37% (t-test; P < 0.001; n = 8), 37% (t-test; P < 0.01; n = 6), or 29% (t-test; P < 0.001; n = 12) after proximal, middle, or distal amputation (Fig. 2e). By contrast, in the case of Gb’dac rdRNAi (0.2 μM), the relative ratios of Ta1 increased to approximately 163% (t-test; P < 0.001; n = 10), 186% (t-test; P < 0.01; n = 6), or 234% (t-test; P < 0.001; n = 6) after proximal, middle, or distal amputation, respectively (Fig. 2e). The
regenerating leg. (p) Regenerated leg of a fifth-instar nymph treated with Gb'dac dsRNA. (q) Higher magnification of panel p showing a tarsus of Gb'dac dsRNA regenerating leg. (r) Regenerated leg of a Gb'Dll dsRNA adult. (s) A Gb'Dll (0.2 μM) dsRNA adult. (t) A Gb'Dll (0.2 μM) dsRNA regenerating leg. (u) Regenerated leg of a Gb'Dll (0.2 μM) dsRNA adult. A long Ta1 was formed. (b–c, f–g, j–k, and n–o) Scanning electron microscopy (SEM) of dsRNA regenerating legs. Red arrowheads in (a–b), (e–f), (i–j), and (m–n) indicate the amputation site. Arrows indicate spurs of the tibiae in (c), (d), (g), and (h). Arrowheads indicate spurs of the tarsi in d and h. Scale bars: 5 mm in (a) and (d). Ta1–3, tarsal segments 1–3; Ti, tibia; Cl, claws.

Figure 1 | Leg phenotypes obtained by rdRNAi against Gb’dac and Gb’DII during regeneration. (a) DsRed2 rdRNAi control adult cricket with a normally regenerated right metathoracic (T3) leg. (b) Control regenerating leg of a fifth-instar nymph. (c) Higher magnification of panel b showing a control-regenerating tarsus. (d) Control regenerated leg of an adult. Normal tarsus consists of tarsal segments (Ta) 1, 2, and 3, and spurs and claws. (e) Gb’dac dsRNAi adult with a short regenerated T3 leg. (f) Regenerating leg of a fifth-instar nymph treated with Gb’dac dsRNAi. (g) Higher magnification of panel f showing a tarsus of Gb’dac dsRNAi regenerating leg. (h) Regenerated leg of a Gb’dac dsRNAi adult showing a normal Ta2 and 3, and claws but lacking Ta1 and having a short tibia. (i) Gb’DII dsRNAi adult (injection of 20 μM Gb’DII dsRNA). (j) Regenerating leg of a fifth-instar nymph treated with Gb’DII (20 μM) dsRNAi. (k) Tarsus of regenerating Gb’DII (20 μM) dsRNAi leg at higher magnification than shown in j. (l) Regenerated leg of a Gb’DII (20 μM) dsRNAi adult. Gb’DII (20 μM) dsRNAi caused extreme reduction of all tarsal structures. (m) A Gb’DII (0.2 μM) dsRNAi adult. (n) Regenerating leg of a fifth-instar nymph treated with Gb’DII (0.2 μM) dsRNAi regenerating leg. (o) Regenerated leg of a Gb’DII (0.2 μM) dsRNAi adult. A long Ta1 was formed. (b–c, f–g, j–k, and n–o) Scanning electron microscopy (SEM) of dsRNAi regenerating legs. Red arrowheads in (a–b), (e–f), (i–j), and (m–n) indicate the amputation site. Arrows indicate spurs of the tibiae in (c), (d), (g), and (h). Arrowheads indicate spurs of the tarsi in d and h. Scale bars: 5 mm in (a) and (d). Ta1–3, tarsal segments 1–3; Ti, tibia; Cl, claws.

relative ratio of tibial length in Gb’DII rdRNAi was not significantly different from that of the control group for any amputation position (Fig. 2f). For Gb’dac rdRNAi, the relative ratios of Ta1 were 23% (t-test; P < 0.001; n = 8), 25% (t-test; P < 0.001; n = 6) and 22% (t-test; P < 0.001; n = 10) after proximal, middle, and distal amputation, respectively (Fig. 2e); the relative ratios of tibial length were reduced to about 58% (t-test; P < 0.01; n = 10), 69% (t-test; P < 0.01; n = 6), and 68% (t-test; P < 0.01; n = 14) after proximal, middle and distal amputation (Fig. 2f). One way-analysis of variance (ANOVA) showed no significant differences in the relative ratio of length of Ta1 between sample groups for different amputation positions (Gb’DII rdRNAi 20 μM, P = 0.6927; Gb’DII rdRNAi 0.2 μM, P = 0.0752; Gb’dac rdRNAi, P = 0.8403). The relative ratios of length of the tibial segment in Gb’dac rdRNAi also did not differ significantly between sample groups of amputation position (ANOVA; P = 0.1335). These results suggest that the distinct tarsal phenotypes observed by Gb’DII rdRNAi (20 μM and 0.2 μM) are independent of tibia amputation position. We conclude that Gb’dac is involved in size determination of the tibia and Ta1, whereas Gb’DII plays essential roles in the formation of the three tarsal segments during regeneration. It is interesting to note that size determination of Ta1 depends on the expression levels of both Gb’dac and Gb’DII.

Mutual transcriptional regulation between Gb’dac and Gb’DII in regenerating tarsal segments. To elucidate expression patterns of Gb’DII and Gb’dac during regeneration, we performed whole-mount in situ hybridization. Tracheal tubes shown in the in situ hybridization figures were artificially stained. In the Gryllus limb bud, after the major leg segments are established, both Gb’dac and Gb’DII are expressed in the presumptive tibial segment and in a proximal area of the presumptive tarsal segment, whereas only Gb’DII is expressed in the distal area of the presumptive tarsal segment11. Expression patterns of Gb’dac and Gb’DII in regenerating legs are essentially similar to those in the limb bud. At 5 dpa, Gb’DII expression was more intense in distal Ta2 and 3 than in Ta1 and a distal region of the tibia (Fig. 3a; n = 18), and Gb’dac was expressed in the proximal region of Ta1 and in the tibial segment, except for the most distal region of the tibia (Fig. 3e; n = 21). Expression of Gb’DII was not detected in the claw. We speculate that intense expression of Gb’DII may suppress expression of Gb’dac in Ta2 and 3, while weak expression of Gb’DII in Ta1 might induce Gb’dac expression. To test this, we observed expression patterns of Gb’dac and Gb’DII in regenerating legs with rdRNAi against Gb’dac (Fig. 3f; n = 7/10), while Gb’dac expression was also substantially reduced in Ta1 but persisted in regenerating tibia (Fig. 3f; n = 13/18). In contrast, in the case of Gb’DII rdRNAi (0.2 μM), weak expression of Gb’DII was present in the whole tarsus (Fig. 3c; n = 8/10), whereas the expression of Gb’dac became intense in the distal tarsus (Fig. 3g; n = 14/14). To confirm that the amounts of Gb’dac and Gb’DII mRNA were
decreased by rdRNAi, we performed qPCR and estimated the ratios of the amount of Gb’dac or Gb’Dll mRNA in comparison with the corresponding control (n = 11) at 5 dpa. The relative ratios of Gb’Dll mRNA were lowered to 29% (t-test; P < 0.05) and 53% (t-test; P < 0.05) in the rdRNAi tarsi against Gb’Dll with 20 μM dsRNA (n = 11) and 0.2 μM (n = 10), respectively (Fig. 3i), indicating that knockdown effects by rdRNAi depend on the concentration of Gb’Dll dsRNA. In the same case, the relative ratio of Gb’dac mRNA was reduced to 54% (t-test; P < 0.01) in rdRNAi tarsi against Gb’Dll (20 μM), while the relative ratio of Gb’dac increased to 127% (t-test; P < 0.05) in rdRNAi tarsi against Gb’Dll (0.2 μM) (Fig. 3i). These results demonstrate that increased Gb’dac expression due to lowering Gb’Dll expression levels may contribute to elongation of the regenerated Ta1.

Next, we found that Gb’dac rdRNAi reduced intense expression of Gb’Dll in Ta2 and 3 (Fig. 3d; n = 10/12), and reduced Gb’dac expression (Fig. 3h; n = 9/11) at 5 dpa. In the case of Gb’dac rdRNAi, the relative ratio of Gb’Dll mRNA decreased to 62% (t-test; P < 0.05; n = 12), while Gb’dac mRNA was depleted to 54% (t-test; P < 0.05) at 5 dpa (Fig. 3i). In blastemal cells at 2 dpa, Gb’Dll mRNA was reduced to 87% (t-test; P = 0.076; n = 10), concomitant with reduction of Gb’dac mRNA to 31% (t-test; P < 0.05) (Fig. 3j). Taken together, these results suggest that Gb’dac expression in Ta2 and 3 is suppressed by high expression of Gb’Dll, which was upregulated by Gb’dac in the early blastemal cells. Thus, we concluded that proteins of Gb’Dll and Gb’dac mutually regulate transcription and formation of the tarsal segments during regeneration.

Effects of Gb’Dll and Gb’dac expression on cell proliferation in the regenerating leg. We observed that expression patterns of Gb’Dll and Gb’dac regulate the size of tarsal segments during regeneration. It is reasonable to consider that leg segment size depends on cell proliferation and/or cell death along the PD axis. Thus, we measured cell proliferation rate in regenerating control legs by EdU-incorporation assays. The relative ratios of the number of EdU-positive cells to the total cells in the presumptive tarsus (Ta) at 3.5 dpa (Ta area in Fig. 4b; n = 5) and 5 dpa (Ta area in Fig. 4c; n = 7) increased to 195% (t-test; P < 0.001) and 149% (t-test; P < 0.01), respectively, compared to the relative ratios for the blastema at 2 dpa (rectangle in Fig. 4a; n = 6) (Fig. 4m). We next examined whether cell proliferation rates in regenerating legs were changed by Gb’Dll and Gb’dac rdRNAi. After injecting EdU into rdRNAi nymphs, we counted the number of EdU-positive cells at 2 dpa (rectangle in Fig. 4d, g, j). Interestingly, the number of EdU-positive cells decreased in blastemas treated with rdRNAi against Gb’Dll (20 μM, Fig. 4d; n = 6) or Gb’dac (Fig. 4j; n = 5), in comparison with the control (Fig. 4n). Conversely, the number of EdU-positive cells increased in blastemas treated with rdRNAi against Gb’Dll (0.2 μM) (Fig. 4g; n = 5) (Fig. 4n). We further examined the effect of Gb’Dll and Gb’dac rdRNAi on EdU incorporation rate into the tarsus (Ta) and tibia (Ti) at 3.5 and 5 dpa. In comparison with data for the control Ta, the relative ratios of the number of EdU-positive cells to total cells decreased to 27% (t-test; P < 0.001) at 3.5 dpa (Ta area in Fig. 4e; n = 5) and 65% (t-test; P < 0.05) at 5 dpa (Ta area in Fig. 4f; n = 5) for Gb’Dll rdRNAi (20 μM), and 15% (t-test; P < 0.001) at 3.5 dpa (Ta area in Fig. 4k; n = 5) and 76% (t-test; P < 0.05) at 5 dpa (Ta area in Fig. 4l; n = 6) for Gb’dac rdRNAi (Fig. 4o). By contrast, the number of EdU-positive cells in Ta treated with rdRNAi against Gb’Dll (0.2 μM) increased to 139% (t-test; P < 0.05) at 5 dpa (Ta area in Fig. 4i; n = 5), but there was no corresponding effect on Ta at 3.5 dpa (Ta area in Fig. 4h; n = 4). Whereas the numbers of EdU-positive cells decreased to 29% (t-test; P < 0.05) (Ti area in Fig. 4k) and 62% (t-test; P < 0.05) (Ti area in Fig. 4l) in tibiae treated with Gb’dac rdRNAi at 3.5 dpa and 5 dpa, respectively, compared with the control tibia (Fig. 4p). Next, we investigated whether apoptotic cell death occurs in regenerating legs. Only a few TUNEL-positive cells were detected in regenerating control legs, and their numbers did not increase in rdRNAi legs (data not shown). Taken together, these data suggest that an increase in proliferation rate in the presumptive tarsus at 3.5 dpa is primarily responsible for the tarsal growth, and that the proliferation is affected mainly by Gb’dac expression.

A regulatory cascade of Gb’Dll and Gb’dac expression in regenerating legs. We found that elongated Ta1 obtained by rdRNAi against
Gb'Dll (0.2 μM) formed due to an increase in cell proliferation induced by Gb'dac expression. Furthermore, we considered that weak expression of Gb'Dll induced by rdRNAi (0.2 μM) upregulates the expression of Gb'dac, which in turn activates cell proliferation. To confirm this, we analyzed phenotypes obtained by dual rdRNAi knockdown accomplished by simultaneous injection of two different dsRNAs. At the sixth instar, a control leg with normal tarsus was obtained by injection of dsRNAi against Gb'dac (Fig. 5d; 5n = 14/16) was changed to either normal or short length (Fig. 5c; n = 17/19) by dual rdRNAi against Gb'dac. In contrast, the short tarsus obtained by Gb'Dll rdRNAi (20 μM) showed no significant change by dual rdRNAi against Gb'dac (Fig. 5d; n = 11/11). These findings demonstrate that the expression of Gb'Dll is induced by Gb'dac in Tb1, but not in Tb2 and 3, where intense expression of Gb'Dll suppresses Gb'dac expression. It should be noted that the intense expression of Gb'Dll was induced by expression of Gb’dac in the blastema during the early stage of regeneration. Thus, these interactions between Gb'Dll and Gb'dac regulate the size of tarsal segments during regeneration.

Regulation of Gb'Dll and Gb'dac on expression of distal patterning genes in the tarsus. We found that Gb'Dll is involved in pattern formation of Tb2 and 3. In order to identify the target genes of Gb'Dll involved in patterning the distal tarsal segments, we observed the expression patterns of Gryllus orthologs of Drosophila tarsal-appendage-patterning genes such as Epidermal growth factor receptor (Gb'Egfr), aristless (Gb'ald), BarH (Gb'BarH), and bric-a-brac (Gb'bab). Gb'Egfr was expressed in the segmental boundaries in the tarsus and claw (Fig. 6a; n = 14), and Gb'ald was expressed in Tb2 and 3 and in the tibia–tarsus boundary (Fig. 6c; n = 6). Gb'BarH was expressed broadly over Tb2 and 3 (Fig. 6i; n = 9), and Gb'bab expression occurred as a narrow circumferential ring in Tb2 (Fig. 6m; n = 10). We next examined the effects of Gb'Dll rdRNAi on the expression patterns of these genes. In regenerating legs treated with rdRNAi against Gb'Dll (either 20 μM or 0.2 μM), the expression of Gb'Egfr was significantly reduced in the two boundaries in the tarsus and claw, but persisted in the tibia–tarsus boundary (Fig. 6b, c; n = 12/14 or 10/12). Furthermore, the expression of Gb'al (Fig. 6i, g; n = 5/5 and 5/5), Gb'BarH (Fig. 6j, k; n = 7/7 and 7/7), and Gb'bab (Fig. 6n, o; n = 3/3 and 8/8) were significantly downregulated in rdRNAi legs against Gb'Dll (both 20 μM and 0.2 μM), while rdRNAi against Gb'Dll (0.2 μM) did not affect the expression of Gb'al in the tibia–tarsus boundary (Fig. 6g). These results suggest that depletion of Gb'Dll mRNA results in downregulation of these distal patterning genes, leading to lack of the distal tarsal segments. We also examined the effects of Gb'dac rdRNAi on the expression of distal patterning genes. The expression of Gb'Egfr (Fig. 6d; n = 13/16), Gb'ald (Fig. 6h; n = 5/8), and Gb'BarH (Fig. 6l; n = 5/8) became weak in Tb2 and 3, but the expression of Gb'Egfr and Gb'ald in the tibia–tarsus boundary was unchanged (Fig. 6d, h). In contrast, Gb'bab expression became broad in Tb1 (Fig. 6p; n = 6/8). Because depletion of Gb'dac mRNA reduces Gb'Dll expression in the distal tarsal segment, these results suggest that the expression of distal patterning genes is downregulated by lowered Gb'Dll mRNA levels, but that Gb'bab transcription in Tb1 is negatively regulated by Gb'dac expression.

Gene expression patterns in a wild-type nymph are schematically illustrated in Figure 6q to show plausible correlations between expression patterns and tarsal segmentation (See also Fig. 8a). In the case of rdRNAi against Gb'Dll (0.2 μM), when Gb'Dll decreases to a certain threshold level, expression of Gb'dac is induced in the distal region, which then suppresses expression of the tarsal-pattern-
Figure 4 | Effects of rdRNAi against Gb'Dll and Gb'dac on cell proliferation. (a–l) Localization of EdU-incorporated cells in blastemal regions at 2 dpa, and in regenerating tibiae and tarsi at 3.5 and 5 dpa for DsRed2 rdRNAi (a–c), Gb’Dll rdRNAi (20 μM) (d–f), Gb’Dll rdRNAi (0.2 μM) (g–i), and Gb’dac rdRNAi (j–l) nymphs. EdU-positive and nuclei-merged cells are shown in green, and nuclei are shown in blue. Rectangles in panels (a), (d), (g), and (j) indicate blastemal regions at 2 dpa; rectangles in panels (b–c), (e–f), (h–i), and (k–l) indicate regenerating tibial regions (Ti) and tarsal regions (Ta). The panels in (a–l) show confocal z-stack images. (m) Relative fold changes in the numbers of EdU-positive cells in the blastemal region at 2 dpa and tarsus regions at 3.5 and 5 dpa in regenerating control legs are plotted, with the number in the blastemal region at 2 dpa set at 1. (n–p) Cell proliferation was analyzed quantitatively in blastemal regions at 2 dpa (n), in regenerating tarsal regions at 3.5 and 5 dpa (o), and in tibial regions at 3.5 and 5 dpa (p) in nymphs injected with Gb’Dll (20 μM), Gb’Dll (0.2 μM), and Gb’dac dsRNA. Relative fold changes in the numbers of EdU-positive cells in the blastemal region at 2 dpa, in regenerating tarsal regions at 3.5 and 5 dpa, and in tibial regions at 3.5 and 5 dpa in rdRNAi legs are plotted (numbers of EdU-positive cells, including controls, set at 1). In panels (m–p), the data are means ± SD. (Student’s t-test; *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant). Scale bars in (a–c) = 100 μm.
Figure 5 | Effects of single or dual rdRNAi against Gb'dac and Gb'Dll on length of the tarsus. (a, b) Effect of single rdRNAi on phenotype of regenerating tarsus at the sixth instar. (c, d) Effect of dual rdRNAi on phenotype of regenerating tarsus at the sixth instar. (a) Tarsus in regenerating leg of a DsRed2 rdRNAi nymph. (b) Distal elongated Ta1 of a nymph treated with Gb'Dll (0.2 μM) single rdRNAi. (c) Phenotype of Ta1 of a nymph treated with dual rdRNAi against Gb'Dll (0.2 μM) and Gb'dac. (d) Phenotype of Ta1 of a nymph treated with dual rdRNAi against Gb'Dll (20 μM) and Gb'dac. Ta1, Ta2, and Ta3 indicate tarsal segments 1, 2, and 3. Arrow and arrowhead (panel a) indicate spurs of the tibia and tarsus, respectively. Scale bar = 1 mm.

Figure 6 | Effects of rdRNAi against Gb'Dll and Gb'dac on expression patterns of tarsal patterning genes, with schematic illustrations. (a–d) Expression patterns of Gb'Egfr in regenerating tarsi at 5 dpa for DsRed2 rdRNAi (a), Gb'Dll rdRNAi (20 μM) (b), Gb'Dll rdRNAi (0.2 μM) (c), and Gb'dac rdRNAi (d) nymphs. (e–h) Expression patterns of Gb'al in regenerating tarsi at 5 dpa for DsRed2 rdRNAi (e), Gb'Dll rdRNAi (20 μM) (f), Gb'Dll rdRNAi (0.2 μM) (g), and Gb'dac rdRNAi (h) nymphs. (i–l) Expression patterns of Gb'BarH in regenerating tarsi at 5 dpa for DsRed2 rdRNAi (i), Gb'Dll rdRNAi (20 μM) (j), Gb'Dll rdRNAi (0.2 μM) (k), and Gb'dac rdRNAi (l) nymphs. (m–p) Expression patterns of Gb'bab in regenerating tarsi at 5 dpa for DsRed2 rdRNAi (m), Gb'Dll rdRNAi (20 μM) (n), Gb'Dll rdRNAi (0.2 μM) (o), and Gb'dac rdRNAi (p) nymphs. (q) Schematic illustration of a plausible relationship between expression patterns of the tarsal patterning genes in a wild-type presumptive tarsus at 5 dpa and in tarsal segments 1–3. See details in the text. (r) Schematic illustration of a plausible relationship between tarsal segment-1-like structure and expression patterns of the tarsal patterning genes in a presumptive tarsus at 5 dpa observed in a nymph treated with rdRNAi against Gb'Dll (0.2 μM). See details in the text. Scale bar = 100 μm.
Gb’dac expression is regulated by expression of Gryllus Dachsous and Fat in the regenerating tibia. We found that short-tibia phenotypes obtained by rdRNAi against Gb’dac resemble those obtained by rdRNAi against Gb’ds/Gb’ft; however, the thick phenotype induced by rdRNAi against Gb’ds/Gb’ft was not observed in the Gb’dac rdRNAi case. Therefore, we speculated that a relationship between Gb’dac and Gb’ds/Gb’ft is involved in regulating cell proliferation in the tibia. We hypothesized that expression of Gb’dac in the regenerating tibia may regulate cell proliferation through a Gb’Ds/Gb’Ft signaling pathway. To test this hypothesis, we analyzed expression of Gb’dac in regenerating legs treated with rdRNAi against Gb’ds or Gb’ft. In regenerating control legs at 2 dpa, Gb’dac expression was observed in the blastema and tibia but was not detected in the most distal tip of the blastema (Fig. 7a; n = 7). In regenerating legs treated with Gb’ds or Gb’ft rdRNAi, expression of Gb’dac was substantially reduced in the blastema at 2 dpa (Fig. 7b; n = 8/10 or Fig. 7c; n = 5/8). Interestingly, at 5 dpa the expression of Gb’dac was significantly reduced in Ta1 and whole tibia in Gb’ds rdRNAi legs (Fig. 7e; n = 16/20) relative to control legs (Fig. 7d; n = 10). The expression of Gb’dac was slightly decreased by Gb’ft rdRNAi at 5 dpa (Fig. 7f; n = 12/16). These changes in expression patterns were confirmed by qPCR (Fig. 7g, h). Relative quantities of Gb’dac transcripts in regenerating tibiae decreased due to rdRNAi against Gb’dac/Gb’ft at both 2 dpa (Fig. 7g) and 5 dpa (Fig. 7h). These results suggest that Gb’dac acts as a downstream factor of Gb’Ds/Gb’Ft signaling, which might control cell proliferation in the tibia.

Discussion

Using an rdRNAi-knockdown approach against Gb’Dll and Gb’dac, we found that these genes are involved in regeneration of the tibia and tarsus after tibial amputation. Based on our experimental data, we propose a model for regulation of leg regeneration by Gb’Dll and Gb’dac, and discuss the following two points: (1) molecular cascades involved in tarsal segmentation during leg regeneration (Fig. 8a), and (2) regulation of cell proliferation in the regenerating tibia by Gb’dac (Fig. 8b).

When a cricket leg is amputated at the middle of the tibia, the whole tarsus and half of the tibia are lost. The regenerated blastema is formed in the distal region of the amputated leg, and blastemal cells proliferate and form the missing structures by intercalation between the most distal region and the remaining part of the leg. Since Dll is expressed by induction of Egfr signaling in the most distal region during limb development and regeneration23, Gb’Dll can be considered key for establishing the distal structures, especially the three tarsomeres. In addition, because Gb’dac is expressed in the tibial segment and in Ta1, it can also be considered to be involved in their formation. We illustrate the expression patterns of the genes studied here in the three tarsomeres, as shown in Fig. 6q, and their changes in the case of rdRNAi against Gb’Dll (0.2 μM) (Fig. 6r). A possible molecular cascade for establishing the three tarsal segments is illustrated in Fig. 8a. The cascade consist of two pathways, depending on the amount of Gb’Dll transcripts: when high expression of Gb’Dll is
Gb'BarH, induced by signaling through receptor (EGFR) signaling and model for transcriptional regulation of tarsal repatterning genes to form regulation of cell proliferation in regenerating tibia.

During the establishment of PD patterning in leg regeneration, cell proliferation appears to be promoted in the presumptive tibia in addition to the regenerating tarsus. Here, we focus on regeneration of the tibia. We found that (1) regenerated tibia treated with rdRNAi against Gb'dac became short, similar to the short-tibia phenotype obtained by rdRNAi against Gb'ds/Gb'ft; (2) no intercalary regeneration occurs in the case of rdRNAi against Gb'dac, as observed in the case of rdRNAi against Gb'ds/Gb'ft (see supplemental section); (3) Gb'dac expression during leg regeneration overlaps with the expression domain of both Gb'ds and Gb'ft in the tibia; and (4) Gb'dac expression is positively regulated by Gb'ds/Gb'ft expression. Based on these results, we proposed a possible signaling cascade in tibial regeneration (Fig. 8b). Previous work showed that rdRNAi against Gb'ds and Gb'ft induces formation of a short and thick tibia with the normal short distal structures, including spines and spurs. It is noteworthy that tibia treated with rdRNAi against Gb'ds/Gb'ft became short, despite the fact that cell proliferation was accelerated. This shortening of the tibia is due to rearrangement of positional values in the amputated tibia. On the other hand, the thickening may be caused by cell proliferation that is probably promoted by cyclin E through inactivation of the Hippo/Warts signaling pathway, including Gb'Yokie (Gb'Yki)/Gb'Scalloped (Gb'Sd) or Gb'Yki/Gb'Mothers against dpp (Gb'Mad), and Gb'Bantam (Fig. 8b). Since the short-tibia phenotype found by knockdown of Gb'dac expression is not associated with tibia thickness, a signaling cascade involving Gb'dac should be different from that involving cyclin E (Fig. 8b). We have so far been unable to establish how Gb'dac expression is regulated by Gb'Ds/Gb'ft signaling. However, we can conclude that Gb'dac expression affects tibial cell proliferation and helps to determine the size of the regenerating tibia along the PD axis, through the Gb'Ds/Gb'ft signaling network.

**Methods**

**Animals.** All adult and nymph two-spotted crickets, Gryllus bimaculatus, were reared under standard conditions.

**Regeneration-dependent RNAi (rdRNAi).** Preparation of double-stranded RNAs (dsRNAs) for Gb'Dl, Gb'dac, Gb'ds, and Gb'ft, and the rdRNAi method, were described previously. After injection of dsRNAs into the abdomen of third-instar nymphs, their tibiae were amputated at the distal position between the second and third spines. Thus, this amputation removed 30% of the distal part of the tibia. The concentration of dsRNA for each gene was 20 μM, except for Gb'Dll injected at a lower dose (0.2 μM). The regeneration processes of RNAi nymph legs were observed in comparison with the negative control injected with dsRNA for DsRed2. DsRed2 dsRNA was prepared as previously described. Dual RNAi is performed by injecting a dsRNA mixture for two target genes, Gb'dac and Gb'Dl (20 or 0.2 μM). The final concentration of each dsRNA was adjusted to 20 μM (or 0.2 μM for lower-concentration Gb'Dll).

**Scanning electron microscopy (SEM).** The cricket rdRNAi legs were fixed in 4% paraformaldehyde and 4% glutaraldehyde in PBS overnight and washed three times in PBS for 15 min at room temperature. The rdRNAi legs were processed through an alcohol series (20, 40, and 60% ethanol in PBT; 80% ethanol in water; 45% ethanol and 45% tert-butyl alcohol in water; each for 40 min) and in 100% ethanol (or 0.2 μM for lower-concentration Gb'Dll).

**Transplantation experiments for nymphal legs.** A transplantation experiment for normal intercalary regeneration was performed as described previously. Briefly, the experiments were performed with nymphs treated with Gb'dac rdRNAi. We used the amputated metathoracic leg stump as a host and the amputated mesothoracic distal figure provides a schematic model for transcriptional regulation of tarsal repatterning genes to form the tarsal segments. After leg amputation, epidermal growth factor receptor (EGFR) signaling and Gb'dac induce a high level of Gb'Dll expression in the blastema. When knockdown, low Gb'Dll expression induces Gb'dac expression in the proximal presumptive tarsal segment 1. In the distal presumptive tarsal segments 2 and 3, high Gb'Dll activity represses Gb'dac expression and induces the expression of Gb'Egfr, Gb'al, Gb'BarH, and Gb'bab, which establish the pattern of tarsal segments 2 and 3 along the proximodistal axis. Gb'dac expression represses Gb'bab expression in the distal tarsal segment 1. (b) A schematic illustration of the Ds/Ft signaling cascades in regeneration of the tibia to regulate tibial cell proliferation. Ds/Ft and Hippo signaling suppresses the activity of the Yki/Sd and Yki/Mad complexes, where Mad activity is regulated by Dpp signaling. Both complexes control growth in part by regulating bantam and Cyclin E, which regulates cell proliferation. Expression of Gb'dac in the presumptive tibia is regulated by downstream genes of the Hippo signaling pathway. Gb'dac activated by formation of a complex with unknown factor(s) may induce cell proliferation.
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Quantitative PCR (qPCR). Total RNA was extracted from the blastemal regions at 2 dpa or from regenerating tibiae and tarsi at 5 dpa of DisRed2 (control), Gb’dac, Gb’dil (20 or 0.2 μM), Gb’ds, and Gb’ft rdRNAi metathoracic (T3) legs. Left and right T3 legs from each individual were used for sampling the blastema or regenerating tibiae and tarsi. qPCR assays were performed in triplicate biological samples. In each assay, 11 nympha at 2 dpa and 10 nymphs at 5 dpa were used for control legs; 12 nymphs at 2 dpa and 10 nymphs at 5 dpa were used for Gb’dac rdRNAi; 11 nymphs (5 dpa) were used for Gb’ds 20 μM rdRNAi legs; 10 nymphs (5 dpa) were used for Gb’dil 0.2 μM rdRNAi legs; 10 nymphs each (2 and 5 dpa) were used for Gb’ds and Gb’ft rdRNAi legs. The ABI 9700 Real-Time PCR System (Applied Biosystems) was used for qPCR as described previously. The qPCR primer sequences were as follows: (forward and reverse, 5’ to 3’): Gb’dil, AACTACTCGGCCTGCACTCCTCGACCT and TCTTGTACTCGCGCTCACTTG; Gb’dil, ACGCAGAAAGGCAAGAAGA and AGTACTGCTCGCTGCTGAGA. We used Gb’actin as an internal control.

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Author contributions
Y.I., T.N., S.N. and T.M. designed the work. Y.I. and T.N. performed all experiments and contributed equally to the work. Y.I., T.N., S.N. and T.M. analyzed the data. Y.I., T.N., S.N. and T.M. prepared all figures and wrote the main manuscript text.

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