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BMP signaling in distal patterning and intercalation during leg regeneration of the cricket, *Gryllus bimaculatus*

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Author contributions

Y.I. and T.M. designed this study. Y.I. performed all of the experiments. Y.I., T.B., H.O., S.N., and T.M. analyzed the data. Y.I. and T.M. prepared all of the figures and wrote the main text of the manuscript. All of the authors contributed to a critical assessment of the manuscript.

Running title: Role of BMP signaling in leg regeneration

Abstract

The cricket, *Gryllus bimaculatus*, is a classic model of leg regeneration following amputation. We previously demonstrated that *Gryllus decapentaplegic* (*Gb'dpp*) is expressed during leg regeneration, although it remains unclear whether it is essential for this process. In this study, double-stranded RNA targeting the *Smad mathers-against-dpp* homolog, *Gb'mad*, was employed to examine the role of Bone morphogenetic protein (BMP) signaling in the leg regeneration process of *Gryllus bimaculatus*. RNA interference (RNAi)-mediated knockdown of *Gb'mad* led to a loss of tarsus regeneration at the most distal region of regenerating leg segments. Moreover, we confirmed that the phenotype obtained by knockdown of Dpp type I receptor, *Thick veins* (*Gb'tkv*), closely resembled that observed for *Gb'mad* RNAi crickets, thereby suggesting that the BMP signaling pathway is indispensable for the initial stages of tarsus formation. Interestingly, knockdown of *Gb'mad* and *Gb'tkv* resulted in significant elongation of regenerating tibia along the proximodistal axis compared with normal legs. Moreover, our findings indicate that during the regeneration of tibia, the BMP signaling pathway interacts with Dachsous/Fat (*Gb'*Ds/*Gb'*Ft) signaling and *dachshund* (*Gb'dac*) to re-establish positional information and regulate determination of leg size. Based on these observations, we discuss possible roles for *Gb'mad* in the

distal patterning and intercalation processes during leg regeneration in *Gryllus bimaculatus*.

Keywords: Dachsous/Fat, Decapentaplegic, *Gryllus bimaculatus*, intercalation, leg regeneration.

INTRODUCTION

Regeneration involves the recognition of missing tissue and restoration of relational structures. Insects and arthropods can regenerate lost appendages, such as legs (Bohn, 1971; French, 1976a; French, 1976b; French, 1978; Truby, 1983). However, there are many aspects of regeneration that remain to be understood. For example, how do tissues that undergo regeneration initiate diverse cellular processes such as cell death and blastemal cell proliferation and differentiation, how do blastemal cells specify positional identities along the proximodistal (P/D) axis and restore a tissue to its appropriate size, and what factors contribute to the mechanism(s) underlying these regeneration processes. To address these questions, we have studied leg regeneration in the classic two-spotted cricket model, *Gryllus bimaculatus* (Mito and Noji, 2008; Wilson-Horch et al., 2017).

A cricket leg is composed of five segments along the P/D axis: the coxa, trochanter, femur, tibia, and tarsus, with the latter divided into three segments. When the tibia of a third instar nymph cricket is amputated at any level of the P/D axis, the leg regenerates and recovers its allometric size and proper shape by the sixth instar (Mito et al., 2002). During the initial period of leg regeneration, regional specialization of blastemal cells is evident along the anteroposterior (A/P), dorsoventral (D/V), and P/D axes. According to the molecular mechanisms proposed for the boundary

model modified by Campbell and Tomlinson (CTBM), ventral and dorsal cells in the anterior compartment close to the A/P boundary express *wingless* (*wg*) and *decapentaplegic* (*dpp*), respectively (Campbell and Tomlinson, 1995; Meinhardt, 1982). Similarly, we previously demonstrated that *Gb'wg* and *Gb'dpp* are expressed in the ventral and dorsal blastemal cells of a leg amputated at the distal tibia, consistent with the CTBM (Mito et al., 2002; Nakamura et al., 2008a; Niwa et al., 2000). Interestingly, this expression profile of *Gb'wg* and *Gb'dpp* is similar to that observed in the leg bud of the *Gryllus* embryo (Mito et al., 2002; Niwa et al., 2000). Because the formation of a P/D axis in a regenerating leg is triggered at a site where ventral *Gb'wg*-expressing cells are predicted to be proximal to dorsal *Gb'dpp*-expressing cells at the A/P boundary according to the CTBM, *Gb'wg* and *Gb'dpp* may play essential roles in the establishment of the P/D axis during leg regeneration (Mito et al., 2002; Nakamura et al., 2008a).

Recently, we applied RNAi to elucidate functions of *Gb'*Wg and *Gb'*Dpp in the *Gryllus* nymphs. Silencing of *Gb'wg* or *Gb'dpp* did not result in an observable phenotype during development or regeneration, probably due to compensation for these losses by another member of the corresponding gene families. However, when expression of an ortholog of beta-catenin, *Armadillo* (*Gb'arm*), was knocked down by RNAi, regeneration was disrupted (Nakamura et al., 2007). These results indicate

that the canonical Wg/Arm signaling pathway plays an essential role in the initiation of the leg regeneration process. Meanwhile, *Mothers-against-dpp* (*Gb'mad*) is the prototype of a family of genes required for signaling by transforming growth factor (TGF)-β-related ligands, and it has been shown to be essential for the BMP signaling pathway (Hamaratoglu et al., 2014; Raftery and Sutherland, 1999; Schmierer and Hill, 2007). Mad is downstream of both the Dpp and BMP type I receptor, *Thick veins* (*Gb'tkv*), and it regulates expression of BMP target genes (Hamaratoglu et al., 2014). It has previously been demonstrated that RNAi targeting of *Gb'dpp* has no effect on leg development and regeneration, while RNAi targeting of *Gb'mad*, *Gb'tkv*, or both *Gb'dpp* and *Gb'glass bottom boat/60A* (*Gb'gbb*), which encodes a BMP family member, leads to precocious adult metamorphosis (Ishimaru et al., 2016). Taken together, these results indicate that the Mad-mediated BMP signaling pathway is critical for ensuring completion of adult metamorphosis.

In the present study, regenerated tibias of cricket legs treated with *Gb'mad* RNAi have been found to be much longer than normal tibias. This phenomenon is potentially due to promotion of cell proliferation in the *Gb'mad* RNAi tibia. *Dachshund* (*Gb'dac*) (Dong et al., 2001; Mardon et al., 1994) encodes a transcriptional co-repressor that has also been found to be associated with cell proliferation of regenerating tibia. Consequently, *Gb'dac* has been shown to contribute to leg growth and determination of

tibial size along the P/D axis (Ishimaru et al., 2015). Thus, the functions of BMP signaling and *Gb'dac* affect tibial cell proliferation, thereby contributing to the determination of length for regenerated tibia. Additionally, we recently demonstrated that Dachsous/Fat (*Gb*'Ds/*Gb*'Ft) signaling (Harvey and Hariharan, 2012; Harvey et al., 2013) plays a crucial role in re-establishing positional information that derives from intercalation of missing structures between the amputated position and the most distal position (Bando et al., 2009; Bando et al., 2011a; Bando et al., 2011b). Based on these findings, we proposed a Ds/Ft gradient (steepness) model in which the Ds/Ft signaling pathway maintains positional information and determines leg size (Bando et al., 2011b; Yoshida et al., 2014).

In the present study, the objective was to employ RNAi targeting of *Gb'dac*, *Gb'ds*, *Gb'ft*, *Gb'tkv*, and *Gb'mad* in a cricket model of leg amputation in order to investigate the mechanisms underlying the initiation of distal pattern formation and tarsus regeneration according to the CTBM and the intercalary pattern formation of tibia regeneration according to the Ds/Ft steepness model. Here we show that the function of BMP signaling is essential for the tarsus regeneration and is involved in the mechanism that controls the distal size of regenerating tibia along the P/D axis.

MATERIALS AND METHODS

Animals

All nymphs of the two-spotted cricket, *Gryllus bimaculatus*, were reared at 28–30 °C with 70% humidity as previously described (Mito et al., 2002; Niwa et al., 2000).

RNAi

Double-stranded RNAs (dsRNAs) designed to target *Gb'mad*, *Gb'tkv*, *Gb'dac*, *Gb'ds*, and *Gb'ft* were prepared as previously described (Bando et al., 2009; Ishimaru et al., 2015; Ishimaru et al., 2016; Nakamura et al., 2007). Briefly, following the injection of dsRNAs into the abdomen of third instar nymphs, tibias were amputated at the distal position between the second and third spines. This amputation removed 30% of the distal part of the tibia. The entire length of regenerated tibia was measured from femoral segment to the tibial spurs at tibial end. Negative controls were injected with dsRNA targeting *DsRed2* which was prepared as previously described (Miyawaki et al., 2004). Dual RNAi targeting was achieved with an injection of two dsRNAs targeting two separate genes. The final concentration of each dsRNA was adjusted to 20 μM. The negative control for the dual RNAi experiments was an injection of buffer instead of dsRNA.

Transplantation of nymph legs

Transplantation experiments to examine the formation of supernumerary legs were performed as described previously (Mito et al., 2002; Nakamura et al., 2008a; Truby, 1986). Briefly, control (*DsRed2*) and *Gb'mad* RNAi were applied to an amputated right metathoracic leg stump, while the amputated distal part of the left mesothoracic leg was used as a graft. To connect the legs, the mesothoracic graft was inserted into the metathoracic leg stump. In addition, a control graft was transplanted onto a control host and an RNAi graft was transplanted onto an RNAi host.

Cell proliferation

Cell proliferation assays were performed by using a Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, Carlsbad, CA, US) (Salic and Mitchison, 2008). Briefly, 5-ethynyl-2'-deoxyuridine (EdU) solution was injected into the abdomen of nymphs at the appropriate analysis stage, and regenerated legs were fixed 4 h later (Bando et al., 2009). Hoechst 33342 was used to stain nuclei.

qPCR

Total RNA was extracted from blastemal regions of control (*DsRed2*) and *Gb'mad* RNAi nymphs at 2 dpa or 5 dpa by using ISOGEN (Wako Pure Chemical Industries Ltd., Osaka, Japan). RNA extraction was performed in triplicate biological samples at 2 dpa or 5 dpa. In each sample,

15 nymphs at 2 dpa and 10 nymphs at 5 dpa were used for control RNAi legs; 17 nymphs at 2 dpa and 10 nymphs at 5 dpa were used for *Gb'mad* RNAi legs. Total RNA was reverse transcribed into cDNA by using the SuperScript III First-Strand Synthesis System (Invitrogen) with an oligo$(dT)_{20}$ primer according to the manufacturer's instructions. The ABI 7900 Real-Time PCR System (Applied Biosystems, Foster, CA, US) with Power SYBR Green PCR Master Mix (Applied Biosystems) was used to perform qPCR as previously described (Ishimaru et al., 2016; Nakamura et al., 2008b). The sequences of the qPCR primers used have been previously described (Bando et al., 2009; Ishimaru et al., 2015; Ishimaru et al., 2016). The *Gb'β-actin* gene was selected as an internal control gene (Bando et al., 2009; Ishimaru et al., 2016). All of the qPCR reactions were performed in triplicate as technical replicates.

RESULTS

Effects of *Gb'mad* on leg regeneration along the P/D axis in crickets

To investigate the function of *Gb'mad* during leg regeneration, a gene knockdown analysis was performed with RNAi. Briefly, *Gb'mad* double-stranded RNA (dsRNA) was injected into third instar nymphs. Subsequently, the right metathoracic legs were amputated at the distal tibia (Fig. 1a). Regenerated legs of sixth instar nymphs were used as controls (Fig. 1b; n = 37/37). The latter provided shapes for the three tarsal

segments and claw, although these structures were shorter than the contralateral leg of the controls. In the sixth instar nymphs that were treated with RNAi targeting *Gb'mad*, the tarsus was not regenerated (Fig. 1b; n = 24/27). This result indicates that the BMP signaling pathway may be involved in distal patterning and tarsus formation during leg regeneration.

Previously, we demonstrated that increased cell proliferation in the presumptive tarsus at 3.5 days post-amputation (dpa) is primarily responsible for tarsal growth (Ishimaru et al., 2015). To examine whether *Gb'mad* contributes to this cell proliferation, *Gb'mad* dsRNA was injected into nymphs. After administration of EdU, the number of EdU-positive cells that were counted in the presumptive tarsus region at 3.5 dpa are shown in Fig. 1c. In comparison with the control presumptive tarsus (n = 7), the relative ratio of EdU-positive cells to total cells decreased by 0.39-fold at 3.5 dpa in the presence of *Gb'mad* RNAi (n = 6) (Fig. 1d). These data suggest that *Gb'mad* RNAi compromises development of the tarsus as a result of decreased cell proliferation at 3.5 dpa. These results indicate that the BMP signaling pathway potentially contributes to regulating the initial growth of a regenerating tarsus.

To examine possible roles for *Gb'mad* during the formation of supernumerary legs, we grafted amputated left mesothoracic legs onto right metathoracic legs within the same nymphs. Among these nymphs, those that received *Gb'mad* RNAi did not form supernumerary legs (n = 14/17),

while supernumerary legs did form in the control nymphs (*n* = 14/15) (Fig. 1e). These results suggest that *Gb'mad* is required to form supernumerary legs and for initiation of tarsal regeneration.

Although regeneration of the tarsus was not observed in the *Gb'mad* RNAi legs, the regenerated tibia was longer than the control tibia (Fig. 1b). Tibial spurs also appeared in the distal position of the regenerated tibia. The relative ratio of tibial length of the regenerated legs to that of the contralateral (normal) legs in the sixth instar controls was reduced by approximately 0.84-fold (Fig. 1g). In contrast, regenerated tibia in the *Gb'mad* RNAi legs were remarkably elongated along the P/D axis, and the relative ratios of tibial length to the contralateral legs and the control regenerated legs were approximately 1.24-fold and 1.47-fold, respectively (Fig. 1g). Furthermore, RNAi targeting of the Dpp type 1 receptor, *Thick veins* (*Gb'tkv*), was performed and a phenotype similar to that of *Gb'mad* RNAi was observed (Fig. 1f; *n* = 8/9). For example, the *Gb'tkv* RNAi nymphs exhibited obvious defects in regeneration of the tarsus, the regenerated tibia were lengthened 1.19-fold (Fig. 1g), and distal structures were observed, including tibial spurs. Taken together, these results indicate that loss of *Gb'mad* and *Gb'tkv* functions leads to a proximal-to-distal elongation of regenerated tibia, whereas distal structures, including tibial spurs, regenerate. Accordingly, we suggest that the BMP signaling pathway

is involved in determining the distal size of regenerated tibia along the P/D axis.

Effects of *Gb'mad* and *Gb'dac* on cell proliferation during leg regeneration

To explore whether cell proliferation contributes to the elongated phenotype of RNAi-targeted *Gb'mad* tibia, EdU incorporation assays were performed. In the *Gb'dac* RNAi nymphs (Fig. 2b, n = 7), the number of EdU-positive cells at 2 dpa was reduced in the blastema compared with the control nymphs (Fig. 2a, n = 6). Conversely, the number of EdU-positive cells increased in the blastema of the *Gb'mad* RNAi nymphs (Fig. 2c; n = 7). When cell proliferation was examined in both models at 5 dpa, the number of EdU-positive cells decreased in the presumptive tibia of the *Gb'dac* RNAi nymphs (Fig. 2e; n = 5) and increased in the presumptive tibia of the *Gb'mad* RNAi nymphs (Fig. 2f; n = 7), compared to the control nymphs (Fig. 2d; n = 6).

Next, the relative ratios of EdU-positive cells to total cells in the blastema and presumptive tibia at 2 dpa and 5 dpa were calculated. Compared to the control legs, the relative ratios decreased by approximately 0.62-fold at both time points for the *Gb'dac* RNAi nymphs and increased 1.97-fold and 1.6-fold in the *Gb'mad* RNAi nymphs, respectively (Fig. 2, g and h). Taken together, these data indicate that

Gb'dac positively affects the proliferation of blastemal and presumptive tibial cells, while *Gb'mad* inhibits cell proliferation and may control the size of regenerating tibia.

Gb'dac **and** ***Gb'mad*** **contribute to regulating the size of regenerating tibia**

Based on the results described above, we hypothesize that the suppression of cell proliferation observed in *Gb'dac* RNAi nymphs results from activation of *Gb*'Mad functions. Consequently, knockdown of *Gb'mad* by RNAi should rescue the shortened tibia phenotype triggered by *Gb'dac* RNAi. To test this hypothesis, we injected both *Gb'dac* and *Gb'mad* dsRNAs into third instar nymphs and then amputated their legs. The relative ratio of tibia length for the regenerated *Gb'dac* single RNAi legs at the sixth instar exhibited an approximate 0.63-fold decrease compared to that of the control regenerated legs (Fig. 3, a and c; n = 19/21). Regarding the tibial phenotypes of the regenerated leg models, injection of *Gb'dac* and *Gb'mad* RNAi rescued the short tibia phenotype associated with *Gb'dac* single RNAi (Fig. 3b; n = 19/24), and the dual RNAi tibia exhibited either normal or a 1.16-fold increase in length (Fig. 3c). These results imply that a functional relationship exists between *Gb'dac* and *Gb'mad*, and this relationship regulates cell proliferation and may contribute to the determination of tibial size in the regenerating legs.

Functional relationship between *Gb'ds*, *Gb'ft*, and *Gb'mad* in regenerating legs

In our previous studies, regenerated tibias of *Gb'ds* and *Gb'ft* RNAi legs were very short and thick (Bando et al., 2009). These results indicate that Ds/Ft signaling is associated with the re-establishment of gradients of positional values across blastemal cells during regeneration and it regulates leg size. In combination with the data above, we further hypothesize that *Gb'mad* contributes to the mechanism regulating positional information via *Gb*'Ds/*Gb*'Ft signaling. To confirm this hypothesis, we analyzed the phenotypes obtained with dual RNAi targeting of *Gb'ds* or *Gb'ft* and *Gb'mad* versus injections of single *Gb'ft* and *Gb'ds* dsRNA (Fig. 4). The relative ratios of tibia length in the RNAi legs to that of the control legs were reduced by 0.51-fold and 0.6-fold for the *Gb'ft* (Fig. 4a; n = 18/20) and *Gb'ds* (Fig. 4b; n = 21/25) single RNAi, respectively (Fig. 4e), while the *Gb'ft* and *Gb'mad* dual RNAi legs (Fig. 4c; n = 20/24) or *Gb'ds* and *Gb'mad* dual RNAi legs (Fig. 4d; n = 28/32) exhibited an increase in the relative ratio by approximately 1.22-fold (Fig. 4e). These results suggest that BMP signaling pathway contributes to the mechanisms that determine distal size of regenerating tibia and the re-establishment of positional information via interactions with Ds/Ft signaling.

Regulation of *Gb'dac*, *Gb'ft*, and *Gb'ds* expression by *Gb'mad*

The elongated tibia obtained with *Gb'mad* RNAi may be the result of *Gb'dac*-associated proliferation and *Gb*'Ds/*Gb*'Ft signaling to re-establish positional information. Thus, we hypothesize that tibial size is determined by the relationship between these factors, and expression of *Gb'dac* and *Gb'ds*/*Gb'ft* in regenerating tibia are regulated via the BMP signaling pathway. To test this hypothesis, levels of *Gb'dac*, *Gb'ds*, *Gb'ft*, and *Gb'mad* mRNA in regenerating legs treated with RNAi targeting *Gb'mad* were analyzed by qPCR. The ratios of these mRNA levels in comparison with control tibia were estimated at 2 dpa and 5 dpa. The relative ratios of *Gb'dac* mRNA increased 2.12-fold at 2 dpa and remained unchanged at 5 dpa (Fig. 5). There was no change in the relative ratios of *Gb'ds* and *Gb'ft* mRNA at 2 dpa (Fig. 5a), while the relative ratios slightly increased 1.15-fold and 1.78-fold, respectively, at 5 dpa (Fig. 5b). Meanwhile, *Gb'mad* mRNA was reduced 0.56-fold and 0.33-fold, respectively (Fig. 5). Taken together, these results indicate that expression levels of *Gb'dac* and *Gb'ft* increase when levels of *Gb'mad* are reduced. Thus, we propose that the BMP signaling pathway may contribute to both cell proliferation and the restoration of positional information in regenerating tibia by regulating the expression of *Gb'dac* and *Gb'ft*.

DISCUSSION

By using an RNAi knockdown approach to target *Gb'mad*, our experimental data demonstrate that the BMP signaling pathway is involved in the regeneration of the tibia and tarsus in cricket legs (Fig. 6). When a cricket leg is amputated in the middle of the tibia, the entire tarsus and half of the tibia are lost. Recognition of the amputated position as the most distal position is called "distalization" (Agata et al., 2007). Subsequently, regeneration of the blastema occurs in the distal region of the amputated leg and blastemal cells proliferate to form the missing structures by intercalating between the most distal region and the remaining part of the leg (i.e., "intercalation") (Bando et al., 2011b; Bando et al., 2013). In the present study, we focused on the role of the BMP signaling pathway in the process of leg regeneration according to the "distalization and intercalation" (D&I) principle (Agata et al., 2007). In relation to this principle, we subsequently discuss the following three points: (1) distal pattern formation during the initial stages of leg regeneration, and (2) regulation of positional information and (3) cell proliferation in the regenerating tibia.

We previously reported that *Gb'wg* and *Gb'dpp* are expressed in regenerating blastemal cells of cricket legs (Mito et al., 2002; Nakamura et al., 2008a), consistent with the CTBM (Campbell and Tomlinson, 1995). Thus, in the present study, we considered that formation of the P/D axis in a regenerating leg is triggered at a site where ventral *Gb'wg*-expressing

cells abut dorsal *Gb'dpp*-expressing cells at the A/P boundary (Mito et al., 2002). Moreover, we previously demonstrated that RNAi knockdown of *Gb'arm* prevents regeneration of a cricket leg, thereby indicating the crucial role that *Gb'arm* has in cricket leg regeneration (Nakamura et al., 2007). In the present study, the *Gb'mad* RNAi crickets exhibited a defect in distal regeneration which led to the absence of a tarsus in the regenerated legs. Thus, we conclude that the canonical Wg/Arm and BMP/Mad signaling pathways play a crucial role in distal patterning as part of the leg regeneration process. Subsequently, a combined activity gradient of Dpp and Wg induces transcription of the downstream gene, *vein* (*vn*), which encodes a ligand of the epidermal growth factor receptor (EGFR) at the distal tip of legs (Campbell, 2002; Galindo et al., 2002). Considering the role of EGFR signaling in distal leg patterning, it is possible that the tarsus is controlled by a distal-to-proximal gradient of EGFR activity. Indeed, *Gb'Egfr* has been shown to be required for the formation of distal leg structures (Hamada et al., 2015; Nakamura et al., 2008a; Nakamura et al., 2008b). Thus, Dpp and Wg signaling pathways may achieve a distal patterning role by regulating activation of EGFR signaling.

The second phenotype observed in the present study involved elongation of the tibial segment in *Gb'mad* RNAi legs. Surprisingly, the regenerated tibias in the *Gb'mad* RNAi crickets were longer than the normal control legs along the P/D axis. Based on this observation, we

conclude that the activity of the BMP signaling contributes to the regulation of tibial size during leg regeneration and it may also contribute to the establishment and maintenance of intercalation by identifying distal position values of a proper tibia. Based on the previous finding that *Gb'ds* and *Gb'ft* RNAi legs are shorter (Bando et al., 2009), it appears that Ds/Ft signaling may be a crucial regulator in re-establishing positional information for determining leg size. Our present data further demonstrate that the shortened phenotype of *Gb'ds* and *Gb'ft* RNAi legs can be rescued in the presence of *Gb'mad* RNAi. In combination, these observations suggest that the BMP signaling pathway is critical for regulating distal positional values and may contribute to the re-establishment of positional information, which is determined by Ds/Ft signaling. Furthermore, the BMP signaling pathway may suppress expression of *Gb'ft* in regenerating blastema. Taken together, these results suggest that functional interactions between Ds/Ft and BMP signaling pathways contribute to the mechanisms that underlie intercalation and determination of leg size. Furthermore, we recently proposed a Ds/Ft steepness model for leg regeneration that depends on the proliferation and differentiation of blastemal cells (Bando et al., 2011b). According to the Ds/Ft steepness model, new positional identities are specified in relation to new segment boundaries, and it is hypothesized that the BMP signaling pathway may play a key role in the specification of the most distal positional values or the scalar values of the

Ds/Ft gradient (steep slope) that could be formed by regenerating blastemal cells within the amputated leg stump (yellow and magenta in Fig. 6).

During the establishment of positional information in the leg regeneration process, cell proliferation is induced in the presumptive tibia by positional disparity and it is necessary to determine tibial size. Here, cell proliferation was found to be accelerated in regenerating tibias of *Gb'mad* RNAi legs, suggesting that the BMP signaling pathway is involved in suppressing cell proliferation in regenerating tibia. Correspondingly, the shortened tibia phenotype of the *Gb'dac* RNAi nymphs may have been due to a decrease in cell proliferation and the short *Gb'dac* RNAi tibia was rescued in the presence of *Gb'mad* RNAi. Thus, *Gb'dac* may contribute to cell proliferation through negatively regulating the function of *Gb'*Mad. Based on these results, we conclude that the functional relationship between BMP signaling pathway and *Gb'dac* affects cell proliferation in the tibia and it determines the size of the regenerated tibia.

In conclusion, the goal of this study was to examine whether *Gb'*Mad functions as a BMP signaling factor during cricket leg regeneration. In *Gb'mad* RNAi nymphs, the tarsus of the regenerated legs was absent and cell proliferation was reduced in the tarsal compartment of the blastema. Thus, *Gb'mad* is essential for regeneration of the tarsus, and Mad-mediated BMP signaling is involved in the mechanism that controls distal specification, including formation of the tarsus. We have provided an

overview of the BMP signaling pathway in the distal patterning and intercalation processes of leg regeneration in *Gryllus bimaculatus* (Fig. 6). Thus, the BMP signaling pathway is: 1) required to establish distal patterning and formation of a regenerating tarsus, 2) in combination with Ds/Ft signaling, it mediates the re-establishment of positional information during tibia regeneration, 3) it may help determine the distal positional values or scalar values according to the Ds/Ft steepness model, and 4) it is essential in the suppression of blastemal cell proliferation in response to *Gb'dac*, thereby maintaining an appropriate leg size.

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FIGURE LEGENDS

Fig. 1. Leg phenotypes obtained after depleting *Gb'mad* and *Gb'tkv* mRNA during regeneration. (a) A Schematic illustration showing the distal amputated position in the tibia of third instar nymph. Dotted lines indicate the amputated parts of leg. (b) Regenerated legs of sixth instar control RNAi (*DsRed2*; RL, *n* = 37/37) and *Gb'mad* RNAi nymphs (*n* = 24/27). The latter show no regeneration of the tarsus. (c) Localization of EdU-incorporated cells in regenerating tarsus regions of *DsRed2* RNAi (upper panel, *n* = 7) and *Gb'mad* RNAi (lower panel, *n* = 6) regenerated legs at 3.5 dpa. Nuclei cells are shown in blue and EdU-positive cells are shown in green. (d) Relative fold changes ± SD in the number of EdU-positive cells in regenerating tarsal regions at 3.5 dpa in *Gb'mad* RNAi legs relative to control *DsRed2* RNAi legs (with the latter set to 1). (e) Formation of supernumerary legs in fifth instar control *DsRed2* RNAi (*n* = 14/17) and *Gb'mad* RNAi nymphs (*n* = 14/15). Arrows indicate supernumerary legs. h, host stump; g, graft. (f) Normal legs (NL) of sixth instar control nymphs and regenerated legs of sixth instar *Gb'tkv* RNAi nymphs (*n* = 8/9). (g) Relative fold changes in lengths of regenerated tibias ± SD in sixth instar control *DsRed2* RNAi legs (RL), *Gb'mad* RNAi legs, and *Gb'tkv* RNAi legs. Relative fold of tibial length in sixth instar control normal legs (NL) was set to 1. Length of regenerated tibia was calculated on the basis of red lines in (b) and (f). Red arrowheads and blue arrows in

(a), (b) and (f) indicate the amputation site and spurs of the tibia, respectively. In panels (d) and (g), data are means ± SD. (Student's *t*-test; *P < 0.05, **P < 0.001). Fe, femur; Ti, tibia; Ta, tarsus. Scale bars in (b), (e) and (f), 1 mm; (c), 100 μm.

Fig. 2. Effects of *Gb'mad* and *Gb'dac* RNAi on cell proliferation. (a-f) Localization of EdU-incorporated cells in regenerating tibial regions at 2 dpa and 5 dpa for control (*DsRed2*) (a and d, *n* = 6 and 6), *Gb'dac* (b and e, *n* = 7 and 5), and *Gb'mad* (c and f, *n* = 7 and 7) RNAi nymphs. EdU-positive cells are shown in green and nuclei are shown in blue. Rectangular outlines in panels (a-f) indicate regenerating tibial regions (Ti). (g, h) Quantitation of cell proliferation detected in regenerating tibial regions at 2 dpa (g) and 5 dpa (h) in nymphs injected with control, *Gb'dac,* or *Gb'mad* dsRNA. Numbers for the controls are set to 1 in each graph. In panels (g) and (h), data are means ± SD. (Student's *t*-test; *P < 0.05, **P < 0.01). Scale bars in (a) and (d), 100 μm.

Fig. 3. Effects of single or dual RNAi targeting of *Gb'dac* and *Gb'mad* on length of regenerated tibia. (a, b) Representative images of regenerated tibia of sixth instar control (*DsRed2*) (a, upper), *Gb'dac* (a, lower, *n* = 19/21), and *Gb'dac* + *Gb'mad* (b, *n* = 19/24) RNAi nymphs. (c) Relative fold changes in regenerated tibia lengths ± SD for sixth instar control (set

to 1), *Gb'dac*, and *Gb'dac* + *Gb'mad* RNAi nymphs. In panel (c), data are means ± SD. (Student's *t*-test; *P < 0.05, n.s., not significant). Ti, tibia. Scale bar in (a) and (b), 1 mm.

Fig. 4. Effects of single or dual RNAi targeting of *Gb'ft*, *Gb'ds*, and *Gb'mad* on length of regenerated tibia. (a, b) Representative images of regenerated tibia of sixth instar control (*DsRed2*) (a and b, upper), *Gb'ft* (a, lower, *n* = 18/20), *Gb'ds* (b, lower, *n* = 21/25), *Gb'ft* + *Gb'mad* (c, *n* = 20/24), and *Gb'ds* + *Gb'mad* (d, *n* = 28/32) RNAi nymphs. (e) Relative fold changes in regenerated tibia lengths ± SD for sixth instar control (set to 1), *Gb'ft*, *Gb'ft* + *Gb'mad*, *Gb'ds*, and *Gb'ds* + *Gb'mad* RNAi nymphs. In panel (e), data are means ± SD. (Student's *t*-test; *P < 0.05). Ti, tibia. Scale bars in (a-d), 1 mm.

Fig. 5. Effects of *Gb'mad* RNAi on expression of *Gb'dac*, *Gb'ft*, and *Gb'ds* mRNA in regenerated legs. (a, b) Transcript levels ± SD for *Gb'mad* (*mad*), *Gb'dac* (*dac*), *Gb'ft* (*ft*), and *Gb'ds* (*ds*) were determined by qPCR at 2 dpa (a) and 5 dpa (b) in nymphs that received injections of control (*DsRed2*; set to 1) or *Gb'mad* dsRNA. In panels (a) and (b), the data are means ± SD of three biological replicates. (Student's *t*-test; *P < 0.05, **P < 0.01, ***P < 0.001, n.s., not significant).

Fig. 6. An illustrated overview of a proposed cricket leg regeneration process and steepness model, with the BMP signaling pathway regulating distal patterning and intercalation. Positional values (PV) are denoted arbitrarily by the numbers 1 to 9. After amputation in the tibia at PV = 4 (the tibial stump is indicated with orange coloring), the blastemal cells detect positional disparity (PV, 4/9). The most distal region is established through distalization mechanisms. A steep slope then leads to intercalary growth until positional continuity is re-established (yellow, PV = 4-9) and epimorphic-like regeneration is established. The results of the present study confirm various roles for the BMP signaling pathway in this model: (1) no tarsus formation is observed in *Gb'mad* RNAi regenerated legs, thereby the BMP signaling pathway is required for distal patterning and tarsus formation; (2) The BMP signaling pathway is involved in establishing positional identities via regulation of Dachsous/Fat (Ds/Ft) signaling. The normal Ds/Ft gradient (dotted line) may be affected by RNAi-mediated knockdown of *Gb'mad* (Mad RNAi). The most distal positional value or the minimum scalar value of the Ds/Ft gradient may be shifted down or up within the *Gb'mad* RNAi leg stump (magenta), respectively; and (3) an interaction between Dachshund (Dac) and BMP signaling pathway is involved in regulating cell proliferation and determines the proper tibial size in regenerating legs.