Leg regeneration is epigenetically regulated by histone H3K27 methylation in the cricket Gryllus bimaculatus

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ABSTRACT
Hemimetabolous insects such as the cricket Gryllus bimaculatus regenerate lost tissue parts using blastemal cells, a population of dedifferentiated proliferating cells. The expression of several factors that control epigenetic modification is upregulated in the blastema compared with differentiated tissue, suggesting that epigenetic changes in gene expression might control the differentiation status of blastema cells during regeneration. To clarify the molecular basis of epigenetic regulation during regeneration, we focused on the function of the Gryllus Enhancer of zeste (Gb'E(z)) and Ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome (Gb'Utx) homologues, which regulate methylation and demethylation of histone H3 lysine 27 (H3K27), respectively. Methylated histone H3K27 in the regenerating leg was diminished by Gb'E(z)RNAi and was increased by Gb'UtxRNAi. Regenerated Gb'E(z)RNAi cricket legs exhibited extra leg segment formation between the tibia and tarsus, and regenerated Gb'UtxRNAi cricket legs showed leg joint formation defects in the tarsus. In the Gb'E(z)RNAi regenerating leg, the Gb'dac expression domain expanded in the tarsus. By contrast, in the Gb'UtxRNAi regenerating leg, Gb'Egfr expression in the middle of the tarsus was diminished. These results suggest that regulation of the histone H3K27 methylation state is involved in the repatterning process during leg regeneration among cricket species via the epigenetic regulation of leg patterning gene expression.

KEY WORDS: Regeneration, Epigenetics, Histone H3K27, Gryllus bimaculatus, Polycomb

INTRODUCTION
Regeneration is a phenomenon in which animals restore lost tissue parts using remaining cells. This phenomenon is observed in various organisms ranging from the sponge to vertebrates, including planarians, insects, fishes and urodeles; however, the regenerative capacity of humans, mice and chicks is limited (Agata and Inoue, 1985; Tweedell, 2010). Blastema cells differentiate into several types of unipotent cells (‘redifferentiation’) to restore the lost tissue part following the expression of tissue patterning genes (‘repatterning’). These differentiated cells and blastema cells display different gene expression patterns. Thus, during the dedifferentiation and redifferentiation processes, epigenetic factors may play a key role in changing gene expression in both cell types.

Epigenetics is defined as heritable changes in gene expression that are not caused by changes in the DNA sequence (Lan et al., 2007; Stewart et al., 2009; Wyngaarden et al., 2011). The epigenetic regulation of gene expression is primarily mediated by the methylation of specific DNA nucleotides and post-translational histone modifications. Methylation of the cytosine DNA base is an irreversible reaction that represses the expression of neighbouring genes via the formation of inactive chromatin. Other epigenetic events include chemical modifications, such as methylation, acetylation, phosphorylation and ubiquitination, of specific amino acid residues of the N-terminal tail of histones H2A, H2B, H3 and H4. Methylation of lysine residue 27 of histone H3 (H3K27) is a well-known epigenetic mark that represses the expression of neighbouring genes via the induction of heterochromatin formation by recruiting Polycomb group proteins. Conversely, demethylation of trimethylated histone H3K27 (H3K27me3) derepresses and promotes gene expression to change heterochromatin into euchromatin.

During tissue regeneration, epigenetic modifications may change during the dedifferentiation and redifferentiation processes (Katsuyama and Paro, 2011; McCusker and Gardiner, 2013; Tamura et al., 2010; Tweedell, 2010). In the frog Xenopus laevis, the regenerative capacity gradually decreases during development, and this decrease is caused by the downregulation of Sonic hedgehog (Shh) expression mediated by epigenetic mechanisms (Tamura et al., 2010; Yakushiji et al., 2007, 2009). By contrast, the regenerative capacity of the newt Cynops pyrrhogaster is not limited by growth because epigenetic modification of the newt Shh locus does not change throughout growth (Yakushiji et al., 2007). In zebrafish, a lost part of the caudal fin is regenerated from the blastema, and the lost fin part is not regenerated in kdm6b1 morphant fish, which encodes a histone H3K27me3 demethylase (Stewart et al., 2009). JmjD3 (Kdm6b) and Utx (Kdm6a), which also encode histone H3K27me3 demethylases, are required for murine skin repair (Shaw and Martin, 2009). The SET/MLL family of histone methyltransferases is essential for stem cell maintenance in
the planarian *Schmidtea mediterranea* (Hubert et al., 2014; Robb and Alvarado, 2014). In *Drosophila* imaginal disc regeneration, the expression of Polycomb group genes is downregulated in the blastema of amputated discs, which suppresses methylation on histone H3K27 (Lee et al., 2005; Repiso et al., 2011; Sun and Irvine, 2014; Worley et al., 2012). Epigenetic regulation of gene expression affects stem cell plasticity in mammals, and the expression of stem cell-related and differentiated cell-related genes is epigenetically altered during the differentiation process via the histone H3K4 and H3K27 methylation states (Barrero and Izpisua Belmonte, 2011). Histone H3K27 methylation by Ezh2 in mammals affects the reprogramming efficiency of induced pluripotent stem cells (iPSCs) derived from fibroblasts *in vitro* (Ding et al., 2014; Hochdelinger and Plath, 2009). These previous studies imply that epigenetic regulation of gene expression plays a key role in dedifferentiation and redifferentiation during regeneration.

The two-spotted cricket *Gryllus bimaculatus*, a hemimetabolous insect, has a remarkable regenerative capacity to restore a missing distal leg part. The cricket leg consists of six segments arranged along the proximodistal (PD) axis in the following order: coxa, trochanter, femur, tibia, tarsus and claw (Fig. 1A). When a metathoracic leg of a *Gryllus* nymph in the third instar is amputated at the distal position of the tibia, the distal missing part is restored after 1 month during four molts that occur subsequent to the amputation. After the amputation of a leg, a blastema forms beneath the wound epidermis, similar to that of other regenerative organisms. The lost part of the tissue is regenerated using blastemal cells and is dependent on the expression of signalling molecules...
such as the Gryllus wingless, decapentaplegic and hedgehog homologues, and leg patterning genes including dachshund (Gb dac), Epidermal growth factor receptor (Gb Egfr), Distal-less (Gb Dll) and BarH (Gb BarH) (Ishimaru et al., 2015; Mito et al., 2002; Nakamura et al., 2007, 2008a,b). The blastemal expression of these genes is activated during regeneration and may be epigenetically regulated during this process. However, the underlying mechanisms regulating gene expression during dedifferentiation and redifferentiation processes in tissue regeneration remain elusive.

In a previous study to identify the molecules that undergo expression changes in the blastema, we performed a comparative transcriptome analysis and found that the expression of several epigenetic modifiers is upregulated in the blastema (Bando et al., 2013). In the present study, we focused on the function of the Gryllus homologues of Enhancer of zeste [Gb E(z)] and Ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome (Gb Utx). Here, we show that Gb E(z) and Gb Utx are involved in the repatterning process during regeneration via the regulation of leg patterning genes.

RESULTS
Gb E(z) and Gb Utx are expressed in regenerating legs

Previously, we reported that the expression of several epigenetic modifiers is upregulated in the blastema during cricket leg regeneration based on comparative transcriptome analysis. The highest RPKM (reads per kilobase per million reads) ratio observed between the blastema and non-regenerative tissue was 8.9 for the highest RPKM (reads per kilobase per million reads) ratio observed in the blastema during cricket leg regeneration based on comparative transcriptome analysis. The modifiers is upregulated in the blastema during cricket leg regeneration.

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Gb E(z) and Gb Utx regulate the histone H3K27 methylation state

To clarify Gb E(z) and Gb Utx functions, we performed RNA interference (RNAi) experiments to reduce their expression. We observed histone H3K27me3 patterns by immunostaining to investigate whether Gb E(z)RNAi and Gb UtxRNAi alter the histone H3K27 methylation state during leg regeneration. In control cricket legs, histone H3K27me3 was detected in the blastema and host stump at 2 dpa and the regenerating tibia and tarsus at 6 dpa (Fig. 2A). In Gb E(z)RNAi cricket legs, fluorescence intensities of histone H3K27me3-positive nuclei were decreased in the blastema and regenerating tarsus at 2 and 6 dpa, respectively. In the Gb UtxRNAi cricket legs, histone H3K27me3-positive nuclei appeared to be increased in the regenerating legs at 2 and 6 dpa. These histological results suggest that Gb E(z) and Gb Utx are necessary for histone H3K27 methylation and histone H3K27me3 demethylation, respectively (Fig. 2A).

To confirm knockdown of endogenous Gb E(z) and Gb Utx mRNA levels by RNAi, we estimated the mRNA ratio of these genes in the Gb E(z)RNAi and Gb UtxRNAi cricket legs compared with control cricket legs (n=15) using quantitative PCR (qPCR). The average ratio of Gb E(z) and Gb Utx mRNA levels at 3 dpa decreased to 0.52±0.01 and 0.56±0.02 (n=3; ±s.d.) in regenerating Gb E(z)RNAi and Gb UtxRNAi tibiae, respectively (Fig. 2B,C), indicating that the RNAi did indeed lower the mRNA levels of these genes.

Gb E(z) is involved in segment patterning during leg regeneration

To examine the function of Gb E(z) during leg regeneration, we performed RNAi and amputated the metathoracic legs of third instar nymphs. In the control cricket adults, regenerated legs were indistinguishable from contralateral intact legs. Three pairs of tibial spurs and several pairs of spines were reconstructed on the tibia. Three tarsomeres and a claw were regenerated adjacent to the tibia. One pair of tarsal spurs (arrowheads in Fig. 3A) was reconstructed at the anterior and posterior ends of tarsomere 1 (Ta1). Notably, no decorative structures were formed on the small tarsomere 2 (Ta2) and middle-sized tarsomere 3 (Ta3) in the regenerated or contralateral intact legs (Fig. 3A).

Gb E(z)RNAi cricket legs were viable, and the lost parts of their amputated legs were regenerated. In the Gb E(z)RNAi adults, the lost sections of the tibia, tarsus and claw were regenerated; however, the leg segment patterns were abnormal (Fig. 3A). We categorised Gb E(z)RNAi regenerated legs into three classes based on leg morphology abnormalities during the sixth instar stage. The class 1 phenotype (23%, n=11/49) was mild; both anterior and posterior tarsal spurs were lost in Ta1, and Ta2 was not regenerated. Most Gb E(z)RNAi regenerated legs were classified as class 2 (55%, n=26/49); three tarsomeres were regenerated, but the tarsal spurs were abnormal. Several spurs were reconstructed in Ta1 at the ventral side in addition to the anterior and posterior sides, where tarsal spurs were formed in the controls (red arrows in Fig. 3A). The regenerated leg class 3 phenotype (13%, n=7/49), which showed the most severe morphological abnormalities, consisted of four leg segments in the tarsus, whereas the controls consisted of three tarsomeres. The second leg segment morphology of the class 3 regenerated tarsus appeared to be equivalent to the Ta1 of the control; one pair of tarsal spurs was reconstructed at the end of the tarsomere (arrowheads in Fig. 3A). We estimate that the third and fourth segments of the class 3 regenerated tarsus were equivalent to Ta2 and Ta3 of the control based on the size of each segment. The first segment of the class 3 regenerated tarsus was ambiguous (red
bracket in Fig. 3A, C); more than two spurs were formed at the end of the leg segment (red arrows in Fig. 3A), which is characteristic of the tibia, and several spines were formed at the dorsal side in this extra leg segment. Regenerated legs of the other 9% of Gb'E(z)RNAi adults (n = 5/49) showed normal morphology, and the morphologies of the regenerated tibiae were normal in all classes. These phenotypes were observed when we performed RNAi against the Gb'E(z)$_C$ region (Fig. 3B; supplementary material Fig. S2), suggesting that these phenotypes were not caused by an off-target effect.

**Regenerated legs in Gb'E(z)RNAi crickets exhibit an extra tibia segment**

To identify the origin of the extra leg segments formed in class 3 Gb'E(z)RNAi crickets, we performed further morphological observation of the extra leg segment, which appeared to be a tibia-like structure. We observed the mesothoracic (T2) leg regeneration process in control and Gb'E(z)RNAi crickets because the tibia and Ta1 morphologies were different in the T2 leg. Specifically, in T2 legs tibial spurs formed on the tibia; however, tarsal spurs did not form at Ta1, which differed from the metathoracic (T3) leg. In the control cricket (n = 20), the lost part of the T2 leg was regenerated after amputation on the tibia. Two pairs of tibial spurs, three tarsomeres and the claw were regenerated, and no tarsal spurs formed in the tarsus (Fig. 4A). In Gb'E(z)RNAi crickets (n = 39), regenerated T2 legs had an extra leg segment between the tibia and tarsus, and two pairs of spurs formed on both ends of the tibia and extra leg segment (72%, n = 28/39), indicating that the extra leg segment observed in the T2 regenerated leg of Gb'E(z)RNAi crickets was the tibia (Fig. 4A, B). Morphologies of the T2 regenerated leg indicated that Gb'E(z)RNAi induced extra tibia segment formation during regeneration.

We changed the amputation position from the tibia to femur, and after the amputation of the cricket leg at the distal position of the femur the lost parts of the femur, tibia, three tarsomerces and claw regenerated in the control adult (Fig. 4C). By contrast, the morphologies of the regenerated legs of Gb'E(z)RNAi adults were abnormal. In class 1, the tibia, Ta1 and Ta2 regenerated as a single short and thick leg segment without joints. Small Ta3 and claws were regenerated following the extra tibia segment (38%, n = 6/16) (Fig. 4C). These morphological observations of Gb'E(z)RNAi...
regenerated legs after amputation at the femur suggest that Gb’ E(z) might suppress extra tibia formation during regeneration regardless of amputation position.

We next performed grafting experiments to induce supernumerary leg formation in control and Gb’ E(z) RNAi crickets. Transplantation of the left mesothoracic tibia onto the right metathoracic tibia resulted in the inversion of the anteroposterior polarity of the graft to the host, and two supernumerary legs were formed at the anterior and posterior sides of the tibia (Mito et al., 2002). In the control cricket (n=22), supernumerary legs formed at both sides of the tibia, comprising tibia, tarsus and claw. In the Gb’ E(z) RNAi cricket (n=23), supernumerary legs formed on both sides of the tibia and, again, consisted of a tibia, extra tibia segment (red arrows in Fig. 4D), tarsus and claw (26%, n=6/23; Fig. 4D,E), indicating that Gb’ E(z) regulates leg segment pattern along the PD axis but does not regulate the polarities along anteroposterior and dorsoventral axes.

Amputation position affects the Gb’ E(z) RNAi phenotype

To elucidate whether the amputation position along the PD axis of the tibia affects the Gb’ E(z) RNAi phenotype, we amputated at the distal, middle or proximal position in the tibiae in Gb’ E(z) RNAi nymphs. In control crickets, the morphologies of regenerated legs amputated at any position were similar (Fig. 5A). However, in the Gb’ E(z) RNAi crickets, the phenotypic rate of class 3 was elevated after amputation at the more proximal position (Fig. 5B). After amputation of the Gb’ E(z) RNAi cricket leg at the proximal position, 62% (n=21/34) of regenerated legs were categorised into class 3, whereas 14% (n=7/49) and 25% (n=10/40) of regenerated legs were categorised into class 3 after amputation at the distal and middle positions, respectively (Fig. 5B). In addition, the length of the extra segment normalised to femur length was also extended after proximal amputation compared with amputation at the middle or distal positions (Fig. 5C). Conversely, the normalised length of the regenerated tibia was shortened after proximal amputation compared with middle or distal amputation (Fig. 5A,C). We assume that Gb’ E(z) target genes may be expressed in a region-specific manner along the PD axis because the amputation position affects the Gb’ E(z) RNAi phenotype ratios.

E(z) function during regeneration is conserved among two cricket species

Tarsus structures and tarsomere numbers are strictly determined according to insect species (Tajiri et al., 2011). To confirm whether the extra tibia segment formation caused by E(z) RNAi is a species-specific phenotype, we tested E(z) RNAi during leg regeneration in the field cricket Mocicogryllus siamensis (supplementary material Fig. S3B). M. siamensis regenerated the lost part of the metathoracic
leg after amputation at the distal tibia, similar to *G. bimaculatus* (supplementary material Fig. S3A,C). Next, we cloned the *M. siamensis* *E(z)* homologue *Ms*′*E(z)* and performed RNAi against *Ms*′*E(z)* in *M. siamensis* nymphs. In *Ms*′*E(z)* RNAi regenerated legs, an extra tibia segment was formed between the tibia and tarsus (red bracket in supplementary material Fig. S3A,D),

![Image](url)

**Fig. 4. Typical regenerated and supernumerary leg phenotypes in control and *Gb*′*E(z)* RNAi crickets.** (A) Regenerated mesothoracic legs of control and *Gb*′*E(z)* RNAi crickets. Tibial spurs are indicated by arrows. The extra tibia segment and its spurs are indicated by the red bracket and red arrow, respectively. (B) Ratios of normal (no phenotype) and RNAi phenotypes (class 1 to 3) of regenerated mesothoracic legs of control and *Gb*′*E(z)* RNAi cricket nymphs at sixth instar. (C) Regenerated legs amputated at the distal femur of control (*n*=10) and *Gb*′*E(z)* RNAi (*n*=16). Tibial spurs and tarsal spurs are indicated by arrows and arrowheads, respectively. Tarsi are indicated by brackets. Ti, tibia; Ta, tarsus; Cl, claw; Tis, tibial spur; Tas, tarsal spur. The extra tibia segment and its spurs are indicated by red brackets and red arrows, respectively. (D) Supernumerary legs in control and *Gb*′*E(z)* RNAi crickets. The boxed region is magnified to the right. Tibial spurs are indicated by arrows; those on extra tibia segments are indicated by a red arrow. (E) Ratios of normal and RNAi phenotypes of supernumerary legs of control and *Gb*′*E(z)* RNAi cricket nymphs at sixth instar.

![Image](url)

**Fig. 5. Effect on extra tibia segment formation of amputation position in *Gb*′*E(z)* RNAi regenerated legs.** (A) Regenerated legs amputated at the distal, middle and proximal positions of control and *Gb*′*E(z)* RNAi crickets. Amputation positions are shown in the left columns. Tarsi and extra tibia segments are indicated by black and red brackets, respectively. Ti, tibia; Ta, tarsus; Cl, claw. (B) Ratios of normal and RNAi phenotypes of control and *Gb*′*E(z)* RNAi crickets amputated at distal, middle and proximal positions at sixth instar. (C) Relative length of each leg segment of the control and *Gb*′*E(z)* RNAi regenerated legs normalised to the femur. Error bars indicate s.d.
promotes leg joint and spur formation at the tarsus during repatterning. To clarify whether Gb'Utx and Gb'E(z) epigenetically regulate gene expression involved in tibia and/or tarsus formation, we examined the Gb'dac, Gb'Egr, Gb'BarH and Gb'Dll expression patterns in the regenerating legs of RNAi cricket nymphs using whole-mount in situ hybridisation. In control regenerating legs at 6 dpa, Gb'dac was expressed in the tibia and tarsus proximal region (Fig. 7A), and Gb'Egr was expressed at the distal position of tibia and the middle and distal positions of the tarsus (arrowheads, Fig. 7A) (Nakamura et al., 2008b). In the tarsus, Gb'BarH and Gb'Dll were expressed in the middle section (arrowhead, Fig. 7A) and the entire tarsus, respectively. In Gb'E(z)RNAi regenerating legs at 6 dpa, the Gb'dac expression domain in the proximal tarsal region was expanded (Fig. 7A). Gb'dac expression in the distal tarsal region (red arrowhead in Fig. 7A) was observed in both the Gb'E(z)RNAi and control regenerating legs (Nakamura et al., 2008b). The Gb'Egr, Gb'BarH and Gb'Dll expression patterns were not altered in the Gb'E(z)RNAi regenerating legs. In the Gb'UtxRNAi regenerating legs, Gb'Egr was expressed at the distal position of the tibia and tarsus (arrowheads, Fig. 7A); however, Gb'Egr was not expressed in the middle position of the tarsus (blue arrowhead), which becomes the Ta1 and Ta2 leg joint. The Gb'dac, Gb'BarH and Gb'Dll expression patterns were not altered in the Gb'UtxRNAi regenerating legs. Overall, these results suggest that Gb'E(z) and Gb'Utx epigenetically regulate Gb'dac and Gb'Egr expression, respectively, in regenerating legs.

We analysed the Gb'dac expression patterns in the control and Gb'E(z)RNAi regenerating legs after amputation at the middle or proximal positions because the Gb'E(z)RNAi phenotypic rate was altered depending on the amputation position. Gb'dac was expressed in the tibia and proximal region of the tarsus of the control regenerating legs amputated at the middle or proximal positions (Fig. 7B). In the Gb'E(z)RNAi regenerating legs, Gb'dac was expressed in the tibia and throughout the tarsus after amputation at the middle and proximal positions (Fig. 7B). The Gb'dac expression domain ratios in the tarsi were calculated (Fig. 7C). Gb'dac expression in the tarsi was significantly expanded in the Gb'E(z)RNAi compared with the control regenerating legs (P<0.01, Fig. 7C). In Gb'E(z)RNAi, Gb'dac expression was significantly expanded in the regenerating leg amputated proximally as compared with the legs amputated at the middle or distally (Fig. 7C), correlating with the Gb'E(z)RNAi phenotype ratios (Fig. 5).

Gb'E(z) and Gb'Utx regulate repatterning but are not involved in dedifferentiation

To determine the effective time window of RNAi against Gb'E(z) and Gb'Utx, we performed RNAi after cricket leg amputation and observed the RNAi phenotypes. In the control, RNAi against the exogenous gene Egfp at any time point resulted in regenerates that were similar to the contralateral intact leg. With Gb'E(z), the phenotype ratio (the percentage that showed an RNAi phenotype versus a normal phenotype) of Gb'E(z)RNAi at 4 and 8 hours post amputation (hpa) was 80%, which is similar to the phenotype ratio at 0 hpa, and subsequently decreased to 50% when we performed Gb'E(z)RNAi at 48 hpa (supplementary material Fig. S4A). For Gb'Utx, the phenotype ratios gradually decreased depending on the timing of RNAi after amputation; the ratio was 60% when the RNAi was performed at 0 hpa, and the ratios were 40% at 4 or 8 hpa and 20% at 12, 24 and 48 hpa (supplementary material Fig. S4A). No additional phenotypes were found by employing Gb'E(z)RNAi or Gb'UtxRNAi at any time point.

similar to the Gb'E(z)RNAi phenotype, indicating that suppression of extra tibia formation during regeneration mediated by E(z) is a conserved mechanism among at least two cricket species.

Gb'Utx is involved in tarsus joint formation during leg regeneration

Utx demethylates histone H3K27me3, whereas this methylation is mediated by E(z); therefore, we performed RNAi against Gb'Utx to analyse its function during leg regeneration. In Gb'UtxRNAi cricket adults, the lost leg segments regenerated; however, the regenerated tarsomers showed various morphological abnormalities in the formation of tarsal spurs (arrowheads in Fig. 6A) or Ta2. In most cases, the tarsal spur at the anterior side was not reconstructed, and the anterior tarsal spur size was smaller than in the control. In several cases, tarsal spurs on both the anterior and posterior sides were not reconstructed. In addition, we also observed leg joint formation defects between Ta1 and Ta2 in class 2 regenerated legs. These phenotypes were observed when RNAi was employed against the Gb'Utx_C region, suggesting that these phenotypes were not caused by off-target effects (Fig. 6B).

**Expression of Gb'dac and Gb'Egr is epigenetically regulated via histone H3K27me3**

These RNAi experiments suggest that Gb'E(z) suppresses extra tibia segment formation between the tibia and tarsus and that Gb'Utx
If $Gb\ E(z)$ and/or $Gb\ Utx$ contribute to dedifferentiation during blastema formation, $Gb\ E(z)^{RNAi}$ and/or $Gb\ Utx^{RNAi}$ would exhibit a regeneration defective phenotype when RNAi is performed prior to amputation. We applied RNAi at the third instar and amputation at the fourth instar in nymphs, allowing a 72 h incubation period prior to amputation. In the other group, RNAi and amputation were performed simultaneously at the fourth instar stage. In control crickets with RNAi against $Egfr$, regeneration occurred in both groups (data not shown). In $Gb\ E(z)^{RNAi}$ crickets, 80% of RNAi crickets showed RNAi phenotypes in the regenerates in both groups (supplementary material Fig. S4B). In $Gb\ Utx^{RNAi}$ crickets, 30% and 50% of crickets showed RNAi phenotypes in the regenerates following RNAi at third and fourth instar, respectively (supplementary material Fig. S4B). No additional phenotypes were observed. No regeneration defects were observed when employing RNAi 72 h before amputation (supplementary material Fig. S4B), suggesting that $Gb\ E(z)$ and $Gb\ Utx$ are involved in the repatterning process but not in the dedifferentiation process to form the blastema.

**DISCUSSION**

Using an RNAi knockdown approach, we determined that $Gb\ E(z)$ and $Gb\ Utx$ mediate the methylation and demethylation, respectively, of histone H3K27 during Gryllus leg regeneration. Regenerated $Gb\ E(z)^{RNAi}$ and $Gb\ Utx^{RNAi}$ legs exhibited extra tibia segment formation and defects in leg joint formation, respectively, caused by the epigenetic regulation of leg patterning gene expression during regeneration.

**$Gb\ E(z)$ regulates patterning of the lost leg section via histone H3K27me3 during leg regeneration**

Following amputation, differentiated cells in the remaining tissue dedifferentiate into blastema cells. Blastema cells proliferate rapidly, then redifferentiate into several types of differentiated cells (Nye et al., 2003; Tamura et al., 2010; Truby, 1985; Tweedell, 2010; Worley et al., 2012). During the dedifferentiation and redifferentiation processes, the expression profiles of differentiated cell-related and stem cell-related genes change epigenetically (Barrero and Iziyusa Belmonte, 2011; Katsuyama and Paro, 2011; Tamura et al., 2010). Our previous study showed that the expression levels of the epigenetic modifiers $Gb\ E(z)$ and $Gb\ Utx$ are upregulated during cricket leg regeneration (Bando et al., 2013). In this study, we examined the roles of these factors in leg regeneration.

In the $Gb\ E(z)^{RNAi}$ class 3 phenotype, regenerated legs exhibited extra tibia segment formation between the tibia and Ta1 (Fig. 3A), implying that $Gb\ E(z)$ epigenetically regulates leg patterning gene expression during tibia regeneration. Previous RNAi experiments showed that $Gb\ dac$ mediates tibia and Ta1 formation during leg regeneration (Ishimaru et al., 2015; Nakamura et al., 2008b). By contrast, $Dll$ and $BarH$ expression is involved in tarsus formation in Drosophila limb development (Kojima, 2004), and $Gb\ Dll$ expression is involved in Gryllus tarsus regeneration (Ishimaru et al., 2015; Nakamura et al., 2008b). The $Gb\ dac$ expression domain in Ta1 expanded in $Gb\ E(z)^{RNAi}$ regenerating legs (Fig. 7 and Fig. 8A), and this ectopic derepression of $Gb\ dac$ expression in the Ta1 distal region by $Gb\ E(z)^{RNAi}$ would lead to the formation of an extra tibia segment (Fig. 8B). By contrast, $Gb\ BarH$ and $Gb\ Dll$ expression was not altered in $Gb\ E(z)^{RNAi}$ regenerating legs, which might explain why the proportion of regenerated legs with extra tibia segments was less than 20% in the $Gb\ E(z)^{RNAi}$ crickets (Fig. 3B), since normal $Gb\ BarH$ and $Gb\ Dll$ expression in the $Gb\ E(z)^{RNAi}$ regenerating leg induces tarsus formation and may suppress extra tibia segment formation during regeneration (Fig. 8A,B).

The class 3 $Gb\ E(z)^{RNAi}$ phenotype ratio increased after amputation of the leg at the proximal position in comparison with amputation at the middle or distal position (Fig. 5). As mentioned above, $Gb\ dac$ is not expressed in the proximal region of the tibia in the developing cricket embryo (Inoue et al., 2002) or in the stump amputated at the proximal position. By contrast, $Gb\ dac$ expression remained in the host stumps amputated at the middle or distal position of regenerating legs. Maintained $Gb\ dac$ expression in the host stump might decrease the $Gb\ E(z)^{RNAi}$ phenotype ratio (i.e. the proportion showing an abnormal regeneration phenotype) and lead to normal regeneration after amputation at the middle or distal position (Fig. 8B). Thus, $Gb\ E(z)$ regulates repatterning of the lost leg section via $Gb\ dac$ expression by modulating histone H3K27me3, which prevents malformations such as extra leg segment formation. $Gb\ E(z)$ may also promote cell proliferation in the leg segment through the regulation of $Gb\ dac$ expression.
Fig. 8. Model for the repatterning process during leg regeneration in cricket. (A) Schematics of Gb dac, Gb Egfr, Gb BarH and Gb Dil expression in control, Gb E(z)RNAi and Gb UtxRNAi regenerating legs. The Gb dac expression domain in Ta1 is indicated by red double-headed arrows. (B) E(z) methylates histone H3K27 to induce heterochromatin formation for gene repression. In controls, Gb dac is expressed in the tibia and Ta1 to reconstruct tibia segments (red double-headed arrows). In Gb E(z)RNAi crickets, Gb dac is expressed in the tibia and Ta1 to reconstruct tibia segments; however, the Gb dac expression domain in Ta1 expanded, which led to the formation of extra tibia segments between tibia and Ta1. After amputation of the leg at the proximal tibia, the Gb dac expression domain expanded widely compared with that following amputation at the distal tibia. Wider Gb dac expression may lead to extra tibia segment formation at high efficiency. Ta3 and claws are reconstructed normally in Gb E(z)RNAi crickets because Gb Dil expression in the tarsus and Gb BarH expression in the tarsus centre were not altered. (C) Utx demethylates histone H3K27me3 to induce euchromatin formation for derepression and activation of gene expression. In controls, Gb Egfr was expressed in the distal regions of tibia and Ta1. In Gb UtxRNAi crickets, Gb Egfr expression in the distal region of Ta1 was diminished (blue arrowhead), which caused leg joint formation and tarsal spur formation defects at Ta1. Ta3 and the claw are reconstructed normally in Gb UtxRNAi crickets because Gb Dil expression in the tarsus and Gb BarH expression in the tarsus centre were not altered.
Blastema cells, which are derived by the dedifferentiation of differentiated cells, are essential for regeneration in multiple organisms, including the cricket. The Wg/Wnt and Jak/STAT signalling pathways are essential for blastema cell formation because RNAi silencing of these signalling pathways causes regeneration defects (Bando et al., 2013; Nakamura et al., 2007). If Gb E(z) and Gb Utx are essential for the dedifferentiation process to form the blastema, RNAi against Gb E(z) and Gb Utx should cause complete regeneration defects. In this study, Gb E(z)RNAi and Gb Utx RNAi exhibited defects in regenerated leg repatterning (Figs 3 and 6). Furthermore, leg regeneration occurred when we performed RNAi against Gb E(z) and Gb Utx 72 h before amputation. Thus, the epigenetic regulation of gene expression via histone H3K27me3 is not required for dedifferentiation into blastema cells during cricket leg regeneration, which is different from the roles of epigenetic control during cell dedifferentiation in mouse, zebrafish and Drosophila (Lee et al., 2005; Shaw and Martin, 2009; Stewart et al., 2009). We also determined the effective time window of Gb E(z)RNAi and Gb Utx RNAi. Both Gb E(z)RNAi and Gb Utx RNAi are effective within 8 h after amputation (supplementary material Fig. S4). In general, RNAi suppresses endogenous gene expression within 24 h in the cricket (Uryu et al., 2013), indicating that pattern formation involved in reconstructing the lost segment in the blastema occurs within 1.3 days after amputation via histone H3K27me3. RNAi experiments targeting Gb E(z) or Gb Utx result in extra tibia formation and joint formation defects; therefore, Gb E(z) and Gb Utx might not be involved in dedifferentiation but prevent malformations during leg regeneration and play a role in the fine-tuning of tarsus shape, respectively.

Epigenetic control of gene expression in cricket versus other organisms

E(z) is a component of the Polycomb repressive complex (PRC) and epigenetically represses gene expression during embryogenesis (Barrero and Izpisua Belmonte, 2011). In mouse limb development, Ezh2 regulates pattern formation via Hox gene expression in an epigenetic manner (Wyngaarden et al., 2011). Similarly, Ezh2 regulates pattern formation via Hox gene expression in an epigenetic manner (Barrero and Izpisua Belmonte, 2011). In mouse limb development, organisms are effective within 8 h after amputation (supplementary material Fig. S4). In general, RNAi suppresses endogenous gene expression within 24 h in the cricket (Uryu et al., 2013), indicating that pattern formation involved in reconstructing the lost segment in the blastema occurs within 1.3 days after amputation via histone H3K27me3. RNAi experiments targeting Gb E(z) or Gb Utx result in extra tibia formation and joint formation defects; therefore, Gb E(z) and Gb Utx might not be involved in dedifferentiation but prevent malformations during leg regeneration and play a role in the fine-tuning of tarsus shape, respectively.

MATERIALS AND METHODS

Animals

Two-spotted cricket (Gryllus bimaculatus) nymphs and adults were reared under standard conditions (light:day=12:12 h, 28°C) (Mito and Noji, 2008). Field crickets (Modicogryllus siamensis) were reared under long-day conditions (light:day=16:8 h, 25°C) (Tamaki et al., 2013).

Cloning of Gryllus E(z) and Utx homologues

Gryllus E(z) and Utx homologues were cloned by PCR with LA-Taq or Ex-Taq in GC buffer (TaKaRa). Primers were designed based on the nucleotide sequence determined from the transcriptome data. Template cDNAs were synthesized using the SuperScript III Reverse Transcription Kit with random primers (Invitrogen) from total RNA extracted from regenerating legs of third instar nymphs or late stage embryos (Bando et al., 2009). Gb E(z) and Gb Utx nucleotide sequences were deposited in GenBank under accession numbers LC012934 and LC012935, respectively.

RNAi

Double-stranded RNAs (dsRNAs) were synthesized using the MEGAScript T7 Kit (Ambion) and adjusted to 20 μM for RNAi. In total, 200 nl dsRNA was injected into the abdomen of cricket nymphs. As a negative control, we injected dsRNA for exogenous genes DsRed2 or Egfp. After dsRNA injection, the legs of the crickets were amputated at the appropriate positions (Mito and Noji, 2008).

Whole-mount in situ hybridisation

Regenerating legs were amputated and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline with 0.1% Tween 20 (PBT) for 6 min at 55°C with a microwave oven. The scab and cuticle were removed using tweezers under a dissecting microscope. The regenerating legs were refixed in 4% PFA/PBT. Whole-mount in situ hybridisation of regenerating legs was conducted as previously described (Bando et al., 2009, 2011). Antisense and sense probes were labelled with digoxigenin.

Immunostaining

Fixed regenerating legs were washed with PBT and blocked with 1% bovine serum albumin (BSA) in PBT for 1 h. Blocked samples were incubated with primary antibody (rabbit polyclonal anti-trimethylated H3K27 antibody; Millipore, 07-449) at 1:500 in 1% BSA in PBT overnight at 4°C. Then, the
samples were blocked with 1% BSA in PBS and incubated with secondary antibody (Alexa Fluor 488-conjugated anti-rabbit IgG antibody; Molecular Probes, A-11008) at 1:500 in 1% BSA/PBT for 3 hr at 25°C. Samples were washed with PBS and incubated with DAPI at 1:1000 in PBS for 15 min (Nakamura et al., 2008b).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.H., T.B. and S.N. designed the experiments; Y.H., T.B. and T.N. performed experiments; Y.H., T.B., T.M. and S.N. analysed experimental data; and Y.H., T.B., Y.I., T.M., H.O., K.T. and S.N. prepared the manuscript.

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Supplementary material

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