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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

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'Utx*^{RNAi}. Regenerated *Gb'E(z)*^{RNAi} cricket legs exhibited extra leg segment formation between the tibia and tarsus, and regenerated *Gb'Utx*^{RNAi} 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'Utx*^{RNAi} 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, 2012). When regenerative animals lose tissue sections, a wound epidermis immediately covers the wound surface. Subsequently, a population of proliferating multipotent cells or pluripotent stem cells develops into a blastema beneath the wound epidermis. The lost tissue is restored using the blastema cells via a repatterning process that depends on positional information and pattern formation genes. In planarians, blastema cells originate from stem cells called neoblasts (Handberg-Thorsager et al., 2008). In other regenerative animals, including insects, differentiated cells lose their cell fate to produce blastema cells ('dedifferentiation') (Konstantinides and Averof, 2014; Tamura et al., 2010; Truby, 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 ubiquitylation, 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

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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; Hochedlinger 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

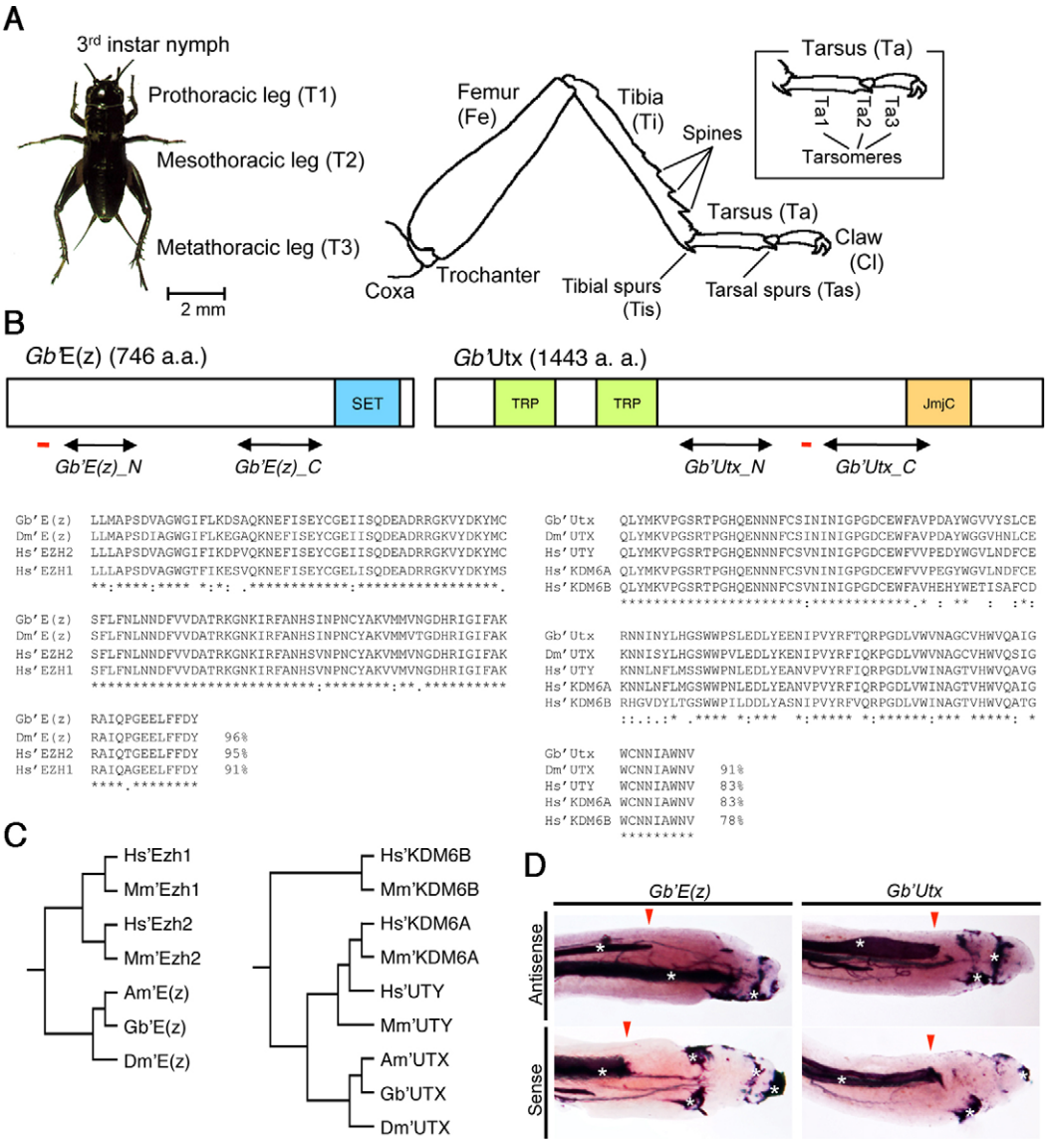

Fig. 1. Isolation of the *Gryllus E(z)* and *Utx* homologues. (A) Dorsal view of *Gryllus* nymph at third instar and schematic of *Gryllus* metathoracic leg. (B) Domain structures and corresponding regions of dsRNAs (double-headed arrow) and amplicons for qPCR (red bar) of *Gb'*E(z) and *Gb'*Utx. E(z) has a SET domain. Utx has TRP (tetratricopeptide repeat) domains and a JmjC domain. Amino acid alignments of the E(z) SET domain and Utx JmjC domain are shown. Identical and similar amino acid residues are indicated by asterisks and dots, respectively. Sequence identities of *Gb'*E(z) and *Gb'*Utx with homologous proteins are indicated by percentage. (C) Phylogenetic tree based on amino acid sequence alignments. Gb, *Gryllus bimaculatus*; Am, *Apis mellifera*; Dm, *Drosophila melanogaster*; Mm, *Mus musculus*; Hs, *Homo sapiens*. (D) Expression pattern of *Gb'E(z)* and *Gb'Utx* in regenerating legs at 6 dpa (*n*=10). Asterisks indicate non-specific staining. Arrowheads indicate the amputation position; regions distal (to the right of) the amputation position are regenerated regions.

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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 *Gb'Utx*, which encodes a histone H3K27 demethylase. *Gb'E(z)*, which encodes a histone H3K27 methyltransferase, and *Gb'Polycomb* (*Gb'Pc*), which encodes a histone H3K27me3-binding protein, were also upregulated in the blastema (Bando et al., 2013).

To further analyse the significance of epigenetic regulation via methylation on histone H3K27 during regeneration, we identified *Gb'E(z)* and *Gb'Utx* full-length transcripts based on transcriptome data. *Gb'E(z)* encodes a 746 amino acid protein, and a histone methyltransferase (SET) domain was found at its C-terminus. Amino acid sequence comparison of the *Gb'*E(z) SET domain with *Drosophila melanogaster* E(z) and *Homo sapiens* EZH2 showed 96% and 95% identity, respectively (Fig. 1B). In *Gryllus* and *Drosophila*, a single *E(z)* gene was found in their genomes; however, two paralogous genes, *Ezh1* and *Ezh2*, were found in the mouse and human genomes (Fig. 1C). *Gb'Utx* encodes a 1443 amino acid protein, and a histone demethylase (JmjC) domain was found at its C-terminus. Amino acid sequence comparison of the *Gb'*Utx JmjC domain with *Drosophila melanogaster* Utx and *Homo sapiens* KDM6A showed 91% and 83% identity, respectively (Fig. 1B). In *Gryllus* and *Drosophila*, a single *Utx* gene was found in their genomes; however, three paralogous genes, *Kdm6a*, *Kdm6b* and *Uty*, were found in the mouse and human genomes (Fig. 1C).

To determine whether *Gb'E(z)* and *Gb'Utx* are expressed during regeneration, we performed whole-mount *in situ* hybridisation with regenerating legs from which the cuticle had been removed. We observed that *Gb'E(z)* and *Gb'Utx* were ubiquitously expressed in regenerating legs at 6 days post amputation (dpa) (Fig. 1D). No significant signal was observed in the negative controls. Ubiquitous expression of *Gb'E(z)* and *Gb'Utx* in regenerating legs at 6 dpa was similar to the expression patterns in developing limb buds and regenerating legs at 2 dpa (supplementary material Fig. S1).

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'Utx*^{RNAi} alter the histone H3K27 methylation state during leg regeneration. In control crickets, 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} crickets, fluorescence intensities of histone H3K27me3-positive nuclei were decreased in the blastema and regenerating tarsus at 2 and 6 dpa, respectively. In the *Gb'Utx*^{RNAi} crickets, 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'Utx*^{RNAi} crickets compared with control crickets (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'Utx*^{RNAi} 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} crickets 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

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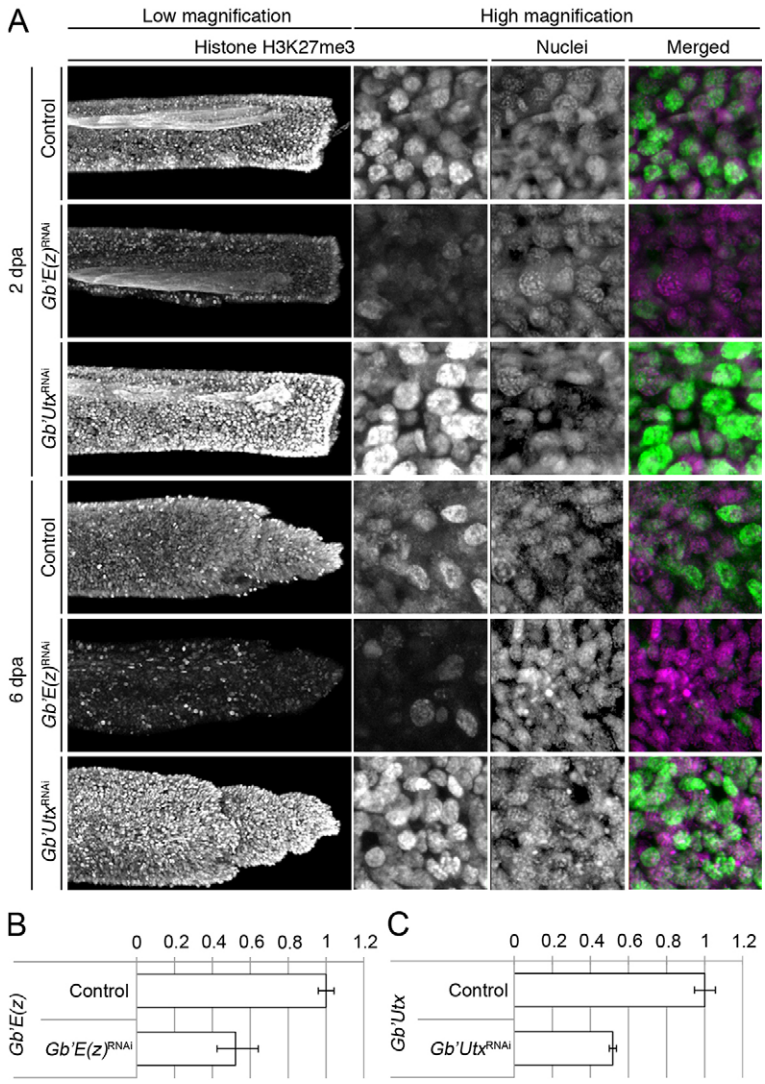

Fig. 2. Localisation of histone H3K27me3 in regenerating legs. (A) Localisation of histone H3K27me3 (green) and nuclei (DAPI, magenta) in regenerating legs of control, *Gb'E(z)*^{RNAi} and *Gb'Utx*^{RNAi} crickets at 2 and 6 dpa (n=10). The right three columns show high-magnification images from the low-magnification images in the left column. Distal portion of the regenerating leg is directed towards the right. (B) Relative *Gb'E(z)* mRNA levels in the control and *Gb'E(z)*^{RNAi} regenerating legs at 3 dpa (n=15). (C) Relative *Gb'Utx* mRNA levels in the control and *Gb'Utx*^{RNAi} regenerating legs at 3 dpa (n=15). Error bars indicate s.d.

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 tarsomeres 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 at the end of a jointless leg segment (25%, n=4/16). In class 2 regenerated legs, the tibia, tarsus and claw regenerated, and a short extra leg segment formed between the tibia and Ta1 (25%, n=4/16). In class 3 regenerated legs, the tibia with tibial spurs regenerated adjacent to the regenerated femur. An extra tibia segment, which was assessed by spur reconstruction, formed between the tibia and tarsus. A thick and short Ta1, the Ta3 and claw regenerated following the extra tibia segment (38%, n=6/16) (Fig. 4C). These morphological observations of *Gb'E(z)*^{RNAi}

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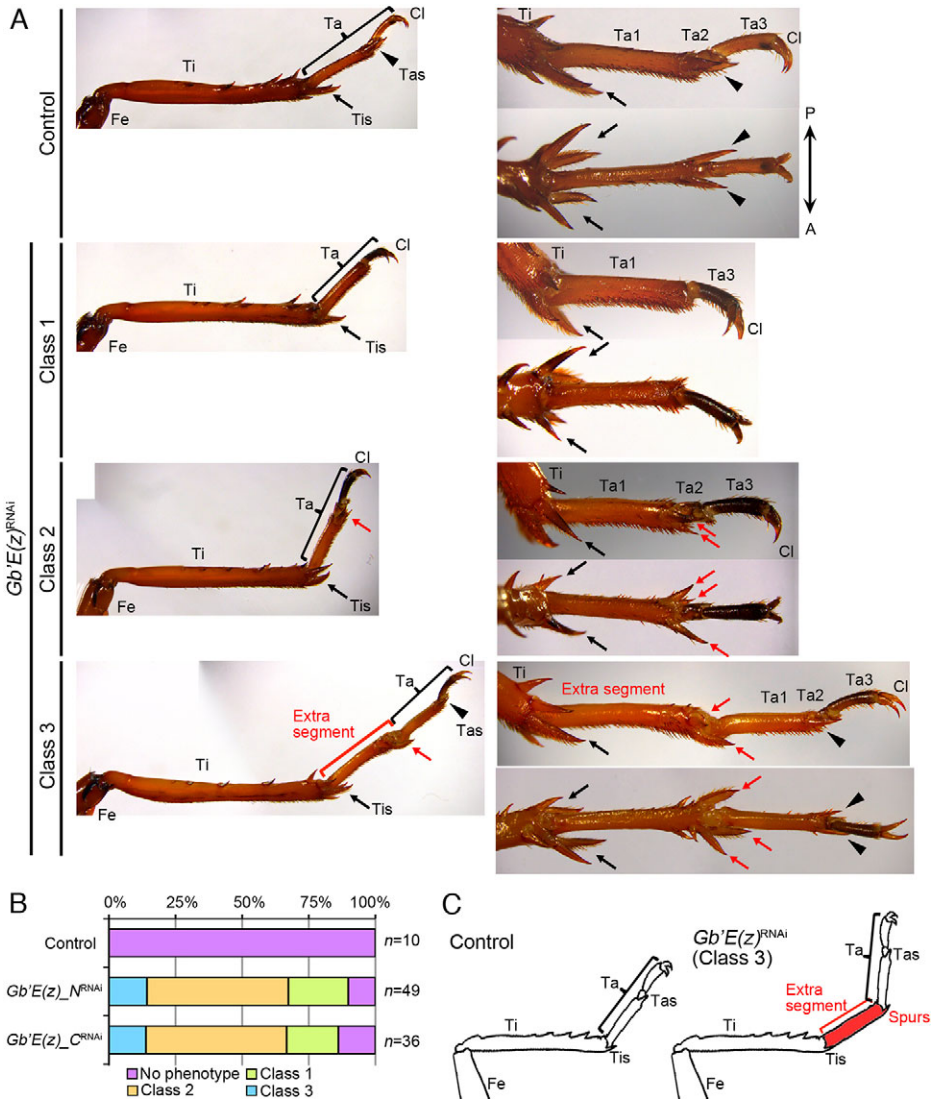

Fig. 3. Typical regenerated leg phenotypes in the control and *Gb'E(z)*^{RNAi} crickets. (A) Regenerated legs in control and *Gb'E(z)*^{RNAi} adults. Lateral views at low magnification are shown in the left column. Lateral and dorsal views at high magnification are shown in the right column in the upper and lower panels, respectively. Tibial spurs and tarsal spurs are indicated by arrows and arrowheads, respectively. Tarsi are indicated by brackets. Fe, femur; Ti, tibia; Ta, tarsus; Cl, claw; Tis, tibial spur; Tas, tarsal spur. The extra tibia segment and its spurs are shown by red brackets and red arrows, respectively. P, posterior; A, anterior. (B) Ratios of normal (no phenotype) and RNAi phenotypes (class 1 to 3) of control and *Gb'E(z)*^{RNAi} cricket nymphs at sixth instar. (C) Schematics of regenerating legs of control and *Gb'E(z)*^{RNAi} crickets. The extra leg segment regenerated between the tibia and tarsus of *Gb'E(z)*^{RNAi} crickets is indicated in red.

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. 5A,C). 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 *Modicogryllus siamensis* (supplementary material Fig. S3B). *M. siamensis* regenerated the lost part of the metathoracic

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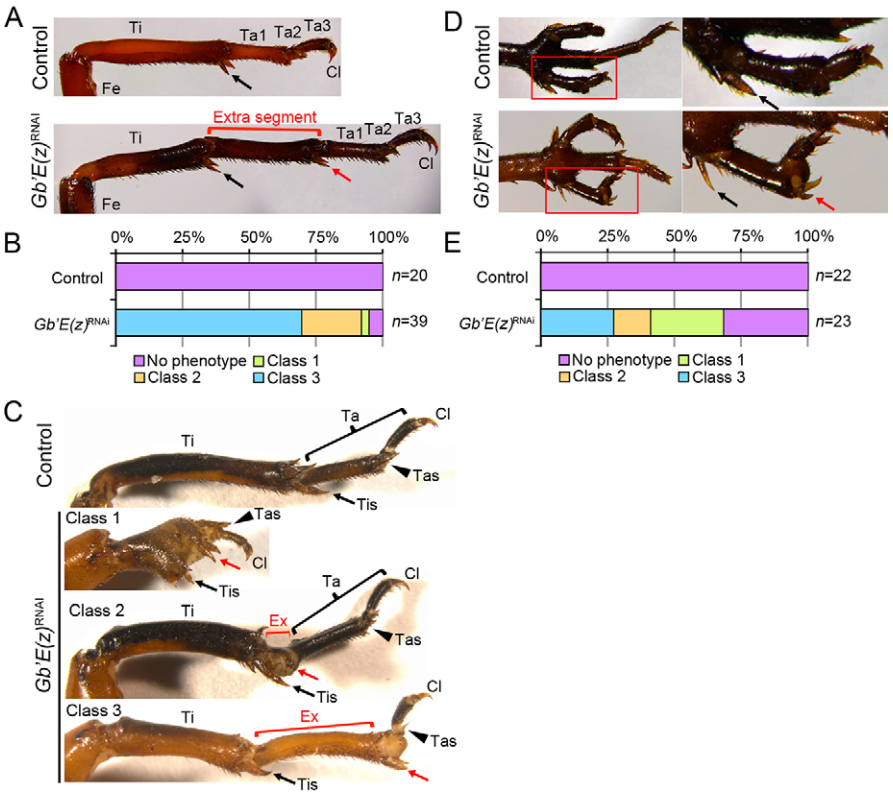

Fig. 4. Typical regenerated and supernumerary leg phenotypes in control and *Gb'E(z)*^{RNAi} crickets. (A) Regenerated mesothoracic legs of control and *Gb'E(z)*^{RNAi} crickets. Tibial spurs are indicated by arrows. The extra tibia segment and its spurs are indicated by the red bracket and red arrow, 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.

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),

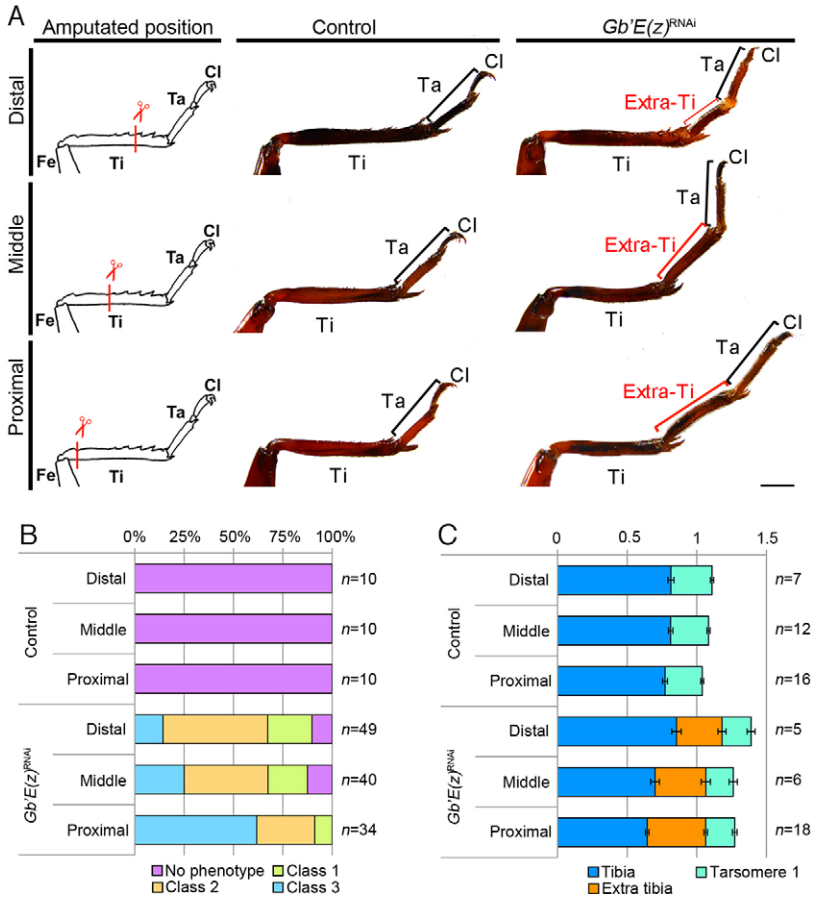

Fig. 5. Effect on extra tibia segment formation of amputation position in *Gb'E(z)*^{RNAi} regenerated legs. (A) Regenerated legs amputated at the distal, middle and proximal positions of control and *Gb'E(z)*^{RNAi} crickets. Amputation positions are shown in the left columns. Tarsi and extra tibia 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.

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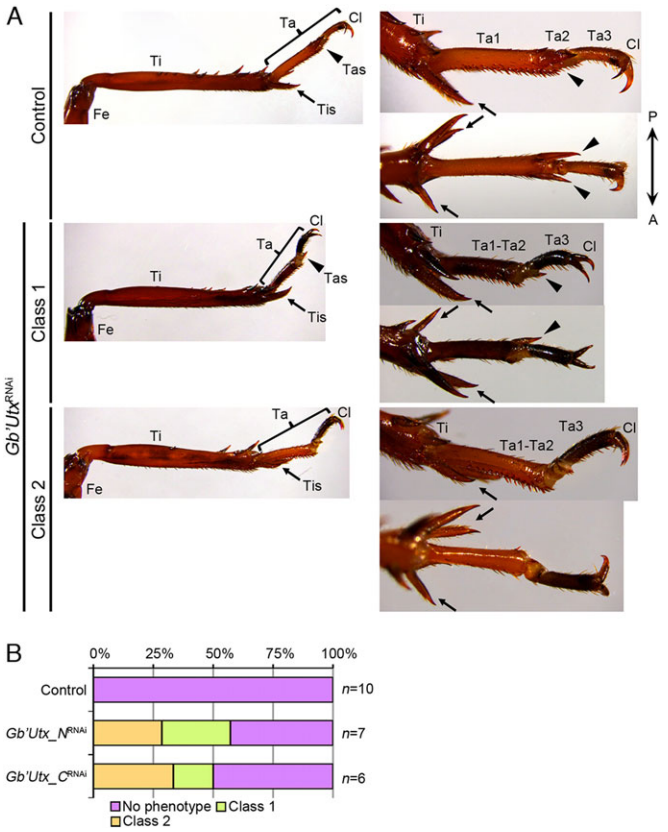

Fig. 6. Typical phenotypes of regenerated legs in control and *Gb'Utx*^{RNAi} crickets. (A) Regenerated legs in the control and *Gb'Utx*^{RNAi} adults. Lateral views at low magnification are shown in the left column. Lateral and dorsal views at high magnification are shown in the right column in the upper and lower panels, respectively. Tibial and tarsal spurs are indicated by arrows and arrowheads, respectively. Fe, femur; Ti, tibia; Ta, tarsus; Cl, claw; Tis, tibial spur; Tas, tarsal spur. (B) Ratio of normal (no phenotype) and RNAi phenotypes (class 1 and 2) of control and *Gb'Utx*^{RNAi} cricket nymphs at sixth instar.

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'Utx*^{RNAi} cricket adults, the lost leg segments regenerated; however, the regenerated tarsomeres 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'Egfr* 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* promotes leg joint and spur formation at the tarsus during repatterning. To clarify whether *Gb'E(z)* and *Gb'Utx* epigenetically regulate leg patterning gene expression involved in tibia and/or tarsus formation, we examined the *Gb'dac*, *Gb'Egfr*, *Gb'BarH* and *Gb'Dll* expression patterns in the regenerating legs of RNAi crickets 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'Egfr* 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'Egfr*, *Gb'BarH* and *Gb'Dll* expression patterns were not altered in the *Gb'E(z)*^{RNAi} regenerating legs. In the *Gb'Utx*^{RNAi} regenerating legs, *Gb'Egfr* was expressed at the distal position of the tibia and tarsus (arrowheads, Fig. 7A); however, *Gb'Egfr* 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'Utx*^{RNAi} regenerating legs. Overall, these results suggest that *Gb'E(z)* and *Gb'Utx* epigenetically regulate *Gb'dac* and *Gb'Egfr* 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} crickets, *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 regenerated legs 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'Utx*^{RNAi} at any time point.

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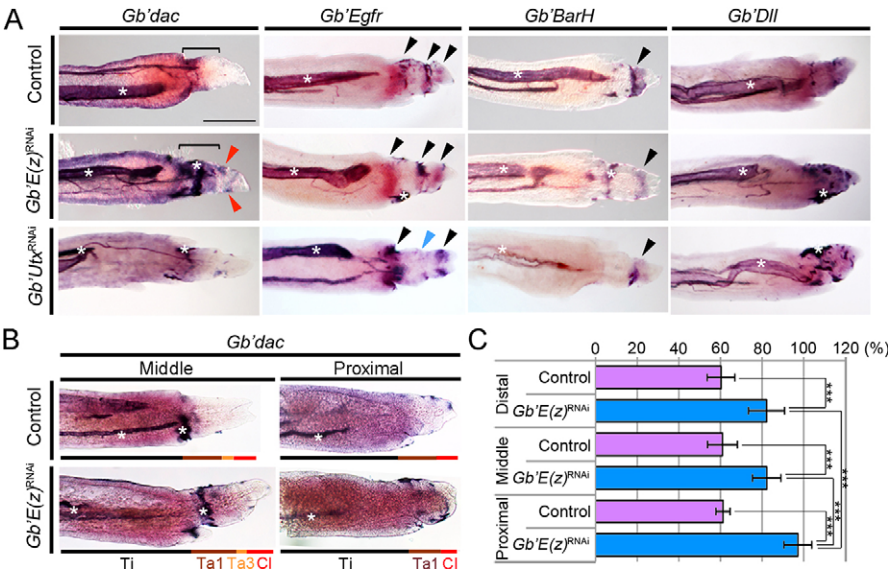

Fig. 7. Expression pattern of leg patterning genes in regenerating legs. (A) *Gb'dac*, *Gb'Egfr*, *Gb'BarH* and *Gb'Dll* expression patterns in control, *Gb'E(z)*^{RNAi} and *Gb'Utx*^{RNAi} regenerating legs at 6 dpa ($n \geq 20$). Ta1 is indicated by brackets. *Gb'Egfr* and *Gb'BarH* expression is indicated by arrowheads. Red arrowheads indicate *Gb'dac* expression in the distal tarsus. Asterisks indicate non-specific staining. Scale bar: 100 µm. (B) *Gb'dac* expression patterns in control and *Gb'E(z)*^{RNAi} regenerating legs amputated at the middle (6 dpa) and proximal (7 dpa) positions. Note that the growth rate of cricket nymphs amputated proximally was slower than that of nymphs amputated distally or in the middle; therefore, we fixed regenerating legs amputated proximally at 7 dpa, whereas other regenerating legs were fixed at 6 dpa. Ti, tibia; Ta1, tarsomere 1; Ta3, tarsomere 3; Cl, claw. Asterisks indicate non-specific staining. (C) The percentage of *Gb'dac* expression within Ta1. ***P<0.01 (Student's *t*-test). Error bars indicate s.d.

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 *Egfp*, regeneration occurred in both groups (data not shown). In *Gb'E(z)*^{RNAi} crickets, ~80% of RNAi crickets showed RNAi phenotypes in the regenerated legs in both groups (supplementary material Fig. S4B). In *Gb'Utx*^{RNAi} crickets, 30% and 50% of crickets showed RNAi phenotypes in the regenerated legs 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 Izpisua 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.

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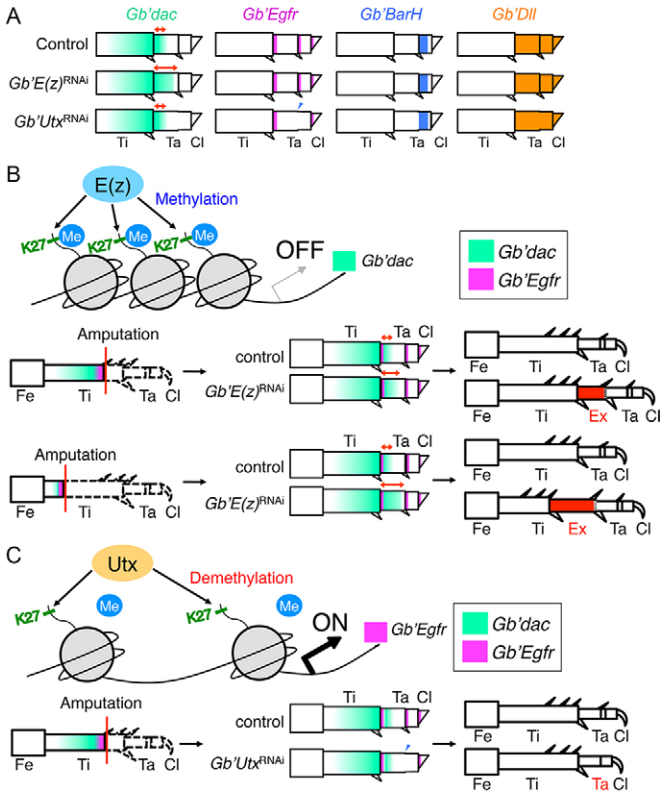

Fig. 8. Model for the repatterning process during leg regeneration in cricket. (A) Schematics of *Gb'dac*, *Gb'Egfr*, *Gb'BarH* and *Gb'Dll* expression in control, *Gb'E(z)*^{RNAi} and *Gb'Utx*^{RNAi} regenerating legs. The *Gb'dac* expression domain in Ta1 is indicated by red double-headed arrows. (B) 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'Dll* 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'Utx*^{RNAi} 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'Utx*^{RNAi} crickets because *Gb'Dll* expression in the tarsus and *Gb'BarH* expression in the tarsus centre were not altered.

Gb'Utx promotes joint formation via histone H3K27me3 during leg regeneration

Gb'Utx^{RNAi} regenerated legs exhibited leg joint formation defects at Ta1 (Fig. 6A). The tarsal spur reconstruction defects were probably caused by joint formation defects between Ta1 and Ta2. These findings in *Gb'Utx*^{RNAi} regenerated legs imply that *Gb'*Utx epigenetically regulates the expression of genes involved in leg joint formation. During leg regeneration, *Gb'Egfr* is expressed in leg joints and in the spur primordia (Nakamura et al., 2008b). In *Gb'Utx*^{RNAi} crickets, *Gb'Egfr* expression in the leg joint in the middle region of the tarsus was diminished. Epigenetic derepression errors might have resulted in *Gb'Utx*^{RNAi} altering *Gb'Egfr* expression in the tarsus, causing leg joint and spur formation defects on the tarsus (Fig. 8A,C). Systemic RNAi against *Gb'Egfr* has been reported to cause defects in Ta3 and claw reconstruction during regeneration (Nakamura et al., 2008b). In *Drosophila*, *Utx* genetically interacts with *Notch* to regulate cell proliferation (Herz et al., 2010), and in *Gryllus* systemic RNAi against *Gb'Notch* causes the formation of a short regenerated tarsus with no leg joint formation (Bando et al., 2011). Differences between the *Gb'Utx*^{RNAi} and *Gb'Egfr*^{RNAi} or *Gb'Notch*^{RNAi} phenotypes indicate that *Gb'*Utx regulates *Gb'Egfr* expression in the middle region of the tarsus and that *Gb'Utx* may not interact with *Gb'Notch* during regeneration nor with *Gb'Egfr* in the other regions. Thus, *Gb'Utx* promotes leg joint restoration via epigenetic regulation of *Gb'Egfr* expression by histone H3K27me3 during leg regeneration.

In comparison with the class 3 phenotype of *Gb'E(z)*^{RNAi}, the morphological defects caused by *Gb'Utx*^{RNAi} were limited, with the notable defect involving leg joint formation between Ta1 and Ta2. In embryonic development, *Gb'E(z)*^{RNAi} embryos exhibit abnormal appendage formation (see below) (Matsuoka et al., 2015); however, *Gb'Utx*^{RNAi} embryos exhibit minor morphological defects in the head segment (supplementary material Fig. S5). In other organisms, Utx is essential for regeneration, wound healing (Shaw and Martin, 2009; Stewart et al., 2009), embryonic development (Shpargel et al., 2014) and embryonic stem cell differentiation (Morales Torres et al., 2013) via the epigenetic regulation of cell proliferation or repair genes. Since the cricket is a hemimetabolous insect, cell proliferation and the production of new cuticles during the molting process are essential; as such, the activation of cell proliferation and subsequent processes might be regulated via various redundant molecular systems in addition to Utx. This might explain why *Gb'Utx*^{RNAi} did not result in marked regeneration defects compared with the regeneration defective phenotype observed in other organisms.

Epigenetic regulation by histone H3K27me3 does not play a role in dedifferentiation

We previously showed that *Gb'E(z)* and *Gb'Utx* expression is upregulated within 24 h after cricket leg amputation (Bando et al., 2013), suggesting that *Gb'E(z)* and *Gb'Utx* might be involved early in the regeneration process, such as during the dedifferentiation into blastema cells. During *Drosophila* imaginal disc regeneration, the expression of Polycomb group genes, including *E(z)*, is directly downregulated by JNK signalling in the wounding edge, increasing the plasticity of differentiated cells for promoting blastema formation (Lee et al., 2005). In addition, *Utx* and *E(z)* expression is activated and suppressed, respectively, after injury to epigenetically regulate the expression of dedifferentiation-related and redifferentiation-related genes to promote blastema formation in *Drosophila* (Katsuyama and Paro, 2011; Repiso et al., 2011). Conversely, during murine skin repair, Utx and Jmjd3 are involved in wound healing by upregulating the expression of repair genes (Shaw and Martin, 2009). The expression of Kdm6 family genes, which encode Utx and Jmjd3 orthologues, is upregulated during amputation to promote blastema cell proliferation in zebrafish (Stewart et al., 2009). During the reprogramming process, in which differentiated cells develop into iPSCs, the expression of differentiation-related genes is suppressed via histone H3K27me3; however, dedifferentiation-related genes are not repressed (Meissner, 2010), possibly through the actions of mammalian Utx homologues (Mansour et al., 2012). These reports imply that derepression of dedifferentiation-related genes via demethylation on histone H3K27me3 is a key event in dedifferentiation and reprogramming, in iPSCs as well as in blastema cells.

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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, *Gb'*E(z) also regulates appendage development in the head, gnathal and thoracic segments and katatrepsis via the epigenetic regulation of homeotic gene expression during *Gryllus* embryonic development (Matsuoka et al., 2015). However, during the regeneration process, E(z) is unlikely to regulate homeotic gene expression because the regenerated mesothoracic or metathoracic legs maintained their identities and showed no homeotic transformations (Fig. 3A and Fig. 4A). Thus, the expression of genes regulated by E(z) appears to depend on the biological context – that is, during embryogenesis or regeneration.

Differences in the regenerative capacity between the newt and frog are attributed to the extent of DNA methylation at a *Shh cis*-regulatory element (Yakushiji et al., 2007, 2009). In zebrafish, the status of DNA methylation is changed in the blastema during fin regeneration (Hirose et al., 2014; Takayama et al., 2014). In addition, DNA methylation has important roles in stem cells, development, tumourigenesis and other processes. However, in insects, epigenetic repression via DNA methylation is limited to processes such as oogenesis in *Drosophila* or caste determination in the honey bee (Glastad et al., 2011; Lyko and Maleszka, 2011). In *Gryllus*, RNAi against DNA methyltransferases (*Gb'Dnmt2* and *Gb'Dnmt3*) or 5-methylcytosine hydroxylase (*Gb'Tet*) does not result in a phenotype (supplementary material Fig. S6), suggesting that the epigenetic regulation of gene expression via DNA methylation is not essential in *Gryllus* leg regeneration. Epigenetic repression of gene expression via DNA methylation is widely utilised in combination with histone H3K27 and H3K9 methylation in vertebrates; however, in insects, histone H3K27 methylation is the dominant alteration involved in epigenetic repression. Methylation on histone H3K9 is another epigenetic alteration involved in repression; therefore, inhibition of histone H3K9me3 may cause other defects during regeneration in insects.

In conclusion, this study demonstrates that strict control of leg patterning gene expression via histone H3K27me3 is essential for proper repatterning during regeneration: *Gb'E(z)* promotes the restoration of lost leg sections, while *Gb'Utx* promotes leg joint restoration. Previously, we identified other factors that regulate methylation states on histones H3K4, H3K9 and H3K36 or that regulate the histone acetylation state in regenerating cricket legs (Bando et al., 2013). Methylation on histone H3K4 is needed to maintain the stem cell population in planarians (Hubert et al., 2014; Robb and Alvarado, 2014), and epigenetic changes on histone H3K4 and H3K9 correlate with cell proliferation during regeneration in the polychaete worm (Niwa et al., 2013), suggesting that the orchestrated regulation of epigenetic histone modification might promote dedifferentiation into blastema cells in *Gryllus*. This report provides a clear contribution to an understanding of the epigenetic regulation of gene expression during tissue regeneration.

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 PBT 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 h at 25°C. Samples were washed with PBT and incubated with DAPI at 1:1000 in PBT 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
Supplementary material available online at
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