Development 136, 2235-2245 (2009) doi:10.1242/dev.035204

Regulation of leg size and shape by the Dachsous/Fat signalling pathway during regeneration

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An amputated cricket leg regenerates all missing parts with normal size and shape, indicating that regenerating blastemal cells are aware of both their position and the normal size of the leg. However, the molecular mechanisms regulating this process remain elusive. Here, we use a cricket model to show that the Dachsous/Fat (Ds/Ft) signalling pathway is essential for leg regeneration. We found that knockdown of *ft* or *ds* transcripts by regeneration-dependent RNA interference (rdRNAi) suppressed proliferation of the regenerating cells along the proximodistal (PD) axis concomitantly with remodelling of the pre-existing stump, making the regenerated legs shorter than normal. By contrast, knockdown of the *expanded* (*ex*) or *Merlin* (*Mer*) transcripts induced over-proliferation of the regenerating cells, making the regenerated legs longer. These results are consistent with those obtained using rdRNAi during intercalary regeneration induced by leg transplantation. We present a model to explain our results in which the steepness of the Ds/Ft gradient controls growth along the PD axis of the regenerating leg.

Key words: Dachsous/Fat, Expanded/Merlin, Legs, Regeneration, Size, Steepness model

INTRODUCTION

Regeneration depends on the recognition of tissue loss and the subsequent restoration of the relevant structure. Many insights into the mechanisms underlying such regeneration processes have been obtained from studies on limb regeneration in urodeles (for reviews, see Nye et al., 2003; Brockes and Kumar, 2008) and hemimetabolous insects (for a review, see Nakamura et al., 2008a). However, numerous questions remain, including how blastemal cells know their positional identities, how they detect positional disparity along the proximodistal (PD) axis, how they accurately restore the leg to its normal size, and what factors govern blastemal cell proliferation in the correct proportions. To address these questions, we have developed an experimental system using the two-spotted cricket *Gryllus bimaculatus* (Mito et al., 2002; Nakamura et al., 2007; Nakamura et al., 2008a; Nakamura et al., 2008b; Mito and Noji, 2009). Using this system, we have studied the molecular biological basis of a number of phenomena that were originally identified in a series of excellent classical studies on insect leg regeneration (Bohn, 1965; French et al., 1976; Meinhardt, 1982). The greatest advantage to our cricket system is that it allows regeneration-dependent RNAi (rdRNAi) to be used for loss-of-function analyses. Regeneration-dependent RNAi is a type of RNAi that occurs specifically after leg amputation in cricket nymphs that have been injected with double-stranded RNA (dsRNA) for a target gene (Nakamura et al., 2008a). In this system, when the metathoracic (T3) tibia of the third-instar nymph (Fig. 1A) is amputated (Fig. 1B), it takes approximately 40 days (six ecdyses) to restore the adult leg. The process begins with the covering of the amputated region by newly formed cuticle. A ligand of Epidermal growth factor receptor (*Gb*'Egfr) is then induced by Decapentaplegic (*Gb*'Dpp) and Wingless (*Gb*'Wg) in a blastema composed of epithelial stem cells, which begins to undergo rapid proliferation to restore the lost portion in the fourth instar (Fig. 1B) (Mito et al., 2002; Nakamura et al., 2008b). In the fifth instar, the tibiae, tibial spurs, tarsi and tarsal claws are restored in miniature (Fig. 1B). In the seventh instar, the amputated legs restore the missing portion to regain a nearly normal appearance (Fig. 1B). As no leg regeneration was observed after amputation in the case of rdRNAi against *Gb'armadillo* (*Gb'arm*), the canonical Wnt pathway should be involved in the initiation of the regeneration (Nakamura et al., 2007).

To identify other genes involved in leg regeneration, we chose candidates that have been implicated in *Drosophila* appendage PD patterning, and examined their functions using the rdRNAi method. We were particularly interested in molecules involved in the Dachsous/Fat (Ds/Ft) signalling pathway, which has been extensively studied (for a review, see Reddy and Irvine, 2008). Based on these *Drosophila* data, we selected a set of 23 genes in the Ds/Ft signalling pathway, as listed in Table 1 and shown in Fig. 1C (Reddy and Irvine, 2008). To determine whether these candidate *Drosophila* genes are involved in cricket leg regeneration, we cloned cDNA fragments of their *Gryllus* homologues (unbroken lines in Fig. 1C). We examined the effect of rdRNAi against each of the 23 target genes on regenerating legs, and found at least 15 genes to be involved in leg regeneration, indicated in yellow in Fig. 1C and listed in Table 1. In this report, we focus mainly on the functions of the *Gryllus* homologue of *ft* (*Gb'ft*), *Gb'ds*, *Gb'four-jointed* (*fj*), *Gb'dachs* (*d*), *Gb'expanded* (*ex*), or *Gb'Merlin* (*Mer*). Others will be published elsewhere. In brief, Ft is a large protocadherin that is evolutionarily conserved from *Drosophila* to mammals (Reddy and Irvine, 2008). In *Drosophila*, the protocadherins Ft and Ds regulate planar cell polarity (PCP), PD patterning and cell proliferation (Mahoney et al., 1991; Clark et al., 1995; Adler et al., 1998; Yang et al., 2002), whereas Fj, encoding a Golgi kinase, regulates Ft signalling by phosphorylating Ft and Ds (Ma et al., 2003; Strutt et al., 2004; Ishikawa et al., 2008). D is an unconventional myosin, acting as a crucial downstream component of the Ds/Ft signalling

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Accepted 28 April 2009

Table 1. Summary of rdRNAi for Fat signalling and related molecules on cricket leg regeneration

Gene	Phenotypes of RNAi					Accession No.
	Enlargement at blastema	Longitudinal regeneration	Circumferential regeneration	Other phenotypes on regeneration leg	Other phenotypes	
14-3-3ε						AB443441
14-3-3ζ						AB443440
14-3-3ε/14-3-3ζ	Enlarged				Nymphal lethal at 3rd to 4th instar	
app					Suppress *Gb'ft* rdRNAi phenotype	AB378066
cycE						AB378067
d		Shortened				AB378068
dco						AB443442
diap1					Nymphal lethal at 3rd to 4th instar	AB378071
*ds**	Enlarged	Shortened	Thick			AB300571, AB300572
ena						AB378069
ex	Slightly enlarged	Lengthened				AB378099
E(z)				Abnormal tarsus		AB378079
fj		Slightly shortened				AB300573
*ft**	Enlarged	Shortened	Thick			AB300569, AB300570
gug†						AB378077, AB378078
hpo						AB378070
mats						AB378072
Mer	Slightly enlarged	Lengthened				AB378073
Pc				Curved tibia, abnormal tarsus		AB444104
Rassf						AB443439
sav						AB378074
sd				Curved tibia, abnormal tarsus	Abnormal antenna and wing	AB443438
wts	Enlarged			Curved tibia, abnormal tarsus	Nymphal lethal at 3rd to 6th instar	AB300574
yki‡					Nymphal lethal at 3rd to 4th instar	AB378075, AB378076

Summary of rdRNAi for Fat signalling and related molecules, based on *Drosophila* data [a recent review (Reddy and Irvine, 2008)], on cricket leg regeneration.
*For *Gb'ft* and *Gb'ds*, AB300569 and AB300571 were used for RNAi, and AB300570 and AB300572 for probe regions of in situ hybridization and target regions of q-PCR.
†For *Gb'gug*, a mixed solution of dsRNAs corresponding to both AB378077 and AB378078 was used for RNAi since each of fragment was too short to RNAi.
‡*Gb'yki* encodes long (AB378076) and short (AB378075) isoforms. AB378075 was used for RNAi.

pathway that influences growth, cell affinity and gene expression during development (Mao et al., 2006). Both *ex* and *Mer* encode cytoplasmic proteins belonging to the Band 4.1 superfamily that are proposed to function together in a redundant manner upstream of Hippo/Warts (Hpo/Wts) signalling to regulate contact inhibition of cell proliferation (Boedigheimer and Laughon, 1993; McCartney et al., 2000; Hamaratoglu et al., 2006). Our experimental results indicated that leg size and shape are regulated through the Ds/Ft signalling pathway, including Ex/Mer, during regeneration. These insights provide cues to understand molecular mechanisms underlying a process of regeneration that was first described a century ago.

MATERIALS AND METHODS

Animals

All adult and nymph two-spotted crickets, *Gryllus bimaculatus*, were reared under standard conditions (Mito et al., 2002; Nakamura et al., 2007; Nakamura et al., 2008b; Mito and Noji, 2009).

Cloning of the *Gryllus* homologues of Ds/Ft-signalling-related genes

Gryllus genes homologous to the *Drosophila* Ds/Ft-signalling-related genes were cloned by a degenerate PCR method or by PCR using gene-specific primers in a reaction mixture containing LA-taq or Ex-taq in a GC buffer (TaKaRa, Kyoto), as described previously (Nakamura et al., 2007). Degenerate primers were designed using the CODEHOP algorithm (http://bioinformatics.weizmann.ac.il/blocks/codehop.html) with conserved regions among insects and mammals, or were designed based on conserved nucleic acid sequences among insect species. Gene-specific primers were designed by probe search (http://probe-search.ccr.tokushima-u.ac.jp/probe_search/index.html) based on the nucleotide sequence of expressed sequence tag (EST) and high-throughput cDNA (HTC) data on DNA Databank of Japan. The primer sequences are listed in Table 2. The sequences reported here were deposited in GenBank. The accession number of each gene is listed in Table 1.

Regeneration-dependent RNAi

Preparation of dsRNA and rdRNAi was as previously described (Nakamura et al., 2007; Nakamura et al., 2008b). Briefly, dsRNAs were synthesized using the MEGAScript Kit (Ambion) and adjusted to 20 μM. After injection of dsRNA into the abdomen of third-instar nymphs, their tibiae were amputated between the second and third spines. Because there are three spines in the tibia, the first being the most distal, 30% of the distal portion of the tibia was removed by the amputation, unless otherwise stated. As a negative control, we injected dsRNA for an exogenous gene, *DsRed2*, which encodes a red fluorescent protein in the nymph. We performed RNAi experiments with more than 20 nymphs twice for each gene (Nakamura et al., 2008b). Expressivity of phenotypes caused by nymphal RNAi was more than 90%, unless otherwise noted. For dual RNAi experiments, a mixture of dsRNAs for the two target genes was used. In the mixture, the final concentration of each dsRNA was adjusted to 10 μM. In a control experiment for the dual RNAi procedure, the dsRNAs were replaced by injection buffers alone.

Table 2. Sequences of primers and template cDNAs for cloning of *Gryllus* homologous genes

Gene	First PCR, forward and reverse	Nested PCR, forward and reverse	Template cDNAs
14-3-3ε	CAGGTGGAAAAAGAGCTTCG TCTCACCATCACCCTGCATA		Nymphs and adults
14-3-3ζ	GGAACTGGGAGGTTGTGCTA TACTTGCTTTGGGGATCAGG		Nymphs and adults
app	CTGATGATGGCCCCACACDSNGGNGTNTT CCGATGATGGACCACACGSWRMARAARCA		48 h AEL and late stage embryo
cycE	CAACCACGHATGCGDGCVATHCTNYT ANTTGCATRTANAHDYYVAGCCAGCC	GAYTGGTTGATBGARGTBTGTGAAGT CCATCHGTVACRTARGCAAAYTC	48 h AEL and late stage embryo
d	CGAGACCGGGTACCCCCARDSNATHAT TCCAGCTCGGACTTGAAGATGTGNSWRTTRWA	CCAAGATGCACTGCTACTTCCTGGANMARWSNMG CGAAGCCGAACATGTCCAGGAYNCCDATRAA	12 hpa regenerating legs
dco	GACTACAAYGTNATGGTRATGG TTGTTTTCYCGRTARGGTATGTG		48 h AEL and late stage embryo
diap1	TCAAACTTCGAGGGAGCTGT ACTCCCAGCTGCTCCAGTAA		Nymphs and adults
ds(CD18/21)	CGTGCAGATCAACGTGCGNGAYRTNAAYG GGCGGGGTAGGTGTCCACRTCRTTNSC	CAGATCAACGTGCGCGACRTNAAYGAYMA GGAAGGTGGGCTGGTTGTCRTTNRYRTC	12 hpa regenerating legs
ds(IC)	GGACGACGGCGAGGAYGANGARAT CGGTGAACACGTGGGCNARNGGYTG	ACGACGAGATCCGGATGATHAAYGA CAGTCCAGCAGGTAGTCCCARTTRTA	12 hpa regenerating legs
ena	GGTGTACGACGACGTCAACAARAARTGGRT CCCGGGCGAAGACGTYNGCRTCNTBYT	CGGCTTCTCCAAGGTGCANHTNTWYCA CGTCCTCCTTGGAGCAGAARTTNARNCC	Late stage embryo
ex	CCATCGTGAGCGACGGNGARTAYNTNT CGAACTTCTTCCGGTCGAANSHNARYTT	TCGACCCCGAGCAGAAGYTNWSNAARTA TCTTCCGGTCGAAGCACARYTTNSYDAT	48 h AEL embryo
E(z)	GTCTCAAACAGCGGAAAAGC TTCTTCTGGCCTCCCCTTAT		Nymphs and adults
fj	GCCTGCCTGCTGTCCATCYTNGCNGCNTT CGGTCGATCCGCTCCAYNARDATYTT	TCCAGGAGGAGCTGAGACCNGCNCANTGGA GGACGGTGCGCTCCCKRAANACRCA	12 hpa regenerating legs
ft(CD2/4)	CCCACCTATCCNAYNGARGT TCCACCGTCIGKNGCNSWDAT	GGCTACCTCCARGTNAAYGTNAC TCGAACACNGGNKSRTGCTCRTT	12 hpa regenerating legs
ft(IC)	CCCGATATCATHGARMGNGA GCTAGGTCCCCARTTNARNARRT	CACTACGACHTNGANAAYGC CTCCGAGCANGTRAANSWRTC	12 hpa regenerating legs
gug(Nter)	GGTCCATGGCCGCCTTCSMNGGNATGTG GGGGGTGTCCTTGTGGGGNARNARNTC	CTGCATGGCCGCCTCCMGNGAYGAYAC CGAACTGCCGCAGGCCYTTNAYRAA	48 h AEL and late stage embryo
gug(Cter)	GGGGTTGATTTCAACTCCTG TGGTTTTGATCCTTCTGGC		Nymphs and adults
hpo	TGTCTTCCAAAAGTGAGTTAAAGAAA TGCCATTTCCAAAGCAGTTA		Nymphs and adults
mats	GGCGATCATCCAAGACGTT AAGGAAAAAGTGTTTCATCATCAAG		Nymphs and adults
Mer	CGAGCAACTGGTCGAGATTT CCCCCATACATGTCAAGGTC		Nymphs and adults
Pc	GTVAARTGGAAGGGHTGGA CBYKCTCNGTYTTRCACTCCC		48 h AEL and late stage embryo
Rassf	ATGTGGAARTGYCAYAARTGCGG TNCGCTGATCRTCRTYYTCYTT		48 h AEL and late stage embryo
sav	GARCTBCCDCTDCCBCCYGGCTGGTC TAGGGATTDGCYGGVACCARNRCRYTGTG		48 h AEL and late stage embryo
sd	CCRGAYATCGAGCAGAGYTT AARTTGATCATRTABTCGCACATSGG		48 h AEL and late stage embryo
wts	CAGCARCCNATHATHATGCA TATRTGYTGYTCCATRAARAAYTT	CGCAAGTNCARAARCCNGT CTTGAAGGCYTGNGGNSWRTA	12 hpa regenerating legs
yki	CGGCCACTACAACTACGTCA TCCAATGCTGCCATATCTGA		Nymphs and adults

Partial cDNA fragments of *Gryllus* homologous genes were cloned by PCR (see Materials and methods).
AEL, after egg laying; h, hour; hpa, hours post-amputation.

Nymph leg transplantation

Transplantation experiments for normal and reverse intercalary regeneration and supernumerary leg formation were performed as described previously (Mito et al., 2002; Nakamura et al., 2007). Briefly, we collected nymphs one day after moulting from the second to the third instar. For transplantation experiments, we used the amputated metathoracic leg stump as the host and the amputated mesothoracic distal portion as the graft. To connect the legs, the mesothoracic graft was inserted into the metathoracic leg stump of the same animal as used for graft preparation under ice-chilled conditions and left for 1 hour. We tried to transplant between control and RNAi legs. However, when a control graft was transplanted to an RNAi host, RNAi was induced in the graft. Conversely, when an RNAi graft was transplanted to a control host, the graft became normal. Thus, we cannot show results for a case that one half of the regenerate is treated with RNAi.

Whole-mount in situ hybridization

Whole-mount in situ hybridization of embryos and regenerating legs were carried out using a semiautomatic hybridization instrument (HYBRIMASTER HS5100, ALOKA, Tokyo) and with InSitu Chip [InSitu Chip (L), ALOKA, Tokyo] (Ogasawara et al., 2006) as previously described (Mito et al., 2002; Nakamura et al., 2007; Nakamura et al., 2008b).

Cell proliferation assay

Cell proliferation assays were carried out using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen) (Salic and Mitchison, 2008). 5-Ethynyl-2′-deoxyuridine (EdU) was injected into the abdomen of cricket nymphs at the appropriate stages after amputation. Regenerating legs were fixed 4 hours after EdU injection. EdU-incorporating cells were detected according the manufacturer's instructions. Propidium iodide (PI) was used for nuclear staining.

Quantitative-PCR (q-PCR)

Total RNA was extracted from the regenerating tibiae of control, *Gb'ft*, *Gb'ds* or *Gb'hpo* rdRNAi nymphs at the third or sixth instar, 2 days post-amputation (dpa), using the RNAqueous-Micro Kit (Ambion). mRNA (1 μg) was reverse transcribed to cDNA using the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. q-PCR was performed with the ABI 7900 Real-Time PCR System (Applied Biosystems) as described previously (Nakamura et al., 2008b). The sequences of the q-PCR primers are as follows (forward and reverse, 5′ to 3′): *ds(IC)*, CGGATGATAAACGAAGGTCCTCTA and GGTTGTCATCATCTGCATCATCTC; *fj*, TCGTGTTCGACTACCTCACG and TTCGTTGTCGAAGAAGACCA; *ft(IC)*, TGCACCACCTCAACCACCT and TCGTCGAGCTGTGGTTGTCC; *hpo*, AAAAGGAACACAGTCATAGGCACA and AATGTCAGCAACACAATCATACCC; *β-actin*, TTGACAATGGATCCGGAATGT and AAAACTGCCCTGGGTGCAT.

RESULTS

Knockdown of *Gryllus* Ds/Ft signalling factors induces abnormal regeneration

To examine the developmental roles of *Gb'ft*, *Gb'ds* and *Gb'fj*, we attempted to knock down their expression by parental, embryonic and nymphal RNA interference (RNAi) (Mito and Noji, 2009). No significant effect was observed in the treated eggs or nymphs, indicating that RNAi against those genes is not effective at these stages. By contrast, rdRNAi was highly effective, reproducible and constant; with this method, we found at least 15 genes (yellow in Fig. 1C) to have phenotypes implicating them in leg regeneration (see Fig. S1 in the supplementary material). We divided the leg phenotypes obtained by rdRNAi into five classes: (1) distal enlargement; (2) abnormal regeneration along the PD axis (short or long); (3) abnormal regeneration along the circumferential axis (thin or thick); (4) others; and (5) no phenotype (Table 1). To denote, for example, a nymph containing injected dsRNA for *Gb'ft*, we use the term '*Gb'ft* RNAi nymph'. Details of nymph phenotypes after RNAi treatment of genes not shown here will be published elsewhere.

To confirm that rdRNAi decreased the amount of *Gb'ft* mRNA in regenerating tibial stumps, we performed q-PCR. We estimated the ratio of the amount of *Gb'ft* mRNA at 2 dpa compared to 0 dpa (n=10). The average ratio at 2 dpa was lowered to 0.30±0.02 (in triplicate, ± standard deviation unless otherwise noted) in regenerating *Gb'ft* RNAi tibiae, indicating that rdRNAi had occurred (see Fig. S2A in the supplementary material). We also estimated the duration of the rdRNAi effect in the nymphal leg. When legs were amputated at the sixth instar after injection of dsRNA at the third instar, the relative ratio of *Gb'ft* mRNA (n=10) at 2 dpa was not lowered to 1.06±0.06 (see Fig. S2A in the supplementary material), indicating that RNAi was no longer effective at the sixth instar. To ensure that there had been no off-target effect, we compared the phenotypes obtained with two dsRNAs corresponding to different regions of the same gene. In the case of rdRNAi against *Gb'ft* or *Gb'ds*, we observed a similar 'short and thick' phenotype (see Fig. S2C,D in the supplementary material) as well, indicating that the phenotypes obtained by rdRNAi are not off-target effects. We also confirmed the specificity by which mRNA was depleted by rdRNAi (see Fig. S2B in the supplementary material): in regenerating *Gb'ft* RNAi tibiae (n=10), the relative ratio of *Gb'ft* mRNA at 2 dpa was reduced (0.40±0.06), whereas that of *Gb'ds* was slightly increased (1.38±0.31). Similarly, in regenerating *Gb'ds* RNAi tibiae (n=10), the ratio of *Gb'ds* mRNA was reduced (0.37±0.12), whereas that of *Gb'ft* was slightly increased (1.28±0.36). In regenerating *Gb'fj* RNAi tibiae (n=3), expression of *Gb'fj* became undetectable by whole-mount in situ hybridization (data not shown). These results suggest that the observed effect of rdRNAi was genuine and specific to each target gene.

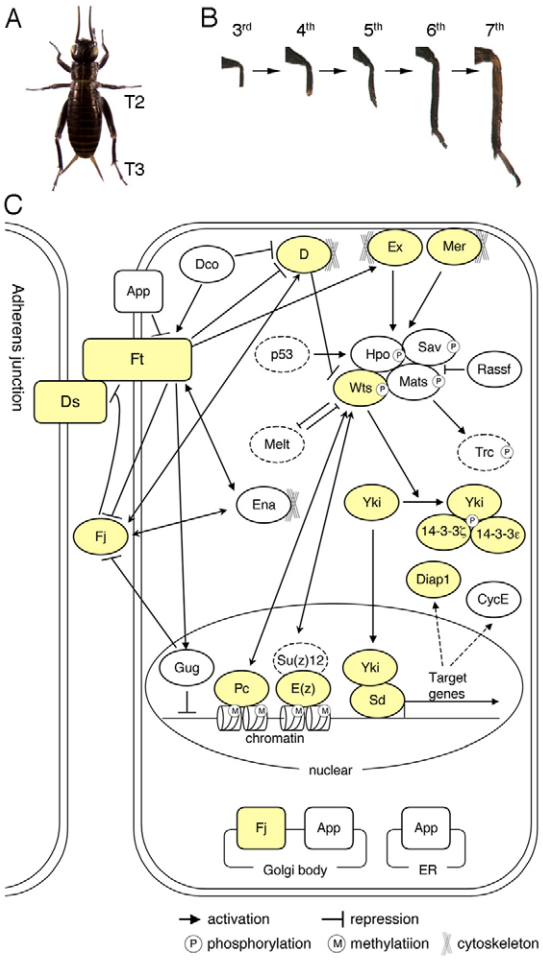

Fig. 1. Regenerating legs of *Gryllus bimaculatus* and the *Drosophila* Ds/Ft signalling pathway. (**A**) Cricket nymph at third instar. Mesothoracic and metathoracic legs are indicated by T2 and T3, respectively. (**B**) Typical processes of cricket leg regeneration. (**C**) Subcellular localization of the Ds/Ft signalling factors and their network in *Drosophila*. The *Gryllus* homologues we cloned are indicated by unbroken lines, with others shown as dashed lines. Yellow indicates factors with RNAi phenotypes that were observed during *Gryllus* leg regeneration.

Gryllus genes encoding Ds/Ft signalling factors are expressed in leg buds and regenerating legs

To observe the expression pattern of *Gb'ft*, *Gb'ds*, *Gb'fj* and *Gb'd* during leg development and regeneration, we performed whole-mount in situ hybridization. *Gb'ft* and *Gb'ds* were intensely expressed in limb buds at early stages (Fig. 2A,B), whereas the expression of *Gb'fj* or *Gb'd* was weak (Fig. 2B; see Fig. S3 in the supplementary material). At later stages, the expression of *Gb'ft* was localized to the proximal region of each leg segment, where it

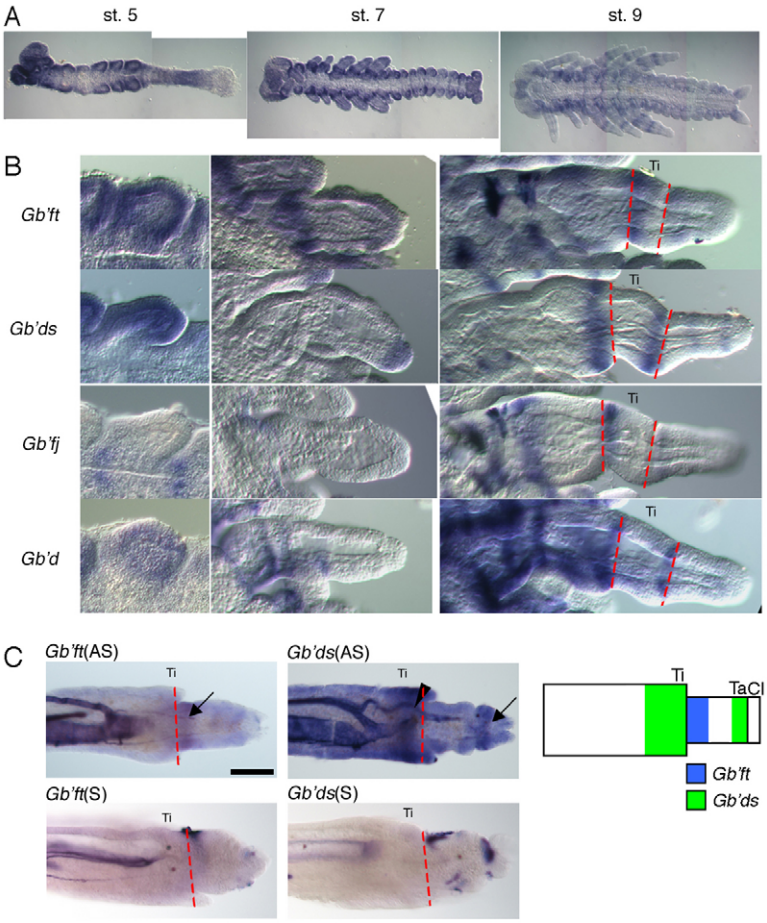

Fig. 2. Expression pattern of *Gb'ft*, *Gb'ds*, *Gb'fj* and *Gb'd* in embryos, and of *Gb'ft* and *Gb'ds* in regenerating legs 5 days after amputation. (**A**) Expression patterns of *Gb'ft* in embryo at stages 5, 7 and 9, obtained by whole-mount in situ hybridization (WISH). *Gb'ft* was expressed in the distal region of the prothoracic limb, the head and abdominal regions at stage 5. At stages 7 and 9, *Gb'ft* was expressed in the antenna, limb, cercus and every abdominal segment. (**B**) Expression patterns of *Gb'ft*, *Gb'ds*, *Gb'fj* and *Gb'd* in developing limbs at stages 5, 7 and 9, obtained by WISH. *Gb'ft* was expressed in the distal region of the limb bud at stage 5, in the proximal region at stage 7 and thereafter in each limb segment with a PD gradient. *Gb'ds* was expressed in the distal region of the limb bud at stage 5, the most distal portion of the limb at stage 7, and in the distal region of each limb segment at stage 9. *Gb'fj* was expressed in the proximal region of each limb segment between stages 7 and 9. *Gb'd* was expressed in the distal region of each limb segment between stages 5 and 9. The red dotted lines indicate segment boundaries. (**C**) Expression pattern of *Gb'ft* and *Gb'ds* in regenerating legs at 5 dpa, obtained by WISH. The red dotted lines indicate the segment boundary between the tibial and tarsal primordia. An arrow indicates the proximal expression of *Gb'ft*. An arrowhead and arrow indicate distal expression of *Gb'ds* in the tibial and tarsal primordia, respectively. As negative controls, we used the corresponding sense RNA probes (bottom). In controls, no significant signal was detected. The observed areas of intense staining represent nonspecific binding of dyes to small cuticular structures and tracheal tubes. (Right) Schematic of the *Gb'ft* and *Gb'ds* expression patterns. Scale bar, 100 μm. Cl, claw; Ta, tarsus; Ti, tibia.

showed a PD gradient (Fig. 2B), whereas *Gb'ds* and *Gb'd* were expressed in the distal region of each segment (Nakamura et al., 2008a). Expression of *Gb'fj* was observed in the proximal region of each segment (Fig. 2B). To determine whether *Gb'ft* or *Gb'ds* are expressed during regeneration, we performed whole-mount in situ hybridization with regenerating legs in which the cuticle had been removed. We observed that *Gb'ft* was expressed in the proximal region of the tarsus primordium at 5 dpa (Fig. 2C), represented by blue in the illustration (Fig. 2C, right), whereas expression of *Gb'ds* was observed in distal regions of the regenerating tibia and tarsus primordia (represented by green, Fig. 2C, right). No significant signal was observed in negative controls (Fig. 2C). The expression pattern of *Gb'ft* and *Gb'ds* in regenerating legs resembled that normally observed in late-stage limb buds (Fig. 2B), suggesting that these patterns are similar in each leg segment. However, *Gb'ex* was expressed in the proximal region of each segment, whereas expression of *Gb'Mer* was ubiquitous in leg buds (data not shown). Based on their expression patterns, we concluded that these genes are involved in pattern formation of the limb during development and regeneration.

The Ds/Ft signalling pathway participates in the control of leg segment size and shape

We observed that the length of regenerated adult tibiae changed in nymphs treated with RNAi against *Gb'd*, *Gb'ds*, *Gb'ex*, *Gb'ft* and *Gb'Mer* (see Fig. S1 in the supplementary material). Because *Gb'ft* and *Gb'ds* had shown different expression patterns in the tibia, we examined whether the short phenotype depended on location of the amputation. When the *Gb'ft* or *Gb'ds* rdRNAi tibia was amputated proximally, we found that the regenerated adult legs became very short and thick, and contained distal structures, including spines and spurs on the tibiae and three joints of the tarsi and claws, whereas the contralateral legs appeared normal. A typical adult with a 'short and thick' regenerate obtained by rdRNAi against *Gb'ds* is shown in Fig. 3B, alongside a control adult (Fig. 3A) for comparison. As the legs of *Gb'ds* rdRNAi crickets (Fig. 3D) resembled those of controls (Fig. 3C), but on a reduced scale, the pre-existing host stump of amputated tibiae may be repatterned as a whole in nymphs treated with RNAi against *Gb'ft*, *Gb'ds* or *Gb'd*. However, the thickening might be due to a PCP defect, because Ft and Ds are known as regulators of PCP (for a review, see Lawrence et al., 2008). As we observed no defect in leg surface structures (see Fig. S4 in the supplementary material), if it is the case, it must be uncoupled from the PCP of the surface structures.

We next determined effects of the location of amputation on regenerated tibial phenotypes at the sixth instar after injection of target-gene dsRNA at the third instar (Fig. 3E). In control regenerations, the missing portion of the leg was restored regardless of the position of amputation. In the *Gb'ft* RNAi nymphs, we found the length of the regenerating tibia to depend linearly on the amputated position. When tibiae were amputated at proximal (30%), middle (50%), or distal (70%) positions, they continued to grow and formed short and thick adult legs. The length of the regenerated tibiae became 40±2% (n=8) of that of the control tibia for proximal amputation, 52±6% (n=6) for middle amputation and 74±14% (n=4) for distal amputation. Thus, tibiae with RNAi against *Gb'ft* were shortened to a degree that was in proportion to the position of amputation (Fig. 3E). In *Gb'ds* or *Gb'd* RNAi nymphs, the length of

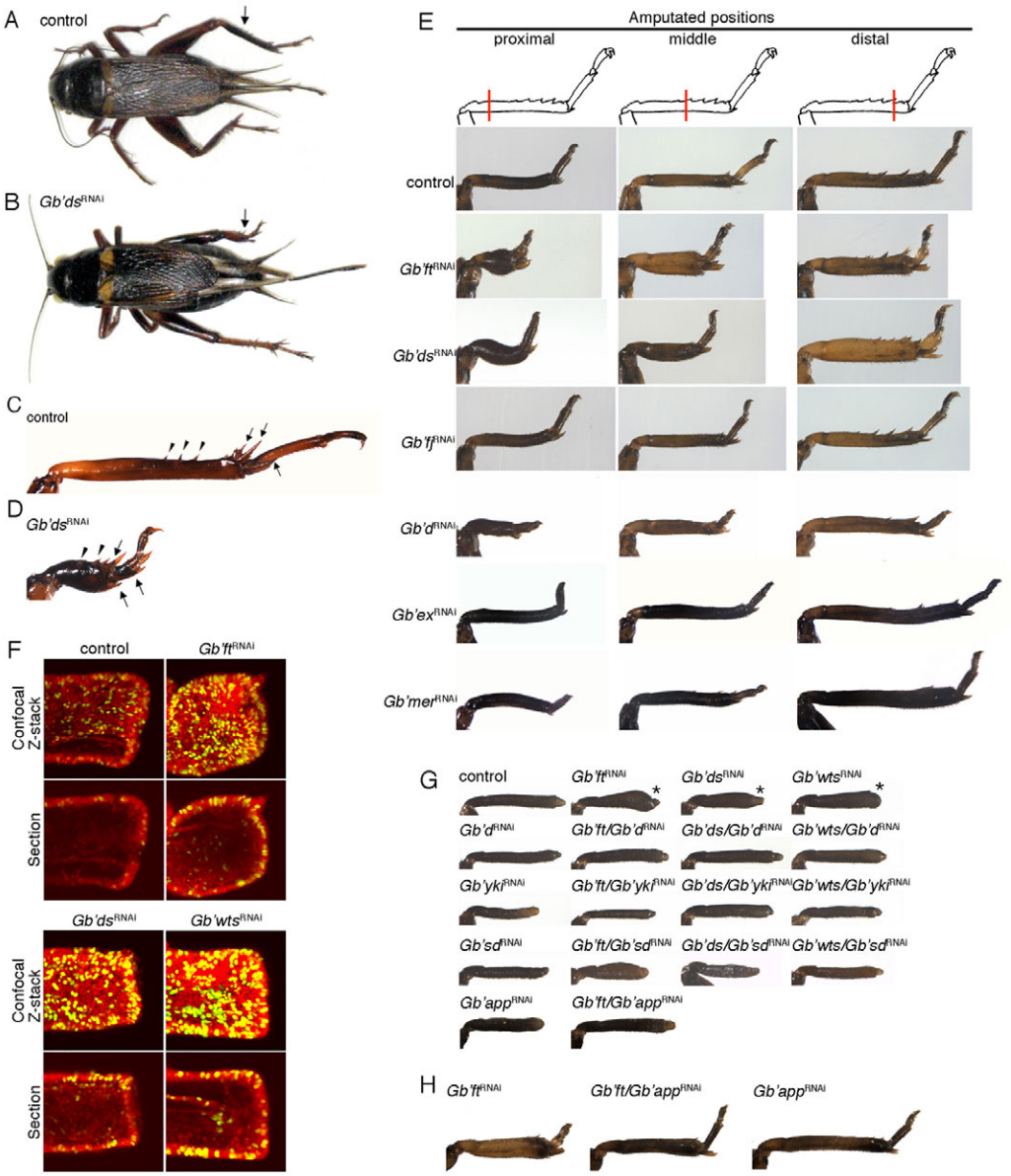

Fig. 3. Effect of rdRNAi against *Gb'ft*, *Gb'ds*, *Gb'fj*, *Gb'd*, *Gb'ex* and *Gb'Mer* on leg size, proliferation of regenerating cells, and distal enlargement phenotypes. (**A**) A control adult cricket with a normally regenerated leg. An arrow indicates the site of amputation position. (**B**) A *Gb'ds* RNAi adult with a short regenerated T3 leg (middle amputated position). Its short leg is shown in D. (**C**,**D**) Control and *Gb'ds* rdRNAi legs at higher magnification. Arrows indicate spurs of the tibia and tarsus, and arrowheads indicate tibial spines. (**E**) Effects of rdRNAi on the size of regenerating legs at sixth instar in control, *Gb'ft*, *Gb'ds*, *Gb'fj*, *Gb'd*, *Gb'ex* and *Gb'Mer* RNAi nymphs. These regenerating legs were amputated at distal, middle or proximal positions at the third instar. Sites of amputation are indicated by red lines (top). (**F**) Effect of rdRNAi on the proliferation of regenerating cells. PI staining (red) and EdU incorporation (green) of regenerating cells in control, *Gb'ft*, or *Gb'ds* RNAi nymphs at 2 dpa. Merged signals appear yellow. The upper and lower panels show confocal *z*-stack images and sections of the distal regions, respectively. Distal is to the right. (**G**) Effect of single or dual RNAi on distal enlargement phenotypes at the fourth instar. Asterisks indicate the enlargement phenotype. (**H**) Effects of single or dual RNAi against *Gb'ft* and *Gb'app*, on regeneration of adult legs.

the regenerated tibiae was also shorter in correspondence with the amputated positions (Fig. 3E). However, no significant change was observed in the length of the tibia of *Gb'fj* RNAi nymphs (Fig. 3E). These results imply that *Gb'ft*, *Gb'ds* and *Gb'd* appear to regulate the size and shape of the leg segments during regeneration.

In contrast to *Gb'ft* and *Gb'ds*, in the *Gb'ex* RNAi nymphs, regenerated tibiae became longer by 25±4% (n=5) than normally regenerated tibiae after distal amputation (Fig. 3E). A similar phenotype was observed even in proximal amputation in the *Gb'ex* RNAi nymphs (Fig. 3E). In *Gb'Mer* RNAi nymphs, the average length of regenerated tibiae exceeded that of normally regenerated tibiae by 22±7% (n=8) after distal amputation (Fig. 3E). This phenotype was not observed in proximal or middle amputations (Fig. 3E), which may indicate redundancy between *Gb'Mer* and *Gb'ex*. These results suggested that *Gb'ex*/*Gb'Mer* transcripts can influence determination of leg size by inhibiting over-proliferation along the PD axis during regeneration (see Fig. S5 in the supplementary material).

Proliferation of regenerating leg cells is regulated through the Ds/Ft/Wts pathway

The short and thick phenotypes of legs treated with rdRNAi against *Gb'ft* or *Gb'ds* appear to be due to abnormal regulation of blastemal cell proliferation. Thus, we next examined the effect of *Gb'ft* or *Gb'ds* rdRNAi on the proliferation of blastemal cells at 2 dpa in normal regeneration, wherein blastemal cells begin to rapidly proliferate so as to restore the lost portion of the leg. We performed EdU incorporation assays (Salic and Mitchison, 2008) in *Gb'ft*, *Gb'ds* and *Gb'warts* (*wts*) RNAi nymphs. As shown in Fig. 3F, EdU-positive cells were localized to the epithelial cell layer underlying the wound surface. At 2 dpa, the number of positive cells in distal longitudinal sections of *Gb'ft*, *Gb'ds* and *Gb'wts* RNAi regenerating legs was increased in comparison with the corresponding control legs (Fig. 3F). When calculated as the ratio of the number of positive cells to the total number of cells in the distal longitudinal section (average length 0.22±0.01 mm and width 0.18±0.01 mm, n=18) the value was 0.22±0.03 (n=5) for control legs, 0.50±0.11 (n=4) for *Gb'ft*-RNAi, 0.48±0.19 (n=4) for *Gb'ds*-RNAi and 0.47±0.18 (n=5) for *Gb'wts*-RNAi nymphs. These data indicate that the proliferation of blastemal cells was enhanced in *Gb'ft*, *Gb'ds* or *Gb'wts* rdRNAi nymphs.

To examine epistasis in the Ds/Ft signalling cascades, we analyzed the phenotypes of dual RNAi knockdown of related genes, accomplished by simultaneous injection of two different dsRNAs. The enlarged phenotype of the *Gb'ft* or *Gb'ds* RNAi nymph was suppressed by dual rdRNAi against *Gb'd* (Fig. 3G), consistent with a previous epistatic test in *Drosophila* (Cho and Irvine, 2004; Cho et al., 2006). The enlarged phenotype of *Gb'wts* RNAi nymphs was also suppressed by dual RNAi against *Gb'd*, which implies that *Gb*'D functions downstream of *Gb*'Wts in leg regeneration (see Discussion). By contrast, we did not observe any phenotype in the *Gb'hippo* (*hpo*), *Gb'salvador* (*sav*) or *Gb'Mob as tumor suppressor* (*mats*) RNAi nymphs, or even in triple knockdown nymphs, although relative amount of *Gb'hpo* transcript in *Gb'hpo* RNAi tibiae was reduced to be 0.28±0.04 (n=10) (see Fig. S2E in the supplementary material). Both 'distal enlarged' and 'short and thick' phenotypes of *Gb'ft* rdRNAi nymphs were suppressed by dual RNAi with *Gb'approximated* (*app*) (Fig. 3G,H), consistent with a previous epistatic test in *Drosophila* (Matakatsu and Blair, 2008). The enlargement phenotypes of *Gb'ft*, *Gb'ds* and *Gb'wts* RNAi nymphs were suppressed dual RNAi against *Gb'yorkie* (*yki*) and weakly by dual RNAi against *Gb'scalloped* (*sd*) (Fig. 3G), which indicates that a transcriptional complex of *Gb'*Yki and *Gb'*Sd functions downstream. These results suggested that the proliferation of regenerating cells is regulated by the Wts pathway within the Ds/Ft signalling pathway.

The Ds/Ft signalling pathway is involved in intercalary regeneration

Classical experiments using insect legs have demonstrated that, when two leg stumps with disparate positional values are placed next to one another, intercalary regeneration occurs to restore the missing positional values (Fig. 4A) (Bohn, 1965; French et al., 1976). In intercalary regeneration, it is known that most of the regenerated cells are derived from the donor or host with the more distal positional identity (Fig. 4A) (Bohn, 1976; French et al., 1976), suggesting that regenerating cells inhibit the proliferation of more proximal cells at the junction, a phenomenon known as 'distal preponderance' (Brockes and Kumar, 2008). The extent and origin of a regenerated portion can be determined by observation of its surface morphology (Fig. 4A). In the case of reverse intercalation, the PD polarity of regenerates can be determined by the orientation of bristles.

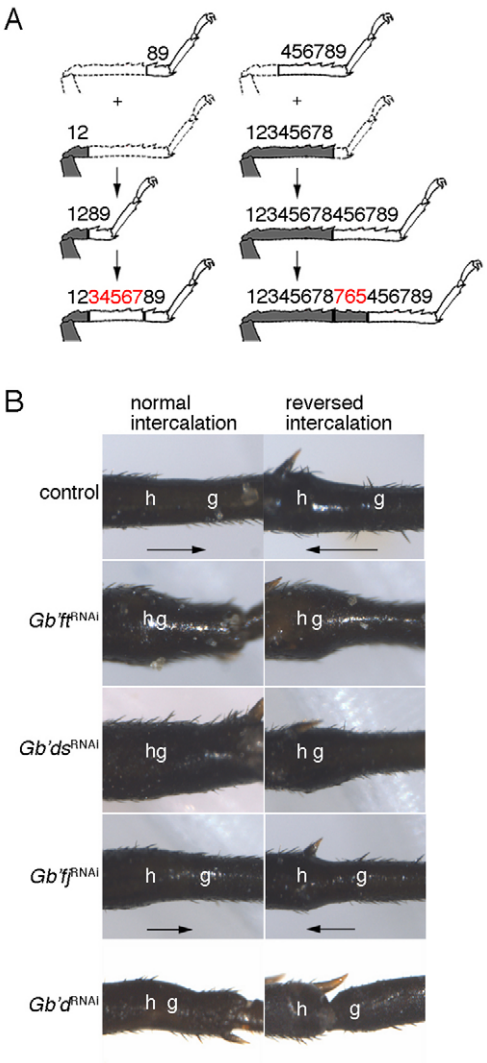

Fig. 4. Effect of rdRNAi against *Gb'ft*, *Gb'ds*, *Gb'fj* or *Gb'd* on intercalary regeneration. (**A**) Schematic of transplantations for intercalary regeneration. The positional values are denoted arbitrarily by the numbers 1 to 9. Normal intercalary experiment (left), includes transplantation of a distal graft (89) to a proximal host (12), resulting in intercalation of the missing elements (3-7). Reverse intercalary experiment (right), entails transplantation of a proximal graft (4-9) to a distal host (1-8), resulting in intercalation of extra elements (765). (**B**) Effect of rdRNAi on intercalary regeneration. Normal (left) and reverse (right) intercalation in control, *Gb'ft* RNAi, *Gb'ds* RNAi, *Gb'fj* RNAi and *Gb'd* RNAi nymphs at the fifth instar. Orientation of surface bristles is indicated by leftward arrows (reverse orientation) and rightward arrows (normal orientation). g, distal graft; h, host stump.

To further explore the role of Ds/Ft signalling factors in intercalary regeneration, we carried out transplantation experiments by grafting an amputated T2 piece to a T3 host in the same animal. In the *Gb'ft* or *Gb'ds* RNAi nymphs, although host-graft jointed regions enlarged, neither normal nor reverse intercalary regeneration to restore the missing region was observed (Fig. 4B). Intercalary regeneration was similarly absent in *Gb'd* RNAi nymphs. Instead, their host-graft jointed regions became thinner than normal, reminiscent of the phenotype of reverse intercalation observed in *Gb'arm* RNAi nymphs (Nakamura et al., 2007). In *Gb'fj* RNAi nymphs, intercalary regeneration occurred normally in

both grafting experiments. These results indicate that *Gb'ft*, *Gb'ds* and *Gb'd* are essential for intercalary regeneration. In the *Gb'ex* or *Gb'Mer* rdRNAi nymphs, both normal and reverse intercalary regeneration took place (see Fig. S6A in the supplementary material), but regenerated cells were derived from both distal and proximal pieces shown by EdU incorporation assay (see Fig. S6B in the supplementary material). These results support the possibility that *Gb'ex* and *Gb'Mer* are involved in the directional contact-dependent inhibition of proliferation leading to a proximal re-specification.

To examine whether circumferential positional information is affected in RNAi nymphs, we performed an additional transplantation experiment to induce supernumerary legs (Mito et al., 2002) (see Fig. S7A in the supplementary material). Supernumerary legs were formed in *Gb'ft*, *Gb'ds*, *Gb'd* and *Gb'fj* RNAi nymphs after three moults subsequent to the transplantation experiment (see Fig. S7B in the supplementary material). These results suggested that circumferential positional information was normal in these RNAi nymphs, and that their regenerating cells retained the ability to proliferate. We also examined the effect of rdRNAi against *Gb'ex* and *Gb'Mer* on the formation of supernumerary legs. We observed normal supernumerary legs in *Gb'ex* RNAi nymphs, but not in *Gb'Mer* RNAi nymphs (see Fig. S7B in the supplementary material), suggesting that *Gb'Mer*, but not *Gb'ex*, may be involved in the regulation of proliferation along the circumferential axis.

Knockdown of the Ds/Ft signalling factors induces re-specification of positional values in amputated legs

In the short *Gb'ft*, *Gb'ds* and *Gb'd* RNAi legs, although their tarsi, tarsal claws and decorative structures were small, they were essentially restored (Fig. 3B,D,E). This may indicate that cells in the distal end of the short tibia possess the most distal positional identity, possibly due to a re-specification of positional value. To test this possibility, we performed transplantation experiments (Fig. 5A). If re-specification would occur in the leg of the *Gb'ft* RNAi nymphs, so as to allow the acquisition of the missing distal positional values in the shortened tibia (Fig. 5A), then it would be expected that, when normal mesothoracic tibiae were amputated proximally and grafted to distally amputated regenerating metathoracic tibial host at the sixth instar, reverse intercalary regeneration would be observed. It should be noted that RNAi is no longer effective in a regenerating host leg of *Gb'ft* RNAi nymph at the sixth instar, when injected with dsRNA at the third instar (see Fig. S2A in the supplementary material). We observed reversed orientation of the surface bristles in the regenerates (Fig. 5B), indicating that a reversed intercalation took place at the graft-host junction. Thus, we concluded that the positional values of the tibial stump cells were re-specified in the short rdRNAi tibia during moulting.

DISCUSSION

Using an RNAi knockdown approach against 23 candidate genes, we identified 15 components of the Ds/Ft signalling pathway that are involved in cricket leg regeneration (Fig. 1C; Table 1). Based on additional data from *Gryllus* and *Drosophila* (Reddy and Irvine, 2008), we propose a model signalling cascade for the regulation of leg regeneration by the Ds/Ft signalling pathway (Fig. 6). As the main components of the Ds/Ft signalling pathways are conserved in vertebrates (Reddy and Irvine, 2008), this signalling cascade may also be involved in vertebrate leg regeneration. In the remainder of this section we will discuss how the Ds/Ft signalling pathway might be involved in leg regeneration.

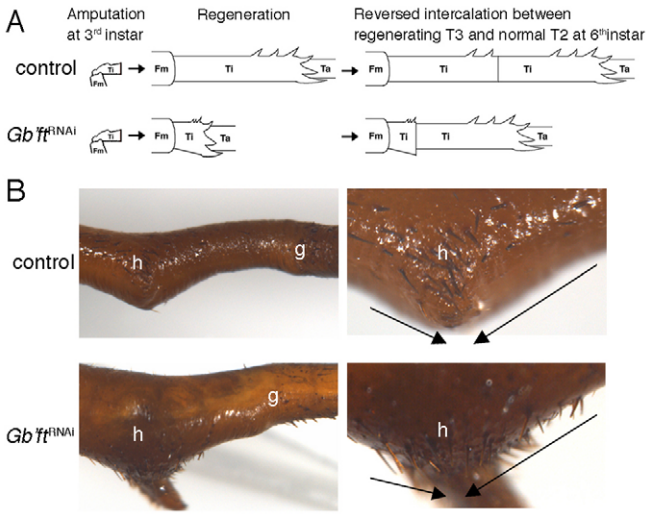

Fig. 5. Schematic of leg transplantation experiments to show re-specification of lost distal positional values in the short tibial stump of the *Gb'ft* RNAi regenerated leg and the experimental results. (**A**) Schematic of leg transplantation experiments. (Top) In the control transplantation, a proximally amputated normal graft is transplanted at the sixth instar to a distally amputated regenerate as a host, obtained by proximal amputation at the third instar. As the lost distal positional values have been restored in the host regenerate, a reverse intercalary regeneration was observed as shown in B. (Bottom) Transplantation of a proximally amputated normal graft to a distally amputated *Gb'ft* rdRNAi short tibia (obtained by proximal amputation at the third instar) as a host at the sixth instar (in which rdRNAi is no longer effective). If the lost distal positional values were restored even in the short tibia, a reverse intercalary regeneration should be observed, as observed in the control. (**B**) Experimental results. Reversed intercalation was observed in both control and *Gb'ft* RNAi short tibia, indicating that restoration of positional values takes place in the stump. In higher magnification images (right column), reverse-oriented bristles (leftward arrows; normal orientation, rightward arrows) were observed in both regenerates. g, normal graft; h, regenerated stump after proximal amputation at the third instar.

The Ds/Ft signalling pathway is involved in leg regeneration

The most typical phenotypes in the present data were the short and thick legs induced by rdRNAi against *Gb'ft* or *Gb'ds* (Fig. 3C,D,E). It is known that the size of each leg segment normally scales with overall body size, a phenomenon known as allometry (Shingleton et al., 2007). Surprisingly, the size of the regenerated legs in the phenotypes we observed did not scale with overall body size (Fig. 3B). Furthermore, the size of the regenerated legs depended upon the site of tibial amputation (Fig. 3D,E). It is noteworthy that, although the expression patterns of *Gb'ft* and *Gb'ds* were different (Fig. 2C), their short and thick phenotypes were similar. This is consistent with the fact that *Drosophila* mutant phenotypes of both *ft* and *ds* in adult legs are short and thick, despite the fact that *ft* and *ds* have distinct expression patterns in *Drosophila* imaginal discs (Garoia et al., 2000; Ma et al., 2003). Thus, we conclude that the activity of Ds/Ft signalling regulates leg segment size and shape during regeneration. Furthermore, we showed that the Ds/Ft signalling pathway may regulate leg size during regeneration through the Hpo signalling pathway (Fig. 6A). This is also supported by the fact that the Hpo signalling pathway is involved in an intrinsic mechanism that restricts organ size (Edgar, 2006; Dong et al., 2007;

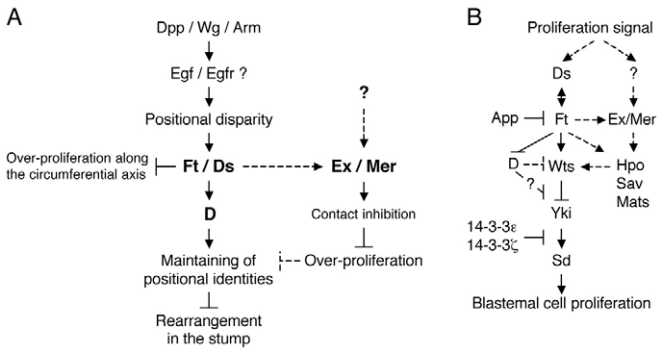

Fig. 6. A plausible Ds/Ft signalling pathway model for leg regeneration. (**A**) After amputation, a ligand (Egf) of Egfr may be induced by Dpp/Wg via Arm in the blastemal cells, establishing the most distal positional value at the amputated surface (Mito et al., 2002; Nakamura et al., 2008b). The positional disparity is linked to regulation of cellular proliferation through the Ds/Ft. D mediates the signal for regeneration along the PD axis, probably through the Hpo/Wts pathway (Fig. 1C; Table 1). The Ds/Ft also regulates proliferation along the circumferential axis. The Ex/Mer is involved in contact-dependent inhibition of proliferation in the stump. Signalling factors that may activate the Ex/Mer are unidentified. (**B**) A plausible genetic cascade of Ds/Ft signalling factors for proliferation of leg blastemal cells. Blastemal cell proliferation is regulated by the activity of Ds/Ft through App and Hpo/Wts signalling factors, as revealed by dual rdRNAi. Dotted lines indicate potential interactions derived from the *Drosophila* data (Reddy and Irvine, 2008).

Pan, 2007; Yin and Pan, 2007; Lawrence et al., 2008) and that the Ds/Ft signalling system defines a cell-to-cell signalling mechanism that regulates the Hpo pathway, thereby contributing to the control of organ size (Cho et al., 2006; Rogulja et al., 2008; Willecke et al., 2008).

Meinhardt pointed out that two processes operate during leg regeneration. One, which operates during the restoration of distal structures, is instructed by a morphogen epidermal growth factor (Egf), which is itself induced by two morphogens, Dpp and Wg, at the amputated surface (Meinhardt, 1982; Mito et al., 2002; Nakamura et al., 2008a). The other, operating in intercalary regeneration, is directly controlled by neighbouring cells at the junction between host and graft, but not by long-range morphogens (Meinhardt, 2007; Nakamura et al., 2008a). It is likely that the Ds/Ft signalling pathway participates in both mechanisms, because rdRNAi against *Gb'ft* or *Gb'ds* affected leg regeneration after either distal amputation or intercalary transplantation. In the case of distal amputation, as the *Gryllus* tarsi and claws were not restored after tibial amputation in the *Gb'Egfr* rdRNAi nymphs (Nakamura et al., 2008a), we have speculated that *Gb'*Egf functions as a morphogen in the leg regeneration, as found in *Drosophila* leg imaginal discs (Campbell, 2002; Galindo et al., 2002). Recently, Rogulja et al. (Rogulja et al., 2008) demonstrated in the *Drosophila* wing disc that the Fat signalling pathway links the morphogen-mediated establishment of gradients of positional values across developing organs to the regulation of organ growth. Thus, we speculate that the Ds/Ft system links the Egf-mediated establishment of gradients of positional values across regenerating blastemal cells to the regulation of regenerate growth.

As *Gb'd* rdRNAi legs exhibited the short-leg phenotypes, but not thick ones (Fig. 3E), and *Gb'd* is epistatic of *Gb'ft* and *Gb'ds*, *Gb*'D may mediate the components of Ds/Ft signalling controlling leg size (Fig. 6A). The enlarged phenotype of *Gb'wts* RNAi nymphs was suppressed by RNAi against *Gb'd* in *Gryllus*, indicating that *Gb'd* is in the downstream of *Gb'wts* (Fig. 6B). This result differs from *Drosophila* data (broken line in Fig. 6B) (Cho et al., 2006). A genetic analysis is necessary to confirm the difference, because the epistatic allele is not null in RNAi experiments. As the phenotype of rdRNAi treatment against *Gb'ds* was weaker than that against *Gb'ft*, as reported in the corresponding *Drosophila* mutants (Matakatsu and Blair, 2006), *Gb*'Ft may interact with factors that are as yet unidentified. Although the effect of rdRNAi against *Gb'fj* on leg size was very mild, we cannot exclude the possible involvement of *Gb'fj* in allometric growth. The short phenotypes were observed in the *Gb'sd* RNAi nymphal legs (see Fig. S1 in the supplementary material), so it remains a possibility that *Gb'sd* is involved in allometric leg growth (Fig. 6A). However, the apparent contribution of the Hpo-Sav-Mats complex is as yet uncertain (indicated by a broken line in Fig. 6B).

We demonstrated that regenerated legs of *Gb'ex* and *Gb'Mer* RNAi adults become longer than normal control legs (Fig. 3E; see Fig. S1 in the supplementary material), and that *Gb'ex* and *Gb'Mer* regulate cell proliferation induced by the presence of positional disparity (see Fig. S6A,B in the supplementary material). These results suggest that *Gb'ex* and *Gb'Mer* are also involved in allometric growth of the leg segment (Fig. 6A). In *Drosophila*, it was reported that Ex and Mer negatively regulate cell growth and proliferation through the Hpo/Wts pathway (Hamaratoglu et al., 2006; Pellock et al., 2007). In mammalian cells, Nf2 (merlin) is known to be a crucial regulator of contact-dependent inhibition of proliferation (Curto and McClatchey, 2008). Thus, we conclude that activities of Ex and Mer may regulate contact-dependent inhibition of proliferation via the Wts signalling pathway to restore the proper leg segment size during regeneration (Fig. 6A).

The Ds/Ft signalling pathway links positional and allometric information to determine regenerated leg size and shape: interpretation according to the Ds/Ft steepness model

A widely accepted model for leg regeneration is the intercalation model, based on positional information (Wolpert, 1969; Wolpert, 1994; Nye et al., 2003; Brockes and Kumar, 2008). This model is based on the intercalation of new structures so as to re-establish continuity of positional values during regeneration. However, on the basis of this model, it is difficult to explain the changes in leg size that were observed in the present study. Thus, we need to extend it to include the control of growth and tissue size during regeneration. Several models have been proposed to explain how organ size is regulated (Garcia-Bellido and Garcia-Bellido, 1998; Day and Lawrence, 2000; Lawrence, 2004). Recently, Lawrence, Struhl and Casal (Lawrence et al., 2008) proposed a model, which we refer to here as the Ds/Ft steepness model, to explain the mechanisms underlying the determination of organ size and PCP, including the Warts/Hippo pathway as the mechanism for controlling growth (Rogulja et al., 2008; Willecke et al., 2008) in the previous model (Casal et al., 2002; Lawrence et al., 2004; Casal et al., 2006). In their model, they hypothesized that: (1) the morphogens responsible for the overall pattern of an organ establish and orient the Ds/Ft system, which then forms a linear Ds/Ft gradient. The nature of the Ds/Ft gradient is unknown, although the number of Ds/Ft trans heterodimers is the key variable; (2) the steepness of the Ds/Ft gradient regulates Hpo target expression and cell proliferation, and its direction provides information used to establish the correct cellular polarity; (3) growth would be expected to cease when the

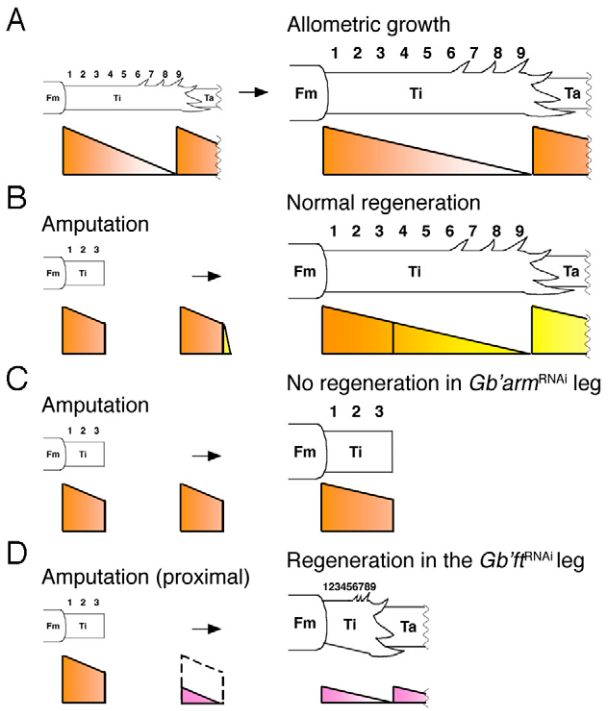

Fig. 7. Schematics of the Ds/Ft steepness model for leg regeneration. (**A**) Normal allometric growth: the positional values (PVs) are denoted arbitrarily by the numbers 1 to 9, whereas the scalar values of the Ds/Ft gradient are indicated by the orange shading in the Ds/Ft steepness model (Lawrence et al., 2008). The scalar value of the Ds/Ft gradient is minimum at the most distal value, PV=9. The steepness of the gradient at each point, measured as a differential across each cell, correlates with the size along the PD axis in the leg. Growth would be predicted to cease when the slope of the gradient fell below a certain threshold value. (**B**) Normal regeneration: after amputation at PV=3 in the tibia (left side, the tibial stump is indicated by orange), blastemal cells detect positional disparity (PVs, 3/9) through the Ds/Ft signalling pathway, and then a steeply sloped Ds/Ft gradient is formed, which leads to intercalary growth until the re-establishment of positional continuity (yellow, PV=4-8), as epimorphic-like regeneration. The pre-existing stump (orange) grows allometrically, retaining the original positional and allometric information (PV=1-3). (**C**) No regeneration: the pre-existing stump grows without restoring the missing portion, having the original positional and allometric information (PVs=1-3). The phenotype was observed in the *Gb'arm* rdRNAi leg (Nakamura et al., 2007). (**D**) Morphallaxis-like regeneration in *Gb'ft*, *Gb'ds* or *Gb'd* rdRNAi nymphs after proximal amputation. No epimorphic-like regeneration takes place by suppression of proliferation of blastemal cells along the PD axis, although remodelling of the stump takes place as morphallaxis-like regeneration. The positional values are re-established in relation to the new tibia-tarsus boundary, in which information about the ultimate tibial size (allometric information) is lost. The normal Ds/Ft gradient, indicated by a dotted line, would shift down with the same slope so as to reset the positional value of the amputated surface to the most distal positional value, or the minimum scalar value of the Ds/Ft gradient. The short leg size induced by rdRNAi against *Gb'ft* is well interpreted with this model (continues in Fig. S8 in the supplementary material).

slope of the gradient declines below a certain threshold value; and (4) the maximum and minimum limits of the system are conserved, while recently divided cells take up intermediate scalar values from their neighbours.

Using their model, we propose a modified Ds/Ft steepness model for leg regeneration acting as follows. Our results indicate that nymphal leg regeneration depends on two major processes (Fig. 7B): proliferation and differentiation of blastemal cells (yellow in Fig. 7B) and growth of the pre-existing stump (orange in Fig. 7B). In each of these processes, new positional identities are specified in relation to new segment boundaries. According to the Ds/Ft steepness model, in normal regeneration, a very steep gradient should be formed in the regenerating blastema (Fig. 7B). The regenerate may grow so as to restore the normal pre-existing steepness. Reassignment of positional identities after amputation will correlate with a similar re-setting of the minimum Ds/Ft scalar value, and the results are consistent with the steepness hypothesis.

Growth of the pre-existing stump is a normal component of leg growth, in which the pre-existing stump cells proliferate according to some allometric signals, which may be related to the maximum scalar value and a slope of the gradient, keeping their original positional information. This was observed in the truncated leg of *Gb'arm* rdRNAi adults (Nakamura et al., 2007) (Fig. 7C).

In the absence of the proliferation and differentiation of blastemal cells, as observed in the *Gb'ft* rdRNAi leg, the minimum scalar value, which is the most distal positional value, would be established at the site of amputation, and the Ds/Ft gradient would be expected, in turn, to shift down with the same slope as the pre-existing one (pink, Fig. 7D). The Ds/Ft steepness model provides an explanation for the observation that the final leg size depends on the amputated position, if we assume that the gradient shifts down with the same slope as that where cells at an amputated position have the minimum scalar value (pink, Fig. 7D; see Fig. S8 in the supplementary material). Thus, the observed re-specification of regeneration legs induced in the legs treated with rdRNAi against *Gb'ft* or *Gb'ds* is as would be predicted by the Ds/Ft steepness model. Thus, it is likely that the Ds/Ft gradient functions to link positional and allometric information to the regulation of leg segment growth. Furthermore, if we assume that the activity of Ex/Mer is related to a threshold value of the slope of the gradient that determines when growth ceases (see Fig. S8 in the supplementary material), we can interpret all rdRNAi phenotypes in the present study consistently with the Ds/Ft steepness model for regeneration (Fig. 7; see Fig. S8 in the supplementary material).

Recently, da Silva et al. (da Silva et al., 2002) found that, in newt leg regeneration, a cell surface protein with a glycosylphosphatidylinositol anchor, Prod 1, is implicated in the local cell-to-cell interactions mediating PD positional identity (Brockes and Kumar, 2008). As the Ds/Ft signalling pathway is conserved in vertebrates (Reddy and Irvine, 2008), the pathway should be involved in vertebrate leg regeneration, probably interacting with the Prod 1 system.

We thank K. D. Irvine, H. Meinhardt, P. A. Lawrence and two anonymous reviewers for critical comments and valuable suggestions, and Y. Tanaka for technical advices. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan to T.M., H.O., and S.N. and by a grant from the Knowledge Clusters Initiative Projects to T.B., H.O. and S.N.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/13/2235/DC1

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