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An extended steepness model for leg-size determination based on Dachsous/Fat trans-dimer system

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What determines organ size has been a long-standing biological question. Lawrence et al. (2008) proposed the steepness hypothesis suggesting that the protocadherin Dachsous/Fat (Ds/Ft) system may provide some measure of dimension to the cells in relation to the gradient. In this paper we extended the model as a means of interpreting experimental results in cricket leg regeneration. We assumed that (1) Ds/Ft trans-heterodimers or trans-homodimers are redistributed during cell division, and (2) growth would cease when a differential of the dimer across each cell decreases to a certain threshold. We applied our model to simulate the results obtained by leg regeneration experiments in a cricket model. The results were qualitatively consistent with the experimental data obtained for cricket legs by RNA interference methodology. Using our extended steepness model, we provided a molecular-based explanation for leg size determination even in intercalary regeneration and for organ size determination.

During animal development, bodies grow to a certain point, following the establishment of a pattern, that defines body size e.g., body height, limb length, etc, and in humans this process can take approximately 20 years. During this growth period, the legs, for example, continue to grow. The observation that the length of the right and left legs is similar after 20 years is intriguing, resulting in the long-standing question: How leg size is determined? To date, no satisfactory explanations have been put forth. In 1970, Lawrence et al.[1] proposed the steepness model that is a model to explain a condition for an organ to stop its growth and fix its size. Certain chemical gradients are assumed to be present in respective organs and these gradients become less steep as the organ grows (Fig. 1a). When the gradient reaches a threshold value it is hypothesized that the organ stops growing. However, this model, for example, reverse intercalary regeneration has not been explained. In 2008, the steepness model was modified as follows[2]: The morphogens responsible for the overall pattern of an organ (such as Decapentaplegic (Dpp)/Bone Morphogenetic Protein (BMP), Hedgehog (Hh) and Wingless (Wg)/Wnt) set up and orient the Dachsous/Fat (Ds/Ft) system, which then provides a linear gradient. In the Ds/Ft steepness model[2], based on the abdominal epidermis of *Drosophila*, they assumed that Ds and Four-jointed (Fj), a Golgi kinase, are expressed in opposing gradients which span the organ and they interact with uniformly expressed Ft molecules to build together a linear gradient of Ds/Ft trans-heterodimers (Fig. 1b). The Ds/Ft system regulates both growth and planar cell polarity (PCP). In the Ds/Ft system, it was assumed that there was a direction to the gradient (the vector) (Fig. 1b) that determines cell polarity, whereas the steepness of the same gradient feeds into the die-or-divide decisions through the Hippo pathway, which is linked to growth[3,4].

On the other hand, Schwank and Basler[5] pointed out that the first evidence for a link between patterning (positional information) and growth came from regeneration studies on cockroach legs[6,7]. This mechanism of intercalary growth, which occurs in various systems, suggested that that pattern formation depending on positional information regulated cell proliferation[5]. In conventional theoretical models[8,9], each cell was assigned a positional value, and the regeneration was explained as recovery of positional-value continuity. The regenerated leg restores almost the same length as the normal leg that was not amputated, indicating that there is a mechanism to detect amputated leg size in the regeneration blastema. They proposed that the Dpp gradient is the "key factor" that controls the final size. However, experiments that have been conducted to date indicated that Dpp is merely one among many growth modulators, and suggests that several other signaling pathways, including the Wg, Hh, Notch, Insulin, and Hippo pathways, also play a role in growth of wing imaginal discs. It is likely that

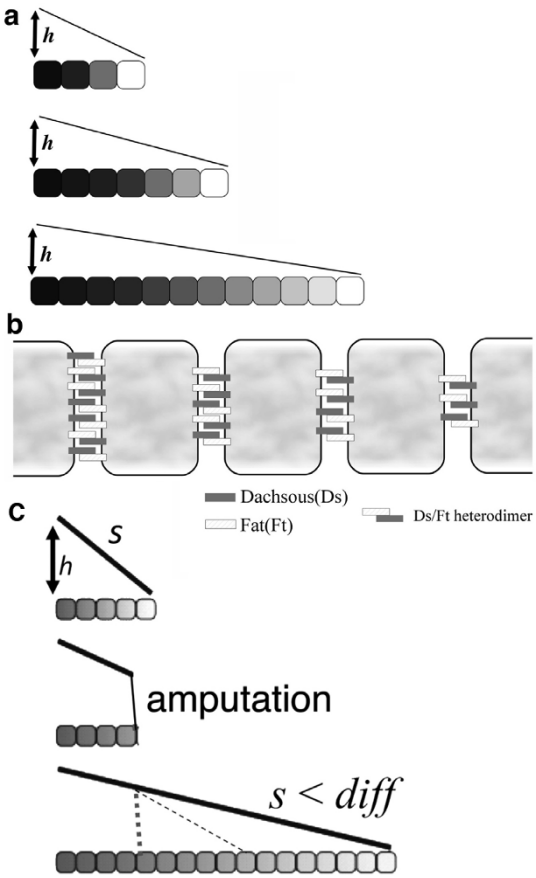

Figure 1 | The steepness hypothesis, the Dachsous/Fat (Ds/Ft) system, and the steepness model for leg regeneration. (a) The steepness hypothesis[2]. The linear gradient of a compound is assumed and depicted by the grayscale shading. The slope of the gradient becomes less steep as the cell chain becomes longer and the cell proliferates until the slope declines to some unknown threshold value. (b) A schematic illustration of the Ds/Ft system. The Ds/Ft heterodimeric bridges are formed by Ds and Ft molecules between cells. The gradient of Ds/Ft heterodimers along the cells is thought to provide cells with a polarity or regeneration cue[27]. In this figure, the amount of Ds/Ft heterodimers decreases from the left to right and this gradient established *steepness* (gradient) in the steepness hypothesis. (c) The steepness model for leg regeneration[13]. The Ds/Ft system might provide a gradient in a leg segment. When a leg is amputated, the steepness of the gradient becomes highest. The missing part is recovered by growth. Growth stops when the slope drops below a certain threshold level, *diff*. Three parameters: *h*, initial height related to final size; *s*, slope related to proliferation of cells; *diff*, threshold value of the slope related to time when growth stops.

they act together with Dpp to regulate the final size of this organ[5]. Schwank et al.[10] recently proposed antagonistic growth regulation by Dpp and Fat drives uniform cell proliferation along the anteroposterior axis of the *Drosophila* wing disc. Thus, the Ds/Ft signaling pathway may act together with Dpp to regulate the final size of the wing disc.

Recently, Bando *et al.*[11] found that the Ds/Ft system was likely to be involved in the mechanism underlying the leg regeneration, including intercalary regeneration, of the cricket *Gryllus bimaculatus*. Since knock-down of the expression of *Gryllus dachsous* (*Gb′ds*) or *Gb′fat* (*Gb′ft*) resulted in shorter regenerated legs, they assumed that the Ds/Ft system was likely involved in determination of leg length. In contrast, when expressions of *Gb′Merlin* (*Gb′Mer*) and *Gb′expanded* (*Gb′ex*), which are upstream components of the Hippo pathway[12], encoding the membrane-associated FERM-domain proteins were knocked down by RNAi during leg regeneration, the regenerated leg became longer than normal[11,13]. To further analyze their data, they applied the Ds/Ft steepness model[2], assuming a gradient of the Ds/Ft tarns-dimer was linear, as shown in Fig. 1c[13], since they did not know any protein distribution of Ds and Ft in the leg segment of the cricket. After amputation of the leg, regeneration started and regeneration (cell proliferation) stopped when the gradient of the Ds/Ft trans-dimers reached a threshold value (*diff*) that determined normal leg length[11]. In the steepness model, there are 3 parameters, *h*: the initial height of the gradient, *s*: slope, and *diff*: threshold value that determines length. Bando *et al.*[13] assumed that the *h* value became low in the case of the Ds/Ft RNAi experiment, in which the regenerated leg was short, while the *diff* value became low in the case of the ex/Mer RNAi experiment, in which the regenerated leg became longer. Furthermore, every experiment including intercalary regeneration of the leg could be interpreted using the steepness model.

In order to propose the extended steepness model taking the Ds/Ft trans-dimers into account, we formulate our idea under the following two simple assumptions in the next section: (1) the Ds/Ft trans-heterodimers or trans-homodimers are redistributed during cell division, and (2) growth would cease when a differential of the dimer across each cell decreases to a certain threshold.

In the case of the cricket leg, we assumed the presence of Ds/Ft trans-homodimer gradients, based on their expression patterns in the leg bud[13]. In the cricket leg bud, expression of the *Gb′ds* was intense in the distal region of each leg segment and showed a negative gradient to the distal direction, while that of *Gb′ft* was opposite, i.e., intense in the proximal region and show a negative gradient to the distal direction, which did not appear linear (although we could not observe any protein distribution). In the leg segment, therefore, gradients of the trans-homodimers may be formed instead of the trans-heterodimers, because formation of trans-homodimers between Ds or Ft is possible, as reported by Halbleib and Nelson[14]. Thus, we performed simulation for developmental and regenerative growth along the proximodistal axis of the insect leg or wing with our extended steepness model, assuming at first a non-linear concentration gradient of the Ds/Ft trans-heterodimers and then that of the Ds/Ft trans-homodimers. We verified our extended steepness model by comparing the simulation results with experimental data obtained for the cricket leg.

Results

Extended steepness models were proposed to simulate development and regeneration of organs. Previously, based on the steepness model, Yoshida[15,16] studied equations developed to study the cellular regeneration and self-maintenance over periods of turnover with the aid of symbolic computation in 2011. In the present report, we extended these studies by constructing at first a simple extended steepness model with one kind of molecule in order to derive a condition for stopping cell proliferation. Figure 2a shows how molecules in the cell membrane redistribute during cell division. We here assume that before cell division, a cell has l molecules on the left side and r molecules on the right side. After cell division, the molecules are redistributed so that the newly created cell membranes have a value of $pl + p'r$, meaning that the assignment ratios from the left and right membranes are p and p', respectively. Furthermore, based on cell proliferation research[17,18], we assumed that the cell stops proliferation when the difference between the values on both the ends, denoted by $|l - r|$, is less than a given threshold value. We term this threshold *diff* in the sequel. Under this assumption, the condition for the cell chain to eventually stop proliferating can be described as follows: for all l and r such that $l > r > 0$, $l - r > |l - (pl + p'r)|$ & $l - r > |r - (pl + p'r)|$, which means that $l - r$ in each cell declines every time it divides. This inequality is found equivalent to

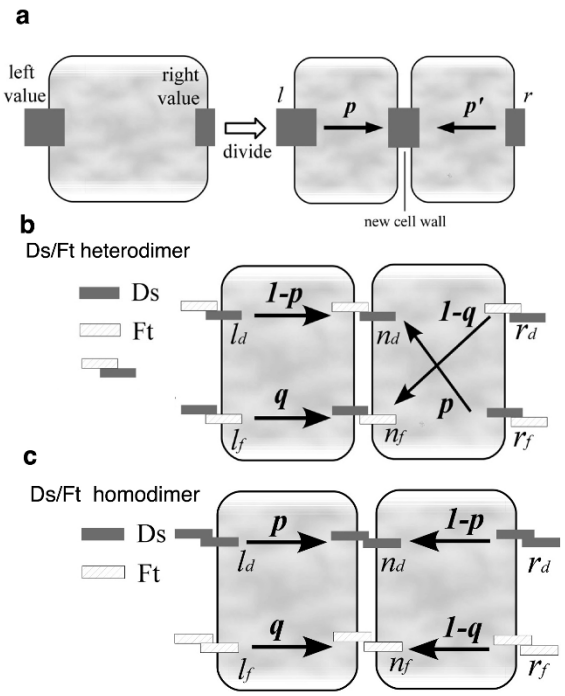

Figure 2 | The extended steepness model. (a) A one-molecule case. During cell division, the left and right *values* (molecules) are assumed to be redistributed to provide the newly created cell wall with a *value* of $pl + p'r$. (b) The extended steepness model: A Ds/Ft trans-heterodimer case. During cell division, the Ds/Ft heterodimers are deemed redistributed on the wall of the newly created cell. We accordingly assume that the left and the right Ds/Ft molecules are redistributed at a $p:1-p$ $(q:1-q)$ ratio. (c) The extended steepness model: A Ds/Ft trans-homodimer case. Simulations were performed assuming gradients of the Ds/Ft trans-homodimer, based on a cell-dividing formula [3] and a cell-proliferation condition $|l_f - r_f| >$ diff & $|l_d - r_d| > diff$. In this model, opposite gradients of Ds and Ft homodimers were assumed.

$$0 < p < 1 \ \ \& \ p' = 1 - p. \qquad \text{(Formula 1)}$$

The same formula also holds for all l and r such that $r > l > 0$. We call p in Formula [1] the *distribution ratio* in that p designates distribution of Ds/Ft heterodimers during cell division in what follows.

Extending the one-molecule model above, we further constructed an extended steepness model with 2 kinds of molecules: Ds and Ft as illustrated in Fig. 2b. According to the Ds/Ft steepness model[2], at first, we assumed that Ds/Ft trans-heterodimers exist between cells and they are expected to be distributed on the newly created cell membrane after cell division. Let l_f and l_d be the amounts of Ft and Ds heterodimers, respectively, on the left-side membrane of the cell on the left. Likewise, let r_f and r_d be the amounts of Ft and Ds heterodimers, respectively, on the right-side membrane of the cell to the right. After cell division, the heterodimers are redistributed on the newly created cell membrane. Accordingly, let n_f and n_d be the amounts of Ft and Ds heterodimer, respectively, on the left-side membrane of the cell on the right. Extending Formula [1] into the Ds/Ft model, we assume that the heterodimers are redistributed in the following way during cell division:

$$\begin{cases} n_f = ql_f + (1-q)r_d, \\ n_d = pr_f + (1-p)l_d, \end{cases} \qquad \text{(Formula 2)}$$

where the left and the right Ds and Ft molecules are redistributed with a $p:1-p$ $(q:1-q)$ ratio so that an eventual halt to cell proliferation is assured.

Under these assumptions, we started calculation using a single cell and increased the cell number through cell division. It should be noted that the amounts of Ds and Ft heterodimers on the right-side membrane of the left cell (namely, the left side of the newly created membrane) are equal to n_f and n_d, respectively, because of the trans-heterodimeric form.

Secondly, we assumed that trans-homodimers exist between cells (Fig. 2c) and performed simulations similarly as did for trans-heterodimers, based on a cell-dividing formula $(n_f, n_d) = (ql_f + (1-q)r_f, pl_d + (1-p)r_d)$, Fomula [3], and a cell-proliferation condition $|l_f - r_f| > diff$ & $|l_d - r_d| > diff$.

Simulations with the extended steepness model. We at first show that the gradient along the cell chain varies in shape according to various distribution ratios, p. We next show that under various gradients, the cell chain eventually stops proliferating and that the cell chain has the ability to regenerate when some part of it is excised. Finally, we show simulations that explain the results of some regeneration experiments using regeneration-dependent RNA interference (rdRNAi)[11].

Simulations were performed using the one-molecule model. Starting with the cell to the left having a value 10 and the cell to the right a value of 0, the cell divided in 2 and the values of the new cell membranes distributed according to $pl + p'r$ (Fig. 2a) satisfying Formula [1]. We assumed that the cell stopped cell proliferation when the difference between the values on both ends is less than 1, that is, we set *diff* as 1. Under these assumptions, the cell chain stops cell proliferation eventually as illustrated in Fig. 3a that depicts the case of $p = 4/5$. Various distribution ratios, p-values describing various gradients along the cell chain are illustrated in Fig. 3b,

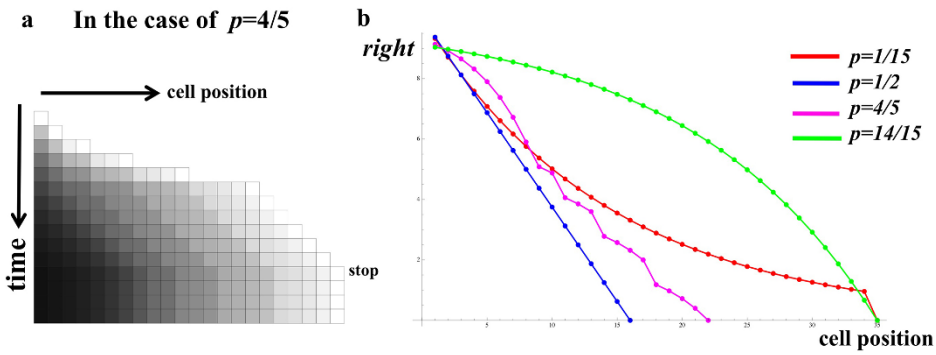

Figure 3 | Simulations with the extended steepness model: one-molecule case. (a) Any p-values make the cell chain stop proliferation as long as $0 < p < 1$. The grayscale denotes the gradient along the cell chain, in which black and white represent values 10 and 0, respectively. The figure shows the case of $p = 4/5$, where starting with a cell, the cell chain increases in number and halts proliferation at the *stop* time. (b) Various p-values yield a variety of gradients of the value along the cell chain. The ordinate denotes the right value at each cell.

where the blue linear line ($p = 1/2$) corresponds to the linear gradient postulated in the steepness hypothesis. In the case of $p = 4/5$ in Fig. 3b, although the curve is not smooth only at a resolution of a few cells, it monotonically decreases. The reason why it is non-monotonous is that depending on values of p or q, some cells stop cell division when their *diff* values happen to become less than the threshold value, while other cells continue dividing to recover the number of cells.

Even though various gradients arise, any can make the cell chain stop proliferating eventually, because every time the cell divides the difference between the values on the left and right becomes smaller according to Formula [1]. We here only show simulations with the initial values to the left, right, and *diff* being 10, 0, and 1, respectively, but the results above hold as long as the initial difference exists and p satisfies Formula [1].

Simulations of leg regeneration were performed using the Ds/Ft trans-heterodimer model. Recently, Bando *et al.* (2009)[11] reported that the Ds/Ft system was involved in cricket leg regeneration by using regeneration-dependent RNAi (rdRNAi) for loss-of-function analyses and they interpreted their data using the steepness model for leg regeneration[13], as shown in Fig. 1c. We therefore simulated their regeneration experiments at first using our Ds/Ft trans-heterodimer model (Fig. 2b). In this simulation, we assumed that each cell stopped cell proliferation when $|l_f - r_d| \le diff$ or $|l_d - r_f| \le diff$, in other words, the cell keeps proliferating when $|l_f - r_d| > diff$ and $|l_d - r_f| > diff$. With a set of initial values $(l_f, l_d, r_f, r_d) = (100,100,0,0)$, and $p = 1/2$, $q = 1/5$, $diff = 1$, a single cell divides into a 64-cell chain (Fig. 4a). We then extracted the first 12 cells corresponding to amputation at level 12. After the amputation, we reset the right-side value of the 12 cell (corresponding to the extremity of the amputated cell chain) to the initial right value, $(r_f, r_d) = (0,0)$. The effect of this reset can be seen at the right end colored white in the first cell row in Fig. 4b–e. The reset procedure was used to interpret data obtained from leg regeneration experiments[19] i.e., cells in the amputated site had the most distal positional value[11,13], which is known as the *distalization* rule[20]. The amputated leg developed into a chain of 60-cell (not shown in the figure). The regenerated chain had the same gradient as the original one, indicating successful normal regeneration (distal outgrowth).

Simulations of experimental results obtained by rdRNAi against *Gb′ft* or *Gb′ds* were performed with the Ds/Ft trans-heterodimer model. In order to simulate the effects of rdRNAi against *Gb′ft* or

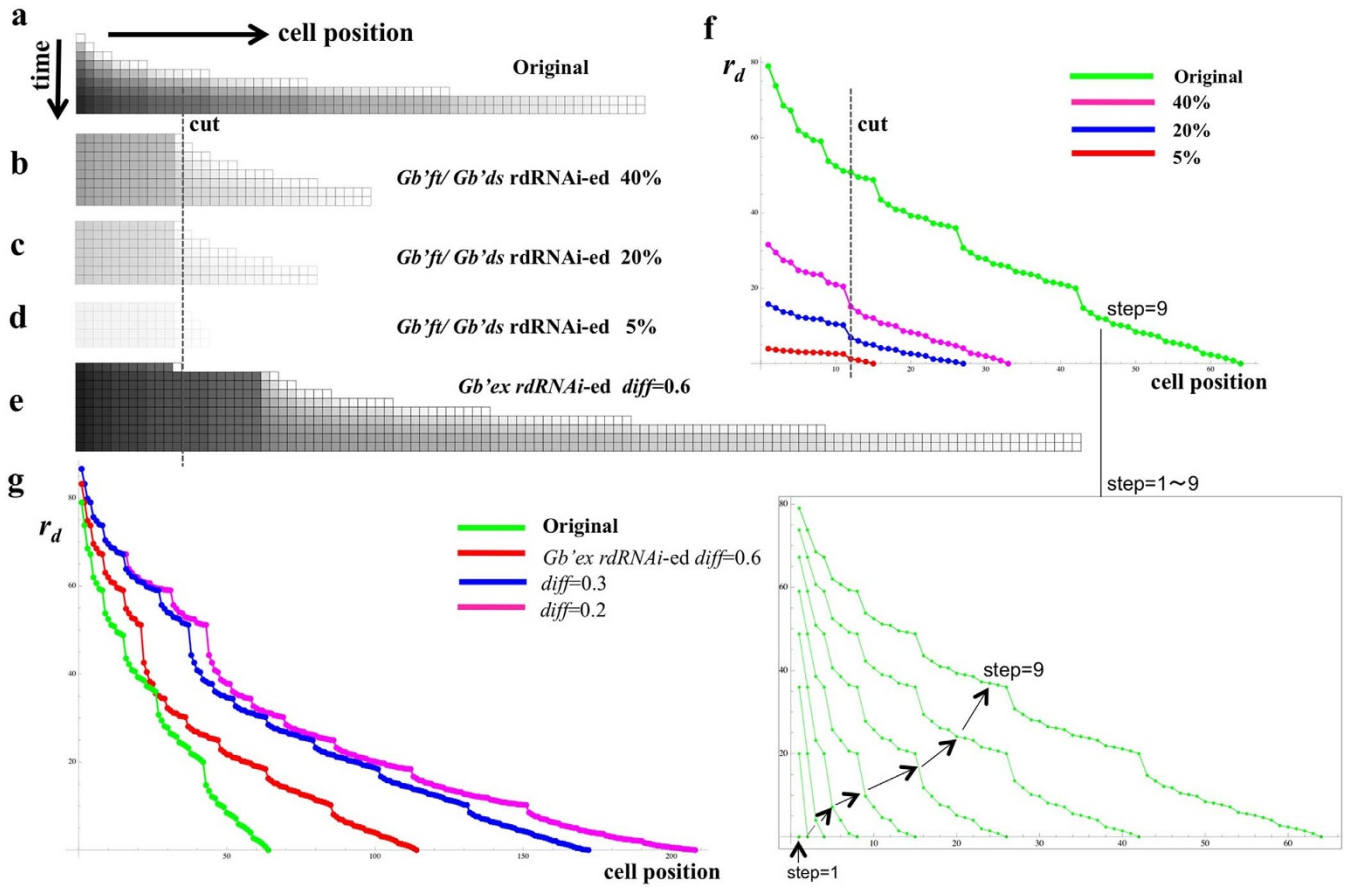

Figure 4 | Simulations of leg regeneration obtained by rdRNAi against Ft or Ds with the extended steepness model with gradients of the Ds/Ft trans-heterodimer. (a–e) The grayscale denotes the gradient along the cell chain, in which black and white represent values of 100 and 0, respectively. (a) Starting with one cell and $(l_f, l_d, r_f, r_d) = (100,100,0,0)$, $p = 1/2$, $q = 1/5$, $diff = 1$, the cell developed into a 64-cell chain. (b) We extracted the first 12 cells, followed by *distalization* (see the text), and then developed them under an influence of *Gb′ft* or *Gb′ds* rdRNAi that lowers the level of Ft or Ds by 40% (60% reduction) within the cell, obtaining a 33-cell chain. (c) A simulation under the influence that lowers the level of Ft or Ds to 20% (80% reduction) to obtain a chain of 27 cells. (d) Reduction of 5% (95% reduction) resulted in a chain of 15 cells. (e) A simulation of under the influence of *Gb′ex* that makes a regenerated leg longer, obtaining a 114-cell chain. In this simulation, we set *diff* at 0.6. (f) The gradients along the cell chains in the *Gb′ft*/*Gb′ds* rdRNAi experiments. The ordinate denotes r_d at each cell. The gradient of the original chain is colored green. The gradients produced by lowering the amounts of Ds/Ft to 40, 20, and 5% are colored magenta, blue, and red, respectively. The outer figure of (f) shows the gradients at the first 9 steps including the final step (=the 9th) on the original chain. (g) The gradients along the cell chains in the *Gb′ex* experiments. The gradients of the *diff*-lowered (0.6, 0.3, and 0.2) chains have the same ordinate-intercept as the original (green) gradient.

Gb′ds, we performed a regeneration simulation from the same amputated chain (the first 12 cells of Fig. 4a) under a low level of Ft or Ds within the cell. When we simulated the RNAi effect by lowering the amount of heterodimers to 40% (down 60%) after amputation, a shorter 33 cell chain was obtained (Fig. 4b). This result is qualitatively consistent with the short leg phenotypes obtained by actual *Gb′ft* or *Gb′ds* rdRNAi experiments[13]. It should be noted that in the Ds/Ft model, we postulated that Ds or Ft molecules acted as heterodimers; hence we assumed that either *Gb′ft* or *Gb′ds* would have the same effects on the amount of Ds/Ft heterodimers present. When we also simulated *Gb′ft* or *Gb′ds* rdRNAi experiments by lowering the amount of Ds/Ft heterodimers to 20% and 5% (reduction of 80% and 95%, respectively), they developed into a chain of 27 and 15 cells, respectively (Fig. 4c and d).

Figure 4f depicts the gradients along the cell chain in the *Gb′ft*/*Gb′ds* rdRNAi experiments. The magenta- (40%), blue- (20%), and red- (5%) colored gradients represent a down shift in the gradient of the original chain (green), indicating that the *Gb′ft*/*Gb′ds* rdRNAi-ed simulation corresponded to Morphallaxis-like regeneration. The outer figure of Fig. 4f shows the gradients at the first 9 steps including the final step (=the 9th) on the original (green) chain. It can be seen that even though the gradient has some bumps at a resolution of a few cells, as a whole it is monotonic and becomes less steep as the cell chain grows longer. This is consistent with the steepness model illustrated in Fig. 1a and c.

Simulations of experimental results obtained by rdRNAi against *Gb′expanded or Gb′Merlin* were performed with the Ds/Ft trans-heterodimer model. Next we simulated long-leg phenotypes obtained by RNAi against *Gb′ex or Gb′Mer* (*Gb′ex*/*Gb′Mer*). In this case, Bando *et al.*[13] assumed that *Gb′ex*/*Gb′Mer* may be related to the threshold value of *diff* for stopping of cell proliferation because regenerated legs under their RNAi condition became longer than the normally regenerated legs. We simulated this effect by setting *diff* to 0.6, 0.3, or 0.2 after the amputation, and obtained longer chains of 114 (Fig. 4e), 172, and 208 cells, respectively. These results were qualitatively consistent with the long-leg phenotypes obtained by the actual *Gb′ex*/*Gb′Mer* rdRNAi experiments[13].

Furthermore, Figure 4g depicts the gradients along the cell chains in the *Gb′ex* rdRNAi experiments, where the gradient of the *diff*-lowered (0.6, 0.3, and 0.2) chains have almost the same ordinate-intercept as the original (green) gradient. This indicated that in the actual experiment, *Gb′ex*-rdRNAi reduced the Ds/Ft amounts along the cell at which point the cell stopped proliferating.

Simulations of experimental results obtained by rdRNAi during reverse intercalary regeneration were performed with the Ds/Ft heterodimer model. We investigated reverse intercalary regeneration in the Ds/Ft model under an influence of *Gb′ex* rdRNAi. Figure 5a illustrates reverse intercalary regeneration and an explanation under the assumption of a Ds/Ft gradient. When a distally amputated leg is grafted with a proximally amputated leg, reverse intercalary regeneration occurs so that value continuity is recovered. Just after grafting, a steep (reverse) Ds/Ft gradient is formed at the junction and this keeps cells proliferating until the gradient is less than a threshold value, resulting in the recovery of the amputated portion (Fig. 5a).

In this simulation, we employed a condition that stopped proliferation, $|l_f - r_d| \le diff$ or $|l_d - r_f| \le diff$, which was the same as in the previous rdRNAi simulations, and used the following set of initial values $(l_f, l_d, r_f, r_d) = (100,100,0,0)$, $p = 1/2$, $q = 1/5$, $diff = 1$. Under this set, the cell chain developed into a 64-cell chain (Fig. 5b). The results of simulations for reverse intercalary regeneration are shown in Fig. 5c–g. Using non-reverse (normal) intercalary regeneration as a reference, we extracted the first 40 cells and the last 15 cells and joined them. They developed into a 69 cell chain with a similar gradient as the original one (Fig. 5c) and the orange curve depicted in Fig. 5g. We next performed simulations of reverse intercalary regeneration by extracting and combining the first 50 and the last 25 cells from the original chain (Fig. 5d). We also simulated the influence of *Gb′ex* rdRNAi that makes a regenerated leg longer by decreasing the value of *diff*. Figure 5d depicts a simulation of reverse intercalary regeneration with no influence of *Gb′ex* rdRNAi with *diff* being 1. The regenerated chain consisted of 83 cells that had a reverse gradient at its inner part as shown by the red curve of Fig. 5g. This reverse gradient was almost the reverse of the 50–60th values of the original chain (colored green), indicating that reverse intercalary regeneration occurred. In contrast, Figure 5e and f depict simulations of reverse intercalary regeneration under the influence of *Gb′ex* rdRNAi, with *diff* being 0.6 and 0.4, respectively. These cells grew into 128 and 170 cells, respectively, whose reverse-gradient portions were shifted as shown in the blue and magenta curves (Fig. 5g). This shift shows that the host part (the proximal part from the amputation) extended to produce a non-reverse gradient, followed by a reverse gradient that is qualitatively consistent with the observation in *Gb′ex* rdRNAi-ed legs[11].

Simulations of experimental results obtained by rdRNAi against *Gb′ft* or *Gb′ds* were performed with the Ds/Ft trans-homodimer model. In this model, instead of assuming formation of the Ds/Ft trans-heterodimer, we assumed formation of the trans-homodimer in the cricket leg segments, illustrated schematically in Fig. 2c. We also assumed that opposite concentration gradients of Ds and Ft molecules in the leg segment, as speculated from their expression patterns in the cricket leg bud[13]. Some simulation results were shown in Fig. 6a–f. Figure 6a shows a result calculated with a set of initial values $(l_f, l_d, r_f, r_d) = (100,0,0,100)$, $p = 1/2$, $q = 1/3$, $diff = 1$, in which a single cell divides into a 100-cell chain. The grayscale denotes the gradient along the cell chain, in which black and white represent values of 100 and 0, respectively. The grayscales in Fig. 6a and b indicate values of r_f and r_d, respectively. In order to simulate regeneration experimental data, we extracted the first 30 cells, followed by *distalization*, and then developed them without influence of *Gb′ft* or *Gb′ds* rdRNAi, obtaining a 98-cell chain with a similar gradient of the original, as shown in Fig. 6c or d. To simulate the influence of *Gb′ft* or *Gb′ds* rdRNAi, the level of Ft or Ds was lowered to 50% in the simulation. In both cases, a chain of cells decreased to be 62 cells (Fig. 6e or f). The gradients along the cell chains in these simulations are shown in Fig. 6g, where the ordinates denote r_f and r_d at each cell. Comparing with the gradients of the original chain (green), the gradients in the regenerated chain without rdRNAi influence (magenta) are similar. However, when the gradients produced by lowering the amounts of Ds and Ft to 50% (blue), the cell chains become short. These results are qualitatively consistent with the short leg phenotype in the regeneration experiments with the cricket leg. Especially, although the gradient of Ds is opposite to that of Ft, the short leg phenotype appears in both cases[13]. Thus, we concluded that we can explain the experimental results obtained in the cricket leg with our extended steepness model assuming the presence of a gradient of either the Ds/Ft trans-heterodimer or trans-homodimer.

Discussion

How size is regulated during animal development is one of the most fundamental questions in biology. It is known that positional information and growth are tightly linked during embryogenesis and regeneration. The steepness model may be the simplest means of interpreting the tight link and the size constraints observed in biological systems. We proposed an extended steepness model that assumed that (1) Ds/Ft trans-heterodeimer or trans-homodimers are redistributed during cell division, and (2) growth would cease when a

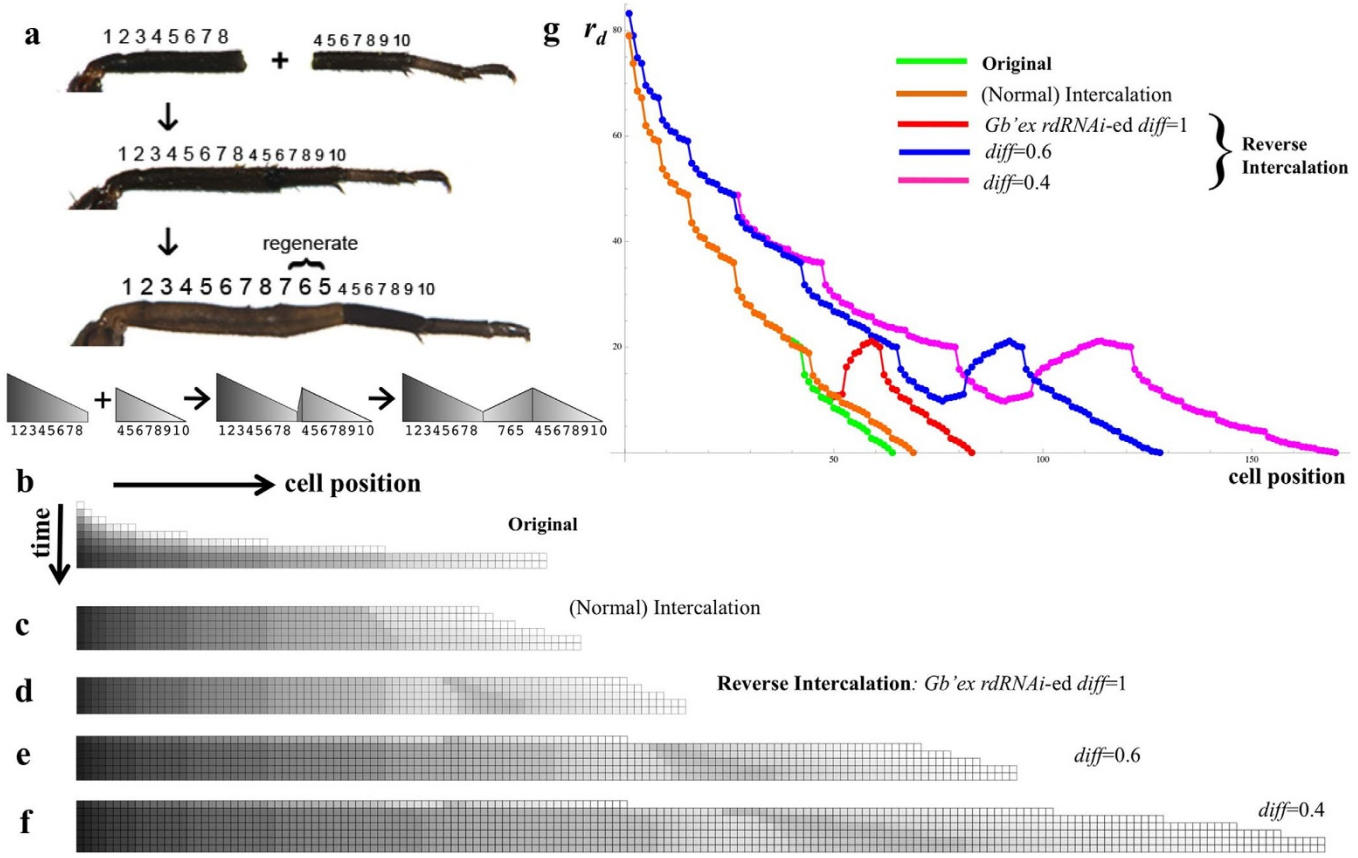

Figure 5 | Simulations of reverse intercalary regeneration obtained by rdRNAi against *expanded* with the extended steepness model with gradients of the Ds/Ft trans-heterodimer. (a) Grafting experiments using legs of the cricket *Gryllus bimaculatus* nymphs (This method was described previously[11]). Briefly, when a distally amputated leg (with the eighth value in the figure) is grafted with a proximally amputated one (with the fourth value) at the 3rd instar, reverse intercalary regeneration occurs such that positional-value continuity is recovered at the 6th instar. Just after the graft, a steep (reserve) Ds/Ft gradient is formed at the junction the middle of the lower panel in (a). This steep gradient maintains cells proliferating until the gradient is less than the threshold, resulting in recovery of the 7-6-5th cells. (b–f) The grayscale denotes the gradient along the cell chain. Black and white represent values of 100 and 10, respectively. (b) Starting with one cell and $(l_f, l_d, r_f, r_d) = (100,100,0,0)$, $p = 1/2$, $q = 1/5$, $diff = 1$, it developed into a 64-cell chain. (c) Non-reverse (normal) intercalary regeneration. Extraction and combination of the first 40 and the last 15 cells yielded a chain of 69 cells with a similar gradient as (b). (d) Reverse intercalary regeneration without the influence of *Gb′ex* rdRNAi, setting *diff* as 1. Extraction and combination of the first 50 and the last 25 cells yielded a chain of 83 cells that has a reverse gradient. (e) In the same manner as (d), setting *diff* to 0.6, a chain of 128 cells was generated with a shifted reverse gradient. (f) In the same manner as (d), setting *diff* to 0.4, a chain of 170 cells was generated. (g) The gradients along the cell chains in the case of (b–f). The ordinate denotes r_d at each cell. The original chain is colored green (5b). The non-reverse (normal) intercalary regeneration is colored orange (5c). The reverse intercalary regenerations with *diff* being 1, 0.6, and 0.4 are colored red, blue, and magenta, respectively, corresponding to Fig. 5d–f.

differential in the dimers across each cell decreases below a certain threshold value. Using this model, we consistently reproduced experimental data obtained from the cricket leg regeneration studies. Especially, we demonstrated that the decrease in the concentration of the Ds/Ft trans-heterodimer or trans-homodimers make the regenerated leg short (Fig. 4 for the heterodimer, Fig. 6 for the homodimer) and an assumption that the *diff* value decreases in the *Gb′ex/Mer* rdRNAi-ed leg is consistent with the longer-leg phenotype (Fig. 4e and g), as observed in the RNAi experiments[11,13]. Furthermore, we demonstrated that normal or reverse intercalary regeneration observed in the cricket leg[11] was successfully simulated with the extended steepness model (Fig. 5c–f). Furthermore, longer reverse intercalary regeneration found in *Gb′ex*-RNAi-ed legs[11,13] was also simulated with our model. Thus, we concluded that our extended steepness model is qualitatively consistent with experimental results observed in the cricket leg regeneration.

In the Ds/Ft steepness model[2], the gradient of the Ds/Ft trans-heterodimer is assumed to be linear. This assumption should be reasonable in the *Drosophila* wing imaginal disc and abdominal epidermis. However, it is not clear that linear concentration of the Ds/Ft trans-heterodimers is present in the cricket leg segment. Actually, expression patterns of the cricket *ds* or *ft* in their limb buds seemed opposite and not linear in the leg segment of the cricket embryo[11]. Thus, we also examined whether the gradient of the Ds/Ft trans-homodimer is functional similarly as that of the Ds/Ft trans-heterodimer in the extended steepness model. Our simulation results clearly showed that the gradient of the trans-homodimers work as a factor determining the leg size.

We also examined whether the linearity of the gradient is a necessary condition or not in the extended steepness model in this paper. From simulations shown in Fig. 3, we concluded that it was not necessary to assume a linear Ds/Ft heterodimer gradient in the steepness model. We found that despite pattern of the Ds/Ft trans-dimers in any organs, if there is a gradient of Ds/Ft trans-dimer, in general the extended steepness model may be applicable to explain why the dimers are involved in their size determination.

In our simulations, there are non-monotonous profiles in Figs. 3b, 4f and g, 5g. It should be noted that the non-monotonous feature appears only at a resolution of a few cells, while the curves are monotonically decreasing as a whole. These local non-monotonous

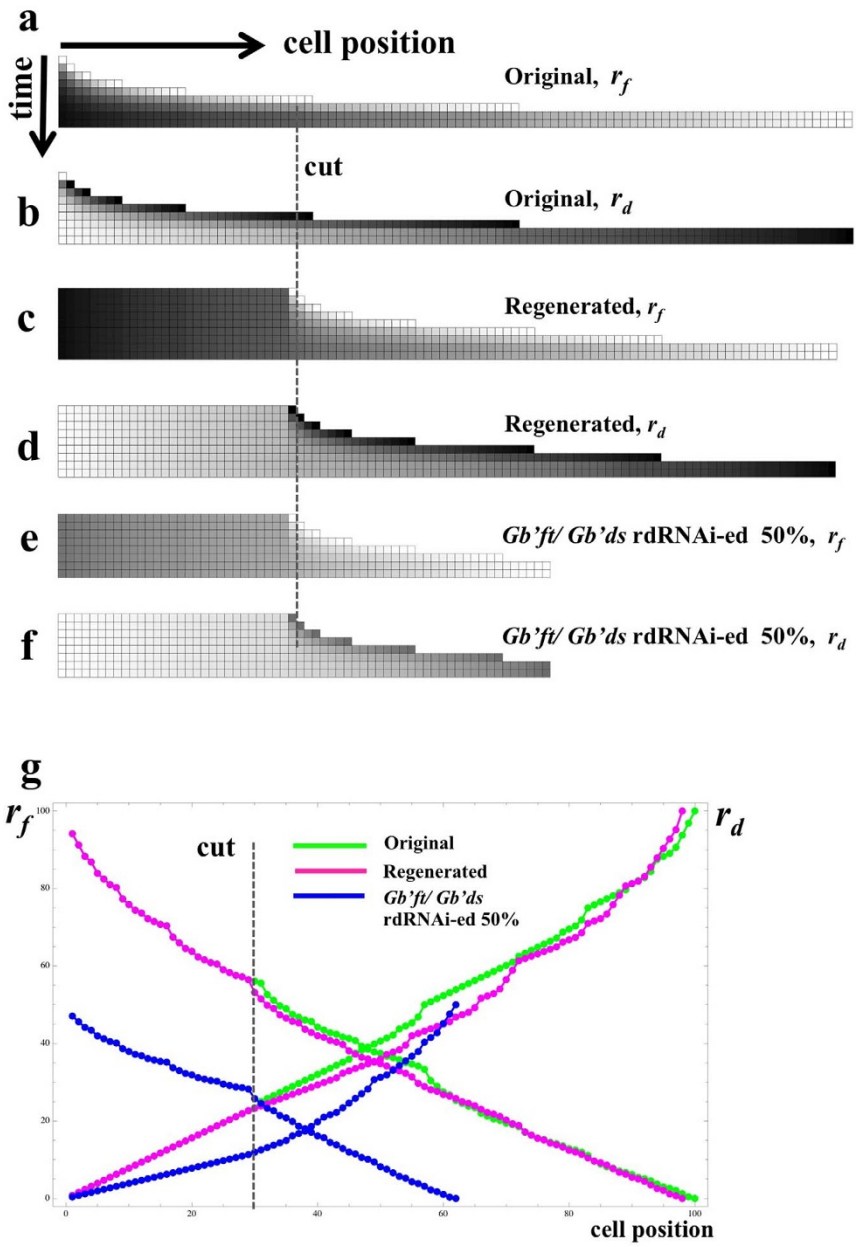

Figure 6 | Simulations of leg regeneration obtained by rdRNAi against Ft or Ds with the extended steepness model with gradients of the Ds/Ft trans-homodimer. (a–f) The grayscale denotes the gradient along the cell chain, in which black and white represent values of 100 and 0, respectively. (a, b) Starting with one cell and $(l_f, l_d, r_f, r_d) = (100,0,0,100)$, $p = 1/2$, $q = 1/3$, $diff = 1$, the cell developed into a 100-cell chain. The grayscale denotes r_f in (a) and r_d in (b). (c, d) We extracted the first 30 cells, followed by *distalization*, and then developed them without influence of *Gb′ ft* or *Gb′ ds* rdRNAi, obtaining a 98-cell chain with a similar gradient of the original. (e, f) A simulation under the influence of rdRNAi that lowers the level of Ft and Ds to 50% to obtain a chain of 62 cells. (g) The gradients along the cell chains in these experiments. The ordinates denote r_f and r_d at each cell. The gradients of the original chain are colored green. The gradients in the regenerated chain without rdRNAi influence are colored magenta. The gradients produced by lowering the amounts of Ds and Ft to 50% are colored blue.

profiles (some bumps) on the curve are due to difference in timings when cells stop dividing, which depend on values of *p*, *q*, and *diff*. In experiments to detect cell proliferation, usually we measure BrdU or EdU incorporation in proliferating cells during development or regeneration. In *Drosophila* wing growth, for example, BrdU incorporation is non-monotonous so that BrdU-labeled cells are scattered in the wing disc[16], which might be consistent with the local non-monotonous profile seen in our model.

Even though to date we do not know how the Ds/Ft system regulates cell proliferation through the Hippo pathway in details. Bando *et al.*[11] found that the Hippo signaling pathway and the ex/Mer signaling system were involved in the Ds/Ft signaling system. It is now generally accepted that the Hippo signaling pathway is involved in the size regulation of the organs in both the invertebrate and vertebrate (a review[21]). It is also known that Mer mediates contact inhibition induced by proliferation by blocking recruitment of Rac to the plasma membrane[22]. In *Drosophila*, Mer functions with ex to promote the endocytosis of many signaling receptors, limiting their accumulation at the plasma membrane, and activating the Hippo signaling pathway[21]. Thus, it is reasonable to consider that ex/Mer may be involved in regulation of the growth control, which may be related to *diff*.

In addition, experiments with *Drosophila* suggested that the steepness of the gradient at each point, measured perhaps as a differential

across each cell, correlated with one dimension of the organ[23–25]. Thus, it is very reasonable to assume that growth would cease when the difference in the number of the molecules decreases to a certain threshold value of *diff* in our model. Since it is known that the Ds/Ft system is conserved in the mammals (reviewed in ref. 26), our extended steepness model would be applicable to size determination of the mammalian organs as well. At this juncture we need more data to confirm our hypothesis. Furthermore, we have to analyze different systems that determine organ size along the other axis, for example, leg size along the circumferential axis or dorsoventral axis.

Methods

To explain our method for simulations, we demonstrate a simple case in the one-molecule model as follows: We start a simulation with a single cell having a value of 3 on the left wall and of 1 on the right. After one cell division, the newly created wall has a value of $1 + 2p$, coming from $3p + 1(1 - p)$ (using $p,p' = 1 - p$ in Formula [1]). After two divisions, the walls of the four-cell chain have values $(3,1 + 4p - 2p^2,1 + 2p,1 + 2p^2,1)$ from the left to the right, where the second and fourth terms come from $3p + (1 + 2p)(1 - p)$ and $(1 + 2p)p + 1(1 - p)$, respectively. Each cell keeps its proliferation as long as the difference between the left and right wall values is equal or greater than the threshold value, *diff*, as mentioned in the Results section. Hence, in the case of $p = 1/3$ and $diff = 1/2$, cells except the first one stop proliferation because the values are (3,19/9,5/3,11/9,1), meaning that the differences between the left and right are (8/9,4/9,4/9,2/9). The cell chain stops the whole proliferation (development) when all the differences are less than *diff*. The development of a cell chain is also illustrated in a previous paper[16], where $p_1 = p$, $p_2 = 1 - p$.

In the Ds/Ft model, each cell has two kinds of wall value, corresponding to the Ds/Ft trans-dimer, on the left and right sides as mentioned in the Results section. The trans- heterodimer and trans-homodimer are redistributed according to Formula [2] and [3], respectively, in the cell division, and the cell chain develops in the same manner as the one-molecule model. The condition for the cell to stop proliferation in the Ds/Ft model is shown in the Results section.

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Author contributions

H.Y., S.N. and T.B. conceived and designed the theoretical models. H.Y. performed all of the simulations. H.Y., T.B., H.O., T.M. and S.N. discussed results to improve the manuscript.

Additional information

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