Sharp boundaries of Dpp signaling trigger local cell death required for *Drosophila* leg morphogenesis

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Morphogens are secreted signaling molecules that govern many developmental processes [1]. In the *Drosophila* wing disc, the TGFβ homolog Decapentaplegic (Dpp) morphogen forms a smooth gradient and specifies cell fate by conferring a defined value of morphogen activity. Thus, neighboring cells have similar amounts of Dpp product, and if a sharp discontinuity in Dpp activity is generated between them, JNK-dependent apoptosis is triggered to restore graded positional information [2, 3]. So far, it has been implicitly assumed that this apoptotic process is only activated when normal signaling is distorted. We have found, however, that a similar process occurs during normal development. We demonstrate here that a rupture in Dpp activity takes place during normal segmentation of the distal legs of *Drosophila*. This sharp boundary of Dpp signaling, independently of the absolute level of Dpp activity, induces a JNK/*reaper*-dependent apoptosis required for the morphogenesis of a particular structure of the leg, the joint. Hence, our results show that Dpp could induce a developmental program not only in a concentration dependent manner, but also by the creation of a sharp boundary of Dpp activity. Furthermore, they show that the same process could be used either to restore a normal pattern in response to artificial disturbance or to direct a morphogenetic process.

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The *Drosophila* leg is an appendage composed of nine segments: coxa, trochanter, femur, tibia and five tarsal segments (T1 to T5), separated by flexible structures named joints. The leg develops from the leg imaginal disc, a group of cells which segregates at the end of embryogenesis, proliferates during the larval stages and evaginates during pupation

to form the adult appendages. During the third instar larval stage, a progressive subdivision of the future leg occurs [4]. This is reflected in the successive formation of folds in the disc, which prefigures the location of each future joint. Hence, cells located in the folds could be considered as the presumptive joint.

On studying leg morphogenesis, we have observed that a reporter construct that reproduces the expression of the pro-apoptotic gene *reaper* (*rpr)* [5] shows a restricted segmental expression pattern in the third instar and prepupal leg disc: it is expressed in concentric rings, 2 to 6 cells wide, precisely located at the presumptive joint in the distal part of the leg, from the tibia to the fifth tarsal segment (Fig. 1A, B, I, J). *rpr* is a potent activator of caspases [6], a unique family of cysteine proteases that execute programmed cell death. Caspases are produced as zymogens and have to be activated at the onset of apoptosis to degrade cellular substrates, thus causing cell death. To see if the expression of *rpr* indicates that apoptotic cells are present in the presumptive joint, we stained third instar and prepupal leg discs with an antibody against an active form of caspase-3, a key component of the effector caspases [6]. We observe groups of cells with activated caspase-3 in the presumptive joints (Fig.1C, D, G, H), sometimes forming half-rings (Fig. 1K), in the distal leg discs. More than 75% of the caspase-3-positive cells at the distal region of the leg are included within the *rpr*-expression stripes (Fig. 1I, J, M-O and Supplementary Information Table 1) and are progressively eliminated from the epithelium sheet by basal epithelial extrusion (Fig. 1H). Outside the *rpr* bands, only a few and isolated nuclei with caspase-3 signal are detected (See Supplementary Information Table 1). As caspases have already been reported to play roles independent of cell death [6], we used Tunel (for TdT-mediated-dUTP-Nick-End Labelling) staining to confirm that these cells are actually

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dying. We observe that the Tunel staining effectively co-localized with the active form of caspase-3, confirming that these cells are apoptotic (Fig. 1L, P-P''). Furthermore, both *rpr* and activated caspase-3 are detected just before and at the time of distal fold formation (Fig.1E-F, G-H) [4]. Taken together, these observations suggest that programmed cell death could play a role in fold formation.

To test this hypothesis, we prevented cell death in the leg distal domain by two methods: first, we expressed the caspase inhibitor *p35* [7] using the GAL4/UAS method [8]. Second, we generated large clones mutants for *Df(3L)H99* (see Methods), a deficiency uncovering the three RHG pro-apoptotic genes *rpr*, *head involution defective* and *grim*, known to eliminate programmed cell death in developing embryos [9]. In both cases we checked that, as expected, the activated caspase-3 positive cells are no longer detected (see Supplementary Information Fig S1A-F). When caspase activation is inhibited in large domains of the leg disc by any of these two methods, the distal epithelial folds in the leg disc and the joints in the adult are altered or absent (Fig. 2B, C, F, G, compare with the wild type in Fig. 2A and 2E, Supplementary Information Fig. S1G, H and data not shown for *Dll*-Gal4/UAS-*p35* adults). These results demonstrate that apoptosis is required for joint formation and that it is controlled by the RHG proteins.

The JNK pathway is known to induce apoptosis in some particular contexts. For example, it is required in the wing disc to remove cells with unbalanced levels of Dpp [2, 3]. In the leg disc, we noticed that *puckered* (*puc*), a target and a repressor of the JNK pathway [10], is expressed similarly to *rpr* (Fig. 2I, J). To test the potential role of the JNK pathway in joint morphogenesis we overexpressed *puc*, resulting in the inhibition of the JNK pathway. We observed the same kind of alterations in folds and joints formation as when apoptosis is

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inhibited (Fig. 2D, H and data not shown for the *ap*-Gal4; UAS-*puc* combination). These results suggest that the apoptosis present in the presumptive joints is mediated by JNK activity and this is supported by the observation that inhibition of the JNK pathway is accompanied by a large reduction of *rpr* levels (Fig. 2K, L).

To look for factors that may mediate or restrict the expression of pro-apoptotic genes in the presumptive joint, we searched for genes with a segmental expression pattern in the leg disc. Interestingly, it has been shown that *dpp* has such pattern in cricket and grasshopper legs [11, 12], and that several BMPs (as BMP-2, BMP-4, GDF-5 and GDF6) are also expressed metamerically in mouse and chicken digits [13]. In *Drosophila,* Dpp expression has never been reported as segmental in the leg disc, however, a split of the dorsal and ventral stripes at very late pupal stages has been described [11, 12]. These results were obtained using a *dpp-lacZ* reporter construct, so we decided to follow the *dpp* expression pattern by *in situ* hybridization with a *dpp* probe. At mid third instar larval stage, *dpp* is expressed, as previously described, in a stripe of cells just anterior to the A/P boundary, stronger in the dorsal than in the ventral part of the disc (See Supplementary Information Fig. S2A). However, we noted that, already at this stage, the *dpp* mRNA expression forms incipient circular bands in each segment of the distal part of the leg disc (See Supplementary Information Fig. S2A'). This expression pattern is maintained until the early prepupal stage. Finally, during disc evagination, the segmental expression pattern of *dpp* is evident. It forms semi-rings both dorsally and ventrally, located at the distal edge of each distal segment (See Supplementary Information Fig. S2B-D).

To visualize the activation pattern of Dpp signaling we have used an antibody against the phosphorylated form of *Mother against decapentaplegic* (P-Mad), a Dpp signal

transducer [14]. At early third instar, when folds in the disc are not yet formed, P-Mad is uniformly present in a dorsal domain (data not shown). As the disc matures, a segmental pattern of phosphorylated P-Mad appears progressively in both dorsal and ventral sides of the third instar larval leg disc (Fig. 3A, B). Although there are numerous folds present in the leg disc at this stage, a gradient of Dpp activity could be observed in z section of the disc (Fig. 3D', D''). At the prepupal stage, it becomes more evident that Dpp signaling forms a proximo-distal gradient in each segment, with its lowest value proximally and the highest distally, thus creating a sharp interface of Dpp activity at the segment boundary (Fig. 3E, F, H-H'). The comparison of *dpp* expression and activity patterns at the prepupal stage suggests that Dpp is produced by a row of cells in the most distal part of the segment and spreads proximally. To check this hypothesis, we looked at the distribution of a GFP-Dpp fusion protein that reveals Dpp distribution [15, 16]. When we expressed this protein at the segment boundary (with the *bib*-Gal4 driver), GFP-Dpp vesicles are readily detected proximally but not distally (Fig. 3K, L, compare with *bib*-Gal4/UAS-GFP expression in Fig. 3I, J), showing that the asymmetric activation pattern of Dpp probably relies on an asymmetric distribution of the Dpp protein. We do not know how this possible unidirectional Dpp spread can be formed. We have found, however, that Dally-like, a heparan sulphate proteoglycane required to "transport" the Dpp protein in the wing disc [17] is expressed proximally to the *rpr* stripe in the tarsal segments (not shown), suggesting that it may account, at least in part, for the Dpp distribution.

We noticed that the most dorsal and ventral domains of *rpr* expression (where its expression is strongest) follow the establishment of the Dpp gradient and straddle the Dpp sharp boundary (Fig. 3B-C, D'-D", F-H'). These results suggest a link between Dpp

signaling and apoptosis induction in the presumptive joint. To test this connection, we reduced or increased Dpp activity in part of the leg disc and analyzed *rpr* and *puc* expression as well as folds and joints formation. We expressed, late in development, either a dominant negative (tkv^{DN}) or a constitutively activated form (tkv^{QD}) of the *dpp* receptor *thick veins (tkv)* [18, 19] (see Methods). When Dpp activity is uniformly reduced (tkv^{DN}) or increased (tkv^{QD}) in the *apterous* (*ap*)-Gal4 domain (which includes most of the T4, the T4/T5 boundary and a few proximal cells of the T5), the joint separating the T4 and T5 segments is grossly altered or missing (Fig. 4B, F and 4C, G; compare with the wild type in 4A, E). In the leg disc, this phenotype correlates with alterations in the T4-T5 fold and with the disappearance of *rpr* expression in the T4-T5 presumptive joint (compare the wild type in Fig. 4I-I'' with 4J-J'' and 4K-K'' and see also Supplementary Information Fig. S3A-A", B-B'' and C-C''). *puc* is similarly down-regulated in these mutant combinations (see Supplementary Information Fig. S3E-E", F-F'' and G-G''). These results show that either a reduction or an increase in Dpp signaling leads to a similar phenotype of absence of *rpr* and *puc* expression and of fold formation. They also suggest that apoptosis and joint morphogenesis, although requiring Dpp signaling, do not depend on the absolute level of Dpp. Rather, our data suggest that these processes result from the generation of abrupt Dpp activity borders.

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We have confirmed this inference by two methods. First, we find that in about 1/3 of the *ap*-Gal4;UAS-tkv^{QD} individuals, the sharp new border of Dpp activity established within T5 induces the ectopic activation of *rpr* and *puc*, the formation of an ectopic fold and the development of an ectopic invagination of the cuticule that could correspond to a "preliminary joint" (Fig. 4D, H, L-L'' and Supplementary Information Fig. S3D-D'' and

H-H''). Second, we have induced, in the distal leg disc, clones that either eliminate (*Mad* mutant clones) or force (tkv^{QD}-expressing clones) Dpp activity. In both cases, new abrupt borders of Dpp activity are generated and we find that in many cases *rpr* expression and fold formation are induced bordering these clones (Fig. 4M, N and data not shown). We note that *rpr* ectopic expression is more frequently induced in the border of tkv^{QD} clones than in *Mad* mutant clones, probably because the latter can only form new sharp boundaries of Dpp activity close to the segment border.

To see if the *rpr* ectopic expression induced by a new border of Dpp activity results in cell death, we looked at the activated caspase-3 distribution either in tkv^{QD} clones or when tkv^{QD} is expressed in the *patched* (*ptc*) domain. We saw that cell death is induced ectopically in these contexts (in 1/3 of the discs observed); however, not all the cells at the interface of different Dpp activity show activated caspase-3 expression (see Supplementary Information Fig. S3I-M), perhaps because dying cells invaginate rapidly. Collectively, these results suggest that, in the presumptive joint, the probable asymmetric movement of Dpp allows the formation of sharp Dpp boundaries that induces a local JNK/*rpr*-dependent apoptosis required for leg joint morphogenesis (Fig. 5). Intriguingly, *rpr* and *puc* expressions are induced both in the cells where Dpp activity is highest and in the neighboring distal cells, where no Dpp activity is detected. How a sharp border of Dpp activity could be translated into the activation of the JNK pathway and induce apoptosis in these two cells is unknown. Possibly, the different cellular properties of these cells, conferred by the sharp discontinuity of Dpp signaling, may lead to a specific interaction between them which results in the activation of the JNK pathway.

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Although our data conform to the model proposed, some observations seem to indicate that the Dpp pathway may not act alone to induce cell death at the presumptive joint. For instance, when we create groups of cells with high Dpp activity, we notice that *rpr* expression and cell death are frequently not induced at the entire new interface delimited by these cells (see for example Fig. 4L'' and S3I-M). Similarly, the *rpr* wildtype expression is wider than the P-Mad activation domain along the dorso-ventral axis. This suggests that other factors may contribute to the induction of apoptosis and formation of the joint. Interestingly, the *Notch* (*N*) pathway has been shown to be necessary and sufficient to form a joint in the legs [20-22]. Preliminary results show that changes in N activity eliminate the P-Mad and *rpr* signals (not shown), suggesting that *N* may be involved in the formation of the Dpp gradient and in the induction of cell death. However, the Dpp pathway may also regulate the outcome of *N* activity: a target of *N*, *Enhancer of Split* (*E*(*spl*)), is expressed at the segment boundaries [21], and we have observed that an *E*(*spl*) reporter construct is ectopically expressed around some of the tkv^{QD}-expressing clones (Fig. 4O, P). This demonstrates, first, that at least the initial steps of joint formation are induced when sharp boundaries of Dpp activity are induced, and second, that there is a complex relationship between the N and Dpp pathways in joint determination. In spite of this, we cannot rule out that *N* may have a Dpp-independent role that contributes to cell death and joint formation in the lateral domain of leg disc, where P-Mad is barely detectable.

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The process of "Dpp border-induced cell death" described here takes place only in the distal joints, from the tibia to the T5. These results highlight that proximal and distal joints are formed by distinct mechanisms. Intriguingly, proximal and distal leg domains have been distinguished previously: the ancestral ground-state limb structure has been

proposed to be a leg-like appendage composed of a proximal segment and a distal tarsus [23]. Similarly, in vertebrates, the autopode (the most distal part of the appendage) is also known to be a more recent structure than the proximal part [24]. This suggests that, in the fly, appendage evolution could account for the differences between distal and proximal joint formation: the ancestral way to form a joint would require apoptosis, while the proximal formation of joints would have evolved by a different mechanism.

Methods

Genetic strains :

The *puc*^{E69}, *rpr*-4kb-*lacZ* , UAS-*tkv*^{QD}, UAS-*tkv*^{DN} , UAS-GFP and UAS-*N*^{DN} constructs are described in Flybase [25]. The UAS-*p35* [7], UAS-*puc2A* [10], UAS-GFP-Dpp [15, 16] and *E(spl)-lacZ* [26] lines have also been previously described. The *rpr-lacZ* construct of the X chromosome is from C. S. Thummel. *DfH99* is a deletion that eliminates the pro-apoptotic genes *reaper*, *grim* and *hid* [9]. Ectopic expression was obtained by using the GAL4/UAS system [8] with the following drivers : *Dll*-Gal4 (MD23 and EM212 [25] lines), *ap*-Gal4 [25], *hh*-Gal4 [14] , *ptc*-Gal4 [25]and *bib*-Gal4[27] (obtained through J. F. de Celis).

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Clonal analysis :

Clones mutant for *Df(3L)H99* were induced in larvae of the following genotypes: *y w*; *Dll*-Gal4 (EM212) UAS-*flp*/+; *Df(3L)H99* FRT2A/*Dpsc*^{S2} *(y+) M*(3)*i*^{55} FRT2A or *y w hs-flp*/+; *Df(3L)H99* FRT2A/*Ubi*-GFP FRT2A. The crosses were maintained at 25°C and the clones identified by the *yellow* cuticular marker or the absence of GFP expression. Clones that ectopically express the activated form of the Tkv receptor and in which *rpr-lacZ* , *E(spl)-lacZ* or activated caspase-3 expression were analyzed, were induced in larvae with the following genotypes: *y w hs-flp*/*rpr-lacZ* or *E(spl)-lacZ*; *act*>*y*$^{+}$>Gal4 UAS-GFP/UAS-*tkv*^{QD}. The clones were induced in larvae at 72-96h after egg laying by a 12 min. heat-shock at 34°C.

Immunostaining, histochemical staining and in situ hybridisation :

The antibody staining was done according to Pérez-Garijo et al [28]. We used rabbit anti-β-galactosidase (Cappel), mouse anti-armadillo and mouse anti-Dlg (from Hybridoma Bank), rabbit anti-P-MAD [14] (gift from T. Tabata), rabbit anti-Dlp (gift from S. Baumgartner), TRITC-phalloïdine (from Sigma) and rabbit anti-cleaved human caspase-3 (Cell Signaling Technology), which recognizes *Drosophila* activated caspase-3 [29]. Secondary antibodies were coupled with Red-X, FITC and Cy5 fluorochromes (Jackson Immunoresearch). *In situ* hybridization was performed according to Azpiazu and Frasch [30]. The RNA probe of *dpp* was synthesized from the BS-*dpp* plasmid provided by A. Macías (bluescript vector containing the cDNA of *dpp*) digested with KpnI and transcribed by T3.

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Tunel staining:

Tunel staining was performed according to Pérez-Garijo et al [28], following the in situ cell death detection kit from Roche.

Temperature shift experiments:

As Dpp is involved in the establishment of proximo-distal axis of the leg until 84h of development [31, 32], the analysis of Dpp requirement for joint formation was carried out using the temperature inducible system Gal4/Gal80^{ts} [33]. 84-108 hours old *ap*-Gal4/+; *tub*-Gal80^{ts}/UAS-*tkv*^{QD} or UAS-*tkv*^{DN} larvae were shifted from 17 to 29°C, and the phenotypes in the legs were analyzed in the adult, so that Dpp signaling in the *ap*-Gal4 domain is altered not before 84 hours of development. At this stage, Dpp is only involved in dorso-

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ventral axis formation and joint formation but not any longer in the establishment of the proximo-distal axis (not shown). Similar experiments were carried out in *ptc*-Gal4 UAS-GFP/+;UAS-*tkv*^{QD}/*tub*-Gal80^{ts} larvae to detect cell death. In this case, early third instar larvae were transferred from 18°C to 30°C and leg discs were fixed 48h later.

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Scoring of the adult phenotype in *ap*-Gal4/ UAS-*tkv*^{QD} flies:

In *ap*-Gal4/UAS-*tkv*^{QD} adults the wildtype T4/T5 joint disappears and an ectopic one is occasionally formed. Both joints were distinguished by measuring the distance of the new joint to the T3/T4 one as a percentage of the distance from T3/T4 to the T5/pretarsus boundary (T3-T5 distance). In the wildtype, T4/T5 is located approximately in the middle of the T3-T5 distance (at 48,4%, n=18). In the mutant context, the occasional crease observed is located at about one third (36,7%, n=15) of the T3-T5 distance.

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References

1. Teleman, A. A., Strigini, M. & Cohen, S. M. Shaping morphogen gradients. *Cell* **105**, 559-62 (2001).

2. Adachi-Yamada, T., Fujimura-Kamada, K., Nishida, Y. & Matsumoto, K. Distortion of proximodistal information causes JNK-dependent apoptosis in Drosophila wing. *Nature* **400**, 166-9 (1999).

3. Adachi-Yamada, T. & O'Connor, M. B. Morphogenetic apoptosis: a mechanism for correcting discontinuities in morphogen gradients. *Dev Biol* **251**, 74-90 (2002).

4. Rauskolb, C. The establishment of segmentation in the Drosophila leg. *Development* **128**, 4511-21 (2001).

5. Jiang, C., Lamblin, A. F., Steller, H. & Thummel, C. S. A steroid-triggered transcriptional hierarchy controls salivary gland cell death during Drosophila metamorphosis. *Mol Cell* **5**, 445-55 (2000).

6. Cashio, P., Lee, T. V. & Bergmann, A. Genetic control of programmed cell death in Drosophila melanogaster. *Semin Cell Dev Biol* **16**, 225-35 (2005).

7. Hay, B. A., Wolff, T. & Rubin, G. M. Expression of baculovirus P35 prevents cell death in Drosophila. *Development* **120**, 2121-9 (1994).

8. Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-15 (1993).

9. White, K. et al. Genetic control of programmed cell death in Drosophila. *Science* **264**, 677-83 (1994).

10. Martin-Blanco, E. et al. puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. *Genes Dev* **12**, 557-70 (1998).

11. Jockusch, E. L., Nulsen, C., Newfeld, S. J. & Nagy, L. M. Leg development in flies versus grasshoppers: differences in dpp expression do not lead to differences in the expression of downstream components of the leg patterning pathway. *Development* **127**, 1617-26 (2000).

12. Niwa, N. et al. Correlation of diversity of leg morphology in Gryllus bimaculatus (cricket) with divergence in dpp expression pattern during leg development. *Development* **127**, 4373-81 (2000).

13. Edwards, C. J. & Francis-West, P. H. Bone morphogenetic proteins in the development and healing of synovial joints. *Semin Arthritis Rheum* **31**, 33-42 (2001).

14. Tanimoto, H., Itoh, S., ten Dijke, P. & Tabata, T. Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs. *Mol Cell* **5**, 59-71 (2000).

15. Entchev, E. V., Schwabedissen, A. & Gonzalez-Gaitan, M. Gradient formation of the TGF-beta homolog Dpp. *Cell* **103**, 981-91 (2000).

16. Teleman, A. A. & Cohen, S. M. Dpp gradient formation in the Drosophila wing imaginal disc. *Cell* **103**, 971-80 (2000).

17. Belenkaya, T. Y. et al. Drosophila Dpp morphogen movement is independent of dynamin-mediated endocytosis but regulated by the glypican members of heparan sulfate proteoglycans. *Cell* **119**, 231-44 (2004).

18. Haerry, T. E., Khalsa, O., O'Connor, M. B. & Wharton, K. A. Synergistic signaling by two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in Drosophila. *Development* **125**, 3977-87 (1998).

19. Nellen, D., Burke, R., Struhl, G. & Basler, K. Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-68 (1996).

20. Bishop, S. A., Klein, T., Arias, A. M. & Couso, J. P. Composite signalling from Serrate and Delta establishes leg segments in Drosophila through Notch. *Development* **126**, 2993-3003 (1999).

21. de Celis, J. F., Tyler, D. M., de Celis, J. & Bray, S. J. Notch signalling mediates segmentation of the Drosophila leg. *Development* **125**, 4617-26 (1998).

22. Rauskolb, C. & Irvine, K. D. Notch-mediated segmentation and growth control of the Drosophila leg. *Dev Biol* **210**, 339-50 (1999).

23. Casares, F. & Mann, R. S. The ground state of the ventral appendage in Drosophila. *Science* **293**, 1477-80 (2001).

24. Shubin, N., Tabin, C. & Carroll, S. Fossils, genes and the evolution of animal limbs. *Nature* **388**, 639-48 (1997).

25. Drysdale, R. A. & Crosby, M. A. FlyBase: genes and gene models. *Nucleic Acids Res* **33**, D390-5 (2005).

26. Nellesen, D. T., Lai, E. C. & Posakony, J. W. Discrete enhancer elements mediate selective responsiveness of enhancer of split complex genes to common transcriptional activators. *Dev Biol* **213**, 33-53 (1999).

27. Hayashi, S. et al. GETDB, a database compiling expression patterns and molecular locations of a collection of Gal4 enhancer traps. *Genesis* **34**, 58-61 (2002).

28. Perez-Garijo, A., Martin, F. A. & Morata, G. Caspase inhibition during apoptosis causes abnormal signalling and developmental aberrations in Drosophila. *Development* **131**, 5591-8 (2004).

29. Yu, S. Y. et al. A pathway of signals regulating effector and initiator caspases in the developing Drosophila eye. *Development* **129**, 3269-78 (2002).

30. Azpiazu, N. & Frasch, M. tinman and bagpipe: two homeo box genes that determine cell fates in the dorsal mesoderm of Drosophila. *Genes Dev* **7**, 1325-40 (1993).

31. Campbell, G. Distalization of the Drosophila leg by graded EGF-receptor activity. *Nature* **418**, 781-5 (2002).

32. Galindo, M. I., Bishop, S. A., Greig, S. & Couso, J. P. Leg patterning driven by proximal-distal interactions and EGFR signaling. *Science* **297**, 256-9 (2002).

33. McGuire, S. E., Le, P. T., Osborn, A. J., Matsumoto, K. & Davis, R. L. Spatiotemporal rescue of memory dysfunction in Drosophila. *Science* **302**, 1765-8 (2003).

Figure legends

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Figure 1. Programmed cell death occurs at the presumptive joints. In this and subsequent figures, pupal leg discs are oriented with the distal region to the right and larval leg disc with the dorsal region up. T1 to T5 indicate tarsal segments. (A, B, E, F) *rpr-lacZ* expression (in blue) in presumptive tarsal joints of wild-type prepupal leg discs. F-actin is stained in red with TRITC-phalloïdin (phal; also in other panels except L). (E, F) High magnifications of the boxes shown in A and B, respectively. (C, D, G, H) Activated caspase-3 signal (casp3; in green) in the presumptive tarsal joints. (G, H) High magnification of the boxes shown in C and D, respectively. Note that both *rpr* and activated caspase-3 are detected before fold formation. The arrowheads point to the place were folds will subsequently form and the arrows the invaginating folds, formed just distally to the *rpr* expression domains. In H, see the extrusion of cells expressing an active form of caspase-3 (in green) and that cells delaminate basally from the epithelium (asterisks). The basal side of the epithelium of the disc is outlined by the white dotted line. (I, J and M-O) Co-localization of *rpr-lacZ* expression (in blue) and caspase-3 activation (in green) in larval and prepupal leg discs. The boxed region in M is shown in detail in N, O. Note that the small groups of cells expressing an active form of caspase-3 are included within the *rpr* expression domain. (K) caspase-3 activation (in green) could be observed in semi-rings in the mid-third instard larval disc. (L, P, P', P'') Co-localization of Tunel staining (in blue) and activated form of caspase-3 (in green). The disc has been stained with an anti-*armadillo* antibody (red) to see the folds. (P-P''). High magnification of the box seen in L. In this and subsequent figures, the scale bar represents 30 µm.

Figure 2. Distal joint morphogenesis requires apoptosis. (A-H) Prepupal discs stained with TRITC-phalloïdin (A-D) and tarsal joints of adult legs (E-H; electron microscope figures) of wild-type (A, E), *Dll*-Gal4; UAS-*p35* (B), *ap*-Gal4; UAS-*p35* (F), *Dll*-Gal4 UAS-*flp*; FRT2A *Df(3L)H99* / FRT2A *M(3)i*^{55} *Ubi*-GFP (C, G) and *Dll*-Gal4; UAS-*puc* (D, H) pupae and adults. Note the abnormal or absent folds (arrowheads) in the mutant discs, compared with the normal folds in the wild type (arrows in A), and the absence of the T4/T5 adult joint in the mutants (arrowheads) compared with the wild-type (arrow in E). In C the mutant tissue is marked by the absence of GFP expression (in green; all the region is mutant) but in G the domain mutant for *Df(3L)H99* has not been marked. In Supplementary Information (Fig. S1G, H), see that the same phenotype is observed in clones mutant for *Df(3L)H99* marked with the *yellow* (*y*) marker. The inset in B outlines the expression of the *Dll* driver. (I, J) *puc-lacZ* expression pattern in the prepupal leg disc (green), stained with phalloïdin (red) to localize the folds. Sagittal (I) and superficial (J) views of the same leg disc. (K-L) Dorsal view of a *rpr-lacZ*/UAS-GFP; *hh*-Gal4 /UAS-*puc* prepupal leg disc. A and P indicate anterior and posterior compartments, respectively. See the absence or reduction of folds (marked with phalloïdin) and of *rpr-lacZ* expression (in blue, arrows) in the P compartment (outlined in L), where the *hh*-Gal4 insertion drives *puc* expression.

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Figure 3. Dpp, P-Mad and *rpr-lacZ* expression patterns in third instar and prepupal leg discs. (A-C, E-G) P-Mad (red) and *rpr-lacZ* expression (blue) in third instar (A-C) and prepupal (E-G, dorsal view) leg discs. The third instar leg disc shows incomplete rings of P-Mad (arrows), stronger in the ventral and dorsal regions, and circles of *rpr* expression (due to the folds of the third instar larval disc, rings of *rpr* expression are not complete at

this focal plane, but can be better visualized in the projection of several Z sections seen in C). In prepupal leg discs (E-G), a gradient of P-Mad is observed (see also D"). (H-H') Detail of the boxed region in F. Note the gradient of P-Mad and that *rpr* expression (outlined in blue in H) straddles the sharp interface of *dpp* activity. (D-D'') Z section of a third instard larval disc showing P-Mad and *rpr* expression. (D) Schematic representation of the z section of the disc, showing the proximal, distal, dorsal and ventral part, and the folds (arrowheads). (D') The disc is stained with phalloïdin (in green), P-Mad (in red) and *rpr*-lacZ (in blue). (D'') Magnifications of the box seen in D'. Note the expression of *rpr* at the presumptive fold (green arrowhead), the partial overlapping of *rpr* and P-Mad domains, and the gradient of P-Mad proximally to the fold (red arrowheads). (I-J) A *bib*-Gal4; UAS-GFP prepupal disc revealing the expression pattern of *big brain* (*bib*) in green (I) and white (J), in the distal part of two consecutive segments (delimited by phalloïdine in red). Distal to the right. The arrowhead in J indicates absence of GFP signal between the segment borders. (K-L) A *bib*-Gal4*;* UAS-GFP-Dpp prepupal disc. Note that Dpp spreads proximally (arrows in L mark the GFP-Dpp dots), but not distally (arrowhead).

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Figure 4. Apoptosis induction and joint formation require a sharp boundary of Dpp signaling. Black or white arrows indicate normal segment boundaries or joints; red arrowheads show the absence of either structure and blue arrowheads ectopic ones. (A-H) Tarsal joints of wild-type (A, E), *ap*-Gal4/UAS-tkv^{DN} (B, F) and *ap*-Gal4/UAS-tkv^{QD} (C, D, G, H) flies. (E-H) High magnifications of the boxes seen in A, B, C, and D, respectively. (I-L") *ap*-Gal4 UAS-GFP *rpr-lacZ* (I), *ap*-Gal4 UAS-GFP *rpr-lacZ*/UAS-tkv^{DN} (J) and *ap*-Gal4 UAS-GFP *rpr-lacZ*/UAS-tkv^{QD} (K, L) pupal discs, where the *ap*-Gal4 domain is

visualized with GFP (green), segment boundaries with phalloïdin (red) and *rpr*-lacZ in blue. I', I'', J', J'', K', K''' and L', L''are magnifications of the boxes shown in I, J, K and L, respectively. See the absence of joints, folds and *rpr* expression when the Dpp activity border is absent (B, C, J- J'', K-K'') and their ectopic formation when a new sharp Dpp boundary is formed (D, L-L''). GFP expression is outlined in I'', J'', K''and L''. (M-P) Tkv^{QD}-expressing clones, marked with GFP (in green in M and O), showing *rpr-lacZ* (M, N) or *E(spl)-lacZ* (O, P) ectopic expression at the boundary of the clones (outlined in white in N and P). Note that a second clone in O, outside the tarsus (in the pretarsus, the central region), does not activate *E(spl).* N and P are magnification of the boxes shown in M and O, respectively.

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Figure 5. Model of joint formation in distal leg segments. Black arrows indicate normal segment boundaries, crossed arrows absence of boundary and blue arrows, ectopic ones. (A) During third instar larvae and prepupal stages, Dpp signaling forms a gradient from proximal to distal in each segment of the distal part of the leg, generating a sharp Dpp activity interface. This border is required to induce JNK- and *rpr*-dependent apoptosis, formation of folds and joints. The absence of this interface through the reduction (B) or the increase (C1) of Dpp activity levels blocks *rpr* expression and neither folds nor joints are formed. If an ectopic border of Dpp activity is generated, it frequently induces de novo *rpr* expression, ectopic folds and a structure looking like an incomplete joint in a new position (C2, in about 1/3 of the leg discs).

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Supplementary information:

Table 1. Quantitative mapping analysis of the site of programmed cell death. We indicate the number of cells expressing activated caspase-3 within and outside the *reaper*-expression domains. n, number of tarsal segments analyzed.

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Fig. S1. Cell death is required in the distal leg for joint formation. (A-C) A pupal leg disc with clones mutant for *DfH99*, marked by the lack of GFP expression (in green; A), showing absence of caspase-3 signal (in blue in B) within the clones (outlined in white). In C there is a merged image, also marked with phalloïdin (in red). (D-F) A *Dll*-Gal4/UAS-p35 UAS-GFP (D-F) pupal leg disc showing absence of caspase-3 signal (in blue in E). The *Dll* expression domain is shown by the GFP expression (in green in F) and outlined in white, and phalloïdin expression is shown in D. (G) A distal leg with *DfH99* clones (marked with *y*) induced at 72-96h after egg laying. (H) Detail of the region boxed in G. Note the absence of joints (red arrows) in the clones. The black arrows indicate the wildtype joints, the asterisks mark some of the mutant *(y)* bristles and the mutant region is delimited by the discontinuous red line.

Fig. S2. *dpp* RNA expression in late leg discs. (A-D) Discs hybridized with a *dpp* RNA probe at mid third instar (A, A') and prepupal (B-D) stages. B and C are, respectively, dorsal and ventral views of the same disc and D is a magnification of the box in B. In A, *dpp* is expressed in dorsal and (weaker) ventral bands. (A') A detailed view of the box in A shows incipient segmental *dpp* stripes (arrows). These segmental bands are clearly shown in prepupal legs (B-D), close to the folds separating the segments.

Fig. S3. Apoptosis induction and joint formation require a sharp boundary of *dpp* signaling. White arrows indicate normal rings of *rpr* expression; red arrowheads show the absence of *rpr* or *puc* expression and blue arrowheads ectopic *rpr* or *puc* expression. (A-D'') *rpr-lacZ* (A, dorsal view), *ap*-Gal4 UAS-GFP *rpr-lacZ*/UAS-tkv^{DN} (B, dorso-lateral view) and *ap*-Gal4 UAS-GFP *rpr-lacZ*/UAS-tkv^{QD} (C, D) pupal discs where the *ap*-Gal4 domain is visualized with GFP (green), P-Mad in red and *rpr-lacZ* in blue. A', A'', B', B'', C', C'' and D', D''are magnifications of the boxes shown in A, B, C and D, respectively. See the absence of *rpr* expression when the Dpp activity border is absent (B-B'' and C-C'') and its ectopic expression when a new sharp boundary of Dpp activity (P-Mad signal) is formed (D-D''). GFP expression is outlined in A'', B'', C'' and D''. (E-H'') *puc* (*pucZ*) expression is modified like *rpr* expression when Dpp activity is altered. *ap*-Gal4 UAS-GFP; puc^{E69}/+ (E), *ap*-Gal4 UAS-GFP /*UAS-*tkv^{DN}*;* puc^{E69}/+ (F) and *ap*-Gal4 UAS-GFP/*UAS-*tkv^{QD} ; puc^{E69}/+ (G, H) pupal discs stained with phalloïdin (in red) to detect segment boundaries and anti-β−galactosidase to detect puc^{E69} expression (in blue). E', E'', F', F'', G', G'' and H', H'' are higher magnifications of the boxes shown in E, F, G and H, respectively. See the absence or reduction of folds and of *puc* expression when the Dpp activity border is absent (F', F'', G' and G'') and the ectopic *puc* expression when a new sharp Dpp activity boundary is formed (H', H''). GFP expression is outlined in E'', F'', G'' and H''. (I-K) Dorsal view of a *ptc*-Gal4 UAS-GFP/+; UAS-tkv^{QD}/tub-$Gal80^{ts}$ pupal leg disc. The region of Dpp activity is marked by GFP expression (in green in I and outlined in K), activated caspase-3 is stained in blue (I, K), and an antibody against Disc-large (Dlg) (in red in I and J) was used to mark the cell membranes. Note the activation of caspase-3 in several cells at

the border of the *tkv*^{QD} expression domain (K, arrows) and the change in cell morphology at this border, corresponding to a ectopic fold (J, arrow). (L, M) *Tkv*^{QD}-expressing clones, marked with GFP (in green in L, outlined in M), showing activated caspase-3 (in red). Note the half-ring of caspase-3 staining around one of the clones. Although caspase-3 can only be seen around one clone in this picture, it can be observed close to the clones, in other focal planes, around about 1/3 of the *tkv*^{QD}-expressing clones. M is a magnification of the box in L. The dotted line delimits GFP expression.

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Table 1.

	n	caspase+ cells inside rpr domain	caspase+ cells outside rpr domain
Third instar larva	22	205	58
Prepupa	30	165	54

n=number of tarsal segments analyzed