A DPP-mediated feed-forward loop canalizes morphogenesis during *Drosophila* dorsal closure

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Development is robust because nature has selected various mechanisms to buffer the deleterious effects of environmental and genetic variations to deliver phenotypic stability. Robustness relies on smart network motifs such as feed-forward loops (FFLs) that ensure the reliable interpretation of developmental signals. In this paper, we show that Decapentaplegic (DPP) and JNK form a coherent FFL that controls the specification and differentiation of leading edge cells during *Drosophila melanogaster* dorsal closure (DC). We provide molecular evidence that through repression by Brinker (Brk), the DPP branch of the FFL filters unwanted JNK activity. High-throughput live imaging revealed that this DPP/Brk branch is dispensable for DC under normal conditions but is required when embryos are subjected to thermal stress. Our results indicate that the wiring of DPP signaling buffers against environmental challenges and canalizes cell identity. We propose that the main function of DPP pathway during *Drosophila* DC is to ensure robust morphogenesis, a distinct function from its well-established ability to spread spatial information.

**Introduction**

Mechanisms that achieve robustness evolved to cope with environmental stress or genomic instability. This buffering process, known as canalization (Waddington, 1959), stores genotypic diversity and minimizes phenotypic plasticity (Paaby and Rockman, 2014). When canalization is overwhelmed, cryptic genetic variations are unleashed for natural selection to act upon (Rutherford and Lindquist, 1998; Rohner et al., 2013). A well-known biological network that conveys robustness is the feed-forward loop (FFL), in which molecule A controls the expression of a branch component B, and A and B together act on a common target (Milo et al., 2002; Mangan and Alon, 2003). FFLs control patterning both in the *Drosophila melanogaster* embryo (Xu et al., 2005), the wing imaginal disc (Zecca and Struhl, 2007), and in the developing eye (Tsuda et al., 2002). In addition, miRNAs have been shown to form FFLs that regulate canalization (Posadas and Carthew, 2014).

Dorsal closure (DC) in the *Drosophila* embryo provides an elegant system to study robustness: hundreds of leading edge (LE) cells differentiate and act in concert to seal the dorsal opening in a process reminiscent of wound healing (Martin and Parkhurst, 2004; Belacortu and Paricio, 2011). LE cells are polarized, display strong adherent junctions, accumulate a dense microtubule network, and produce a trans-cellular actomyosin cable and filopodia (Jacinto et al., 2000, 2002; Kaltschmidt et al., 2002; Jankovics and Brunner, 2006; Fernández et al., 2007; Millard and Martin, 2008; Solon et al., 2009). The closure dynamics are highly reproducible at a given temperature, indicating that DC is a robust and quantifiable process (Kiehart et al., 2000; Hutson et al., 2003).

Two major developmental pathways control DC: the stress response pathway JNK acts upstream and induces the bone morphogenetic protein homologue Decapentaplegic (DPP; Glise and Noselli, 1997; Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar and Hafen, 1997). These two signaling pathways are crucial for DC since embryos mutant for either JNK or DPP pathway components fail to close dorsally and exhibit a dorsal open phenotype (Affolter et al., 1994; Glise et al., 1995). However, how JNK and DPP contribute to DC and how the signals are integrated in a robust manner remain unclear (Riesgo-Escovar and Hafen, 1997; Martin and Parkhurst, 2004; Ríos-Barrera and Riesgo-Escovar, 2013).

Here we report that DPP and JNK are wired in a coherent FFL that controls LE cell identity and differentiation.

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Abbreviations used in this paper: ANOVA, analysis of variance; Brk, Brinker; DC, dorsal closure; DPP, Decapentaplegic; FFL, feed-forward loop; Jar, Jaguar; LE, leading edge; tkv, thick veins; UAS, upstream activation sequence.
Figure 1. **DPP signaling is required for Jupiter, Jar, and Zasp52 LE expression during DC.** (A–C) Embryos at stage (S) 12 (A), 13 (B), and 15 (C) displaying Jupiter::GFP (green; gray in A’, B’, and C’), Jar (red; gray in A’, B’, and C’), and Zasp52 (blue; gray in A’, B’, and C’). Bars, 50 µm. (D–G) Control (D and E) and tkvΔ (F and G) stage 12 embryos marked for Jupiter::GFP (green in D and F; gray in D’ and F’), Jar (red in D and F; gray in D’ and F’), and E-Cadherin (blue in D and F), or Zasp52 (green in E and G) and E-Cadherin (magenta in E and G). Bars, 10 µm. (H–H’) Plot profile of Jupiter::GFP (n = 8), Jar (n = 8), and Zasp52 (n = 10) intensity in control and tkvΔ embryos. AS, amnioserosa; LE, leading edge; Lat.E, lateral epidermis. (Two-way ANOVA and Bonferroni post-hoc test: ***, P < 0.001.) Accumulation of Jupiter::GFP, Jar, and Zasp52 at the LE is lost in tkvΔ embryos (arrowheads). Error bars are means ± SEM.
mechanistic level, we provide evidence that derepression by the transcription factor Brk is sufficient to mediate DPP input. We show that the DPP/Brk indirect branch of the FFL does not pattern the LE but can filter unwanted JNK signaling so that the developmental JNK input remains preserved. Interestingly, although the DPP/Brk indirect branch of the FFL is dispensable for DC at 25°C, it is critical at 32°C. We propose that DPP function during DC is to ensure the robust interpretation of the positional information provided by JNK. By being wired into the FFL, DPP signaling acts as a filter rather than a positional signal and fosters the canalization of morphogenesis.

Results

DPP is required for Jupiter, Jaguar (Jar), and Zasp52 accumulation at the LE

We first analyzed three markers that display a strong accumulation at the LE during DC: the myosin VI homologue Jar (Kellerman and Miller, 1992), the microtubule binding molecule Jupiter (Morin et al., 2001; Karpova et al., 2006), and Zasp52, which promotes integrin-mediated adhesion (Morin et al., 2001; Jani and Schöck, 2007). To determine whether DPP signaling is required for their accumulation, we analyzed these three markers in embryos mutant for the DPP receptor thick veins (tkv) at stage 12, during which morphological defects are not yet detected. We observed that the LE accumulation of all three markers is lost in tkv mutant embryos compared with controls (Fig. 1, D–G; see Fig. 1, H–H′ for quantifications). Therefore, LE accumulation of all three targets requires DPP activity.

We next wondered how DPP mediates its effect on the markers. Indeed, DPP is known to induce two classes of targets that are both repressed by brinker (brk). Upon DPP action, Brk is transcriptionally repressed (Jaźwińska et al., 1999), leading to the induction of the first set of targets. The expression of the second set, however, requires the concomitant activation by the SMAD family of transcriptional activators (Affolter and Basler, 2007). Interestingly, loss of Brk is sufficient to rescue DC in the absence of pathway activation, suggesting that the DPP targets required for DC are expressed upon Brk derepression only (Marty et al., 2000). We hence tested whether removing Brk activity in the absence of DPP activation rescues Jar, Jupiter, and Zasp52 expression at the LE. To do so, we generated embryos double mutant for brk and tkv, to simultaneously disable DPP activation and prevent repression by Brk (Fig. S1 A). In these embryos, Jar, Jupiter, and Zasp52 expression is restored to wild type (Fig. 2, A–F′). In addition, brk overexpression represses
JNK and DPP form a coherent FFL that regulates cell differentiation. [A] Experimental design. The wild-type (WT) cell (black rectangle) secretes DPP (red dots) that induces its pathway in all cells (red nuclei). The absence of target (green) in the Prd>BskDN cell abutting the wild-type cell indicates the presence of a JNK/DPP FFL ([B–C']) Prd-Gal4, UAS-bskDN, Dpp-lacZ embryos marked for Jupiter:GFP (green in B; gray in B') or Zasp52::GFP (green in C; gray in C'), phospho-Mad (red in B and C; gray in B' and C'), and lacZ (blue in B and C; gray in B' and C'). The brackets indicate the BskDN domain, where DPP-lacZ (blue) is off. Anti-phospho-Mad (red) indicates that all cells receive DPP. Jupiter (B) and Zasp52 (C) in green are excluded from the BskDN territory, even though DPP signaling is active (arrowheads), indicating that JNK acts also in parallel of DPP. [D–D'] Prd-Gal4, UAS-bskDN, Dpp-lacZ embryos marked for Jupiter:GFP (green in D; gray in D') and lacZ (blue in D; gray in D'). The brackets indicate the BskDN domain, where DPP-lacZ (blue) is off. Anti–phospho-Mad (red) indicates that all cells receive DPP. Jupiter (B) and Zasp52 (C) in green are excluded from the BskDN territory, even though DPP signaling is active (arrowheads), indicating that JNK acts also in parallel of DPP. [E] Prd-Gal4, UAS-hepACT, Dpp-lacZ embryos marked for Zasp52::GFP and lacZ. All the markers are lost in the entire BskDN territory (brackets in B–D or dotted lines in E). [F] Prd-Gal4, UAS-hepACT, Jupiter::GFP (green in F; gray in F'), Jar (red in F; gray in F'), and lacZ (blue in F; gray in F'). [G–H] Prd-Gal4, UAS-hepACT, Dpp-lacZ embryos marked for lacZ (magenta in G and H; gray in G') and Zasp52 (green in G; gray in G') or Zasp52::GFP (green in H). Ectopic JNK activity (dotted lines) induces Jar, Jupiter, and Zasp52 accumulation (arrowheads). Bars, 10 µm.

The three markers (Fig. S1, B–B'). We conclude that repression of brk alone is sufficient for the accumulation of Jar, Jupiter, and Zasp52 at the LE.

DPP does not delineate Jupiter, Jar, and Zasp52 expression pattern

DPP is the best example of a secreted morphogen, a factor that patterns gene expression in a concentration-dependent manner (Nellen et al., 1996). In the wing imaginal disc, Brk activity dictates the boundaries of the DPP targets Salm and Omb, whose expression patterns expand in brk+ clones (Jażwińska et al., 1999). In contrast, at the LE, the expression patterns of Jar, Jupiter, and Zasp52 remain unchanged in tkv brk or brk embryos (Fig. 2, E–F'; and Fig. S1, C–H'). In addition, the phospho-Mad pattern is broader than the Jupiter, Jar, and Zasp52 pattern, suggesting that, instead of delineating the boundaries of the expression of these targets, DPP may fulfill a function different from its well-established patterning activity (Fig. 2, G and H; Dorfman and Shilo, 2001). We further confirmed that ectopic activation of the DPP pathway in paired stripes fails to induce these targets outside the LE, indicating that DPP does not define the boundary of the expression patterns of the three markers during DC (Fig. 2, G–H'). What then, is the factor that limits their expression pattern, and what is the biological significance of DPP control of Jar, Jupiter, and Zasp52?
JNK and DPP are wired into a coherent FFL that controls LE cell differentiation

JNK acts upstream of DPP and determines LE identity (Glise and Noselli, 1997; Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar and Hafen, 1997). To test whether JNK activates the targets in parallel to DPP, we expressed a dominant-negative form of the JNK homologue basket (bsk) in paired stripes so that cells in the paired domain are deficient for JNK signaling but still receive DPP from their wild-type neighbors by diffusion (Fig. 3 A). We reasoned that if the expression of the markers does not require JNK activity in parallel to DPP, the markers should remain expressed in the cells in which JNK is affected as long as they receive DPP. We found that DPP produced by the neighboring cells efficiently induces Mad phosphorylation in the paired domain, yet the targets are not expressed (Fig. 3, B–E). Therefore, JNK acts both upstream and in parallel to DPP to control Jar, Jupiter, and Zasp52. To confirm that JNK directs the pattern of Jar, Jupiter, and Zasp52, we induced ectopic JNK signaling in paired stripes and used DPP-lacZ as a reporter of JNK activity. All the cells in which DPP-lacZ is induced also express Jar, Jupiter, and Zasp52 (Fig. 3, F–H). These observations indicate that JNK and DPP form a coherent FFL, in which JNK induces DPP, and both signals are absolutely required for target gene expression.

We next asked whether the FFL controls LE cell differentiation. We selectively inactivated in paired stripes, either JNK by using bsk
(4, A–D”) or DPP input by overexpressing brk (Fig. 4, E–H”) and analyzed microtubule polarization, actomyosin cable, filopodia formation, and junctional integrity. Impairing either JNK or DPP signal affects the hallmarks of LE cell differentiation: First, microtubules fail to polarize and accumulate (Fig. 4, A–D”; see Fig. 4, I–N for quantifications). We conclude that both branches of the FFL are absolutely required for LE cell differentiation and morphogenesis.

A prediction of this model is that ectopic JNK, but not ectopic DPP, should redirect lateral cells to the LE cell identity and path of differentiation. We tested this prediction by inducing either JNK activity or DPP signaling in stripes (Fig. 5, A–D” and E–H’, respectively). As expected for an FFL, ectopic JNK induces ectopic accumulation of microtubules (Fig. 5, A–A”) and actin (Fig. 5, B–B”) as well as E-Cadherin and β-catenin (Fig. 5, C–D”). Conversely, ectopic activation of the DPP pathway has no effect on microtubules, actin, E-Cadherin, or β-catenin accumulation (Fig. 5, E–H’). Altogether, these data indicate that we identified a novel FFL that plays a pivotal role in LE cells specification and differentiation.
The JNK/DPP FFL can filter unwanted JNK signaling

FFLs can act as filters of short bursts of signaling (Milo et al., 2002; Mangan and Alon, 2003), which are random noises that make biological processes error prone if unchecked. In this paradigm, signaling robustness is achieved in that the synchrony between the two branches of the FFL is absolutely required for a response to occur. If the direct signal switches off before the indirect signal fires, no response can be elicited. We reasoned that in the JNK/DPP FFL, brk-mediated repression is the sentinel that prevents unwanted JNK activity from specifying ectopic LE identity. To test this hypothesis, we needed to first produce a source of ectopic JNK signal that is nonuniform and subsequently verify whether the FFL can indeed filter out such unwanted JNK activity to canalize LE identity. A previous study and our observations indicate that puc mutant embryos display a salt-and-pepper pattern of ectopic JNK activation throughout the lateral epidermis, suggesting the presence of nonuniform, ectopic JNK signal that varies in strength (Martín-Blanco et al., 1998). To test whether the FFL can filter the ectopic JNK signal in puc embryos, we generated puc brk double mutants and found that the ectopic JNK expression and the morphological defects are magnified compared with puc single mutants, suggesting that more cells respond erroneously to the action of the unwanted JNK signal when the FFL is disabled (Fig. 6, A–D). A critical aspect of the FFL is that the filtering ability depends on the delay between the activation of the direct and the indirect branch: any signal shorter than the delay is filtered out. We reasoned that the uneven JNK activity pattern reflects signal duration and could provide us with a nice system to test whether transient and robust JNK inputs are discriminated by the FFL: weak Jun staining corresponds to short accumulation of Jun and reveals transient signaling; strong Jun staining corresponds to an accumulation of Jun synthesis over
Discussion

We present a novel mechanism that weaves two classic signaling pathways into an FFL to canalize morphogenesis. This FFL is coherent as both JNK and DPP act positively and belong to the “and” type, as either signal alone does not trigger a response. Both experimental and computational evidence indicate that the general function of the indirect branch of a coherent FFL is to filter the input received by the direct branch (Mangan and Alon, 2003). Here, we find that during DC, patterning information is given by JNK, and the DPP/Brk branch filters this spatial information. In the presence of ectopic JNK generated by puckered loss of function, Brk filters out unwanted JNK signaling in two thirds of the cells displaying weak, but not strong, JNK activation. This is a prediction of the FFL model in which the network filters out only short bursts of signal and not longer, more robust signaling events. Interestingly, under normal laboratory conditions, at 25°C, Brk activity is not required for DC to proceed normally; LE markers are patterned correctly, and the dynamics of DC are nearly wild-type. Conversely, when embryos are subjected to thermal stress, at 32°C, Brk becomes critical to prevent the presence of ectopic LE cells in the lateral epidermis and to ensure proper closure dynamics. These observations provide strong evidence to support that DPP function during DC is to provide robustness to the system: under difficult conditions, phenotypic variation remains minimal, and cell identity remains canalized.

miRNAs are major players in the canalization of cell decisions in the face of environmental challenges (Posadas and Carthew, 2014): mir-7 stabilizes gene expression and allows the correct determination of sensory organs in flies subjected to temperature fluctuations (Li et al., 2009). miRNAs are
prediction is that DPP-mediated FFL filters JNK inputs that are on a long time scale: DPP would not only filter out JNK noise but could also filter out authentic JNK signaling that is important for nonpatterning functions. JNK is the main messenger of stress, and mechanisms must exist to distinguish stress-related and development-related JNK inputs within a given cell. This would explain why brk mutants close normally in favorable conditions. Environmental perturbations such as temperature excess are bound to have pleiotropic effects on biological systems. The FFL appears as the generic remedy to enforce robustness at several levels. Factors acting at specific kinetics form the indirect branches of FFLs adapted to specific needs: miRNAs cancel noise, and DPP ensures the proper interpretation of JNK signaling.

DPP is one of the main architects of fly development and as such fulfills many functions during embryogenesis: DPP specifies dorsal tissues, including the amnioserosa early and the dorsal epidermis at midembryogenesis (Ferguson and Anderson, 1992; Xu et al., 2005) and also directs dorsal tracheal migration (Vincent et al., 1997). At stage 5, DPP induces zerknült, and both DPP and Zerknült control the amnioserosa-specific gene Race, thus forming a coherent FFL (Xu et al., 2005). In addition,
DPP also controls the spatial distribution of targets such as Ustaped, in both the dorsal epidermis and the amnioserosa (Lada et al., 2012). This regulation is important for the interaction between these two tissues that is critical for DC. Recently, a study reported how DPP can protect from JNK-induced apoptosis in the dorsal epidermis (Beira et al., 2014). They show that the DPP pathway repressor Schnurri directly represses the pro-apoptotic gene reaper. Therefore, JNK fails to induce reaper expression or apoptosis in the pannier domain. This indicates that JNK and DPP signaling pathways are reiteratively integrated during Drosophila embryogenesis. To get a full picture of this network, we will also need to integrate the two negative feedback loops mediated by Puc and scarface that dampen JNK activity (Martin-Blanco et al., 1998; Rousset et al., 2010). A likely possibility is that these feedback loops improve fidelity in signaling. Altogether, the dorsal epidermis provides an elegant model system to understand how different inputs are integrated to modulate cell decisions during development. Although some of these functions are paramount to cell specification, we show that some, such as the JNK/DPP-FFL, can also counteract deleterious environmental stimuli and canalize development, a function distinct from DPP well-established, non–cell-autonomous patterning activity.

Materials and methods

Fly strains and genetics

We used the following lines: Canton-S (wild type), tkv<sup>Δ</sup> (amorphic allele; Bloomington Stock Center [BL] 34509), Brk<sup> ΔΔ</sup> (loss-of-function allele, see Jazwińska et al., 1999), gift from M. Affolter (University of Basel, Basel, Switzerland). Puc<sup>ΔΔ</sup> (loss-of-function allele, see Martin-Blanco et al., 1998), Pd-Gal (an upstream activation sequence [UAS]-<sup>Δ</sup>act [BL 36537], gift from M. Grammont (Université de Lyon, Lyon, France), UAS-brk<sup>ΔΔ</sup> (BL 6409), UAS-hep<sup>ΔΔ</sup> (BL 9306), UAS-brk [brk coding sequence under the control of a promoter containing UAS sequence], gift from J. de Celis (Centro de Biología Molecular “Severo Ochoa,” Madrid, Spain), UAS-GFP<sup>Δ</sup> (BL 4775), Jupiter: GFP [GFP knock-in; BL 6836], Zasp52::GFP [GFP knock-in; BL 6838], and DPP-lacZ<sup>ΔNDEL</sup> [lacZ-ΔNLS coding sequence cloned after the BS 3.0 promoter of DPP, see Blackman et al., 1991]. Unless otherwise indicated, all crosses were performed at 25°C.

Immunofluorescence and quantification

We used standard techniques of immunohistofluorescence as described in Ducuing et al. (2013). Embryos were dechorionated with bleach, fixed in 4% PFA–heptane. Samples were incubated with primary antibodies, with fluorescent-coupled secondary antibodies and mounted in Vectashield.

We used the following primary antibodies: rabbit anti-lacZ (1:1,000; Cappel), mouse anti-lacZ (1:250; G4644; Sigma-Aldrich), guinea pig anti-brk (1:500; gift from G. Morata, Centro de Biología Molecular “Severo Ochoa,” Madrid, Spain), mouse anti-Jar 3C7 (1:100; Kellerman and Miller, 1992), rabbit anti-pMad (1:500; gift from P. ten Dijke, Leiden University, Leiden, Netherlands), rat anti–DE-Cadherin (1:333; Devel-Swiss et al., 1999), gift from M. Affolter (University of Basel, Basel, Switzerland), mouse anti–Armadillo (1:250; DSHB), mouse anti-Armadillo (1:250; Devel-Swiss et al., 1999), mouse anti-Brk (1:500; gift from G. Morata, Centro de Biología Molecular “Severo Ochoa,” Madrid, Spain), mouse anti-lacZ (1:250; G4644; Sigma-Aldrich), guinea pig anti-Brk (1:500; gift from G. Morata, Centro de Biología Molecular “Severo Ochoa,” Madrid, Spain), mouse anti-Jar 3C7 (1:100; Kellerman and Miller, 1992), rabbit anti-pMad (1:500; gift from P. ten Dijke, Leids Universitair Medisch, Leiden, Netherlands), rat anti–DE-Cadherin (1:333; Devel-Swiss et al., 1999), mouse anti-Armadillo (1:250; DSHB), mouse anti–a-tubulin (1:1,000; 16199; Sigma-Aldrich), rabbit anti-Jun (1:10; Santa Cruz Biotechnology, Inc.), and rabbit anti-Zasp52 (1:400; gift from F. Schäck, McGill university, Montreal, Quebec J. For Brk, pMad, Jar, and Zasp52, antigen was a full-length protein. Secondary antibodies are from Invitrogen and were used at 1:500. We used the following secondary antibodies: Alexa Fluor donkey anti–mouse 488, Alexa Fluor goat anti–mouse 633, Alexa Fluor goat anti–rat 546, Alexa Fluor donkey anti–rabbit 488, Alexa Fluor goat anti–rat 546, and Alexa Fluor goat anti–guinea pig 488. For 32°C experiments, embryos were first grown at 25°C and then shifted for 4 h at 32°C and immediately fixed after.

Phalloidin staining

Embryos were dechorionated with bleach and fixed in a 1:1 mix of 4% PFA–heptane. After PFA removal, embryos were stuck on double-sided tape, immerged in 0.1% Triton X-100 and PBS with Rhodamine Phalloidin (1:500; Sigma-Aldrich), and hand devitellinized with a needle. Devitellinized embryos were quickly rinsed twice with 0.1% Triton X-100 and mounted in Vectashield.

Image processing

Images were acquired on the acousto-optical beam splitter confocal laser-scanning microscope (SP5; Leica) with the following objectives: HC Plan Fluotar 20×, 0.5 multi-immersion (numerical aperture: 0.7), HCX Plan Apochromat 40× 1.25–0.75 oil (numerical aperture: 1.25), and HCX Plan Apochromat 63× 1.4–0.6 oil (numerical aperture: 1.4) using the acquisition software LAS AF (Leica) at the PLATIM imaging facility and analyzed with Image (National Institutes of Health). Unless otherwise indicated, all images are projections of confocal sections.

Live imaging

Unless otherwise indicated, all crosses were performed at 25°C. Stage 10 or 11 embryos were staged and aligned in Halocarbon oil 27 (Sigma-Aldrich) and then imaged at 25°C or 32°C with a spinning disk (Leica), with a 20× dry objective (numerical aperture: 0.4) and a camera (Key3; Andor Technology) using the acquisition software MetaMorph (Molecular Devices). brk<sup>ΔΔ</sup>/FM7 females were crossed with Jupiter::GFP males. In addition, wild-type females were crossed with Jupiter::GFP males as controls. Brk mutant embryos were identified by the absence of spontaneous movements at stage 17 and confirmed by the absence of hatching. For every sample, the length and width over time were normalized with the maximal length or maximal width, respectively.

Quantification and statistical analyses

We used the Prism software (GraphPad Software) to generate graphs. For Figs. 1, 4, 6, and 7 M, bar graphs represent means ± SEM. For Figs. 7 (N and N') and S4, graphs represent the mean. Mann–Whitney's U test was used to determine significant differences for Figs. 4 and 6 (D and G). For Figs. 1 (H–H'), 6 F, and 7 M, we used a two-way analysis of variance (ANOVA) followed by a Bonferroni posthoc test. **, P < 0.01; ***, P < 0.001.

Online supplemental material

Fig. S1 describes the experimental strategy used to determine whether the three targets belong to the derepressed only or to the derepressed and induced class of DPP targets as well as the effects of the overexpression and the loss of function on the targets' expression. Fig. S2 reports the effects of temperature on brk mutants. Fig. S3 displays the analysis of the dynamics of DCs in brk mutants at 25°C and 32°C. Video 1 is a live recording of the closure of embryos representative of the controls and brk mutants we analyzed at 25°C. Video 2 is a live recording of the closure of embryos representative of the controls and brk mutants we analyzed at 32°C. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201410042/D1C.

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**Figure S1. Effects of Brk loss of function and overexpression on Jupiter, Jar, and Zasp52.** (A) Experimental strategy. As indicated on the figure, DPP pathway has two sets of targets: targets that only require Brk repression (derepressed targets) and targets that required Brk repression and subsequent activation by the Mad–Medea complex (derepressed and induced targets). In brk\(^{-}\), tkv\(^{-}\) double mutant embryos, only derepressed targets are restored. [B–B’] Prd::Gal4, UAS-brk, Jupiter::GFP embryos marked for Jupiter::GFP (green in B; gray in B’), Jar (red in B; gray in B’), and Zasp52 (blue in B; gray in B’). Brk overexpression leads to a decrease of Jupiter::GFP, Jar, and Zasp52 expression. (C–H’) Control (C, D, and G) and brk\(^{M68}\) (E, F, and H) embryos marked for Jupiter::GFP (green in C and E; gray in C’ and E’), Jar (red in C and E; gray in C’ and E’), and E-Cadherin (blue in C and E) or Zasp52 (gray in D and F) or for Jar (gray in G’ and H’), E-Cadherin (gray in G’ and H’), and Ftz (fushi tarazu)-lacZ to detect the balancer chromosome (blue in G and H; gray in G’ and H’). The Jupiter::GFP, Jar, and Zasp52 expression pattern is similar in both control and brk\(^{M68}\) embryos. Bars, 10 µm. WT, wild type.
Figure S2. **Extended data of Fig. 7 (A–L)**. (A) Control and brk<sup>M68</sup> mutant embryos at 25°C or 32°C and stained for tubulin, cadherin, and lacZ. Bars, 50 µm. Brk<sup>M68</sup> embryos exhibit closure and head involution defects only at 32°C. (B–E') Control and brk<sup>−</sup> embryos at 25°C or 32°C marked for Jar (gray in B–E') and E-Cadherin (gray in B–E'). (F–I') Control and brk<sup>−</sup> embryos at 25°C or 32°C marked for Zasp52 (gray in F–I') and Tubulin (gray in F–I'). Bars, 10 µm. At 25°C control and brk<sup>−</sup> embryos have similar expression pattern. Only brk<sup>M68</sup> embryos at 32°C display ectopic Jar, Zasp52, and microtubule accumulation. (J–M) Zasp52 pixel intensity at the leading edge (LE) or in the lateral epidermis (Lat. Ep.) using the ImageJ plot profile function of the corresponding control (J and L) or brk<sup>M68</sup> (K and M) embryos at 25°C (J and K) or 32°C (L and M). Only brk<sup>−</sup> embryos at 32°C exhibit ectopic Zasp52 in the lateral epidermis that reaches Zasp52 LE levels. A.U., arbitrary unit.
Figure S3. Extended data of Fig. 7 (N and N’). (A–D) Still image of Jupiter::GFP/+ (A and C) and brkM68, Jupiter::GFP/+ (C and D) embryos imaged at 25°C or 32°C. Bars, 50 µm. See also Videos 1 and 2. (E and E’) Dorsal opening length over time. Only brkM68 embryos at 32°C exhibit slower closure dynamics. AS, amnioserosa. (F) Area under the curve of dorsal opening length and width graphs. (G) Brk fragility index defined as the difference of the mean Area Under the Curve (AUC) of brk− minus the mean Area Under the Curve of control for a given temperature. (H) Brk fragility index. At 32°C, brk− embryo robustness is much more affected than at 25°C.
Video 1. **Control and Brk mutant embryos (25°C).** Live imaging of a Jupiter::GFP/+ and brk<sup>M68</sup>; Jupiter::GFP/+ embryo raised and imaged at 25°C with a spinning-disk confocal microscope (Leica). Jupiter::GFP is in gray. Frames were acquired every 15 min for 6 h. The closure dynamic is similar in both genotypes. Bar, 50 µm.

Video 2. **Control and Brk mutant embryos (32°C).** Live imaging of a Jupiter::GFP/+ and brk<sup>M68</sup>; Jupiter::GFP/+ embryo raised at 25°C and imaged at 32°C with a spinning-disk confocal microscope (Leica). Jupiter::GFP is in gray. Frames were acquired every 15 min for 6 h. Closure is delayed in brk<sup>M68</sup>; Jupiter::GFP/+ embryos. Bar, 50 µm.