Development 138, 3021-3031 (2011) doi:10.1242/dev.059980
© 2011. Published by The Company of Biologists Ltd

Apical deficiency triggers JNK-dependent apoptosis in the embryonic epidermis of *Drosophila*

Golnar Kolahgar*,†, Pierre-Luc Bardet*,‡, Paul F. Langton, Cyrille Alexandre and Jean-Paul Vincent§

SUMMARY

Epithelial homeostasis and the avoidance of diseases such as cancer require the elimination of defective cells by apoptosis. Here, we investigate how loss of apical determinants triggers apoptosis in the embryonic epidermis of *Drosophila*. Transcriptional profiling and in situ hybridisation show that JNK signalling is upregulated in mutants lacking Crumbs or other apical determinants. This leads to transcriptional activation of the pro-apoptotic gene *reaper* and to apoptosis. Suppression of JNK signalling by overexpression of Puckered, a feedback inhibitor of the pathway, prevents *reaper* upregulation and apoptosis. Moreover, removal of endogenous Puckered leads to ectopic *reaper* expression. Importantly, disruption of the basolateral domain in the embryonic epidermis does not trigger JNK signalling or apoptosis. We suggest that apical, not basolateral, integrity could be intrinsically required for the survival of epithelial cells. In apically deficient embryos, JNK signalling is activated throughout the epidermis. Yet, in the dorsal region, *reaper* expression is not activated and cells survive. One characteristic of these surviving cells is that they retain discernible adherens junctions despite the apical deficit. We suggest that junctional integrity could restrain the pro-apoptotic influence of JNK signalling.

KEY WORDS: Epithelial integrity, JNK (Basket), Reaper, Apical-basal polarity, Apoptosis, *Drosophila*

INTRODUCTION

Apoptosis is an integral part of development; for example, in tissue sculpting or for the removal of cells that are no longer needed (for reviews, see Baehrecke, 2002; Meier et al., 2000). This form of apoptosis is often referred to as programmed cell death because it is a scheduled part of development. Apoptosis is also required for tissue homeostasis both in embryos and adults. Such homeostatic apoptosis contributes to the elimination of misspecified or defective cells (Pazdera et al., 1998; Werz et al., 2005). For example, detachment from an epithelium often leads to apoptosis (e.g. Gibson and Perrimon, 2005; Hughes and Krause, 2001), ensuring that loose cells do not create havoc. Although the cellular machinery that executes the apoptotic programme is relatively well characterised, our understanding of the upstream signals that trigger homeostatic apoptosis is still fragmentary. In order to approach this problem, we have investigated the apoptotic response that follows from widespread epithelial disruption.

The establishment and maintenance of epithelial integrity rely on cells acquiring apicobasal polarity. Three protein complexes that are conserved in many species are involved in this process. Two are located on the apical side: the Crumbs complex (Bulgakova and Knust, 2009), which includes Crumbs (Crb), Pals1-associated tight junction protein (Patj) and Stardust (Sdt); and the PAR complex (Suzuki and Ohno, 2006), comprising Bazooka (Par3), Par6 and Atypical protein kinase C (aPKC) (reviewed by Muller and Bossinger, 2003; Wang and Margolis, 2007) [for a more precise functional description of the apical complexes, see St Johnston and Ahringer (St Johnston and Ahringer, 2010)]. It is thought that these two complexes cooperate to counteract the basal determinant complex, which comprises Lethal giant larvae [Lgl, or L(2)gl], Discs large (Dlg) and Scribble (Scrib), thereby leading to stable apicobasal polarisation and to the formation of adherens junctions in a defined subapical belt termed the zonulae adherens (ZA) (Bilder et al., 2003; Hutterer et al., 2004; Plant et al., 2003; Tanentzapf and Tepass, 2003; Yamanaka et al., 2003). E-cadherin is a major component of adherens junctions and interfering with E-cadherin activity leads to dramatic epithelial disruption, highlighting the importance of this adhesion protein (Gumbiner, 1996; Takeichi, 1995). In addition to cell-cell adhesion, the integrity of mature epithelia also requires adhesion to the matrix, which is mediated by integrins (Bökel and Brown, 2002).

Various forms of epithelial disruption lead to apoptosis. Best understood is the process of anoikis, whereby loss of integrin signalling due to detachment from the basal lamina triggers apoptosis (reviewed by Frisch and Screaton, 2001). Failure to establish or maintain adherens junctions is also thought to cause apoptosis (Hermiston and Gordon, 1995; Shen and Kramer, 2004). This particular process is still poorly understood and indeed has been difficult to study because of confounding effects from anoikis and additional signals from the local microenvironment (Shen and Kramer, 2004; Westhoff and Fulda, 2009; Westhoff et al., 2008). Finally, disruption of apicobasal polarity can also trigger apoptosis: in *Drosophila* embryos, removal of apical determinants, such as Crb, Sdt or Baz, leads to widespread cell death (Abrams et al., 1993; Knust et al., 1993; Muller and Wieschaus, 1996; Tepass et al., 1990). In imaginal discs, removal of Crb, Sdt or Baz does not substantially affect cell polarity as redundant determinants maintain the apical domain after its initial establishment. By contrast, mutations in basolateral determinants lead to epithelial disorganisation in imaginal discs. Imaginal discs lacking basolateral

MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

*These authors contributed equally to this work
†Present address: Wellcome Trust/Cancer Research UK Gurdon Institute, Tennis Court Road, Cambridge CB2 1QN, UK
‡Present address: Institut Curie, Bâtiment de Biologie du Développement, U934/UMR3215, 26, rue d'Ulm 75248 Paris Cedex 05, France
§Author for correspondence (jvincen@nimr.mrc.ac.uk)

Accepted 10 May 2011

determinants are characterised by ectopic proliferation and neoplastic overgrowth (reviewed by Bilder, 2004). Mutations in basolateral determinants have also been reported to cause JNK-dependent apoptosis in mosaic situations (Brumby and Richardson, 2003; Igaki et al., 2006). However, this is now thought to be due to competition from the surrounding wild-type tissue (Bilder, 2004; Adachi-Yamada and O'Connor, 2002; Menéndez et al., 2010). Because studies of imaginal tissues are complicated by secondary cell interactions such as cell competition, we have sought to establish a simple and tractable system with which to study the specific features of epithelial disruption that feed into the apoptotic machinery.

The embryonic epidermis of *Drosophila* is a monolayer epithelium with a well-defined polarity. It undergoes little growth, if any (Foe et al., 1993; Martinez Arias, 1993), and is not thought to be subject to cell competition. As mentioned above, it is well established that apical disruption leads to apoptosis (e.g. in *crb* or *sdt* mutants of *Drosophila*) but the underlying mechanism has remained unknown (Abrams et al., 1993; Tepass et al., 1990). Anoikis is unlikely to contribute in this instance as genetic ablation of integrin signalling is not associated with apoptosis or tissue loss in the embryonic epidermis of *Drosophila* (Devenport and Brown, 2004). Therefore, this system is well suited to begin dissecting the intrinsic effect of epithelial disruption on apoptosis. In *Drosophila*, apoptosis is generally mediated by one or more of the five pro-apoptotic proteins Reaper (Rpr), Hid (Wrinkled – FlyBase), Grim, Sickle (Skl) and Jafrac (Abrams et al., 1993; Nordstrom et al., 1996; Srinivasula et al., 2002; Tenev et al., 2002; White et al., 1994; Wing et al., 2002). These proteins, which are also known as inhibitor of apoptosis protein (IAP) antagonists, act by binding Diap1 (Thread – FlyBase), which normally keeps caspases inactive. IAP antagonists in turn act by targeting Diap1 for proteasomal degradation (Ditzel et al., 2003; Steller, 2008; Zachariou et al., 2003). This allows apical caspases to become active and to initiate the apoptotic cascade. In *crb* mutants that are also deficient at the *H99* locus, which encodes *rpr*, *hid* and *grim*, cell death no longer occurs even though epithelial organisation is still disrupted. Therefore, apoptosis is a downstream consequence of loss of polarity and not the other way around (Bilder et al., 2003; Tanentzapf and Tepass, 2003). The specific features of *crb* mutants that trigger apoptosis remain unknown.

Here, we first describe the spatiotemporal pattern of cell death in *crb* mutant embryos. In such mutants, most cells of the epidermis undergo *rpr*-dependent apoptosis, but a subset located in a dorsal band does not express *rpr* and survives. This behaviour is also seen in mutants lacking other apical determinants, whereas no excess apoptosis is seen in basolateral mutants. Microarray and in situ expression analyses reveal that several transcriptional targets of JNK signalling are activated both in dying and protected cells of *crb* mutants. Genetic analysis shows that JNK signalling is essential for *rpr* expression and hence for apoptosis in apically compromised cells. We find that the (dorsal) surviving cells of *crb* mutant embryos maintain residual junctional integrity and suggest that this could protect them from the potentially lethal effect of JNK signalling. In addition, endogenous Puckered (Puc), a feedback inhibitor of JNK signalling (Martin-Blanco et al., 1998), also prevents activation of *rpr* expression in these dorsal cells. Therefore, apical deficit triggers JNK signalling, which in turn activates *rpr* expression and apoptosis in a context-dependent manner.

MATERIALS AND METHODS

Fly stocks and genetics

As wild type, *yw* was used. As marked balancers, we used *TTG* (*TM3, twistGal4>UASGFP*), *TDY* (*TM6, Dfd-YFP*), *CKG* (*Cyo, Kr-Gal4>UASGFP*) and *CTG* (*Cyo, twistGal4>UASGFP*). Other stocks (numbered for clarity) were: (1) crb^{2}/*TTG*; (2) *Def(H99),* crb^{2}/*TDY*; (3) baz^{xi106}, *FRT9.2/FM7*$^{KrGal4,\ UASGFP}$ (from Y. Bellaiche, Institut Curie, Paris); (4) sdt^{7D22}/*FM7*^{ftzlacZ} (from E. Knust, Max-Planck Institute, Dresden, Germany); (5) *pnrGal4*^{MD231}/*TM3* (from G. Morata, University Autonoma, Madrid, Spain); (6) *pnrGal4*^{MD231}, crb^{2}/*TDY*; (7) *UAS-CD8-GFP,* crb^{2}/*TM6*; (8) puc^{E69}/*TM6* (Martin-Blanco et al., 1998); (9) puc^{E69}, crb^{2}/*TDY*; (10) $l(2)gl^{4}$/*CKG; UAS CD8 GFP,* crb^{2}/*TDY*; (11) shg^{g317}/*CKG; UAS CD8 GFP,* crb^{2}/*TDY* (shg^{g317} obtained from N. Gorfinkiel, University of Cambridge, UK); (12) $l(2)gl^{27S3}$, *FRT40A/CTG* [$l(2)gl^{27S3}$ from Brumby et al. (Brumby et al., 2004)]; (13) *hsflp;* Adv^{1}/*CyO*; (14) *P{ovoD1-18}2La P{ovoD1-18}2Lb P{neoFRT}40A/Dp(?;2)bw[D], S[1] wg[Sp-1] Ms(2)M[1] bw[D]/CyO*; (15) dlg^{M52}, *FRT101/FM7*^{lacZ} (Woods and Bryant, 1991); (16) *w ovoD1 FRT101/Y; hsflp38*; (17) egr^{1} (Igaki et al., 2002); (18) Kr^{9} (from H. Skaer, University of Cambridge, UK); (19) *Def(3L)XR38,* crb^{2}/*TTG*; (20) *Def(3L)X14,* crb^{2}/*TTG*; (21) *tubulinGal4,* crb^{2}/*TM3*; (22) *UAS* Puc^{14C} (Martin Blanco et al., 1998); and (23) *UAS-crb-RNAi* (VDRC line 39178).

Embryo stainings

Embryos were dechorionated in 50% bleach for 2 minutes, washed and then fixed for 20 minutes in 6% paraformaldehyde diluted in PBS for in situ hybridisation, or for 5 minutes in 37% formaldehyde for phalloidin staining, or in 4% paraformaldehyde diluted in borate-buffered saline (BBS) supplemented with 0.1 mM $CaCl_2$ for E-cadherin staining. After fixation, embryos were devitellinised in methanol or by hand (for phalloidin stainings).

Primary antibodies were mouse anti-Engrailed [mAb4D9, Developmental Studies Hybridoma Bank (DSHB), 1/200], rabbit anti-cleaved Caspase 3 (Cell Signaling, 1/100), chicken anti-β-galactosidase (Abcam, 1/1500), chicken anti-GFP (Abcam, 1/1500), rabbit anti-GFP (Molecular Probes, 1/1000), rabbit anti-Sas (1/5000, from E. Knust), mouse anti-Fasciclin III (7G10, DSHB, 1/100), mouse anti-Dlg (4F3, DSHB, 1/50), rat anti-E-cadherin (DCAD2, DSHB, 1/20) and mouse anti-Sxl (M18, DSHB, 1/50). Secondary antibodies were anti-mouse, anti-rabbit or anti-chicken coupled to Alexa Fluor 488, 555 or Cy5 (Molecular Probes) or to Biotin (Jackson). Rhodamine-coupled phalloidin (Molecular Probes) was used at 1/30, and TOPRO-3 (Molecular Probes) was used at 1/20,000. Stained embryos were mounted in Vectashield (Vector Laboratories) and images were obtained using a SP5 laser-scanning confocal microscope (Leica) and assembled in Photoshop (Adobe) and Volocity (Improvision).

In situ hybridisation

RNA expression patterns were visualised using digoxigenin-labelled antisense RNA probes prepared from 1-2 μg linearised DNA templates. Hybridisation and detection were performed according to standard protocols. Sheep anti-digoxigenin coupled to alkaline phosphatase was used (Roche, 1/2000).

Cuticle preparation and rescue analysis

Embryos were collected for 12 hours at 25°C on a grape juice plate and allowed to age until cuticle formation, ~24 hours post-laying. Embryos were dechorionated in 50% bleach, mounted in 1:1 lactic acid:Hoyer's and then incubated overnight at 65°C. For rescue experiments, females of the genotype *w;;tubGal4,* crb^{2}/*TM3* were crossed to males from an F1 of the genotype *w; UAS-X/CyO*$^{wg\text{-}lacZ}$*; UAS-CD8GFP,* crb^{2}/+ (where *X* refers to the particular gene to be overexpressed). GFP-positive embryos were preselected before mounting.

Affymetrix microarray and data analysis

Mutant (crb^{2}) and control (crb^{2}/TTG) embryos were sorted under a dissecting fluorescence microscope. They were aged to stage 10, 11 and 12, washed twice in double-distilled water, snap-frozen in liquid nitrogen and stored at –80°C until use. Total RNA of three biological

replicates for each condition (25-35 embryos each) was extracted and purified using the RNeasy kit (Qiagen). Subsequent RNA processing was performed at the Affymetrix Microarray Facility of the NIMR, London, UK. RNA quality was assessed using a 2100 Agilent Bioanalyzer. Total RNA (40-80 ng/μl) underwent two rounds of amplification and the resulting labeled RNA was hybridised onto the GeneChip Drosophila 2.0 Array (Affymetrix) following the manufacturer's instructions. Data analysis was performed at the Bloomsbury Centre, UCL, London, UK. Differential expression was assessed with the LIMMA Bioconductor package (Gentleman et al., 2004). The Benjamini-Hochberg (FDR) test for multiple corrections was used with a *P*-value cut-off of 0.05 to correct for multiple testing. The *t*-tests were performed on GCRMA-normalised data. Probesets called absent by the MAS5 algorithm in more than 16 samples were considered to be unexpressed in all samples and were filtered out. The data *P*-values were corrected using the Bonferroni multiple testing correction. An adjusted *P*-value cut-off of 0.05 and log fold change of 1 (fold change 2) were used as thresholds to select differentially expressed genes.

RESULTS

Cell death is patterned in *crb* mutant embryos

At the end of embryogenesis, epidermal cells normally secrete a protective cuticle on their apical (outer) surface. Segmentally repeated bands of cells also secrete hair-like protrusions called denticles on both the ventral and dorsal surfaces. In *crb* mutant embryos, most epidermal cells undergo apoptosis (Abrams et al., 1993; Knust et al., 1993; Tepass and Knust, 1993). However, some cells do survive and assemble into multicellular rosettes with their apical surface facing toward the lumen, where, at the end of embryogenesis, they deposit cuticle, including denticles (Tanentzapf and Tepass, 2003). Therefore, whereas wild-type epidermal cells secrete a continuous sheet of cuticle, surviving epidermal *crb* mutant cells form crumbs of cuticle (Tepass et al., 1990). To examine whether these surviving cells arise at random or whether specific groups of epidermal cells reproducibly escape death, we mapped the pattern of apoptosis in *crb* mutant embryos. Apoptotic cells were recognised with an anti-activated Caspase 3 antibody or with Apoliner, a fluorescent reporter of caspase activity (Bardet et al., 2008). As spatial landmarks along the anteroposterior and dorsoventral axes, we used an anti-Engrailed antibody to mark the posterior part of each segment or an enhancer trap in the *pannier* gene, which is expressed in the dorsal epidermis (Heitzler et al., 1996; Herranz and Morata, 2001).

Staining with anti-activated Caspase 3 antibodies revealed the presence of apoptotic cells in wild-type embryos from stage 11 [5.5-7.5 hours after egg laying (AEL)]. They were mainly detected in the head region and at the posterior of the germ band, although dying cells were also seen sporadically in the trunk (Fig. 1A). In *crb* mutant embryos, excess immunoreactivity was readily seen at stage 11, in the head region (bracket, Fig. 1B) as well as in the trunk, especially in the area between each Engrailed stripe (arrows, Fig. 1B). Beyond stage 11, as the segmental pattern of cell death intensified, it became apparent that the dorsal region remains largely Caspase 3 negative. This region corresponds roughly to the domain of *pannier* expression as marked with *pannier-Gal4 UAS-GFP* (Fig. 1D,F). The dorsoventral bias was confirmed with Apoliner, an RFP-GFP fusion protein that resides at cell membranes in live cells and releases its GFP moiety upon caspase activation (Bardet et al., 2008); thus, live cells appear yellow, whereas dying cells appear red because the GFP moiety is relatively unstable after cleavage. At stage 14 (10.5-11.5 hours AEL), the ventral area appeared generally red, an indication of caspase activity, whereas the dorsal region remained a coherent sheet of yellow cells (Fig. 1H). At this stage, the whole ventral epidermis, including the cells within the Engrailed stripes, appeared to undergo apoptosis (data not shown).

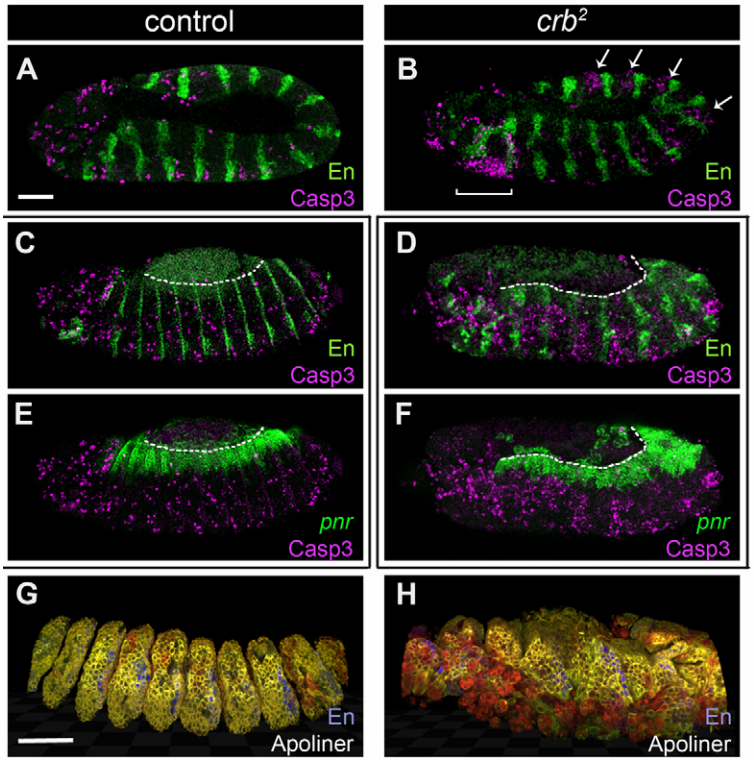

Fig. 1. The ventral epidermis undergoes apoptosis in *crb* mutant *Drosophila* embryos. (**A**,**B**) Anti-Caspase 3 immunoreactivity (magenta) at stage 11 in control (A, *crb* heterozygous) and *crb* homozygous (B) embryos. At this stage, activated Caspase 3 is already clearly elevated in the head region (bracket) and a moderate segmental increase can be seen in the trunk (arrows). Anti-Engrailed (En, green) was used as a reference along the anteroposterior axis. (**C-F**) At stage 13, anti-Caspase 3 staining has further increased in the trunk region. These embryos were triple stained for activated Caspase 3, Engrailed and GFP expressed under the control of *pannier-Gal4*. Activated Caspase 3 and Engrailed are shown in C (control, *crb* heterozygous) and D (*crb* homozygous), whereas activated Caspase 3 and GFP are shown in E (*crb* heterozygous) and F (*crb* homozygous). The domain of *pannier* expression is largely devoid of Caspase 3-positive cells in *crb* mutants. The dashed line marks the dorsal edge of the epidermis. (**G**,**H**) Stage 14 control (G, *crb* heterozygous) and *crb* homozygous (H) embryos expressing Apoliner under the control of the ubiquitous *tubulin-Gal4* driver. With this sensor, live cells appear yellow. A band of live cells remains on the dorsal side, whereas no live cells remain ventrally in the *crb* mutant. (A-F) Projections of a confocal stack; (G,H) Generated by 3D rendering. See also Fig. S1 in the supplementary material. Scale bars: 50 μm (in A for A-F; in G for G,H).

The above observations suggest that the cells in the dorsal epidermis of *crb* mutant embryos undergo relatively little apoptosis and could contribute to the remnants of cuticle (including denticles) seen at the end of embryogenesis. To test this, *pannier-Gal4* and *UAS-GFP* were used to mark dorsal cells and to follow their fate up to a time when denticle precursors can be recognised. Embryos were examined at stage 16, when the actin bundles that serve as a template for denticle formation can be recognised with Rhodamine-phalloidin (Dickinson and Thatcher, 1997). At this stage, the surviving epidermal cells of *crb* mutant embryos had assembled into rosettes. Most of these rosettes were GFP-positive (50 out of 55 rosettes counted in five embryos), indicating that they originated from the dorsal epidermis, and were often seen to produce inward-pointing actin bundles, anticipating dorsal denticles (see Fig. S1 in the supplementary material). We conclude that, in the absence of *crb*, most of the ventral trunk epidermis undergoes apoptosis, initially in a segmental manner and subsequently along the whole anteroposterior axis. By contrast, most cells of the dorsal epidermis appear to survive.

Apoptosis in *crb* mutant embryos requires transcriptional activation of *rpr*

RNA in situ hybridisation was used to determine the expression pattern of characterised pro-apoptotic genes in the ventral epidermis of *crb* mutant embryos. *rpr* and *skl* transcripts were upregulated whereas the expression of *hid* and *grim* was similar to that in control heterozygous embryos (Fig. 2A-J). At stage 11, expression of *rpr* had a clear segmental aspect (Nordstrom et al., 1996), with higher expression between the stripes of Engrailed expression (Fig. 2D). Upregulation of *rpr* and *skl* expression was confined to the ventral epidermis; expression remained silent in the dorsal region. Therefore, the pattern of *rpr* expression in the trunk epidermis of *crb* mutant embryos broadly correlates with the pattern of apoptosis.

Next, we sought to determine which pro-apoptotic genes are required to mediate apoptosis in *crb* mutants. Single mutations are not available for each of the pro-apoptotic genes. However, various deficiencies can be used to remove these genes in combination (Peterson et al., 2002; White et al., 1994). Some of these

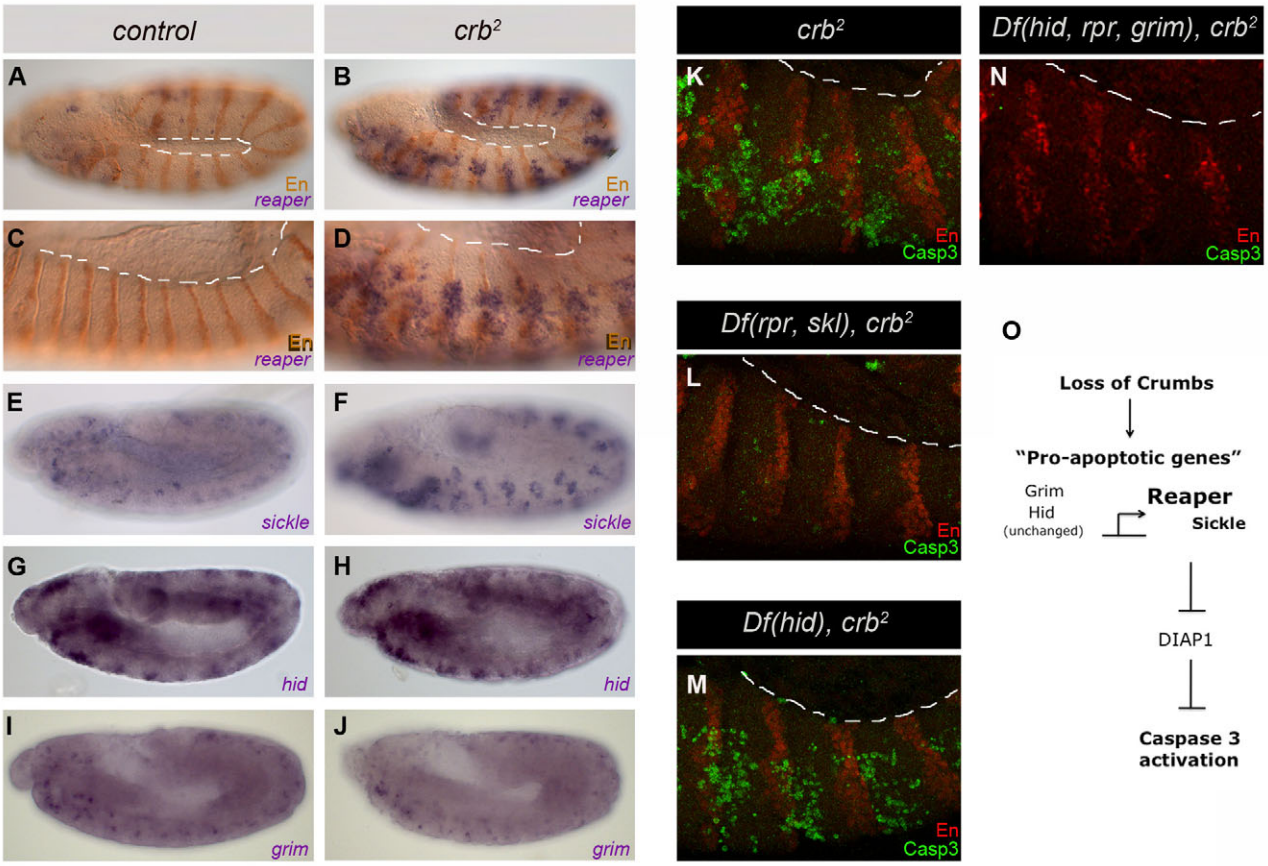

Fig. 2. Apoptosis in the epidermis of *crb* mutant embryos relies on the transcriptional activation of *rpr*. (**A-J**) Expression of the pro-apoptotic genes *rpr* (A-D), *skl* (E,F), *hid* (G,H) and *grim* (I,J) in control and *crb* mutant *Drosophila* embryos at stage 11 (A,B,E-J) or 13 (C,D). Whereas *hid* or *grim* are similarly expressed in the two genotypes, *rpr* and *skl* are both upregulated in the *crb* mutant background. Co-staining with anti-Engrailed (A-D) provides a spatial landmark, showing that there is a general correlation between the pattern of *rpr* expression and that of Caspase 3 activation (see K). (**K-N**) Caspase 3 immunoreactivity in stage 13 *crb* mutant embryos lacking various combinations of pro-apoptotic genes. Engrailed (red) marks the posterior of each segment. Almost no anti-Caspase 3 staining can be detected in *Def(XR38), crb* (L), which removes *rpr* and *skl*, or in *Def(H99), crb* (N), where the contribution of *hid*, *rpr* and *grim* is absent. By contrast, the levels of Caspase 3 staining are similar in *crb* and *Def(X14), crb* embryos (where the contribution of *hid* is removed) (M). The dorsal edge of the epidermis is marked with a dashed line. (**O**) Model of the effect of *crb* loss of function on pro-apoptotic gene expression.

deficiencies were recombined with a *crb* mutation and activated Caspase 3 immunoreactivity was assessed in the resulting embryos. No staining was detected in *crb* mutant embryos that are also deficient at the *H99* locus (i.e. in the absence of *hid*, *rpr* and *grim*, Fig. 2N), even though *skl* is not affected by this deficiency. Removal of *rpr* and *skl* also prevented apoptosis in *crb* mutants (Fig. 2L), whereas the loss of *hid* had no noticeable effect (Fig. 2M). We conclude that loss of *crb* activity leads to the activation of *rpr* transcription, which is required for subsequent apoptosis. Although not essential, *skl* could also contribute (see Fig. 2O). We next aimed to characterise the upstream events that link the loss of epithelial integrity to the transcriptional activation of *rpr*.

JNK signalling is upregulated in *crb* mutant embryos

We obtained several lines of evidence (not shown) that Yorkie and the upstream Hippo pathway, which have been implicated in the regulation of apoptosis (Hariharan, 2006; Pan, 2007), do not mediate the apoptotic response of *crb* mutant embryos. We therefore turned to an unbiased approach to identify relevant mediators. Microarray analysis of whole embryos was used to further characterise the transcriptional response to the loss of *crb* activity. Fifty-one genes were found to be upregulated by more than 2-fold at stage 12 (G.K. and J.-P.V., unpublished). As expected, they included *rpr* and *skl*. Among the remainder, ten were found to be present within a collection of transcriptional targets of the Jun N-terminal kinase (JNK) pathway (S. Noselli, personal communication). This suggests that JNK signalling is upregulated in *crb* mutant embryos. In the wild type, JNK signalling is confined to the dorsal-most cells of the epidermis, where it controls dorsal closure, a morphogenetic process that requires dorsal edge cells to elongate and migrate towards the midline (Harden, 2002; Jacinto et al., 2002; Noselli and Agnes, 1999). Several genes are transcriptionally activated by JNK signalling in these dorsal-most cells. One is *decapentaplegic* (*dpp*), which encodes a signalling molecule of the TGFβ family (Affolter et al., 1994). A second target is *puckered* (*puc*), which encodes a dual VH1 phosphatase that dephosphorylates JNK and hence acts as a feedback inhibitor of the pathway (Martin-Blanco et al., 1998; Ring and Martinez Arias, 1993). In addition, expression of *scarface*, which encodes a protease-like protein, has recently been shown to be a reliable reporter of JNK activity (Rousset et al., 2010; Sorrosal et al., 2010). We used all three genes to assess the spatial characteristics of JNK signalling in *crb* mutant embryos.

RNA in situ hybridisation revealed a general increase of *dpp* and *scarface* expression in *crb* mutant embryos (Fig. 3B,E). Increased *puc* expression was also seen upon staining *crb* mutant embryos carrying a single copy of a *puc-lacZ* reporter (puc^{E69}) (Martin-Blanco et al., 1998) with anti-β-galactosidase antibodies (see Fig. S2 in the supplementary material). For all three genes, expression in dorsal cells was increased and expression also became detectable in the ventral region, indicating a general upregulation of JNK activity. At stage 13, upregulation of *dpp* expression had a segmental character. Segmental upregulation of JNK signalling at this stage was further confirmed in *crb* homozygotes carrying *puc-lacZ*. Co-staining with anti-β-galactosidase, anti-activated Caspase 3 and anti-Engrailed showed that, in the ventral epidermis at stage 13, *puc* expression was mostly activated between the Engrailed stripes (see Fig. S2 in the supplementary material), where apoptosis is first initiated. Because of the subsequent disruption of the epidermis, expression patterns could not be interpreted at later stages. We conclude that loss of *crb* activity causes an increase of JNK signalling activity, both in the ventral region where apoptosis is activated and in the dorsal domain where it is not.

In some circumstances, JNK signalling can be activated as a consequence of apoptosis (Kuranaga et al., 2002). To determine whether activation of JNK signalling in *crb* mutant embryos lies downstream of apoptosis, JNK reporter gene expression was assessed in *crb* mutants that are unable to execute the apoptosis programme because they are also deficient at the *H99* locus [*Def(H99), crb*^{2}]. Embryos of this genotype were processed to detect *dpp or scarface* transcripts. These two JNK target genes were still upregulated despite the block to apoptosis (Fig. 3C,F). Therefore, activation of the JNK pathway in *crb* mutant embryos is not a consequence of apoptosis.

JNK signalling is necessary for the activation of apoptosis in the ventral epidermis of *crb* mutant embryos

Is JNK signalling an essential component of the cascade that mediates *rpr* upregulation in *crb* mutant embryos? To address this question, *rpr* expression was assessed in *crb* embryos that are prevented from activating JNK signalling by overexpression of *puc* with the *tubulin-Gal4* driver. Both *rpr* expression (Fig. 4A,B) and activated Caspase 3 immunoreactivity (Fig. 4C,D) were consistently brought back down to wild-type levels. Apicobasal polarity, as assayed by anti-E-cadherin and anti-Dlg staining, was not rescued by *puc* overexpression (data not shown). Therefore, it

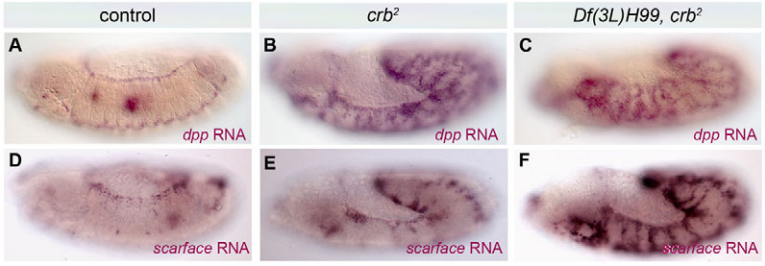

Fig. 3. Transcriptional targets of JNK signalling are upregulated in the epidermis of *crb* mutant embryos. (**A-F**) Expression pattern of *dpp* and *scarface* at stage 13, as determined by RNA in situ hybridisation in control (A,D), *crb* single-mutant (B,E) and *Def(H99) crb* double-mutant (C,F) *Drosophila* embryos. In control embryos, JNK-dependent expression of *dpp* and *scarface* can be detected in the most dorsal epidermal cells, which abut the amnioserosa (A,D). In the absence of Crb, expression of *dpp* and *scarface* expands laterally in a segmented fashion (B,E). This is also true when apoptosis is suppressed in *Def(H99) crb* double mutants (C,F). See also Fig. S2 in the supplementary material.

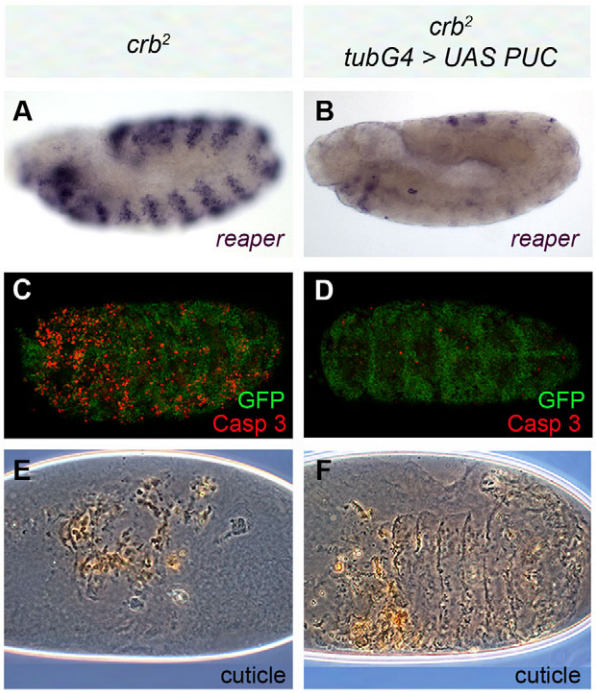

Fig. 4. JNK activity is required for apoptosis in *Drosophila crb* mutant embryos. Comparison of *crb* embryos with *crb* embryos overexpressing *puc* under the control of the *tubulin-Gal4* driver (JNK signalling prevented). (**A**,**B**) Puc overexpression prevents activation of *rpr* expression as assayed by RNA in situ hybridisation at stage 11 (lateral views). In Puc-overexpressing *crb* mutants, expression of *rpr* is similar to that in wild-type embryos (not shown). (**C**,**D**) Puc overexpression also prevents caspase activation (red), as shown here in ventral views of stage 13 embryos. GFP is expressed from a UAS transgene activated by *tubulin-Gal4*. (**E**,**F**) Puc overexpression also rescues the cuticle phenotype of *crb* mutants. Whereas *crb* mutant embryos deposit 'crumbs' of cuticle (refracting objects in E) at the end of embryogenesis, a continuous sheet of cuticle is apparent in rescued embryos (F). Dorsal closure is defective (as is the case in wild-type embryos made to overexpress Puc), but segmentally repeated denticle belts can be discerned. See also Fig. S3 in the supplementary material.

is unlikely that Puc suppresses apoptosis by simply restoring apicobasal polarity. Caspase 3 immunoreactivity was also reduced, although to a lesser extent, upon overexpression of a dominant-negative form of JNK [Bsk^{DN}; JNK is also known as Basket (Bsk) – FlyBase] (see Fig. S3 in the supplementary material). Surprisingly, however, Eiger (Egr; *Drosophila* tumor necrosis factor), which acts upstream in the JNK cascade (Igaki et al., 2002), does not appear to be involved because expression of *scarface* (and *rpr*) was upregulated in *egr crb* double mutants, as it is in *crb* single mutants (see Fig. S4 in the supplementary material). Nevertheless, our results suggest that JNK signalling is an essential component of the mechanism that induces *rpr* expression, and hence apoptosis, in the ventral epidermis of *crb* mutant embryos.

As expected from the anti-apoptotic effect of Puc, *crb* mutant embryos made to overexpress Puc deposited considerably more, and better organised, cuticle than control *crb* mutants (Fig. 4E,F). Segmentally repeated denticle belts were readily recognisable in *puc*-rescued embryos. The level of rescue was considerably better than that obtained by simply preventing apoptosis [compare the cuticle phenotype in Fig. 4F with that of *Def(H99), crb* embryos as

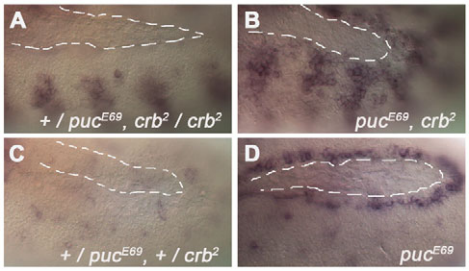

Fig. 5. Endogenous Puc restrains *rpr* expression. (**A**) Expression of *rpr* in a stage 11 homozygous *crb* mutant *Drosophila* embryo that is also heterozygous for puc^{E69}. The pattern is seemingly identical to that in single *crb* mutants (compare with Fig. 2B). (**B**) In a puc^{E69} *crb* double mutant embryo of the same stage, expression of *rpr* expands into the dorsal epidermis. Dashed line marks the boundary between the dorsal epidermis and the amnioserosa. (**C**) Expression of *rpr* in a $+/puc^{E69}$, *+/crb* embryo remains low, as it does in the wild type. (**D**) Expression of *rpr* is specifically activated in the dorsal-most cells of homozygous puc^{E69} embryos. Genotypes: (A) puc^{E69}, crb^2/+, crb^2; (B) puc^{E69}, crb^2; (C) puc^{E69}, crb^2/*TDY*; (D) puc^{E69}.

shown in figure 3b of Tanentzapf and Tepass (Tanentzapf and Tepass, 2003)]. Therefore, the action of Puc might not be limited to suppressing apoptosis; it could, in addition, act on the cytoskeleton to prevent complete dismantling of the ventral epidermis (see Fig. S5 in the supplementary material).

Endogenous Puc is required to prevent *rpr* expression in the dorsal epidermis

As shown above, overexpressed Puc prevents *rpr* expression in *crb* mutants. We next examined whether endogenous Puc contributes to inhibiting *rpr* expression in the dorsal epidermis of *crb* embryos. Indeed, in *crb* mutants that are also homozygous for *puc-lacZ* (puc^{E69}, a hypomorphic *puc* allele), *rpr* expression appeared in the dorsal band of cells that normally resists apoptosis in *crb* mutants. As early as stage 11, striped *rpr* expression, which is normally only present in the ventral epidermis of *crb* mutant embryos (Fig. 2B, Fig. 5A), was seen to extend into the dorsal epidermis of puc^{E69}, *crb* homozygotes (Fig. 5B). Therefore, in a *crb* mutant background, reduction of Puc allows JNK signalling to activate *rpr* expression in the dorsal region, which normally does not express this pro-apoptotic gene.

In wild-type embryos, JNK signalling is activated in a thin row of cells located at the dorsal edge of the epidermis. These cells undergo JNK-dependent cytoskeletal rearrangement and lead the process of dorsal closure. In puc^{E69} homozygotes, these cells instead began to express *rpr* (Fig. 5D). Therefore, reduction of Puc leads to *rpr* expression in cells that activate JNK signalling as part of their normal developmental programme.

Disruption of the apical, but not the basolateral, domain causes JNK activation and apoptosis in the ventral epidermis

So far, we have provided evidence that loss of *crb* triggers the activation of JNK signalling and *rpr* transcription in the ventral epidermis. To examine whether activation of JNK signalling and the ensuing activation of *rpr* expression are specifically due to the loss of *crb* or are a general consequence of apical domain disruption, the effect of mutations in genes encoding other apical determinants was assessed. In both *sdt* and *baz* mutant embryos, expression of *rpr* (Fig. 6A-C) and *scarface* (data not shown) was

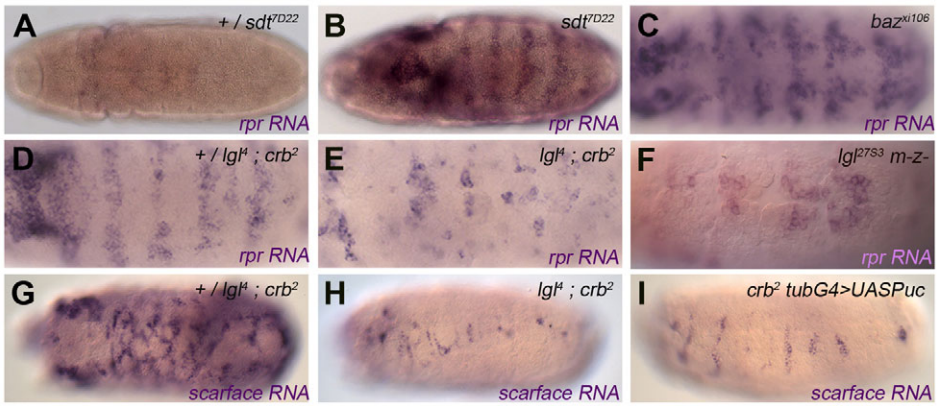

Fig. 6. Loss of apical, but not basolateral, identity causes JNK-dependent *rpr* expression. (**A-C**) Expression of *rpr* in control (A), *sdt* (B) and *baz* (C) *Drosophila* embryos. Upregulation is seen in both *sdt* and *baz* mutants. (**D**,**E**) Removal of zygotic *lgl* activity partially prevents upregulation of *rpr* expression in *crb* mutants. To ensure identical treatments, double-mutant embryos (E) were compared with single *crb* mutants that are heterozygous at the *lgl* locus (D). (**F**) Complete absence of a basolateral determinant (maternal zygotic *lgl* mutant) does not cause widespread *rpr* expression. (**G**,**H**) Upregulation of *scarface* expression in *crb* mutant embryos (G; the embryo shown is also heterozygous at *lgl*) is prevented if zygotic *lgl* activity is completely removed (H; *lgl; crb*). (**I**) Inhibition of JNK signalling by *puc* overexpression prevents *scarface* expression in a *crb* background. Ventral views of stage 11 (A-F) and stage 13 (G-I) embryos are shown, with expression determined by RNA in situ hybridisation. See also Figs S6-S8 in the supplementary material. Genotypes: (A) *sdt*^{7D22}/*FM7* ^{ftzlacZ}; (B) *sdt*^{7D22}; (C) *baz*^{xi106}; (D,G) *lgl*^{4}, *FRT40A/CyO*$^{KrG4>UASGFP}$; *UAS-CD8-GFP, crb*^{2}; (E,H) *lgl*^{4}, *FRT40A; UAS-CD8-GFP, crb*^{2}; (F) *lgl*^{27S3} *(M–Z–)*; (I) *w; UAS Puc*^{14C}*/+; tubulin-Gal4, crb*^{2} *e/UAS-CD8-GFP, crb*^{2} *e.*

upregulated in a similar pattern as in *crb* mutants. Therefore, in the embryonic epidermis, disruption of the apical domain leads to upregulated JNK signalling, which on the ventral side triggers activation of *rpr* expression and apoptosis. We cannot determine with certainty whether loss of apical determinants leads to apoptosis in imaginal discs because loss or reduction of Crb activity in this tissue does not lead to apical disruption, nor to apoptosis (see Fig. S6 in the supplementary material).

Does removal of basolateral components also trigger JNK signalling and apoptosis in the embryonic epidermis? Because of maternal contribution, removal of zygotic *lgl* has no noticeable effect. However, in maternal/zygotic *lgl* mutant embryos, the apical domain expands and the epidermis takes on a multilayered appearance (Bilder and Perrimon, 2000). In such embryos, we did not detect excess expression of *rpr* (Fig. 6F) or *scarface* (data not shown). This result was confirmed in embryos lacking both maternal and zygotic Dlg, another basolateral determinant (Woods and Bryant, 1991). As with *lgl* mutants, no excess expression of *rpr* or *scarface* could be detected (see Fig. S7 in the supplementary material). Likewise, no upregulation of *rpr* was seen in embryos lacking maternal and zygotic *scrib* (*scrib*^{1}; data not shown). We conclude that removal of at least three key basolateral determinants does not lead to JNK signalling or to upregulation of *rpr*. This is in apparent contrast to the situation in imaginal discs, where mosaic loss of *lgl* or other basolateral determinants triggers JNK signalling and apoptosis (e.g. Agrawal et al., 1995; Brumby and Richardson, 2003; Igaki et al., 2006). However, this is likely to be due to cell competition (Menéndez et al., 2010) or the experimental setup (the *puc-lacZ* reporter, which is included in many experimental set-ups, boosts the propensity of basolaterally deficient cells to undergo apoptosis; see Fig. S8 in the supplementary material). We conclude, therefore, that basolateral determinants are not intrinsically required for the survival of epithelial cells.

To distinguish between the effects of apical versus basolateral disruption on JNK signalling in the embryonic epidermis, we took advantage of the observation that the apical domain is substantially better organised in *lgl crb* double mutants than in *crb* single-mutant embryos (Tanentzapf and Tepass, 2003) (see Fig. S9 in the supplementary material). This partial rescue of epithelial integrity correlated with a reduction of *rpr* and *scarface* expression (Fig. 6E,H) to levels similar to those seen in control embryos (*lgl/+; crb/+*, not shown). A similar restoration to normal levels of *scarface* (Fig. 6I) and *rpr* (Fig. 4B) expression was seen in *crb* embryos overexpressing *puc*. Therefore, removal of zygotic *lgl* activity, which partially restores apical organisation in *crb* mutants, prevents JNK signalling and substantially reduces epidermal apoptosis in this background. These results establish that, in the embryonic epidermis, the loss of apical, not basolateral, character leads to JNK signalling and apoptosis.

Junctional integrity puts a brake on JNK-dependent apoptosis in the dorsal epidermis

In the dorsal epidermis of *crb* mutants, JNK signalling is activated, yet this does not lead to *rpr* expression and apoptosis. One distinctive feature of the dorsal epidermis is that junctional integrity seems relatively resistant to the loss of *crb*. For example, at stage 13, anti-E-cadherin staining in the dorsal epidermis remained in a honeycomb pattern (although less marked than at equivalent positions in the wild type), whereas it was punctate and weak in the ventral region (Fig. 7A-D,G,H). This suggests that, in the dorsal epidermis, adherens junctions are weakened but not completely disrupted by the loss of *crb*. The reason underlying this dorsoventral difference is unknown, but the correlation between junctional integrity and cell survival suggests that residual junctional integrity could prevent JNK signalling from triggering *rpr*. If this were the case, one would expect apoptotic cells to start appearing in the dorsal epidermis of *crb* mutants following further junctional weakening. To test this expectation, we examined *crb* mutant embryos lacking zygotic activity of *shotgun* (*shg*), the gene that encodes E-cadherin (Tepass et al., 1996; Uemura et al., 1996). First, it is important to mention that, in single *shg* zygotic mutant embryos, the dorsal epidermis remains a coherent sheet in which relatively few *rpr*-expressing cells are detected (Fig. 7K), probably

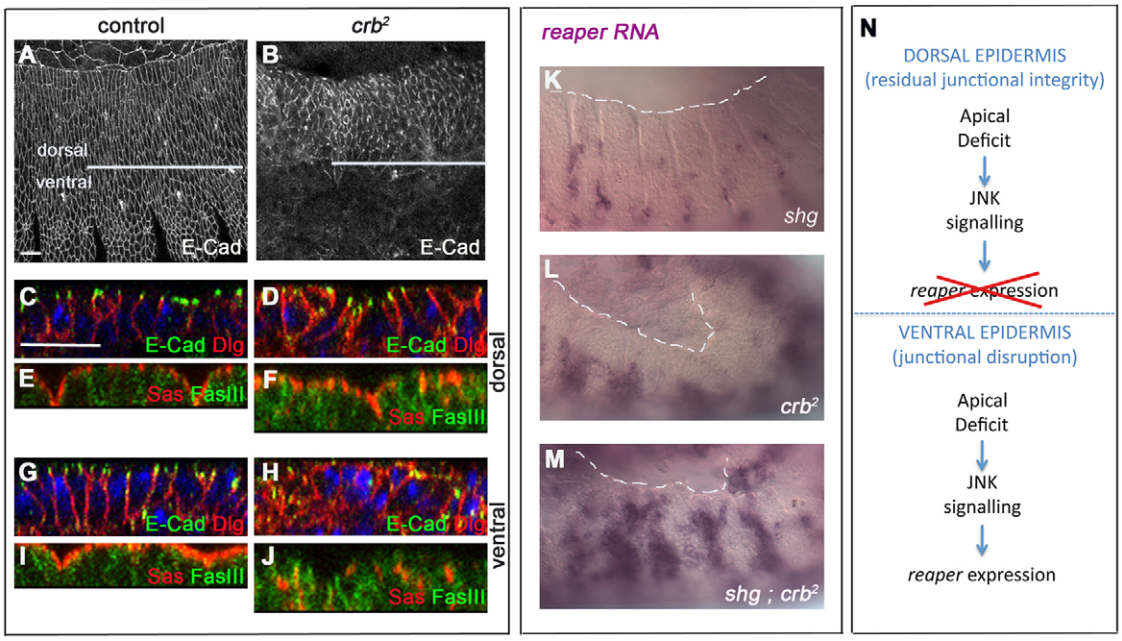

Fig. 7. JNK signalling and junctional disruption activate *rpr* expression. (**A**,**B**) Distribution of E-cadherin (E-Cad) at stage 13 in a wild-type (A) and a *crb* mutant (B) *Drosophila* embryo. Stacks of confocal micrographs are shown. Note the residual honeycomb pattern in the dorsal epidermis of the *crb* mutant. (**C-J**) Optical transverse sections through the dorsal and ventral epidermis (as indicated) stained for (C,D,G,H) E-cadherin (green), Dlg (a basolateral marker, red) and nuclei (TOPRO-3, blue) and stained for (E,F,I,J) Stranded at second (Sas, red) and Fasciclin III (FasIII, green). (**K**) *rpr* expression is mildly upregulated in the ventral-lateral epidermis of embryos homozygous for a *shg* antimorphic allele (embryo shown is also heterozygous for *crb*). No *rpr* upregulation is seen in the dorsal region. (**L**) Expression of *rpr* in a stage 13 homozygous *crb* mutant embryo that is also heterozygous for the same *shg* antimorphic allele. Expression is undistinguishable from that in a single *crb* mutant. (**M**) In a *shg crb* double mutant embryo of the same stage, expression of *rpr* expands into the dorsal epidermis. White dashed line marks the boundary between the dorsal epidermis and the amnioserosa. (**N**) Diagram highlighting the correlation between junctional disruption and the ability of JNK signalling to activate *rpr* expression. See also Fig. S9 in the supplementary material. Genotypes: (A,C,E,G,I) crb^{2}/*TTG*; (B,D,F,H,J) crb^{2}; (K) shg^{g317}; *UAS-CD8-GFP*, crb^{2}/*TDY*; (L) shg^{g317}/$CyO^{KrG4>UASGFP}$; *UAS-CD8-GFP*, crb^{2}; (M) shg^{g317}; *UAS-CD8-GFP*, crb^{2}. Scale bars: 10 μm.

owing to maternal contribution (Tepass et al., 1996; Uemura et al., 1996). In these embryos, loss of adhesion could be seen in the ventral epidermis, as indicated by the appearance of holes at the end of development. Even though loss of zygotic *shg* activity has no apparent effect on the dorsal epidermis, in *shg crb* double mutant embryos, segmental *rpr* expression appeared to creep from the ventral to the dorsal region (Fig. 7M). Numerous Caspase 3-positive cells were detectable in the dorsal epidermis (data not shown), and cuticle secretion was further reduced compared with that in single *crb* mutant embryos (data not shown). This suggests that the dorsal epidermal cells that survive in *crb* mutants undergo apoptosis in *shg crb* double mutants. The upregulation of *rpr* expression in the dorsal domain of *shg crb* double mutants was somewhat variable. However, it must be noted that only a partial reduction of E-cadherin can be achieved because of the presence of maternal protein, which cannot be removed because E-cadherin is required for oogenesis.

DISCUSSION

In this study we have shown that apical, but not basolateral, disruption leads to apoptosis in a developing epithelium. We also showed that JNK signalling is a key intermediate in the signal transduction mechanism that triggers apoptosis in response to the loss of apical determinants. Apical disruption leads to activation of JNK signalling, which in turn activates transcription of the pro-apoptotic gene *rpr*. Moreover, *rpr* expression is not activated in apically disrupted embryos that are prevented from activating JNK signalling. Interestingly, in *bicoid*-deficient embryos and other segmentation mutants, cell fate misspecification requires activation of a different pro-apoptotic gene, *hid* (Werz et al., 2005). Therefore, as previously suggested (Werz et al., 2005), distinct quality control pathways might exist to ensure that different forms of defective cells are removed from developing epithelia. JNK signalling has been shown to mediate apoptosis in a variety of other situations (Leppa and Bohmann, 1999), including after DNA damage (Luo et al., 2007; McEwen and Peifer, 2005) or during cell competition (Adachi-Yamada and O'Connor, 2002; Moreno et al., 2002). However, JNK signalling does not necessarily cause apoptosis. Indeed, this pathway modulates many other cell activities, such as proliferation, differentiation and morphogenesis. What conditions determine whether JNK triggers apoptosis or not is an important issue. Another obvious question raised by our findings concerns the nature of the mechanism that triggers JNK signalling following the loss of apical determinants.

Distinct effects of apical and basolateral disruption

We have shown that, in the embryonic epidermis, JNK signalling is activated by the loss of apical, not basolateral, determinants. In fact, reduction of *lgl* activity prevents JNK activation in *crb* mutant embryos. Similarly, *Scrib* knockdown prevents JNK activity in the mouse mammary epithelium (Zhan et al., 2008), suggesting that the loss of the basolateral domain could have a general anti-JNK (and perhaps anti-apoptotic) activity. Although JNK activation has been

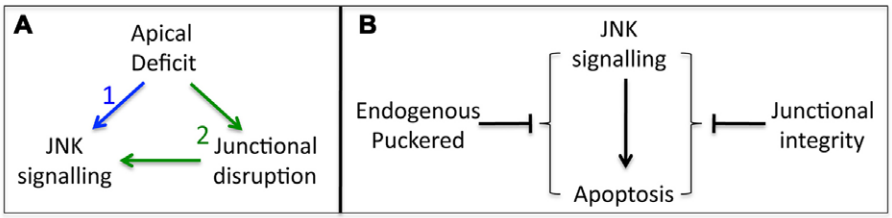

Fig. 8. Signalling upstream and downstream of JNK. (**A**) Apical disruption leads to JNK activation, either as a direct consequence of altered cell polarity (1, blue arrow), or indirectly, via the disruption of junctional integrity (2, green arrow). (**B**) Two mechanisms restrain the ability of JNK signalling to trigger apoptosis. Junctional integrity could act either at the level of JNK signalling or further downstream. As a feedback inhibitor, Puc is likely to limit the extent of JNK signalling. However, we cannot exclude the possibility that Puc has additional activities.

documented in tissues that lack a basolateral determinant (Brumby and Richardson, 2003; Igaki et al., 2006), we suggest that this might be an indirect consequence of cell competition, which triggers JNK signalling (Adachi-Yamada and O'Connor, 2002; Moreno et al., 2002), or of the specific experimental conditions (partial reduction of Puc activity). Overall, our results suggest the existence of an apical domain-dependent activity that modulates JNK signalling in the embryonic epidermis of *Drosophila*. This activity could be similar to that postulated to be at work in cultured MDCK cells (Kim et al., 2007), but is likely to be distinct from the process that leads to apoptosis in response to mosaic disruption of the basolateral domain in imaginal discs.

Signalling upstream of JNK

The mechanism underlying the activation of JNK signalling by loss of apical determinants remains unknown. For example, we cannot tell at this point whether there is an apically localised activity that directly modulates JNK signalling (path 1, blue arrow in Fig. 8A) or whether a more indirect route is at work. Since apical organisation is required for the establishment of adherens junctions, it is conceivable that the effect of apical disruption on JNK signalling is mediated by junctional disruption (path 2, green arrows in Fig. 8A). This possibility is compatible with the absence of ectopic JNK activation in basolateral mutants, in which E-cadherin remains localised to patches at the cell surface (Bilder and Perrimon, 2000; Blankenship et al., 2007; Grawe et al., 1996). However, one would have to invoke that slight junctional disruption is sufficient to trigger JNK signalling, as this pathway is upregulated in the dorsal region of *crb* mutants, where the extent of junctional disruption is relatively mild (see Fig. 7B,N). We have been unable to discriminate between paths 1 and 2, partly because of the current difficulty in eliminating adherens junctions from early *Drosophila* embryos. Future work will require novel means of interfering specifically with adherens junctions. Considering the lack of involvement of Egr, it will also be necessary to identify the upstream components of JNK signalling that respond to epithelial disruption.

Signalling downstream of JNK

Overexpression of Puc, a feedback inhibitor of JNK signalling, prevents apoptosis in the ventral epidermis of *crb* embryos. This is clear evidence that JNK signalling is required for apical deficit to trigger apoptosis. However, it is well established that JNK signalling does not necessarily lead to apoptosis. This is particularly well illustrated by the situation at the dorsal edge of wild-type embryos, where JNK is highly active without triggering apoptosis. Moreover, in *crb* mutants, a 6- to 10-cell-wide band of dorsal tissue is refractory to the pro-apoptotic influence of JNK signalling. Therefore, additional conditions must be met for JNK signalling to activate *rpr* expression and trigger apoptosis. We have identified two situations when refractory cells succumb to the pressure of JNK signalling. One involves the reduction of Puc and the other the removal of zygotic E-cadherin activity (Fig. 8B). The first situation suggests that endogenous Puc can limit the ability of JNK to activate *rpr* expression and trigger apoptosis. The important role of Puc in preventing cell death is also highlighted by the extensive apoptosis seen in embryos lacking both maternal and zygotic Puc activity (McEwen and Peifer, 2005). Puc could act solely by limiting the extent of JNK signalling, thus preventing the very high level of signalling required for *rpr* expression. Alternatively, or in addition, Puc could have an activity that specifically prevents certain genes, such as *rpr*, from being spuriously activated. In any case, it is likely that the regulatory relationships between JNK, Puc and apoptosis are influenced by the cellular context (e.g. the state of adherens junctions; see Fig. 8).

As we have shown, JNK signalling triggers *rpr* expression (and apoptosis) more readily if adherens junctions are weakened or disrupted. Therefore, junctional integrity could also protect epithelial cells from the pro-apoptotic effects of JNK signalling. Dorsoventral differences in junctional integrity and remodelling have been noted in the embryonic epidermis of *Drosophila* (Campbell et al., 2009; Harris and Tepass, 2008; Laprise et al., 2006; Roeth et al., 2009; Tepass et al., 1996; Uemura et al., 1996) and these might explain why these two regions respond differently to the loss of *crb*. Our results suggest that residual junctional integrity in the dorsal epidermis prevents JNK signalling from activating *rpr* expression. It is conceivable that a protective signal emanates from adherens junctions. Alternatively, junctional disruption could interfere with the ability of Puc to rein in the effect of JNK signalling on *rpr* expression. Although differential junctional dynamics between the dorsal and ventral epidermis could determine the propensity to undergo apoptosis, we cannot exclude the possibility that other dorsoventral determinants are at work too.

Conclusion

We have shown that loss of apical polarity leads to apoptosis by activating JNK signalling and causing junctional disruption. We expect that this response, which is readily detectable in the *crb* mutant condition, might reflect a process that ensures the removal of abnormal and damaged cells during epithelial homeostasis. It is hoped that understanding the machinery that links epithelial disruption to JNK signalling and the transcription of pro-apoptotic genes will suggest means of reactivating this pathway in pathological situations.

Acknowledgements
We thank S. Noselli (CNRS, Nice) for discussions and the sharing of unpublished data; Bob Butler (NIMR) for microarray support; Sonia Shah (UCL Bloomsbury Centre) for statistical analysis; Eugenia Piddini (The Gurdon Institute, Cambridge) in whose laboratory some of the work was done; and Yohanns Bellaiche and Nic Tapon for perceptive and constructive comments on the manuscript. We have received numerous reagents from the Bloomington *Drosophila* Stock Center, the *Drosophila* Genomics Resource Center (DGRC), the Developmental Studies Hybridoma Bank (DSHB), the *Drosophila* Genetic Resource Center of the National Institute of Genetics in Kyoto, and colleagues listed in the Materials and methods. This work was largely supported by the Medical Research Council (MRC). P.-L.B. was supported in part by a fellowship from the Association pour la recherche contre le cancer (ARC). Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.059980/-/DC1

References
Abrams, J. M., White, K., Fessler, L. I. and Steller, H. (1993). Programmed cell death during Drosophila embryogenesis. *Development* **117**, 29-43.
Adachi-Yamada, T. and O'Connor, M. B. (2002). Morphogenetic apoptosis: a mechanism for correcting discontinuities in morphogen gradients. *Dev. Biol.* **251**, 74-90.
Affolter, M., Nellen, D., Nussbaumer, U. and Basler, K. (1994). Multiple requirements for the receptor serine/threonine kinase thick veins reveal novel functions of TGF beta homologs during Drosophila embryogenesis. *Development* **120**, 3105-3117.
Agrawal, N., Kango, M., Mishra, A. and Sinha, P. (1995). Neoplastic transformation and aberrant cell-cell interactions in genetic mosaics of lethal(2)giant larvae (lgl), a tumor suppressor gene of Drosophila. *Dev. Biol.* **172**, 218-229.
Baehrecke, E. H. (2002). How death shapes life during development. *Nat. Rev. Mol. Cell Biol.* **3**, 779-787.
Bardet, P. L., Kolahgar, G., Mynett, A., Miguel-Aliaga, I., Briscoe, J., Meier, P. and Vincent, J. P. (2008). A fluorescent reporter of caspase activity for live imaging. *Proc. Natl. Acad. Sci. USA* **105**, 13901-13905.
Bilder, D. (2004). Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. *Genes Dev.* **18**, 1909-1925.
Bilder, D. and Perrimon, N. (2000). Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* **403**, 676-680.
Bilder, D., Schober, M. and Perrimon, N. (2003). Integrated activity of PDZ protein complexes regulates epithelial polarity. *Nat. Cell Biol.* **5**, 53-58.
Blankenship, J. T., Fuller, M. T. and Zallen, J. A. (2007). The Drosophila homolog of the Exo84 exocyst subunit promotes apical epithelial identity. *J. Cell Sci.* **120**, 3099-3110.
Bökel, C. and Brown, N. H. (2002). Integrins in development: moving on, responding to, and sticking to the extracellular matrix. *Dev. Cell* **3**, 311-321.
Brumby, A., Secombe, J., Horsfield, J., Coombe, M., Amin, N., Coates, D., Saint, R. and Richardson, H. (2004). A genetic screen for dominant modifiers of a cyclin E hypomorphic mutation identifies novel regulators of S-phase entry in Drosophila. *Genetics* **168**, 227-251.
Brumby, A. M. and Richardson, H. E. (2003). scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in Drosophila. *EMBO J.* **22**, 5769-5779.
Bulgakova, N. A. and Knust, E. (2009). The Crumbs complex: from epithelial-cell polarity to retinal degeneration. *J. Cell Sci.* **122**, 2587-2596.
Campbell, K., Knust, E. and Skaer, H. (2009). Crumbs stabilises epithelial polarity during tissue remodelling. *J. Cell Sci.* **122**, 2604-2612.
Devenport, D. and Brown, N. H. (2004). Morphogenesis in the absence of integrins: mutation of both Drosophila beta subunits prevents midgut migration. *Development* **131**, 5405-5415.
Dickinson, W. J. and Thatcher, J. W. (1997). Morphogenesis of denticles and hairs in Drosophila embryos: involvement of actin-associated proteins that also affect adult structures. *Cell Motil. Cytoskeleton* **38**, 9-21.
Ditzel, M., Wilson, R., Tenev, T., Zachariou, A., Paul, A., Deas, E. and Meier, P. (2003). Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. *Nat. Cell Biol.* **5**, 467-473.
Foe, V. E., Odell, G. M. and Edgar, B. A. (1993). Mitosis and morphogenesis in the Drosophila embryo: point and counterpoint. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 149-300. New York: Cold Spring Harbor Laboratory Press.
Frisch, S. M. and Screaton, R. A. (2001). Anoikis mechanisms. *Curr. Opin. Cell Biol.* **13**, 555-562.
Gentleman, R., Carey, V., Dudoit, S., Irizarry, R. and Huber, W. (2005). *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. New York: Springer.
Gibson, M. C. and Perrimon, N. (2005). Extrusion and death of DPP/BMP-compromised epithelial cells in the developing Drosophila wing. *Science* **307**, 1785-1789.
Grawe, F., Wodarz, A., Lee, B., Knust, E. and Skaer, H. (1996). The Drosophila genes crumbs and stardust are involved in the biogenesis of adherens junctions. *Development* **122**, 951-959.
Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345-357.
Harden, N. (2002). Signaling pathways directing the movement and fusion of epithelial sheets: lessons from dorsal closure in Drosophila. *Differentiation* **70**, 181-203.
Hariharan, I. K. (2006). Growth regulation: a beginning for the hippo pathway. *Curr. Biol.* **16**, R1037-R1039.
Harris, K. P. and Tepass, U. (2008). Cdc42 and Par proteins stabilize dynamic adherens junctions in the Drosophila neuroectoderm through regulation of apical endocytosis. *J. Cell Biol.* **183**, 1129-1143.
Heitzler, P., Haenlin, M., Ramain, P., Calleja, M. and Simpson, P. (1996). A genetic analysis of pannier, a gene necessary for viability of dorsal tissues and bristle positioning in Drosophila. *Genetics* **143**, 1271-1286.
Hermiston, M. L. and Gordon, J. I. (1995). In vivo analysis of cadherin function in the mouse intestinal epithelium: essential roles in adhesion, maintenance of differentiation, and regulation of programmed cell death. *J. Cell Biol.* **129**, 489-506.
Herranz, H. and Morata, G. (2001). The functions of pannier during Drosophila embryogenesis. *Development* **128**, 4837-4846.
Hughes, S. C. and Krause, H. M. (2001). Establishment and maintenance of parasegmental compartments. *Development* **128**, 1109-1118.
Hutterer, A., Betschinger, J., Petronczki, M. and Knoblich, J. A. (2004). Sequential roles of Cdc42, Par-6, aPKC, and Lgl in the establishment of epithelial polarity during Drosophila embryogenesis. *Dev. Cell* **6**, 845-854.
Igaki, T., Kanda, H., Yamamoto-Goto, Y., Kanuka, H., Kuranaga, E., Aigaki, T. and Miura, M. (2002). Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway. *EMBO J.* **21**, 3009-3018.
Igaki, T., Pagliarini, R. A. and Xu, T. (2006). Loss of cell polarity drives tumor growth and invasion through JNK activation in Drosophila. *Curr. Biol.* **16**, 1139-1146.
Jacinto, A., Woolner, S. and Martin, P. (2002). Dynamic analysis of dorsal closure in Drosophila: from genetics to cell biology. *Dev. Cell* **3**, 9-19.
Kim, M., Datta, A., Brakeman, P., Yu, W. and Mostov, K. E. (2007). Polarity proteins PAR6 and aPKC regulate cell death through GSK-3beta in 3D epithelial morphogenesis. *J. Cell Sci.* **120**, 2309-2317.
Knust, E., Tepass, U. and Wodarz, A. (1993). crumbs and stardust, two genes of Drosophila required for the development of epithelial cell polarity. *Dev. Suppl.* **1993**, 261-268.
Kuranaga, E., Kanuka, H., Igaki, T., Sawamoto, K., Ichijo, H., Okano, H. and Miura, M. (2002). Reaper-mediated inhibition of DIAP1-induced DTRAF1 degradation results in activation of JNK in Drosophila. *Nat. Cell Biol.* **4**, 705-710.
Laprise, P., Beronja, S., Silva-Gagliardi, N. F., Pellikka, M., Jensen, A. M., McGlade, C. J. and Tepass, U. (2006). The FERM protein Yurt is a negative regulatory component of the Crumbs complex that controls epithelial polarity and apical membrane size. *Dev. Cell* **11**, 363-374.
Leppa, S. and Bohmann, D. (1999). Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. *Oncogene* **18**, 6158-6162.
Luo, X., Puig, O., Hyun, J., Bohmann, D. and Jasper, H. (2007). Foxo and Fos regulate the decision between cell death and survival in response to UV irradiation. *EMBO J.* **26**, 380-390.
Martin-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A. M. and Martinez-Arias, A. (1998). puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. *Genes Dev.* **12**, 557-570.
Martinez Arias, A. (1993). Development and patterning of the larval epidermis of Drosophila. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 517-608. New York: Cold Spring Harbor Laboratory Press.
McEwen, D. G. and Peifer, M. (2005). Puckered, a Drosophila MAPK phosphatase, ensures cell viability by antagonizing JNK-induced apoptosis. *Development* **132**, 3935-3946.
Meier, P., Finch, A. and Evan, G. (2000). Apoptosis in development. *Nature* **407**, 796-801.
Menéndez, J., Pérez-Garijo, A., Calleja, M. and Morata, G. (2010). A tumor-suppressing mechanism in Drosophila involving cell competition and the Hippo pathway. *Proc. Natl. Acad. Sci. USA* **107**, 14651-14656.
Moreno, E., Basler, K. and Morata, G. (2002). Cells compete for decapentaplegic survival factor to prevent apoptosis in Drosophila wing development. *Nature* **416**, 755-759.
Muller, H. A. and Wieschaus, E. (1996). armadillo, bazooka, and stardust are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in Drosophila. *J. Cell Biol.* **134**, 149-163.

Muller, H. A. and Bossinger, O. (2003). Molecular networks controlling epithelial cell polarity in development. *Mech. Dev.* **120**, 1231-1256.
Nordstrom, W., Chen, P., Steller, H. and Abrams, J. M. (1996). Activation of the reaper gene during ectopic cell killing in Drosophila. *Dev. Biol.* **180**, 213-226.
Noselli, S. and Agnes, F. (1999). Roles of the JNK signaling pathway in Drosophila morphogenesis. *Curr. Opin. Genet. Dev.* **9**, 466-472.
Pan, D. (2007). Hippo signalling in organ size control. *Genes Dev.* **21**, 886-897.
Pazdera, T. M., Janardhan, P. and Minden, J. S. (1998). Patterned epidermal cell death in wild-type and segment polarity mutant Drosophila embryos. *Development* **125**, 3427-3436.
Peterson, C., Carney, G. E., Taylor, B. J. and White, K. (2002). reaper is required for neuroblast apoptosis during Drosophila development. *Development* **129**, 1467-1476.
Plant, P. J., Fawcett, J. P., Lin, D. C., Holdorf, A. D., Binns, K., Kulkarni, S. and Pawson, T. (2003). A polarity complex of mPar-6 and atypical PKC binds, phosphorylates and regulates mammalian Lgl. *Nat. Cell Biol.* **5**, 301-308.
Ring, J. M. and Martinez Arias, A. (1993). puckered, a gene involved in position-specific cell differentiation in the dorsal epidermis of the Drosophila larva. *Dev. Suppl.* **1993**, 251-259.
Roeth, J. F., Sawyer, J. K., Wilner, D. A. and Peifer, M. (2009). Rab11 helps maintain apical crumbs and adherens junctions in the Drosophila embryonic ectoderm. *PLoS ONE* **4**, e7634.
Rousset, R., Bono-Lauriol, S., Gettings, M., Suzanne, M., Speder, P. and Noselli, S. (2010). The Drosophila serine protease homologue Scarface regulates JNK signalling in a negative-feedback loop during epithelial morphogenesis. *Development* **137**, 2177-2186.
Shen, X. and Kramer, R. H. (2004). Adhesion-mediated squamous cell carcinoma survival through ligand-independent activation of epidermal growth factor receptor. *Am. J. Pathol.* **165**, 1315-1329.
Sorrosal, G., Perez, L., Herranz, H. and Milan, M. (2010). Scarface, a secreted serine protease-like protein, regulates polarized localization of laminin A at the basement membrane of the Drosophila embryo. *EMBO Rep.* **11**, 373-379.
Srinivasula, S. M., Datta, P., Kobayashi, M., Wu, J. W., Fujioka, M., Hegde, R., Zhang, Z., Mukattash, R., Fernandes-Alnemri, T., Shi, Y. et al. (2002). sickle, a novel Drosophila death gene in the reaper/hid/grim region, encodes an IAP-inhibitory protein. *Curr. Biol.* **12**, 125-130.
St Johnston, D. and Ahringer, J. (2010). Cell polarity in eggs and epithelia: parallels and diversity. *Cell* **141**, 757-774.
Steller, H. (2008). Regulation of apoptosis in Drosophila. *Cell Death Differ.* **15**, 1132-1138.
Suzuki, A. and Ohno, S. (2006). The PAR-aPKC system: lessons in polarity. *J. Cell Sci.* **119**, 979-987.
Takeichi, M. (1995). Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* **7**, 619-627.
Tanentzapf, G. and Tepass, U. (2003). Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. *Nat. Cell Biol.* **5**, 46-52.
Tenev, T., Zachariou, A., Wilson, R., Paul, A. and Meier, P. (2002). Jafrac2 is an IAP antagonist that promotes cell death by liberating Dronc from DIAP1. *EMBO J.* **21**, 5118-5129.
Tepass, U. and Knust, E. (1993). Crumbs and stardust act in a genetic pathway that controls the organization of epithelia in Drosophila melanogaster. *Dev. Biol.* **159**, 311-326.
Tepass, U., Theres, C. and Knust, E. (1990). crumbs encodes an EGF-like protein expressed on apical membranes of Drosophila epithelial cells and required for organization of epithelia. *Cell* **61**, 787-799.
Tepass, U., Gruszynski-DeFeo, E., Haag, T. A., Omatyar, L., Torok, T. and Hartenstein, V. (1996). shotgun encodes Drosophila E-cadherin and is preferentially required during cell rearrangement in the neurectoderm and other morphogenetically active epithelia. *Genes Dev.* **10**, 672-685.
Uemura, T., Oda, H., Kraut, R., Hayashi, S., Kotaoka, Y. and Takeichi, M. (1996). Zygotic Drosophila E-cadherin expression is required for processes of dynamic epithelial cell rearrangement in the Drosophila embryo. *Genes Dev.* **10**, 659-671.
Wang, Q. and Margolis, B. (2007). Apical junctional complexes and cell polarity. *Kidney Int.* **72**, 1448-1458.
Werz, C., Lee, T. V., Lee, P. L., Lackey, M., Bolduc, C., Stein, D. S. and Bergmann, A. (2005). Mis-specified cells die by an active gene-directed process, and inhibition of this death results in cell fate transformation in Drosophila. *Development* **132**, 5343-5352.
Westhoff, M. A. and Fulda, S. (2009). Adhesion-mediated apoptosis resistance in cancer. *Drug Resist. Updat.* **12**, 127-136.
Westhoff, M. A., Zhou, S., Bachem, M. G., Debatin, K. M. and Fulda, S. (2008). Identification of a novel switch in the dominant forms of cell adhesion-mediated drug resistance in glioblastoma cells. *Oncogene* **27**, 5169-5181.
White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller, H. (1994). Genetic control of programmed cell death in Drosophila. *Science* **264**, 677-683.
Wing, J. P., Karres, J. S., Ogdahl, J. L., Zhou, L., Schwartz, L. M. and Nambu, J. R. (2002). Drosophila sickle is a novel grim-reaper cell death activator. *Curr. Biol.* **12**, 131-135.
Woods, D. F. and Bryant, P. J. (1991). The discs-large tumor suppressor gene of *Drosophila* encodes a guanylate kinase homolog localized at septate junctions. *Cell* **66**, 451-464.
Yamanaka, T., Horikoshi, Y., Sugiyama, Y., Ishiyama, C., Suzuki, A., Hirose, T., Iwamatsu, A., Shinohara, A. and Ohno, S. (2003). Mammalian Lgl forms a protein complex with PAR-6 and aPKC independently of PAR-3 to regulate epithelial cell polarity. *Curr. Biol.* **13**, 734-743.
Zachariou, A., Tenev, T., Goyal, L., Agapite, J., Steller, H. and Meier, P. (2003). IAP-antagonists exhibit non-redundant modes of action through differential DIAP1 binding. *EMBO J.* **22**, 6642-6652.
Zhan, L., Rosenberg, A., Bergami, K. C., Yu, M., Xuan, Z., Jaffe, A. B., Allred, C. and Muthuswamy, S. K. (2008). Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell* **135**, 865-878.