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 Aypical 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, 2010). It is thought that these two complexes cooperate to counteract the basal determinant complex, which comprises Lethal giant larvae [Lgl, or L(2)g1], 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...
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; Tapass et al., 1990). Anoikis is unlikely to contribute in this instance since 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 (SkI) and Jfrac (Abrams et al., 1993; Nordstrom et al., 1991); (16) l(2)gl4/CKG; UAS CD8 GFP, crb2/TTG; (10) pucE69/TM6, crb2/TDY; (8) UAS-CD8-GFP, crb2/TMD; (6) crb2/TDY; (5) pnrGal4MD231/TMD (from G. Morata, University Autonoma, Madrid, Spain); (6) pnrGal4MD231, crb2/TDY; (7) UAS-CD8-GFP, crb2/TMD; (8) pucE69/TM6 (Martin-Blanco et al., 1998); (9) pucE69, crb2/TDY; (10) I(2)g3/+; CGK; UAS CD8 GFP, crb2/TDY; (11) shg3+/+; CGK; UAS CD8 GFP, crb2/TDY (shg3+/+ obtained from N. Gorinkiel, University of Cambridge, UK); (12) I(2)g3/FRT40A/Cig/I(2)g3 from Brumby et al. (Brumby et al., 2004)); (13) hsflp; Adsc/Cyo; (14) P[oovoD1-18]/2La P[oovoD1-18]/2Lb P[neoFRT40A/Dpy(2)bw]/Dpy(2)bw, S[1] wg[Sp-1] Ms(2)M[1] bw[1]/Cyo[15]; dlg[532]; FRT101/FM{b}[562] (Woods and Bryant, 1991); (16) w; oovoD1 FRT101/Y; hsflp88; (17) egger[1] (Igaki et al., 2002); (18) Kr[1] (from H. Skaer, University of Cambridge, UK); (19) def3(L)X38, crb2/TMD; (20) Def3(L)X38, crb2/TMD; (21) tubulinGal4, crb2/TMD; (22) UAS Puc[24] (Martin-Blanco et al., 1998); and (23) UAS-crb-RNAi (VDRC line 39178).

**Materials and Methods**

**Fly stocks and genetics**

As wild type, yw was used. As marked balancers, we used TTG (TM3, twissGal4–UASGFP), TTY (TM6, Dfd–YFP), CGK (Cyo, Kr-Gal4–UASGFP) and CTG (Cyo, Kr-Gal4–UASGFP) and rpr (Cyo, Kr-Gal4–UASGFP) and CTG (Cyo, Kr-Gal4–UASGFP). Other stocks (numbered for clarity) were: (1) crb2/TTG; (2) Def(T99); crb2/TDY; (3) huz1106, FRT9.2/FM{b}KrGal4–UASGFP (from Y. Ballestica, Institut Curie, Paris); (4) shg122/FM{b}puc14C (from E. Knust, Max-Planck Institute, Dresden, Germany); (5) pnrGal4MD231/TMD (from G. Morata, University Autonoma, Madrid, Spain); (6) pnrGal4MD231, crb2/TDY; (7) UAS-CD8-GFP, crb2/TMD; (8) pucE69/TM6 (Martin-Blanco et al., 1998); (9) pucE69, crb2/TDY; (10) I(2)g3/+; CGK; UAS CD8 GFP, crb2/TDY; (11) shg3+/+; CGK; UAS CD8 GFP, crb2/TDY (shg3+/+ obtained from N. Gorinkiel, University of Cambridge, UK); (12) I(2)g3/FRT40A/Cig/I(2)g3 from Brumby et al. (Brumby et al., 2004)); (13) hsflp; Adsc/Cyo; (14) P[oovoD1-18]/2La P[oovoD1-18]/2Lb P[neoFRT40A/Dpy(2)bw]/Dpy(2)bw, S[1] wg[Sp-1] Ms(2)M[1] bw[1]/Cyo[15]; dlg[532]; FRT101/FM{b}[562] (Woods and Bryant, 1991); (16) w; oovoD1 FRT101/Y; hsflp88; (17) egger[1] (Igaki et al., 2002); (18) Kr[1] (from H. Skaer, University of Cambridge, UK); (19) def3(L)X38, crb2/TMD; (20) Def3(L)X38, crb2/TMD; (21) tubulinGal4, crb2/TMD; (22) UAS Puc[24] (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 CaCl₂ 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 Hybridity Board (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 456 (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 Velocity (Improvision).

**In situ hybridisation**

Embryonic 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 at 12 hours for 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, crb2/TTG3 were crossed to males from an F1 of the genotype w; UAS-X;CyO{p}[b]ac[1]–; UAS-CD8GFP, crb2+/+ (where X refers to the particular gene to be overexpressed). GFP-positive embryos were preselected before mounting.

**Affymetrix microarray and data analysis**

Mutant (crb2) and control (crb2/+) 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 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 panner 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 panner expression as marked with panner-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 panner-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 panner 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, panner-Gal4 and UAS-GFP were used to mark dorsal cells and to follow their fate up to a time when dentine precursors can be recognised. Embryos were examined at stage 16, when the actin bundles that serve as a template for dentine 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 deficiencies are summarised in Fig. 2K-N.

**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 dorsal region (Affolter et al., 1994). A second target is puckered (puc), which encodes a dual VHI 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<sup>2699</sup>) (Martín-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<sup>2</sup>]. 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
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 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. 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 [BskDN; 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 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. S3 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 pucE69, 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 pucE69, 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 pucE69 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
Apical deficit triggers apoptosis

**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. Uregulation 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+/+;FM7>F:Baz; (B) sdt+/sdt2D52/+/+; (C) baz+/SIG; (D,G) lgl+/+, FRT40A/CyO;P[UAS-Puc14C];UAS-Crbd189-GFP;crb+/+; tubulin-Gal4, crb+/+. (E) lgl/+, FRT40A; UAS-Crbd189-GFP; crb+/+; (I) lgl+/+, UAS-Puc14C/+; tubulin-Gal4, crb2 e/UAS-Crbd189-GFP, crb2 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 scr (scrib; data not shown). We conclude that removal of at least three key basolateral determinants does not lead to JNK signalling or upregulation of rpr. This is in apparent contrast to the situation in imaginal discs, where mosaics 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 functional 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...
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
Apical deficit triggers apoptosis

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; Grabe 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.


Apical deficit triggers apoptosis


