An in vivo model of apoptosis: linking cell behaviours and caspase substrates in embryos lacking DIAP1

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Summary
The apoptotic phenotype is characterised by dynamic changes in cell behaviours such as cell rounding and blebbing, followed by chromatin condensation and cell fragmentation. Whereas the biochemical pathways leading to caspase activation have been actively studied, much less is known about how caspase activity changes cell behaviours during apoptosis. Here, we address this question using early Drosophila melanogaster embryos lacking DIAP1. Reflecting its central role in the inhibition of apoptosis, loss of DIAP1 causes massive caspase activation. We generated DIAP1-depleted embryos by either using homozygous null mutants for thread, the gene coding DIAP1, or by ectopically expressing in early embryos the RGH protein Reaper, which inhibits DIAP1. We show that (1) all cells in embryos lacking DIAP1 follow synchronously the stereotypic temporal sequence of behaviours described for apoptotic mammalian cells and (2) these cell behaviours specifically require caspase activity and are not merely a consequence of cellular stress. Next, we analyse the dynamic changes in the localisation of actomyosin, Discs large, Bazooka and DE-cadherin in the course of apoptosis. We show that early changes in Bazooka and Discs large correlate with early processing of these proteins by caspases. DE-cadherin and Myosin light chain do not appear to be cleaved, but their altered localisation can be explained by cleavage of known regulators. This illustrates how embryos lacking DIAP1 can be used to characterise apoptotic changes in the context of an embryo, thus providing an unprecedented in vivo model in which thousands of cells initiate apoptosis simultaneously.

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Key words: Apoptosis, Live imaging, Blebbing, Epithelial polarity, Caspase substrates

Introduction
Drosophila melanogaster provides an excellent model in which to investigate programmed cell death, for its ease of study and because of the high level of conservation of apoptotic mechanisms with mammals (Adrain et al., 2006; Cashio et al., 2005). Here we focus on analysing the apoptotic phenotype of embryos lacking DIAP1, one of the Drosophila inhibitor of apoptosis proteins (IAPs). IAPs were initially characterised in baculovirus as proteins, such as p35, that suppressed apoptosis in infected host cells. Since then IAPs have been found in Caenorhabditis elegans, Drosophila and vertebrates. IAPs contain baculovirus IAP repeat (BIR) domains, which can suppress apoptosis by directly binding caspases. Most IAPs also contain a RING domain, which provides E3 ubiquitin ligase activity (Stennicke et al., 2002; Vaux and Silke, 2005).

In Drosophila, DIAP1 constitutes a central point of control during apoptosis. DIAP1 binds to and inhibits the apical caspase DRONC as well as the effector caspases Drice and Dcp-1 (Hawkins et al., 1999; Meier et al., 2000; Wang et al., 1999). There is evidence that binding of DIAP1 to caspases induces their ubiquitylation-dependent degradation (Chai et al., 2003; Wilson et al., 2002). Inhibition of the caspase cascade by DIAP1 is relieved by pro-apoptotic factors such as Reaper, Grim and Hid, which are clustered in region H99 of the genome. In embryos deficient for H99, developmental apoptosis is completely abolished (White et al., 1994). The RGH (Reaper, Grim and Hid) proteins have been reported to induce cell death by a variety of mechanisms. First, they are able to bind to the second BIR domain of DIAP1 and thereby release the caspases bound to it (Goyal et al., 2000; Wang et al., 1999; Wu et al., 2001). Second, the RGH proteins stimulate DIAP1 protein degradation by regulating its ubiquitylation (Holley et al., 2002; Ryoo et al., 2002; Wilson et al., 2002; Yoo, 2005; Yoo et al., 2002). Third, the RGH proteins act as general translational repressors, and because DIAP1 has a shorter half-life than most caspases, this promotes cell death (Colon-Ramos et al., 2006; Holley et al., 2002; Yoo et al., 2002). In the absence of DIAP1, the caspase cascade is fully activated. For example, loss of thread, the gene coding for DIAP1, causes massive apoptosis in early Drosophila embryos (Wang et al., 1999). Caspase activation in the absence of DIAP1 requires Dark, the homologue of mammalian Apaf-1 (Quinn et al., 2000; Rodriguez et al., 1999). Dark and DRONC form an apoptosome (Yu et al., 2006), which in turn is thought to activate effector caspases such as Drice or Dcp-1.

Although there have been spectacular advances in understanding the pathways that lead to caspase activation, how caspases accomplish the cellular changes of apoptosis is less well understood. Caspase activation promotes the proteolytic cleavage of many proteins (Fischer et al., 2003), and this is
thought to cause the characteristic phenotypes displayed by the apoptotic cells, such as cell rounding and blebbing, chromatin condensation and cell fragmentation (Mills et al., 1999). Although the consequences of cleaving some individual proteins have been well characterised (e.g. Adrain et al., 2004; Brancolini et al., 1997; Coleman et al., 2001; Freeman, 1996; Lane et al., 2002; Lane et al., 2001; Lowe et al., 2004; Sebbagh et al., 2001; Slee et al., 2001), the majority of reported caspase cleavages have not been linked to a specific apoptotic phenotype. It seems likely that only some of the cleaved substrates are key to producing the apoptotic phenotype whereas others will be ‘innocent bystanders’ (Martin, 2002). In addition, some cleavage events might be required for the early phenotypes of apoptosis (cell rounding and blebbing), whereas others might be responsible for the later phenotypes (such as chromatin condensation and cell fragmentation). Because it is difficult to obtain synchronous populations of mammalian cells entering the apoptotic program, it has been hard to investigate the timing in these systems (Mills et al., 1999).

We have established an alternative method to address these questions using early Drosophila embryos lacking DIAP1 (i.e. embryos homozygous null for thread, or ectopically expressing Reaper). We confirm that the sequence of extranuclear changes described for mammalian cells (Mills et al., 1999) occurs in embryos lacking DIAP1. Crucially, all cells enter apoptosis simultaneously, which enabled us to identify specific caspase cleavages and relate them in vivo to the behaviour of several components of the cytoskeleton and epithelial polarity. This establishes embryos lacking DIAP1 as a powerful in vivo model to investigate the consequence of caspase cleavage in apoptotic cells.

**Results**

**Cells initiate apoptosis synchronously in DIAP1-null mutants**

Our interest in the role of thread in regulating cell behaviour arose when we recovered thread in a screen designed to find genes required for boundary formation in Drosophila embryos (D. Chandraratana, The role of DIAP1 in cellular integrity during embryogenesis, PhD thesis, University of Cambridge, 2004). The product of the engrailed segment polarity gene is normally expressed in a series of regular stripes in the ectoderm of wild-type embryos (Fig. 1A). In embryos deficient for a genomic region including the thread gene and those homoygous for th5, a null mutation in thread, the Engrailed-expressing cells are found rounded-up and completely dispersed (Fig. 1C), at the stage when wild-type embryos are completing germ-band extension (a morphogenetic movement of convergence and extension following gastrulation). Embryos mutant for th5, a thread hypomorph mutation, exhibit an intermediate phenotype (Fig. 1B).

Abnormal cell behaviours are not limited to the Engrailed cell population. Ectodermal cells expressing the pair-rule gene eve round up and disperse in th5 mutants (Fig. 1D,E). The other embryonic layers are also affected: Twist and Forkhead-expressing cells normally invaginate to form the ventral furrow (mesoderm) (Fig. 1F) and the anterior and posterior midgut rudiments (endoderm) (Fig. 1H), respectively. In th5 mutants, these invaginations regress after the beginning of germ-band extension, and presumptive mesodermal and endodermal cells end up at the surface of the embryo (Fig. 1G,1).

We labelled cell nuclei with HistoneYFP to document the kinetics of cell dispersal and found that it is surprisingly swift: in HistoneYFP[th5] embryos, cells start dispersing on average 13 minutes after cephalic furrow formation (13±2 minutes, n=7 at 23°C), which corresponds to approximately 20 minutes after the end of cellularisation. Note that the end of cellularisation (abbreviated as C) is used as time zero for all time-lapse work hereafter. Cell dispersal is completed within minutes. An example is shown in Fig. 1K and supplementary material Movie 1 [wild-type (WT) control shown in Fig. 1J].

Examining the morphology of th5 embryos by Nomarski optics and comparing it with WT, we could not detect any defects during cellularisation or prior to the onset of cell rounding (supplementary material Movie 2 and Fig. 1L versus Fig. 1M, C+16 minutes). Strikingly, cell rounding is associated with a strong contraction of the ectoderm followed by a rapid disappearance of all morphogenetic furrows and invaginations (supplementary material Movie 2 and Fig. 1M, C+18 minutes).

In summary, cellularisation and initial gastrulation movements (such as ventral and cephalic furrow formation and posterior midgut invagination) are initially normal in th5 embryos, until the moment when cells round up and morphogenesis is arrested, consistent with previous description (Wang et al., 1999). In addition, we show that the ectoderm contracts and that the cells disperse, indicating that cells lose basal as well as lateral adhesion. Cell rounding and dispersal occurs very rapidly and reproducibly. Thus, th5 embryos provide an unprecedented model system in which thousands of cells initiate apoptosis simultaneously, allowing us to characterise the sequence of apoptotic cellular changes in the context of an embryo.

**Cells in DIAP1 mutants follow the canonical progression of apoptotic phenotypes**

To analyse the progression of apoptosis in th5 mutant embryos, we collected embryos at the end of cellularisation and aged them 30 minutes (T1), 1 hour (T2), 1.5 hours (T3) or 2 hours (T4) prior to fixation. We followed the changes in cell shape in these fixed embryos using Nomarski optics, and related them to apoptotic progression, either by using an antibody that detects the activated form of Drice to monitor caspase activation (Yoo et al., 2002), or by performing TUNEL labelling to detect DNA fragmentation.

T1 showed a mixture of embryos: some in which cells had not yet dispersed (Fig. 2A,C,D, T1) and some in which cells had just started to round up and disperse (not shown). Prior to cell dispersal, at the beginning of germ-band extension, th5 embryos exhibit wild-type Engrailed stripes (Fig. 2A, T1). Same stage embryos showed a subset of cells with detectable levels of activated Drice (Fig. 2C, T1), but no TUNEL staining (Fig. 2D, T1). This shows that just prior to cell rounding and dispersal, caspase activation is initiated but not DNA fragmentation. By 1 hour after the end of cellularisation (T2), all embryos have undergone cell dispersal (Fig. 2A, T2) and all cells are positive for activated Drice (Fig. 2C, T2) and TUNEL (Fig. 2D, T2). The yolk cell is fragmented and distributed at the surface of the embryos (Fig. 2A, T2). By 1.5 hours after cellularisation (T3), the cells appear smaller and more numerous (Fig. 2A, T3, see inset). This is caused by cells fragmenting into smaller bodies rather than cell division, because (1) mitotic cells are not detectable using an antibody

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against phosphorylated Histone H3 (supplementary material Fig. S1) and (2) the number of Engrailed-positive nuclei remains the same (Fig. 2A, inset T2 versus T3). Note that although the number of nuclei is unchanged in these embryos, they are considerably reduced in size compared with 30 minutes earlier. This is also evident in embryos stained for activated Drice or TUNEL (Fig. 2C,D, T3). This indicates that the nuclei have condensed, which is a canonical phenotype of apoptosis (Martelli et al., 2001). By 2 hours after cellularisation (T4), En expression is lost (not shown).

Another feature of apoptotic cells is the appearance of small membranous extensions called blebs, which are extended and retracted dynamically from the cell surface early in apoptosis (Charras et al., 2006; Mills et al., 1999). Previous work showed, by scanning electron microscopy, that small blebs appear in th4 mutant embryos once cells have rounded up (Wang et al., 1999). To follow these blebs dynamically, we labelled the cell plasma membrane using the membrane marker SrcGFP (Kaltschmidt et al., 2000) in live th5 mutant embryos. We detected small dots that appear and disappear dynamically from the time cells start rounding up (Fig. 2E and supplementary material Movie 3). Although we cannot rule out alternate explanations of these dots (such as bulk membrane endocytosis), we think that they are likely to correspond to the blebs observed by Wang et al. (Wang et al., 1999). These apparent blebs are absent prior to cell dispersal but appear as cells lose their columnar shape (Fig. 2E, arrow in frame C+25 minutes). The blebbing intensifies until all the cells have

![Fig. 1.](image-url) Cells round up and disperse swiftly in th5 mutant embryos. (A-C) Stage 10 embryos stained for Engrailed. In wild-type (A), the germ-band is fully extended and the Engrailed-expressing cells are arranged in segmental stripes in the ectoderm. In hypomorphic th4 mutants (B), germ-band extension fails halfway, and the Engrailed-expressing cells in the dorsal-most part of the ectoderm are found dispersed. In th5-null mutants (C), germ-band extension fails completely, and all Engrailed-expressing cells are dispersed. The pale regions correspond to fragments of the yolk cell emerging at the surface of the embryo. (D-I) Stage 8 embryos showing distribution of Even-skipped (Eve), Twist (Twi) and Forkhead (Fkh) to mark the ectoderm, mesoderm and endoderm, respectively. In wild-type embryos (D), Eve-expressing cells are arranged into pair-rule stripes. These cells are found completely dispersed in th5 embryos (E). (F) Ventral view showing Twist-expressing cells at the ventral midline and underneath, where the mesoderm has invaginated. In th5 embryos (G), the mesoderm invagination collapses and the Twist-expressing cells reappear at the surface of the embryo. Fkh-expressing cells mark the anterior (arrow) and posterior midgut rudiment in wild type. Note that at stage 8, the posterior Fkh domain has already invaginated (arrowhead) (H). In th5 embryos, the Fkh-expressing cells reappear at the surface of the embryo (I). (J-M) Kinetics of cell dispersal in live embryos, observed either by labelling nuclei using HistoneYFP (J,K, see supplementary material Movie 1), or in unlabelled embryos filmed under Nomarski optics (L,M, see supplementary material Movie 2). Times are indicated from the end of cellularisation (abbreviated as C). In both movies, no defects are detected until the beginning of germ-band extension, at which point the morphogenetic folds of the embryos start to regress (C+21-minute frame in K and C+18-minute frame in M), then the embryos contract abruptly and the cells round up and disperse in a few minutes (C+26-minute frame in K and C+25-minute frame in M). Note that in K, the nuclei within the field of view at C+26 minutes are entirely different from those within the field of view at C+21 minutes, showing that the nuclei have dispersed extensively. In J and K, anterior, posterior, ventral and dorsal sides are labelled, and the arrow indicates the posterior transverse furrow. In L and M, the arrow marks the cephalic furrow, and the arrowhead indicates the posterior midgut invagination.
Fig. 2. Progression of the apoptotic phenotype in \(th^5\) embryos. (A-D) Wild-type or homozygous \(th^5\) embryos were hand-selected at the end of cellularisation and fixed after ageing for another 30 minutes (T1), 1 hour (T2) or 1.5 hours (T3). Fixed embryos are stained for either Engrailed (A), activated Drice (B,C) or TUNEL (D), and photographed using Nomarski optics. (A) T1 shows a \(th^5\) embryo that is still morphologically normal: it has initiated germ-band extension and exhibits regular Engrailed stripes. T2 shows a \(th^5\) embryo that has completed cell dispersal: the cells have lost their columnar shape and adopted a rounded shape, and as a consequence these appear larger (compare close-ups for T1 and T2). The Engrailed cells have scattered and the yolk cell has fragmented and emerged at the surface of the embryo. The embryo in T3 shows a striking change in cell and nuclear shapes (the latter revealed by the nuclear localisation of the Engrailed protein). The nuclei have condensed and the cell bodies are smaller and more numerous, indicating that the cells have fragmented (compare close-ups for T2 and T3). (B) No staining for the activated form of Drice is detected in wild-type embryos before stage 10 (T3) at which two nuclei are found positive in the amnioserosa (arrow). This is consistent with the fact that there is no apoptosis in early wild-type embryos and that the amnioserosa is the first tissue in which apoptosis is detected at stage 10-11 (Abrams et al., 1993). Similarly, no staining was detected in early embryos stained for TUNEL (not shown). (C) T1: just prior to cell dispersal, discrete cells already show detectable levels of activated Drice. Activated Drice staining patterns appear random, except in the head, where an anterior spot is always detected. T2: once cell dispersal is complete, activated Drice is detected in every cell (unstained areas are yolk fragments). T3: the nuclei have condensed (compare close-ups for T2 and T3). (D) TUNEL staining is not detected in any \(th^5\) homozygous embryos prior to cell dispersal (T1). However, once cell dispersal is completed (T2), all nuclei are positive for TUNEL. In T3, nuclei condensation is clearly seen (compare close-ups for T2 and T3). (E) Stills from a time-lapse movie (supplementary material Movie 3) of a \(th^5\) homozygous embryo in which cell membranes have been labelled with SrcGFP and imaged by confocal microscopy. Times are indicated from the end of cellularisation. The arrow in the first frame indicates the cephalic furrow. In the following frames, arrowheads label examples of the blebs observed in this movie. The embryo appears normal at first (C+10 minutes), then the cephalic furrow starts to regress, signalling the onset of cell shape changes. The embryo contracts abruptly, the cells undergo extreme cell shape changes and small membranous blebs start to form (C+25 minutes). The cells reach a more regular, rounded shape and the blebs form dynamically at the cell surface (C+40 minutes). Later, cells start fragmenting and blebbing decreases in intensity (C+55 minutes). Towards the end of the movie (C+65 minutes), most cells are fragmenting, and blebbing is detectable in only a fraction of the cell bodies. The timing of cell fragmentation in this movie matches the timing of cell fragmentation in the fixed samples (T3). (F) Summary of the sequence of apoptotic phenotypes in \(th^5\) embryos. Note that two hours after the end of cellularisation (T4), Engrailed expression is not detectable anymore in fixed \(th^5\) embryos (data not shown).
adopted a rounded morphology (C+40 minutes). Blebbing then remains constant until cells start deforming again and fragment (C+55 minutes). Cell fragmentation is accompanied by a decrease in blebbing, and later on, the majority of cell bodies do not show any blebbing activity (C+65 minutes). Cell fragmentation as observed in SrcGFP movies coincides with the time when cell fragmentation and nuclear condensation is observed in fixed samples. These observations suggest that blebbing occurs in th\(^5\) embryos with a very precise timing, appearing when cells start rounding up, and disappearing when cells fragment, with a maximum duration of 1 hour at room temperature.

In conclusion, our observations show an ordered sequence of apoptotic phenotypes in th\(^5\) embryos (Fig. 2F): first, caspase activation occurs shortly before cell dispersal when the embryo is morphologically normal. Then, by the time cells have rounded up and dispersed, every cell has initiated DNA fragmentation (monitored by TUNEL). Cells start blebbing at the onset of dispersal and continue to bleb for approximately 1 hour. Cell fragmentation and nuclear condensation occurs concomitantly with a reduction, and then loss, of blebbing activity. This indicates that the sequence of extranuclear changes described for mammalian cells (Mills et al., 1999) occurs in the context of an embryo.

**Caspase activation is required for the cell behaviours observed in embryos lacking DIAP1**

To test whether all of the dynamic shape changes in th\(^5\) embryos are a consequence of caspase activation, we overexpressed in these the general caspase inhibitor p35, which blocks cell death in Drosophila, C. elegans and mammalian cells (Hay et al., 1994; Miller, 1997). Taking advantage of the UAS/Gal4 system (Brand and Perrimon, 1993), we used the maternal driver tubulinGal4VP16 to overexpress UASp35 early and ubiquitously in th\(^5\) mutant embryos. The majority of th\(^5\) homozygous embryos overexpressing UASp35 develop normally, and have a normal pattern of Engrailed stripes, indicating that the abnormal cell behaviours are completely rescued (supplementary material Fig. S2). As expected, rescued embryos show absence of TUNEL staining, confirming that cell death is blocked effectively in embryos expressing p35 (supplementary material Fig. S2) (see also Rodriguez et al., 2002). This demonstrates that every aspect of the th\(^5\) phenotype, including cell shape changes and dispersal, requires caspase activation.

We then attempted to mimic the cellular phenotypes observed in th\(^5\) mutant embryos by overexpressing components of the cell death pathway with tubulinGal4VP16. As a control, we also expressed these components in the Drosophila eye with GMRGal4 (supplementary material Table S1). We find that overexpression of Hid or Reaper, two regulators of apoptosis that inhibit DIAP1 function (Holley et al., 2002; Ryoo et al., 2002; Wilson et al., 2002; Yoo, 2005; Yoo et al., 2002), produces th\(^5\)-like phenotypes in early embryos (supplementary material Table S1 and see Fig. 3). Reaper and Hid\(^{a5}\) (a non-phosphorylatable form of Hid, see legend of supplementary material Table S1) gave the strongest phenotypes. By contrast, overexpression of DRONC, the main apical caspase in Drosophila (Meier et al., 2000; Quinn et al., 2000), and p53, which activates apoptosis in response to cellular stress (Jin et al., 2000), do not cause significant phenotypes in early embryos, whereas they do in the eye (supplementary material Table S1). We can rule out that GMRGal4 is more effective than tubulinGal4VP16, because Hid\(^{a5}\) and Reaper give strong overexpression phenotypes in both tissues (supplementary material Table S1). Together, these observations indicate that whereas inhibition of DIAP1 is very effective at initiating apoptosis in early embryonic cells, Dronc and p53 are not sufficient to do so. Because inhibition of DIAP1 is known to trigger caspase activation, it is likely that caspase activity is required to induce the cell behaviours observed in embryos overexpressing Reaper or Hid\(^{a5}\). In support of this, we found that the abnormal cell behaviours are rescued when coexpressing p35 and Reaper in early embryos (not shown).

In conclusion, the above results show that caspase activation is required for the phenotypes observed in embryos lacking DIAP1 (either th\(^5\) mutant embryos or embryos overexpressing Reaper) and rules out a non-apoptotic role of DIAP1 in this context.

**Cell shape changes in embryos lacking DIAP1 are a specific effect of caspase activation and not a consequence of cell stress**

Our above results show that caspase activation directly affects cell behaviours in DIAP1-depleted embryos, but is it a specific effect or is it a mere consequence of cell stress? To test this, we used Ricin to produce a cellular stress distinct from apoptosis. Expression of Ricin in tissues leads to non-apoptotic cell death by irreversible inhibition of translation (Lord et al., 1994), and UASricin has been shown to inhibit translation in many Drosophila tissues (Allen et al., 2002; Moffat et al., 1992). We checked that expression in early embryos using tubulinGal4VP16 also results in translation inhibition: Engrailed protein levels (monitored by antibody staining) were strongly reduced, whereas transcription levels (assessed by in situ hybridisation with an engrafted probe) were normal (supplementary material Fig. S3). We then compared in more detail embryos overexpressing UASricin with those overexpressing UASreaper (abbreviated hereafter as Ricin\(^{\text{OVER}}\) and Reaper\(^{\text{OVER}}\), respectively), in order to identify the cell shape changes that are specific to the apoptotic program.

In Reaper\(^{\text{OVER}}\) embryos, the ectoderm contracts and the cells lose lateral adhesion as well as their basal connection with the yolk, round up and rearrange in multiple cell layers (supplementary material Movie 4 and Fig. 3B,E,F). This dramatic release is very similar to the release observed in th\(^5\) embryos. The only difference is that it happens at the end of celluarisation instead of shortly after the beginning of germ-band extension. Cell dispersal occurs in Reaper\(^{\text{OVER}}\) embryos (supplementary material Fig. 3S), but is less extensive than in th\(^5\) embryos, probably because of this timing difference (see Discussion). The time when phenotypes are seen in Reaper\(^{\text{OVER}}\) embryos corresponds to the time at which UAS constructs start to be expressed under the control of the tubulinGal4VP16 driver. We can rule out the possibility that th\(^5\) is not a null mutation because the phenotype of th\(^5\) embryos is indistinguishable from the phenotype of embryos homozygous for deficiencies removing the thread locus, such as Df(3L)brm11 or Df(3L)th117 (not shown) (Wang et al., 1999). We therefore propose that Reaper is inhibiting a maternal pool of DIAP1 protein when expressed at
cellularisation. In th5 embryos, this maternal pool would provide function until the beginning of germ-band extension, approximately 20 minutes later.

In contrast to ReaperOVER embryos, embryos overexpressing Ricin do not show th5-like phenotypes. RicinOVER embryos start displaying abnormal morphologies at the end of...
cellularisation, then morphogenetic movements cease and development arrests just prior to germ-band extension in most embryos (supplementary material Movie 5 and Fig. 3C,G). In these embryos, cell shapes become slightly irregular compared with wild type but the cells stay columnar (Fig. 3D versus Fig. 3G) and no cell dispersal is observed (supplementary material Fig. S3). This is dramatically different from the cell shape changes observed in either ReaperOVER or th5 embryos.

The differences in cell shape changes between Ricin and Reaper overexpression are matched by differences in the integrity of the adherens junctions and the cytoskeleton. We looked at the dynamics of DE-cadherin localisation in both RicinOVER and ReaperOVER embryos, using DEcadGFP as a reporter (Oda and Tsukita, 2001) (Fig. 3H-J). In ReaperOVER embryos (supplementary material Movie 6 and Fig. 3I) the ectoderm contracts and then DEcadGFP is delocalised in dots. In RicinOVER embryos, however, DEcadGFP is not delocalised and remains in the lateral membranes (supplementary material Movie 7 and Fig. 3J). Actin distribution as detected by phalloidin staining (Fig. 3K-P) also reveals clear differences. In ReaperOVER embryos, actin is delocalised and eventually concentrates on one side of the cells (Fig. 3L,O). By contrast, actin is cortical in RicinOVER embryos as in wild type (Fig. 3M,P versus Fig. 3K,N).

Developmental arrest in ReaperOVER embryos correlates with an activation of the caspase cascade, as in th5 embryos: in the first hour after the end of cellularisation, all ReaperOVER embryos showing a cell-rounding phenotype were positive for activated Drice (supplementary material Fig. S3), and a subset were also positive for TUNEL (not shown). By contrast, same stage RicinOVER embryos did not exhibit any cells positive for activated Drice or TUNEL (not shown). Together, these results demonstrate that cell stress caused by Ricin is not sufficient to induce the dramatic cell shape changes and cytoskeletal remodelling produced by caspase activation, namely cell release and the delocalisation of markers such as actin or DE-cadherin. Thus, caspase activation must specifically target cytoskeleton and epithelial polarity components to achieve these dynamic cell shape changes.

Hyperactivation and delocalisation of the actomyosin cytoskeleton in DIAP1 mutants

To characterise further how caspase activation modifies cell behaviours, we examined the actomyosin cytoskeleton in th5 embryos. In wild-type embryos at the beginning of germ-band extension, actin (revealed by phalloidin staining) is found apically at the cell cortex (Fig. 4A). In th5 mutant embryos fixed at the time when the embryo contracts and the cells disperse, we found subpopulations of cells showing elevated levels of actin at their cortex (Fig. 4B). This cortical staining is less regular than in wild-type cells, adopting a fibrous appearance. Later on, in embryos in which cells have just rounded up, we find a significant proportion of the cells with a wild-type actin distribution; i.e. a regular cortical staining (Fig. 4C, right-side of panel). The remaining cells exhibit a dramatic delocalisation of actin, with loss of cortical staining and the presence of a dense spot of actin on one side of the cell (Fig. 4C, left-side of panel). In older th5 embryos (Fig. 4D), all cells have delocalised their actin, as observed in ReaperOVER embryos (Fig. 3L,O). These observations suggest that when the embryo contracts and cells round up, actin localisation remains normal except in a subset of cells, which show elevated cortical actin. Actin is delocalised later, after the cells have rounded up.

Next, we followed Myosin II dynamics using green fluorescent protein (GFP)-tagged Spaghetti-Squash (SqhGFP), the Drosophila homologue of Myosin light chain (MLC) (Royou et al., 2002) (supplementary material Movies 8 and 9 and Fig. 4F,G). In wild-type embryos at the beginning of germ-band extension, SqhGFP forms apical cables at the anteroposterior membranes of cells (Bertet et al., 2004; Zallen and Wieschaus, 2004) (supplementary material Movie 8 and Fig. 4F). In th5 mutant embryos, cables form at the onset of germ-band extension as in wild-type (supplementary material Movie 9 and Fig. 4G). When the embryo starts contracting, however, the SqhGFP cables become thicker and take on a fibrous appearance, which is reminiscent of the actin distribution in th5 embryos at the same stage (Fig. 4, compare Fig. 4G, C+38 minutes, with Fig. 4B). Later on, when the cells have completely rounded up, the cables disappear and SqhGFP is found relocalised in punctate structures (Fig. 4G, C+50 minutes).

The high levels of actin and Myosin II detected in subpopulations of cells in contracting th5 embryos, as well as the cell blebbing activity (see Fig. 2E), suggest that the actomyosin cytoskeleton is hyperactivated in these embryos. In support of this, it has been shown in mammalian cells that Caspase-3 cleaves the kinase ROCK, making it constitutively active (Coleman et al., 2001; Sebbagh et al., 2001). Truncated ROCK then activates Myosin II light chain through phosphorylation. To ask whether caspase activation resulted in an increase in MLC phosphorylation in th5 embryos, we prepared extracts of WT and th5 homozygous embryos selected 1 hour after the end of cellularisation (C+1 hour), and analysed the extracts in western blots probed with an antibody recognising the phosphorylated form of Drosophila MLC (Lee and Treisman, 2004). We found a significant elevation in the amount of the phosphorylated form of MLC in th5 embryos versus WT (Mann-Whitney U-test, P<0.001, n=12) (Fig. 4E). This is consistent with hyperactivation of the actomyosin cytoskeleton in th5 embryos.

Discs large (Dlg), Bazooka (Baz) and DE-cadherin are delocalised in DIAP1 mutants

To characterise how caspase activation modifies epithelial characteristics in DIAP1 mutant cells, we looked at three markers for protein complexes that specify distinct epithelial domains: Baz (the fly homologue of Par3), DE-cadherin and Dlg. Baz is part of the Baz/PKC/Dpar-6 complex required for apical polarization, DE-cadherin is the main component of adherens junctions, and Dlg is part of the LGL/Dlg/Scribble complex that marks the lateral membrane below the adherens junctions (Tepass et al., 2001) (Fig. 5J).

In early wild-type embryos, Baz is localized apically at the cell cortex (Fig. 5A). During germ-band extension, cells undergo intercalation and Baz tends to be enriched in cortical fibres at the dorsoventral cell membranes (Fig. 5A) (Zallen and Wieschaus, 2004). In th5 embryos just after dispersal, Baz is still localised in cortical ‘fibres’ (Fig. 5B). A bit later, the fibrous-like localisation of Baz staining is lost, but the staining remains cortical (Fig. 5C, right-hand side). Later on, when cells have started fragmenting, Baz appears completely delocalised, with a cytoplasmic staining (Fig. 5D).
Analysis of apoptotic cell behaviours in Drosophila embryos lacking DIAP1

We followed the fate of adherens junctions during cell dispersal using time-lapse movies of embryos expressing DEcadGFP (Fig. 5E,F). We could not detect any difference in the behaviour or localisation of DEcadGFP between WT and th5 embryos, up to the onset of cell dispersal (Fig. 5E,F). The cephalic furrow forms normally (Fig. 5F, C+10 minutes), but shortly afterwards the cells within the furrow start to be misaligned (Fig. 5F, C+16 minutes). The cephalic furrow then regresses and DEcadGFP appears to move apically (arrowhead, C+24 minutes). The embryo at this stage undergoes strong contractions, and the apical DEcadGFP signal coalesces in places, indicating a constriction of the apical domain (arrowhead, C+48 minutes). Finally, DEcadGFP loses its apical localisation and is found in dots in the cytoplasm (Fig. 5F, C+64 minutes), as observed for ReaperOVER embryos (Fig. 3I).

An antibody against Dlg was used to mark the lateral domain (Fig. 5G). In th5 embryos fixed just at the onset of dispersal, the ectodermal tissue exhibits patches of cells in which Dlg appears to move apically (arrowhead, C+10 minutes). In the same embryo (left-side of C), some cells show a dramatic delocalisation of actin, with a very faint cortical staining, and dense accumulation of actin in one dense spot at the cortex. In older embryos (D), all cells have delocalised their actin. (E) Embryos lacking DIAP1 were collected 1 hour after cellularisation and extracts were analysed by western blotting using an antibody against the phosphorylated form of Myosin light chain (three independent extracts of 40 embryos each were loaded for each genotype). The blot was reprobed with an antibody against tubulin as a loading control (boxed bands). Band intensities were quantified and the ratio of phospho-MLC to tubulin is indicated in the graph. Shaded boxes show average values across the three samples for each genotype, whereas dotted boxes show the minimum and maximum values found for each genotype. (F,G) Stills from time-lapse supplementary material Movies 8 and 9 of WT and th5 mutant embryos labelled with SqhGFP. (F) Wild-type embryos undergoing germ-band extension exhibit a planar polarisation of SqhGFP, with cables running parallel to the dorsoventral (D/V) axis (C+20-50 minutes). In th5 embryos (G), SqhGFP planar polarisation is initially normal (C+20 minutes), and then an enrichment of Myosin II is seen in the cables compared with wild-type embryos (C+33 minutes). This enrichment culminates when the embryo contracts, and large regions of the embryo show a strong accumulation of Myosin II in cables as well as in fibrous aggregates at the cell cortices (C+38 minutes). When cells have rounded up (C+50 minutes), Myosin II is found in small dots that are likely to correspond to the membraneous blebs observed in the srcGFP movie (supplementary material Movie 3). Larger dots are also seen, which are likely to be remnants of the basal actomyosin II rings present in wild-type embryos between ectodermal cells and the yolk cell.

We followed the fate of adherens junctions during cell dispersal using time-lapse movies of embryos expressing DEcadGFP (Fig. 5E,F). We could not detect any difference in the behaviour or localisation of DEcadGFP between WT and th5 embryos, up to the onset of cell dispersal (Fig. 5E,F). The cephalic furrow forms normally (Fig. 5F, C+10 minutes), but shortly afterwards the cells within the furrow start to be misaligned (Fig. 5F, C+16 minutes). The cephalic furrow then regresses and DEcadGFP appears to move apically (arrowhead, C+24 minutes). The embryo at this stage undergoes strong contractions, and the apical DEcadGFP signal coalesces in places, indicating a constriction of the apical domain (arrowhead, C+48 minutes). Finally, DEcadGFP loses its apical localisation and is found in dots in the cytoplasm (Fig. 5F, C+64 minutes), as observed for ReaperOVER embryos (Fig. 3I).

An antibody against Dlg was used to mark the lateral domain (Fig. 5G). In th5 embryos fixed just at the onset of dispersal, the ectodermal tissue exhibits patches of cells in which Dlg
localisation is normal, and others in which Dlg is completely delocalised, showing a cytoplasmic staining (Fig. 5H). Normal Dlg localisation corresponds to cells that are still columnar, and delocalisation to cells that have rounded up. Later on, all cells show a cytoplasmic staining for Dlg (Fig. 5I).

The above results show that Baz, Dlg and DE-cadherin are delocalised early in the apoptotic sequence in th5 embryos. Dlg and Baz, but not DE-cadherin, are processed in embryos lacking DIAP1

A large number of caspase substrates have been identified in the past years (Fischer et al., 2003). It is not always clear, however, which substrates are cleaved at a given time in the apoptotic sequence, and which of the cleavages cause specific phenotypic changes. Embryos lacking DIAP1 give us an
opportunity to characterise these substrates and assign them to a given stage of apoptosis. Below, we ask whether delocalisation of Dlg, Baz and DE-cadherin at the onset of apoptosis can be explained by processing by caspases.

We prepared extracts of th5 homozygous embryos either 1 or 3 hours after the end of cellularisation, and monitored the molecular changes in Dlg, Baz and DE-cadherin on western blots (Fig. 6). In WT embryos, Dlg is detected as a doublet of approximately 120 and 100 kDa (Fig. 6A,B). This is consistent with previous reports showing that two main Dlg isoforms are found in embryonic extracts: a 120 kDa form and a 97 kDa isoform that lacks the SAP97-type N-terminus (Mendoza et al., 2003; Siegrist and Doe, 2005). These two forms are strongly reduced in early th5 embryos, and are replaced by faster migrating forms: a major band of approximately 85 kDa, and two minor bands of 35 and 20 kDa (Fig. 6A,B). In late th5 embryos, both the WT forms and the faster forms are reduced in quantity (Fig. 6A, C+3 hours), indicating that degradation occurs, albeit slowly. To check that these bands were dependent upon caspase activation, we also prepared extracts of ReaperOVER embryos and compared them with th5 extracts (Fig. 6B). The same faster migrating bands were detected in both extracts (Fig. 6B), indicating that these bands are caspase cleavage products. We next performed an analysis based on the known consensus for caspase cleavages (see details in Materials and methods). This analysis predicts that a single cleavage at DARD200 is responsible for the main cleavage product of 85 kDa detected in th5 and ReaperOVER extracts.

In WT, Baz, which has a predicted molecular weight (MW) of 157 kDa, migrates as a band of high MW at approximately 200 kDa (Fig. 6C). In th5 extracts, the full-length 200 kDa band disappears completely and is replaced by a faster migrating form of approximately 100 kDa and a fainter doublet at around 80 kDa. We hypothesise that these bands represent caspase cleavage products. The concentration of these products decreases in late embryos, showing that these are slowly degraded (Fig. 6C, C+3 hours). Analysis of the putative cleavage sites in the Baz sequence (see Materials and Methods) predicts that a single caspase cleavage at ASRD984 could generate the 100 kDa form.

DE-cadherin behaves differently from Baz and Dlg. In WT, DE-cadherin migrates as a main 120 kDa band as well as a fainter 180 kDa band (Fig. 6D). These two forms are consistent with the major 150 kDa and minor 200 kDa bands previously described (Oda et al., 1994). The minor form is thought to correspond to the DE-cadherin precursor before proteolytic cleavage, whereas the major form represents the mature form of DE-cadherin, which associates with Armadillo (the homologue of beta-catenin) and alpha-catenin. In th5 extracts, no apparent cleavage products were detected (Fig. 6D). Moreover, the mature form of DE-cadherin (approximately 120 kDa on our gel) is found in similar quantities in WT compared with th5 extracts, either 1 hour or 3 hours after the end of cellularisation. This suggests that DE-cadherin is not cleaved by caspases in early Drosophila embryos.

This work shows that extracts of embryos lacking DIAP1 can be very easily used to find caspase substrates. The fact that it is possible to examine these extracts immediately after the initiation of the caspase cascade makes cleavage detection easier (protein fragments are not degraded yet), and increases the chance of finding primary cleavages (rather than cleavage because of subsequent degradation). We propose that Baz and Dlg are primary substrates of caspase cleavage in Drosophila, whereas DE-cadherin is not.

**Discussion**

In this study, we induce apoptosis in early embryos at two distinct developmental time points: cellularisation and germ-band extension, using either embryos overexpressing Reaper or embryos null mutant for thread. Both experiments produce embryos depleted for DIAP1 because (1) th5 is a null mutant in DIAP1 and (2) Reaper overexpression has been demonstrated to dramatically decrease DIAP1 levels by stimulating its ubiquitin-dependent degradation (Holley et al., 2002; Ryoo et al., 2002; Wilson et al., 2002; Yoo, 2005; Yoo et al., 2002). As a consequence, embryonic cells enter apoptosis with remarkable synchrony in both experiments. We demonstrate that the embryonic cells go through a sequence of apoptotic cell behaviours similar to those described for mammalian cells. These behaviours are directly caused by the massive caspase activation occurring in embryos lacking DIAP1 (they are fully rescued by coexpression of caspase inhibitor p35). Also, these behaviours are caused by a specific action of caspases, because non-apoptotic cell stress produced by translation inhibition (using Ricin) does not mimic them. Taking advantage of the synchrony with which the embryonic cells enter apoptosis, we identify Dlg and Baz as putative early caspase substrates. This shows that embryos lacking DIAP1 provide a powerful model in which to analyse in detail the kinetics of subcellular and molecular changes during apoptosis.

**Early apoptotic phenotypes in embryos lacking DIAP1**

The earliest phenotype detected in embryos lacking DIAP1 is the very abrupt rounding up and dispersal of the embryonic cells. The extent of cell dispersal is less in embryos overexpressing Reaper compared with th5 mutants (supplementary material Fig. S3), presumably because of the difference in the time when the two types of embryos initiate cell rounding. Indeed, in th5 embryos the morphogenetic movements of mesoderm invagination and germ-band extension have already started when cells lose their basal and lateral attachments: this might explain the more extensive cell dispersal because of the sudden release of the mechanical tension generated by these morphogenetic movements. Aside from these differences, both types of embryos lacking DIAP1 go through strong contractions when the cells enter apoptosis (see, for example, supplementary material Movies 2 and 6). At the same time, high levels of actin and Myosin II are detected in a subset of the cells (Fig. 4). This is reminiscent of the contraction and the formation of actomyosin cables described for non-apoptotic cells surrounding an apoptotic cell in epithelia (Rosenblatt et al., 2001). Because caspase activation is initiated first in a subset of cells in th5 embryos (Fig. 2C), it is possible that these first apoptotic cells send a signal to the neighbouring cells to contract and assemble actomyosin cables as hypothesised by Rosenblatt et al. (Rosenblatt et al., 2001), which would explain the tissue behaviour of embryos lacking DIAP1.

We have examined the delocalisation of epithelial polarity markers in embryos lacking DIAP1. Dlg is delocalised as the cells round up (Fig. 5). This delocalisation is likely to be the direct consequence of cleavage of the Dlg protein (as detected
in thread mutant embryonic extracts, Fig. 6). Baz, the homologue of Par3, which localises at the apical membrane, was still found at the cortex in cells that had already rounded up (Fig. 5). However, shortly afterwards Baz was found delocalised in the cytoplasm. This delocalisation is consistent with cleavages in the Baz protein in thread embryonic extracts (Fig. 6). The third epithelial marker we looked at was DE-cadherin, which labels the adherens junctions. DE-cadherin was found delocalised in dots (Fig. 5), but no cleavage product was found that could explain this delocalisation. This suggests that there must be components required for proper cadherin localisation that are themselves cleaved by caspases. Baz is one candidate because it is required to maintain the DE-cadherin complexes in a medio-apical position (Harris and Peifer, 2004). Based on this study, Baz cleavage would be expected to delocalise DE-cadherin (Fig. 4). Another candidate that could be responsible for DE-cadherin delocalisation is Armadillo, the homologue of beta-catenin, which binds the cytoplasmic tail of cadherin to link it to the actin cytoskeleton. In support of this, early embryos mutant for Armadillo exhibit a pattern of
cadherin delocalisation similar to the one observed in thread mutant embryos (Harris and Peifer, 2004). Moreover, beta-catenin is cleaved during apoptosis in mammalian systems (Brancolini et al., 1997) and Armadillo has been found recently to be cleaved by the caspase-3 homologue Drice in DIAP1 mutant embryos (T. Kessler and A. Muller, personal communication).

Concomitant with these delocalisation events and these cleavages, the epithelial cells in embryos lacking DIAP1 undergo a dramatic loss of intercellular adhesion, round up and start blebbing. These events are clearly a consequence of caspase activation and are distinct from cellular stresses such as translational inhibition. A major challenge lies in establishing which caspase cleavage substrates are responsible for these dramatic phenotypes. Delocalisation of DE-cadherin alone would be sufficient to produce loss of lateral adhesion. As mentioned above, processing of Armadillo and/or Baz could both be responsible for DE-cadherin delocalisation. Dlg delocalisation is not known to affect intercellular adhesion; however, loss of Dlg would be sufficient for the cells to lose their columnar shape and round up (Bilder et al., 2000). In addition to the loss of lateral adhesion and cell rounding, cells also lose basal contacts, both in late cellularised embryos (Reaper overexpression) or in embryos initiating germ-band extension (thread-null mutants). At these stages, the columnar epithelial cells are still linked to the yolk cell by cytoplasmic bridges, and they sever this membranous link later on, through a process related to cytokinesis (Mazumdar and Mazumdar, 2002). The abrupt loss of basal contact in embryos lacking DIAP1 suggests that the cells undergo premature cytokinesis. This could be explained if the caspase cascade activates the actomyosin cytoskeleton. In support of this, it was shown in mammalian cells that Caspase-3 cleaves the kinase ROCK to make it constitutively active (Coleman et al., 2001; Sebbagh et al., 2001), and that truncated ROCK activates MLC through phosphorylation. Hyperactivation of the actomyosin cytoskeleton could also explain the onset of cell blebbing in embryos lacking DIAP1, because MLC phosphorylation has been implicated in apoptotic membrane blebbing in mammalian cells (Mills et al., 1998). In support of this, we detected an increase in the phosphorylated form of MLC in th5 extracts (Fig. 4E). Note that no cleavage product of the phospho-MLC was detected in these western blots. The hyperphosphorylation of MLC might also explain the increase in cortical actin and Myosin II observed in a subset of cells in th5 embryos at the onset of apoptosis (Fig. 4).

In an attempt to address the functional contribution of Myosin II hyperphosphorylation to the DIAP1 mutant phenotype, we injected th5 embryos with Y27632 (supplementary material Fig. S4). This drug inhibits ROCK in mammalian cells and in Drosophila embryos, and as a consequence inhibits MLC activity (Ishizaki et al., 2000; Royou et al., 2002). We found a partial rescue of the th5 phenotype, with injected embryos exhibiting a reduction in the multilayering of cells (supplementary material Fig. S4). This suggests that Myosin II contributes to this aspect of the DIAP1 phenotype: it could be consistent with a role in premature cytokinesis and contraction of the tissue, which would both increase multilayering. Consistent with the requirement of Dlg, Baz and DE-cadherin in epithelial integrity, however, cell rounding and dispersal still occur in these embryos (supplementary material Fig. S4 and not shown). This experiment underlines the limit of single gene rescue in this system in which several components are targeted by the apoptotic program.

**Embryos lacking DIAP1 as a model system to investigate caspase cleavages**

Together with Wang et al. (Wang et al., 1999), our study shows that the cells in embryos lacking DIAP1 undergo a series of stereotypic changes, namely cell rounding, cell blebbing, DNA fragmentation, cell fragmentation and nuclear condensation. These characteristics are remarkably similar to the phenotypes described for mammalian apoptotic cells (Mills et al., 1999). As in mammalian cells, our work shows that one can distinguish between early apoptotic phenotypes, namely cell release and membrane blebbing, the latter lasting approximately 1 hour-or-so after cell release, and later phenotypes, when cells start to produce intact fragments called apoptotic bodies, associated with nuclear condensation. Remarkably, the blebbing phase lasts close to 1 hour both in embryos lacking DIAP1 and in mammalian cells (Mills et al., 1999). The actomyosin cytoskeleton has been implicated both in apoptotic cell blebbing (see above) and in apoptotic body formation (Lane et al., 2005) and nuclear disintegration (Croft et al., 2005). The cause of the transition from an apoptotic cell extending small membranous blebs, to cells fragmenting into apoptotic bodies is not known.

It has been hypothesised that considering the remarkable conservation of the apoptotic phenotypes and of the biochemical pathways leading to caspase activation, that a conserved group of substrates must exist (Martin, 2002). However, to our knowledge, the homologues of Dlg and Baz have not been found to be a substrate of caspases in mammalian systems. A clearer picture should emerge upon completion of proteomic analyses of apoptotic cells in different organisms (Adrain et al., 2004). We propose that thread-null mutant embryos are an ideal in vivo model in which to apply proteomics technology. First, one can compare embryos lacking DIAP1 in which thousands of cells are entering apoptosis in a remarkably synchronous fashion, with wild-type embryos in which apoptosis is absent [developmental apoptosis does not occur before stage 11 in embryogenesis (Abrams et al., 1993), i.e. 2 hours after apoptosis has started in embryos lacking DIAP1]. Second, there is no need for an external agent to induce apoptosis, because DIAP1 is removed genetically. Third, as we have shown, a clear sequence of apoptotic phenotypes occur in a 2-hour window, which allows one to collect extracts at distinct time-points with the aim of identifying substrates cleaved by caspases in the early versus late phases of apoptosis as defined above.

**Materials and Methods**

**Drosophila strains**

The w1118 strain was used as wild type. Two alleles of thread were employed: th4, a hypomorphallele, and th5, a null allele (Hay et al., 1995). The UAS/Gal4 system (Brand and Perrimon, 1993), ubiquitous expression in early embryos was driven by the maternal driver tubulinGal4VP16 (a gift from D. St Johnston, University of Cambridge, UK) (see also Hacker and Perrimon, 1998). Expression in the eye was driven by GMRGal4 (Freeman, 1996). The UAS strains used were: UASScrGFP (Kalschenidt et al., 2000), UASHid::VP16 (Belleauche et al., 2001), UASp53 (Zhou et al., 1997), UASp33 (Jin et al., 2000), UASsNDronc and UASsDronc (Meier et al., 2000), UAShid and UASreaper (Zhou et al., 1997), UAShind (Bergmann et al., 2002) and UASrinc (Allen et al., 2002). Other
transgenic strains were DEcadGFP (Oda and Tsukita, 2001) and SqhGFP42; SqhGFP40 (abbreviated as SqhGFP) (Royou et al., 2002). LacZ and GFP expression from the balancer chromosomes TM3hblacZ and TM3krrGal4UASGFP (Casso et al., 1999) were used to genotype the embryos. We built the recombiant chromosomes tubulinGal4VP16.th5, UASp35.th5 and UASp40.th5.

UAS/GAL4 overexpression assays

All overexpression experiments were performed at 25°C. For the rescue experiment with p35, tubulinGal4VP16.th5/TM3hblacZ females were crossed with UASp35.th5/TM3hblacZ males. For overexpression in embryos (supplementary material Table S1), tubulinGal4VP16 females were crossed with UAS males. Note that levels of expression vary amongst embryos because of mosaic expression of tubulinGal4VP16. Bicoid and the germline (D. St Johnston, personal communication). This phenomenon is observed with all tubulinGal4VP16 lines tested so far. For overexpression in the eye (supplementary material Table S1), GMRGal4 females were crossed with UAS males.

Immunocytochemistry and immunofluorescence on whole mount embryos

For all stainings, embryos were fixed for 20 minutes in 4% phosphate-buffered saline/formaldehyde. For actin stainings, embryos were then deionised manually and stained immediately, without dehydration, using Alexa Fluor-488-conjugated phalloloid (Molecular Probes). For all other stainings, embryos were deionised using methanol.

Immunocytochemistry and immunofluorescence on fixed embryos were performed according to standard protocols [as described by N. Patel in Goldstein (Goldstein, 1994)]. Primary antibodies were: En [1:50, 4D9; Developmental Studies Hybridoma Bank (DSHB)], Eve (1:5000; provided by M. Frasch, University of Erlangen-Nuremberg, Germany), activated Drice (1:1000; provided by A. Müller, University of Dundee, UK), phosphoryistone H1 (1:300; Upstate Biotechnology Dlg) (1:200; 4F3; DSHB), Baz (1:200; provided by A. Wodarz, University of Düsseldorf, Germany), Fkh (1:1000; provided by P. Carrera, University of Göttingen, Germany), β-gal (1:5000; Cappel), Twist (1:5000; provided by S. Roal, University of Cologne, Germany). Secondary antibodies (1:250) were: goat anti-mouse Alexa Fluor-488 and -594, goat anti-rabbit Alexa Fluor-488 and -594 (Molecular Probes), biotinylated donkey anti-rabbit, anti-guinea pig and goat anti-mouse (Jackson Immunoresearch).

Fixed time-lapses

Embryos from the th5/TM3hblacZ stock were collected at 25°C on grape juice plates every hour and left at age to 2.5 hours to reach late cellularisation. Embryos at the end of cellularisation were hand-selected and either fixed immediately (T0) or fixed after aging for 0.5 hours (T1), 1 hour (T2), 1.5 hours (T3) or 2 hours (T4), and then stained (that β-gal staining was used to identify th5 embryos).

Injection of Y-27632

Y-27632 is a specific inhibitor of the ROCK family of protein kinases (Ishizaki et al., 1994). Injection of Y-27632 under a dissecting microscope (Leica) activated Drice (1:1000; provided by M. Frasch, University of Erlangen-Nuremberg, Germany), activated Drice and time-lapses were performed using an in situ cell death detection kit (TUNEL Label and Enzyme, Roche) for confocal analysis, or prepared for light microscopy (see below). Embryos were left to develop until 4 hours. Time-lapse movies of live embryos were collected at room temperature (confocal movies at 19-21°C, Nomarski movies at 22-23°C) and time points were taken at either 1-minute (supplementary material Movies 2, 4, 5 and 6) or 30-second intervals (supplementary material Movies 1, 3 and 8), using either wide-field or confocal microscopy (see below). Embryos were left to develop until cuticle formation after each movie to check that the embryos did not die of anoxia. The following genotypes were used (see Movies 1-9 in supplementary material): HistoneYFP[th5]; UASH2BmYFP+/+; tubulinGal4VP16.th5/th5 (Fig. 1, Movie 1); th5/th5 (Fig. 1, Movie 2); SrcGFP[th5]+/UASsrcGFP+; tubulinGal4VP16.th5/th5 (Fig. 2, Movie 3); ReaperOVR; tubulinGal4VP16/UASreaper (Fig. 3, Movie 4); RicinOVR; tubulinGal4VP16/UASricin (Fig. 3, Movie 5); DEcadGFP ReaperOVR; tubulinGal4VP16; DEcadGFP/UASreaper (Fig. 3, Movie 6); DEcadGFP RicinOVR; tubulinGal4VP16; DEcadGFP/UASricin (Fig. 3, Movie 7); DEcadGFP +/UASGFP42; SquhGFP40 (Fig. 4, Movie 8); SquhGFP[th5]+/UASgfp40.th5/SquhGFP40.th5 (Fig. 4, Movie 9).

Time-lapse movies of live embryos

Embryos were selected at cellularisation, mounted on 0.2-permeable membrane (Sartorius) and covered in V oltalef oil (Attachem). Movies were recorded at room temperature at 25°C. Nomarski microscopy was used at 22-23°C and time points were taken at either 1-minute (supplementary material Movies 2, 4, 5 and 6) or 30-second intervals (supplementary material Movies 1, 3 and 8), using either wide-field or confocal microscopy (see below). Embryos were left to develop until cuticle formation after each movie to check that the embryos did not die of anoxia. The following genotypes were used (see Movies 1-9 in supplementary material): HistoneYFP[th5]; UASH2BmYFP+/+; tubulinGal4VP16.th5/th5 (Fig. 1, Movie 1); th5/th5 (Fig. 1, Movie 2); SrcGFP[th5]+/UASsrcGFP+; tubulinGal4VP16.th5/th5 (Fig. 2, Movie 3); ReaperOVR; tubulinGal4VP16/UASreaper (Fig. 3, Movie 4); RicinOVR; tubulinGal4VP16/UASricin (Fig. 3, Movie 5); DEcadGFP ReaperOVR; tubulinGal4VP16; DEcadGFP/UASreaper (Fig. 3, Movie 6); DEcadGFP RicinOVR; tubulinGal4VP16; DEcadGFP/UASricin (Fig. 3, Movie 7); DEcadGFP +/UASGFP42; SquhGFP40 (Fig. 4, Movie 8); SquhGFP[th5]+/UASgfp40.th5/SquhGFP40.th5 (Fig. 4, Movie 9).

Image acquisition

Nomarski images (immunofluorescence and supplementary material Movies 2, 4 and 5) were taken on an upright Zeiss AxioPlan2 wide-field microscope with an Orca cooled CCD camera (Hamamatsu) controlled by the Openlab software (Improvision) (objective: 20×; Plan-Neofluar 0.5 NA, 40×; Plan-Neofluar 0.75 NA, 63× oil Plan-Achromat (1.4 NA)). Fluorescent images (immunofluorescence and supplementary material Movies 1, 3, 6 and 7) were collected on an MRC1024 Bio-Rad confocal microscope coupled to an upright Nikon Eclipse E800 microscope (objective: 40× oil-Plan-Fluo (1.3 NA), 60× oil Plan-Apo (1.4 NA)). A single optical Z-slice is shown in supplementary material Movies 1, 3, 6 and 7. For supplementary material Movies 8 and 9, images were collected on an inverted microscope (Zeiss Axiovert) with a Perkin-Elmer Spinning Disk confocal microscope (objective: 63× oil Plan-Neofluar).


Table S1. Overexpression phenotypes of cell-death-pathway components in embryos and adult *Drosophila* eye

<table>
<thead>
<tr>
<th>UAS construct</th>
<th>Overexpression in early embryos (tubulinGal4VP16)</th>
<th>Overexpression in the eye (GMRGal4)</th>
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<tbody>
<tr>
<td>UASp53 on II</td>
<td>No phenotype</td>
<td>Reduced and rough eye</td>
</tr>
<tr>
<td>UASp53 on III</td>
<td>No phenotype</td>
<td>Mild rough eye phenotype</td>
</tr>
<tr>
<td>UAS Ndronc</td>
<td>pc in late embryos</td>
<td>Lethal at pharate stage</td>
</tr>
<tr>
<td>UASproDronc</td>
<td>Weak phenotype in late embryos</td>
<td>Reduced and rough eye</td>
</tr>
<tr>
<td>UASHid on II</td>
<td>Weak phenotype in early embryos</td>
<td>Very reduced and rough eye</td>
</tr>
<tr>
<td>UASHidΔ380</td>
<td>Mild phenotype in early embryos</td>
<td>Lethal at pharate stage</td>
</tr>
<tr>
<td>UASHidala5</td>
<td>Strong phenotype in early embryos</td>
<td>Lethal at pharate stage</td>
</tr>
<tr>
<td>UASreaper</td>
<td>Strong phenotype in early embryos</td>
<td>Lethal at pharate stage</td>
</tr>
<tr>
<td>UASricin</td>
<td>Strong phenotype in early embryos</td>
<td>Lethal at pharate stage</td>
</tr>
</tbody>
</table>

Overexpression of UASp53 causes cell death in the *Drosophila* eye but not in early embryos. Similarly, overexpression of either UASNdronc (a constitutively active form of DRONC) or UASproDronc (a form containing the pro-domain) does not produce ectopic cell death in early embryos (assayed by TUNEL stainings), nor does it cause cell dispersal (assayed by Engrailed staining). In some late embryos (stage 11 and later), we observed a slight elevation of TUNEL-positive cells, as well as some disruption in the pattern of the Engrailed stripes, suggesting that these constructs have some activity in older embryos. Two different UASHid insertions gave some ectopic cell death in early embryos, as well as partial phenotypes of cell dispersal, similar to those observed for tk' alleles (see Fig. 1B). A mutated form of Hid, UASHidala5 had a much stronger effect, with all cells becoming TUNEL-positive, associated with a complete cell dispersal phenotype. This phenotype is identical to the phenotype of embryos overexpressing UASreaper. In UASHidala5, five phosphoacceptor residues of MAPK phosphorylation sites have been changed to non-phosphorylatable Ala residues (Bergmann et al., 1998; Bergmann et al., 2002), suggesting that the cells that do not die in tubulinGal4VP16/UASHid embryos are protected from Hid killing by MAPK signalling.

References
