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The cleaved-Caspase-3 antibody is a marker of Caspase-9-like DRONC activity in *Drosophila*

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The cleaved-Caspase-3 antibody is a popular tool in apoptosis research in *Drosophila*. As the antibody was raised against cleaved human Caspase-3, it was assumed that it detects cleaved DRICE and DCP-1, Caspase-3-like effector caspases in *Drosophila*. However, as shown here, strong immunoreactivity persists in apoptotic models doubly mutant for *drICE* and *dcp-1*. In contrast, mutants of the apoptosome components DRONC (Caspase-9-like) and ARK (Apaf-1 related) do not label with the cleaved-Caspase-3 antibody. By peptide blocking experiments and further genetic studies, we provide evidence that the cleaved-Caspase-3 antibody recognizes multiple proteins including DCP-1 and likely DRICE, but also at least one additional unknown protein, all of which require DRONC for epitope exposure. The unknown substrate may be involved in non-apoptotic functions of DRONC. Because the cleaved-Caspase-3 antibody not only detects cleaved Caspase-3-like proteins in *Drosophila*, but also other proteins in a DRONC-dependent manner, it is more accurate to consider the cleaved-Caspase-3 antibody as a marker for DRONC activity, rather than effector caspase activity.
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The cleaved-Caspase-3 (Asp175) antibody (referred to as cleaved-Caspase-3 antibody) from Cell Signaling Technology (Danvas, MA, USA) is a polyclonal antibody obtained from rabbit that was raised against a peptide in the large subunit of the human effector caspase, Caspase-3, amino-terminal to Asp175.[1] The antibody does not detect unprocessed Caspase-3. However, after proteolytic cleavage between Asp175 and Ser176, separating the large and small subunits leads to the activation of Caspase-3, the epitope is exposed and can be detected by cleaved-Caspase-3 antibody, thus making the antibody a marker for cleaved and active Caspase-3 in dying cells. A similar antibody, termed CM1, has previously been described;[2,3] however, the CM1 antibody is no longer available and is not a subject of this analysis.

Apoptosis in *Drosophila* is under the control of pro-apoptotic genes *reaper*, *hid* and *grim*.[4–6] (reviewed in[7]) The products of these genes trigger apoptosis through the induction of proteolytic degradation of inhibitor of apoptosis proteins (IAPs), most notably DIAP1,[8–11] to activate a caspase program. Of the seven caspase genes in *Drosophila*,[7] only the putative initiator caspase DRONC which is most similar to mammalian Caspase-9, and the Caspase-3-like effector caspases DRICE and DCP-1 have been implicated in developmental apoptosis.[12–18] After its release from DIAP1 inhibition, DRONC becomes a part of the apoptosome through interaction with ARK, also known as HAC-1 and D-APAF-1, the APAF-1-related gene in *Drosophila*.[19–21] The apoptosome cleaves and activates the effector caspases DCP-1 and DRICE.

The cleaved-Caspase-3 antibody has become a very popular tool for detection of dying cells in *Drosophila* (see for example references [3,13,22–27]). As it has been raised against an epitope of human Caspase-3, it was proposed that the antibody would cross-react with the cleaved Caspase-3-like effector caspases DRICE and DCP-1 in *Drosophila*.[3] However, this has never been rigorously tested. Previously, it was shown that the antibody does not loose its immunoreactivity in *drICE* single mutants,[15] thus suggesting that it is not specific for cleaved DRICE. However, partial redundancy with DCP-1 (ref. [13]) may account for this result. Here, we show that the cleaved-Caspase-3 antibody still labels cells induced to die, which are double mutants for defined null alleles of *dcp-1* and *drICE*. In contrast, the antibody requires the activity of the apoptosome components DRONC and ARK for immunoreactivity. Subsequent analysis demonstrates that the cleaved-Caspase-3 antibody detects at least one additional putative DRONC substrate that may be involved in non-apoptotic processes. Because the cleaved-Caspase-3 antibody is not entirely specific for cleaved DRICE and DCP-1, but requires DRONC for its immunoreactivity, we propose that it is more accurate to refer to this antibody as a marker of DRONC activity in *Drosophila*.

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Abbreviations: APAF-1, apoptosis peptidase-activating protein-1; ARK, apoptosis peptidase-activating protein-1-related killer; D-APAF-1, *Drosophila* apoptosis peptidase-activating protein-1; DCP-1, death caspase-1; ΔN, delta N-terminus; DIAP-1, *Drosophila* inhibitor of apoptosis protein-1; DRICE, *Drosophila* interleukin-converting enzyme; DRONC, *Drosophila* Nedd2-like caspase; ETD, Glu–Thr–Asp; GMR, glass multimer reporter; HAC-1, homolog of APAF-1 and Ced4-1; IAP, inhibitor of apoptosis protein; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP-nick end labeling
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Results

Labeling by the cleaved-Caspase-3 antibody persists in *dcp-1 drICE* double mutants. To evaluate the specificity of the cleaved-Caspase-3 antibody, we analyzed eye imaginal discs from third instar larvae. In wild-type eye imaginal discs, the antibody detects a few dying cells scattered throughout the disc (Figure 1a). Surprisingly, in eye discs doubly mutant for the null alleles *dcp-1^{Prev}* and *drICE$^{\Delta 1}$* (ref.[14,15]), the cleaved-Caspase-3 antibody still labels cells (Figure 1b). Labeling in *dcp-1^{Prev} drICE$^{\Delta 1}$* double mutants occurs in clusters (Figure 1b), similar to what has been observed previously when cell death was blocked by the expression of Caspase-3 inhibitor P35.[3] These observations would suggest that the cleaved-Caspase-3 antibody still detects an epitope in the absence of the Caspase-3-like proteins DCP-1 and DRICE. Nevertheless, as apoptosis at this stage does not occur in a defined pattern, we were uncertain about the specificity of these labeling signals.

Therefore, we used *GMR-hid* transgenes, a well characterized apoptotic model,[5] to further examine the specificity of the cleaved-Caspase-3 antibody. Through *GMR*-driven expression of the pro-apoptotic gene *hid* specifically in the posterior half of the developing eye, *GMR-hid* transgenes induce apoptosis in two distinct waves as shown by cleaved-Caspase-3 antibody and TUNEL labeling[28] (Figure 1c,d). To evaluate the specificity of the cleaved-Caspase-3 antibody, we examined *GMR-hid* eye discs that were doubly mutant for *dcp-1^{Prev}* and *drICE$^{\Delta 1}$*. Consistent with the expectation, loss of *dcp-1^{Prev}* and *drICE$^{\Delta 1}$* completely abrogates TUNEL-positive apoptosis in *GMR-hid* discs (Figure 1f). Surprisingly, however, *dcp-1^{Prev} drICE$^{\Delta 1}$* double mutant *GMR-hid* eye discs still showed strong immunoreactivity with cleaved-Caspase-3 antibody (Figure 1e). Thus, the cleaved-Caspase-3 antibody does not or not only detect DRICE and/or DCP-1. We do note, though, that the labeling appearance of the cleaved-Caspase-3 antibody changes in the absence of DCP-1 and DRICE (compare Figure 1c and e). The labeling signal is no longer restricted to two distinct waves (Figure 1c), but rather fills the entire posterior compartment of the eye disc and is confined to interommatidial cells (Figure 1e). A similar change of labeling pattern has been reported for CM1 antibody labeling upon expression of the caspase inhibitor P35.[3] This change of the labeling pattern is likely because of the fact that cells in *dcp-1^{Prev} drICE$^{\Delta 1}$* double mutant *GMR-hid* eye discs do not die (Figure 1f) and thus, maintain the epitope detected by cleaved-Caspase-3 antibody. However, it is important to note that this analysis demonstrates the detection of an epitope in the absence of Caspase-3-like proteins DCP-1 and DRICE by cleaved-Caspase-3 antibody.

Immunoreactivity of the cleaved-Caspase-3 antibody depends on the apoptosome components DRONC and ARK. There are two possibilities why the cleaved-Caspase-3 antibody labels *dcp-1 drICE* double mutant cells,

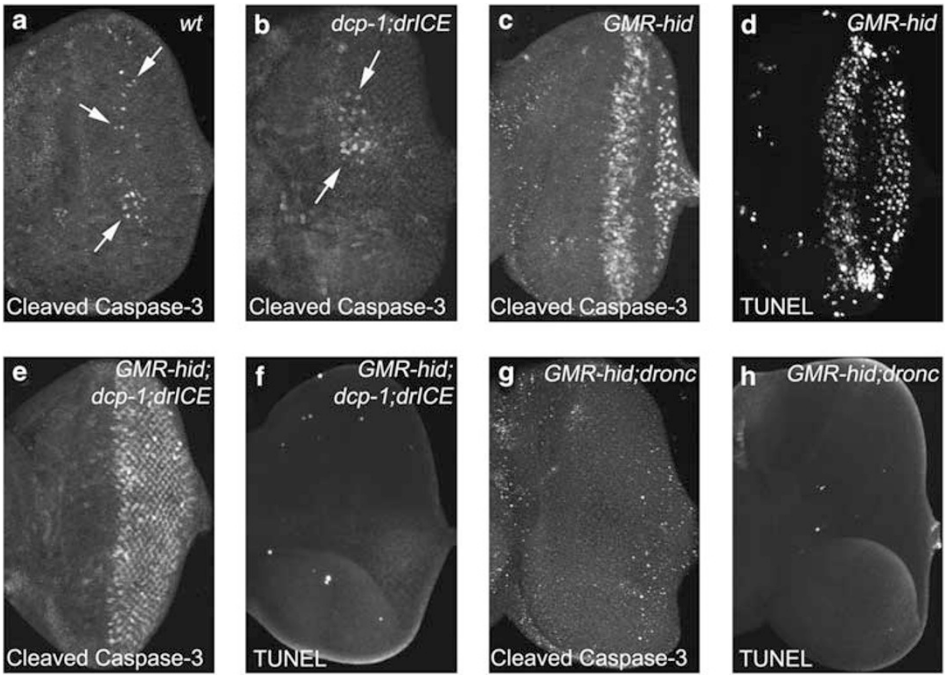

Figure 1 The cleaved-Caspase-3 antibody is a marker for DRONC activity. Shown are eye imaginal discs of third instar larvae. Posterior is to the right. *GMR-hid* is a transgenic insertion on the X chromosome.(**a**) Wild-type (wt) disc labeled with cleaved-Caspase-3 antibody. Arrows point to a few immunopositive cells.(**b**) A disc doubly mutant for the null alleles *dcp-1^{Prev}* and *drICE$^{\Delta 1}$* labeled with cleaved-Caspase-3 antibody. Arrows point to a few immunopositive cells.(**c**) and (**d**) *GMR-hid* eye discs in otherwise wild-type background labeled with (**c**) cleaved-Caspase-3 antibody and (**d**) TUNEL. Note the strong signals in the posterior half of the eye discs. (**e**) and (**f**) *GMR-hid* eye discs doubly mutant for *dcp-1^{Prev}* and *drICE$^{\Delta 1}$* labeled for (**e**) cleaved-Caspase-3 antibody and (**f**) TUNEL. Although TUNEL labeling is completely blocked, cleaved-Caspase-3 antibody still delivers a strong signal in the posterior half of the eye disc.(**g**) and (**h**) *GMR-hid* eye discs mutant for the null allele *dronc^{I24}*. Both (**g**) cleaved-Caspase-3 antibody and (**h**) TUNEL labeling are blocked by loss of DRONC. Genotypes: (a) wild-type; (b) *dcp-1^{Prev}/dcp-1^{Prev}*; *drICE$^{\Delta 1}$/drICE$^{\Delta 1}$*; (c) and (d) *GMR-hid/GMR-hid*; $+/+$, $+/+$; (**e**) and (**f**) *GMR-hid/GMR-hid*; *dcp-1^{Prev}/dcp-1^{Prev}*; *drICE$^{\Delta 1}$/drICE$^{\Delta 1}$*; (**g**) and (**h**) *GMR-hid/GMR-hid*, *dronc^{I24}/dronc^{I24}*

although they are not apoptotic. First, the antibody may not detect an apoptotic epitope; or second, the antibody may detect an apoptotic epitope generated upstream or in parallel to DCP-1 and DRICE. To distinguish between these possibilities, we examined *GMR-hid* eye discs mutant for the apoptosome components DRONC and ARK, which act upstream of DRICE and DCP-1. In *dronc* and *ark* mutant *GMR-hid* eye discs, both TUNEL and cleaved-Caspase-3 antibody labelings are blocked (Figure 1 g,h; Figure 3a,b). These data confirm that the cleaved-Caspase-3 antibody indeed detects an apoptotic epitope in *Drosophila*. Furthermore, because it fails to detect the apoptotic epitope in *dronc* and *ark* mutants, but not in *dcp-1 drICE* double mutants, it is more accurate to consider the cleaved-Caspase-3 antibody as a marker for DRONC activity, rather than effector caspase activity, in dying *Drosophila* cells.

The tripeptide ETD is the apoptotic epitope detected by cleaved-Caspase-3 antibody. The epitope detected by the cleaved-Caspase-3 antibody depends on DRONC activity. It may be possible that the antibody directly recognizes activated DRONC. Alternatively, it is also possible that the cleaved-Caspase-3 antibody detects an epitope generated through the cleavage of an unknown substrate by active DRONC, independently of DRICE and DCP-1.

To distinguish between these two possibilities, we aligned the residues from the catalytic cysteine (Cys163) to the cleavage site at Asp175 of human Caspase-3 (Caspase-3 peptide) with the corresponding regions of the *Drosophila* caspases (Figure 2a; see also ref. [3]). The most C-terminal residues of the Caspase-3 peptide, ETD, are conserved in DRICE and DCP-1 (Figure 2a). Similar to Caspase-3, this is the cleavage site for activation of at least DRICE,[29] and possibly DCP-1. It is interesting to note that the N-terminal, two-third of the Caspase-3 peptide, bears highest similarity to DRONC; six out of nine residues are conserved (Figure 2a). This part of the Caspase-3 peptide is less well-conserved in DRICE, DCP-1 and the remaining *Drosophila* caspases. Although cleavage between the large and small subunits of DRONC is not necessary for its activity,[30,31] and may not occur *in vivo*, we considered the possibility that antibodies directed against the N-terminal part of Caspase-3 peptide may directly detect active DRONC in dying cells.

We used blocking peptides to evaluate which epitopes of the Caspase-3 peptide are detected by the cleaved-Caspase-3 antibody in dying *Drosophila* cells. The sequences of the blocking peptides are shown in Figure 2b and underlined in Figure 2a. Blocking peptide A (TETD) is derived from DRICE and DCP-1 and blocking peptide B (CRGDEYDLG) corresponds to the region of highest similarity in DRONC (Figure 2a,b). Peptide C is a control peptide corresponding to the residues immediately adjacent to peptide B in DRONC (Figure 2a,b). Peptide D is another control peptide, which is very similar to peptide A and is derived from the prodomain of DRONC at position 113. If the prodomain of DRONC is cleaved at this site, it will expose ESD at its C-terminus, which is very similar to the C-terminus of the Caspase-3 peptide (ETD, peptide A) and may, thus, be detected by the cleaved-Caspase-3 antibody.

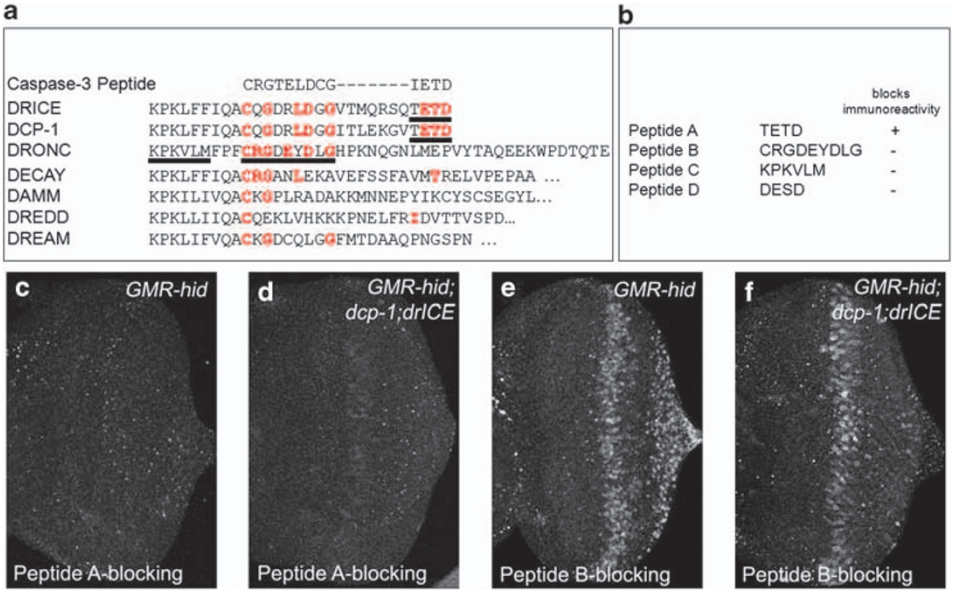

Figure 2 A peptide containing ETD blocks cleaved-Caspase-3 immunoreactivity.(**a**) Amino-acid sequence alignment of the residues from catalytic Cys163 to the cleavage site at Asp175 of human Caspase-3 (Caspase-3 peptide) and the corresponding regions of the *Drosophila* caspases. Residues highlighted in red are identical in the Caspase-3 peptide. For DRICE, DCP-1 and DRONC cleavage has been demonstrated following the last residue (**d** and **e**) of the sequence shown. For DECAY, DAMM, DREDD and DREAM cleavage is uncertain and the end of the sequence shown does not imply cleavage (indicated by …). Underlined sequences were used in blocking peptides (compare with **b**). (**b**) Amino-acid sequences of the blocking peptides. Only peptide A blocks immunoreactivity of the cleaved-Caspase-3 antibody. (**c** and **d**) Preincubation of cleaved-Caspase-3 antibody with peptide A completely abrogates its immunoreactivity in *GMR-hid* eye discs (**c**) and *GMR-hid* eye discs mutant for *dcp-1* and *drICE* (**d**). (**e** and **f**) Preincubation of cleaved-Caspase-3 antibody with peptide B has little or no effect on its immunoreactivity in *GMR-hid* eye discs (**e**) and *GMR-hid* eye discs mutant for *dcp-1* and *drICE* (**f**). Similar results were obtained for peptides **c** and **d** (data not shown)

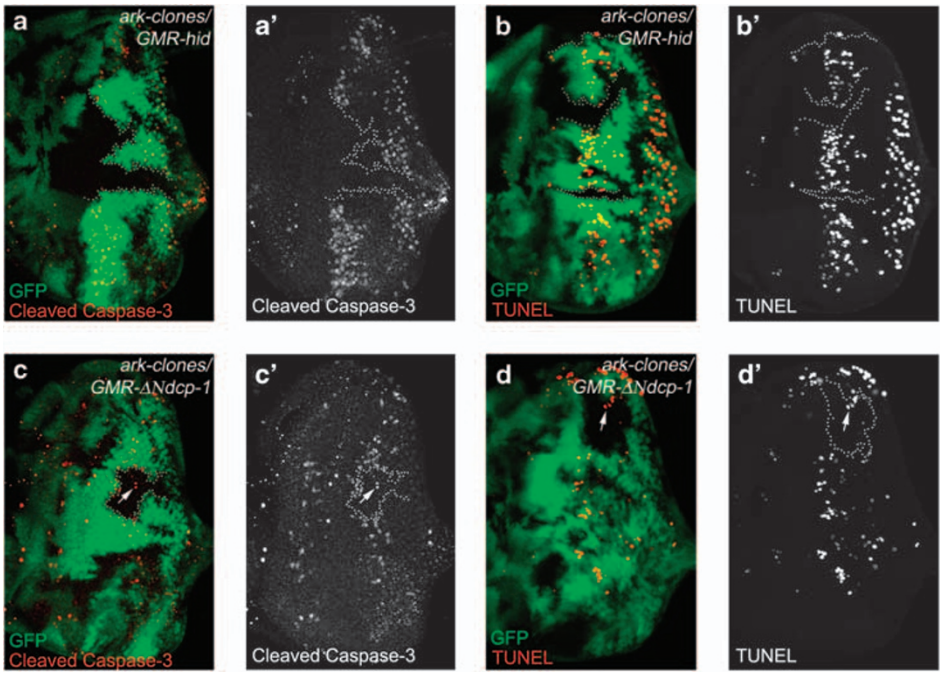

Figure 3 Expression of a *ΔN-dcp-1* transgene in *ark* mutant clones partially restores cleaved-Caspase-3 immunoreactivity. (**a**, **a'**, **b**, **b'**) *GMR-hid* eye discs containing *ark* mutant clones were labeled with cleaved-Caspase-3 antibody (**a**, **a'**) and TUNEL (**b**, **b'**). *ark* mutant clones are marked by absence of GFP. A few clonal boundaries are indicated by stippled lines. Both the cleaved-Caspase-3 and TUNEL signals are lost in *ark* clones. (**a'**) and (**b'**) are the cleaved-Caspase-3 and TUNEL channels only. Genotype: *GMR-hid ey-FLP*; *FRT42D ark^{G8}/FRT42D* P(*ubi-GFP*). (**c**, **c'**, **d**, **d'**) *GMR-ΔN-dcp-1* eye discs containing *ark* mutant clones were labeled with cleaved-Caspase-3 antibody (**c**, **c'**) and TUNEL (**d**, **d'**). *ark* mutant clones are marked by the absence of GFP. In *ark*$^{+}$ tissue, marked by GFP (green), cleaved-Caspase-3 and TUNEL signals are easily detectable. In *ark* mutant clones (see outline of clonal boundaries by stippled lines), the number of both cleaved-Caspase-3- and TUNEL-positive cells is reduced, but a few are present (arrows). (**c'**) and (**d'**) are the cleaved-Caspase-3 and TUNEL channels only. Genotype: *ey-FLP*; *FRT42D ark^{G8}/FRT42D* P(*ubi-GFP*); *GMR-ΔN-dcp-1*

The blocking peptides were mixed with the cleaved-Caspase-3 antibody 60 min before incubation with the eye imaginal discs. The results of the blocking experiments are summarized in Figure 2b, and for peptides A and B shown in Figure 2c–f. Peptide A is sufficient to block the entire immunoreactivity of the cleaved-Caspase-3 antibody in *GMR-hid* and in *dcp-1 drICE* double mutant *GMR-hid* eye discs (Figure 2c,d). In contrast, peptide B does not abrogate immunoreactivity of the antibody in these discs (Figure 2e,f). The control peptides C and D also fail to block cleaved-Caspase-3 immunoreactivity (Figure 2b; data not shown).

These data demonstrate that the cleaved-Caspase-3 antibody specifically detects the epitope ETD in apoptotic cells. Among the *Drosophila* caspases, this epitope is only present in DRICE and DCP-1, therefore making it very likely that the antibody does indeed detect these effector caspases. In contrast, the fact that the DRONC-derived peptides B, C and D fail to block immunoreactivity suggest that it is unlikely for the antibody to directly detect active DRONC. Therefore, because the cleaved-Caspase-3 antibody does not lose immunoreactivity in *dcp-1 drICE* double mutants (Figure 1e), it detects at least one other protein, which exposes the ETD epitope in a DRONC-dependent manner.

Activation of DCP-1 independently of ARK restores cleaved-Caspase-3 immunoreactivity. The analysis presented in Figure 2 suggests, but does not prove, that the cleaved-Caspase-3 antibody does indeed detect cleaved DCP-1 and DRICE. To directly test this possibility, we expressed a *GMR-ΔN-dcp-1* transgene in *ark* mutant background. *ΔN-dcp-1* lacks the N-terminal prodomain of DCP-1. It is thought that prodomain-depleted ΔN-DCP-1 readily promotes autoprocessing, and consistent expression under *GMR* control induces an eye ablation phenotype.[32] This eye ablation phenotype is caused by the induction of apoptosis (Figure 3c,d). As mentioned above, *ark* mutant clones in *GMR-hid* background fail to induce TUNEL-positive apoptosis (Figure 3b) and the cleaved-Caspase-3 antibody does not have any immunoreactivity in *ark* clones (Figure 3a) suggesting that neither DCP-1 nor DRICE nor the unknown DRONC substrates are cleaved in *ark* mutant clones. Therefore, we tested whether expression of *ΔN-Dcp-1* in the absence of apoptosome activity, that is, in *ark* clones, can restore cleaved-Caspase-3 antibody labeling. Compared with wild-type tissue (marked by GFP in Figure 3c,c'), the number of cleaved-Caspase-3 positive cells is strongly reduced in *ark* mutant clones (Figure 3c,c') suggesting that activation of ΔN-DCP-1 is at least partially dependent on the apoptosome. However, about 50% of *ark* mutant clones ($n = 32$) in *GMR-ΔN-Dcp-1* background contain cleaved-Caspase-3 immunoreactive cells (arrows in Figure 3c, c'). This is unlikely to be a labeling artifact, because these cells also are TUNEL-positive (Figure 3d, d'). Therefore, because DRONC is inactive in *ark* mutant background suggesting that the cleaved-Caspase-3 labeling signal is not caused by the unknown DRONC substrate, this analysis demonstrates that

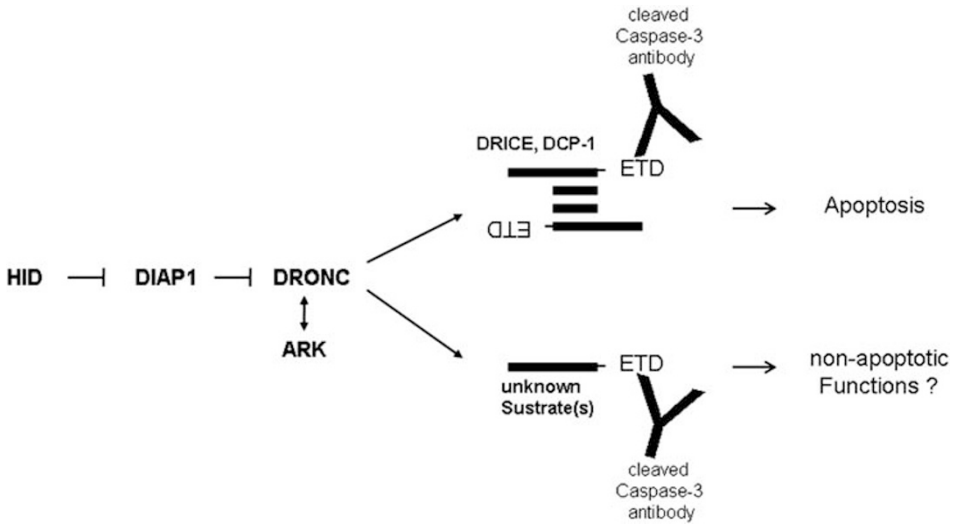

Figure 4 Specificity of the cleaved-Caspase-3 antibody. HID-mediated release of DRONC from DIAP1-inhibition triggers the formation of apoptosome through association with ARK. After DRONC cleavage, the apoptotic substrates DRICE and DCP-1 form a heterotetramer, exposing the ETD epitope at the C-terminus of the large subunits of these proteins. In addition, DRONC has at least one additional substrate, which likely exposes an ETD epitope after cleavage. This substrate is unknown and may mediate non-apoptotic functions of DRONC. The cleaved-Caspase-3 antibody (Y shape) detects the exposed ETD epitope of cleaved DCP-1, DRICE and the unknown DRONC substrate

the cleaved-Caspase-3 antibody does indeed detect cleaved DCP-1. It is also possible that the cleaved-Caspase-3 antibody detects cleaved DRICE in this experiment, because DCP-1 can proteolytically process DRICE, at least *in vitro*.[29,32] A similar analysis with DRICE could not be carried out because *GMR-drICE* and *GMR-ΔN-drICE* do not cause an eye ablation phenotype.[32] Nevertheless, whether DCP-1 cleaves DRICE *in vivo* or not, given the sequence similarity of DCP-1 and DRICE at the C-terminus of the large subunit and the peptide blocking data of Figure 2, it suggests that the antibody may also detect cleaved DRICE.

Discussion

The findings of this study are summarized in Figure 4. In addition to DCP-1 and likely DRICE, the cleaved-Caspase-3 antibody also recognizes at least one, perhaps even several additional proteins containing the ETD epitope. Because exposure of this epitope is dependent on apoptosome activity, it is very likely that the protein is a substrate of DRONC. However, cleavage of this protein by DRONC is not sufficient to induce apoptosis because *dcp-1 drICE* double mutants in which this epitope is present, are not apoptotic (Figure 1e,f). Consistently, this protein is unlikely to be a caspase because none of the remaining *Drosophila* caspases carries the ETD epitope (Figure 2a).

Nevertheless, these findings are interesting from a different point of view. In addition to induction of apoptosis, DRONC and caspases in general also have non-apoptotic functions (reviewed in [33]). For example, apoptosis-induced compensatory proliferation is dependent on a non-apoptotic function of DRONC.[16,34,35] It is at present unknown how DRONC is mediating this response. Identification of DRONC substrates involved in non-apoptotic functions may be very helpful in elucidating the mechanism by which DRONC exerts its non-apoptotic functions. Unfortunately, database searches using ETD as query to identify potential non-apoptotic DRONC substrates were not successful. Other approaches such as immunoprecipitation using the cleaved-Caspase-3 antibody will be necessary to identify this substrate.

This study also demonstrates that caution should be taken about the specificity of antibodies. As shown here in *Drosophila*, it is likely that the cleaved-Caspase-3 antibody not only detects cleaved Caspase-3 in mammals, but also additional proteins containing the ETD epitope. In *Drosophila*, exposure of this epitope is apoptosome-dependent and thus, the antibody is a useful reagent for detecting apoptosis. But in other model organisms this may not be the case and may result in mis-interpretations.

In summary, we demonstrated in this paper that the cleaved-Caspase-3 antibody not only detects active effector caspases DRICE and DCP-1 in apoptotic cells in *Drosophila*, but also additional potential DRONC substrates which may be involved in non-apoptotic processes. Therefore, we propose that it is most useful to interpret the labeling information obtained with the cleaved-Caspase-3 antibody as the activity of Caspase-9-like initiator caspase DRONC in *Drosophila*.

Materials and Methods

The cleaved-Caspase-3 (Asp175) antibody was purchased from Cell Signaling Technology (catalog # 9661). 1 *GMR-hid*, *GMR-ΔN-dcp-1*, *dronc*^{I24}, *ark*^{G8}, *drICE*$^{\Delta 1}$ and *dcp-1*^{Prev} are described elsewhere.[5,12,14,15,32,36] For details about making *ark* clones, see Srivastava *et al.* (ref. [36]). Immunohistochemistry and TUNEL labeling was done as described.[22,28,37] Photographs were taken by confocal microscopy.

Blocking peptides A, B, C, and D were obtained from ProImmune (Oxford, UK). They were dissolved in a small amount of 0.1% ammonium bicarbonate and diluted to a final concentration of 1 mg/ml with sterile, distilled water according to the manufacturer's instructions. 1 μl of the peptide solution was mixed with 1 μl of cleaved-Caspase-3 antibody in labeling solution for 60 min at room temperature before incubation with the eye discs.

Conflict of interest

The authors declare no conflict of interest.

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