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**Drosophila IAP1-Mediated Ubiquitylation Controls Activation of the Initiator Caspase DRONC Independent of Protein Degradation**

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**Abstract**

Ubiquitylation targets proteins for proteasome-mediated degradation and plays important roles in many biological processes including apoptosis. However, non-proteolytic functions of ubiquitylation are also known. In *Drosophila*, the inhibitor of apoptosis protein 1 (DIAP1) is known to ubiquitylate the initiator caspase DRONC in *vivo*. Because DRONC protein accumulates in *diap1* mutant cells that are kept alive by caspase inhibition (“undead” cells), it is thought that DIAP1-mediated ubiquitylation causes proteasomal degradation of DRONC, protecting cells from apoptosis. However, contrary to this model, we show here that DIAP1-mediated ubiquitylation does not trigger proteasomal degradation of full-length DRONC, but serves a non-proteolytic function. Our data suggest that DIAP1-mediated ubiquitylation blocks processing and activation of DRONC. Interestingly, while full-length DRONC is not subject to DIAP1-induced degradation, once it is processed and activated it has reduced protein stability. Finally, we show that DRONC protein accumulates in “undead” cells due to increased transcription of *drone* in these cells. These data refine current models of caspase regulation by IAPs.

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**Introduction**

Ubiquitylation describes the covalent attachment of ubiquitin, a 76 amino acid polypeptide, to proteins which occurs as a multi-step process (reviewed in [1,2]). E1-activating enzymes activate ubiquitin and transfer it to E2-conjugating enzymes. E3-ubiquitin ligases mediate the conjugation of ubiquitin from the E2 to the target protein. Repeated ubiquitylation cycles lead to the formation of polyubiquitin chains attached on target proteins. Polyubiquitylated proteins are delivered to the 26S proteasome for degradation. However, non-proteolytic roles of ubiquitylation have also been described (reviewed in [3,4]). From E1 to E3, there is increasing complexity. For example, the *Drosophila* genome encodes only one E1 enzyme, termed UBA1, which is required for all ubiquitin-dependent reactions in the cell [5]. In contrast, there are hundreds of E3-ubiquitin ligases which are needed to confer substrate specificity.

Programmed cell death or apoptosis is an essential physiological process for normal development and maintenance of tissue homeostasis in both vertebrates and invertebrates (reviewed in [6]). A highly specialized class of proteases, termed caspases, are central components of the apoptotic pathway (reviewed in [7]). The full-length form (zymogen) of caspases is catalytically inactive and consists of a prodomain, a large and a small subunit. Activation of caspases occurs through dimerization and proteolytic cleavage, separating the large and small subunits. Based on the length of the prodomain, caspases are divided into initiator (also known as apical or upstream) and effector (also known as executioner or downstream) caspases [7]. The long prodomains of initiator caspases harbor regulatory motifs such as the caspase activation and recruitment domain (CARD) in CASPASE-9. Through homotypic CARD/CARD interactions with the adapter protein APAF-1, CASPASE-9 is recruited into the apoptosome, a large multi-subunit complex, where it dimerizes and auto-processes leading to its activation [8,9]. Activated CASPASE-9 cleaves and activates effector caspases (CASPASE-3, -6, and –7), which are characterized by short prodomains. Effector caspases execute the cell death process by cleaving a large number of cellular proteins [10].

In *Drosophila*, the initiator caspase DRONC and the effector caspases DrICE and DCP-1 are essential for apoptosis [11–18]. Like human CASPASE-9, DRONC carries a CARD motif in its prodomain [19]. Consistently, DRONC interacts with ARK, the APAF-1 ortholog in *Drosophila* (also known as DARK, HAC-1 or...
Author Summary

The *Drosophila* inhibitor of apoptosis 1 (DIAP1) readily promotes ubiquitylation of the CASPASE-9-like initiator caspase DRONC in *in vitro* and *in vivo*. Because DRONC protein accumulates in *diap1* mutant cells that are kept alive by effector caspase inhibition—producing so-called "undead" cells—it has been proposed that DIAP1-meditated ubiquitylation would target full-length DRONC for proteasomal degradation, ensuring survival of normal cells. However, this has never been tested rigorously *in vivo*. By examining loss and gain of *diap1* function, we show that DIAP1-mediated ubiquitylation does not trigger degradation of full-length DRONC. Our analysis demonstrates that DIAP1-mediated ubiquitylation controls DRONC processing and activation in a non-proteolytic manner. Interestingly, once DRONC is processed and activated, it has reduced protein stability. We also demonstrate that "undead" cells induce transcription of *dronc*, explaining increased protein levels of DRONC in these cells. This study re-defines the mechanism by which IAP-mediated ubiquitylation regulates caspase activity.

D-APAF-1) [20-22] for recruitment into an apoptosome-like complex which is required for DRONC activation [20,23-31]. After recruitment into the ARK apoptosome, DRONC cleaves and activates the effector caspases DrICE and DCP-1 [25,31–34].

Caspases are subject to negative regulation by inhibitor of apoptosis proteins (IAPs) (reviewed in [35,36]). For example, DRONC is negatively regulated by *Drosophila* IAP (DIAP1) [37,38]. *dronc* mutations cause a dramatic cell death phenotype, in which nearly every mutant cell is apoptotic, suggesting an essential genetic role of *dronc* for cellular survival [39–41]. DIAP1 is characterized by two tandem repeats known as the Baculovirus IAP Repeat (BIR), and one C-terminally located RING domain [42]. The BIR domains are required for binding to caspases [37,38,43]. The RING domain provides DIAP1 with E3-ubiquitin ligase activity, required for ubiquitylation of target proteins [35,36]. Importantly, the BIR domains can bind to caspases independently of the RING domain [37,43].

Usually, IAPs bind to and inhibit activated, i.e. processed caspases, including CASPASE-3, CASPASE-7 and CASPASE-9 as well as the *Drosophila* caspases DrICE and DCP-1 (reviewed in [35,36]). However, a notable exception to this rule is DRONC. DIAP1 binds to the prodomain of full-length DRONC [37,38,43]. This unusual behavior suggests an important mechanism for the control of DRONC activation. Indeed, it has been shown that the RING domain of DIAP1 ubiquitylates full-length DRONC *in vitro* [38,44]. It has also been proposed that DIAP1 ubiquitylates auto-processed DRONC [33]. These ubiquitylation events are critical for the control of apoptosis, as homozygous *dronc* mutants which lack a functional RING domain (diap1RING) are highly apoptotic [41]. Because the BIR domains are intact in diap1RING mutants, binding of DIAP1 to DRONC is not sufficient for inhibition of DRONC under physiological conditions, and ubiquitylation is the critical event for DRONC inhibition.

Although the importance of DIAP1-mediated ubiquitylation of DRONC is well established, it is still unclear how this ubiquitylation event leads to inactivation of DRONC and of caspases in general. Because DRONC protein accumulates in *diap1* mutant cells that are kept alive by expression of the effector caspase inhibitor P35, generating so-called ‘undead’ cells, it has been proposed that DIAP1-mediated ubiquitylation triggers proteasomal degradation of full-length DRONC in living cells, thus protecting them from apoptosis [33,38,45,46]. However, degradation of full-length DRONC in living cells has never been observed and non-degradative models have also been proposed [44]. Furthermore, ubiquitylation of mammalian CASPASE-3 and CASPASE-7 has been demonstrated *in vitro* [47–49]. However, evidence for proteasome-dependent degradation of these caspases *in vivo*, i.e. in the context of a living animal, is lacking. In fact, a non-degradative mechanism has been demonstrated for the effector caspase DrICE in *Drosophila* [50].

Here, we further characterize the role of ubiquitylation for the control of DRONC activation. Consistent with a previous report [44], we find that ubiquitylation of DRONC by DIAP1 is critical for inhibition of DRONC’s pro-apoptotic activity. Using loss and gain of *diap1* function, we provide genetic evidence that DIAP1-mediated ubiquitylation of full-length DRONC regulates this initiator caspase through a non-degradative mechanism. We find that the conjugation of ubiquitin suppresses DRONC processing and activation. Interestingly, once DRONC is processed and activated, it has reduced protein stability. Finally, we show that *dronc* transcripts accumulate in P35-expressing ‘undead’ cells, accounting for increased DRONC protein levels in these cells. These data refine the current model of caspase regulation by IAPs.

Results

Overexpression of DIAP1 fails to suppress apoptosis of *Uba1* mutant cells

It has previously been shown that complete loss of ubiquitylation due to mutations of the E1 enzyme *Uba1* causes apoptosis in eye imaginal discs as detected by an antibody that recognizes cleaved, i.e. activated, CASPASE-3 (CASP3*) [5,51,52] (see also Figure 1A). Because ubiquitylation of DRONC does not occur in *Uba1* mutants, we hypothesized that inappropriate activation of DRONC accounts for the apoptotic phenotype of *Uba1* mutants. To test this possibility, we targeted *dronc* overexpression by RNA interference (RNAi) in *Uba1* mutant cells in eye imaginal discs using the MARCM system and labeled for apoptosis using CAS3* antibody. In this system, *Uba1* mutant cells expressing *dronc* RNAi are positively marked by GFP. Consistent with our hypothesis, knock-down of *dronc* strongly reduces apoptosis in *Uba1* mutant clones (Figure 1B). Furthermore, we tested clones doubly mutant for *Uba1* and *ark*, the *Drosophila* ortholog of APAF-1 that is required for DRONC activation (see Introduction). Apoptosis induced in *Uba1* mutant clones is strongly suppressed if *ark* function is removed (Figure S1). These observations suggest that the apoptotic phenotype in *Uba1* clones is caused by inappropriate activation of DRONC, presumably due to lack of ubiquitylation.

However, the protein levels of DIAP1 are increased in *Uba1* mutant clones [5,52]. There are two possibilities to explain the apoptotic phenotype in *Uba1* mutants despite increased DIAP1 levels. First, the DIAP1 levels may not be sufficiently increased to inhibit DRONC. Alternatively, binding of DIAP1 to DRONC alone may not be sufficient for inhibition of DRONC; instead, ubiquitylation by DIAP1 is required to block DRONC activation, as previously suggested [44]. To distinguish between these two possibilities, we strongly overexpressed *dronc* in *Uba1* mutant clones in eye discs using the MARCM system and imaged for apoptosis by CAS3* labeling. Surprisingly, despite massive expression of *dronc* (>20 fold over wild-type levels; Figure 1C†), apoptosis still proceeds in *Uba1* mutant clones (Figure 1C†), even though expression of the same transgene can block strong apoptotic phenotypes in several apoptotic paradigms (Figure S2). Apparently, overexpression of DIAP1 is not sufficient to inhibit DRONC and to protect *Uba1* mutant cells from apoptosis.
Because DIAP1 is the key regulator of DRONC and because DRONC is required for the apoptotic phenotype of Uba1 mutant cells, as evidenced by knock-down of dronc (Figure 1B), our data provide genetic evidence that binding of DIAP1 is not sufficient for DRONC inhibition in Uba1 mutant cells.

Consistent with this view, it has previously been shown that DIAP1 does ubiquitylate full-length DRONC in vitro [33,38,44]. We tested whether DIAP1 can also ubiquitylate DRONC in vivo. Because the available DRONC antibodies failed to immunoprecipitate endogenous DRONC, we transfected DRONC-V5 along...
with DIAP1* or DIAP1RING mutants (CA6, lacking the last six C-terminal residues, and F437A changing a critical Phe residue in the RING domain to Ala [53]) and His-tagged Ubiquitin into Drosophila S2 cells. Ubiquitylated proteins were affinity purified under denaturing conditions using Ni columns. The eluates were subsequently examined by immunoblotting with anti-V5 antibodies to detect ubiquitylated forms of DRONC. Under these conditions, DIAP1 readily ubiquitylates full-length DRONC in S2 cells (Figure 2), whereas the RING mutants DIAP1CA6 and DIAP1F437A were significantly impaired in their ability to ubiquitylate DRONC (Figure 2). These results indicate that DIAP1 ubiquitylates full-length DRONC in a RING-dependent manner in cultured cells.

**Overexpression of DIAP1 does not induce degradation of DRONC**

Because DIAP1 readily ubiquitylates DRONC, it has been postulated that DIAP1-mediated ubiquitylation leads to proteasomal degradation of DRONC [33,38,45]. However, this has never been rigorously tested in vivo. Therefore, we examined, whether overexpression of diap1 in wild-type animals can influence DRONC protein levels in vivo. To this end, we generated clones overexpressing diap1 (marked by absence of GFP) in eye discs, and analyzed the protein abundance of DRONC. Interestingly, despite high expression of diap1 (Figure 3A), the levels of DRONC remained unchanged and were not influenced by DIAP1 (Figure 3A). The anti-DRONC antibody used in this assay is specific for DRONC (Figure S3). Importantly, the diap1 transgene used produces a functional DIAP1 protein that is able to inhibit apoptosis in several paradigms (Figure S2). Therefore, these data suggest that overexpressed DIAP1 does not target DRONC for degradation in living cells.

**REAPER-induced loss of DIAP1 does not increase DRONC protein levels**

Because of the surprising observation that overexpressed DIAP1 does not cause degradation of DRONC, we tested whether removal of DIAP1 changes DRONC protein levels. Expression of the IAP antagonist reaper (rpr) induces DIAP1 degradation and apoptosis [54–58]. Therefore, we examined whether RPR-induced degradation of DIAP1 changes DRONC protein levels. If DIAP1 targets DRONC for degradation, we would expect that DRONC protein levels would accumulate in response to rpr expression. Expression of rpr in eye imaginal discs posterior to the morphogenetic furrow (MF) using the GMR promoter (GMR-rpr) is well suited for this analysis. The MF is a dynamic structure that initiates at the posterior edge of the eye disc and moves towards the anterior during 3rd instar larval stage [59,60] (Figure 4A, arrow). Expression of rpr by GMR is induced in all cells posterior to the MF [61] (red in Figure 4A). Therefore, GMR-rpr eye discs provide a continuum of all developmental stages in which cells close to the MF have only recently induced rpr expression, while cells towards the posterior edge of the disc have been exposed to rpr progressively longer. Therefore, if DIAP1 accumulates during any of these stages, we should be able to detect it. In wild-type eye discs, DRONC protein is homogenously distributed throughout the disc. Only in the MF, higher levels of DRONC are detectable (arrowhead in Figure 4B). This high expression of DRONC in the MF serves as an orientation mark. DIAP1 protein levels are low anterior to the MF, but increase in the MF (arrowhead) and posterior to it in wild-type discs (Figure 4B). In GMR-rpr eye discs, overall DIAP1 levels are reduced in the rpr-expressing domain posterior to the MF (Figure 4C), but particularly strongly reduced in the CAS3* positive area (Figure 4C, D*, arrow) consistent with previous reports [54–58]. However, accumulation of DRONC is not observed (Figure 4C, D*). In contrast, it appears that DRONC levels are also reduced. They are still high in the MF (Figure 4C, arrowhead), but drop immediately thereafter.

We also related DRONC levels to caspase activation. In the MF, where CAS3* activity is not detectable, DRONC is still high (Figure 4D, D*; arrowhead), but in the CAS3* positive area, DRONC levels are reduced (Figure 4D, D*; arrow). These data indicate that loss of DIAP1 does not cause accumulation of DRONC protein implying that DIAP1 does not induce degradation of DRONC. In contrast, it appears that DIAP1 stabilizes DRONC at least under these conditions (see Discussion).

**“Undead” diap1 mutant cells induce transcription of dronc**

Finally, we analyzed DRONC protein levels in diap1RING mutants which cannot ubiquitylate DRONC [44]. It has previously been shown that clones of the RING mutant diap1P250-A1 accumulate DRONC protein [45,46] implying that ubiquitylation by the RING domain of DIAP1 causes degradation of DRONC. We repeated these experiments and indeed confirmed that DRONC levels are increased in diap1P250-A1 mutant clones (Figure S4). Thus, these results appear inconsistent with the data presented in Figure 3 and Figure 4.
in which manipulating DIAP1 levels did not provide evidence for DIAP1-mediated degradation of DRONC. However, one caveat with the \textit{diap1}\textsuperscript{22-8s} experiment was the use of the caspase inhibitor P35 which kept \textit{diap1}\textsuperscript{22-8s} mutant cells in an ‘undead’ condition [45]. It has been pointed out that the ‘undead’ state may change the properties of the affected cells (reviewed by [62]) and in fact abnormal induction of transcription in ‘undead’ cells has been reported [45,63–66]. Thus, to explain the conflicting results between the \textit{diap1}\textsuperscript{22-8s} data [45] and our data shown here, we hypothesized that P35-expressing ‘undead’ \textit{diap1}\textsuperscript{22-8s} clones induce \textit{dronc} transcription, leading to accumulation of DRONC protein. To test this hypothesis, we used a transcriptional \textit{lacZ} reporter containing 1.33 kb of regulatory genomic sequences upstream of the transcriptional start site of the \textit{dronc} gene fused to \textit{lacZ} [dronc\textit{1.33-lacZ}] [67,68]. Compared to controls (Figure 5A, 5A') and consistent with the hypothesis, \textit{dronc1.33-lacZ} reporter activity is increased in ‘undead’ cells in the dorsal half of the eye (Figure 5D). Expression of P35 alone does not trigger transcription of \textit{dronc} (Figure 5E) suggesting it is not the mere presence of P35 which causes \textit{dronc} transcription, but the ‘undead’ nature of the affected cells.

We also produced ‘undead’ cells in eye imaginal discs by co-expression of the IAP-antagonist \textit{hid} and the caspase inhibitor P35 in the dorsal half of the eye disc using a dorsal \textit{eye-} (DE-) \textit{GAL4} driver (Figure 5C). Similar to wing discs, \textit{dronc} reporter activity is increased in ‘undead’ cells in the dorsal half of the eye (Figure 5D). Expression of \textit{p35} alone does not trigger transcription of \textit{dronc} (Figure 5E) suggesting it is not the mere presence of P35 which causes \textit{dronc} transcription, but the ‘undead’ nature of the affected cells.

These observations may explain why DRONC protein accumulates in ‘undead’ \textit{diap1}\textsuperscript{22-8s} mutant cells, but they still do not rule out the possibility that DRONC protein accumulates in \textit{diap1}\textsuperscript{22-8s} mutants due to lack of ubiquitylation and thus degradation. To clarify this issue we examined \textit{dronc1.33-lacZ} and DRONC levels in \textit{diap1}\textsuperscript{22-8s} mutant clones without simultaneous \textit{p35} expression. Without the inhibition of apoptosis by P35, \textit{diap1}\textsuperscript{22-8s} clones die rapidly. Nevertheless, we were able to recover wing discs which contained small \textit{diap1}\textsuperscript{22-8s} mutant clones. In these clones, neither \textit{dronc1.33-lacZ} nor DRONC levels are detectably

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\textbf{Figure 3. Overexpression of \textit{diap1} does not trigger degradation of DRONC.} Shown is an eye imaginal disc from a third instar larva. Posterior is to the right. \textit{diap1}-overexpressing clones are marked by absence of GFP and can be detected using anti-DIAP1 antibodies in magenta (A"'). The boundary between \textit{diap1}-expressing clones and normal tissue is indicated by a white stippled line in (A'). DRONC levels are unchanged (A'). (A) and (A"') are merged images. Genotype: \textit{UAS-diap1/hs-FLP; tub>GFP>GAL4}. doi:10.1371/journal.pgen.1002261.g003
Dronc protein levels are reduced in the CAS3*-positive area (arrow). Arrowheads mark the MF. The brackets indicate the extent of 
mGMR expression. (D–D') 2×GMR-rpr eye disc labeled for cleaved CASPASE 3 (CAS3*) (D') and DRONC (D'). DRONC protein levels are reduced in the CAS3*-positive area (arrow). Arrowheads mark the MF. The brackets indicate the extent of 
mGMR expression. doi:10.1371/journal.pgen.1002261.g004

Figure 4. Loss of DIAP1 in GMR-rpr eye discs does not alter DRONC protein levels. (A) Schematic illustration of the GMR-reaper (GMR-rpr) eye imaginal disc from 3rd instar larvae. The morphogenetic furrow (MF, arrowhead) initiates at the posterior (P) edge of the disc and moves towards the anterior (A) (arrow). The GMR enhancer is active posterior to the MF (bracket) and thus expresses rpr posterior to the MF (red area). (B–B') Eye disc showing normal protein distribution of DIAP1 (B') and DRONC (B'). Both DIAP1 and DRONC levels are increased in the MF (arrowhead). (B) is the merged image of DIAP1 and DRONC labeling. (C–C') Eye discs expressing two copies of GMR-rpr eye disc labeled for DIAP1 (C') and DRONC (C'). Arrowheads mark the MF. DIAP1 levels are markedly reduced posterior to the MF (C', arrow). Surprisingly, DRONC protein levels are also reduced (C', arrow). The brackets indicate the extent of 
mGMR expression. (D–D') 2×GMR-rpr eye disc labeled for cleaved CASPASE 3 (CAS3*) (D') and DRONC (D'). DRONC protein levels are reduced in the CAS3*-positive area (arrow). Arrowheads mark the MF. The brackets indicate the extent of 
mGMR expression.

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 Ubiquitylation controls processing and thus activation of DRONC in vivo

Our in vivo analysis implies that DIAP1-mediated ubiquitylation does not trigger proteosomal degradation of DRONC. To identify the role of ubiquitylation for regulation of DRONC activity, we analyzed the fate of DRONC protein in RING mutants of diap1. Of note, these mutants retain their ability to bind to DRONC, because DRONC binding is not mediated by the RING domain, but by the BIR2 domain [37,38,43]. The RING mutant diap1<sup>1s</sup> is especially suitable for this analysis because a premature stop codon results in deletion of the entire RING domain but leaves the BIR domains intact [44] (Figure 6A), thus abrogating its E3 activity, but not caspase binding. Importantly, diap1<sup>1s</sup> is characterized by a strong apoptotic phenotype, suggesting inappropriate caspase activation [41,45]. Indeed, we showed previously that diap1<sub>BIR</sub> mutant phenotypes are dependent upon DRONC, because dronc mutant suppresses diap1<sub>BIR</sub> phenotypes [11]. Therefore, ubiquitylation of DRONC by DIAP1 is critical to maintain cell survival.

We examined the cause of the diap1<sup>1s</sup> apoptotic phenotype. First, as a control, we determined whether the diap1<sup>1s</sup>-F gene produces a stable protein in vivo. We chose to analyze stage 6–9 embryos, because normal developmental cell death starts at stage 11 [69]; therefore, stage 6–9 diap1<sup>1s</sup>-F mutant embryos allow analysis of DIAP1 in the absence of upstream apoptotic signals. In immunoblots of embryonic extracts obtained from a cross of heterozygous diap1<sup>1s</sup>-F males and females, the DIAP1<sup>33-1s</sup> protein is easily distinguished from wild-type DIAP1<sup>+</sup> protein due to its faster electrophoretic mobility (Figure 6A, top panel). The presence of the DIAP1<sup>33-1s</sup> protein suggests that the apoptotic phenotype in diap1<sup>1s</sup>-F mutant embryos is not caused by instability of the mutant protein. Interestingly, the protein levels of DIAP1<sup>+</sup> and RING-deleted DIAP1<sup>33-1s</sup> are similar (Figure 6A, top panel) suggesting that loss of the RING domain does not influence the protein stability of DIAP1 in the absence of pro-apoptotic signals.

Next, we analyzed DRONC protein in extracts from diap1<sup>1s</sup>-F mutant embryos. Consistent with the data in Figure 4 and Figure 5, we do not detect a significant increase in the protein levels of DRONC in these embryos (Figure 6A, middle panel). However, a significant amount of DRONC is present in a processed form in extracts of stage 6–9 diap1<sup>1s</sup>-F mutant embryos which is absent in control extracts from wild-type embryos (Figure 6A, middle panel). Therefore, DRONC processing, and thus activation, occurs in RING-depleted diap1<sup>1s</sup>-F mutant embryos despite the fact that the BIR domains of DIAP1 are unaffected. The processed form of DRONC in this mutant of MW ~36 kDa corresponds to the major apoptotic form of DRONC composed of the large subunit and the prodomain of DRONC [70]. This finding, and the one presented in Figure 1, confirms that binding of DIAP1 to DRONC is not sufficient to prevent processing and activation of DRONC. Instead, the RING domain is required to control DRONC processing. Because the RING domain contains an E3-ubiquitin ligase activity [45,55–58] and because ubiquitylation of full-length DRONC does not trigger proteosomal degradation (Figure 3, Figure 4, and Figure 5), we conclude that ubiquitylation of DRONC by the RING domain of DIAP1 is necessary to inhibit DRONC processing and thus activation.

To further characterize the role of ubiquitylation in the regulation of DRONC processing, we performed an immunoblot analysis with extracts from wild-type and Uba1 mosaic imaginal discs, which, under our experimental conditions, are about half mutant for Uba1 and half wild-type. Immunoblot analysis demonstrated that a significant amount of DRONC is processed in Uba1 mosaic discs (Figure 6B). Thus, these data further support the notion that ubiquitylation of full-length DRONC is necessary for inhibition of DRONC processing.

Discussion

In this paper, we provide three take-home messages. First, we provide genetic evidence that binding of DIAP1 to DRONC is not sufficient for inhibition of DRONC. Instead, ubiquitylation of DRONC controls its apoptotic activity, consistent with the apoptotic phenotype of diap1<sub>BIR</sub> mutants, that retain caspase binding abilities. Second, DIAP1-mediated ubiquitylation of full-length DRONC does not lead to its proteosomal degradation; rather, ubiquitylation directly controls processing and activation of DRONC. Interestingly, processed and active DRONC shows reduced protein stability. Third, ‘undead’ cells accumulate dronc transcripts.

Binding of DIAP1 is not sufficient for Dronc inhibition

Based on biochemical studies in vitro and overexpression studies in cultured cells, many of cancerous origin, it was initially proposed that binding of IAPs to caspases through their BIR domains is sufficient to inhibit caspsases [71–80]. However, when ubiquitylation of caspases by IAPs was described [38,44,47,48], it was unclear what role ubiquitylation would play for control of caspase activity, especially since for none of them, ubiquitin-mediated degradation has been observed (see below). Because the RING domain is also required for auto-ubiquitylation of DIAP1 [54–58], mutations of the RING domain would be expected to increase DIAP1 protein levels and protect cells from apoptosis. However, the opposite phenotype, massive apoptosis, was observed [41]. Nevertheless, despite the biochemical studies showing that the BIR domains of DIAP1 are sufficient for interaction with DRONC [37,38,43], one could argue that
Figure 5. “Undead” diap1 mutant cells trigger transcription of dronc. Shown are 3rd instar larval wing (A,B,F) and eye imaginal discs (C,D,E) labeled for DRONC protein levels (blue) and dronc transcriptional activity (red) using the dronc1.33-lacZ reporter (β-GAL labeling). (A,A’) Co-labeling for DRONC protein (A) and dronc reporter activity (A’) of a wild-type wing disc expressing the dronc1.33-lacZ transgene. (B-B’’) A diap122-8s mosaic non-degradative ubiquitylation of DRONC.
wing disc expressing p35 under nub-GAL4 control in a dronc1.33-lacZ background. A mutant clone in the wing pouch is highlighted by an arrow in the GFP-only channel (B). DRONC protein (B') and β-GAL immunoreactivity as readout of dronc1.33-lacZ activity (B") are increased in the same cells and overlap (B"'). Please note that the dronc1.33-lacZ reporter produces nuclear β-GAL while DRONC protein appears cytoplasmic. (C) GFP expression in the eye imaginal disc indicates the dorsal expression domain (arrow) of the dorsal eye (DE)-GAL4 driver [95]. (D) Increased dronc reporter activity in the dorsal half of the eye imaginal disc (arrow) in undead cells obtained by co-expression of hid and p35 using DE-GAL4. (E) Expression of p35 alone by DE-GAL4 does not induce dronc reporter activity. (F-F') A diap122-8s mosaic wing disc in a dronc1.33-lacZ background which does not express p35. diap122-8s mutant clones are marked by the absence of GFP (F). An arrow points to a representative diap122-8s clone in the wing pouch. In the same position, neither DRONC protein (F') nor dronc reporter activity (F") are increased. Note, that this clone is present in the wing pouch which has the capacity to upregulate DRONC and dronc transcription in the ‘undead’, p35-expressing condition (see panel B’). Genotypes: (A) dronc1.33-lacZ/+; (B) ubx-FLP; nub-GAL4 UAS-p35/ dronc1.33-lacZ, diap122-8s FRT80/ubi-GFP FRT80. (C) DE-GAL4 UAS-GFP/+; (D) UAS-p35 UAS-hid/dronc1.33-lacZ; DE-GAL4. (E) UAS-p35/dronc1.33-lacZ; DE-GAL4. (F) ubx-FLP; nub-GAL4/dronc1.33-lacZ; diap122-8s FRT80/ubi-GFP FRT80.

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DIAP1RING mutants have lost the ability to interact with DRONC in vivo. While we cannot exclude this possibility due to the inability of our antibodies to immunoprecipitate endogenous proteins, another experiment supports the notion that ubiquitylation is necessary for DRONC inhibition: when wild-type diap1 was strongly overexpressed in an ubiquitylation-deficient Uba1 mutant background, DRONC-dependent apoptosis was not inhibited (Figure 1C), suggesting that binding of DIAP1 is not sufficient for inhibition of DRONC. Instead, ubiquitylation is critical for inhibition of DRONC activity.

DIAP1 does not control protein levels of full-length DRONC

The current model holds that DIAP1-mediated ubiquitylation leads to proteasomal degradation of full-length DRONC in living cells [33,38,45]. However, our data do not support this model in vivo. This model is based on biochemical ubiquitylation studies without in vivo validation and does not take into account that ubiquitylation often serves non-proteolytic functions [1,3,4]. Both overexpression and loss of diap1 does not cause a detectable alteration of the protein levels of DRONC (Figure 3, Figure 4, Figure 5), arguing against a degradation model. The only example where DRONC accumulation has been observed is in P35-expressing ‘undead’ diap1RING mutant cells [45,46], and we showed here that the ‘undead’ nature of these cells causes transcriptional induction of dronc (Figure 5). Together, these observations argue against a degradation model of full-length DRONC and favor a non-traditional (non-proteolytic) role of ubiquitylation regarding control of DRONC activity. Similarly, DIAP1-mediated ubiquitylation of the effector caspase DrICE.

**Figure 6. Ubiquitylation controls processing of DRONC.** (A) Top: schematic outline of the domain structure of DIAP1* (wild-type) and RING-deleted DIAP133-1s. Not drawn to scale. Immunoblots of embryonic extracts of stage 6–9 wild-type (wt) and heterozygous diap133-1s mutants were probed with anti-DIAP1 (upper panel) and anti-DRONC antibodies (middle panel). The RING-depleted diap133-1s allele produces a stable protein (DIAP133-1s) that is detectable by its faster electrophoretic mobility (upper panel). In RING-deleted diap133-1s embryos a significant portion of processed DRONC is present (middle panel) which likely accounts for the apoptotic phenotype of diap133-1s embryos [41]. These extracts were obtained from a cross of heterozygous males and females. Thus, only one quarter of the embryos is homozygous mutant for diap133-1s; yet, processed DRONC is easily detectable. The anti-DRONC antibody is specific for the large subunit of DRONC. Lower panel: loading control. (B) Extracts of imaginal discs from wild-type (wt) and mosaic Uba1 imaginal discs were analyzed by immunoblotting using an antibody raised against the small subunit of DRONC. Clones of the temperature sensitive allele Uba190 were induced at the permissive temperature in first larval instar and then shifted to the non-permissive temperature (30°C) during third larval instar 12 hours before dissection (see Material and Methods). This treatment ensures that approximately 50% of the mosaic disc is mutant for Uba1. Although only 50% of the disc tissue is mutant for Uba1, processed DRONC is easily detectable. Lower panel: loading control. doi:10.1371/journal.pgen.1002261.g006
inactivates this effector caspase through a non-degradative mechanism [50].

Interestingly, in GMR-rpr eye imaginal discs, DRONC protein levels appear to be reduced in apoptotic cells compared to living cells (Figure 4C, 4D). Due to the apoptotic activity of REAPER, DRONC is present in its processed and activated form. Reduced protein stability of DRONC has also been reported after incorporation into the ARK apoptosome where it is processed and activated [46]. Combined, these observations suggest that while DIAP1-mediated ubiquitylation of full-length DRONC does not trigger its degradation, processed and activated DRONC has reduced protein stability and may indeed be degraded. It is unclear whether degradation of activated DRONC is mediated by DIAP1, as proposed previously [33]. In GMR-rpr eye imaginal discs, reduced DRONC levels correlate with a reduction of DIAP1 protein (Figure 4C, 4D). This correlation indicates that DIAP1 may actually stabilize DRONC protein, at least in part. Alternatively, because DRONC and DIAP1 form a complex [37], REAPER-induced degradation of DIAP1 may result in co-degradation of complexed DRONC in the process. Further studies are needed to determine the cause of decreased DRONC stability in apoptotic cells.

In addition to Drosophila DRONC, mammalian CASPASE-3 and CASPASE-7 have been reported to be ubiquitylated in vitro [47,48]. However, proteasome-mediated degradation of these caspases in vivo has not been reported. Although a decrease of CASPASE-3 levels has been noted upon overexpression of XIAP, this was shown for an artificial CASPASE-3 mutant, in which the order of the subunits was reversed and the Cys residue in the active site changed to Ser [47]. This catalytically inactive CASPASE-3 mutant is not proteolytically processed [47]. Therefore, physiological in vivo evidence for IAP-mediated degradation of mammalian caspases is lacking.

Moreover, the phenotype of a RING-deleted XIAP mutant mouse is consistent with our data [49]. The XIAPRING mutant, which was generated by a knock-in approach in the endogenous XIAP gene, is characterized by increased caspase activity [49]. Intriguingly, the protein levels of CASPASE-3, CASPASE-7 and CASPASE-9 did not significantly change in the XIAPRING mutant despite the fact that ubiquitylation of CASPASE-3 was reduced. However, processing of these caspases was easily detectable in XIAPRING mutants [49]. These data are very similar to the ones presented here for diap133D4 (Figure 6), and together strongly suggest that non-proteolytic ubiquitylation controls caspase processing and activity in both vertebrates and invertebrates.

Non-proteolytic roles of ubiquitylation have been described in recent years and are involved in many processes including signal transduction, endocytosis, DNA repair, and histone activity (reviewed in [1,3,4]). Two types of ubiquitylation lead to non-proteolytic functions. Monoubiquitylation is involved in endocytosis, DNA repair and histone activity. In fact, mammalian CASPASE-3 and CASPASE-7 have been found to be mono-ubiquitylated in vitro [48]. However, it is unclear whether DRONC is monoubiquitylated by DIAP1. The presence of high molecular-weight ubiquitin conjugates in vitro (Figure 2) suggests that DRONC may be polyubiquitylated, at least under the experimental conditions [38,44].

Polyubiquitylation serves both proteolytic and non-proteolytic functions depending on the Lysine (K) residue used for polyubiquitin chain formation. In general, if polyubiquitylation occurs via K48, the target protein is subject to proteasome-mediated degradation. If it occurs on a different Lys residue, such as K63, non-proteolytic functions are induced [1,3,4]. The best studied examples of both K48- and K63-linked polyubiquitylation are in the NF-kB pathway (reviewed in [3,81]). While K48-polyubiquitylation leads to proteasomal degradation, K63-linked polyubiquitin chains act as scaffolds to assemble protein complexes required for NF-kB activation [3,81]. It is unknown what type of polyubiquitin chain is attached to DRONC, but it is possible that it is not K48-linked. Interestingly, in this context it has been shown that auto-ubiquitylation of DIAP1 preferentially involves K63-linked polyubiquitin chains [82]. By analogy, DIAP1 may ubiquitylate DRONC through formation of K63-linked polyubiquitin chains. This will be an interesting avenue to explore in future experiments.

Conjugated monoubiquitin and polyubiquitin chains can serve as docking sites for factors containing ubiquitin-binding domains (UBD) [2,4,83]. The UBD-containing factors interpret the ubiquitylation status of the target protein, and trigger the appropriate response. For example, K48-linked polyubiquitin chains are recognized by Rad23 and Drk2 which deliver them to the proteasome [2]. Other forms of ubiquitin conjugates are recognized by different UBD-containing factors which control the activity of the ubiquitylated protein. Therefore, it is possible that an as yet unknown UBD-containing protein binds to ubiquitylated DRONC and controls its activity. For example, such an interaction could block the recruitment of ubiquitylated DRONC into the ARK apoptosome. Another possibility is that ubiquitylation could block dimerization of DRONC, which is required for activation of DRONC [34].

“Undead” cells trigger dronc transcription

‘Undead’ cells can be obtained by expression of the effector caspase inhibitor P35 [84]. In these cells, apoptosis has been induced, but cannot be executed due to effector caspase inhibition. Nevertheless, the initiator caspase DRONC is active in ‘undead’ cells and can promote non-apoptotic processes [51]. Recent work has suggested that ‘undead’ cells may alter their cellular behavior. For example, ‘undead’ cells change their size and shape, and have some migratory abilities to invade neighboring tissue [62]. They are also able to promote proliferation of neighboring cells causing hyperplastic overgrowth [15,45,63–66] (reviewed by [85,86]). Transcription of the TGF-β/BMP member decapentaplegic (dpp), the Wnt-homolog wingless (wg), and the p53 ortholog dp35 has also been reported in ‘undead’ cells [45,64–66]. Intriguingly, while dpp and wg are usually not expressed in the same cells [87], ‘undead’ cells co-express them ectopically, clearly indicating an altered transcriptional program.

As part of this altered transcriptional program, we showed that ‘undead’ cells also stimulate transcription of the initiator caspase dronc (Figure 5). Interestingly, p35 expression in normal cells does not induce dronc transcription suggesting that it is not the mere presence of P35 that induces dronc transcription, but instead the ‘undead’ condition of the affected cells. This transcriptional induction of dronc provides an explanation why DRONC protein levels are increased in ‘undead’ cells. It may also help to explain another observation regarding ‘undead’ cells. It has been demonstrated that although these cells are unable to die, they maintain the apoptotic machinery indefinitely [62,88]. Therefore, as part of this maintenance program, ‘undead’ cells stimulate dronc transcription. This is likely not restricted for dronc. Martin et al. (2009) [62] also showed that DrICE protein levels remain high in ‘undead’ cells which may also be caused by increased dICE transcription. It is also possible that the induction of dp35 by ‘undead’ cells [66] is part of this maintenance program, because we have shown that Dp53 induces expression of hid and rpr [89] and a positive feedback loop between dp35, hid and dronc exists in ‘undead’ cells [66]. This may all occur at a transcriptional level.
The mechanism by which ‘undead’ cells stimulate expression of dpp, ago, p35, dronc and potentially drICE are currently unknown and are avenues for future research.

Material and Methods

Drosophila genetics

Fly crosses were conducted using standard procedures at 25°C. The following mutants and transgenes were used: Uba1D6 [5]; arkG8 [26]; diap122-8s and diap120-1s [44]; vps25N55 [90]; droncIR (dronc inverted repeats) [91]; GMR-ptr [92]; droncI29 [67,68], ubx-FLP [93], nub-GAL4 [94], DE- (dorsal eye) GAL4 [95], and UAS-hid [96]. ubx-FLP is sub-GAL4 UAS-FLP, UAS-p35 and UAS-diap1 were obtained from Bloomington. Uba1D6 is a temperature-sensitive allele which at 25°C is a hypomorphic allele, but at 30°C is a null allele [5]. In the experiments in Figure 1, Figure 6B, and Figure S1, Uba1D6 and Uba1D6 arkG8 mosaic larvae were incubated at 25°C; 12 hours before dissection the temperature was shifted to 30°C. This treatment allows recovery of Uba1D6 null mutant clones, which otherwise are cell lethal.

Generation of mutant clones and expression of transgenes

Mutant clones were induced in eye-antennal imaginal discs using the FLP/FRT mitotic recombination system [97] using ey-FLP [90]. In this case, clones are marked by loss of GFP. Expression of diap1 and dronc RNAi in Uba1D6 clones (Figure 1) was induced from UAS-diap1 or UAS-droncIR transgenes using the MARCM system [99]. Here, clones are positively marked by GFP. For induction of diap1-expressing clones in Figure 3, the UAS-diap1 transgene was crossed to hs-FLP, tub-GAL80,GAL4 (ts = FRT). Clones are marked by the absence of GFP. MARCM clones and diap1-overexpressing clones were induced in first instar larvae by heat-shock for one hour in a 37°C water bath. Expression of UAS-p35 in diap122-8s mosaic discs was accomplished by ubx-GAL4.

Immunohistochemistry

Eye-antennal imaginal discs from third instar larvae were dissected using standard protocols and labeled with antibodies raised against the following antigens: DIAP1 (a kind gift of Hermann Steller and Hyung Don Ryoo); cleaved CASPASE-3 (CAS3* (Cell Signaling Technology) and anti-il-GAL4 (Promega). The DRONC antibody used for immunofluorescence was raised against the C-terminus of DRONC in guinea pigs [44]. This antibody is specific for DRONC (Figure S3). Cy3- and Cy5-fluorescently-conjugated secondary antibodies were obtained from Jackson ImmunoResearch. In each experiment, multiple clones in 10–20 eye and wing imaginal discs were analyzed, unless otherwise noted. Images were captured using an Olympus Optical FV500 confocal microscope.

Ubiquitylation assays

Schneider S2 cells were co-transfected with pMT-DRONC C>A V3, pAc-DIAP1 (wt or CA6, F437A, respectively, described in [30]) and pAc His-HA-Ub at equal ratios. Expression of DRONC was induced overnight with 350 μM CuSO4. Cells were lysed under denaturing conditions and ubiquitylated proteins were purified using Ni2+-NTA agarose beads (QIAGEN). Immunoblot analysis was performed with α-V3 (Serotec) and α-DIAP1 antibodies [37,43].

Immunoblot analysis

For the immunoblots in Figure 6A, embryos were collected, decortonated and snap frozen in liquid nitrogen. Embryos were sonicated in Laemmli SDS loading buffer while being frozen. The equivalent of 30 lyzed embryos was loaded per lane. Immunoblots were done using standard procedures. The anti-DRONC antibody used in Figure 6A is a peptide antibody raised against the large subunit of DRONC. The anti-DRONC antibody used in Figure 6B was raised against the C-terminus of DRONC in guinea pigs.

Supporting Information

Figure S1 Loss of arch suppresses apoptosis in Uba1 clones. Uba1 ark mosaic eye-antennal disc labeled for cleaved CASPASE-3 (CAS3*) antibody (red). These discs were incubated at 30°C 12 hours before dissection (see Material and Methods). Absence of GFP marks the location of Uba1 ark clones (see arrows). There is scattered apoptosis detectable. However, this occurs throughout the disc and does not correlate with the positions of the Uba1 ark double mutant clones. Genotype: ey-FLP; FRT42D Uba1ark FRT42D Uba1ark GBP. (TIF)

Figure S2 UAS-diap1 rescues GMR-hid and apoptosis induced in vps25 mutants. Because the UAS-diap1 transgene failed to suppress apoptosis in Uba1 clones (Figure 1C), we tested its ability to inhibit the strong apoptotic phenotype in two other paradigms. (A) Overexpression of the IAP-antagonist hid specifically in the fly eye under GMR promoter control gives rise to a strong eye ablation phenotype due to massive induction of apoptosis [100]. (B) Coexpression of UAS-diap1 partially suppresses the GMR-hid eye ablation phenotype [42]. (C) vps25 mutant clones induce a strong apoptotic phenotype. vps25 can encode an component involved in endosomal protein sorting [90]. The apoptotic phenotype of vps25 and Uba1 as well as other phenotypes caused by inactivation of these genes are very similar, and both mutants were obtained in the same genetic screen [5,90]. The left panel is the merge of GFP and anti-cleaved CASPASE-3 (CAS3*) labeling, the right panel (C) displays only the CAS3* channel. White arrows mark a few clones as examples. (D) Overexpression of diap1 completely suppresses the strong apoptotic phenotype of vps25 mutant clones. The experimental conditions applied here are identical to the Uba1 experiment in Figure 1C. The left panel is the merge of GFP and anti-cleaved CASPASE-3 (CAS3*) labeling, the right panel (D’) displays only the CAS3* channel. Genotype: hs-FLP UAS-GFP/UAS-diap1; FRT42D vps25N55/FRT42D tub-Gal80; tub-GAL4. Genotypes: (A) GMR-hid GMR-GAL4. (B) UAS-diap1; GMR-hid GMR-GAL4. (C) ey-FLP; FRT42D vps25N55/FRT42D P[ubi-GFP]. (D) ey-FLP; FRT42D vps25N55/FRT42D P[ubi-GFP]. (TIF)

Figure S3 Specificity of the anti-DRONC antibody. The specificity of the anti-DRONC antibody used for immunofluorescence in Figure 3, Figure 4, and Figure 5 was verified in dronc29 mosaic eye (A) and wing (B) imaginal discs. The dronc29 allele contains a premature STOP codon at position 53 [11]. dronc29 clones were induced using the MARCM system, hence they are positively marked by GFP (arrows). The anti-DRONC antibody does not produce labeling signals in the mutant clones (arrows in A’ and B’, and the merge in A’ and B’), demonstrating that it is specific for DRONC. Genotype: hs-FLP; dronc29 FRT80/ubi-GFP FRT80. (TIF)

Figure S4 “Undead” diap122-8s cells accumulate DRONC protein autonomously. (A, A’) Using MARCM, p35-expressing, ‘undead’ diap122-8s mutant clones (green) were induced in eye discs and labeled for DRONC protein (red). DRONC protein
automonomously accumulates in P35-expressing 

\( \text{dia}p{\text{-}}1^{22-8s} \) clones (arrows). Similar results were obtained in wing discs (data not shown). Genotype: hs-FLP tub-GAL4 UAS-GFP/+; UAS-p35/+. 

\( \text{dia}p{\text{-}}1^{22-8s} \) FRT80/tub-GAL80 FRT80. 

(TIF)

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Author Contributions

Conceived and designed the experiments: AB PM TVL YF. Performed the experiments: TVL YF SW MS MB. Analyzed the data: AB PM TVL YF SW MS MB. Contributed reagents/materials/analysis tools: AB PM TVL YF SW MS MB. Wrote the paper: TVL AB.

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