THE EMBO JOURNAL

Manuscript EMBO-2009-72789

Metabolic regulation of Drosophila apoptosis through inhibitory phosphorylation of Dronc

Chih-Sheng Yang, Michael J. Thomenius, Eugene C. Gan, Wanli Tang, Christopher D. Freel, Leta K. Nutt and Sally Kornbluth

Corresponding author: Sally Kornbluth, Duke University Medical Center

Review timeline:

Submission date:	05 October 2009
1st Editorial Decision:	12 November 2009
Revision received:	19 June 2010
2nd Editorial Decision:	14 July 2010
Accepted:	15 July 2010

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 November 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. First, I would like to apologise for the delay in getting back to you with a decision. Unfortunately, two of the referees were not able to get back to us with their reports as quickly as initially expected.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see all three referees consider the study as being an interesting one in principle, but they all feel that some further experimentation will be needed before they can support publication here. While all three referees put forward the need for further controls and deeper analysis of the study at the present level of insight referee 1 feels that at least some more in vivo data in flies would be required to strengthen the evidence for the physiological significance of your findings. Taking together all these considerations we will thus be happy to consider a revised manuscript that addresses the referees' concerns in an adequate manner and to their satisfaction. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your

revision.

With kind regards,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This study presents the intriguing observation that metabolic changes might regulate caspases and apoptosis in Drosophila, extending the authors' previous data on the regulation of caspase 2 in vertebrates. They present evidence that the initiator caspase Dronc may be phosphorylated by CAMKII in response to changes in NADPH levels, and that this influences the cells' response to apoptosis. All in all this is an exciting piece of work. Even more so because it appears that this mechanism of cell death regulation is not cell-type specific but instead operates in various tissues, however closer examination is needed to corroborate this assumption. Since Dronc mutant flies exist, it would be great to reconstitute such flies with a phospho-mutant, or phospo-mimetic Dronc rescue construct.

As it stands, the manuscript makes some interesting findings. However, the manuscript will profit from a deeper and more consolidated analysis, addressing the same issue from various angles and corroborating the physiological implication. While these data are interesting, they somewhat lack experimental rigour (see below). Unfortunately, the authors have used experimental settings that are not generally used to study regulation of cell death, making it difficult to appreciate the full extent of the metabolic regulation of cell death. Moreover, some experiments seem not to be anchored in controls, and for people not familiar with the use of DHEA it is difficult to know whether DHEA really does in Drosophila cells what it is supposed to do in other systems - unfortunately no such controls are provided.

Specific points:
1) The authors should establish that DHEA and malate are actually affecting NADPH levels in this system. Alternatively, if the effects of these compounds in S2 cells are already well established, the authors should, at least, provide references.

2) The authors rule out any contribution of Rpr, Grim and Hid. However, this is not sufficiently addressed. Since some of the IAP-antagonists are expressed in living cells the author should perform qPCR to exclude the possibility that Malate affects expression of RGH. Furthermore, does malate affect DIAP1 levels?

3) The author imply that cellular energy crisis contribute to the regulation of apoptosis. How widely is this applicapable? Unfortunately, the authors provide little in vivo data that would indicate that this operates under physiological conditions.

4) The legend for figure 3A states that cells were treated with DIAP1 dsRNA; the authors need to clarify this as the text states that death was induced with DHEA.

5) Somewhat surprisingly, the authors state that z-VAD, which generally blocks all caspases and calpains, does not block Dronc. The authors need to back this up with references or experimental evidence. Moreover, the need for zVAD in fig 3C is not clear; it is well established that p35 blocks caspases other than Dronc, so p35 expression should be used instead. Also, Figure 3C uses Dronc autoprocessing as a readout for Dronc activity, when they would be better off using Dronc-mediated cleavage of drICE as a readout, as in figure 3E.

6) The assay used in figure 4B is confusing: it is not clear what the observed loss of GFP reflects. This experiment should be repeated using PI staining or some other more established assay for cell

death.

7) The decrease in the interaction between Dark and Dronc, as shown in Figure 4D, is not too convincing: the difference in the amount of Dronc co-precipitated is minimal. It is not clear whether wild type Dronc is actually phosphorylated uner the conditions used here; more convincing data might be obtained by performing the IPs from cells treated with DHEA +/- malate, ie the conditions used in fig 4A, where there is a clear difference in Dronc phosphorylation status.

8) PKA and CKI are ruled out as Dronc-kinases on the basis of chemical inhibitor experiments; are these inhibitors known to inhibit the Drosophila kinases?

9) Although the data shown in figure 4E implies that CamKII can regulate cell death, labelling experiments (as in fig 4A) +/- CamKII dsRNA need to be carried out to explicitly demonstrate that CamKII phosphorylates Dronc in vivo, and that the presence of malate has no effect under these conditions.

10) The assay used in figure 5 is somewhat unusual. The basis of the assay is Dronc-induced death of a subset of neurons that would normally produce a neuropeptide hormone to induce wing expansion and sclerotisation. The indirect readout of wing expansion may misrepresent the underlying defects. An alternative assay, given that expression of Dronc alone gave no phenotype in the eye, could involve looking at sensitisation to GMR-Rpr or to UV-irradiation.

All in all I feel that the initial observation is really interesting but that the physiological relevance of NADPH-regulation of Dronc activity is not sufficiently addressed. Perhaps an obvious system to investigate this would be in the oocyte, where nutrient availability is known to regulate cell death, and thus it could be envisaged that cellular energy levels impact upon apoptosis here.

In summary, the data shown are very interesting, and more rigorous analyses, with better-established assays, would turn this into a very nice paper.

Referee #2 (Remarks to the Author):

Here, Kornbluth and colleagues present data to argue that manipulation of NADPH levels leads to CAMKII-mediated phosphorylation of Dronc at Ser130, thereby suppressing the activation/activity of this caspase. Conversely, dephosphorylation of Dronc at this site leads to enhanced activation and apoptosis. This is an interesting study, which somewhat parallels the previous work of this group on caspase-2 phosphorylation and inhibition in oocytes by Cam Kinase II. However, due to the distinct mechanism of caspase activation in the fly, the current work is not merely a translation of their previous work into a different species. However, there are significant gaps in the current story that would preclude publication as the paper stands at present.

Specific comments

1. There is a lot of switching back and forth between DHEA as a driver of apoptosis and CHX, although the way in which these highly dissimilar agents promote apoptosis is likely to be distinct. Some observations are made using CHX and others using DHEA, which is irritating. The suppressive effect of malate on apoptosis in S2 cells is much more convincing in the DHEA-driven context rather than the CHX-driven context. Yet, the authors have not demonstrated some key findings in the DHEA-initiated scenario. For example, in Figure 2, the authors have examined the effect of Men knockdown on the rescue afforded by Malate on CHX-induced cell death, but this has not been done for DHEA-induced cell death, despite the fact that Malate appears to have a much better protective effect on DHEA-induced death than it does on CHX-driven death. Similarly, the authors have not examined Dronc processing in response to DHEA and the effect that Malate has on this (in this particular context, I also feel that zVAD should not be added to the experiment). The same comment applies to the type of experiment presented in Figure 4B where the effect of a non-phosphoralatable Dronc mutant on CHX-induced cell death, in the presence and absence of malate,

has been examined.

2. Phosphorylation of endogenous Dronc, either constitutively, or in response to malate treatment, has not been demonstrated. The authors should really try to offer evidence that endogenous Dronc (or even an overexpressed catalytically-inactive full length Dronc) is phosphorylated in S2 cells. And that the Ser130A mutant is not phosphorylated under the same conditions. As it stands, an artificial prodomain-only construct has been used, there could be several additional sites on Dronc that are phosphorylated.

3. In the CamKII knockdown experiments presented in Figure 4E, this experiment should again be repeated using DHEA as the cell death trigger.

4. The authors should also examine endogenous or overexpressed full-length Dronc phosphorylation in control versus CamKII-deficient cells (i.e where this has been knocked down). As it stands, they have shown that CamKII can phosphorylate the Dronc prodomain in vitro at S130 but this does not demonstrate that this is the kinase responsible in S2 cells. At a minimum, the authors could use the S2 cell lysate syatem they used in Figure 4A, where the lysates have been generated from control versus CamKII-deficient cells.

5. Using recombinant Dronc in vitro, does recombinant CamKII suppress the activity of Dronc towards a synthetic substrate or towards in vitro transcribed/translated Drice?

6. Quantitation of the degree of apoptosis is weak throughout the paper and many effects are represented only by photomicrographs, which can be misleading. Flow cytometry analysis (or similar) of cell death should be performed for all key observations (Figs. 1E and 1G, Figs 2A and B, Fig. 4E) and absolute percentages of cell death should be shown.

Referee #3 (Remarks to the Author):

This is a very interesting manuscript on metabolic regulation of apoptosis in Drosophila. The main conclusion from this study is that high NADPH levels result in a inhibitory phosphorylation of the initiator caspase Dronc. Consequently, decrease in NADPH levels lead to dephosphorylation of Dronc, allowing it to be activated by Dark which leads to apoptosis. They also report that phosphorylation of Dronc is accomplished by CamKII.

Many of the key findings are well-supported by the data and the paper is of overall high quality. In particular, the authors provide strong evidence that NADPH levels regulate apoptosis in S2 cells downstream of DIAP1. They use two methods to modulate NADPH levels, DHEA to decrease and malate to increase NADPH. Four assays which together demonstrate apoptotic cell-death; one for cell number - "cell-density", one for cell viability - PI, and two apoptotic markers - membrane blebbing and DEVDase activity all support their conclusions. They go on to show that malate protects cells from cycloheximide and DIAP1 dsRNA as well. They also show that Dronc and Dark dsRNA can protect cells from DHEA induced death. All together this is strong evidence that NADPH levels protect cells by preventing the activation or activity of caspases.

The authors' evidence that NADPH protection is mediated by decreasing Dronc activation by Dark is a bit less convincing (Fig. 3). Here they switch to cycloheximide as the death stimulus and show a slight delay of Dronc processing at time points where the difference in apoptosis between malate treated versus untreated cells is small according to their cell density assay. Why not show the 12 hr time point when, according to figure 2A, there is a dramatic difference between malate treated versus untreated cells? Why not test drice processing in the same assay instead of switching to DIAP1 dsRNA? Indeed, the authors have not shown inhibition of DEVDase activity by malate for either of these stimuli, unlike DHEA. It is still quite possible that most of the protective effect of malate lies downstream of caspases for these stimuli.

The authors go on to demonstrate that exogenously expressed Dronc prodomain is phosphorylated at

Ser130, and that DHEA treatment results in the loss of this phosphorylation. They also show that this loss is prevented by malate, again implicating NADPH levels. The new assay in figure 2B demonstrates that Dronc S130A is a better killer than wild type Dronc and that malate does not appear to protect against it. This experiment indicates that phosphorylation of Ser130 mediates malate's protection from cycloheximide-induced apoptosis. However, it seems that an alternative explanation could be that Dronc S130A is a better killer that overwhelms malate's downstream protection, and it would be helpful if the authors could address this possibility.

Overall, this is a nice study and of broad interest and significance as it provides new insights into the regulation of apoptosis, and also because there is recent evidence that angiostatin (an endogenous anti-angiogenic agent that can induce apoptosis) targets malate dehydrogenase. However, the ms would be improved if the authors could clarify the specific issues raised. Also, Fig. 3 could be improved; the constant switching of stimuli and time points does not allow a reader to easily follow the authors' arguments - why not focus on one stimulus and one set of time points for these experiments?

Minor points:

The authors do not show what proportion of Dronc is phosphorylated at Ser130 (IP followed by a 2-D gel?). If only a small portion of Dronc is phosphorylated, this would not be easily reconciled with the proposed mechanism of protection. The authors should clarify this at least in the discussion.

The legend of Figure 3A figure does not describe the experiment shown.

1st Revision - authors' response 19 June 2010

Responses to Reviewer #1:

Specific points:
1) The authors should establish that DHEA and malate are actually affecting NADPH levels in this system. Alternatively, if the effects of these compounds in S2 cells are already well established, the authors should, at least, provide references.

New Figure 1D We appreciate the reviewer's comment. After careful review of the literature, we found that DHEA and malate had been broadly used in mammalian cells, but not in *Drosophila* cells. Accordingly, to examine the metabolic effects of DHEA and malate in S2 cells, we measured cellular NADPH levels in untreated S2 cells and cells treated with DHEA ± malate. As shown in Fig. 1D, DHEA treatment resulted in a 60% decrease in NADPH levels, while malate co-treatment significantly abrogated this reduction, confirming that those two reagents can regulate cellular NADPH levels in the experimental system we utilized.

2) The authors rule out any contribution of Rpr, Grim and Hid. However, this is not sufficiently addressed. Since some of the IAP-antagonists are expressed in living cells the author should perform qPCR to exclude the possibility that Malate affects expression of RGH. Furthermore, does malate affect DIAP1 levels?

New Figure 2F and G To address this, we monitored DIAP1 levels in S2 cells upon malate treatment. As shown, malate treatment affected neither the steady state levels of DIAP1 (**New Fig. 2F**), nor the half-life of DIAP1 (**New Fig. 2G**). Since the RHG family proteins regulate DIAP1 levels, the constancy in DIAP1 levels in response to malate strongly suggests that malate is unlikely to affect the expression of the RHG family proteins. As we show that malate significantly suppresses CHX-induced apoptosis in **New Fig. 2C and D,** this novel level of regulation cannot be dependent on *de novo* induction of the RHG proteins.

3) The author imply that cellular energy crisis contribute to the regulation of apoptosis. How widely is this applicapable? Unfortunately, the authors provide little in vivo data that would indicate that this operates under physiological conditions.

New Figure 6E, G, H and New Supplementary Figure 8 To more thoroughly examine how NADPH levels control apoptosis *in vivo*, we artificially generated an NADPH deficiency in different tissues by expressing UAS-RNAi hairpins targeted against NADPH-generating enzymes and examined if this would enhance the phenotype caused by overexpression of WT, but not unphosphorylatable Dronc. As shown in Fig. 6, downregulation of malic enzyme (MEN) aggravated the wing expansion phenotype induced by the elav-driven overexpression of wild-type Dronc (**New Fig. 6E**), suggesting that NADPH suppresses the cell killing activity of WT Dronc. In contrast, phosphomutant Dronc (S130A) is not sensitive to cellular NADPH levels, as coexpression of MEN RNAi hairpins did not alter the phenotype induced by phosphomutant Dronc (**New Fig. 6E**). These data are consistent with the conclusion that NADPH suppresses Dronc in neuronal cells through the metabolically-mediated phosphorylation of Dronc at S130.

In our original experiments, although we found that S130A Dronc produced a more severe phenotype than WT Dronc when expressed in neurons under the control of elav-Gal4 drivers, we did not observe any differential phenotype when these same constructs were expressed in the eye (neither WT nor mutant protein produced a marked phenotype). These observations suggest that metabolism might limit apoptosis in some tissues, but not others. To address this issue further, we examined the effects of NADPH deficiency on the eye phenotype induced by GMR-driven overexpression of Dronc. As our Dronc transgenes were not strong enough to generate an eye phenotype in heterozygous animals (1× UAS-Dronc), we crossed them with either DIAP1 deficiency flies (Df(3L)st-f13) or flies constitutively expressing rpr in the eye tissues(GMR-rpr), as suggested. As shown in **New Supplementary Fig. 8**, GMR-driven overexpression of WT or phosphomutant Dronc (S130A) in heterozygous animals (1× UAS-Dronc) resulted in no additional eye defects in a DIAP1 deficiency background, while they caused similar levels of eye ablation when they were co-expressed with GMR-rpr. In addition, GMR-driven overexpression of Dronc (WT or phosphomutant) in homozygous animals (2× UAS-Dronc) led to similar levels of eye ablation. Collectively, these data indicate that Dronc's activity is not regulated by phosphorylation at S130 (or by extension, metabolism) in the fly eye. Importantly, to show definitively that NADPH production could modulate apoptosis *in vivo* in neurons, but not eye tissue, we used the UAS-Gal4 system to knock down G6PDH, the major NADPH-generating enzyme (via the pentose phosphate pathway). As shown, NADPH deficits caused delayed maturation and wing expansion defects (**New Fig. 6G**), when we expressed the UAS-G6PDH RNAi hairpins in neuronal tissues using elav-Gal4 drivers. In contrast, we observed no significant cell death in the eye when the same RNAi constructs were expressed by GMR-Gal4 (**New Fig. 6H**), indicating tissues in the eye are not susceptible to metabolic cell death triggered by NADPH depletion. Thus, genetically engineered loss of NADPH had the same tissue-restricted effects as expression of mutant Dronc. Together, our data demonstrate that caspase(s) are suppressed by physiological levels of NADPH in neuronal cells, so that NADPH deficiency results in aberrant cell death. However, Dronc activity does not appear to be controlled by metabolically-mediated phosphorylation in the eye.

4) The legend for figure 3A states that cells were treated with DIAP1 dsRNA; the authors need to clarify this as the text states that death was induced with DHEA.

We apologize for the error. The figure legend has now been corrected to describe the corresponding experiments and all data using DHEA as the apoptotic stimulus have been moved to Fig. 1 to avoid confusion.

5) Somewhat surprisingly, the authors state that z-VAD, which generally blocks all caspases and calpains, does not block Dronc. The authors need to back this up with references or experimental evidence. Moreover, the need for zVAD in fig 3C is not clear; it is well established that p35 blocks caspases other than Dronc, so p35 expression should be used instead. Also, Figure 3C uses Dronc autoprocessing as a readout for Dronc activity, when they would be better off using Dronc-mediated cleavage of drICE as a readout, as in figure 3E.

New Figure 2E Our initial data showed that Dronc molecules could cleave each other in trans between p20 and p10 to generate the Pr1 fragment even in the presence of zVAD, suggesting that zVAD might not be able to block Dronc's catalytic activity. However, a more detailed examination of the kinetics of Dronc processing now show that although the generation of the Pr1 fragment is not completely inhibited, the autoprocessing of Dronc is delayed by zVAD, indicating this pan-caspase inhibitor can suppress Dronc's activity in S2 cells, albeit not completely. Therefore, as suggested by

the reviewer, we have now used both Dronc-mediated cleavage of drICE and Dronc autoprocessing as readouts to monitor the ability of malate to modulate CHX-induced Dronc activation in the absence of caspase inhibitor. As shown in **New Fig 2E**, malate significantly delayed CHX-induced processing of Dronc and drICE, confirming that malate can suppress Dronc in absence of DIAP1. Therefore, Dronc is very likely to be a direct target of metabolic regulation.

6) The assay used in figure 4B is confusing: it is not clear what the observed loss of GFP reflects. This experiment should be repeated using PI staining or some other more established assay for cell death.

New Supplementary Figure 8 We apologize for the confusion. In Fig. 4E (original Fig. 4B), we used the loss of GFP as a readout for cell viability. Similar assays have been used to monitor cell death in *Drosophila* SL2 cells [Zimmermann et al. J Cell Biol. 2002 Mar 18; 156(6):1077-87] and mammalian cells [Palacios et. al. Oncogene. 2006 Oct 5; 25(45):6133-9]. We have now correlated this loss with measurements of caspase activity to validate the assay. Specifically, we treated S2 cells with CHX to induce apoptosis; cells collected at different time points were used to assess the percentage of GFP-positive cells by FACS and then lysed to measure cellular DEVDase (caspase) activity. As shown in **New Supplementary Fig. 8**, the percentage of GFP-positive cells decreased as caspase activity increased. Moreover, blocking CHX-induced caspase activity inhibited the accompanying loss of GFP, confirming that the loss of GFP is due to caspase-dependent cell death. Therefore, the loss of GFP can be used in our system, as reported previously, as a proxy to assess cell viability.

7) The decrease in the interaction between Dark and Dronc, as shown in Figure 4D, is not too convincing: the difference in the amount of Dronc co-precipitated is minimal. It is not clear whether wild type Dronc is actually phosphorylated uner the conditions used here; more convincing data might be obtained by performing the IPs from cells treated with DHEA +/- malate, ie the conditions used in fig 4A, where there is a clear difference in Dronc phosphorylation status.

New Figure 4B, C and 5A To address this, we have repeated this experiment multiple times. The original panel has been replaced with new representative data (**New Fig. 5A**). We observe that Dark consistently pulls down more phosphomutant Dronc (S130A) than the wild-type Dronc. The difference, albeit small, was consistent among more than 3 individual experiments. To monitor the phosphorylation status of Dronc at S130, we produced a new affinity-purified phospho-specific antibody (Dronc pS130) directed against this phosphosite. Antibody validation is presented in **New Fig. 4B and C**. To determine whether Dronc pS130 recognized phosphorylated Dronc from S2 cells, FLAG-tagged full-length enzymatically inactive Dronc (C318A) was immunoprecipitated on anti-FLAG resin. Precipitates treated with or without l-phosphatase were analyzed by immunoblotting using FLAG and Dronc pS130 antibody. As shown in **New Fig. 4B**, phosphatase treatment removed the signal.
To further examine the specificity of the Dronc pS130 antibody, catalytically-inactive Dronc (C318A ± S130A) was analyzed by immunoblotting using this antibody. As shown, this antibody recognized only wild-type Dronc (C318A), while an extra mutation (S130A) removed this signal, demonstrating that our phospho-specific antibody specifically recognizes phosphorylated S130 and that this residue is phosphorylated in S2 cells (**New Fig. 4C**).

Similarly, as shown in **New Fig. 5A**, HA-tagged wild-type Dronc (C318A) was detected by our phospho-specific antibody, while the additional phospho-mutation (S130A) abrogated this signal, demonstrating that wild-type Dronc (C318A) is phosphorylated at S130 in this assay.

Unfortunately, the buffer conditions used to preserve the phosphorylation status (and to extract sufficient levels of protein for analysis) were too harsh for co-immunoprecipitation, so we could not do the precise experiment suggested by the reviewer.

8) PKA and CKI are ruled out as Dronc-kinases on the basis of chemical inhibitor experiments; are these inhibitors known to inhibit the Drosophila kinases?

New Supplementary Figure 7 We have repeated this experiment with 30 mM PKA inhibitor H-89 [Zhao et. al. Nature. 2007 Nov 8; 450(7167):252-8] and 8 mM CK1 inhibitor IC261 [Mennella et. al. J Cell Biol. 2009 Aug 24; 186(4):481-90], both of which have been shown to inhibit *Drosophila* kinases in the published literature, and malate still protected treated cells from CHX-induced apoptosis (**New Supplementary Fig. 7A**). These data indicate that PKA and CK1 are

unlikely to be involved in the metabolic regulation of apoptosis. To further assess the involvement of PKA and CK1, we specifically down-regulated these two kinases with double-stranded (ds)RNA. Consistent with data using chemical inhibitors, neither PKA nor CK1 dsRNA were able to abrogate malate's protective effects (**New Supplementary Fig. 7B**), confirming that PKA and CK1 are not the relevant kinases in the metabolic control of Dronc.

9) Although the data shown in figure 4E implies that CamKII can regulate cell death, labelling experiments (as in fig 4A) +/- CamKII dsRNA need to be carried out to explicitly demonstrate that CamKII phosphorylates Dronc in vivo, and that the presence of malate has no effect under these conditions.

New Figure 5C To determine whether CaMKII is the kinase targeting Dronc S130 *in vivo*, we treated S2 cells with control GFP dsRNA and CaMKII dsRNA. Lysates from treated cells were then analyzed by immunoblotting using our new phospho-specific antibody toward Dronc S130 described above. As shown, CaMKII dsRNA significantly reduced the phosphorylation at Dronc S130 even in the presence of malate, demonstrating that CaMKII is likely the relevant kinase targeting to Dronc S130 *in vivo*.

10) The assay used in figure 5 is somewhat unusual. The basis of the assay is Dronc-induced death of a subset of neurons that would normally produce a neuropeptide hormone to induce wing expansion and sclerotisation. The indirect readout of wing expansion may misrepresent the underlying defects. An alternative assay, given that expression of Dronc alone gave no phenotype in the eye, could involve looking at sensitisation to GMR-Rpr or to UV-irradiation.

New Figure 6C, E, G, H, and New Supplementary Figure 8 We appreciate the reviewer's comments and provide more evidence to address this concern. Indeed, most apoptosis assays in the transgenic flies are done in the eye. However, our detailed analysis demonstrated that eye tissues are not subject to the metabolic control, as described above. Therefore, in Fig. 6, we use a Dronc-mediated wing expansion defect as readout to assess Dronc's activity in transgenic flies. We acknowledge that wing expansion defects could result from different neuronal abnormalities. To further examine the cause of the wing expansion phenotype we observed, we analyzed the hemolymph from the Dronc-expressing flies by immunoblotting using anti-bursicon antibody [Peabody et. al. J Neurosci. 2008 Dec 31; 28(53):14379-91]. As shown, flies expressing Dronc with elav-Gal4 lacked bursicon in the hemolymph, while coexpression of the caspase inhibitor p35 restored bursicon levels and rectified the accompanying wing expansion defects (**New Fig. 6C**). These data confirm that the wing expansion defects results from caspase-dependent bursicon deficiency. Therefore, wing expansion defects can be used as readouts to assess neuronal caspase activity in transgenic flies.

General concerns:
Since Dronc mutant flies exist, it would be great to reconstitute such flies with a phospho-mutant, or phospo-mimetic Dronc rescue construct.

We have tried to rescue dronc mutant animals with transgenic lines with the UAS-Dronc insertion on the second chromosome. Heterozygous animals expressing those UAS-Dronc constructs under elav-Gal4 show wing expansion defects, but result in no eye ablation when the expression is driven by GMR-Gal4. However, no construct we tested could rescue homozygous Dronc mutants (data not shown). We noticed that the UAS-Dronc constructs used for the published rescue experiments are able to induce eye ablation in heterozygous animals, while ectopic expression of our UAS-Dronc constructs with GMR-Gal4 resulted in no eye phenotype in heterozygous animals (and a weak eye phenotype in homozygous individuals, see New Supplementary Fig. 8), suggesting our UAS constructs are weaker then those previously published. Therefore, we need to generate more transgenic lines and are not able to fully address this in the given time frame. However, we do provide more physiological data supporting the idea that NADPH levels control Dronc's activity *in vivo* (**New Fig. 6E, G and H**). We appreciate the reviewer's comments and will keep pursuing this.

As for the phosphomimmetic mutants, our biochemical assays show that neither D nor E mutants at S130 could mimic S130 phosphorylation. Instead, they function more like phosphomutant (A mutant) as they increase the interaction between Dark and Dronc. In addition, we have some preliminary data (not shown here) that phospho-Dronc, like caspase 2, binds 14-3-3 and it is well established that neither D nor E mutant proteins bind 14-3-3 like the actual phosphate group.

Unfortunately, the authors have used experimental settings that are not generally used to study regulation of cell death, making it difficult to appreciate the full extent of the metabolic regulation of cell death.

New Figure 6C, E, G, H, and New Supplementary Figure 8 We now have provided more evidence demonstrating that neuronal but not eye tissues are subject to metabolic control of apoptosis (**New Fig. 6E, G, H and New Supplementary Fig. 8**). We also present the level of bursicon in hemolymph as a more direct readout (**New Fig. 6C**). Please see replies to points #3 and #10 for more experimental details.

For people not familiar with the use of DHEA it is difficult to know whether DHEA really does in Drosophila cells what it is supposed to do in other systems - unfortunately no such controls are provided.

New Figure 1D The pharmacological effects of DHEA and malate have been examined and presented in **New Fig. 1D**. Please see the replies to point #1 for more experimental details.

All in all I feel that the initial observation is really interesting but that the physiological relevance of NADPH-regulation of Dronc activity is not sufficiently addressed. Perhaps an obvious system to investigate this would be in the oocyte, where nutrient availability is known to regulate cell death, and thus it could be envisaged that cellular energy levels impact upon apoptosis here.

Rather than try a host of tissues (including oocytes) for Dronc WT and mutant expression, we chose to validate the involvement of metabolism in the neuronal death we observed by altering NADPH levels in different ways (malic enzyme and G6PDH ablation) and by measuring the output of these neurons (bursicon) in the presence of the caspase inhibitor p35 to validate that there was apoptotic death occurring. Our data demonstrates that NADPH levels could modulate Dronc activity even in the absence of DIAP1. As Koto *et al.* shows DIAP1 proteins are cleared in the sensory organ precursor (SOP) cell lineage during a specific developmental stage, allowing the caspases to exert their non-apoptotic functions. Based on our data we would propose a possible mechanism where NADPH fine-tunes caspase activity in the absence of DIAP1 by maintaining the proteolytic activity of Dronc at a low level, allowing the cleavage of developmental substrates, but not the processing of apoptotic-relevant substrates such as drICE.

Responses to Reviewer #2:

Specific points:
1. and 3. There is a lot of switching back and forth between DHEA as a driver of apoptosis and CHX, although the way in which these highly dissimilar agents promote apoptosis is likely to be distinct.

New Figure 1E and F To be more consistent with our apoptotic stimuli, all data using DHEA as an apoptotic trigger were combined into Fig.1. To further elucidate the signaling pathway underling DHEA-induced cell death, we performed a loss-of-function analysis using dsRNA to specifically remove individual component in the fly caspase cascade. As shown in **Fig. 1E**, DHEA requires Dronc and Dark to induced cell death, as Dronc or Dark dsRNA completely blocked DHEA-induced cell death. Moreover, lysates from Dronc-deficient cells treated with DHEA were analyzed by immunoblotting (**New Fig. 1F**). DHEA induced Dronc and drICE activation in cells treated with control GFP dsRNA, as assessed by their processing. However, downregulation of Dronc completely blocked drICE processing, confirming that Dronc is essential for DHEA-initiated apoptotic signaling. Consistent with previously published reports, we were also able to show that CHX-mediated apoptosis requires Dronc. Together, our data now demonstrate that DHEA and CHX both promote apoptosis through the Dronc-drICE cascade. Because the locus of metabolic control appears to be Dronc, it is reasonable that apoptosis triggered by both reagents should be metabolically-regulated.

Some observations are made using CHX and others using DHEA, which is irritating. The suppressive effect of malate on apoptosis in S2 cells is much more convincing in the DHEA-driven

context rather than the CHX-driven context. Yet, the authors have not demonstrated some key findings in the DHEA-initiated scenario. For example, in Figure 2, the authors have examined the effect of Men knockdown on the rescue afforded by Malate on CHX-induced cell death, but this has not been done for DHEA-induced cell death, despite the fact that Malate appears to have a much better protective effect on DHEA-induced death than it does on CHX-driven death.

New Figure 1D, 2C, 2D and New Supplementary Figure 1 We have now repeated many key experiments using DHEA as a death trigger. In particular, we provide evidence in **New Supplementary Figure 1** to show that malate must be metabolized by malic enzyme to protect from DHEA-induced death. Moreover, we also provide additional evidence showing that DHEA and malate directly modulate cellular NADPH levels by the assessment of cellular NADPH levels in lysates. As shown in **New Fig. 1D**, DHEA significantly reduced cellular NADPH levels, which was largely restored by malate addition (please see reply to reviewer #1 point #1 for more detail). Together, our data demonstrate that malate also suppresses DHEA-induced apoptosis through modulating NADPH levels.

To further examine the suppressive effect of malate in the CHX-driven context, we have repeated those experiments and present quantitative data in **New Figure 2C and D**. As shown, malate also significantly suppressed CHX-induced apoptosis, as assessed by cell density and cellular caspase activity.

Similarly, the authors have not examined Dronc processing in response to DHEA and the effect that Malate has on this (in this particular context, I also feel that zVAD should not be added to the experiment). The same comment applies to the type of experiment presented in Figure 4B where the effect of a non-phosphoralatable Dronc mutant on CHX-induced cell death, in the presence and absence of malate, has been examined.

In the CamKII knockdown experiments presented in Figure 4E, this experiment should again be repeated using DHEA as the cell death trigger.

New Figure 1G To address this concern, Dronc processing from cells treated with DHEA ± malate was examined by immunoblotting. As suggested, no caspase inhibitors were added. Similar to the CHX-driven death data (**New Fig. 2E**), malate blocked Dronc and drICE autoprocessing upon DHEA treatment (**New Fig. 1G**), indicating that the metabolic checkpoint likely acts on Dronc or upstream to Dronc. On the other hand, malate blocked CHX-induced apoptosis (**New Fig. 2C and D**). As CHX globally blocks translation, including new synthesis of the RHG proteins, and the degradation of endogenous DIAP1 is not affected (**New Fig. 2G**), the direct target for malate-mediated suppression was likely located downstream of DIAP1. Together, these data suggest Dronc is a direct target modulated by NADPH levels. Our current study describes this novel level of "post-DIAP1" regulation. As CHX treatment allowed us to focus on regulation of the caspase cascade itself, CHX is used as the primary apoptotic trigger throughout this study.

2. Phosphorylation of endogenous Dronc, either constitutively, or in response to malate treatment, has not been demonstrated. The authors should really try to offer evidence that endogenous Dronc (or even an overexpressed catalytically-inactive full length Dronc) is phosphorylated in S2 cells. And that the Ser130A mutant is not phosphorylated under the same conditions. As it stands, an artificial prodomain-only construct has been used, there could be several additional sites on Dronc that are phosphorylated.

New Figure 4B-D To examine the phosphorylation status of endogenous Dronc at S130, we developed a new phospho-specific (Dronc pS130) antibody toward this site. Antibody validation is presented in **New Fig. 4B and C.** Please see replies to reviewer #1 point #7 for detailed descriptions on antibody characterization. Lysates from cells treated with DHEA ± malate were analyzed by immunoblotting using Dronc pS130. As shown in **New Fig. 4D**, a DHEA-induced decrease in cellular NADPH resulted in dephosphorylation of endogenous Dronc, while replenishing NADPH with malate restored Dronc phosphorylation at S130, confirming that the phosphorylation of endogenous Dronc is modulated by cellular NADPH levels.

4. The authors should also examine endogenous or overexpressed full-length Dronc phosphorylation in control versus CamKII-deficient cells (i.e where this has been knocked down). As it stands, they have shown that CamKII can phosphorylate the Dronc prodomain in vitro at S130

but this does not demonstrate that this is the kinase responsible in S2 cells. At a minimum, the authors could use the S2 cell lysate syatem they used in Figure 4A, where the lysates have been generated from control versus CamKII-deficient cells.

New Figure 5C We have provided additional evidence to support the conclusion that CaMKII is the kinase targeting to Dronc S130 *in vivo*, as CaMKII dsRNA significantly decreased the phosphorylation at this site even in the presence of malate (**New Fig. 5C**). The same concern was also suggested by reviewer #1. Please refer to our replies to reviewer #1 point #9 for additional details.

5. Using recombinant Dronc in vitro, does recombinant CamKII suppress the activity of Dronc towards a synthetic substrate or towards in vitro transcribed/translated Drice?

New Figure 5E We appreciate the reviewer's helpful comments. Given the phosphorylation is located within the prodomain, the most likely effect of Dronc phosphorylation is to stop it's dimerization by Dark, not to inhibit its enzymatic activity.
We now provide evidence to rule out the possibility that Dronc phosphorylation at S130 affects its catalytic activity. To address this, recombinant Dronc was produced in bacteria where high concentrations triggers artificial dimerization/autoprocessing, activating recombinant Dronc as previously described [Muro et. al. J Cell Sci. 2004 Oct 1; 117(21):5035-41; Dorstyn et. al. Cell Death Differ. 2008 Mar; 15(3):461-7]. The proteolytic activity of CaMKII-treated and mock treated Dronc toward *in vitro* translated ^{35}S-labeled drICE was then assessed. As shown in **New Fig. 5E**, Dronc was phosphorylated at S130 after incubating with CaMKII, as analyzed by immunoblotting using Dronc pS130 antibody. The amount of recombinant Dronc in each group was the same as shown with Dronc antibody. Interestingly, CaMKII-treated and unphosphorylated Dronc cleaved similar amounts of drICE, indicating that once Dronc is dimerized and activated, phosphorylation at S130 has no effect on its proteolytic activity.

6. Quantitation of the degree of apoptosis is weak throughout the paper and many effects are represented only by photomicrographs, which can be misleading. Flow cytometry analysis (or similar) of cell death should be performed for all key observations (Figs. 1E and 1G, Figs 2A and B, Fig. 4E) and absolute percentages of cell death should be shown.

New Figure 2B, D, 3D, 5C and Figure 4E We have repeated many experiments and measured cellular DEVDase (effector caspase) activity as a quantitative readout for apoptosis. Malate's inhibitory effects on DIAP1 dsRNA and CHX-induced apoptosis now have been quantified and shown in **New Fig. 2B and D**. As shown, malate significantly blocked caspase activation induced by either CHX or DIAP1 dsRNA. We now also quantitatively represent malate's effects on the kinetics of CHX-induced caspase activation in **New Fig. 3D**. As shown, malate suppressed CHX-induced caspase activity in cells pretreated with control (GFP) dsRNA, while malic enzyme (MEN) dsRNA not only accelerated caspase activation but also desensitized S2 cells to malate's protection, confirming that malate needs to be metabolized into NADPH to confer its protective effects against CHX-induced apoptosis. As we show that CaMKII dsRNA reduced Dronc phosphorylation at S130 (**New Fig. 5C**), **Fig. 4E** should now recapitulate the quantitative data for original Fig. 4E (Fig. 5B in revised manuscript), as in both cases cells were expressing dephosphorylated Dronc (S130A).

Responses to Reviewer #3:

Major concerns:
The authors' evidence that NADPH protection is mediated by decreasing Dronc activation by Dark is a bit less convincing (Fig. 3). Here they switch to cycloheximide as the death stimulus and show a slight delay of Dronc processing at time points where the difference in apoptosis between malate treated versus untreated cells is small according to their cell density assay. Why not show the 12 hr time point when, according to figure 2A, there is a dramatic difference between malate treated versus untreated cells? Why not test drice processing in the same assay instead of switching to DIAP1 dsRNA?

New Figure 2E We thank the reviewer for the helpful comments. We have examined both Dronc and drICE processing and gained more clear data shown in **New Fig. 2E**. As shown, malate significantly delayed the processing of both caspases. After 6 h of CHX treatment, almost all Dronc and drICE were processed and activated, while malate addition maintained ~50% of Dronc in its full-length form and completely blocked drICE processing, indicating the intracellular proteolytic activity of Dronc is greatly compromised by malate. Given that caspase activation occurs prior to cell death, we, therefore, examined CHX-treated cells for the first 6 h after treatments were applied when the cleavage/processing of caspases were used for readout, while data showing the loss of cell density were collected 9-12 h post treatment.

Indeed, the authors have not shown inhibition of DEVDase activity by malate for either of these stimuli, unlike DHEA. It is still quite possible that most of the protective effect of malate lies downstream of caspases for these stimuli.

New Figure 2B, D, E We have now repeated these experiments and have provided additional evidence to support the idea that malate's protection against CHX and DIAP1 dsRNA-induced apoptosis is targeting the caspase cascade, as malate addition suppresses 1) DEVDase activity (**New Fig. 2B and D**) and 2) caspase processing (**New Fig. 2E**).

The new assay in figure 2B demonstrates that Dronc S130A is a better killer than wild type Dronc and that malate does not appear to protect against it. This experiment indicates that phosphorylation of Ser130 mediates malate's protection from cycloheximide-induced apoptosis. However, it seems that an alternative explanation could be that Dronc S130A is a better killer that overwhelms malate's downstream protection, and it would be helpful if the authors could address this possibility.

New Fig. 2E and 5E As shown, malate addition significantly delays the processing of Dronc and its substrate drICE, indicating a metabolic checkpoint occurs at or upstream of the caspase cascade (**New Fig. 2E**).
We also examine how metabolically-mediated Dronc phosphorylation at S130 affects its proteolytic activity. Since unphosphorylated Dronc (mimic to Dronc S130A) cleaves similar amounts of drICE as phosphorylated Dronc does, Dronc S130A *per se* is unlikely to be a better killer (**New Fig. 5E**).

Also, Fig. 3 could be improved; the constant switching of stimuli and time points does not allow a reader to easily follow the authors' arguments - why not focus on one stimulus and one set of time points for these experiments

New Figure 1G and 2E We apologize for the confusion. We have repeated these experiments with CHX and DHEA. All data using DHEA as an apoptotic stimulus are now moved to Fig. 1, and CHX is utilized throughout the paper afterwards. As the kinetics of cell death induced by these two reagents varies, caspase processing is monitored for 6 h in CHX-treated cells and for 12 h in DHEA-treated cells.

Minor points:
The authors do not show what proportion of Dronc is phosphorylated at Ser130 (IP followed by a 2-D gel?). If only a small portion of Dronc is phosphorylated, this would not be easily reconciled with the proposed mechanism of protection. The authors should clarify this at least in the discussion.

Information regarding this concern has been added to the Discussion section of the manuscript. To address, we have performed a 2D-immunoblot with our new phospho-specific antibody against Dronc S130 (Dronc pS130 antibody). The new antibody is validated and characterized in **New Fig. 4B and C**. Please see our replies to reviewer #1 point #7 for experimental details. Dronc antibody detected at least major 6 foci, suggesting that endogenous Dronc is subject to extensive post-translational modifications in S2 cells (data not published). Dronc pS130 antibody indicated that 3 out of 6 spots on the 2D gel contain phosphorylated Dronc at S130, although the signal is weak possibly due to the low antibody titer. With this limitation in mind, we acknowledge that the data might not be definitive. We are still trying to improve the quality of our reagents and data. However, the fact that Dronc pS130 antibody detected 3 major foci indicates that several species of endogenous Dronc is phosphorylated at S130.

The legend of Figure 3A figure does not describe the experiment shown.

We apologize for the error. The figure legend is now corrected to fit the corresponding experiments and all data using DHEA as death trigger are now moved to Fig. 1 to avoid the confusion.

2nd Editorial Decision 14 July 2010

Thank you for sending us your revised manuscript. Our original referees 1 and 2 have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, I was wondering whether you would like to consider addressing the minor issues suggested by the referees (see below). Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

With kind regards,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

There are two minor issues that must be addressed prior to publication:

1) It appears that Dronc is reguated in a phosphorylation-dependent manner only in certain tissues. For example, this phosphorylation event does not seem to play a role in Dronc mediated cell death in the eye. The authors have indicated this tissue specificity. However, their description is misleading and must be corrected: 'Blockade of NADPH production aggravated the death-inducing activity of Dronc in neuronal but not in the eye tissues of transgenic flies; similarly, non-phosphorylatable Dronc was more potent than wild-type Dronc in triggering neuronal, but not eye apoptosis'. The authors should be aware that the cell death in the eye is due to neuronal cell death. Rpr eyes, for example, remain red because the neuronal photoreceptors die but not the pigment cells. Therefore it is not correct to make the distinction between neuronal cells and eye cells! This has to be corrected as it is inaccurate as it stands.

2) molecular weight markers are missing throughout and must be included in all immunoblot analysis. Molecular weight markers provide vital additional information.

Referee #2 (Remarks to the Author):

The authors have thoroughly revised the paper and have adequately responded to all of my previous concerns.

There are a number of typos throughout the paper (this is not a comprehensive list) that should be attended to.

For example:

p.6, paragraph 3, line 3, 'singaling' pathway.
p.7, last line, 'immunoprcipitated'
p. 8., line 2, 'phospatase'
p.9, line 2, 'phoshorylation'

2nd Revision 14 July 2010

Thanks for the good news! We have corrected the text to address concern #1 of the first reviewer and have also corrected the typos cited by reviewer #2 (as well as other remaining typos we could find). We'd like to respectfully decline to go back and put MW markers on all of the figures. Of course, we have this information, but these are by and large well-characterized proteins and it would require us remaking all of the (many!) figures. I hope that's ok. We are submitting the revised version online today. Thanks so much for your help.

