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A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death

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(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 03 May 2011

Thank you very much for submitting your paper on the role of calreticulin exposure and ATP secretion in cancer cell death for consideration to The EMBO Journal editorial office.
I do enclose the scientific assessments from three scientists for your information. From the remarks it becomes obvious that refs#1 and #2 express interested in the study, though pending on substantial further experimentation to substantiate and functionally validate the claims. However, ref#3 is less enthusiastic based on limited exploration of underlying molecular-mechanistic insight. Together with similar demands from ref#1 that further requests the validation of your findings by knockdown studies in alternative cell lines as well as crucial experiments in fact in tumor models, it would need significant time and efforts to win the referee's essential support.

Given their constructive criticisms and seeing the potential merits of the paper, we still decided to offer you the chance to extend the current dataset during one round of major amendments. We do realize that this entails significant and challenging work and would thus be prepared to extend the three-month deadline upon your request. I do urge you to carefully consider your option and take the demands from our referees serious to avoid disappointments much later in the review process.

Please do not hesitate to contact me in case of further questions (preferably via E-mail).

Finally, I do have to formerly remind you that it is EMBO_J policy to allow one round of revisions only and that the final decision on acceptance or rejection depends on the content and strength of the revised version of your study.

Yours truly,
Editor

Referee reports

Referee #1:

The authors of the manuscript ÑA novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death" report that photodynamic therapy (PDT) leads to immunogenic apoptosis, is able to induce DC maturation in vitro and can serve as an anti tumor vaccine in vivo. They further show that PTD leads to the exposure of calreticulin (CRT) and the release of ATP in an ROS- and PERK-dependent fashion. CRT exposure following PDT (in contrast to anthracycline induced CRT exposure) would be independent of eIF2 phosphorylation, caspase activation and cytosolic calcium but rely (similar as anthracycline induced exposure) on PERK, Bax/Bak and actin activity. Furthermore, the authors propose that both ATP and CRT translocate via a secretory pathway and PI3-Kinase dependent exocytosis and that CRT would bind to CD91 surface receptors.
The manuscript is well written and the experiments conducted are well described. Nevertheless the structure of the data presentation is somehow confusing. There is no continuous flow in the text with regard to the description of the conducted experiments, a circumstance that forces the reader to jump between the figures to follow the text. Some effort might be undertaken to ameliorate the manuscript with regard to this.

Major points:
The authors describe a new pathway for PDT-induced CRT exposure and ATP release that depends on PERK and PI3 kinase. Nevertheless the mechanism that is linking these events is missing. As the authors excluded the involvement of eIF2a it would be interesting to look for the alternative substrate of PERK.
The major findings are exclusively based on experiments with mouse embryonic fibroblasts that are generally clones, meaning that cloning artifacts constitute a significant risk. It would therefore be necessary to conduct additional experiments re-complementing the cells with an expression of the respective protein. In addition RNAi might be used to validate these findings in other cell lines employed in this paper (T24, CT26 and HeLa).
The involvement of PI3 kinase in the exposure of CRT and the release of ATP is exclusively based on experiments employing rather unspecific chemical inhibitors. These experiments have to be validated by RNAi-mediated knockdown or overexpression of dominant negative contructs targeting PI3 kinase.
The paper would gain significant impact if the major findings were consolidated by in vivo experiments that remain the gold standard for anti tumor immunity studies. Therefore RNAi-mediated depletion of PERK, PI3 kinase and CD91 (to validate their importance for PTD induced immunogenicity) as well as of caspase 8 (to exclude its importance for PTD induced immunogenicity) should be performed on murine tumor cells, followed by vaccination studies to assess the immunogenicity of cell death induced in such cells by PTD.

Minor points:
The presentation of the data should be consistent. Therefore figures containing WB might include a densitometric analysis for the analyzed proteins. Statistical evaluations of densitometric data should be accompanied by representative immunoblot results (Fig 2c; 7a,b; Fig8 d,e,f). Most of the immunoblots are missing molecular weight markers.
Some of the immunoblots are of questionable quality. In general, the same blot/exposure should provide information on controls and experimental samples, which apparently is not always the case.
Concerning the ATP release it would be necessary to show the actual concentration of released ATP. One would expect a decreased intracellular ATP level when ATP is released (Fig3 a,b; Fig4c,d; Fig7c, d).
The labels of the figures should indicate the parameter measured (e.g.: Fig.2 E: PS exposure; 3 c: IL-1b...)
The immunostaining in Figure 2a (misslableld as 2b in the legend and in the text) is not convincing as neither the membrane is stained separately nor a vital dye has been employed to exclude cells with corrupted membrane integrity. In my opinion, the data does not allow for any interpretation concerning surface exposure of CRT. Therefore the experiment has to be redone with either or both of the before mentioned co-stainings.
An effect of non-activated hypericin has to be excluded rigorously. In the present form of the manuscript, it is not clear if controls have been treated with this drug or if they have been left

untreated.

Referee #2:

The authors describe for the first time the immunogenicity of cell death following ER-associated photodynamic therapy (hypericin and light-induced ROS-mediated loss of function of SERCA2) in vitro and in vivo and detail the molecular pathway of the ER stress response. The demonstrated concept is of general interest and quite novel.

The manuscript is very clearly and well written, even though it could be shortened since many aspects are redundantly expressed throughout the article.

Major comments:

1. Fig. 8: the fact that CD91 is involved for ecto-CRT exposure following MTX or PHOX-PDT treatment is an important finding. The authors should attempt to restore the decreased ecto-CRT obtained in MEF-CD91-/- by transfecting those cell lines with the cDNA encoding CD91. Moreover, these findings should be substantiated in tumor cell lines knock in with shRNA CD91.

2. Fig. 1: Raw data showing dot plots and gates for human DC expressing CD86 and CD83 (a representative FACS staining) should be shown as well as raw data showing phagocytosis. If no IL-10 is produced when DC are loaded with PHOX PDT dying cells (in contarst to LPS for instance), please show this crucial piece of information.

3. Fig. 3A. Show raw data without normalization for ATP release since the precise amounts that can be measured are important overtime.

Minor comments:

Fig. 8AB panels are dispensable.
I. Martins and Kroemer G and coll. have first shown that ATP release precedes PS exposure at preapoptptic stages (Cell Cycle, 2009). Please correct the discussion part.
Page 6: "anthracyclines induce immunogenic apoptosis at concentrations greatly exceeding those used in humans": not exactly. They also induce an immunogenic cell death at therapeutic dosages.

Referee #3:

The authors report on secretion of CRT and ATP from cancer cells under photodynamic therapy which promotes ROS-mediated ER stress. CRT and ATP secretion occurs before apoptotic events and it might be PERK-dependent (calreticulin). Secretion of CRT and ATP may be crucial to modulate immunogenicity of cancer cell death.

This is potentially an interesting study. The paper is long, difficult to read mostly because of incredible number of abbreviations used, not reader friendly. The paper is very long, intro and discussion should be reduced to avoid repetitions. The title is misleading - what is "novel pathway" reported? The authors only show the exposure of CRT and ATP in cancer cells. Most importantly the study lacks mechanists novelty. Cell surface exposure of CRT has been reported by many, the role of ROS, calcium and ER-to-Golgi pathway has been shown before. Immunogenic effects of CRT are also known. The role of CD91 in binding to CRT has also been reported previously. As authors state themselves under Discussion "...our observations strengthen the value of combining robust ER stress with ROS production to induce immunogenic cell death" but they, unfortunately, failed to provide new mechanists information. The n number for many experiments is too low to be acceptable. A potential role for PERK/BAX/BAK in CRT secretion is intriguing, new and of potential interest but unfortunately has not been explored. Does PERK and CRT form complexes? Do they go to cell surface? How? Why PERKS why not ATF6 which interacts with CRT?

Additional points:
1.Ecto-CRT, exo-CRT, end-CRT nomenclature should not be used as this is confusing to others in the field. The authors should refer to ecto-CRT as cell surface or extracellular calreticulin.

2.Why is ERp57 not involved?
3.Why binding of CRT to CD91 is intriguing to the authors?
4.What is the rationale behind using human bladder carcinoma T24 cells? How universal are reported findings to other cancer cells?
5.The n value for many experiments is very low (n=2), figures 2, 7, 8.
6.Figures 1A and B are likely mixed up.
7.Figure 2F is confusing - have the authors blotted with anti-KDEL antibodies? If so how were specific proteins identified and selected from the blots for presentation? MW only?
8.The authors claim no increase in CRT expression under ER stress conditions; this contradicts many other studies indicating that ER stress (and UPR) induces CRT expression. This needs to be reconciled.
9.Have the authors tested for a potential role of other forms of ROS-induced ER stress?
10.Figures need to be labeled with appropriate figure number.
11.Figure 3D - BSA sample is impossible to evaluate- better quality gel is needed.
12.How does PERK promotes CRT induction (what does induction mean here?) is intriguing but unanswered question.
13.The authors should directly measure ER luminal calcium before concluding that ER calcium does not play a role.
14.With respect to ATP secretion from PERK and BAX/BAK deficient cells the authors state that it "...seemed normal". This needs to be clarified or data need to be included.

1st Revision - authors' response 20 December 2011

Point-by-point response to the referees' comments

Referee #1:

The authors of the manuscript "A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death" report that photodynamic therapy (PDT) leads to immunogenic apoptosis, is able to induce DC maturation in vitro and can serve as an anti tumor vaccine in vivo. They further show that PTD leads to the exposure of calreticulin (CRT) and the release of ATP in an ROS- and PERK-dependent fashion. CRT exposure following PDT (in contrast to anthracycline induced CRT exposure) would be independent of eIF2α phosphorylation, caspase activation and cytosolic calcium but rely (similar as anthracycline induced exposure) on PERK, Bax/Bak and actin activity. Furthermore, the authors propose that both ATP and CRT translocate via a secretory pathway and PI3-Kinase dependent exocytosis and that CRT would bind to CD91 surface receptors.

The manuscript is well written and the experiments conducted are well described. Nevertheless the structure of the data presentation is somehow confusing. There is no continuous flow in the text with regard to the description of the conducted experiments, a circumstance that forces the reader to jump between the figures to follow the text. Some effort might be undertaken to ameliorate the manuscript with regard to this.

-We thank the reviewer for highlighting this point. In this revised version of the manuscript we have made an effort to ameliorate the structure of the work, by clustering together experiments related to a particular approach and explained under the same sub-division in the result section. By doing so the flow in the text and the interpretations/conclusions of the relevant experiments are easier to grasp. Moreover, we shortened the manuscript as much as we could and reduced possible redundant parts.

Major points:
-The authors describe a new pathway for PDT-induced CRT exposure and ATP release that depends on PERK and PI3 kinase. Nevertheless the mechanism that is linking these events is missing. As the authors excluded the involvement of eIF2a it would be interesting to look for the alternative substrate of PERK.

- We are indeed very interested in finding the eIF2alfa independent pathways that PERK regulates in our immunogenic apoptosis paradigm. We have performed some experiments (Fig. S10 B,C) describing an ER budding assay (Kim et al. J Cell Biol, 179:951, 2007), pointing to a potential role of PERK in regulating secretory pathway mechanims after Hyp-PDT. While the data in MEFs and cancer cells are consistently showing a reduced extracellular secretory protein content in the absence of PERK (Fig. S10 B,C), we feel that the role of PERK in the secretory pathway needs to be further and more thoroughly validated by performing additional experiments and by using different methodological approaches. This is evidently beyond the scope of the present study.

-*The major findings are exclusively based on experiments with mouse embryonic fibroblasts that are generally clones, meaning that cloning artifacts constitute a significant risk. It would therefore be necessary to conduct additional experiments re-complementing the cells with an expression of the respective protein. In addition RNAi might be used to validate these findings in other cell lines employed in this paper (T24, CT26 and HeLa).*
The involvement of PI3 kinase in the exposure of CRT and the release of ATP is exclusively based on experiments employing rather unspecific chemical inhibitors. These experiments have to be validated by RNAi-mediated knockdown or overexpression of dominant negative constructs targeting PI3 kinase.

-Although in our previous manuscript several pathways were already validated in human cancer cells, like T24 and HeLa cells, we agree that expanding the molecular mechanisms defined in MEFs to other cancer cell lines is important. Hence we reinforced the study by adding substantial amount of experiments in CT26 cancer cells, as they provide the parallel approach for the in vivo immunization experiments and which we have expanded as well. Thus, in this revised version of our manuscript, as suggested by the reviewer, shRNA-mediated knockdown of caspase-8, PERK, PI3K p110 alfa and LRP1 was performed to validate the novelty of the pathway for ectoCRT induction and ATP secretion by phox-ER stress. Additionally, we chose a CHO LRP1+/+ a LRP1-/- and the LRP1-/- cell line reconstituted with the LRP1 cDNA, since the reconstitution of LRP1 in MEFs was technically difficult to obtain due to the large size of the cDNA.
Here we show that also in CT26 cells the knockdown of PERK reduces significantly both ectoCRT and ATP secretion after Hyp-PDT (Fig. 5B, E), thus confirming the results obtained in the MEFs. Moreover, the caspase-8 shRNA did not affect ectoCRT induction after Hyp-PDT, while blocking as previously reported MTX-induced effects (Fig. 6C). These results, along with the results in HeLa cells using the overexpression of CrmA and in T24 using caspase inhibitor zVAD-fmk, strongly supports the concept that caspase signaling is dispensable for immunogenic apoptosis induced by phox-ER stress.
As suggested by the reviewer we also validated the results for PI3K inhibition. We used a shRNA approach against the PI3K p110 alfa subunit, since this is the predominantly wortmannin-inhibitable subunit, and found that depletion of PI3K p110 alpha reduces the ability of Hyp-PDT to mobilize both ectoCRT and to secrete ATP (Fig. 4G,H).
Additionally, we explored the role of LRP1 in surface tethering of CRT by different approaches; i) we silenced LRP1 by shRNA in CT26 cells and confirmed the decreased surface exposure of CRT (Fig. 7G), ii) we used CHO LRP1-/- cells and saw similar results as in MEFs and CT26 cell lines, and moreover reconstitution of LRP1 in the CHO cells restored ectoCRT (Fig. S13C).
In conclusion, by using the reviewer's suggested approach we answered the constructive criticisms and could rule out clonal artifacts or unspecific effect of wortmannin in our assays and further substantiated/reinforced the previous data. These data moreover (see point below) were confirmed *in vivo* by the additional immunization studies performed. The dispensability of caspase signaling in our paradigm of immunogenic apoptosis is a new finding/concept emerged by our study since the few known immunogenic apoptosis inducers have been shown to rely on caspase activity to mobilize critical DAMPs, like CRT, to the surface. This point has been evidenced in the discussion of the revised manuscript.

-*The paper would gain significant impact if the major findings were consolidated by in vivo experiments that remain the gold standard for anti tumor immunity studies. Therefore RNAi-mediated depletion of PERK, PI3 kinase and CD91 (to validate their importance for PTD induced immunogenicity) as well as of caspase 8 (to exclude its importance for PTD induced immunogenicity) should be performed on murine tumor cells, followed by vaccination studies to assess the immunogenicity of cell death induced in such cells by PDT.*

We took this very important suggestion under serious consideration and we agree with the reviewer that testing the novelty of the DAMP pathway in *in vivo* settings is a golden standard of validating anti-tumor immunity related conclusions. Hence, we immunized the mice with PBS or Hyp-PDT treated control shRNA CT26 and CT26 cells stably expressing shRNA against caspase 8, PERK, PI3K p110 alpha and LRP1. As far as the control shRNA CT26 cells are concerned, we reproduced our initial observations detailed in Fig. 1E, for Hyp-PDT showing that these dying cells are capable of priming the immune system for CT26-associated antigens. Next, we also confirmed *in vivo*, the dispensability of caspase-8 for Hyp-PDT induced immunogenic apoptosis and anti-tumor immunity (Fig. 8B). Importantly, we also confirmed that PERK and PI3K are vital for immunogenicity of the Hyp-PDT treated cancer cells (Fig. 8B). LRP1 knockdown produced a milder reduction in the immunogenicity, likely because secreted CRT is also functioning in anti-tumor immunity as recently reported (Bajor et al., 2011 Clin Exp Immunol; Wang et al., 2011 Intern J Cancer). In conclusion, as requested by the reviewer we added the *in vivo* data that reinforce the molecular pathways defined in our *in vitro* settings.

Minor points:

-*The presentation of the data should be consistent. Therefore figures containing WB might include a densitometric analysis for the analyzed proteins. Statistical evaluations of densitometric data should be accompanied by representative immunoblot results (Fig 2c; 7a,b; Fig8 d,e,f). Most of the immunoblots are missing molecular weight markers.*

- The representative immunoblot results (Fig 2, D; Fig 4, A & D; Fig 7, B-C) have now been shown for the respective densitometric analyses. Also, all of the immunblots now carry the necessary molecular weight markers.

-*Some of the immunoblots are of questionable quality. In general, the same blot/exposure should provide information on controls and experimental samples, which apparently is not always the case.*

- We apologize for this flaw. In this revised version of the manuscript, it has been ensured that the same exposures of the blot account for the representation of both controls and experimental samples. Effort has been made, wherever possible to provide the full rows/lanes of a protein across the gel with minimal or no cropping of the lanes to further emphasize on same exposure for a blot serving as a representative.

-*Concerning the ATP release it would be necessary to show the actual concentration of released ATP. One would expect a decreased intracellular ATP level when ATP is released (Fig3 a,b; Fig4c,d; Fig7c, d).*

- The actual concentrations of the secreted ATP have now been shown. Indeed, in early and mid apoptotic stages it has been reported that the ATP release is accompanied by decrease in intracellular ATP levels however in the pre-apoptotic stage after Hyp-PDT we interestingly do not observe this phenomenon. We suspect that this increased intracellular ATP content might be due to the increased mitochondria-ER coupling, a link that has been reported previously (Bravo et al. 2011) and also something that we have observed in some of our ongoing studies (Verfaillie et al. unpublished data). This has been added/discussed in the main text.

-*The labels of the figures should indicate the parameter measured (e.g.: Fig.2 E: PS exposure; 3 c: IL-1b...)*

- The necessary labeling has now been done in the revised version.

-*The immunostaining in Figure 2a (misslableld as 2b in the legend and in the text) is not convincing as neither the membrane is stained separately nor a vital dye has been employed to exclude cells*

with corrupted membrane integrity. In my opinion, the data does not allow for any interpretation concerning surface exposure of CRT. Therefore the experiment has to be redone with either or both of the before mentioned co-stainings.

- Firstly we apologize for the mislabeling of the figure; this has now been corrected. Secondly, as per the suggestion of the reviewer we performed the experiment again and employed Sytox Green as en exclusion dye to account for cells with corrupted membrane integrity. The idea was that a cell with a corrupted membrane would have a cyan nucleus due to the overlap between DAPI and Sytox Green nuclear staining. Following this, for surface exposure of CRT, we selected cells that had blue rather than cyan nucleus and such cells have now been presented in Fig 2, A.

-An effect of non-activated hypericin has to be excluded rigorously. In the present form of the manuscript, it is not clear if controls have been treated with this drug or if they have been left untreated.

- We apologize for this misunderstanding. All the 'untreated controls' or CNTRs, in fact underwent hypericin incubation without light based activation (hence untreated). Thus the presence of non-activated hypericin has been accounted for. We have now mentioned this important information in the relevant materials and methods section.

Referee #2:

The authors describe for the first time the immunogenicity of cell death following ER-associated photodynamic therapy (hypericin and light-induced ROS-mediated loss of function of SERCA2) in vitro and in vivo and detail the molecular pathway of the ER stress response. The demonstrated concept is of general interest and quite novel. The manuscript is very clearly and well written, even though it could be shortened since many aspects are redundantly expressed throughout the article.

- We thank the reviewer for this positive assessment of our work. We have now substantially shortened the manuscript as much as we can and have made an effort to ameliorate all the redundancies in the manuscript.

Major comments:

1. Fig. 8: the fact that CD91 is involved for ecto-CRT exposure following MTX or PHOX-PDT treatment is an important finding. The authors should attempt to restore the decreased ecto-CRT obtained in MEF-CD91-/- by transfecting those cell lines with the cDNA encoding CD91. Moreover, these findings should be substantiated in tumor cell lines knock in with shRNA CD91.

-As suggested by the reviewer we have further explored and validated the role of LRP1 in surface tethering of CRT by additional approaches;
i) we silenced LRP1 by shRNA in the CT26 cancer cell line and confirmed the decreased surface exposure of CRT (Fig. 7G),
ii) since the reconstitution of LRP1 in MEFs was technically difficult to obtain due to the large size of the cDNA, we chose a CHO LRP1+/+ a LRP1-/- and the LRP1-/- cell line reconstituted with the LRP1 cDNA. By this approach we show that as found in LRP1-/- MEFs and shRNA LRP1 transduced CT26 cells, CHO LRP1-/- cells display increased levels of secreted CRT (exo-CRT). Moreover reconstitution of LRP1 in the CHO cells restored surface tethering of CRT (ectoCRT) (Fig. S13C).
All together these data validate the new finding that LRP1 serves as a privileged CRT surface docking site in cancer cells.

2. Fig. 1: Raw data showing dot plots and gates for human DC expressing CD86 and CD83 (a representative FACS staining) should be shown as well as raw data showing phagocytosis. If no IL-10 is produced when DC are loaded with PHOX PDT dying cells (in contarst to LPS for instance), please show this crucial piece of information.

- The raw data showing the dot plots and gates for human DC phenotypic maturation (Fig S4) and phagocytosis (Fig S3 and S6) has now been shown. We have included the IL-10 data within the results (Fig 1, F).

3. Fig. 3A. Show raw data without normalization for ATP release since the precise amounts that can be measured are important overtime.

- The actual concentrations of the secreted ATP have now been shown.

Minor comments:

-Fig. 8AB panels are dispensable.

- As suggested, we have now removed these sub-panels.

-I. Martins and Kroemer G and coll. have first shown that ATP release precedes PS exposure at preapoptptic stages (Cell Cycle, 2009). Please correct the discussion part.

- The data presented in Martins et al. Cell Cycle (2009; Figure 1) shows that increased secretion of ATP was always accompanied by an increase in the $AnnV^+/PI^-$ cell population for all agents including immunogenic cell death inducers like mitoxantrone and oxaliplatin. In fact the authors conclude in that paper that '*extracellular ATP accumulates in the supernatant of dying tumor cells'*.

Moreover, based on their results the authors also conclude that '*These results do not solve the question whether ATP is passively released or actively extruded from cancer cells as they respond to chemotherapeutic stress'*. Therefore, to the best of our belief and derived from the data presented in the manuscript of Martins and co-workers, we cannot conclude that those observations are described in pre-apoptotic stages.
Instead, here we clearly show that Hyp-PDT induced secretion of ATP that is clearly an active process occurring during the pre-apoptotic phase of the cell death process and requiring a functional secretory pathway.

-Page 6: "anthracyclines induce immunogenic apoptosis at concentrations greatly exceeding those used in humans": not exactly. They also induce an immunogenic cell death at therapeutic dosages.

- We apologize for this fault. This has been corrected in the revised version.

Referee #3:

The authors report on secretion of CRT and ATP from cancer cells under photodynamic therapy which promotes ROS-mediated ER stress. CRT and ATP secretion occurs before apoptotic events and it might be PERK-dependent (calreticulin). Secretion of CRT and ATP may be crucial to modulate immunogenicity of cancer cell death.

This is potentially an interesting study. The paper is long, difficult to read mostly because of incredible number of abbreviations used, not reader friendly. The paper is very long, intro and discussion should be reduced to avoid repetitions.

-We agree that the list of abbreviations is extensive, on the other hand the study is centered around the differential location of immunomodulatory molecules which are emitted and therefore a difference should be made between their intracellular and surface exposed or secreted forms. Therefore, the use of prefixes like 'ecto' or 'endo' or 'exo' (see also below). To simplify this nomenclature we now use 'secreted ATP' instead of exo-ATP. We have also invested major effort in reducing the overall length of the manuscript and reducing redundancies.

-The title is misleading - what is "novel pathway" reported? The authors only show the exposure of CRT and ATP in cancer cells. Most importantly the study lacks mechanists novelty. Cell surface exposure of CRT has been reported by many, the role of ROS, calcium and ER-to-Golgi pathway has been shown before. Immunogenic effects of CRT are also known.

-We respectfully disagree with the reviewer comment. Novel here indicates the pre-apoptotic and caspase-independent, overlapping emission of two crucial DAMPs, in a dose associated manner after phox-ER stress. To the best of our knowledge no pathway for ATP was shown in pre-apoptotic stage and no combined mechanisms for DAMPs emission have been described so far. Moreover, although we did not establish the complete molecular connections between ER stress-associated BAX/BAK and DAMPs, we do provide strong mechanistic evidence implicating PERK and PI3K in governing the emission of these two DAMPs. Moreover, it is the first instance of caspase-independent CRT surface exposure, making this pathway applicable to tumor cells with defect in caspase signaling.
The dispensability of caspase signaling in our paradigm of immunogenic apoptosis is a new finding/concept emerged by our study since the few known immunogenic apoptosis inducers have been shown to rely on caspase activity to mobilize critical DAMPs, like CRT, to the surface. This point has been evidenced in the discussion of the revised manuscript.

-The role of CD91 in binding to CRT has also been reported previously. As authors state themselves under Discussion "...our observations strengthen the value of combining robust ER stress with ROS production to induce immunogenic cell death" but they, unfortunately, failed to provide new mechanists information.

- The role of CD91 binding to CRT has been shown after thrombospondin treatment (see Pallero et al, Faseb J 2008) as a co-receptor but not in pre-apoptotic stage preceding immunogenic apoptosis. Moreover, the possible effects of depletion of LRP1/CD91 on immunogenicity had not been demonstrated before, which we have now shed further light upon in this revised version of the manuscript.

-The n number for many experiments is too low to be acceptable.

- The n-number of various experiments has been raised to three independent experiments or triplicates, wherever applicable.

-A potential role for PERK/BAX/BAK in CRT secretion is intriguing, new and of potential interest but unfortunately has not been explored. Does PERK and CRT form complexes? Do they go to cell surface? How? Why PERKS why not ATF6 which interacts with CRT?

-As shown in Fig. S8B (and previously in Fig. S4C) there is no PERK or BiP, CNX present at the cell surface after Hyp-PDT treatment resulting into the surface exposure of CRT. This result rules out the possibility that PERK and CRT form complexes at the surface or that they are co-translocated. Besides, as shown here, we believe that PERK is endowed with a regulatory function in the secretory pathway, making this ER sensor unique for DAMPs exposure following ER stress. ATF6 in fact seems to undergo a PERK-dependent translocation to the Golgi as reported recently (Teske et al., 2011 Mol Biol Cell).

Additional points:
1.Ecto-CRT, exo-CRT, end-CRT nomenclature should not be used as this is confusing to others in the field. The authors should refer to ecto-CRT as cell surface or extracellular calreticulin.

- Ecto-CRT, exo-CRT and endo-CRT nomenclatures are being used to differentiate between the surface exposed, secreted/released and intracellular forms of CRT without increasing the manuscript length and redundancies. Since some researchers have been debating on whether ecto-CRT and exo-CRT represent established differentially localized forms of CRT, we thought it would be best to differentiate them to clearly indicate three different localizations to the readers at the outset.

2.Why is ERp57 not involved?

-This study shows that various steps involved in the 'canonical' pathway of CRT surface exposure do not apply for Hyp-PDT mechanisms, including Erp57 co-translocation. The reason why is not fully understood but it may underlie the type of ER stress behind CRT surface translocation.

3. Why binding of CRT to CD91 is intriguing to the authors?

- This is the first instance where depletion of a surface receptor (CD91) has been shown to reduce surface exposure of CRT in the pre-apoptotic stage of cancer cells undergoing immunogenic apoptosis. Moreover, we have shown that to a certain extent, LRP1 also affects the immunogenicity of Hyp-PDT treated cells *in vivo* thereby making the role of CD91/LRP1 intriguing.

4.What is the rationale behind using human bladder carcinoma T24 cells? How universal are reported findings to other cancer cells?

- T24 cells were used in the current study since these cells have been consistently used for the previous apoptosis pathway and micro-array studies associated with Hyp-PDT (see Buytaert et al., Oncogene 2008). To create a link between our earlier findings and this one, we needed a common cancer cell line, a purpose served by T24 cells. However, we had also included results for HeLa cells in this manuscript. In the revised version of this manuscript, effort has been made to extent the major findings of the manuscript to an additional cancer cell line i.e. the murine colon carcinoma CT26 cells. We consider that the work done in three cancer cell line should account for a broad applicability of our findings to various other cancer models.

5.The n value for many experiments is very low (n=2), figures 2, 7, 8.

- The n-number of various experiments mentioned has been raised to three independent experiments.

6. Figures 1A and B are likely mixed up.

- This flaw has been ameliorated.

7. Figure 2F is confusing - have the authors blotted with anti-KDEL antibodies? If so how were specific proteins identified and selected from the blots for presentation? MW only?

- In this figure, the immunoblotting was carried out with an antibody directed against the KDEL sequence. The proteins are labeled as per the molecular weight at which the corresponding KDEL sequences were detected according to the manufacturer's instructions (Abcam, UK). For CRT, as it can be seen, a band was detected at 63kDa in CRT WT MEFs which disappeared in CRT KO MEFs thereby indicating that this KDEL band was derived from the CRT protein. All 5 KDEL bands that according to the manufacturer the antibody can detect, have been shown.

8. The authors claim no increase in CRT expression under ER stress conditions; this contradicts many other studies indicating that ER stress (and UPR) induces CRT expression. This needs to be reconciled.

- While we did not always see consistent increase in endo-CRT amounts after Hyp-PDT in all the experiments performed we do agree with the reviewer that this might be a very strong statement to make. Hence, this statement has been removed from the current revised version of the manuscript. On the other hand, we are analyzing short kinetics during which UPR-mediated transcriptional expression of CRT may not be detectable. Of note in Fig. 3D, endoCRT levels appear to be increased after 24h, but not after 1 h.

9.Have the authors tested for a potential role of other forms of ROS-induced ER stress?

- We tested PDT *via* Photofrin as a modality which is also known to induce ROS-based ER stress and we did not find it to be superior to Hyp-PDT (Figure S8, C).

10.Figures need to be labeled with appropriate figure number.

- We have done this in revised version.

11.Figure 3D - BSA sample is impossible to evaluate- better quality gel is needed.

- A more high contrast scan of the BSA sample in the gels has now been added.

12.How does PERK promotes CRT induction (what does induction mean here?) is intriguing but unanswered question.

-Induction always referred to ectoCRT not to CRT. As explained above and as our preliminary data point to, we believe that PERK is important for maintaining proper secretory pathway functionality after phox-ER stress (see also Fig. S10B,C). Clearly, more studies are required to substantiate this assumption. This is of course beyond the scope of this manuscript.

13. The authors should directly measure ER luminal calcium before concluding that ER calcium does not play a role.

-We agree with the reviewer that ER luminal Ca2+ measurements would be important to perform. On the other hand, since SERCA-overexpressing DKO cells, with demonstrated rescued levels of ER-Ca2+ (Scorrano et al., Science 2003) did not restored ectoCRT after phox-ER stress, we indirectly (along with BAPTA-AM experiments) conclude that cytosolic Ca2+ release is not a major determinant of this process. To make this point less speculative, we removed any conclusions related to ER-Ca2+.

14. With respect to ATP secretion from PERK and BAX/BAK deficient cells the authors state that it "...seemed normal". This needs to be clarified or data need to be included.

- We apologize for this misunderstanding. The intracellular ATP levels of the treated MEF cell lines were found not significantly changed irrespective of presence/absence of BAX/BAK or PERK. This has been modified in the main text. The actual data could be included in a supplemental figure, in case it is found required.

Acceptance letter 21 December 2011

I have heard already back from one of the original referees (see comments below) and I am happy to convey that the editorial office will be in touch with you shortly regarding formal acceptance of your study.

Sincerely,
Editor
The EMBO Journal

Referee comments:

The present paper unravels a novel molecular pathway underlying immunogenic apoptosis induced by photodynamic therapy. The authors nicely addressed most if not all the concerns raised by myself and the other reviewers

