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Critical Role for Hyperpolarization-Activated Cyclic Nucleotide-Gated Channel 2 in the AIF-mediated Apoptosis

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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 28 July 2010

Thank you very much for submitting your research paper for consideration to The EMBO Journal editorial office. Three expert referee's initially agreed to assess merits and suitability of your paper for our journal. As I did receive two rather consistent reports that reveal both strength as well as important current weaknesses of the study, I am in the position to make a decision at this point to prevent unnecessary further delays. As you will see, both experts emphasize the conceptual advance provided by your study that links HCN2 channels to apoptotic responses, importantly with confirmations in primary neuron cultures emphasizing general significance. Both refs still ask for significant technical improvements that will have to be thoroughly addressed before a final decision can be reached. Conditioned on such experimentation we would be happy to re-assess a modified version of your work in the near future.

Further, I do have to formerly remind you that it is EMBO_J policy to allow a single round of major revisions only and that the ultimate decision on acceptance or rejection entirely depends on the content and strength of the final version that might be indeed assessed by the original referees.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors identified HCN2 as an important Ca channel regulated by PKC and PKC inhibitors, respectively. This is an interesting observation with possible clinical relevance (anticancer treatments). The papers is nicely written. I have only little criticism.

1. Figure 1A: Authors may consider a negative control (HEK293 cells?). This information could also be included later in the manuscript (Fig. 4F). Also, it would be nice to see how depolarization would look like in this assay.
2. Figure 1B: It is not clear whether cells were treated with STS or PKC412 in this experiment.
3. Figure 1C-E: It should be indicated when STS/PKC412 was added. Ideally, recordings before adding the drug should be shown.
4. Figure 2B: It would be nice to see a HCN4 immunoblot.
5. Figure 2D: Compared to Fig. 2C, this panel is not really convincing.
6. Figure 3: Do HEK293 undergo death upon STS/PKC412? A death assay should be included here as panel D.
7. Figure 4A, 4B, 4G: Why is the calcium influx much more faster under these conditions?
8. Figure 4C: The effect of PMA alone should be shown.
9. Figure 4E: It is not clear whether cells were treated with STS or PKC412 in this experiment.
10. Figure 4F: Genetically unmodified cells should be included.
11. Figure 5C and 5E: Control immunoblots are missing.

Referee #2 (Remarks to the Author):

In this paper, Norberg et al. convincingly show that a specific hyperpolarization-activated calcium channel, HCN2, can be activated by PKC inhibitors, leading to calcium influx, activation of calpain and release of AIF from mitochondria. Thus, the authors delineate a linear pathway that links PKC activation to caspase-independent apoptosis in two different non-small cell lung cancer cell lines, as well as in primary neurons. The author should consider improving their paper in the following points:

Major
- The authors rely on the use of DiBAC, which is a polarization-sensitive due, indeed. Being an anion, DiBAC is usually excluded from the cells. DIBAC enters only enters depolarized cells where it binds to intracellular proteins or membranes. Thus, increased depolarization results in more influx of the anionic dye and thus an increase in fluorescence. In the absence of controls (untreated cells), in Fig. 1A and 1B, it is impossible to understand what the data mean. So this must be corrected. Ideally the authors should also use additional methods (like patch clamping) to measure the plasma membrane potential.
- None of the immunoblots has size markers. None of the PCR reactions has size markers. None of the fluorescence microphotographs has size bars. Altogether, this absence of detail and accuracy generates the impression of sloppiness.
- The authors only use one single siRNA to deplete HCN2. This is not acceptable, because siRNAs can have off-target effects. The authors should use at least two distinct non-overlapping siRNAs specific for HCN2 to reproduce their findings. Moreover, they should re-transfect HCN2-depleted cells with a non-interferable HCN2 cDNA and show that the phenotype can be restored.
- The data in Fig. 2E are no impressive. The upper and the lower panels for "cleaved Atg5" have been derived from two different experimental conditions. Hence, it is incorrect to use one single loading control. Where is the uncleaved Atg5?
- In Fig. 2F, it appears that only a very minor fraction of AIF (likewise less than 10%) is cleaved upon treatment with staurosporine. However, the authors say that in these conditions, most cells release mitochondrial AIF-GFP fusion protein (Fig. 2G). So can calpain-mediated AIF cleavage truly account for the observed AIF release? Or do AIF and AIF-GFP behave differently. This should be clarified.
- A similar discrepancy applies to Fig. 5E and F. While a minor fraction of AIF has undergone calpain cleavage, the majority of the cells condense their nuclei. Does this mean that only a small

fraction of AIF needs to be cleaved and released from mitochondria to induce chromatin condensation. Or is the cleavage dispensable for AIF release in this particular model? Indeed, it might be possible that calcium influx induces mitochondrial permeability transition (with bursting of the outer membrane) and release of AIF without the need of calpain-mediated cleaved. Does calpain inhibition affect the frequency of nuclear apoptosis? And does inhibition of permeability transition (for instance with cyclosporin A) do so?

Minor
- The English is poor and requires extensive editing.
- The last paragraph of the Introduction rehashes the Abstract. This paragraph should be shortened.
- No bands are visible for HCN2 and HCN4 in Fig. 4A. `
- The fluorescence data in Fig. 2A should be quantified by cytofluormetry.
- In Fig. 2G, there is non-transfected, sham-transfected or unrelated siRNA-transfected control.
- No bands are visible in the lower panel of Fig. 4C. Everything is blak.

1st Additional Editorial Correspondence 28 July 2010

Following on my earlier mail, I did receive final comments from the third referee that I do enclose below for your information.
Similar to the more technical criticisms of ref#1 and #2, this referee also demands a much more thorough analysis, particularly on the mechanism of Calcium/HCN2-mediated apoptosis. Again, the comments are really clear and propose essential experiments that should provide such important clarifications.

I thus kindly ask you to consider and address these thoroughly before submitting the revised for final assessment here.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT:

Ref#3 comments:

In this MS Norberg & coll. report data suggesting that Hyperpolarization-activated Cyclic Nucleotide-gated channel 2 (HCN2) plays a role in apoptosis induced by PKC inhibitors and Staurosporine (STS) in lung carcinoma cells and in primary cortical neurons. The model presented here is not novel, as they have already established in a previous report from the same team that an increase in intracellular Ca2+ is required for the release of AIF from mitochondria (Cell Death Differ. 2008 Dec;15(12):1857-64.). The novelty here is the contribution of HCN2. These results are intriguing and potentially interesting. However, they are not always convincing. Indeed, there are still a lot of gaps in the mechanism and it is necessary to show more direct connections between the various steps existing in the proposed apoptotic pathway. As such, the results only suggest the existence of an unclear role of Ca2+ and HCN2 in PKC inhibitors and STS-mediated apoptosis, and do not pinpoint a predominant mechanistic basis for the suggested AIF apoptotic activity.

Comments
1.- Today is not enough to provide data on AIF cleavage and AIF redistribution from mitochondria to cytosol to demonstrate the implication of this protein in apoptosis (Figures 2F, 2G, and 5E). In this sense, the authors do not provide any specific analysis on AIF-mediated apoptosis and, consequently, the apoptotic findings and conclusions included in the manuscript are over-interpreted.
2.- The putative AIF mitochondrial release was only investigated by immunofluorescence in conditions where HCN2-expressing cells are strongly shrinking (Figure 2G), so that it is very

difficult to assess the release of mitochondrial proteins into the cytosol. Moreover, once released, AIF was reported to migrate into the nucleus but I do not see any obvious co-localization of AIF with the nucleus in cells expressing HCN2.
3.- In Figures 2H and 5F, the nuclear condensation associated to STP-mediated death is not related to the AIF apoptotic action. Indeed, the supposed apoptotic activity of AIF provokes a partial chromatin condensation in the nucleus. As a matter of fact, the authors depict in these Figures the typical caspase-dependent nuclear features. This confirms previous reports demonstrating that STS induced a caspase-dependent cell death process. The role of AIF is irrelevant (Proc Natl Acad Sci U S A. 2006 Aug 1;103(31):11573-8; J. Cell Biol. 2002 Dec 23;159(6):923-9; EMBO J. 2003 Sep 1;22(17):4385-99).
4.- The assessment of caspase-3/7 activity through a fluorogenic assay is a pretty rough method (Figure 2I). There are many other methods that are simple and accurate. For example, it is relatively easy to measure caspase-3/7 activation by Western blot. This also applies for other caspases.

1st Revision - Authors' Response 14 September 2010

Reviewer #1:

We appreciate all your comments and are confident that the changes made to our manuscript in response to your critique have significantly strengthened and clarified our experimental work. Below, please find our response to your questions concerning our manuscript.

Major points:

Comment 1. Figure 1A: Authors may consider a negative control (HEK293 cells?). This information could also be included later in the manuscript (Fig. 4F). Also, it would be nice to see how depolarization would look like in this assay.

Response: According to the Referee's suggestion a negative control was included in new Fig 1A. In order to demonstrate depolarization and the functionality of the probe, DiBAC-loaded cells were first exposed to STS to induce hyperpolarization and subsequently to KCl to depolarize the membrane (see the new supporting info Fig S8A).

Comment 2. Figure 1B: It is not clear whether cells were treated with STS or PKC412 in this experiment.

Response: A new figure 1B has been included which more clearly states the treatment.

Comment 3. Figure 1C-E: It should be indicated when STS/PKC412 was added. Ideally, recordings before adding the drug should be shown.

Response: All Ca^{2+} and polarization recordings were started approx. 100 sec before the drug administrations. Since, in the original version it was not clearly stated, we have now included arrows that indicate the time of addition of a certain drug.

Comment 4. Figure 2B: It would be nice to see a HCN4 immunoblot.

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Response: We completely agree with the Reviewer's comment. In fact, we have tried three different commercial antibodies and none of them gave any signal in Western Blotting experiments. However, we are still using three different techniques (RT-PCR, immunostaining and FACS) to assess the expression of HCN4. These data are included into new Figure 2A and 2B.

Comment 5. Figure 2D: Compared to Fig. 2C, this panel is not really convincing.

Response: We agree with the Referee and, therefore, have now repeated the experiments several times. Obtained results are included in Figure 2D, middle panel.

Comment 6. Do HEK293 undergo death upon STS/PKC412? A death assay should be included here as panel D.

Response: Driven by the Reviewer's comment, the level of cell death was measured using FACS analysis of AnnexinV/PI stained cells. The results revealed that HEK cells do not undergo death within 24h of treatment with either of STS or PKC412. However, certain minor morphological changes were observed, suggesting that eventually these cells will die, but in a delayed manner. Of note, the observed AIF-mediated nuclear condensation occurred already after 2-8 hours of treatment. Thus, the absence of HCN2 channel significantly delays the kinetics of cell death. Further, we could show that expression of HCN2 in HEK cells sensitizes them to death induced by either STS or PKC412. These results are included as new Figure 4D. In addition, processing of several caspases and cleavage of PARP in HEK293 cells were analyzed by Western Blotting. As seen in supporting info Fig S7B, none of the caspases were processed and neither PARP cleavage was detected.

Comment 7. Figure 4A, 4B, 4G: Why is the calcium influx much more faster under these conditions?

Response: Under these conditions calcium influx occurs faster since the treatment with either PKC412 or STS triggers two events: hyperpolarization and dephosphorylation (Fig 4C). The latter is required for the prolonged Ca^{2+} import and the subsequent apoptotic signaling. Thus, the consequence of the exposure of cells to either PMA or TPA prior to STS/PKC412 is that the channel remains in a phosphorylated state (Fig 4C, right lane) and can only be opened faster and transiently.

Accordingly, in Fig 4G, where hyperpolarization was induced by decreasing the extracellular $[K^+]$ level, a Ca^{2+} transients through the HCN channel occurred due to the phosphorylation of the channel at Thr549. However, when this putative site is not phosphorylated (as for the T549A mutant), the channel responds with a more prolonged Ca^{2+} increase. Hence, the physiological Ca^{2+} signaling mediated by HCN channels appears to be of rapid and transient nature, meanwhile a prolonged Ca^{2+} influx is required for HCN2's role in cell death signaling.

Comment 8. Figure 4C: The effect of PMA alone should be shown.

Response: The effect of both PKC activators (PMA and TPA) alone is now shown in supporting information (Fig S8B and S8C).

Comment 9. Figure 4E: It is not clear whether cells were treated with STS or PKC412 in this experiment.

Response: We apologize for a typing error and in the revised version it is clarified. In this particular case it was STS.

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Comment 10. Figure 4F: Genetically unmodified cells should be included.

Response: It has been included in new figure 5F.

Comment 11 Figure 5C and 5E: Control immunoblots are missing.

Response: It has been included as new figure 6C and 6E.

Reviewer #2:

We appreciate all your comments and are confident that the changes made to our manuscript in response to your critique have significantly strengthened and clarified our experimental work. Below, please find our response to your questions concerning our manuscript.

Major points.

Comment 1. The authors rely on the use of DiBAC, which is a polarization-sensitive due, indeed. Being an anion, DiBAC is usually excluded from the cells. DIBAC enters only enters depolarized cells where it binds to intracellular proteins or membranes. Thus, increased depolarization results in more influx of the anionic dye and thus an increase in fluorescence. In the absence of controls (untreated cells), in Fig. 1A and 1B, it is impossible to understand what the data mean. So this must be corrected. Ideally the authors should also use additional methods (like patch clamping) to measure the plasma membrane potential.

Response: According to the Reviewer's suggestion, cells were analyzed using whole cell patch-clamp technique. Upon drug addition all analyzed cells were hyperpolarized. The results have been added as new supporting info Fig S2. In addition, several experiments were performed using DiBAC-loaded cells that were first exposed to STS and then to KCl. With this approach we could successfully show the functionality of DiBAC as a polarization sensitive dye in the experimental model used. Accordingly cells hyperpolarized upon STS exposure and depolarized in response to KCl (supporting info FigS8A).

Comment 2. None of the immunoblots has size markers. None of the PCR reactions has size markers. None of the fluorescence microphotographs has size bars. Altogether, this absence of detail and accuracy generates the impression of sloppiness.

Response: In the revised version all requested information was included (please see the Figure in the MS and in the supporting information).

Comment 3. The authors only use one single siRNA to deplete HCN2. This is not acceptable, because siRNAs can have off-target effects. The authors should use at least two distinct non-overlapping siRNAs specific for HCN2 to reproduce their findings. Moreover, they should re-transfect HCN2-depleted cells with a non-interferable HCN2 cDNA and show that the phenotype can be restored.

Response:

Driven by the Reviewer's criticism, we have now performed experiment using two different non-overlapping siRNAs (target sequence of siRNA1; CCAGCGCGGUGAUGAGGAU and of siRNA2; GUAGGUAGCCGUAGUUGGA) and could reproduce our findings that the HCN2 channel is mediating the influx of Ca^{2+} (Supporting Information Fig 9A).

Further, a rescue experiment was performed in which HCN2 was siRNA downregulated and subsequently re-transfected with mHCN2. Ca^{2+} recordings revealed that the observed phenotype could successfully be restored (supporting info S5).

Comment 4. The data in Fig. 2E are no impressive. The upper and the lower panels for "cleaved Atg5" have been derived from two different experimental conditions. Where is the uncleaved Atg5?

Response: To address this concern several additional experiments were performed. A new Figure 3A has been included in the revised version of the manuscript, which shows both the full length and cleaved Atg5. Moreover, these samples were analyzed on the same gel and clearly show that Atg5 cleavage is enhanced by STS and suppressed when HCN2 channel is downregulated.

Comment 5. In Fig. 2F, it appears that only a very minor fraction of AIF (likewise less than 10%) is cleaved upon treatment with staurosporine. However, the authors say that in these conditions, most cells release mitochondrial AIF-GFP fusion protein (Fig. 2G). So can calpain-mediated AIF cleavage truly account for the observed AIF release? Or do AIF and AIF-GFP behave differently. This should be clarified.

Response: Indeed, a minor fraction of AIF is proteolytically processed as detectable by Western Blotting. This was always the case for certain cell lines, such as U1810, HeLa, H661 cells; however, in SH-SY5Y neuroblastoma cells and rat liver mitochondria a significant ~50% cleavage has been observed. The reason for this variety in different experimental model systems is unclear. Up to date it is unknown how much of AIF must be processed and translocated to the nucleus to induce apoptosis. Accordingly, as a result of fractionation experiment, a small amount of AIF was detected in the nuclear fraction. Importantly, the presence of AIF in the nuclei was suppressed after HCN2 downregulation. These results are included as new Figure 3D. It seems that a small amount of nuclear AIF is sufficient to induce apoptosis. Hence, AIF appears to act in a similar fashion as other known proteins involved in apoptosis, like cytochrome *c*. Similarly as for AIF, the whole pool of cytochrome *c* is never released from the mitochondria during apoptotic signaling.

The statement that most cells release AIF-GFP means only the transfected ones. We have reported earlier (Norberg *et al*. Free Radic Biol Med. 2010, 48(6):791-7) that AIF-GFP is integrated into mitochondrial membrane in a similar fashion as endogenous AIF and thus requires processing in order to be released.

To address the question whether the calpain-AIF pathway was contributing to the observed death, we performed several experiments where cells were treated with either STS or PKC412 in the presence or absence of siRNA against AIF, caspase-inhibitor (zVAD-fmk.) and a selective calpain inhibitor (PD150606). Cell death was analyzed by two different methods, FACS (AnnexinV/PI staining), and nuclear apoptosis (DAPI). The results have been included as new Figure 3E and in supporting information FigS6. Both approaches used, revealed that calpain inhibitors and siRNA against AIF can suppress the cell death significantly, meanwhile, the pan-caspase inhibitor had no effect. Thus, we are confident that the cell death observed using our model systems is indeed dependent on calpain-AIF axis of apoptosis.

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Comment 6. A similar discrepancy applies to Fig. 5E and F. While a minor fraction of AIF has undergone calpain cleavage, the majority of the cells condense their nuclei. Does this mean that only a small fraction of AIF needs to be cleaved and released from mitochondria to induce chromatin condensation? Or is the cleavage dispensable for AIF release in this particular model? Indeed, it might be possible that calcium influx induces mitochondrial permeability transition (with bursting of the outer membrane) and release of AIF without the need of calpain-mediated cleaved. Does calpain inhibition affect the frequency of nuclear apoptosis? And does inhibition of permeability transition (for instance with cyclosporin A) do so?

Response: In fact, parts of comment 5 and 6 are quite similar. To prevent the repetition addressing significant component of comment 6, we put the answer to comments 5 and 6 together (please see above). To investigate whether MPT was involved in the release of AIF from the mitochondria, cells were pre-treated with a selective inhibitor of the mitochondrial calcium uniporter (Ru360), which blocks mitochondrial Ca^{2+} uptake and, therefore, MPT induction. As no protective effect was observed when nuclear apoptosis in the presence of Ru360 was analyzed, it allows us to conclude that MPT is not contributing significantly to the release of AIF in this particular experimental system. These results have been included in new supporting information figure S6.

Minor:
Comment 1. The English is poor and requires extensive editing.

Response: One of our co-authors (Olga Korenovska) is native English speaking person, who carefully read the manuscript in order to improve the quality of English.

Comment 2. The last paragraph of the Introduction rehashes the Abstract. This paragraph should be shortened

Response: We have shorten the paragraph according to the Reviewer's comment

Comment 3. No bands are visible for HCN2 and HCN4 in Fig. 4A.

Response: We assume that the Reviewer is talking about the old Fig 2A and not Fig. 4A. A new RT-PCR results have been included, in which the bands are clearer (see new Fig 2A).

Comment 4. The fluorescence data in Fig. 2A should be quantified by cytofluormetry.

Response: These data has been included in new Figure 2A, right panels.

Comment 5. In Fig. 2G, there is non-transfected, sham-transfected or unrelated siRNA-transfected control.

Response: All these controls have been included in new Figure 2G.

Comment 6. No bands are visible in the lower panel of Fig. 4C. Everything is black.

Response: A shorter exposure of the same blot, where the bands are more visible, has been included.

Reviewer #3:

We appreciate all your comments and are confident that the changes made to our manuscript in response to your critique have significantly strengthened and clarified our experimental work. Below, please find our response to your questions concerning our manuscript.

Comment 1. Today is not enough to provide data on AIF cleavage and AIF redistribution from mitochondria to cytosol to demonstrate the implication of this protein in apoptosis (Figures 2F, 2G, and 5E). In this sense, the authors do not provide any specific analysis on AIF-mediated apoptosis and, consequently, the apoptotic findings and conclusions included in the manuscript are over-interpreted.

Response: To address the question whether AIF is involved in the observed cell death, we performed several experiments employing two different methods. First, FACS analysis on AnnexinV/PI stained cells treated with either siRNA against AIF, caspase-inhibitor (zVAD-fmk.) or a selective calpain inhibitor (PD150606) prior to STS or PKC412. The results revealed that the downregulation of AIF or calpain inhibition significantly suppressed the cell death. More than 10,000 cells were analyzed by FACS in each experiment. The results have been included as new figure 3E.

Secondly, nuclear apoptosis (DAPI) under the same conditions was investigated and the obtained results supported the FACS data (Supporting information Fig S6). Thus, these different approaches revealed that siRNA against AIF and calpain inhibition can suppress the cell death significantly meanwhile the pan-caspase inhibitor had no effect. Therefore, we are confident that the cell death observed in our model system is indeed a result of AIF.

Comment 2. The putative AIF mitochondrial release was only investigated by immunofluorescence in conditions where HCN2-expressing cells are strongly shrinking (Figure 2G), so that it is very difficult to assess the release of mitochondrial proteins into the cytosol. Moreover, once released, AIF was reported to migrate into the nucleus but I do not see any obvious co-localization of AIF with the nucleus in cells expressing HCN2.

Response: To address this comment, multiple experiments to analyze whether AIF translocated to the nucleus have been performed. Indeed, it its difficult to analyze cells that are shrinking, therefore, we used a biochemical approach e.g. nuclear fractionation followed by Western Blot analysis of cells treated with siRNA against HCN2. The results revealed that the presence of AIF in the nucleus is significantly suppressed upon HCN2 downregulation. These results have been included as new figure 3D. In addition, we performed more careful assessment of AIF-nuclear translocation in HCN2-expressing cells using confocal microscopy which revealed nuclear localization of AIF (see supporting info Fig. 9B):

Comment 3. In Figures 2H and 5F, the nuclear condensation associated to STP-mediated death is not related to the AIF apoptotic action. Indeed, the supposed apoptotic activity of AIF provokes a partial chromatin condensation in the nucleus. As a matter of fact, the authors depict in these Figures the typical caspase-dependent nuclearfeatures. This confirms previous reports demonstrating that STS induced a caspase-dependent cell death process. The role of AIF is irrelevant (Proc Natl Acad Sci U S A.2006 Aug 1;103(31):11573-8; J. Cell Biol. 2002 Dec 23;159(6):923-9; EMBO J. 2003 Sep 1;22(17):4385-99).

Response: In response to the Reviewer's comment it should be clearly stated that AIF-mediated apoptosis is not a "classical" cell death mechanism. Indeed, as the Reviewer indicates, in most cases caspases are the main effectors. However, AIF has still been reported to be critical for cell death of certain cell types, including neurons, retina and NSCLCs. As evidence we would like to

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mention only few out of many available references: Trends Biochem Sci. 2010, 35:278-87; Oncogene. 2004, 23:6282-91; Nat Cell Biol. 2003, 5:97-9; J Cell Biol. 2002, 158:507-17; Oncogene. 2002, 21:65-77; Am J Pathol. 2001, 158:1271-8; Nature. 2001, 410:549-54.

In addition, nuclear condensation/fragmentation was assessed. In these experiments, we could never observe the caspase-related changes (see referee-only supporting info B). Instead, the nuclear condensation but not the fragmentation has been observed. Depicted is a typical caspase-dependent fragmentation, as described earlier (J Exp Med. 2000, 192:571-80).

Further, we analyzed the processing of caspase-2, -3, -8, -9 and PARP cleavage upon STS or PKC412 exposure of U1810 cells and, again, could not observe any processing/activation of any of these proteases or PARP cleavage. These results have been included as new supporting information fig S7. In addition, no caspase-3/-7-like activity was detected (supporting information fig S7).

In summary, by employing four different experimental approaches, including FACS, nuclear condensation, caspase processing and activity assays we are confident that, although caspases are important for most examples of apoptosis, they are not involved in death of NSCLC U1810 cells, induced by STS or PKC412.

Comment 4. The assessment of caspase-3/7 activity through a fluorogenic assay is a pretty rough method (Figure 2I). There are many other methods that are simple and accurate. For example, it is relatively easy to measure caspase-3/7 activation by Western blot. This also applies for other caspases.

Response: According to the Reviewer's comments we have performed several experiments were caspase processing using Western Blot was investigated. Upon STS or PKC412 exposure of U1810 cells, no processing/activation of caspase-2, -3, -8, -9 or PARP cleavage were detected. Moreover, the pan-caspase inhibitor zVAD-fmk did not protect these cells from cell death. These observations allow us to conclude that caspases are not involved in this cell death pathway. In addition, please, see the response to comment 3.

Additional Editorial Correspondence 15 September 2010

I am currently assessing your revised paper and had to notice that you provided 'figures for referee's only'. According to our transparent review policy, all information that contributed to final acceptance of a manuscript should similarly be available for the scientific community. As these experiments had been requested as controls from our referees, I do suggest that you incorporate them at least into the supplementary information.
I am looking forward to receive your final version that should than be ready for ultimate acceptance.

Yours sincerely

Editor
The EMBO Journal

Addtional Correspondence 16 September 2010

Based on your request, we are sending a modified version of the MS, point-by-point reply as well as a new set of Supplementary Information.

We are sorry for inconvenience, but we followed the Instruction for the authors, which we received on July 28. It is written that "data not intended to be published should be uploaded as separate 'referee-only' supporting figure".
In the revised version these data are included in the Supplementary Information Figure S9.

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