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Cold stress-induced ferroptosis involves the ASK1-p38 pathway

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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 10 April 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings. However, they also raise a number of - often overlapping - issues that would need to be addressed before publication. None of them feels that the current dataset fully and conclusively supports a general role of ASK1-p38 in ferroptosis. Referee 2 and 3 suggest several experiments to further test the hypothesis that cold stress induces indeed ferroptosis and not another form of cell death.

From the analysis of these comments it becomes clear that significant revision is required before the manuscript becomes suitable for publication in EMBO report. However, given the constructive comments from the referees, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

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Referee #1:

This manuscript reports two important findings. First, that cold stress activates ferroptosis, as judged by the inhibition by fer1, DFO, U0126, but not ZVAD, NSA etc. Second, that this induction of lipid peroxidation mediated ferroptosis activated ASK1-p38, which is required for death. Both of these findings are highly significant and will be of great interest to many readers. My only concern is in extending these findings to erastin-induced ferroptosis, the authors suggest that in Fig 6D that the ASK1-p38 pathway is also necessary for erastin-induced ferroptosis. While there is a modest protective effect, if this were the sole effector pathway, and it was completely ablated by knockdown, we would expect a much stronger protection. Thus, either the pathway is not essential for erastin-induced ferroptosis, or it was not effectively ablated. However, the protective effect of siASK1 in Figure 2B on cold stress induced ferroptosis was more profound. In addition, the knockdown of ASK1 in Fig 5C in response to erastin treatment appears complete. Therefore, the most plausible explanation for the presented data is that in the context of erastin-induced ferroptosis, the ASK1-p38 pathway contributes to lethality, but is not required. I suggest the authors either clarify this point in the text, or they perform additional experiments to support their contention that ASK1-p38 is essential for elastin-induced ferroptosis. I think doing the former would be simpler.

Referee #2:

In this work Hattori et al. establish two novel links: (1) between sustained cold stress in tissue culture and activation of the ASK1-p38 pathway, and (2) between cold stress and the induction of ferroptosis, a non-apoptotic cell death pathway. However, the authors do not convincingly establish that activation of the ASK1-p38 pathway is necessary 'to mediate' the execution of ferroptosis downstream of lipid ROS accumulation either in the A549 cell model employed throughout most of the work, or in general in other models of ferroptosis. This paper would be stronger focusing either on the link between cold stress and the ASK1-p38 pathway, or on cold stress and the induction of ferroptosis, and revisions along one of these two lines are recommended.

Major Comments:

1: The data in Figures 1 and 2 are convincing that cold stress activates the ASK1-p38 pathway and that this leads to cell death, in A549 cells. Some degree of caution is warranted here as results are reported for only a single cell line. Are these results concerning cold stress and activation of the ASK1 pathway generalizable to additional cell lines? Could this response be specific to A549? This also applies to most biochemical results, below, as well.

2: Experiments in Figure 3 are convincing that cold stress induces a form of non-apoptotic cell death, and in Figure 4 that this cell death is ferroptosis, given that it is completely suppressed by the canonical suppressors Fer-1 and DFO. ASK1 and p38 phosphorylation are also clearly downstream of ferroptosis-associated lipid ROS accumulation, based on the Western blots in Figure 4C and D. Up to this point, everything is solid. However, the core experiments in this paper, in Figure 6, fail to convincingly demonstrate that ASK1 activation is necessary for ferroptosis, downstream of lipid ROS accumulation. The rescue of cell death by a single small molecule p38 inhibitor or two ASK1 siRNAs appears weak or nonexistent, especially at 10 uM erastin. How are we to interpret these results in light of the claim (in the title and Results) that "These lines of evidence strongly suggest that the ASK1-p38 pathway is a general signal mediator of ferroptosis" when cell death is barely (SB202190) or not at all (siASK) suppressed by inhibition of this pathway? Clearly, the ASK1-p38 pathway is not 'generally' required for death as cells will die with 10 uM erastin just fine even when the ASK1 pathway is inhibited. Additionally, there is no insight provided into how activation of ASKk1-p38 might promote death downstream of lipid peroxidation and membrane damage (which, it should be noted, itself seems to provide a sufficient explanation for why cells die in response to erastin or cold stress).

2b: Are the results presented in Figure 6D really from 12 independent biological replicate experiments, as indicated by the legend? Or is this 12 technical replicates within a single biological replicate? This must be clarified as it impacts the interpretation of the two-way ANOVA (i.e. technical replicates are obviously meaningless to use in an analysis of variance) and in turn our understanding of the effects of ASK1 silencing. Perhaps at 5 uM and 1.25 uM erastin the results are not actually different?

3: If activation of the ASK1-p38 pathway is truly required downstream of lipid peroxidation to execute ferroptosis then silencing or inhibition of ASK1 should prevent ferroptosis due to direct inactivation of GPX4. This can be achieved using RSL3 or another covalent inhibitor described by the Stockwell lab. Showing that siASK1 or the small molecule inhibitors blocked ferroptosis, to a similar extent as Fer-1 or DFO, in response to direct GPX4 inhibition would be convincing that Ask1-p38 act downstream of lipid peroxidation to promote death. Conversely, if siASK1 has no effect on ferroptosis induced by direct GPX4 inhibition, this might indicate that this pathway is operating in parallel to mediate some form of stress response that partially impinges on the kinetics of ferroptotic cell death, but not the ultimate fate.

Minor comments:

1: The methods section mentions that all experiments were repeated three times. Yet, as noted above, in some Figure legends n values between 3 and 12 are indicated. This requires clarification. Are the results shown in each figure from one of the three biologicla replicate experiments, showing 3-12 technical replicates? Or do these results reflect the mean of 3-12 independent biological replicate experients? If the former, it would be more appropriate to merge multiple technical replicates into a single biological replicate and average accross biological replicates. Also, mean +/- standard deviation would be more informative than mean +/- SEM with respect to the dispersion of the data between biological replicates.

2: One hallmark of ferroptosis induced by erastin is depletion of glutathione. Does old stress deplete glutathione?

3: Fig 6A and D. The cell viability for 2.5 uM Erastin treatment on control sample is vastly different (100% viable in DMSO co-treatment vs. 20% viable in control siRNA). Is the control siRNA treatment toxic to the cells?

4: The introduction could be shorter. No need to introduce all the different irrelevant cell death pathways (e.g. pyroptosis)

Referee #3:

In the work that resulted in the presented manuscript, Hattori and Ishikawa et al. investigated the role of ASK and p38 in cold stress. The authors demonstrate that ASK1 is required for cold stress-induced necrosis of A549 cells, and that during this process, Thioredoxin-ASK1 interactions are affected. By means of inhibitors, the authors demonstrate that cold stress-mediated LDH release is sensitive to p38 inhibition, but not to other control inhibitors. Several experiments in this work need further validation and/or additional experiments that justify the authors conclusions. A major drawback of this study is, that the authors tend to interpret cold stress-induced necrosis as ferroptosis. More experiments are required to conclude this!

Major concerns:

- The conclusion that this cold stress-induced necrosis is in fact ferroptosis is problematic, given the provided data (e.g. in Fig. 4). Simply the use of Fer-1 and an iron chelator does not classify this pathway as ferroptosis. Fig. 5 provides much better data to suggest this, but a good classification should not miss HT1080 cells or other classical "ferroptosis-sensitive cell lines" that are treated with RSL3. Would RSL3-treatment activate ASK/pASK and would RSL3-treated cells upon knockdown of ASK lose the sensitivity to ferroptosis?

- Another important control is to take the ASK1-deficient cells and knock down GPX4, and this should be controlled by A549 cells that lack GPX4 (if these are viable). Knockout/down of ACSL4 might be another approach. Please revise the 6F according to these potentially new findings.

- How reproducible is the result in Fig. 3F? There is no good reason to believe that NSA might function in murine cells! Maybe the authors looked at HT29 cells here and it is just a labelling mistake?

Minor remarks:

-The experiment in Fig. 6E is a little confusing. Could the authors provide a western blot to demonstrate the efficacy of the two ASK1-knockdowns?

- Please refer to regulated necrosis rather than programmed necrosis, as the latter term should only be used for embryonically inevitable cell death (compare PMID 24582829).

- In tissues and cell culture models, ferroptosis causes necrosis in a non cell-autonomous manner (PMID 27668796 and Ref 29). Did the authors experience similar findings in their assays? Please discuss in some more detail!

- Ferroptosis is a rapidly moving field. Some rather new data suggest phosphotidylethanolamine to be specifically peroxidized during ferroptosis (PMIDs 27842066, 27842070) and NADPH-abundance to be downstream of lipid peroxidation (PMIDs 26971867, 26853626) Please also discuss your data in the light of these findings.

1st Revision - authors' response [in italics] 07 July 2017

Referee #1:

This manuscript reports two important findings. First, that cold stress activates ferroptosis, as judged by the inhibition by fer1, DFO, U0126, but not ZVAD, NSA etc. Second, that this induction of lipid peroxidation mediated ferroptosis activated ASK1-p38, which is required for death. Both of these findings are highly significant and will be of great interest to many readers. My only concern is in extending these findings to erastin-induced ferroptosis, the authors suggest that in Fig 6D that the ASK1-p38 pathway is also necessary for erastin-induced ferroptosis. While there is a modest protective effect, if this were the sole effector pathway, and it was completely ablated by knockdown, we would expect a much stronger protection. Thus, either the pathway is not essential for erastin-induced ferroptosis, or it was not effectively ablated. However, the protective effect of siASK1 in Figure 2B on cold stress induced ferroptosis was more profound. In addition, the knockdown of ASK1 in Fig 5C in response to erastin treatment appears complete. Therefore, the most plausible explanation for the presented data is that in the context of erastin-induced ferroptosis, the ASK1-p38 pathway contributes to lethality, but is not required. I suggest the authors either clarify this point in the text, or they perform additional experiments to support their contention that ASK1-p38 is essential for elastin-induced ferroptosis. I think doing the former would be simpler.

We thank the reviewer for his/her positive assessment of our work and for constructive comments. As s/he mentioned, the fact is that ASK1-p38 contributes only partially to the erastin-induced cell death because siRNAs effectively ablate ASK1 as shown in the newly added Figure 6E. Therefore, we have toned down our claim throughout the manuscript and we have replaced the schematic model in Figure 6H.

Referee #2:

In this work Hattori et al. establish two novel links: (1) between sustained cold stress in tissue culture and activation of the ASK1-p38 pathway, and (2) between cold stress and the induction of ferroptosis, a non-apoptotic cell death pathway. However, the authors do not convincingly establish that activation of the ASK1-p38 pathway is necessary 'to mediate' the execution of ferroptosis downstream of lipid ROS accumulation either in the A549 cell model employed throughout most of the work, or in general in other models of ferroptosis. This paper would be stronger focusing either on the link between cold stress and the ASK1-p38 pathway, or on cold stress and the induction of ferroptosis, and revisions along one of these two lines are recommended.

We thank the reviewer's constructive comments. Based on his/her comments, we employed several experiments using other cell lines as mentioned below in order to strengthen the link between cold stress and the ASK1-p38 pathway, and also ferroptosis-like cell death and the ASK1-p38 pathway.

Major Comments:

1: The data in Figures 1 and 2 are convincing that cold stress activates the ASK1-p38 pathway and that this leads to cell death, in A549 cells. Some degree of caution is warranted here as results are reported for only a single cell line. Are these results concerning cold stress and activation of the ASK1 pathway generalizable to additional cell lines? Could this response be specific to A549? This also applies to most biochemical results, below, as well.

We agreed the reviewer's comment; hence, we have analyzed using other cell lines such as HEK293, HepG2 and HT-1080 cells. In all type of cells, we observed cold stress-induced cell death that is inhibited by Fer-1 (Figure EV2). Dfx and U0126 also clearly suppressed cold-induced death in HT-1080 cells (Figure EV2D and E). Furthermore, ASK1-knockdown in HEK293A cells and p38 inhibition in HEK293A, HepG2 and HT-1080 cells attenuated the cold-induced cell death (Figure 2E-I), which suggests that cold stress induces ASK1-p38 axis-dependent cell death in multiple cell lines. We also observed that an inactive analog SB202474 exhibited marginal but significant inhibitory effects with unknown mechanisms, especially in HEK293A cells (Figure 2G). These results were reflected in the schematic model (Figure 6H).

2: Experiments in Figure 3 are convincing that cold stress induces a form of non-apoptotic cell death, and in Figure 4 that this cell death is ferroptosis, given that it is completely suppressed by the canonical suppressors Fer-1 and DFO. ASK1 and p38 phosphorylation are also clearly downstream of ferroptosis-associated lipid ROS accumulation, based on the Western blots in Figure 4C and D. Up to this point, everything is solid. However, the core experiments in this paper, in Figure 6, fail to convincingly demonstrate that ASK1 activation is necessary for ferroptosis, downstream of lipid ROS accumulation. The rescue of cell death by a single small molecule p38 inhibitor or two ASK1 siRNAs appears weak or nonexistent, especially at 10 uM erastin. How are we to interpret these results in light of the claim (in the title and Results) that "These lines of evidence strongly suggest that the ASK1-p38 pathway is a general signal mediator of ferroptosis" when cell death is barely (SB202190) or not at all (siASK) suppressed by inhibition of this pathway? Clearly, the ASK1-p38 pathway is not 'generally' required for death as cells will die with 10 uM erastin just fine even when the ASK1 pathway is inhibited. Additionally, there is no insight provided into how activation of ASKk1-p38 might promote death downstream of lipid peroxidation and membrane damage (which, it should be noted, itself seems to provide a sufficient explanation for why cells die in response to erastin or cold stress).

We apologize that we exaggerated our claim in the initial manuscript. The fact is that ASK1-p38 pathway is partially involved in erastin- and RSL3-induced ferroptosis, especially in A549 cells (Figure 6D-G). Additionally, we observed that p38 activity is also partially involved in erastin- and RSL3-induced ferroptotis in HEK293A and HT-1080 cells, even though ASK1 might not be a regulator in those cells (Figure EV3 and 4). Therefore, we have toned down our claim throughout the manuscript and we have replaced the schematic model in Figure 6H. We have not determined how p38 MAPK regulates cell death so far; however, there is a possibility that p38 MAPK induces the nuclear translocation of AIF, which was reported as one possible mechanism to induce ferroptotic cell death (Cell Metabolism 8, 237ñ248 (2008), Toxicology 257, 1ñ9 (2009), Cell Biology International 36, 339ñ344 (2012)). We have added the sentence with appropriate references regarding this point in the discussion part.

2b: Are the results presented in Figure 6D really from 12 independent biological replicate experiments, as indicated by the legend? Or is this 12 technical replicates within a single biological replicate? This must be clarified as it impacts the interpretation of the two-way ANOVA (i.e. technical replicates are obviously meaningless to use in an analysis of variance) and in turn our understanding of the effects of ASK1 silencing. Perhaps at 5 uM and 1.25 uM erastin the results are not actually different?

We are sure that the results are from 12 independent biological replicates. However, we have performed similar experiments again using 6 kinds of siRNA and wide range of erastin from low concentration to high concentration. We observed the significant difference at 1.25 M erastin (Figure 6D).

3: If activation of the ASK1-p38 pathway is truly required downstream of lipid peroxidation to

execute ferroptosis then silencing or inhibition of ASK1 should prevent ferroptosis due to direct inactivation of GPX4. This can be achieved using RSL3 or another covalent inhibitor described by the Stockwell lab. Showing that siASK1 or the small molecule inhibitors blocked ferroptosis, to a similar extent as Fer-1 or DFO, in response to direct GPX4 inhibition would be convincing that Ask1-p38 act downstream of lipid peroxidation to promote death. Conversely, if siASK1 has no effect on ferroptosis induced by direct GPX4 inhibition, this might indicate that this pathway is operating in parallel to mediate some form of stress response that partially impinges on the kinetics of ferroptotic cell death, but not the ultimate fate.

We thank the reviewer's constructive suggestions. We have analyzed using RSL3 and siRNAs targeting to GPX4 using A549, HEK293A and HT-1080 cells. The results in Figure 6G show that ASK1 is partially involved in RSL3-mediated ferroptosis in A549 cells. However, RSL3- or GPX4 deficiency-dependent ferroptosis were not suppressed by ASK1 knockdown in HEK293A and HT-1080 cells, where p38 inhibition partially suppressed the cell death (Figure EV3-EV5). These results suggest that ASK1 participates in only a part of ferroptotic pathway in a cell type-specific manner. p38 MAPK involvement, however, was observed in multiple cell lines, which suggests that other MAP3Ks contribute to ferroptosis in HEK293A and HT-1080 cells. These results were reflected in the schematic model (Figure 6H).

Minor comments:

1: The methods section mentions that all experiments were repeated three times. Yet, as noted above, in some Figure legends n values between 3 and 12 are indicated. This requires clarification. Are the results shown in each figure from one of the three biologicla replicate experiments, showing 3-12 technical replicates? Or do these results reflect the mean of 3-12 independent biological replicate experients? If the former, it would be more appropriate to merge multiple technical replicates into a single biological replicate and average accross biological replicates. Also, mean +/- standard deviation would be more informative than mean +/- SEM with respect to the dispersion of the data between biological replicates.

We apologize to trouble you to understand the meaning of N. As we stated in the methods section, all experiments were independently repeated at least three times; hence, N values indicated in the figure legends represents the exact number of experiments we repeated. To clarify this point, we have added the sentence "N in each figure legend represents biological replicates." in the methods section. In regards to error bars, we believe that it is appropriate to use inferential error bar such as SEM to compare with control samples (The Journal of Cell Biology 177, 7ñ11 (2007)).

2: One hallmark of ferroptosis induced by erastin is depletion of glutathione. Does old stress deplete glutathione?

We thank the reviewer's suggestion. We have measured the glutathione levels after cold stress and revealed that the total glutathione rather increased in response to cold stress (Figure EV1F); hence, we concluded that cold-induced cell death is not induced by the depletion of glutathione.

3: Fig 6A and D. The cell viability for 2.5 uM Erastin treatment on control sample is vastly different (100% viable in DMSO co-treatment vs. 20% viable in control siRNA). Is the control siRNA treatment toxic to the cells?

It is, indeed, vastly different because of the difference of the experimental systems. We represented in the replaced Figure 6D that three different control siRNA-treated cell exhibited exactly the same tendency: all three type of cells became more susceptible than the parent cells. These results indicate that siRNA transfection appears to affect the cell viability.

4: The introduction could be shorter. No need to introduce all the different irrelevant cell death pathways (e.g. pyroptosis)

We thank the reviewer for the suggestion. We have deleted several sentences in the introduction.

Referee #3:

In the work that resulted in the presented manuscript, Hattori and Ishikawa et al. investigated the role of ASK and p38 in cold stress. The authors demonstrate that ASK1 is required for cold stress-induced necrosis of A549 cells, and that during this process, Thioredoxin-ASK1 interactions are affected. By means of inhibitors, the authors demonstrate that cold stress-mediated LDH release is sensitive to p38 inhibition, but not to other control inhibitors. Several experiments in this work need further validation and/or additional experiments that justify the authors conclusions. A major drawback of this study is, that the authors tend to interpret cold stress-induced necrosis as ferroptosis. More experiments are required to conclude this!

We thank the reviewer's comments. We have changed "ferroptosis" to "ferroptosis-like cell death" to tone down in the context of cold stress-induced cell death.

Major concerns:

- The conclusion that this cold stress-induced necrosis is in fact ferroptosis is problematic, given the provided data (e.g. in Fig. 4). Simply the use of Fer-1 and an iron chelator does not classify this pathway as ferroptosis. Fig. 5 provides much better data to suggest this, but a good classification should not miss HT1080 cells or other classical "ferroptosis-sensitive cell lines" that are treated with RSL3. Would RSL3-treatment activate ASK/pASK and would RSL3-treated cells upon knockdown of ASK lose the sensitivity to ferroptosis?

We thank the reviewer's constructive suggestions. We observed ASK1 and p38 activation responding to RSL3 treatment in HT-1080 cells (Fig EV4A), which suggests that ASK1-p38 axis may regulate ferroptosis in HT-1080 cells. Unexpectedly, however, ASK1-deficiency did not prevent RSL3-induced ferroptosis in HT-1080 (Figure EV4), suggesting that ASK1 is not a global regulator of ferroptosis. Meanwhile, we observed that ASK1 is involved in RSL3-mediated death in A549 cells. These results recapitulate that ASK1 is a cell type-specific regulator for ferroptotic cell death.

ï Another important control is to take the ASK1-deficient cells and knock down GPX4, and this should be controlled by A549 cells that lack GPX4 (if these are viable). Knockout/down of ACSL4 might be another approach. Please revise the 6F according to these potentially new findings.

We thank the reviewer's suggestion. We have performed GPX4 knockdown experiments and revealed that ASK1 is not involved in GPX4 deficiency-dependent ferroptosis in HT-1080 cells as evidenced by the double knockdown of ASK1 and GPX4 (Figure EV5). These results were consistent with the data of RSL3-dependent ferroptosis model shown in Figure EV4F. Unfortunately, we could not observe GPX4 deficiency-dependent cell death in A549 cells. Therefore, we added "other pathways" in the schematic model shown in Figure 6H.

- How reproducible is the result in Fig. 3F? There is no good reason to believe that NSA might function in murine cells! Maybe the authors looked at HT29 cells here and it is just a labelling mistake?

We apologize that we have incorrectly performed this experiment using L929. We have re-analyzed using HT-29 cells and the result is presented in Figure 3F.

Minor remarks:

- The experiment in Fig. 6E is a little confusing. Could the authors provide a western blot to demonstrate the efficacy of the two ASK1-knockdowns?

We apologize that we did not provide the data showing the knockdown efficiency. We newly added the data in Figure 6E.

- Please refer to regulated necrosis rather than programmed necrosis, as the latter term should only be used for embryonically inevitable cell death (compare PMID 24582829).

We thank the reviewer's suggestion. Programmed necrosis was replaced by regulated necrosis throughout the manuscript.

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- In tissues and cell culture models, ferroptosis causes necrosis in a non cell-autonomous manner (PMID 27668796 and Ref 29). Did the authors experience similar findings in their assays? Please discuss in some more detail!

We apologize that we have not analyzed ferroptotic cell death at the single cell levels; hence, we have not observed non cell-autonomous mechanism so far. However, we believe that it is an important issue to be solved, and we added a sentence related to this point in the discussion part.

- Ferroptosis is a rapidly moving field. Some rather new data suggest phosphotidylethanolamine tob e specifically peroxidized during ferroptosis (PMIDs 27842066, 27842070) and NADPH-abundance to be downstream of lipid peroxidation (PMIDs 26971867, 26853626) Please also discuss your data in the light of these findings.

We thank the reviewer's suggestion. We have already made a brief discussion on ASK1 activation mechanism at ER-associated compartment based on the recent studies (PMIDs 27842066, 27842070). Although we have not analyzed yet, NADPH depletion may lead to the activation of ASK1-p38, or vice versa, which is also an intriguing question to be elucidated. We have added a sentence related to this point in the discussion part.

2nd Editorial Decision 18 July 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the report from the two referees that were asked to re-evaluate your study (you will find enclosed below). As you will see, both support the publication of your manuscript in EMBO reports. However, referee #1 has still a couple of minor concerns, we would ask you to address in a final revised version of the manuscript. Further, I have the following editorial requests that need to be addressed:

The title is currently too long (it should not have more than 100 characters including spaces). Please shorten the title (also taking into account the concerns of referee #1 regarding the title).

Please also add a short running title to the title page (next to the key words).

Please remove the tables showing the statistical analyses from the figures, mark the statistics/p-values in the panels, move the tables to an Appendix file, and put callouts to these tables into the figure legends. The Appendix should be a single pdf file labeled Appendix. The Appendix includes a table of content on the first page, all tables and their legends. Please follow the nomenclature Appendix Table Sx throughout the text and figure legends, and also label the tables according to this nomenclature. For more details please refer to our guide to authors.

Was statistical testing performed for the data shown in panels 3A, B, C, and E? Please add this to the panels.

As the Western blot panels show significantly cropped images, we would like to ask you if it would be possible to provide the original source data for these that will then be published together with the paper (with the aim of making primary data more accessible and transparent to the reader). The source data will be published in a separate source data files online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of the entire gels or blots) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

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Referee #2:

We thank the Authors for addressing in a thoughful manner all the issues raised in the initial review. We are supportive of publication based on the important finding that cold stress can induce ferroptosis, and based on the observation that ASK1-p38 is activated. Given the inhibition of cell death by Fer-1 and Dfx, and the accumulation of lipid peroxides in response to cold stress, we feel that it would be appropriate to describe this death as ferroptosis, not 'ferroptosis-like', a term that may only create confusion (i.e. either it is ferroptosis, or it isn't. By the evidence presented here, it is).

A note of caution: based on the weak or non-existent effect of ASK silencing on ferroptosis in 2/3 tested cell lines and contexts (e.g. in response to erastin and RSL3 in 293T and HT-1080) we remain skeptical that the ASK1-p38 pathway 'mediates' ferroptosis in any meaningful way, as suggested by the title and implied at various points in the Abstract and Discussion. The Ask1/p38 is clearly activated by cold stress and in response to other pro-ferroptotic conditions, as shown. ASK/p38 activation is clearly downstream of lipid ROS. And death in response to cold stress is clearly ferroptosis, as shown. But, objectively, complete silencing or inhibition of ASK1/p38 has little impact on erastin or RSL3-induced death in 2/3 tested cell lines, arguing that it is not essential to mediate anything, but rather a correlate of ferroptosis and very likely a failed attempt by the cell to respond to the lethal stress in some way. We encourage the authors to consider a different title that might not get so far ahead of the reported results and better reflect the most solid findings of this paper (e.g. "Cold stress induces ferroptosis and activates the ASK1-p38 pathway").

Other points:
-page 6. Line 9-10. What, specifically, remains elusive about the mechanism? This statement is very vague.

-Page 7. Line 3. "..insight on the downstream pathway of lipid peroxide remains..." seems like a word is missing or that this could be rephrased.

-Page 10, line 1. "..two types of p38 inhibitiors..". Those two inhibitors are structurally analogs, they are not different 'types'. Indeed, while not essential, the results might be strengthened by using a structurally-distinct p38 inhibitor. What if this chemical scaffold acts as an antioxidant?

-In Figure 3A was only 10 uM zVADfmk sufficient to block apoptosis induced by STS or another positive control? Many studies use much higher concentrations of zVAD (50 or 100 uM). Not necessary to address, just a point to think about.

-Page 12, line 1. '..evokes ferroptosis pathways..' Is there more than one?

- Page 18, line 15. "the ASK1-p38 pathway is one of the regulator..." Should be 'regulators'. In general, the manuscript could use a solid copy-edit to fix many similar small errors of a similar nature.

Referee #3:

The manuscript has been significantly improved during the revision process. It now adds significant novel findings - especially the data provided in Fig EV4 A - to our understanding of regulated necrosis. The authors are congratulated to an important piece!

2nd Revision - authors' response 19 July 2017

Response to the reviewers

Referee #2:

We thank the Authors for addressing in a thoughful manner all the issues raised in the initial review. We are supportive of publication based on the important finding that cold stress can induce

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ferroptosis, and based on the observation that ASK1-p38 is activated. Given the inhibition of cell death by Fer-1 and Dfx, and the accumulation of lipid peroxides in response to cold stress, we feel that it would be appropriate to describe this death as ferroptosis, not 'ferroptosis-like', a term that may only create confusion (i.e. either it is ferroptosis, or it isn't. By the evidence presented here, it is).

We thank the reviewer's suggestion. We have replaced ferroptosis-like cell death with ferroptosis regarding to cold stress-induced cell death throughout the manuscript.

A note of caution: based on the weak or non-existent effect of ASK silencing on ferroptosis in 2/3 tested cell lines and contexts (e.g. in response to erastin and RSL3 in 293T and HT-1080) we remain skeptical that the ASK1-p38 pathway 'mediates' ferroptosis in any meaningful way, as suggested by the title and implied at various points in the Abstract and Discussion. The Ask1/p38 is clearly activated by cold stress and in response to other pro-ferroptotic conditions, as shown. ASK/p38 activation is clearly downstream of lipid ROS. And death in response to cold stress is clearly ferroptosis, as shown. But, objectively, complete silencing or inhibition of ASK1/p38 has little impact on erastin or RSL3-induced death in 2/3 tested cell lines, arguing that it is not essential to mediate anything, but rather a correlate of ferroptosis and very likely a failed attempt by the cell to respond to the lethal stress in some way. We encourage the authors to consider a different title that might not get so far ahead of the reported results and better reflect the most solid findings of this paper (e.g. "Cold stress induces ferroptosis and activates the ASK1-p38 pathway").

We thank the reviewer's thoughtful comments. We have replaced the title as "Cold stress induces ferroptosis partly through ASK1-p38 pathway".

Other points:

-page 6. Line 9-10. What, specifically, remains elusive about the mechanism? This statement is very vague.

We have replaced as "However, the precise..." (page 7 line 9).

-Page 7. Line 3. "..insight on the downstream pathway of lipid peroxide remains..." seems like a word is missing or that this could be rephrased.

We have replaced as "...the downstream signaling..." (page 8 line4).

-Page 10, line 1. "..two types of p38 inhibitiors..". Those two inhibitors are structurally analogs, they are not different 'types'. Indeed, while not essential, the results might be strengthened by using a structurally-distinct p38 inhibitor. What if this chemical scaffold acts as an antioxidant?

We understand that it is better to use structurally-distinct inhibitors; however, we believe that the data provide the meaningful information to some extent. We have deleted the phrase "types of" (page 11 line 1).

-In Figure 3A was only 10 uM zVADfmk sufficient to block apoptosis induced by STS or another positive control? Many studies use much higher concentrations of zVAD (50 or 100 uM). Not necessary to address, just a point to think about.

We have analyzed that 10 M Z-VAD-FMK is strong enough to inhibit STS-dependent caspase 3 activation using A549 cells; hence we believe that cold stress induces cell death in a caspase 3-independent manner.

-Page 12, line 1. '..evokes ferroptosis pathways..' Is there more than one?

We have deleted "s" (page 13 line 1).

- Page 18, line 15. "the ASK1-p38 pathway is one of the regulator..." Should be 'regulators'. In general, the manuscript could use a solid copy-edit to fix many similar small errors of a similar nature.

We have added "s" in several sentences (page 15 line 3, page 19 line15, page 20 line 8).

Referee #3:

The manuscript has been significantly improved during the revision process. It now adds significant novel findings - especially the data provided in Fig EV4 A - to our understanding of regulated necrosis. The authors are congratulated to an important piece!

We appreciate the reviewer's positive assessment. Considering the comments from referee #2, we have replaced "ferroptosis-like cell death" with "ferroptosis" again in the context of cold-induced cell death. However, we have sustained moderate descriptions about the involvement of ASK1-p38 pathway in ferroptosis throughout this revised manuscript.

3rd Editorial Decision 31 July 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - exact statistical test results, e.g., P values = x but not P values < x;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical mechods were used to determine sample size in advance.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the analysis in most of the experiments; however, the data sets in which the LDH release levels were less than 20% at least in one control samples of Figure 2E were excluded, which was pre-established.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, we mentioned precisely in the methods section and figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	No statistical methods were used to assess it.
Is there an estimate of variation within each group of data?	Yes, we provided tha data as means +/- SEM.
Is the variance similar between the groups that are being statistically compared?	In case of student's t-test, we performed welch correction (unequal variances t-test). Otherwise, we hypothesized that the variance is similar between the groups.

C- Reagents

Question	Answer
6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We have provided the information in the methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We have provided the information in the methods section.

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D- Animal Models

Question	Answer
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9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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E- Human Subjects

Question	Answer
11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

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