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Mitochondria control store-operated Ca2+ entry through Na+ and redox signals

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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 12 August 2015

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by two referees whose comments are shown below. As you will see, while the referees express interest in the work and topic in principle, they do not offer strong support for publication in The EMBO Journal - at least at the current stage of analysis.

I will not repeat all individual points of criticism here, but while both referees find the reported link between Na+ flux, mitochondrial redox state and SOCE activation intriguing, it becomes clear that they also find the depth of analysis in the study to be too limited for them to recommend publication in The EMBO Journal at present. Clearly, an extensive amount of further experimentation would be required to address the issues issues raised by the referees and to bring the study to the level of insight and significance required for publication here. Furthermore, the outcome of such experiments cannot be predicted at this point and would thus lie outside the scope and the timeframe of a revision.

Given these negative opinions from the referees, I am afraid that we are unable to offer further steps towards publication in The EMBO Journal at this stage. If you should decide to undertake the efforts to extensively address all the concerns raised by our referees, we could be willing to look at

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an amended version of the manuscript, but this would have to be considered as a new independent submission at that point.

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REFEREE REPORTS

Referee #1:

This is potentially interesting study linking the activity of the mitochondrial Na+-Ca2+ exchanger NCLX with mitochondrial redox potential, which in turn regulates the CRAC current and SOCs. Regulation of CRAC-mediated Ca2+ influx by the mitochondria has been known for quite some time and is generally attributed to reduction of Ca2+-dependent inactivation of Orai1 by mitochondrial Ca2+ uptake. The present study reports on an alternative mechanism that can operate in parallel with direct regulation of CRAC by Ca2+. In addition, the study provides a potential molecular mechanism for several recent studies reporting regulation of STIM1 and Orai1 activity by cellular redox potential. However, the studies appear somewhat preliminary by not exploring in depth how NCLX -mediated changes in mitochondria redox potential affect the component of SOCs. In addition, the results point to potentially two modes by which NCLX affect SOCs/CRAC activity, one dependent and one independent of the effect of NCLX on mitochondrial redox.

Comments:

1. It is interesting that the effect of NCLX knockout on CRAC is observed with 20 and even 50 mM BAPTA in the pipette solution. It is difficult to see how the model in Figure 8 operates under these conditions. It is unlikely that the mitochondrial matrix has any Ca2+ left or that redox is affected by changes in matrix Ca2+. At such high BAPTA mitochondrial Ca2+ is likely very low already and not affected much by NCLX. This does not exclude an effect of NCLX on mitochondria ROS and regulation of CRAC, but raises the possibility that a component of reduced CRAC/SOCs due to NCLX knockdown is independent of mitochondrial redox state. This possibility is further suggested by the additional reduction in SOCs by knockdown of NCLX in cells incubated with NMDG (Figure 5). This possibility should be examined more rigorously. For example, analyze more carefully and quantitavely the level of STIM1 and the Orai1 and their clustering. Additionally, the authors should determine if NCLX knockdown affect STIM1-Orai1 Co-IP in untreated and store depleted cells and/or the time corse and extent of FRET between STIM1-Orai1.

2. It is surprising that the authors did not perform similar experiments with expressed STIM1 and Orai1. This should be tested and then use the STIM1 and Orai1 cysteine mutants that are redox resistant and show that the CRAC current by the mutants is not affected either by NCLX knockdown, Na+ influx or mCatalase expression.

3. It is difficult to understand the ouabain experiments. It seems that pre-incubation in the absence of external Na+ should have depleted all internal Na+ prior to addition of external Ca2+. Additionally, if Na+ remained elevated, it is not clear how the authors excluded the possibility that the enhance Ca2+ influx is mediated by plasma membrane NCX. It would be helpful to show if ouabain treatment has the same effect of CRAC current.

4. If the interpretation offered by the authors for the removal of Na+ and ouabain experiments is correct, then elevation of pipette Na+ should enhance CRAC current both in control cells and cells treated with siNCLX. Under these conditions removal of external Na+ and ouabain should have no effect on the current and the current should not be affected by manipulation of the mitochondria redox potential.

5. Figure 7: The authors should determine how expression of mCat affect the inhibition of SOCs/CRAC due to removal of external Na+ and treatment with ouabain.

6. For the experiments in Figure 7 to be interpreted as suggested, mCatalase activity should be Ca2+ dependent. Is it?

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Referee #2:

This was a difficult paper to read and understand, for many reasons, including poor description of the experimental rationale, protocols and results. In addition, many of the experimental paradigms suffer from variables that are not well considered or controlled. Finally, some key experiments are missing.

1. The basic observation is that genetic knockdown of the mitochondrial Na/Ca2+ exchanger NCLX reduces store-operated Ca2+ influx, mediated in the cell types examined presumably completely by ORAI, since the only Ca2+ currents measured appear to be consistent with CRAC currents. (Thus, the speculation later that Trp channel activation contributes to the elevated Na seems like it must be incorrect). The Ca2+ and Na+ current measurements are critical, because knockdown of the NCLX is likely wreaking havoc with cell health, as the mitochondria must be overloaded with Ca2+. Accordingly, plasma membrane potential is likely quite different between the control and knockdown cells, and the Ca2+ influx measured by fluorescence will reflect that in part. The authors compound this potential problem by conducting experiments with altered extracellular cation composition. What is missing the CRAC current measurements, where membrane potential and ionic compositions are controlled with changes of Na or Li in the pipette solution to determine if there is indeed an effect, in the physiological range, of Na on CRAC currents.

2. The authors need to do a better job of reviewing the literature regarding the effects of redox on SOCE (CRAC) near the beginning of the manuscript to help establish context. Madesh's group reported that mitochondrial ROS enhanced SOCE by glutathionylation of STIM1. Bogeski's group, referred to as supporting their model, reported that EXTRACELLULAR ROS inhibited ORAI. Here, the authors suggest that mitochondrial ROS via the activity of the NCLX, inhibits ORAI mediated Ca2+ influx, which seems to not mesh with published data.

3. A major problem is the CRAC measurements, which are critical to interpretation, as noted above. They are quantitatively really miniscule....the current responses to Gd3+ inhibition are barely perceptible, at a level that could be accounted for by effects on seal resistance....and they are smaller than the small numbers indicated in the bar charts. Most investigators have not been able to record CRAC currents in 293 cells, and certainly have not been able to determine with enough biophysical confidence that the currents recorded are indeed CRAC currents. Nothing here has proven these currents are ORAI dependent. It is not obvious to this reviewer that the authors can be confident that these are CRAC currents and not a Gd sensitive Na "leak" current...certainly the authors measure a Na+ leak with their fluorescence probes.

4. Also surprising is the model, since it assumes, I think, a rapid cycling of Ca2+ across the mitochondrial inner membrane under nearly resting conditions, such that inhibition of Ca2+ efflux can have rapid and pronounced effects. Under basal conditions, the cycling is generally low because of gatekeeping of MCU.

5. Regarding Na influx measured here. The authors should be careful with their terminology. In particular the phrase "store-dependent activation of Na influx" is really quite misleading. There is no demonstration here of a store dependence. My guess is that Na influx would be equally triggered by raising cytoplasmic Ca2+ concentration by other means, unrelated to the content of the stores. Furthermore, the CRAC channel is impermeable to Na under physiological conditions.

6. The authors state that SOCE "requires" Na influx. I don't understand this conclusion. NCLX Na affinity is sitting right at resting [Na], as the authors note. Why should higher Na be "required"? Furthermore, there is no rationale for the musings that small increments of Na above basal levels should strongly activate Na/Ca2+ exchange activity...has a regulatory role for Na been demonstrated?

7. Finally, the stated premise (and running title) that NCLX regulates CRAC is misleading. At the end of the day, although I'm not sure it's explicitly stated anywhere, the authors really think that mitochondrial Ca2+ concentration, and how it drives production of ROS, are what regulate CRAC.

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What is shown here are ways to manipulate matrix Ca2+ by manipulating Na efflux from the mitochondria. There is no evidence presented here to show physiological "regulation" of the NCLX that would support this premise.

Specifics, major and minor

1. Fig 1b..what are the units, both for the Ca signal and for the rates? Fura can be calibrated. Furthermore, all data seem to be normalized, but it might be expected that baseline Ca2+ differs in different paradigms, for example following ouabain treatment.
2. Similarly for the Na dye fluorescence. We have no idea at all what the magnitude of changes in [Na] that correspond to the signals observed, so the significance is lost.

3. Fig 2. There is no description in Methods regarding the ORAI and STIM imaging.

4. Fig 2. Regarding the conclusion that normal puncta formation occurs in the NCLX knockdown cells, the figure is not at all convincing. As noted above, my suspicion would be that cells with NCLX knocked down are unhealthy because of mito Ca2+ overload. I would expect this to affect ER Ca2+ homeostasis as well as puncta formation. Thus, it needs to be demonstrated well that the cells remain healthy and that the ORAI-STIM dance occurs normally.

5. Fig 2e current densities seem to be discrepant with the other data.

6. Fig 3a....why didn't the Ca2+ go back to baseline? Is it because the PMCA is not operating well because the cells are not healthy?

7. Fig 4. I don't see the point of doing the experiments with 50 vs 20 mM BAPTA....20 mM is a huge concentration.

8. Figure 5. Obviously, there is a Na influx pathway in the cells employed. It becomes activated during store depletion. How it becomes activated is not clear. It is most likely related to the Ca2+ transient associated with the store depletion protocol. Whatever the pathway is, it is also permeable to Li. The conclusion that this somehow equates to evidence in favor of involvement of the NCLX is premature and unwarranted. It simply reflects the fact that the plasma membrane in permeable to both Na and Li.
a. Given that, it would not be surprising, from a thermodynamics perspective, for the NCLX to operate more strongly when Na influx into the cell is enhanced. However, it seems surprising that in the whole cell configuration, enough Na could enter the cell in the face of continuous dialysis to change Na concentration to such a degree to have a major effect on NCLX activity.
9. Furthermore, the correlation between Na entry, Na influx into mitochondria with inhibition of SOCE is simply that...a causal relationship is not demonstrated.

10. Fig 6a...the Ca2+ transient associated with store depletion well over by 6 min, yet the Na pump has not pumped the Na back down to control levels. Either Na isn't what's being recorded, or the cells are sick.

11. Fig 6c. Here, I question again what the dye is measuring. Why would [Na] fall by activation of a Ca2+ signal? This is unprecedented, I think. Furthermore, its's observed in both the presence and absence of extracellular Na.

12. Fig 6g. There is no correction for non-mitochondrial corona red fluorescence.

13. Fig 7a,b. Normally one would expect enhanced mitochondrial ROS production with Ca2+ entry into the matrix, resulting in more oxidized situation, whereas enhanced expected NADH production would favor a more reduced environment. The transient redox change observed correlates with the Ca2+ transient, would be most consistent with the major signal being driven by enhanced NADH production. The authors focus on the ROS and suggest that that the opposite takes place based on the roGFP signal. Why?
14. They show that KD of NCLX promotes a less reducing response...I think their model is that now mitochondria have more Ca2+ in them, which drives more ROS production...is this correct? Thus, they propose that NCLX activity would LIMIT "free radical bursts".

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15. Fig 7c. The authors show only normalized data so it is impossible to know, quantititatively, what catalase expression does to redox potential of the mitochondria, but one might expect it to be constitutively altered. Thus, interpretation of changes associated with mitochondrial Ca2+ transients is a bit probelematic. In addition, the controls here seem to behave differently thatn those in panel a,b

1st Revision - authors' response 05 June 2016

Response to reviewers

We would like to thank the reviewers for their thorough review and constructive suggestions on our manuscript that we have fully addressed as described below:

Reviewer 1

1) It is interesting that the effect of NCLX knockout on CRAC is observed with 20 and even 50 mM BAPTA in the pipette solution. It is difficult to see how the model in Figure 8 operates under these conditions. It is unlikely that the mitochondrial matrix has any Ca2+ left or that redox is affected by changes in matrix Ca2+. At such high BAPTA mitochondrial Ca2+ is likely very low already and not affected much by NCLX. This does not exclude an effect of NCLX on mitochondria ROS and regulation of CRAC, but raises the possibility that a component of reduced CRAC/SOCs due to NCLX knockdown is independent of mitochondrial redox state. This possibility is further suggested by the additional reduction in SOCs by knockdown of NCLX in cells incubated with NMDG (Figure 5). This possibility should be examined more rigorously. For example, analyze more carefully and quantitatively the level of STIM1 and the Orai1 and their clustering. Additionally, the authors should determine if NCLX knockdown affect STIM1-Orai1 Co-IP in untreated and store depleted cells and/or the time course and extent of FRET between STIM1-Orai1.

The reviewer raises a very important issue. As a result, we have now completely revised the analysis of the STIM1/Orai1 interaction using high resolution confocal and FRET analysis shown in Fig. 2 and concluded that there is no change in the STIM1/Orai1 interaction following knockdown of NCLX. This is consistent with Orai1 being the target of redox regulation by NCLX as shown in the new Fig. 8.

2) It is surprising that the authors did not perform similar experiments with expressed STIM1 and Orai1. This should be tested and then use the STIM1 and Orai1 cysteine mutants that are redox resistant and show that the CRAC current by the mutants is not affected either by NCLX knockdown, Na^+ influx or mCatalase expression.

We thank the reviewer for raising this important issue. While we have mainly focused on endogenous SOCE and CRAC, following the reviewer suggestion we have demonstrated that the effect of NCLX knockdown on SOCE and CRAC currents are also evident when STIM1 and Orai1 are overexpressed in HEK293T cells. We have also identified Cysteine-195 on Orai1 as the target of NCLX-mediated ROS regulation (see new Fig. 8). This represents a substantive addition to the impact of the paper.

3) *It is difficult to understand the Ouabain experiments. It seems that pre-incubation in the absence of external Na^+ should have depleted all internal Na^+ prior to addition of external Ca^{2+}. Additionally, if Na^+ remained elevated, it is not clear how the authors excluded the possibility that the enhance Ca^{2+} influx is mediated by plasma membrane NCX. It would be helpful to show if Ouabain treatment has the same effect of CRAC current.*

We apologize for our failure to describe the rationale for the *Ouabain* experiment. We now have clarified how the Ouabain experiments were performed. In fact, the incubation with Ouabain was performed in the presence of Na^+ and therefore following the inhibition of the Na^+/K^+ ATPase, cytosolic Na^+ is increased as now demonstrated by direct Na^+ measurements in the cytosol (please see Supplementary Fig. 4). Further, we now include a new set of experiments in which CRAC currents were measured in the presence or absence of increasing concentrations of cytosolic Na^+

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delivered through the pipette and show that concentrations of Na^+ between 10-15 mM are required for full activation of CRAC currents (Fig. 5h).

4) *If the interpretation offered by the authors for the removal of Na^+ and Ouabain experiments is correct, then elevation of pipette Na^+ should enhance CRAC current both in control cells and cells treated with siNCLX. Under these conditions removal of external Na^+ and Ouabain should have no effect on the current and the current should not be affected by manipulation of the mitochondria redox potential.*

This was another important issue that has allowed us to significantly clarify and improve the paper. Our results suggest that Na^+ is acting through NCLX. We show that the full activation of NCLX is dependent on Na^+ influx (Fig. 6c) therefore NCLX is tuned to sense physiological changes in Na^+ and its activity is strongly affected by these changes. Our findings are based on several previous studies showing that the affinity of NCLX for Na^+ is low and is therefore strongly activated by Na^+ influx (Paucek and Jaburek 2004, Nita, Hershfinkel et al. 2014). We are also relating to this issue in the introduction and discussion see (pp. 3 and 14-15).

5) *Figure 7: The authors should determine how expression of mCat affect the inhibition of SOCE/CRAC due to removal of external Na^+ and treatment with Ouabain.*
Following the reviewer's suggestion, we have performed the experiments and show that mCat rescues SOCE even when Na^+ is omitted, consistent with the signaling cascade and the ability of mCat to rescue the redox response in the absence of Na^+ as shown in Supplementary Fig. 8.
6) *For the experiments in Figure 7 to be interpreted as suggested, mCatalase activity should be Ca^{2+}-dependent. Is it?*

This comment by the reviewer again allows us to clarify and enhance the paper's impact. There are several studies which show regulation of mCat (Naziroglu 2012, Littlejohns, Pasdois et al. 2014). However, the H_2O_2 clearing rate of catalase is mainly dependent on the levels of expression of catalase (Rodriguez, Carrico et al. 2000). Therefore, over-expressed catalase targeted to the mitochondria (as described here) is expected to enhance H_2O_2 detoxification in the mitochondria generated by abnormal mitochondrial Ca^{2+} rise. We have now clarified this issue and revised the discussion accordingly (p.16-17).

Reviewer 2
This was a difficult paper to read and understand, for many reasons, including poor description of the experimental rationale, protocols and results. In addition, many of the experimental paradigms suffer from variables that are not well considered or controlled. Finally, some key experiments are missing.

We accept that the original paper was not well described and the experimental paradigms were not clear. As suggested by the reviewer, we have now revised the text and figure legends to improve the overall clarity and offer a strong rationale for the experiments performed.

1) *The basic observation is that genetic knockdown of the mitochondrial Na^+/Ca^{2+} exchanger NCLX reduces store-operated Ca^{2+} influx, mediated in the cell types examined presumably completely by ORAI, since the only Ca^{2+} currents measured appear to be consistent with CRAC currents. (Thus, the speculation later that Trp channel activation contributes to the elevated Na^+ seems like it must be incorrect). The Ca^{2+} and Na^+ current measurements are critical, because knockdown of the NCLX is likely wreaking havoc with cell health, as the mitochondria must be overloaded with Ca^{2+}. Accordingly, plasma membrane potential is likely quite different between the control and knockdown cells, and the Ca^{2+} influx measured by fluorescence will reflect that in part. The authors compound this potential problem by conducting experiments with altered extracellular cation composition. What is missing the CRAC current measurements, where membrane potential and ionic compositions are controlled with changes of Na^+ or Li^+ in the pipette solution to determine if there is indeed an effect, in the physiological range, of Na^+ on CRAC currents.*

We thank the reviewer for these important comments which have prompted us to be able to clarify and greatly improve the paper. We would respectfully point out that our studies do not suggest that

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"*store-operated Ca^{2+} influx is mediated, in the cell types examined, presumably completely by Orai1*. Ca^{2+} measurements using the SOCE protocol while blind to increased cytosolic Na^+ could contain a small contribution of Ca^{2+} entry through non-selective cation channels. Note that the bulk of ions passed by TrpC channels will be Na^+ ions. For CRAC measurements, the patch clamp protocol with strong cytosolic Ca^{2+} buffering is not ideal for revealing TrpC currents, especially when small endogenous currents are involved. Regarding the role of membrane potential, the use of whole-cell patch clamp electrophysiology would eliminate any contribution of membrane potential to the decrease in SOCE/CRAC upon NCLX knockdown. As suggested by the reviewer, we have performed additional experiments with varying concentrations of Na^+ in the patch pipette and reveal that Na^+, at physiological range (10-15mM), is required for optimal CRAC function (Fig. 5h). We also show that store-depletion is accompanied by a rise in cytosolic Na^+ (Fig. 6a-b) and mitochondrial Na^+ (Fig. 6c-d)

2) *The authors need to do a better job of reviewing the literature regarding the effects of redox on SOCE (CRAC) near the beginning of the manuscript to help establish context. Madesh's group reported that mitochondrial ROS enhanced SOCE by glutathionylation of STIM1. Bogeski's group, referred to as supporting their model, reported that EXTRACELLULAR ROS inhibited ORAI. Here, the authors suggest that mitochondrial ROS via the activity of the NCLX, inhibits ORAI mediated Ca^{2+} influx, which seems to not mesh with published data.*

We apologize that the former paper did not accurately describe some of the former work. We have now revised this part in the introduction and discussion relating to the literature on redox regulation of SOCE. Specifically, the Madesh and Bogeski studies (Bogeski, Kummerow et al. 2010, Mancarella, Wang et al. 2011) invoked by the reviewer are not necessarily contradictory to our findings. ROS-mediated enhancement of STIM1 glutathionylation and subsequent enhancement of SOCE might represent an alternative mechanism that counteracts direct ROS-mediated inhibition of Orai1. Bogeski and Co-workers identified Cys195 located on the second extracellular loop of Orai1 as the target of extracellular ROS. This is the same cysteine that mediates the inhibitory effects of endogenous ROS on Orai1 in our studies (Fig. 8). Because the superoxide anion is quickly dismutated into hydrogen peroxide, which is liposoluble and can cross biological membranes, H_2O_2 is likely the specific ROS acting on cysteine-195 in agreement with our experiments overexpressing mitochondria-targeted catalase (Fig. 7).

3) *A major problem is the CRAC measurements, which are critical to interpretation, as noted above. They are quantitatively really miniscule....the current responses to Gd^{3+} inhibition are barely perceptible at a level that could be accounted for by effects on seal resistance....and they are smaller than the small numbers indicated in the bar charts. Most investigators have not been able to record CRAC currents in HEK293T cells, and certainly have not been able to determine with enough biophysical confidence that the currents recorded are indeed CRAC currents. Nothing here has proven these currents are ORAI dependent. It is not obvious to this reviewer that the authors can be confident that these are CRAC currents and not a Gd^{3+} sensitive Na^+ "leak" current...certainly the authors measure a Na^+ leak with their fluorescence probes.*

We thank the reviewer for this constructive criticism. Please also see our response to Comment #2 from reviewer 1. We agree with the reviewer that the endogenous CRAC currents are small. However, we feel it is important to study native channels and use overexpression only to shed light on native conditions. We also agree that most investigators have not been able to record native CRAC currents in HEK293T cells. However, a few laboratories including our own have been able to reliably record such currents from HEK293T cells as well as from a number of primary cells with great confidence. These currents have been extensively characterized in our studies (including in HEK293T cells) with inhibitors, molecular knockdown of Orai1 and STIM1 and different patch clamp protocols and recording conditions/pipette and bath solutions (Zhang, Zhang et al. 2014). Briefly, we do not record from cells where seal resistance is below 16GΩ and Na^+ leak in divalent-free (DVF) solutions is accounted for and subtracted by a first DVF pulse run before store depletion has taken place. Further, the I/V relationships of these currents, the reversal potential and the depotentiation in DVF bath solutions are unlikely those of a coincidental "leak" current. Nevertheless, we now show huge CRAC currents in HEK293T cells co-expressing STIM1 and Orai1 which are also inhibited by NCLX knockdown and rescued when cysteine-195 is mutated to serine (C195S; Fig. 8d-i). Note that we also extended our analysis to two additional cysteines on Orai1 (C126S and C143S, see Fig. 8 j-l)

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We believe these overexpression experiments demonstrate that CRAC currents are regulated by NCLX and mitochondrial ROS to the level requested by this reviewer.

4) *Also surprising is the model, since it assumes, I think, a rapid cycling of Ca^{2+} across the mitochondrial inner membrane under nearly resting conditions, such that inhibition of Ca^{2+} efflux can have rapid and pronounced effects. Under basal conditions, the cycling is generally low because of gatekeeping of MCU.*

This is another insightful comment by the reviewer, raising an important issue which is often ignored: the mitochondrial Ca^{2+} transient initiated by MCU reflects only ~5% of the total mitochondrial Ca^{2+}. Approximately 95% of the mitochondrial Ca^{2+} pool is an insoluble/soluble pool consisting mainly of Ca^{2+} phosphate salt. The solubility of the latter pool is strongly pH-dependent and therefore any change in matrix pH, by metabolic activity for example, will trigger a matrix Ca^{2+} change. Mitochondrial Ca^{2+} sensitive dyes are however less than optimal (Pizzo, Drago et al. 2012). Therefore, many of these changes are often unnoticed when undertaking mitochondrial Ca^{2+} imaging. Indeed, our pervious and current results show that the apparent mitochondrial Na^{+} fluxes (which in contrast to Ca^{2+} are unbuffered) are much longer than the mitochondrial Ca^{2+} response (Palty, Silverman et al. 2010, Nita, Hershfinkel et al. 2014), indicating that NCLX is pumping out Ca^{2+} for much longer intervals than the "apparent" mitochondrial Ca^{2+} transients. Finally in contrast to MCU, NCLX is dually linked to Ca^{2+} and Na^{+} signaling which we show here is critical for SOCE response. Thus, mitochondrial Ca^{2+} and Na^{+} responses that trigger redox changes could be considered, at least partly, to be independent of MCU.

5) *Regarding Na^{+} influx measured here. The authors should be careful with their terminology. In particular the phrase "store-dependent activation of Na influx" is really quite misleading. There is no demonstration here of a store dependence. My guess is that Na^{+} influx would be equally triggered by raising cytoplasmic Ca^{2+} concentration by other means, unrelated to the content of the stores. Furthermore, the CRAC channel is impermeable to Na^{+} under physiological conditions.*

We fully agree that CRAC channels are impermeable to Na^{+} under physiological conditions and propose in our discussion and model that Na^{+} influx is mediated through a distinct pathway that may well be Ca^{2+}-activated as suggested by the reviewer. Our assertion is supported by several studies cited in our manuscript that store-dependent Ca^{2+} influx is associated with Na^{+} influx through TRP channels, some isoforms of which (e.g. TRPC5) are Ca^{2+}-activated (Poburko, Liao et al. 2007, Baryshnikov, Pulina et al. 2009) (p.4). Following the reviewer's suggestion, we have revised the terminology to store-depletion associated, instead of Na^{+} dependent $Ca2^{+}$ influx.

6) *The authors state that SOCE "requires" Na^{+} influx. I don't understand this conclusion. NCLX Na^{+} affinity is sitting right at resting [Na], as the authors note. Why should higher Na^{+} be "required"? Furthermore, there is no rationale for the musings that small increments of Na^{+} above basal levels should strongly activate Na^{+}/Ca^{2+} exchange activity...has a regulatory role for Na^{+} been demonstrated?*

Please see our response to reviewer 1. We and others have previously shown that the affinity of NCLX for Na^{+} is physiologically tuned and regulated to sense and respond to small changes in cytosolic Na^{+}. Our results are consistent with previous studies indicating that the Na^{+} change is associated with store depletion and activates NCLX leading to redox control which is required for the full activation of CRAC that we now show in Fig. 9 is linked to the redox sensitive cysteine on Orai1.

7) *Finally, the stated premise (and running title) that NCLX regulates CRAC is misleading. At the end of the day, although I'm not sure it's explicitly stated anywhere, the authors really think that mitochondrial Ca^{2+} concentration, and how it drives production of ROS, are what regulate CRAC. What is shown here are ways to manipulate matrix Ca^{2+} by manipulating Na^{+} efflux from the mitochondria. There is no evidence presented here to show physiological "regulation" of the NCLX that would support this premise.*

We thank the reviewer for raising this issue. We agree that NCLX-mediated control of Na^{+} and Ca^{2+} underlines a communication between mitochondria and CRAC channels and we have revised this accordingly.

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Specifics, major and minor

1) and 2) *Fig 1b..what are the units…*

Calibration of Ca^{2+} concentration was done in quite a few studies. However, studies that addressed Ca^{2+} calibration validity with Fura-2 showed that it is flawed because of several reasons. Among them is the need to use Ca^{2+} inophore (Reynolds and Dubyak 1986) and the non-linear nature of the equation used for Ca^{2+} calibration. Therefore considering the pitfalls and inaccurate nature of the calibration most studies are avoiding this step and the same holds for Na^{+}. We are using the very conventional F_1/F_0 presentation mode which provide a ratio between basal and response values using raw data.

3) *Fig 2. There is no description in Methods regarding the ORAI and STIM imaging*.

We apologize for this. Details on how imaging was performed are now included and is now described in the methods section; pp. 22-23.

4) *Fig 2. Regarding the conclusion that normal puncta formation occurs in the NCLX knockdown cells, the figure is not at all convincing.*

We have revised this part thoroughly with new imaging experiments which now include FRET measurements of STIM1/Orai1 interactions upon NCLX knockdown (Fig. 2). Please see also our response to reviewer1

5) *Fig.2. Regarding current densities in Fig.2e that seem to be discrepant with other data.*

We thank the reviewer for this comment. Note that in the revised version of the paper it is now Fig 1. In fact, the representative I-V curves shown in figure 1d, lower pannel are taken from the representative time course traces in figure 1d, upper panel and 1e and are therefore a match. However, these representative I-V curves do not match the mean value in the bar graph from figure 1f which represent mean+/-SE from 13 independent recordings per experimental condition. In this revised version, we have clarified in the legend to Fig.1 that what is shown in Fig. 1d-e are representative I-V curves and time courses, while Figure 1f is statistical analysis of peak currents at -100mV from 13 different recordings.

6) *Fig 3a....why didn't the Ca^{2+} go back to baseline? Thank* you for raising this issue.

The traces in this figure were not good representative traces. Therefore, we replaced them in Fig. 3a with traces which are better reflecting the typical Ca^{2+} response of our measurements .

7) *fig 4. I don't see the point of doing the experiments with 50 vs 2*0 mM…

While we agree with the reviewer that 20mM BAPTA is already a huge concentration, a very close and tight interaction might require even stronger buffering. Since experiments were already performed, we think it appropriate to include these data.

8) *Figure 5. Obviously, there is a Na^{+} influx pathway in the cells employed…*

Please note that the unique functional properties of NCLX compared to other NCX members is that it catalyzes Li^{+}/Ca^{2+} exchange whereas the rest are inert to Li^{+} . Therefore the fact that Li^{+} support NCLX regulation of SOCE strongly supports a role for NCLX. However the rate of Li^{+}/Ca^{2+} exchange by NCLX is a bit slower then Na^{+}/Ca^{2+} exchange and this unique property is remarkably reflected on the effect of Li^{+} acting by NCLX on SOCE. (Carafoli, Tiozzo et al. 1974, Palty, Silverman et al. 2010, Nita, Hershfinkel et al. 2012, Nita, Hershfinkel et al. 2014). This issue is addressed in the results and in the discussion (pp. 10, 15)

9*) Furthermore, the correlation between Na^{+} entry, Na^{+} influx into mitochondria…*

Please see our comment to reviewer 1 and note that we have now complemented the imaging with electrophysiological data and report a dependence of SOCE on cytosolic Na^{+}, supporting the requirement for a rise in cytosolic Na^{+} which then acts through NCLX to fully activate SOCE and CRAC currents.

10) *The Ca^{2+} transient associated with store depletion well over by 6 min, yet the Na^{+} pump has not pumped the Na^{+} back*.

Thank you for raising this important issue. The traces in Fig. 6d were not good representative traces. Therefore, we replaced them with traces which better reflect the typical Na^{+} response of our measurements.

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11) *Here, I question again what the dye is measuring. Why would [Na^+] fall by activation of a Ca^{2+} signal*?
Thanks you for raising this issue. This change is apparent in the presence or absence of Na^+ and therefore a fluorescence artifact. We now address this in the methods section (p.21).

12*) There is no correction for non-mitochondrial Corona red fluorescence*.
Several studies show that corona red is selectively targeted to the mitochondria (Yang, Pan et al. 2004, Baron, Caplanusi et al. 2005). Nevertheless to ascertain that it reaches mitochondria in our experimental setup, we co-stained cells with Corona red and Mitotracker green and show excellent mitochondrial co-localization of both dyes (see supplementary Fig. 7)

13) *Normally one would expect enhanced mitochondrial ROS production with Ca^{2+} entry into the matrix, resulting in more oxidized situation, whereas enhanced expected NADH production would favor a more reduced environment.*
Following the reviewer suggestion we have also monitored the change in NADH/NAD^+ ratio in mitochondria and show in Fig. 7b that the reduced form NADH is strongly diminished following NCLX knockdown, consistent with increased oxidative load triggered by reduction in NCLX expression.

14) *They show that KD of NCLX promotes a less reducing response…*
Indeed, we agree with the terminology suggested by the reviewer; NCLX reduced the oxidative load or bursts and knocking it down will increase them. We have clarified this and revised the text accordingly (p. 12).

15) *The authors show only normalized data so it is impossible to know..,*
The data are in fact calibrated to max and min oxidation for each trace at the end of the experiments by superfusing the cells with excesses H_2O_2 and DTT. Please note that we show representative traces that are prone to variability. Following the reviewer suggestion we rechecked the basal redox state of SiNCLX vs SiNCLX+mcatalase expressing cells and found that for siNCLX it is slightly higher (more oxidized), However, it is not a significant difference. We present them now as supplementary Fig. 8c.

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2nd Editorial Decision 25 July 2016

Thank you for submitting a revised version of your manuscript and my apologies for the extended duration of the re-review. Your manuscript has now been seen by the two original referees and their comments are shown below.

As you will see ref #1 finds that all criticisms have been sufficiently addressed and recommends the manuscript for publication, while ref#2 still has a number of concerns, particularly around the functional/physiological context of the reported effects. In light of the rather conflicting recommendations from the referees I conducted a round of cross-referee comments and received the following feedback from ref #1:

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'Obviously my opinion is different from that of the other reviewer. Some of the comments were raised in the original review and some are new. I thought that the authors did address the major criticism rather well and that the manuscript does address a long-standing issue in the field of the relationship between Ca2+ influx channels and mitochondrial Ca2+ homeostasis. The way I read the comments is that reviewer #2 raises two major points; does Na+ change during cell stimulation and what is the mechanism by which Na+ enter the cells during stimulation. It is true that the authors did not analyze Na+ influx rigorously in the HEK cells that they used but I do not see this as a major problem since this is only a model system to describe the relationship between Ca2+ influx by Orai1 and mitochondrial Ca2+. Changes in cellular Na+ in various stimulated native cells from neurons to muscle cells to secretory epithelial cells is extensively documented in the literature and thus I view the findings in the manuscript in this context. As to the suggestion that Na+ influx is mediated by TRPC channels, this is only a suggestion and not a central point of the manuscript since Na+ influx by any transporter, whether by TRPC channels, the NaCl, NaK2Cl , Na-Glucose, Na-amino acids cotransporters or Na/H+ exchanger will affect cytoplasmic Na+ and thus mitochondrial Na+/Ca2+ exchange by NCLX. The other points are minor and can be addressed by changes to Figure presentation or the text. '

At the same time ref #2 remained critical and stated the following (after seeing the comments from ref #1)

'In response to the other reviewer.... if we take the line of arguments far enough, then experiments would not seem to be necessary...we could simply reason our way to the conclusions. The fact is that they do not record a change in Na concentration in these cells but nevertheless observe an effect regarding NCLX. This goes to the heart of the manuscript. I agree that Na concentration rises in many cells associated with cell activation, but that is not the case here, and yet NCLX phenomena are observed. So, experimentally there is a major disconnect. The reviewer wants us to ignore this by postulating that this is simply a "model system", but since there is no rise in Na here, then it is not a very good model at all. Other issues were also raised in my review.
Consequently, I don't believe that the authors have adequately addressed issues that I raised in my review that I believe go to the heart of the conclusions and the model that is developed. '

In light of both the referee reports and these additional comments, I would like to invite you to submit a revised version of the manuscript in which you comment on the remaining concerns raised by ref #2 and - if possible - address them with further experimental date. From our side, we value the strong support from ref #1 in this case but would nonetheless ask you to discuss the issues of broader functional relevance raised by ref #2.

REFEREE REPORTS

Referee #1:

The author did really a good job addressing the reviewers' comments. The additional experiments provided clear several points and extend the significance of the finding in highlighting how ROS generated by the mitochondria regulates Orai1 and the role of NCLX in this activity. I am satisfied with the revised version and find the manuscript suitable for publication in EMBO Journal.

Referee #2:

I am not convinced of this model, in which it is proposed that calcium store-depletion activates a Na influx that is sufficient to drive Ca2+ out of the mitochondrial matrix, affecting ROS production there that in turn regulates ORAI channel gating.

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1. First, the lack of Na concentration dependence in the whole cell recordings is a major problem. An effect is observed only at resting [Na]i. This suggests that the model is not correct

2. There is no delta [Na] in these cells when they're activated (unless the Na pump is inhibited). Fig 6a contradicts Fig S4. The data suggest if there is any delta [Na], it's minimal in the studies..if the authors wish to argue otherwise, calibrations are required. Furthermore, CRAC is Na-impermeable. In addition, the authors attribute Na influx to Trp channels, but that was not shown to be activated in the present studies that recorded, according to the authors, only and specifically CRAC currents. This is the second major problem.....Trp mediated Na influx, measured electrically and with optical indicators, appears to be absent in the cells used.

3. There are no measurements of mito Ca2+ when stores are depleted and Ca2+ is re-introduced. This is important, because under basal conditions the uniporter permeability is low and rate limiting, in contrast to the authors' assertions...and even up to reasonably high cyto [Ca], for example a couple of μM, the NCLX can keep up fine at resting [Na]. It will only become limiting when cyto Ca2+ rises high enough to activate the uniporter (several μM). Thus, the question is, does mito Ca2+ rise during the re-introduction phase of SOCE sufficiently to activate Na/Ca2+ exchange that is rate limited by [Na]? AND: does any of this work under physiological conditions in which complete store depletion is not part of the protocol?

4. The results of Fig 7a and 7b seem very counterintuitive, given that the probes are reporting on mito properties, presumably responding to Ca, since under these conditions, mito [Ca], as shown in Fig S6, is bigger in the KD cells. On the one hand, the authors claim that the oxidation rate is reduced with NCLX KD, but on the other they also suggest less NADH production, i.e. less reduction.

5. Fig 1A requires quantification

6. Why should the results in Fig 1b and 1c be so different?

7. What is the consequence of NCLX knockdown on resting mito [Ca2+]? Are they over-loaded?

8. Fig 3b-c... the representative current traces are large by comparison with the averages, whereas the error bars are small with a small n. Seems like a contradiction.

9. State the cell type used for experiments in Fig. S3.

10. Fig 5h... the current densities are HUGE, and much bigger than any others before this....is this due to over-expression?

11. Fig 7a. figure legends say that rates are plotted, but the numbers shown are not rates. If they are rates, the trace in 7a suggests that there is no difference.
ALSO, the result is bi-phasic...which is being reported ?

12. Fig 8b. in patching, how does the experimenter know which cells are expressing the transfected catalase?

2nd Revision - authors' response 20 September 2016

A point by point response to reviewers' comments:

We would like to thank the reviewers for their thorough review and constructive suggestions on our manuscript. We have now fully addressed the remaining comments, as described below:

Reviewer 2
1. First, the lack of Na+ concentration dependence in the whole cell recordings is a major problem. An effect is observed only at resting [Na]i. This suggests that the model is not correct

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This is an important issue raised by reviewer 2. Previous studies performed in our lab in Min6 cells demonstrated that mitochondrial Ca2+ efflux rates are Na+ dose dependent (Nita, Hershfinkel et al., 2014). Nevertheless, to address reviewer 2 comment, we conducted a similar experiment in HEK293T cells that shows Na+ concentration dependence of NCLX activity, which is now shown in Fig. 6f. The Na+ effect reviewer 2 mentioned in her/his comment that is observed only at resting [Na]i is on CRAC current measurements, not on NCLX activity. Since knockdown of NCLX or NMDG+ replacement do not fully abrogate CRAC currents, we don't think these data necessarily mean that the model is incorrect. In other words, CRAC currents would be expected to be inhibited when cytosolic Na+ drops below the physiological 5-10 mM or when NCLX is impaired; however, if cytosolic Na+ increases and NCLX activity is enhanced, CRAC current activity need not to be enhanced as well. Consisted with this model, we now show that NCLX activity is highly tuned by small and physiologically relevant changes in cytosolic Na+

2. *There is no delta [Na] in these cells when they're activated (unless the Na pump is inhibited). Fig 6a contradicts Fig S4. The data suggest if there is any delta [Na], it's minimal in the studies..if the authors wish to argue otherwise, calibrations are required. Furthermore, CRAC is Na-impermeable. In addition, the authors attribute Na influx to Trp channels, but that was not shown to be activated in the present studies that recorded, according to the authors, only and specifically CRAC currents. This is the second major problem.....Trp mediated Na influx, measured electrically and with optical indicators, appears to be absent in the cells used.*

We thank the reviewer for noticing this discrepancy, which is an unfortunate mistake on our part. In fact, Fig.S4 corresponds to Ouabain alone without ATP and TG. As expected, Ouabain (through inhibition of the Na+/K+ ATPase) triggers a rise in [Na]i that is not observed in control non-treated cells. We have now revised Fig. S4.

In the manuscript, we provided no evidence to either support or rule out TRPC channels as the mediators of Na+ entry into the cytosol from the extracellular space. We merely speculated that TRPC channels might be involved but believe that additional studies, beyond the scope of this manuscript, should address this issue. While previous studies demonstrated that TRPC6 activation results in Na+ entry in response to activation of Ca2+ entry in purinergically stimulated smooth muscle and HEK cells (Goel, Sinkins et al., 2005, Poburko, Liao et al., 2007, Soboloff, Spassova et al., 2005), we cannot discount that other pathways may contribute. For example, we have previously shown (Nita et al., 2014) that the voltage-gated Na+ channel is mediating Na+ influx required for the activation of NCLX in pancreatic beta cells. Collectively, our Na+ and Ca2+ recordings in cytosol and mitochondria, our data with Ouabain, Li+ and NMDG+ replacement and our data with NCLX knockdown suggest that cytosolic Na+ is required for NCLX activity and optimal CRAC current activation. The data overall support the model that NCLX works under physiological cytosolic Na+ concentrations to maintain optimal CRAC activity. Increased cytosolic Na+ would enhance NCLX-mediated Ca2+ shuttling to keep up with agonist-induced rise in cytosolic and mitochondrial Ca2+. Please note that we don't claim that CRAC is dependent on Na+ but our data supports a model whereby CRAC is regulated by NCLX via changes in cytosolic Na+ concentrations, a point that is further supported by data presented in the new fig 6f. We added a paragraph in the discussion that relate to this issue (p. 16, 17) and revised the scheme accordingly.

3. *There are no measurements of mito Ca2+ when stores are depleted and Ca2+ is re-introduced. This is important, because under basal conditions the uniporter permeability is low and rate limiting, in contrast to the authors' assertions...and even up to reasonably high cyto [Ca], for example a couple of μM, the NCLX can keep up fine at resting [Na]. It will only become limiting when cyto Ca2+ rises high enough to activate the uniporter (several μM). Thus, the question is, does mito Ca2+ rise during the re-introduction phase of SOCE sufficiently to activate Na/Ca2+ exchange that is rate limited by [Na]? AND: does any of this work under physiological conditions in which complete store depletion is not part of the protocol?*

In this work, we examined the effects on store operated Ca2+ entry induced by emptying stores with the SERCA pump blocker, Thapsigargin combined with ATP. To establish physiological relevance, per reviewer 2 suggestion, we have now monitored the Ca2+ responses in mitochondria using purinergic stimulation by ATP alone with or without Na+ and show a very similar effect to ATP + TG (Fig. 6e). This figure also shows measurements of mito [Ca2+] when stores are depleted and Ca2+ is reintroduced as suggested by reviewer 2. Consistent with our model, absence of Na+ led to

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lower mitochondrial Ca2+ efflux than the control (with Na+) and the combination of using NMDG+ ringer together with silencing NCLX resulted in the lowest mitochondrial Ca2+ efflux rate. We also revised the version of results; see (p.12).

4. *The results of Fig 7a and 7b seem very counterintuitive, given that the probes are reporting on mito properties, presumably responding to Ca, since under these conditions, mito [Ca], as shown in Fig S6, is bigger in the KD cells. On the one hand, the authors claim that the oxidation rate is reduced with NCLX KD, but on the other they also suggest less NADH production, i.e. less reduction.*

Fig. 7b shows that the knockdown of NCLX causes an increase of oxidative load (please see arrows for directions of oxidation/reduction). Consistent with this, the knockdown of NCLX is also followed by a shift form the reduced NADH to NAD+. These results are consistent with an increase in oxidative burden leading to oxygen radical formation following knockdown of NCLX expression.

5. *Fig 1A requires quantification.*

We thank the reviewer for this suggestion. We updated the figures and added quantification of the western blot (see Fig. 1a, lower panel).

6. *Why should the results in Fig 1b and 1c be so different?*

Thank you for raising this important issue. In Fig. 1b, we monitor the cytosolic Ca2+ using Fura-2. In Fig. 1c we use the plasma membrane-targeted Calcium sensor, GCamp5. We have employed the same experimental paradigm in cells expressing GCamp5 to determine the localized change in Ca2+ concentration at the plasma membrane. One likely explanation is that this localized Ca2+ influx signal is monitored before the intervention of many other pumps and transporters that are further changing cytosolic Ca2+ regulation. Furthermore, the dynamic range and affinity and sensitivity to Ca2+ of Gmap5 versus Fura-2 are quite different which may contribute to the differences between the results obtained with these two indicators.

7. *What is the consequence of NCLX knockdown on resting mito [Ca2+]? Are they over-loaded?*

We thank the reviewer for raising this important issue. We examined the non-normalized data of fig. S6, which shows that shNCLX transfected cells are not overloaded with [Ca2+] (See new fig.S6). This finding is also consistent with our pervious study showing that the knockdown of NCLX expression was not followed by a change in mito resting [Ca2+] (Palty, Silverman et al., 2010).

8. *Fig 3b-c... the representative current traces are large by comparison with the averages, whereas the error bars are small with a small n. Seems like a contradiction.-*

We thank the reviewer for raising this issue. What is represented in the bar graphs are Na+ CRAC currents recorded in DVF solutions. The basal current recorded in the first DVF pulse immediately after break-in (before store depletion has taken place) is always subtracted from the second DVF pulse, thus explaining why the currents are smaller. This has been clarified in p. 6-7.

9. *State the cell type used for experiments in Fig. S3.*

Thank you for your comment. The cells are HEK293T and we updated it in the text.

10. *Fig 5h... the current densities are HUGE, and much bigger than any others before this....is this due to over-expression?*

Indeed, the legend to fig. 5h and the results section mention that these recordings were performed with cells ectopically expressing STIM1 and Orai1, thus generating large CRAC currents.

11. *Fig 7a. figure legends say that rates are plotted, but the numbers shown are not rates. If they are rates, the trace in 7a suggests that there is no difference.*
ALSO, the result is bi-phasic...which is being reported?

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Thank you for noticing. We updated the figure legend. The numbers are not rates, but redox changes and we have revised it accordingly. Please note that several other studies also report a biphasic mitochondrial oxygen radical response; see for example (Dooley, Dore et al., 2004). Therefore our results which are highlighted by the rectangle in traces from fig. 7a and clarified in the legend to fig. 7 are consistent with previous studies.

12. *Fig 8b. in patching, how does the experimenter know which cells are expressing the transfected catalase?*

Our transfection efficiency in HEK293T cells is ~90%. To further ascertain that we are focusing on transfected cells, mCatalase vector is routinely co-transfected with a plasmid encoding eGFP for identification of transfected cells. This has been clarified in legend to fig. 7.

Reviewer 1
We would like to thank reviewer 1 for his positive response on the revised version of the MS.

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3rd Editorial Decision 04 October 2016

Thank you for submitting a revised version of your manuscript. This version and your point-by-point response to the remaining concerns have now been seen by one of the original referees and his/her comments are included below. As you will see the referee supports publication of your work in The EMBO Journal; however, before I can go on to officially accept your study and transfer the manuscript files to our production team I have to ask you to address the following editorial points in a final revision:

-> Please include a scale bar in all microscopy images and mention the size of the bar in the corresponding figure legend.

-> During our routine test for text plagiarism we noticed that several sections in the Material and Methods are rather similar to previously reported work (see attached screenshots). Since we appreciate having extensive methods in our papers this is not a problem per se but I would ask you to include a reference to the original manuscript in the attached examples.

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REFEREE REPORT

Referee #1:

After reading this manuscript for the third time and the response to the remaining reviewers' comments, I remain convinced that the message of the manuscript is strong and timely. The major remaining concerns have been addressed by additional control experiments that confirm the main conclusion that NCLX-mediated pathway coordinates Na+ and Ca2+ signals to effect mitochondrial redox control over SOCE. In my opinion this version of the manuscript is suitable for publication in EMBO.

3rd Revision - authors' response 24 November 2016

Enclosed below are the revisions that we have made according to the journal guidelines as detailed:

1. Please include a scale bar in all microscopy images and mention the size of the bar in the corresponding figure legend.

We included a scale bar in all microscopy images: Fig 1, Fig 2, Fig EV1, and Appendix Fig 3 and added it also to the figure legends.

2. During our routine test for text plagiarism we noticed that several sections in the Material and Methods are rather similar to previously reported work (see attached screenshots). Since we appreciate having extensive methods in our papers this is not a problem per se but I would ask you to include a reference to the original manuscript in the attached examples.

We added more references – In the part of "Fluorescent Ca^{2+} and Na^{+} imaging" (p. 22), we added Palty et al, 2010 and Bisaillon et al, 2010.
In the part of "Föster Resonance Energy Transfer (FRET) Measurements" (p. 24) we added Cai et al, 2016; Navarro-Borelly et al, 2008a; Wang et al, 2014.

4th Editorial Decision 07 December 2016

Thank you for sending the revised version of your manuscript to us.

There are still some editorial points that need to be addressed before I can accept your manuscript for publication here:

- please provide an author checklist. You can download the checklist on our website (here: http://emboj.embopress.org/authorguide)

- please provide the appendix figures as a single document and including a TOC and appendix figure legends => the appendix figure legends thus need to be removed from the main text. Appendix figure callouts need to be 'Appendix Figure S1' etc, please amend your main text accordingly

- please provide further labeling of figure EV1 - the 'before iono' and 'after iono' are now missing

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Israel Sekler, Mohamed Trebak
Journal Submitted to: EMBOJ
Manuscript Number: 2015-92481R2

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?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We calculated the sample size to be used in our research using methods showing on the website below: <http://www.biomath.info>: a simple website of the biomathematics division of the Department of Pediatrics at the College of Physicians & Surgeons at Columbia University.
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?	In priciples smaples were not exlueded unless a celar thechnical problem was found.
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.	Yes, samples were cultured and prepared at the same time. Samples were randomized. For example, from the same plate of cells coverslips were randomized taken for the experiment.
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.	Most of the the experiments were independently carried by several investigators that were carried at differeret dates.
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
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The results of the experiments are presented as the means ± SEM of ≥4 experiments (n), using 5–30 cells in each condition. Statistical significance for all experiments was determined using either unpaired student's t-test or 1-way ANOVA test followed by Tukey post hoc analysis. Values of $p < 0.05$ were considered significant.
Is there an estimate of variation within each group of data?	Within each group of data there is no variation.
Is the variance similar between the groups that are being statistically compared?	There is no difference in the variance between the groups tested.

C- Reagents

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).	Anti-NCLX polyclonal antibody was generated in our lab as previously described (Palty et al, 2010) while anti-STIM1 monoclonal antibody was from BD biosciences, Anti-Orai1 polyclonal antibody was from Alomone labs, and anti-HSC 70 was from Santa Cruz Biotechnology.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All of our cell lines were purchased from ATCC and were derived from the original frozen stocks. We frequently check our cell lines for mycoplasma in our lab using a detection PCR kit (Hy Laboratories Ltd. Rehovot, Israel).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. 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

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No