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# STIM1 is required for attenuation of PMCA-mediated Ca2+ clearance during T Cell Activation

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**Review timeline:**

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| Submission date: | 06 July 2011 |
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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 08 August 2011

Thank you for submitting your manuscript to the EMBO Journal. Three referees have now seen your study and their comments are provided below.

As you can see, the referees appreciate the findings reporting that STIM1 regulates PMCA activity. However, they also find that further experiments are needed in order to provide support for that STIM1 directly regulates PMCA activity and to further delineate the contribution of mitochondria to Ca2+ clearance. Should you be able to address the raised concerns in full we would consider a revised manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision only and that it is important to address the concerns raised at this stage.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

This manuscript reports on the role of STIM1 in the regulation of the plasma membrane Ca2+ pump PMCA. The central finding is that STIM1 and PMCA are recruited to the immunological synapse (IS) upon T cell stimulation, at which STIM1 inhibits PMCA activity. Inhibition of PMCA activity depends on the STIM1 PR domain.

Establishing regulation of PMCA activity by STIM1 can be novel and will provide considerable advance in understanding STIM1 function and Ca2+ homeostasis in the IS. Although the findings in this manuscript are consistent with such a regulation, considerable additional work is needed to establish direct regulation of PMCA activity by STIM1.

1. The increase in PMCA mRNA expression indicates that the main increase is in PMCA4 (likely the regulated 4b). anti-PMCA4b antibodies are available and the authors need to determine whether it is PMCA4b that is upregulated and specifically interacts with STIM1. The authors can also express the PMCA1a and 4b isoforms and determine whether STIM1 specifically regulates the activity of the PMCA4a. Such experiments can strengthen the conclusion of regulation of PMCA by STIM1.

2. Effects of mitochondria and STIM1 on PMCA activity needs to be differentiated better. A minimal set of experiments is to inhibit mitochondria Ca2+ influx by treating the cells with FCCP and with ruthenium red during application of external Ca2+ and assay Ca2+ clearance by PMCA in resting and stimulated T cells.

3. To better analyze the role of STIM1 the authors should determine if expression of constitutively clustering STIM1 mutants that can activate Orai1 and that is not able to activate Orai1 and determine if they can recruit STIM1 to the IS and inhibit PMCA activity. If they do not, then determine if they affect PMCA recruitment by cell stimulation.

4. Although the STIM1 mutant that lack the C terminus that includes the PR domain can activate Orai1, it is not clear that this mutant is recruited to the same microdomain in the IS as STIM1. To address this problem and better establish the role of the STIM1 PR domain the authors shod test the effect of a STIM1 that lacks only the PR domain. Additional analysis should determine whether a STIM1 cytoplasmic fragment that includes and lack the PR domain can interact with and affect PMCA activity.

5. It is likely that inhibition of Ca2+ clearance is confined to the IS. Analysis of global Ca2+ underestimates the inhibition and does not report Ca2+ clearance in other parts of the cells. The authors should analyze their records more carefully to compare global and local changes in Ca2+ during Ca2+ addition and removal.

6. The correlation between STIM1 expression and inhibition of Ca2+ clearance is not tight. For example, expression of YFP-STIM1 in untreated cells to a level less than double STIM1, which is much lower than the upregulation induced by PHA inhibits Ca2+ clearance to the same extent as PHA treatment. Reduction of STIM1 by only 50% eliminated the inhibition of Ca2+ clearance. The authors should explain and discuss this discrepancy.

Referee #2

The paper by Ritchie and Soboloff describes STIM1 inhibition of PMCA-mediated Ca2+ clearance in T cells. By measuring the rate of Ca2+ clearance following a pulse of Ca2+ influx through open CRAC channels, this study shows that PHA-activated T-cells exhibit a slower rate of Ca2+ clearance compared to control cells. Because PMCA is the major player for Ca2+ clearance in T-cells, authors attribute attenuation of Ca2+ clearance in PHA-activated cells to STIM1-induced inhibition of PMCA activity. Although the experiments are well described and the paper is reasonably well-written, this study suffers from a fatal flaw in that the contribution of mitochondria to the apparent prolongation of Ca2+ clearance is completely ignored. A sizeable body of well-established literature (Thayer and Miller, 1990; Friel and Tsien, 1994; Hoet et al, 1997; Babcock and Hille, 1998) shows that mitochondria have a large capacity to sequester and release Ca2+. Importantly, release of Ca2+ from mitochondria can significantly influence the time course of Ca2+ recovery in the type of experiments described in this paper. Unfortunately, there is nothing in this study that assures the reader that the slow relaxation of Ca2+ following removal of extracellular Ca2+ is not due to the slow release of Ca2+ sequestered by mitochondria during the preceding Ca2+ pulse. PHA-activated cells are known to exhibit larger CRAC-mediated Ca2+ influx (e.g., Fig 6A, Fomina, Cahalan et al, J Cell Biol, 2000), and therefore would naturally be expected to exhibit a higher degree of mitochondrial loading (and subsequent release), easily explaining the slower apparent rate of Ca2+ clearance seen here.

A relatively straightforward way to eliminate the confounding effects of mitochondrial Ca2+ release is to redo the experiments in the presence of the mitochondrial inhibitors, antimycin and oligomycin. In the absence of such experiments, the most reasonable explanation is that the effects described here are not due to STIM attenuation of PMCA activity, but rather due to the contamination of the measured Ca2+ signal from mitochondrial Ca2+ efflux. The fact that only the secondary phase of Ca2+ clearance seems attenuated in all experiments here seems consistent that mitochondrial Ca2+ release is contaminating the observed clearance. In fact, the observation that the initial phase of clearance is unaffected would strongly seem to suggest that PMCA activity, is in fact, unchanged by T cell activation.

Referee #3

The manuscript by Ritchie and Soboloff entitled "STIM1 is required for attenuation of PMCA-mediated Ca2+-clearance during T cell activation" deals with the regulation of intracellular Ca2+-levels representing the balance between Ca2+-influx and extrusion. Particularly, the plasma membrane Ca2+-ATPase (PMCA) is proposed to play a critical role in Ca2+ clearance by modulation via STIM1 expression levels. Moreover, both functional and physical links are presented suggesting PMCA inhibition by a STIM1 domain located close to the cytosolic C-terminal end.

The proposed finding of PMCA as a novel interaction candidate of STIM1 is interesting together with the observed inhibitory effect. The several experimental approaches used seem to be carefully carried out. I would like the authors to address the following points, which might strengthen their conclusions.

Main points

1) Generally, the effect of attenuated Ca2+ clearance should be compared between cells that exhibit "iso" -SOCe values. The attenuation of Ca2+ clearance is in some experimental approaches observed when the preceding Ca2+ entry is increased. (better visible by a steeper slope than the relative delta SOCe value). To exclude saturation of PMCA rather than inhibition by STIM1, I would like to see a comparison of control and PHA (or OKT3) stimulated cells which reach similar intracellular Ca2+ levels following addition of extracellular Ca2+. Furthermore, the observed loss of the PHA-dependent attenuation of Ca2+ clearance in STIM1 knock-down cells which exhibit smaller SOCe should be examined under similar Ca2+ entry levels as of control cells. Such conditions might be obtained by adjusting Ca2+ entry by nM La3+ or altered extracellular Ca2+ concentrations as already utilized in Fig. 6B. In such "iso" Ca2+ level cells, attenuated Ca2+ clearance should persist only in those cells that exhibit a higher STIM1 expression level.
2) To derive a specific involvement of STIM1 in PMCA inhibition from stimulation of T cells with

PHA, it was important to demonstrate that over-expression of YFP-STIM1 alone allowed to similarly reconstitute the attenuation of Ca2+ clearance. As the domain proposed to mediate the inhibitory effect of STIM1 on PMCA has been identified according to the constructs used as aa 597-666, I would like to see whether its over-expression mimics the action of full-length STIM1.
3) As an additional specific control, it would be nice to show that siRNA knock-down of PMCAs (e.g. 1/4) similarly reduces Ca2+-clearance as STIM1 overexpression.
4) It seems that a certain level of STIM1 over-expression is required before PMCA inhibition gets visible. This should be at least stated in the Concluding Remarks.

Minor points

1) P6, l7 from the bottom: "the possibility that loading of "; For clarity, better "decreased loading".
2) P6, l5: As mentioned in the text, PHA by itself induces T cell receptor activation and Ca2+ influx. Thus, I would have expected that this is accompanied by store depletion, which however, seems not to be the case as evident following TG treatment. Further, EGTA is not the best reagent to buffer Ca2+ levels to 300µM; Such a concentration can be simply reached by direct addition of 300µ Ca2+, neglecting the typical 10-30µM free Ca2+ present in purified water.
3) P9, l5: "was independent of SOCe"; For clarity, better "was independent of SOCe magnitude".
4) P9, l2 from the bottom: While it is shown that both STIM1-WT and STIM1-deltaP reconstitute SOCE to similar levels, the question remains which SOCe levels are reached under PHA stimulation.
5) P10, l4: the proline-rich region (aa 601-629) is within the portion deleted (aa 597-666) based on what is described in the methods section, but it´s not directly shown that only this region is responsible.
6) P11, l6: "be restricted to"
7) P11, l5 from the bottom: better"after partial STIM1 knockdown".
8) Fig.4A and 5A: It is unclear whether western blots demonstrating effects of STIM1 knockdown have been obtained from control or PHA stimulated cells. The latter condition is more relevant.
9) Fig. 4G and H: I do not understand why Half life depicted for Ctl-BTP2 is longer than that of PHA-BTP2 despite inhibition of Ca2+ clearance of the latter?
10) Fig. 6A: WT is misleading, as it is actually scrambled siRNA.
11) Fig. 6C: Co-localization should also be shown for additional treatment with TG.

1st Revision - authors' response 23 November 2011

**Response to Reviewer 1**

*1. The increase in PMCA mRNA expression indicates that the main increase is in PMCA4 (likely the regulated 4b). anti-PMCA4b antibodies are available and the authors need to determine whether it is PMCA4b that is upregulated and specifically interacts with STIM1. The authors can also express the PMCA1a and 4b isoforms and determine whether STIM1 specifically regulates the activity of the PMCA4a. Such experiments can strengthen the conclusion of regulation of PMCA by STIM1.*

We agree with the reviewer that our analysis of STIM-PMCA expression and interaction in our original manuscript was somewhat biased towards the study of STIM1. In the revised manuscript, we used PMCA4 specific antibodies to determine its ability to bind to STIM1 (Fig 3D; page 8, first para). In addition, we have used PMCA4 siRNA to assess its specific contribution to $[Ca^{2+}]_c$ clearance (Fig 2H,I; page 6, last para). We trust that these additions satisfy this reviewer's concerns.

*2. Effects of mitochondria and STIM1 on PMCA activity needs to be differentiated better. A minimal set of experiments is to inhibit mitochondria Ca2+ influx by treating the cells with FCCP and with ruthenium red during application of external Ca2+ and assay Ca2+ clearance by PMCA in resting and stimulated T cells.*

The reviewer suggests that we better differentiate the relative contribution of mitochondria to $Ca^{2+}$ clearance. It is not our intention to suggest that mitochondria do not contribute to $Ca^{2+}$ homeostasis at the immunological synapse; we simply provide new data that PMCA function is also compromised. In support of this conclusion, we have provided new data using the specific and potent mitochondrial uniporter inhibitor RU360 (see (Baughman et al, 2011). As depicted in figure 2, RU360 dramatically inhibited mitochondrial $Ca^{2+}$ loading without blocking PHA-induced inhibition of $Ca^{2+}$ clearance.

*3. To better analyze the role of STIM1 the authors should determine if expression of constitutively clustering STIM1 mutants that can activate Orai1 and that is not able to activate Orai1 and determine if they can recruit STIM1 to the IS and inhibit PMCA activity. If they do not, then determine if they affect PMCA recruitment by cell stimulation.*

In order to address this concern, we made use of the constitutively active $STIM1_{D76A}$ (Spassova et al, 2006) and the Orai1-binding but non-activating YFP-$STIM1_{\Delta 441-448}$ (Wang et al, 2010). In addition, to assess PMCA4 localization simultaneously with the localization of both YFP-STIM1 and alexafluor-594-labelled PHA, we generated a CFP-PMCA4 construct via chimeragenesis. As depicted in figure S3, STIM1 activation mutations have no effect on the ability of STIM1 to either inhibit PMCA function or co-localize with PHA. These findings further distinguish STIM1-mediated PMCA inhibition from Orai1 activation, a point now made clear in the accompanying discussion (Page 9).

*4. Although the STIM1 mutant that lack the C terminus that includes the PR domain can activate Orai1, it is not clear that this mutant is recruited to the same microdomain in the IS as STIM1. To address this problem and better establish the role of the STIM1 PR domain the authors should test the effect of a STIM1 that lacks only the PR domain. Additional analysis should determine whether a STIM1 cytoplasmic fragment that includes and lack the PR domain can interact with and affect PMCA activity.*

We agree with the reviewer that, based on the evidence available in the original manuscript, we had overstated in claiming that the proline-rich domain of STIM1 was responsible for inhibition

of PMCA function. Therefore, we have renamed the construct previously labeled $STIM1_{\Delta P}$ to $STIM1_{\Delta 597}$ to better reflect its identity. Further, we have now generated a new STIM1 construct specifically deleting the proline-rich domain, now correctly and accurately termed $STIM1_{\Delta P}$. Interestingly, loss of the proline-rich domain caused a near-complete loss of STIM1-mediated inhibition of $[Ca^{2+}]_c$ clearance (Fig 4E,F), yet had no effect on the localization of STIM1 at the IS (Fig 4H). In addition, we noted that there is also a recently identified microtubule end-binding domain located outside of the proline-rich domain but downstream of the $STIM1_{\Delta 597}$ truncation (Honnappa et al, 2009). The potential contribution of this domain to control of $[Ca^{2+}]_c$ clearance was assessing using a non-microtubule end-binding STIM1 moiety containing I644N and P645N point mutations (termed STIM1-NN; generously provided to us by Dr. Anna Akhmonova; Erasmus Medical Center; Netherlands). Interestingly, this mutation had no effect on STIM1's ability to inhibit $[Ca^{2+}]_c$ clearance (Fig 4E,F). The manuscript has been modified to consider these new findings (page 10, first para).

The reviewer also requested that we examine cytosolic fragments of STIM1. After extensive analysis, we were unable to demonstrate that any cytosolic fragment of STIM1 could inhibit $[Ca^{2+}]_c$ clearance. This included studies of the full length fragment, a STIM1 fragment containing the constitutively activating 4EA mutation (Korzeniowski et al, 2010) and the STIM1-Orai1-activating region or SOAR (Yuan et al, 2009). The fact these STIM1 fragments can activate Orai1 but cannot modulate PMCA function further distinguishes these 2 disparate effects of STIM1. While we cannot fully explain the failure of cytosolic fragments that contain the proline-rich domain to inhibit PMCA, we would speculate that PMCA interaction requires a STIM1 configuration that cannot be achieved unless the protein is anchored to the ER, a concept now discussed in the manuscript findings (page 10, last para).

*5. It is likely that inhibition of Ca2+ clearance is confined to the IS. Analysis of global Ca2+ underestimates the inhibition and does not report Ca2+ clearance in other parts of the cells. The authors should analyze their records more carefully to compare global and local changes in Ca2+ during Ca2+ addition and removal.*

As requested, we have reanalyzed our data to distinguish between global vs. local changes in $[Ca^{2+}]_c$ in PHA-activated cells finding a modestly greater inhibition of $[Ca^{2+}]_c$ at the IS than in the rest of the cell (Fig S5). This is now discussed in the manuscript (page 14, top).

*6. The correlation between STIM1 expression and inhibition of Ca2+ clearance is not tight. For example, expression of YFP-STIM1 in untreated cells to a level less than double STIM1, which is much lower than the upregulation induced by PHA inhibits Ca2+ clearance to the same extent as PHA treatment. Reduction of STIM1 by only 50% eliminated the inhibition of Ca2+ clearance. The authors should explain and discuss this discrepancy.*

We agree with the reviewer that the relationship between STIM1 expression and inhibition of $[Ca^{2+}]_c$ clearance is not 100% linear. The concluding remarks have been changed to discuss this observation (page 14, bottom).

**Response to Reviewer 2**

*The paper by Ritchie and Soboloff describes STIM1 inhibition of PMCA-mediated Ca2+ clearance in T cells. By measuring the rate of Ca2+ clearance following a pulse of Ca2+ influx through open CRAC channels, this study shows that PHA-activated T-cells exhibit a slower rate of Ca2+ clearance compared to control cells. Because PMCA is the major player for Ca2+ clearance in T-cells, authors attribute attenuation of Ca2+ clearance in PHA-activated cells to STIM1-induced inhibition of PMCA activity. Although the experiments are well described and the paper is reasonably well-written, this study suffers from a fatal flaw in that the contribution of mitochondria to the apparent prolongation of Ca2+ clearance is completely ignored. A sizeable body of well-established literature (Thayer and Miller, 1990; Friel and Tsien, 1994; Hoet et al, 1997; Babcock and Hille, 1998) shows that mitochondria have a large capacity to sequester and release Ca2+. Importantly, release of Ca2+ from mitochondria can significantly influence the time course of Ca2+ recovery in the type of experiments described in this paper. Unfortunately, there is nothing in this study that assures the reader that the slow relaxation of Ca2+ following removal of extracellular Ca2+ is not due to the slow release of Ca2+ sequestered by mitochondria during the preceding Ca2+ pulse. PHA-activated cells are known to exhibit larger CRAC-mediated Ca2+ influx (e.g., Fig 6A, Fomina, Cahalan et al, J Cell Biol, 2000), and therefore would naturally be expected to exhibit a higher degree of mitochondrial loading (and subsequent release), easily explaining the slower apparent rate of Ca2+ clearance seen here.*

*A relatively straightforward way to eliminate the confounding effects of mitochondrial Ca2+ release is to redo the experiments in the presence of the mitochondrial inhibitors, antimycin and oligomycin. In the absence of such experiments, the most reasonable explanation is that the effects described here are not due to STIM attenuation of PMCA activity, but rather due to the contamination of the measured Ca2+ signal from mitochondrial Ca2+ efflux. The fact that only the secondary phase of Ca2+ clearance seems attenuated in all experiments here seems consistent that mitochondrial Ca2+ release is contaminating the observed clearance. In fact, the observation that the initial phase of clearance is unaffected would strongly seem to suggest that PMCA activity, is in fact, unchanged by T cell activation.*

We recognize and understand this reviewer's conviction that mitochondria serve an important role as modulators of $[Ca^{2+}]_c$. While we do not deny this, the current study reveals an additional and separate role for STIM1 as a modulator of PMCA activity. In support of this conclusion, we have provided new data using the specific and potent mitochondrial uniporter inhibitor RU360 to inhibit mitochondrial $Ca^{2+}$ loading, without blocking PHA-induced attenuation of $[Ca^{2+}]_c$ clearance (Fig 2). Please note that this strategy was chosen to minimize interference with ATP production which would also impact PMCA function.

I would also like to draw the reviewer's attention to 4 specific observations in our manuscript that we do not believe can be explained through a mitochondrial-dependent mechanism. First, in figure 5, we show that BTP2 blocks $Ca^{2+}$ entry to levels similar to that of STIM1 siRNA (in both control and PHA-activated cells), yet fails to attenuate PHA-induced inhibition of $Ca^{2+}$ clearance. Second, in figure 6, we show that $STIM1_{\Delta 597}$ fails to attenuate PHA-induced inhibition of $[Ca^{2+}]_c$ clearance in Jurkat cells stably expressing STIM1 shRNA despite fully restoring Orai1 activation. Hence, PHA-induced inhibition of $Ca^{2+}$ clearance requires full-length STIM1, a requirement that is not dependent on $[Ca^{2+}]_c$. Third, a STIM1 moiety that cannot activate Orai1 ($STIM1_{\Delta 441\text{-}448}$) is fully capable of inhibiting $[Ca^{2+}]_c$ clearance (Fig S3). Finally, in response to comments by reviewer 3, $[Ca^{2+}]_c$ clearance was measured after transfection with siRNA targeting PMCA4 (Fig 2). Importantly, this had dramatic effects on the second phase of

$[Ca^{2+}]_c$ clearance, similar to overexpressing STIM1 or activating T cells with PHA or anti-CD3 crosslinking. This suggests that PMCA activity does indeed impact the second phase of $[Ca^{2+}]_c$ clearance.

**Response to Reviewer 3**

*Main points:*

*1) Generally, the effect of attenuated Ca2+ clearance should be compared between cells that exhibit "iso" -SOCe values. The attenuation of Ca2+ clearance is in some experimental approaches observed when the preceding Ca2+ entry is increased. (better visible by a steeper slope than the relative delta SOCe value). To exclude saturation of PMCA rather than inhibition by STIM1, I would like to see a comparison of control and PHA (or OKT3) stimulated cells which reach similar intracellular Ca2+ levels following addition of extracellular Ca2+. Furthermore, the observed loss of the PHA-dependent attenuation of Ca2+ clearance in STIM1 knock-down cells which exhibit smaller SOCe should be examined under similar Ca2+ entry levels as of control cells. Such conditions might be obtained by adjusting Ca2+ entry by nM La3+ or altered extracellular Ca2+ concentrations as already utilized in Fig. 6B. In such "iso" Ca2+ level cells, attenuated Ca2+ clearance should persist only in those cells that exhibit a higher STIM1 expression level.*

Although we agree with the reviewer that store-operated $Ca^{2+}$ entry is increased by some of the manipulations in our study, this increase is quite modest. Irrespective, in an effort to eliminate this difference, we performed new experiments in which we shorted the amount of time that $[Ca^{2+}]_e$ was elevated while measuring both $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ (Fig 2A,B). Despite minimizing differences in maximal $[Ca^{2+}]_c$ between control and PHA-activated cells, PHA-induced inhibition of $[Ca^{2+}]_c$ clearance could still be detected. Regarding the comparison of STIM1 knockdown cells with 'matched' controls, we respectively point out to the reviewer that this has been done using BTP2 to inhibit $Ca^{2+}$ entry in scrambled RNA-transfected Jurkat cells (Fig 5D-F). Again, whereas BTP2 inhibited SOCe to similar levels as STIM1 siRNA, PHA activation still compromised $[Ca^{2+}]_c$ clearance.

*2) To derive a specific involvement of STIM1 in PMCA inhibition from stimulation of T cells with PHA, it was important to demonstrate that over-expression of YFP-STIM1 alone allowed to similarly reconstitute the attenuation of Ca2+ clearance. As the domain proposed to mediate the inhibitory effect of STIM1 on PMCA has been identified according to the constructs used as aa 597-666, I would like to see whether its over-expression mimics the action of full-length STIM1.*

As discussed above in response to reviewer 1, we generated a number of cytosolic STIM1 constructs, none of which could mimic inhibition of PMCA function by full-length STIM1. Given these findings, we now believe that anchoring to the ER is an important prerequisite for this phenomenon, likely by bringing STIM1 near the PM and placing it in a configuration appropriate for modulation of PMCA function. This concept is now discussed within the manuscript (page 10, last para).

*3) As an additional specific control, it would be nice to show that siRNA knock-down of PMCAs (e.g. 1/4) similarly reduces Ca2+-clearance as STIM1 overexpression.*

As requested, we have tested PMCA4 siRNA (Fig 2) which dramatically inhibited $[Ca^{2+}]_c$ clearance in a manner similar to (albeit greater than) the effects of STIM1 overexpression and T cell activation. Based on this finding, we now conclude that PMCA function does indeed

contribute to both phases of $[Ca^{2+}]_c$ clearance, a point now made clear within the revised manuscript (page 6, last para).

*4) It seems that a certain level of STIM1 over-expression is required before PMCA inhibition gets visible. This should be at least stated in the Concluding Remarks.*

As requested, our concluding remarks have been modified to consider the apparent threshold of STIM1 overexpression required for inhibition of PMCA function (page 14, bottom).

*Minor points*

*1) P6, l7 from the bottom: "the possibility that loading of "; For clarity, better "decreased loading".*

Changed as requested.

*2) P6, l5: As mentioned in the text, PHA by itself induces T cell receptor activation and Ca2+ influx. Thus, I would have expected that this is accompanied by store depletion, which however, seems not to be the case as evident following TG treatment. Further, EGTA is not the best reagent to buffer Ca2+ levels to 300µM; Such a concentration can be simply reached by direct addition of 300µ Ca2+, neglecting the typical 10-30µM free Ca2+ present in purified water.*

While we agree with the reviewer that PHA should induce store depletion, we would expect this depletion to be transient in the absence of Tg. Regarding the use of EGTA to buffer $Ca^{2+}$ levels to 300 µM, this was done because cells were maintained continuously in growth media to minimize the number of manipulations prior to treatment. This media contains 1.8 mM $Ca^{2+}$, requiring the use of EGTA to decrease $[Ca^{2+}]_o$.

*3) P9, l5: "was independent of SOCe"; For clarity, better "was independent of SOCe magnitude".*

Changed as requested.

*4) P9, l2 from the bottom: While it is shown that both STIM1-WT and STIM1-deltaP reconstitute SOCE to similar levels, the question remains which SOCe levels are reached under PHA stimulation.*

We acknowledge the reviewer's concern as to whether or not SOCe levels between $STIM1_{WT}$ and $STIM1_{\Delta 597}$ were the same after PHA stimulation, not shown in the original manuscript. This data has now been added to figure 6F, showing that, although PHA does increased SOCe, it does so identically in cells expressing $STIM1_{WT}$ and $STIM1_{\Delta 597}$.

*5) P10, l4: the proline-rich region (aa 601-629) is within the portion deleted (aa 597-666) based on what is described in the methods section, but it´s not directly shown that only this region is responsible.*

We agree with the reviewer that, based on the evidence available in the original manuscript, we had overstated in claiming that the proline-rich domain of STIM1 was responsible for inhibition of PMCA function. Therefore, we have renamed the construct previously labeled $STIM1_{\Delta P}$ to $STIM1_{\Delta 597}$ to better reflect its identity. In addition (as discussed at length in response to comments by reviewer 1), we have now generated a new construct in which the proline-rich domain is specifically deleted, now correctly and accurately termed $STIM1_{\Delta P}$. Interestingly, loss of the proline-rich domain caused a near complete loss of STIM1-mediated inhibition of $[Ca^{2+}]_c$ clearance (Fig 4E,F).

*6) P11, l6: "be restricted to"*

Changed as requested.

*7) P11, l5 from the bottom: better"after partial STIM1 knockdown".*

Changed as requested.

*8) Fig.4A and 5A: It is unclear whether western blots demonstrating effects of STIM1 knockdown have been obtained from control or PHA stimulated cells. The latter condition is more relevant.*

As requested, these Westerns have been replaced with new data showing the degree of STIM1 knockdown in both control and PHA-stimulated cells.

*9) Fig. 4G and H: I do not understand why Half life depicted for Ctl-BTP2 is longer than that of PHA-BTP2 despite inhibition of Ca2+ clearance of the latter?*

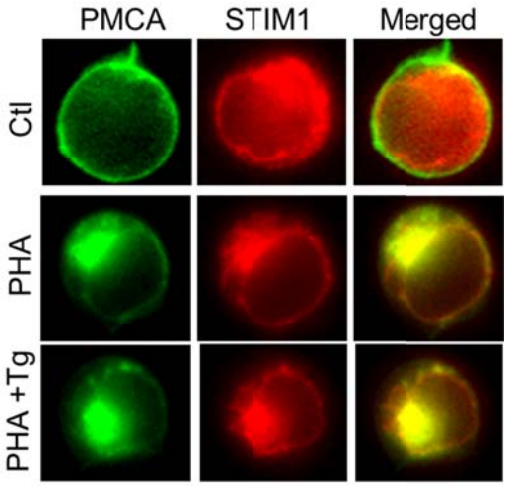
We thank the reviewer for uncovering this unfortunate labeling error; corrected in the current submission.

*10) Fig. 6A: WT is misleading, as it is actually scrambled siRNA.*

Changed as requested.

*11) Fig. 6C: Co-localization should also be shown for additional treatment with TG.*

Interestingly, we did in fact perform this experiment on the cells depicted in figure 6C of the original submission (Fig. 3F of the revised manuscript). Although this had no effect on co-localization of STIM1 and PMCA, the addition of Tg caused a curious morphological shift, such that the IS seemed to move (see accompanying fig). While interesting, we considered this phenomenon beyond the scope of our study. However, the fact that Tg does not affect co-localization of STIM1 and PMCA is now reported in the manuscript as data not shown (page 6, middle para).

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12 December 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked referees #1 and 3 to review the revised version and I have now received their comments back.

As you can see below, both referees appreciate the introduced changes and support publication here. I am therefore very pleased to proceed with its acceptance here. Please check the supplemental figure legend S3 (referee #3) if there has been a mix up in the legend. If so please send us an amended supplemental file.

Thank you for submitting your interesting study to the EMBO Journal. You will receive the formal acceptance letter shortly

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The authors did an excellent job addressing my comments. In particular, it is quite important to demonstrate the selective role of PMCA1a and PMCA4b and that STIM1 acts primarily to regulate PMCA4b.

Referee #3

The authors have adequately responded to all of the points raised in my reviewing comments. No further remarks.

One short point:
Fig. S3 In the legend YFP-STIM1 D76A and YFP-STIM1 delta 441-448 have been mixed up.

 