Docking of Syk to FcεRI is enhanced by Lyn but limited in duration by SHIP1

William Kanagy, Cédric Cleyrat, Mohamadreza Fazel, Shayna Lucero, Marcel Bruchez, Keith Lidke, Bridget Wilson, and Diane Lidke

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RE: Manuscript #E21-12-0603
TITLE: Docking of Syk to FcεRI is enhanced by Lyn but limited in duration by SHIP1

Dear Dr. Lidke:

Two reviewers have evaluated the above referenced manuscript. While they find the work of interest and a potential advance to the field, some questions were raised that preclude its acceptance at this time. Should you decide to revise the work, based on their review, I suggest that you and your co-authors focus particularly on addressing the concerns on whether the scale of the observed change in off-rate has an impact on the net signaling activity of Syk, and on the kinetics of the TT-beta construct in the cells carrying or lacking SHIP1. Other significant concerns include ensuring that the cells are capable of responding with robust calcium signaling given the modest calcium responses observed, as well as confirming that Syk cannot trigger the receptor in Lyn KO cells.

Sincerely,

Avery August
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Lidke,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

Kanagy et al. are seeking to understand how positive and negative signals are integrated at "signalosomes" formed in lymphocytes. This is an important question as most receptors seem to be capable of recruiting multiple signaling proteins, often with antagonistic effects, and how net signaling outcomes are determined in this setting is largely mysterious. The system the authors study comprises FcεR1 receptors aggregated by polyvalent antigen reactive with IgE. I sometimes wonder if this is the best model for receptor triggering, since so much receptor is engaged on every cell simultaneously, presumably in the absence of cell-cell contact, and I wonder if this is what happens in vivo. Nevertheless, it seems like a reasonable place to begin a coarse-grained unravelling of the signaling-integration problem.

The existing paradigm, then, is that FcεR1 phosphorylation is initiated by Lyn, followed by the recruitment of Syk and e.g. SHIP1, proteins that have antagonistic effects on signaling. At the heart of their study is the finding that in the absence of Lyn, an FcεR1-expressing rat basophilic lymphocyte (RBL) cell line is still capable of degranulation, albeit with somewhat reduced sensitivity to antigen (by ~10-fold). In attempting to explain this, the authors then seek to determine what's recruited to the signalosomes using imaging approaches. They propose that, in the absence of Lyn, the limited signaling activity of Syk results in under-phosphorylation of the β-chain of FcεR1, leading to lower recruitment of SHIP1 which, being a negative regulator, favours net-positive signaling by Syk. Most importantly, they propose that the effect of SHIP1 is to accelerate the dissociation of Syk from the signalosome.

The authors' work emphasizes the crucial role of the Src family kinases in the initial receptor phosphorylation event leading to Syk recruitment, reinforcing the existing paradigm, since other Src kinases compensate for the absence of Lyn. It's interesting that the β-chain of FcεR1 specifically recruits SHIP1, but this was apparently already known. My reading of the manuscript, then, is that the one really new observation is that the binding off-rate for Syk is larger in the presence of SHIP1. However, I felt that this effect was relatively small, i.e. a difference in off-rate of ~0.3 s-1 versus ~0.5 s-1, given that the off-rates for protein interactions of this type can vary over several orders of magnitude. It seems to me that the much larger effect of the presence of SHIP1 in the signalosome would derive from its ability to alter the local membrane signaling environment (i.e., phosphoinositide content) via its enzymatic activity. On the other hand, it is interesting that differences in the activities of the Syk and Lyn kinases result in qualitative changes in the recruitment of SHIP1 to the receptor. But it seems to me to be a shame that the imaging data, which can only be used for binary comparisons, wasn't complemented with more conventional biochemical data showing e.g. actual recruitment of SHIP and Syk to specific subunits of the receptor. Instead, only correlations could be derived.

Overall, I felt that the case for the authors' proposed mechanism wasn't persuasively made. It would be necessary to show that the scale of the observed change in off-rate does indeed impact on the net signaling activity of Syk. For now it can only be speculated that this is the case.

Regarding the requirements detailed on the MBoC Author Submission Checklist, I felt that the authors had not completely satisfied requirement II, by not indicating how often key imaging experiments were done or how representative the data is.

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1. Introduction, para 1 - I'm not so sure that immune receptors share the general triggering mechanism of receptor aggregation. For the TCR at least, it's now thought that triggering occurs at the level of individual receptors engaging single pMHC.
2. Introduction, para 2 - the statement here and throughout that Syk family kinases can directly phosphorylate ITAMs needs some careful qualification, I think. It's important to differentiate between what's possible before the kinase has been recruited to the receptor versus what can happen after its recruitment. As far as I'm aware, no-one has shown that Syk kinases are active to any significant degree as ab initio drivers of receptor phosphorylation, coming from the cytoplasm.
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to my knowledge.
12. Discussion, para 1; are Lck and Src really likely to instigate signaling when Lyn is present?

Reviewer #2 (Remarks to the Author):

This is an interesting paper investigating requirements for signaling through FcER1 in RBL cells, a commonly used model for mast cells. In the absence of Lyn, RBL cells can still degranulate, although delayed compared to WT. FcER1g phosphorylation is partial and delayed and only the C-terminal Y of the ITAM is sufficient in the absence of Lyn, suggesting function as a hemi-ITAM or in conjunction with the FcER1b subunit ITAM. While Syk activity contributes to FcER1g phosphorylation, an unidentified Src family kinase was required for the delayed signaling in the absence of Lyn. The FcER1b ITAM was shown to require Lyn for recruitment of both Syk and SHIP1, and recruitment of Syk to the crosslinked FcER1b was only sufficient to trigger degranulation in the absence of SHIP1. In WT Lyn background, the effect of SHIP1 on Syk recruitment to FcER1 complex was an acceration of the koff, potentially due to alterations in phosphoinositol lipids that support Syk recruitment to the membrane. The functional relevance of the very interesting change in Syk recruitment kinetics is not demonstrated.

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We thank the reviewers for their overall positive feedback and constructive comments. In particular, we thank both for prompting us to show a functional effect of changes in Syk off-rate. We have provided new data showing that the reduction in Syk off-rate is translated into increased Syk and LAT phosphorylation. Below we address their individual concerns. Major changes in the manuscript have been underlined.

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The modulation of FcεRI:Syk interactions by SHIP1 is a novel finding of this work. However, we would like to emphasize several other important observations. First, the ability of FcεRI to signal in the absence of Lyn and Fyn was unexpected and the mechanism supporting this activity was unclear. It could have been the activity of another SFK phosphorylating the γ-ITAM. On the other hand, it has been suggested by others that Syk itself can directly phosphorylate BCR ITAMs (see response to point 2 below for more). We show - in intact, living mast cells - it is a combination of the two processes. The γ-ITAM must *first be phosphorylated* to initiate Syk recruitment. But if γ-phosphorylation is weak, ITAM-bound Syk can itself increase γ-phosphorylation to levels that support activation of cellular processes. Importantly, we provide direct evidence that the γ-ITAM is a substrate for Syk kinase activity, but β is not. Second, we show that Syk can bind to the phosphorylated β subunit in live cells (an interaction previously discounted; (Furumoto et al., 2004; Wilson et al., 1995)), and that this interaction is capable of productive signaling. Our results show the importance of balancing Syk signaling with negative regulators, since the removal of just one

negative signaling molecule, SHIP1, allowed for β-initiated Syk signaling to proceed. Third, our data supports the ability of Syk to bind monophosphorylated γ-ITAMS in *trans*. This has implications for Syk's ability to interact with the diverse range of ITAM-bearing receptors, as well as hemITAMs. Altogether, this work shows that the components of ITAM signaling are not inherently specific but require tight control by negative regulators to keep signaling in check. We have revised the abstract to make these points more clear.

The observed change in Syk off-rate is indeed small. However, in our previous work (Schwartz et al., 2017), we showed that introduction of a mutation in the Syk SH2 linker region (Syk-Y130E) resulted in a small *increase* in Syk off-rate (i.e., more transient binding), from 0.625 s^{-1} to 0.871 s^{-1}. This increase in off-rate resulted in reduced Syk phosphorylation, modulated cytokine production and complete loss of degranulation. We emphasize this to highlight the sensitive nature of protein interaction dynamics. In the current paper, we report that a *decrease* in off-rate is seen when SHIP1 is removed from the signalosome. While also a small change, this is on the same order (but in the opposite direction) as seen for the Syk-Y130E. Therefore, we believe that this change in lifetime is significant. We also now include data showing that under the SHIP1 knockout conditions, where Syk off-rate is reduced, the phosphorylation of Syk is increased (see below).

It seems to me that the much larger effect of the presence of SHIP1 in the signalosome would derive from its ability to alter the local membrane signaling environment (i.e., phosphoinositide content) via its enzymatic activity. On the other hand, it is interesting that differences in the activities of the Syk and Lyn kinases result in qualitative changes in the recruitment of SHIP1 to the receptor. But it seems to me to be a shame that the imaging data, which can only be used for binary comparisons, wasn't complemented with more conventional biochemical data showing e.g. actual recruitment of SHIP and Syk to specific subunits of the receptor. Instead, only correlations could be derived.

We were also surprised to find a change in Syk off-rate when SHIP1 was absent since SHIP1 is a lipid phosphatase. Two possible hypotheses come to mind. First, and related to the reviewer's comment, the lipid environment may stabilize Syk binding at the membrane. Such a mechanism is supported by work from the Cho lab showing that SH2 domains can interact with phosphortyrosines and PIPs simultaneously and the PIP interaction stabilizes the protein at the membrane (Park, et al. 2016, *Molecular Cell* DOI: 10.1016/j.molcel.2016.01.027). Second, Syk and SHIP1 may compete for binding to the receptor. This seems less likely since Syk binding is predominantly through the γ-ITAM and SHIP1 only binds the β-ITAM. We have reworded the paragraph in the discussion related to these hypotheses.

A great number of previous studies have looked at protein-protein interactions along the FcεRI signaling pathway using biochemical (IP) methods (Benhamous et al., 1993; Eiseman & Bolen, 1992; Furumoto et al., 2004; Jouvin et al., 1994; Sanderson et al., 2010; Wilson et al., 1995; Yamashita et al., 2008). These biochemical approaches are clearly important; however, we argue that measurements of protein interactions in intact, living cells has its advantages and can be highly quantitative. The process of cell lysing and sample preparation for IP can bias results if interactions are weak. For example, the Syk-Y130E mutant mentioned above was reported to not bind ITAMs based on IP data (Keshvara et al., 1997; Zhang et al., 2008). Our cellular imaging revealed that Syk-Y130E does bind FcεRI, but with altered kinetics. In addition, several studies concluded that Syk does not bind the β-ITAM due to the difficulty to co-IP the proteins (Furumoto et al., 2004) or lack of signaling by TT-β (Wilson et al., 1995), yet we were able to capture this interaction by microscopy. Because we were interested in capturing protein behavior in intact cells, we focused on the imaging approaches. By comparing across TT-ITAMs and γ-mutants, we are able to dissect the binding to individual ITAMs using imaging approaches. The binary nature of the data

mentioned demonstrates the "all-or-none" phenotype for protein recruitment that we observed in many cases, not a limitation of the imaging approach. Note that we have added additional quantification for the recruitment assays (Supplemental Figure 1), which supports the reported outcomes. Importantly, fluorescence microscopy allowed us to quantify and compare Syk recruitment capacity between conditions with *exquisite detail and identify subtle differences when present - not only at the single cell but even the single FcεRI aggregate level*. This is seen in Figures 5&6 where we determined that the amount of Syk recruited to individual receptor aggregates in RBL-Lyn^{KO} cells, while not lost, was reduced and delayed as compared to RBL-WT cells.

Overall, I felt that the case for the authors' proposed mechanism wasn't persuasively made. It would be necessary to show that the scale of the observed change in off-rate does indeed impact on the net signaling activity of Syk. For now it can only be speculated that this is the case.

We thank the reviewer for prompting us to provide evidence of off-rate changes impacting Syk activity. We have now included data showing that in RBL-SHIP1^{KO} cells, where Syk binding lifetime is longer, the phosphorylation of Syk as well as its downstream target LAT is significantly increased in response to DNP_{25}-BSA crosslinking (new Figure 8D&E). In our previous work, we found that reduced interaction time led to reduced Syk phosphorylation. Therefore, these two results together strengthen the idea that controlling interaction kinetics is critical to proper regulation of signaling.

Regarding the requirements detailed on the MBoC Author Submission Checklist, I felt that the authors had not completely satisfied requirement II, by not indicating how often key imaging experiments were done or how representative the data is.

Thank you for pointing out this shortcoming. We have made every effort to ensure that the information on experimental replicates is now properly reported. Figure legends list the number of samples/replicates and the statistical analysis performed. We also provide information on the number of cells imaged for recruitment data set along with additional analysis to support the statements in the text (Supplemental Figure 1).

Minor comments

1. Introduction, para 1 - I'm not so sure that immune receptors share the general triggering mechanism of receptor aggregation. For the TCR at least, it's now thought that triggering occurs at the level of individual receptors engaging single pMHC.

We agree with the reviewer that other mechanisms can be at play, including the extent of receptor clustering, receptor conformational changes, and mechanotransduction. We have attempted to highlight these mechanisms as playing possible roles, but agree a bit more detail is warranted. We have modified the second sentence in the first paragraph to avoid the emphasis on aggregation: "Immunoreceptors share a general mechanism of activation, whereby receptor-antigen engagement initiates the formation of a signaling complex, or signalosome, to promote the recruitment and interaction of positive and negative signaling molecules (Chakraborty & Weiss, 2014; Geahlen, 2009; Kalesnikoff & Galli, 2008; Sigalov, 2004; Siraganian et al., 2002, 2010; Suzuki, 2017; Turner & Kinet, 1999)." We have also expanded the text in the second paragraph to provide more clarity and have added additional references.

2. Introduction, para 2 - the statement here and throughout that Syk family kinases can directly phosphorylate ITAMs needs some careful qualification, I think. It's important to differentiate between what's possible before the kinase has been recruited to the receptor versus what can happen after its recruitment. As far as I'm aware, no-one has shown that Syk kinases are active to any significant degree as ab initio drivers of receptor phosphorylation, coming from the cytoplasm.

The studies cited in the introduction have shown that Syk is capable of phosphorylating ITAMs/hemITAMs on its own, in the absence of SFKs. How this plays out is unclear from these studies. Some of the studies are performed *in vitro* where Syk would presumably be acting on the ITAM from solution. In contrast, our results argue that Syk must first bind to two phosphorylated ITAM tyrosines (whether in *cis* or in *trans*) *before* it can further phosphorylate the γ ITAM.

We have reworded the sentence in the introduction: "The requirements for SFKs in ITAM phosphorylation is also not clear, since multiple studies have shown that Syk is also capable of phosphorylating ITAM/hemITAM tyrosines, independent of SFKs (Bauer et al., 2017; Huysamen et al., 2008; Mukherjee et al., 2013; Rogers et al., 2005; Rolli et al., 2002)."

We have also revised the section in the Results, SFK phosphorylation of FcεRI is needed for Syk recruitment (p. 6), to make our interpretation of our results more clear.

3. Introduction, para 3 - I'm not certain that the FcεR1 signaling cascade always results in allergy and asthma.

We have clarified the language: "Aggregation of FcεRI by multivalent antigen initiates a signaling cascade that ultimately leads to mast cell degranulation and cytokine production and is often associated with allergy and asthma."

4. I was struck by how weak the calcium signaling response was in Fig. 1D, given the scale presented alongside it, even for RBL-WT cells. Are the authors sure their cells are healthy? I would recommend using PMA/ionomycin to show that the cells are capable of responding well, and this just reflects the surprising weakness of stimulus (but in this case, can it be that this is really a suitable model stimulus?).

Thank you for pointing out this concern. While we observed relative changes in calcium response, the absolute values were indeed smaller than in previous studies. Upon reflection, we realized that this data was acquired with a new camera and that we had not recalibrated the system. We have made this correction and now include properly analyzed data. Reanalyzed data and newly acquired data were consistent. The changes in magnitude and lag time for calcium release by wild type are now consistent with our previous measurements (Travers et al., 2019). We also acquired data for PMA stimulation as suggested by the reviewer. PMA stimulation results show that both cell lines are capable of rapid and significant calcium release (Figure R1).

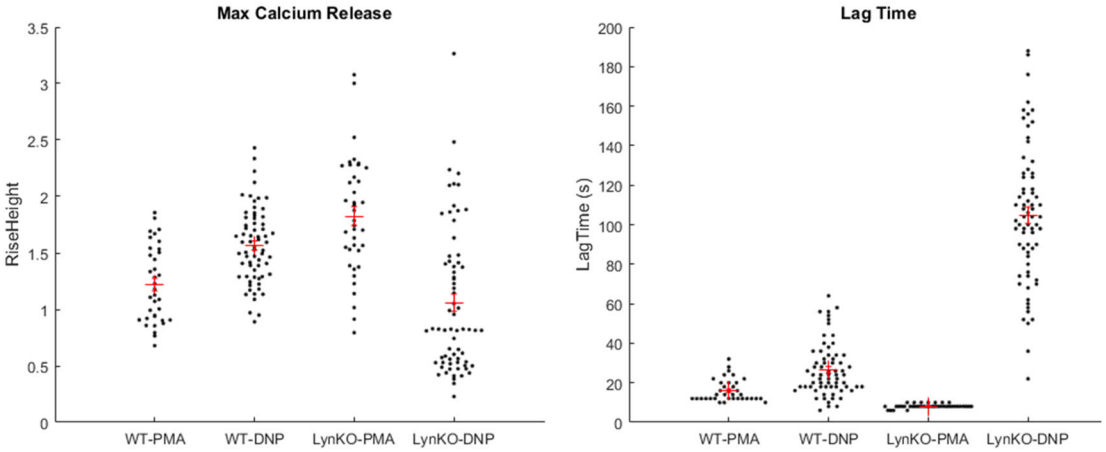

Figure R1. Quantification of calcium response curves between RBL-WT and –Lyn^{KO} cells. RBL-WT (PMA n = 38; DNP n = 67), RBL-Lyn^{KO} (PMA n = 41; DNP n = 65). Quantification of calcium response curves to extract the relative, maximum increase in the Fura-2 ratio per cell after antigen crosslinking (Max Calcium Release-left) and the time between antigen crosslinking and calcium release (Lag Time-right). Red cross and error bars report the mean and SEM, respectively. 50 nM PMA (Phorbol 12-myristate 13-acetate) or 1 mg/mL DNP_{25}-BSA was used for respective treatments.

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Supplementary Figure 2A now includes western blot data showing that dasatanib blocks FcεRI γ-phosphorylation in RBL-Lyn^{KO} cells in a dose dependent manner. This correlates well with Supplementary Figure 2B showing that dasatanib also decreases degranulation output. It also supports the results in Figure 3 showing that the tandem SH2-domains of Syk (a truncation mutant of Syk lacking the kinase domain, Syk(SH2)^{SYFP2}) is still recruited to receptor aggregates in RBL-Lyn^{KO} cells, while dasatanib blocks Syk(SH2)^{SYFP2} recruitment. The experiment in Figure 3 was performed in RBL-Lyn/Syk^{dKO} cells. Altogether, this data indicates that kinase activity of Syk is not involved in the initial Syk recruitment, rather a dasatanib sensitive kinase (i.e, a SFK) is needed to start the phosphorylation of ITAMs for Syk recruitment. We have also modified the legend to improve the description of Figure 3.

6. Top of p.7; Figure 4B should be Figure 4C

Thank you. We have made this correction.

7. Bottom of p.7; where is the data showing that RBL-LynKO cells expressing FcεRIγ-FY had similar kinetics to RBL-LynKO cells?

This data is found in Figure 5C. We have added this figure callout to the sentence on p. 8: "RBL-Lyn/γ^{dKO} cells expressing FcεRIγ-FY had similar Syk recruitment kinetics as RBL-Lyn^{KO} cells (Figure 5C)."

8. Bottom of p.9; is it worth commenting on why the track lengths in the resting state are already longer for the SHIP1KO cells versus the WT cells? Is this reflective of tonic signaling?

In the original version, we noted this increase in fraction of Syk in resting RBL-SHIP^{KO} cells, but discussion was spread across multiple places and we did not make a nice connection for the reader. We have now consolidated and expanded comments on this point (p. 10): "Interestingly, the distribution in non-treated RBL-SHIP^{KO} cells is more similar to activated RBL-WT cells. Fitting these distributions revealed that the off-rate for k_s is reduced in RBL-SHIP^{KO} (k_s = 0.323 s^{-1} ± 0.004), such that the absence of SHIP1 allows for longer-lived interactions of Syk with FcεRI (Figure 8B). Consistent with the right-shifted track length distributions (Figure 8A), we found that the fraction of long-lived Syk events at the membrane is higher in RBL-SHIP1^{KO} cells, for resting and activated cells (Figure 8C). These results reveal that SHIP1 regulates both the amount and duration of Syk binding to FcεRI. An increase in membrane-resident Syk in cells lacking SHIP1 suggests that the regulatory behavior of SHIP1 acts independent of receptor crosslinking and plays a role in preventing aberrant, constitutive FcεRI signaling. The idea that SHIP1 acts to prevent aberrant signaling is supported by previous work showing that even in the resting state phospho-SHIP1 is present at the cell membrane in RBL cells (Mahajan et al., 2014). "

9. Discussion, para 1; I don't think it's possible to claim robust calcium release in LynKO cells - they're very significantly weaker than WT (see my comments above also about the absolute scale of the responses).

As noted above, after recalibration of our instrument we are confident in the calcium readouts for the RBL-Lyn^{KO} cells. There is a clear calcium response in these cells, but it is indeed weaker than in WT. We have removed the clarifier "robust" from the text. We thank the reviewer again for identifying this shortcoming.

10. Discussion, para 1; F et al.??

We have corrected this error.

11. Discussion, para 1; the statement "Our work supports the idea that Syk can directly phosphorylate ITAMs" needs contextualization. My reading of this statement is that Syk can do this from the cytoplasm, which isn't shown here or elsewhere to my knowledge.

Our data shows that Syk cannot act on γ from the cytoplasm but must first be recruited to a phosphorylated ITAM. See also response to point #2 above. We have reworded this section in the discussion to make the idea more clear: "We found that the γ ITAM is a substrate for Syk kinase activity.

However, we also show that Syk cannot bind to unphosphorylated FcεRI so that SFK activity is required to first phosphorylate the ITAM and initiate Syk recruitment. In the absence of Lyn only low-level γ phosphorylation is achieved, but this is sufficient to promote Syk binding and facilitate Syk amplification of γ phosphorylation in a positive feedforward manner."

12. Discussion, para 1; are Lck and Src really likely to instigate signaling when Lyn is present?

This is an excellent question. New data in Supplemental Figure 3 shows that overexpression of either Lck or Src can increase both FcεRIγ and Syk phosphorylation in response to receptor aggregation in RBL-Lyn^{KO} cells. Therefore, these two family members are capable of instigating FcεRIγ signaling. This is perhaps not surprising since SFKs a have been shown to compensate for each other in other situations. For example, Fyn can compensate for Lyn in mast cells (Hernandez-Hansen et al., 2004; Parravicini et al., 2002) and Lyn can substitute for Lck in T cells (Borna et al., 2020). Also, previous work showed that with transfection of the FcεRI complex into Jurkat T cells, Lck can phosphorylate the receptor ITAMs after aggregation (Adamczewski et al., 1995). In the case of RBL-WT cells, the lower expression of Lck and Src, along with the high kinase activity of Lyn, may indicate that Lck and Src are not needed to support FcεRI signaling when Lyn is present. Thus, it is important to consider the relative abundance of receptors and downstream molecules in any signaling network.

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Major question

1. Does the recruitment of Syk to clusters formed by the TT-β also show the longer interaction in the presence of Lyn without SHIP1 compared to with SHIP1? This is the situation for which SHIP1 is shown to have a clear functional effect. I looked, but didn't find other data on role of SHIP1 in RBL model, just WT mast cells, where Fyn can compensate for positive roles of Lyn in FcER1 signaling, but not the negative effects related to SHIP1 recruitment. If there is data on this point in the literature then that may suffice, otherwise it would be ideal to extent the kinetic measurements to the functional setting, or show the SHIP1 KO effect on the WT FcER1 signaling as established, although the effect will likely be quantitative rather than all or none as in the TT-b setting, which seems cleanest.

We thank the reviewer for prompting us to provide evidence of off-rate changes impacting Syk activity.

We have included new data showing that there is a functional relevance for the change in Syk dynamics (new panels in Figure 8). We were able to show that the longer Syk lifetime (reduced off-rate) is translated into an increase of both Syk and LAT phosphorylation (see also Reviewer #1 response). We believe that this addresses the concern about needing to show a functional effect of SHIP1 on Syk.

There is literature about the negative regulation of immunoreceptors by SHIP1, including BCR, FcεRI and other Fc receptors. One of the first papers to examine SHIP1 in mast cells used SHIP1-/- bone marrow derived mast cells (BMMCs) from mice (Huber et al., 1998). This study found that knockout of SHIP1 increased degranulation, indicating that SHIP1 did play a negative regulatory role. More recently, work in RBL cells showed that SHIP1 is recruited to FcεRI aggregates in a Lyn-dependent and antigen-dependent manner and that the SHIP1 recruitment was correlated with reduced FcεRI signaling outcomes (Mahajan et al., 2014). They showed that at optimal doses of antigen, phospho-SHIP1 was less colocalized with FcεRIβ; however at sub- or supra-optimal doses (where signaling and degranulation are lower) there was more phospho-SHIP1 colocalization with the receptor. Our results showing that complete knockout of SHIP1 allows for enhanced signaling, either by allowing for TT-β aggregation to propagate signal or by increased Syk/LAT phosphorylation, is consistent with these previous results.

We agree that determining the off-rate for Syk interactions with TT-β would be interesting. We have spent a considerable effort to make this measurement over the past several months. To do this in the cleanest system possible, we first generated RBL cells with a double knock out for FcεRIγ and SHIP1. We then compared TT-β crosslinking the RBL-γKO and RBL-γ/SHIP1-dKO cells. Despite clear evidence using confocal microscopy that Syk-mNG is recruited to TT-β aggregates (Figure 6B), we did not observe a shift in the track length CPD plot between resting and activated for either cell line. We are not completely sure of why there is not a change with activation in this case. The Syk-mNG recruitment appears weaker for TT-β then it does for intact FcεRI or TT-γ. It could be that the recruitment of β is inherently weaker and our SPT assay is not sensitive to these small changes. Since we would need more time to parse out what is going on, we request that this data not be required for the current manuscript. We believe that the new results showing increased Syk and LAT phosphorylation in RBL-SHIP1^{KO} cells now provide the requested link between lifetime and functional effects.

RE: Manuscript #E21-12-0603R
TITLE: "Docking of Syk to FcεRI is enhanced by Lyn but limited in duration by SHIP1"

Dear Dr. Lidke:

Please ensure that all parts of the manuscript meet the requirements listed in the MBoC Author Submission Checklist, including the legends to figures 3, 7 and figure 8 (i.e., clearly indicate number of times western blot experiments were performed), and characterization of cell lines, including KO lines.

Once this is done, I will be able accept the paper without returning it to the reviewers.

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Avery August
Monitoring Editor
Molecular Biology of the Cell

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RE: Manuscript #E21-12-0603RR
TITLE: "Docking of Syk to FcεRI is enhanced by Lyn but limited in duration by SHIP1"

Dear Dr. Lidke:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
Avery August
Monitoring Editor
Molecular Biology of the Cell

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