EMBO
reports

Manuscript EMBOR-2014-38801

Functional Analysis of Related CrRLK1L Receptor-like Kinases in Pollen Tube Reception

Sharon A. Kessler, Heike Lindner, Daniel S. Jones and Ueli Grossniklaus

Corresponding author: Ueli Grossniklaus and Sharon A. Kessler, University of Zuerich

Review timeline:

Submission date:	02 April 2014
Editorial Decision:	03 April 2014
Correspondence:	07 April 2014
Correspondence:	11 April 2014
Editorial Decision:	29 April 2014
Revision received:	13 October 2014
Editorial Decision:	31 October 2014
Revision received:	10 November 2014
Accepted:	11 November 2014

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.)

Editor: Barbara Pauly

1st Editorial Decision 03 April 2014

Thank you for submitting your manuscript to our editorial office. I have now had a chance to carefully read your manuscript and to discuss it with the other members of our editorial team. I am sorry to inform you that the outcome of these discussions is not a positive one, as we find the manuscript not well suited for publication in EMBO reports.

In assessing your manuscript we acknowledge that you have investigated the functional similarities and/or differences of three members of the CrRLK1L family of plant receptor-like kinases in mediating pollen tube reception. By swapping the extra- and intra-cellular domains of FER, ANX1, and HERK1 you provide evidence that while these three proteins seem to employ the same intracellular signaling pathways, their extracellular domains are not interchangeable, arguing that they are activated by different ligands. You further show that while the intracellular kinase domain of FER is essential for pollen tube reception, kinase activity per se is not required. While we appreciate that these findings represent interesting starting point for further investigations, we also feel that the study as it stands does not offer the kind of biological insights, for example into the different ligands that activate the different RLKs or into why the kinase activity of FER is not required for its signaling activity that we and our readership would look for in an EMBO reports paper and we have therefore decided not to subject it to in-depth peer review here.

© European Molecular Biology Organization

Please note that we can only publish a very small fraction of the many manuscripts that are submitted to our journal and that we therefore have to make a rather stringent selection on which ones to send out for peer review. I am sorry to have to disappoint you on this occasion, and hope that this will not prevent you from considering EMBO reports for publication of your work in the future.

Correspondence - authors 07 April 2014

Thank you for your quick response regarding the submission of our MS EMBOR-2014-38801V1, reporting functional analyses of members of CrRLK1L receptor-like kinase (RLK) family. I must say that I was surprised by your decision not to have our MS reviewed in-depth. The decision comes very unexpectedly in the light of the enthusiastic responses we have gotten from many colleagues, and the fact our MS reports the very first structure-function analysis of any member of this important plant-specific RLK family.

The CrRLK1L family of RLKs has received a lot of attention over the last couple of years. FERONIA, its first functionally characterized member, was found to play roles in hormone signaling (brassinosteroids, ehtylene, abscisic acid), resistance against powdery mildew, innate immunity, cell elongation and growth, root hair development, as well as pollen tube reception. Given that FERONIA and other members of the CrRLK1L family play such important and diverse roles in plants, it is of crucial importance to learn more about the underlying molecular mechanisms.

In our MS we report the very first structure-function analysis of this family of RLKs and gain important insights that will be crucial for many future experiments, i.e. that FERONIA's kinase activity is not required but that FERONIA has to get phosphorylated for activity; and that the extracellular domains of even its closest relatives are not functionally equivalent, suggesting distinct ligands. These results are based on a large number of constructs that were tested in functional assays by transgenic complementation of the feronia mutant pollen tube reception phenotype.

You wrote that you would expect us to report, e.g. which ligands are bound by the different RLKs of this family to make the manuscript of potential interest to EMBO Reports. Unlike in animals, where many signaling pathways have been described in great detail, we know only about a handful of bonafide RLK-ligand pairs, despite the over 600 RLKs in the Arabidopsis genome. The likely ligand of FERONIA was published less than two months ago, seven years after the publication of the FERONIA RLK! Given the sparse knowledge about RLK-ligand interactions in general and the fact that there are nearly 40 potential ligands of the class recently identified, your suggestion would amount to a completely new project.
In the light of the broad interest in the CrRLK1L family of RLKs and the fact that we report the first study of this kind, we strongly believe that our MS would be very well received by the readership of EMBO Reports. We thus kindly ask you to reconsider your decision and would greatly appreciate it if you had the manuscript reviewed in-depth.

Correspondence - editor 11 April 2014

Many thanks for your patience while we were considering your response on our decision not to send out your study for in-depth review. After having discussed the case again with my colleagues we have decided to indeed send it out to have it reviewed by experts in the field.

I will get back to you with a decision as soon as I have received their feedback.

2nd Editorial Decision 29 April 2014

Thank you for the submission of your research manuscript to our editorial office. Please find below the referees' comments that we have now received on your manuscript.

As you will see, all referees agree on the potential interest of the findings and, in principle, support publication of the study in EMBO reports. However, they also point out some issues that would need to be addressed before publication. Referees 1 and 2 feel that some controls are missing, for example to support the claim that the kinase activity of FERRONIA is not needed for pollen tube reception. Reviewer 3 states that stronger evidence for the localization of the (chimeric) proteins at the plasma membrane and with the expected topology should be provided. This reviewer also suggests testing whether the complementation ability of the different ICDs/ECDs extends to root hair development.

Overall, given these evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, also with regard to the novelty of the findings at the time of the submission. The length of the revised manuscript should not exceed roughly 29,000 characters (including spaces and references). Currently, and with almost 44,000 characters, your manuscript exceeds this limit and I would kindly ask you to shorten the text where possible. This might be achieved by combining the results and discussion sections, which avoids unnecessary redundancies. If you feel that the additional data requested by the reviewers would make the manuscript too long you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

This manuscript reports on the functional similarities and differences between the three CrRLK1L members FER, ANX1 and HERK1 in pollen tube reception. Although the intracellular domains of these kinases are interchangeable, the extracellular domains appear not to be, suggesting that the intracellular domains engage similar downstream signalling components, while they are activated by different upstream signals (potentially ligands). In addition, the authors show that the kinase activity of FER is not required for its function in pollen tube reception.
The manuscript presents a short but clear study that provides functional insight into the role of different CrRLK1L and their domains in pollen tube reception, and thus fits the EMBO Reports short-format papers that emphasize functional insight over extensive mechanistic detail. Plant RLKs play a role in the early steps of many different signalling pathways, and in many cases both upstream and downstream components remain to be identified. Therefore, understanding of the functional divergence/conservation of extra-, and intracellular RLK domains is of importance and wide-interest. I suggest the manuscript could be suitable for publication in EMBO Reports, after the following points are taken into account:

Major points:

© European Molecular Biology Organization

• Figure 4, complementation of the fer-1 mutation with CrRLK1L proteins ANX1, HERK1, or FER-ANX1 and FER-HERK1 chimeras: The authors compare the percentage of unfertilized ovules for several independent T1 transformants with untransformed control plants. Certain constructs complement the fer-1 mutant phenotype, whereas others do not. Although the authors detect a fluorescent signal in plants transformed with the different constructs at the same location as FER-GFP (Fig 3), the authors should show total protein accumulation to check if the levels of the GFP tagged proteins are also comparable. The different GFP fusion proteins might have different stability.
• In addition to the above, the authors do not show complementation by the wild-type FER. It would be important to see how complementation by the chimeras compares to the wild type FER.
• Figure 5, complementation of the fer-1 mutation with mutant versions of FER: Same two points as listed above.
• In line with the points above: In the last paragraph of Results section 'Functional analysis of the FERONIA intracellular domain': The authors say 'Changing all three to Ala led to production of stable FER protein that was localized in the filiform apparatus...', but do not show these data.
• Finally, since all ICDs from the tested RLKs could complement the fer phenotype (even the distantly related HERK1), it seems important to test also a chimera with an ICD from an RLK outside of the CrRLK 1L family, such as BRI1, CLV1 or FLS2 for example. Basically, is the presence of an ICD from any RLK sufficient?

Minor points:

• Figure 1AB shows pollen tube growth in wild type and fer-1 ovules. As this has been published by the authors before (Escobar-Restrepo et al. (2007) Science 317: 656-660), I do not see the necessity to include this picture as one of the main figures of the manuscript.
• Page 6, second paragraph: 'Domain swap analysis of FERONIA and related proteins': percentages of identity and similarity between the different domains could be summarized in a table, and added to Figure 2.
• Page 6, second paragraph: "However, the ECDs of all three CrRLK1L proteins contain malectin-like domains"; this should refer to Figure 1D, not 1C.
• Page 7, second paragraph: 'Domain swap analysis of FERONIA and related proteins' the authors refer to Fig 3D for localization of the ANX1(ECD)-FER(ICD)-GFP protein, but these data are actually shown in Fig 3E.
• Page 8, last paragraph: 'Functional analysis of the FERONIA intracellular domain': While in the Discussion it is mentioned that the changes of Ser and Thr to Asp are an attempt to mimic constitutive phosphorylation, this is not mentioned in the Results section.
• Page 9, first paragraph: 'endoplasmic reticulin' should be 'endoplasmic reticulum'.
• Page 9, first paragraph: 'THE' should be 'THE1'.
• Page 11, first paragraph: I would rather write "...by the presence of an extracellular domain potentially involved in ligand-binding" as not all RLKs bind a ligand.
• Page 11, first paragraph: 'FLAGILLIN-SENSITIVE2' should be 'FLAGELLIN SENSING2'.
• Page 11, first paragraph: ref. 34 is not the correct reference for the statement about the heterodimerisation of BAK1 with BRI1 and FLS2. You could cite Liebrand et al., Trends Plant Sciences 2014, for example.
• Last paragraph of the Discussion: The first sentence is an over-statement. It should rather be "Taken together, our results suggest that the three members of the CrRLK1L subfamily...". Also, the last sentence should be more specific: "..., indicating that FER might rather act as an important co-receptor recruiting other co-receptors or downstream targets to mediate signal transduction during PT reception". This is important as FER was shown to be a ligand-binding RLK at least in the case of root cell elongation where it binds to RALF.

Referee #2:

Kessler et al. reports the molecular genetic characterization of a family of receptor kinases in Arabidopsis thaliana. They have previously discovered that a receptor kinase protein, FERONIA, functions as the female determinant of pollen tube arrest in the ovule prior to fertilization. In this

study, they performed a structural-function study of this receptor kinase family via transgene-mediated complementation of the feronia fertilization defect phenotype. Various residues known to be phosphorylated or predicted to be required for kinase activity were mutated to non-phosphorylatable residue, Ala or the phosphomimetic residue, Asp and the effects of those point mutations on the ability of complementing the fertilization defect of the ovule were examined. They also performed a series of domain swapping experiments of this protein family in order to understand whether the extracellular domain or intracellular domain of those receptor protein kinases can be interchangeable. They concluded that whereas the intracellular kinase domain can be interchangeable among the protein family, the ligand-binding extracellular domain cannot. Overall, the datasets shown in this manuscript are interesting and the results provide a significant advancement to the field. We would recommend accepting this manuscript for publication in EMBO Reports after addressing specific comments provided below.

(1) One of the major points they claim in this report concerns the effect of a 'kinase dead' version of FERONIA. They concluded from this that kinase activity is not required for the FERONIA function in fertilization. We strongly recommend that the effect of the Lys-to-Arg mutation at the kinase active site has to be more carefully interpreted. They assume that this Lys-to-Arg mutation completely kills kinase activity; however this is based on their previously published in-gel kinase activity assay showing the absence/reduction of autophosphorylation signal (Escobar-Restrepo et al, 2007). The in-gel kinase activity assay is qualitative and does not support that this mutation completely abolishes kinase activity since it is not providing a Km value nor Vmax of kinase activity. We are also wondering if they had also performed Lys-to-Ala or Met, substitutions to neutral residues, not to Arg, which is a positive charge like Lys, whether the results might have been different. If the role of a receptor kinase is to perceive external signal and initiate a signal cascade and amplify the signal to downstream events, it is also possible that reduced activity of kinase of FERONIA can still effectively transmit signal via phosphorylation. Thus an effect of reduced kinase activity can be masked in the complex biological system represented by the in planta phenotype. In this context, they should provide a peptide sequence alignment of kinase domains of FERONIA and other known kinases and reference literatures in order to show how they identified the Lys residue of the predicted active site involved in ATP binding within FERONIA. They reported on this Lys-to-Arg mutation effect in E. coli expressed kinase (Escobar-Restrepo et al, 2007), but this previous paper also lacks citations for rationale of identifying the Lys residue, and therefore the reader cannot evaluate whether this particular Lys is indeed the same position as other known kinases such as those from mammalian systems. Also the Lys residue involved in the ATP binding site in protein kinase was proposed to form its salt bridge with Glu. In this context, it is prudent to clarify their choice of a conservative change of Lys to Arg to study the effect of mutations at this residue. The authors may also wish to look at Selva et al ("Mitogen-activated protein kinase stimulation by a tyrosine kinase negative EGFR" JBC 268:2250-2254, 1993) since these investigators reported that although a mutated Arg721EGF-R is kinase negative in vitro, a low level of EGF-stimulated tyrosine phosphorylation of this mutated receptor is observed in vivo. Thus, the question of whether the kinase is really kinase dead in planta becomes a quantitative question, rather than a qualitative one. Bottom line is that the authors should examine a series of mutations other than arginine, such as alanine, which are more distantly related chemically to lysine, to back up the claims that the mutation causes a truly dead kinase. In planta measurements would be nice but I would not require them for acceptance of this work.
(2) I was also wondering whether they did the kinase assay with the STS mutant FERONIA proteins to compare in vivo effect and in planta function in a similar way as they discussed with the Lys-to-Arg mutation effect of the ATP binding site. Their experiments of STS-to-AAA or STS-to-DDD mutation study indicate that none of the single residue substitution mutations cause altered effects on the complementation assay but that triple residue substitutions could have dramatic effects in planta. It would be nice to have some comparison and correlations between the in vitro kinase assay and in planta complementation results.
(3) Please add the data of the complementation of the feronia mutant with the wildtype FERONIA transgene in Fig 4 and Fig 5.
For these reasons, I would suggest that the authors put greater emphasis on the results of the excellent domain swap experiments rather than whether or not the kinase activity is needed for biological function, and discuss the caveats on deadness in vivo compared to in vitro with the Lys-to-Arg mutation versus other less conservative mutations, as suggested by the previous work with EGFR.

© European Molecular Biology Organization

Other points:
"In mice, the EPIDERMAL GROWTH FACTOR (EGF) family member ErbB3 has a non-functional kinase domain, and acts in a complex with other EGF proteins with active kinase domains." Please verify the meaning of this, i.e., that EGF is a ligand and that EGFR is the receptor. This line should perhaps read "In mice, the EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) family member ErbB3 has a non-functional kinase domain, and acts in a complex with other EGFR proteins with active kinase domains."

Referee #3:

Kessler et al examine the potential of the predicted extracellular (ECD) and intracellular (ICD) domains of ANX1 and HERK1, two CrRLK1L proteins with respectively high and low homology to FER, to substitute the predicted corresponding predicted ECD and ICD of FER. The functional assay used consists in transforming the <i>fer</i> mutant and assessing whether the chimeric proteins complement the <i>fer</i> phenotype.
Kessler et al provide evidence that the predicted ECD of FER cannot be substituted by the predicted ECD of ANX1 or HERK1. In contrast, the ICD of ANX1 or HERK1 is functionally equivalent to that of FER.
In conclusion, Kessler et al suggest that FER, ANX1 and HERK1 have common downstream signaling pathways but distinct upstream components. Furthermore, mutagenesis experiments indicate that the kinase activity of the ICD is not necessary for FER function.
Although this paper does not provide new insights on how FER perceives and signals in response to the arriving pollen tube, it nevertheless reveals (and this I believe is the main finding of this report) that the predicted ICD of ANX1 and HERK1 can indeed substitute that of FER, suggesting indeed that they have common downstream signaling components. This is of wide interest in the field. In this context, this manuscript would benefit if it would also report whether the predicted ICD and ECD of ANX1 and HERK1 are able to substitute FER ICD and ECD, respectively, in the context of root hair development.

Major points.
The more sensitive points raised by this work concern the negative results obtained with the predicted ECD of ANX1 and HERK1, which both fail to complement fer when fused to the predicted ICD of FER. One interpretation, favored by Kessler et al, is that indeed the FER predicted ECD cannot be replaced by the predicted ECD of ANX1 and HERK1 and thus FER uses specific upstream signaling components.
Another way to interpret the data is that the chimeric proteins are not behaving the way the authors expect them to behave. Indeed, it is assumed throughout the manuscript that FER, ANX1 and HERK1 are localized to the plasma membrane through their transmembrane domain while the ECD and ICD are exposed to the extracellular and intracellular space. Furthermore, authors state that HERK1-GFP and ANX1-GFP fusion proteins showed a subcellular localization very similar to that of FER-GFP in synergids (when the respective genes are under the control of pFER). These affirmations are poorly substantiated if only based on Figure 3. Although HERK1-GFP and ANX1-GFP certainly localize to the micropolar end of the synergids, it is impossible from these data to ascertain that they are indeed localized to the plasma membrane in the same topology expected for FER. I checked what is known about the subcellular localization of these proteins. In the case of FER, Escobar-Restrepo et al (2007) showed that FER is associated with the plasma membrane in onion cells and leaves (Figure 4 of Escobar-Restrepo et al 2007). Concerning the synergids, Escobar-Restrepo et al provide data similar to those in Figure 3A. These results do not ascertain that FER is indeed associated with the PM of synergids and they do not address at all that the FER protein is arranged with the expected topology. FER could be sticking to the PM of onion or leaf cells and yet not spanning it via the TM. Biochemical experiments are needed to address these questions. These experiments would indeed also confirm that the predicted ICD is indeed intracellular and the predicted ECD is indeed extracellular. Another point that is little discussed in Escobar-Restrepo et al 2007 and indeed in this manuscript concerns the signal peptide in the N terminal part of the protein (Figure 1 mentions the acronym "SP" but does not refer to it in the legend of the figure). Is it cleaved? What about the signal peptide of ANX1 and HERK1? Depending on the answer one could imagine that the SP of ANX1 or HERK1 are not processed in the same manner as FER. This could change the topology of the protein, particularly in its

extracellular domain. In the case of FER, the amino acids 12-31 are predicted to contain a transmembrane region (http://smart.embl-heidelberg.de/smart/show_motifs.pl). Thus, the ECD of FER could be anchored to the PM in two places thus generating a bent ECD. The uncertainties concerning how FER, ANX1 and HERK1 are associated with the PM and what is their topology in this context need to be discussed in the text as they might change the interpretation.
The concern regarding the exact subcellular localization and association with the PM of FER, ANX1 and HERK1 is further corroborated concerning the subcellular localization of ANX1, which does not appear to be strictly membrane associated. Indeed Miyazaki et al 2009 mentioned that they could detect ANX1 in the plasma membrane and cytoplasm. In Boisson-Dernier et al 2009 ANX1 seems to also be found in the cytoplasm (although they use the strong ACA9 pollen-specific promoter which could mask the genuine localization of the protein).
In conclusion, Kessler et al should discuss these issues and should at least make sure that their chimeric constructs are able to make proteins that are associated with the PM. This could be done as in figure 4A-4E of Escobar-Restrepo et al 2007.
Minor points
1) Figure 1D has a wrong or confusing color code. For example, the construct ANX1[ECD]-FER[ICD] is schematized with FER ECD (Green) and ANX1 ICD (red).
2) Same problem with Figure 3. For example, the text says "Primary transformants expressing the ANX1 ECD fused to the ICD of FER showed expression of the GFP fusion protein in the filiform apparatus of synergids in multiple transformants (Figure 3D)". Yet, Figure 3D is labeled "FER[ECD]-ANX1[ICD]". Shouldn't it be "ANX1[ECD]-FER[ICD]" ?
3) I could not find where in the text it is specified that the acronym "ECD" stands for "extracellular domain".

1st Revision - authors' response 13 October 2014

Detailed Responses to the Reviewers' Comments

We would first like to thank the reviewers for their positive assessment of our work and for their constructive criticisms. We have carefully read and addressed all their comments in the revised manuscript. The original comments of the reviewers are in blue while our response is in black.

Referee #1:

This manuscript reports on the functional similarities and differences between the three CrRLK1L members FER, ANX1 and HERK1 in pollen tube reception. Although the intracellular domains of these kinases are interchangeable, the extracellular domains appear not to be, suggesting that the intracellular domains engage similar downstream signalling components, while they are activated by different upstream signals (potentially ligands). In addition, the authors show that the kinase activity of FER is not required for its function in pollen tube reception.

The manuscript presents a short but clear study that provides functional insight into the role of different CrRLK1L and their domains in pollen tube reception, and thus fits the EMBO Reports short-format papers that emphasize functional insight over extensive mechanistic detail. Plant RLKs play a role in the early steps of many different signalling pathways, and in many cases both upstream and downstream components remain to be identified. Therefore, understanding of the functional divergence/conservation of extra-, and intracellular RLK domains is of importance and wide-interest. I suggest the manuscript could be suitable for publication in EMBO Reports, after the following points are taken into account:

Major points:

• Figure 4, complementation of the *fer-1* mutation with CrRLK1L proteins ANX1, HERK1, or FER-ANX1 and FER-HERK1 chimeras: The authors compare the percentage of unfertilized ovules for several independent T1 transformants with untransformed control

plants. Certain constructs complement the *fer-1* mutant phenotype, whereas others do not. Although the authors detect a fluorescent signal in plants transformed with the different constructs at the same location as FER-GFP (Fig 3), the authors should show total protein accumulation to check if the levels of the GFP tagged proteins are also comparable. The different GFP fusion proteins might have different stability.

The pFER constructs show varying degrees of sporophytic expression in the T1 generation (see Figure 2) due to position effects. Sporophytic expression does not correlate with the ability of constructs to complement the *fer* PT reception phenotype. There are only 2 synergid cells in each ovule; therefore, total protein accumulation will mostly reflect sporophytic expression, which is not relevant to the gametophytic phenotype. Thus, we feel that comparing GFP expression levels in the synergids by microsopy gives a more relevant estimation of protein expression levels than a Western blot for total protein levels in seedlings or entire flowers.

• In addition to the above, the authors do not show complementation by the wild-type *FER*. It would be important to see how complementation by the chimeras compares to the wild type *FER*.

As suggested, data for *pFER::FER-GFP* primary transformants was added to Figure 3.

• Figure 5, complementation of the *fer-1* mutation with mutant versions of FER: Same two points as listed above.

As suggested, data for *pFER::FER-GFP* primary transformants was added to Figure 4.

• In line with the points above: In the last paragraph of Results section 'Functional analysis of the FERONIA intracellular domain': The authors say 'Changing all three to Ala led to production of stable FER protein that was localized in the filiform apparatus...', but do not show these data.

Due to space constraints, we elected to show GFP fusion protein localization data for non-complementing constructs only. The ones that do complement are expected to function in the relevant tissue.

• Finally, since all ICDs from the tested RLKs could complement the *fer* phenotype (even the distantly related HERK1), it seems important to test also a chimera with an ICD from an RLK outside of the CrRLK 1L family, such as BRI1, CLV1 or FLS2 for example. Basically, is the presence of an ICD from any RLK sufficient?

We constructed a new domain swap construct with the FER ECD and BRI1 ICD as suggested by Referee #1. The protein was localized to the region of the filiform apparatus similar to FER and the other constructs, but did not complement the *fer* PT reception phenotype (Figures 2G and 3, last paragraph of page 6).

Minor points:

• Figure 1AB shows pollen tube growth in wild type and *fer-1* ovules. As this has been published by the authors before (Escobar-Restrepo et al. (2007) Science 317: 656-660), I do not see the necessity to include this picture as one of the main figures of the manuscript.

These panels were moved to Expanded View Figure 1.

• Page 6, second paragraph: 'Domain swap analysis of FERONIA and related proteins': percentages of identity and similarity between the different domains could be summarized in a table, and added to Figure 2.

© European Molecular Biology Organization

The original Figure 2 was moved to Expanded View Figure 2 and a Table comparing the domains was added to Figure 1.

• Page 6, second paragraph: "However, the ECDs of all three CrRLK1L proteins contain malectin-like domains"; this should refer to Figure 1D, not 1C.

We thank Referee #1 for noting this. It was changed to Figure 1B to reflect the altered figure arrangement.

• Page 7, second paragraph: 'Domain swap analysis of FERONIA and related proteins' the authors refer to Fig 3D for localization of the ANX1(ECD)-FER(ICD)-GFP protein, but these data are actually shown in Fig 3E.

This is now corrected in the revised text.

• Page 8, last paragraph: 'Functional analysis of the FERONIA intracellular domain': While in the Discussion it is mentioned that the changes of Ser and Thr to Asp are an attempt to mimic constitutive phosphorylation, this is not mentioned in the Results section.

This is now also mentioned in the result section.

• Page 9, first paragraph: 'endoplasmic reticulin' should be 'endoplasmic reticulum'.

Corrected

• Page 9, first paragraph: 'THE' should be 'THE1'.

Corrected

• Page 11, first paragraph: I would rather write "...by the presence of an extracellular domain potentially involved in ligand-binding" as not all RLKs bind a ligand.

Corrected

• Page 11, first paragraph: 'FLAGILLIN-SENSITIVE2' should be 'FLAGELLIN SENSING2'.

Corrected

• Page 11, first paragraph: ref. 34 is not the correct reference for the statement about the heterodimerisation of BAK1 with BRI1 and FLS2. You could cite Liebrand et al., Trends Plant Sciences 2014, for example.

Reference was changed to Liebrand, et al.

• Last paragraph of the Discussion: The first sentence is an over-statement. It should rather be "Taken together, our results suggest that the three members of the CrRLK1L subfamily...". Also, the last sentence should be more specific: "..., indicating that FER might rather act as an important co-receptor recruiting other co-receptors or downstream targets to mediate signal transduction during PT reception". This is important as FER was shown to be a ligand-binding RLK at least in the case of root cell elongation where it binds to RALF.

The suggestion made by Referee #1 was incorporated into the final paragraph.

Referee #2:

Kessler et al. reports the molecular genetic characterization of a family of receptor kinases in *Arabidopsis thaliana*. They have previously discovered that a receptor kinase protein, FERONIA, functions as the female determinant of pollen tube arrest in the ovule prior to fertilization. In this study, they performed a structural-function study of this receptor kinase family via transgene-mediated complementation of the *feronia* fertilization defect phenotype. Various residues known to be phosphorylated or predicted to be required for kinase activity were mutated to non-phosphorylatable residue, Ala or the phosphomimetic residue, Asp and the effects of those point mutations on the ability of complementing the fertilization defect of the ovule were examined. They also performed a series of domain swapping experiments of this protein family in order to understand whether the extracellular domain or intracellular domain of those receptor protein kinases can be interchangeable. They concluded that whereas the intracellular kinase domain can be interchangeable among the protein family, the ligand-binding extracellular domain cannot. Overall, the datasets shown in this manuscript are interesting and the results provide a significant advancement to the field. We would recommend accepting this manuscript for publication in EMBO Reports after addressing specific comments provided below.

(1) One of the major points they claim in this report concerns the effect of a 'kinase dead' version of FERONIA. They concluded from this that kinase activity is not required for the FERONIA function in fertilization. We strongly recommend that the effect of the Lys-to-Arg mutation at the kinase active site has to be more carefully interpreted. They assume that this Lys-to-Arg mutation completely kills kinase activity; however this is based on their previously published in-gel kinase activity assay showing the absence/reduction of autophosphorylation signal (Escobar-Restrepo et al, 2007). The in-gel kinase activity assay is qualitative and does not support that this mutation completely abolishes kinase activity since it is not providing a Km value nor Vmax of kinase activity. We are also wondering if they had also performed Lys-to-Ala or Met, substitutions to neutral residues, not to Arg, which is a positive charge like Lys, whether the results might have been different. If the role of a receptor kinase is to perceive external signal and initiate a signal cascade and amplify the signal to downstream events, it is also possible that reduced activity of kinase of FERONIA can still effectively transmit signal via phosphorylation. Thus an effect of reduced kinase activity can be masked in the complex biological system represented by the in planta phenotype. In this context, they should provide a peptide sequence alignment of kinase domains of FERONIA and other known kinases and reference literatures in order to show how they identified the Lys residue of the predicted active site involved in ATP binding within FERONIA. They reported on this Lys-to-Arg mutation effect in *E. coli* expressed kinase (Escobar-Restrepo et al, 2007), but this previous paper also lacks citations for rationale of identifying the Lys residue, and therefore the reader cannot evaluate whether this particular Lys is indeed the same position as other known kinases such as those from mammalian systems.

A kinase domain alignment including both plant and non-plant kinases is provided in Expanded View Figure E4 to show that FER, ANX1, and HERK1 all have the conserved Lys in Subdomain II along with a glycine-rich motif in subdomain I and the conserved Glu in Subdomain III. Thus, we are confident that we have chosen the correct amino acid to disrupt kinase activity.

Also the Lys residue involved in the ATP binding site in protein kinase was proposed to form its salt bridge with Glu. In this context, it is prudent to clarify their choice of a conservative change of Lys to Arg to study the effect of mutations at this residue. The authors may also wish to look at Selva et al ("Mitogen-activated protein kinase stimulation by a tyrosine kinase negative EGFR" JBC 268:2250-2254, 1993) since these investigators reported that although a mutated Arg721EGF-R is kinase negative *in vitro*, a low level of EGF-stimulated tyrosine phosphorylation of this mutated receptor is observed *in vivo*. Thus, the question of whether the kinase is really kinase dead *in planta* becomes a quantitative question, rather than a qualitative one. Bottom line is that the authors should examine a series of mutations other than arginine, such as alanine, which are more

distantly related chemically to lysine, to back up the claims that the mutation causes a truly dead kinase. *In planta* measurements would be nice but I would not require them for acceptance of this work.

Referee #2 makes a good point about our choice of mutation in the dead kinase construct. Although our interpretation of the reference cited above is that other kinases are able to phosphorylate the dead kinase version of EGF-R, which allows it to transmit the EGF signal, a minute amount of residual kinase activity cannot be excluded. Therefore, we made two additional mutations in FER, a Lys-to-Ala mutation as suggested and a Lys-to-Glu mutation, which is commonly used in studies of plant Ser/Thr kinases. Both of these mutated versions of FER were able to complement the *fer-1* PT reception phenotype in the T1 generation (Figure 4).

(2) I was also wondering whether they did the kinase assay with the STS mutant FERONIA proteins to compare *in vivo* effect and *in planta* function in a similar way as they discussed with the Lys-to-Arg mutation effect of the ATP binding site. Their experiments of STS-to-AAA or STS-to-DDD mutation study indicate that none of the single residue substitution mutations cause altered effects on the complementation assay but that triple residue substitutions could have dramatic effects *in planta*. It would be nice to have some comparison and correlations between the *in vitro* kinase assay and *in planta* complementation results.

Given our results that kinase activity is not necessary for FER function, we felt that kinase assays on the activation loop mutations were not necessary for this publication.

(3) Please add the data of the complementation of the *feronia* mutant with the wildtype *FERONIA* transgene in Fig 4 and Fig 5.

As suggested, T1 data with the wild-type *pFER::FER-GFP* was added to Figures 3 and 4.

For these reasons, I would suggest that the authors put greater emphasis on the results of the excellent domain swap experiments rather than whether or not the kinase activity is needed for biological function, and discuss the caveats on deadness *in vivo* compared to *in vitro* with the Lys-to-Arg mutation versus other less conservative mutations, as suggested by the previous work with EGFR.

We have adjusted to discussion to include the new results with the additional FER mutants we made.

Other points:

"In mice, the EPIDERMAL GROWTH FACTOR (EGF) family member ErbB3 has a non-functional kinase domain, and acts in a complex with other EGF proteins with active kinase domains." Please verify the meaning of this, i.e., that EGF is a ligand and that EGFR is the receptor. This line should perhaps read "In mice, the EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) family member ErbB3 has a non-functional kinase domain, and acts in a complex with other EGFR proteins with active kinase domains."

We corrected this sentence according to Referee #2's suggestion.

Referee #3:

Kessler et al examine the potential of the predicted extracellular (ECD) and intracellular (ICD) domains of ANX1 and HERK1, two CrRLK1L proteins with respectively high and low homology to FER, to substitute the predicted corresponding predicted ECD and ICD of FER. The functional assay used consists in transforming the *fer* mutant and assessing whether the chimeric proteins complement the *fer* phenotype.

Kessler et al provide evidence that the predicted ECD of FER cannot be substituted by the predicted ECD of ANX1 or HERK1. In contrast, the ICD of ANX1 or HERK1 is functionally equivalent to that of FER.

In conclusion, Kessler et al suggest that FER, ANX1 and HERK1 have common downstream signaling pathways but distinct upstream components. Furthermore, mutagenesis experiments indicate that the kinase activity of the ICD is not necessary for FER function.

Although this paper does not provide new insights on how FER perceives and signals in response to the arriving pollen tube, it nevertheless reveals (and this I believe is the main finding of this report) that the predicted ICD of ANX1 and HERK1 can indeed substitute that of FER, suggesting indeed that they have common downstream signaling components. This is of wide interest in the field. In this context, this manuscript would benefit if it would also report whether the predicted ICD and ECD of ANX1 and HERK1 are able to substitute FER ICD and ECD, respectively, in the context of root hair development.

Since FER is involved in many aspects of plant development, it would indeed be interesting to test whether the constructs we used show differences in their behavior in distinct contexts. However, this would require that all constructs are first recovered in a *fer/fer* homozygous background. For constructs that do not complement the PT reception phenotype, homozygotes can only be recovered at extremely low frequency due to the almost complete gametophyte lethality of fer-1 or in a background in which FER can be expressed conditionally during reproduction. Given that large number of lines and constructs we generated, this would be a formidable taks well beyond the scope of the present study, which therefore focuses completely on pollen tube guidance.

Major points.

The more sensitive points raised by this work concern the negative results obtained with the predicted ECD of ANX1 and HERK1, which both fail to complement *fer* when fused to the predicted ICD of FER. One interpretation, favored by Kessler et al, is that indeed the FER predicted ECD cannot be replaced by the predicted ECD of ANX1 and HERK1 and thus FER uses specific upstream signaling components.

Another way to interpret the data is that the chimeric proteins are not behaving the way the authors expect them to behave. Indeed, it is assumed throughout the manuscript that FER, ANX1 and HERK1 are localized to the plasma membrane through their transmembrane domain while the ECD and ICD are exposed to the extracellular and intracellular space. Furthermore, authors state that HERK1-GFP and ANX1-GFP fusion proteins showed a subcellular localization very similar to that of FER-GFP in synergids (when the respective genes are under the control of pFER). These affirmations are poorly substantiated if only based on Figure 3. Although HERK1-GFP and ANX1-GFP certainly localize to the micropolar end of the synergids, it is impossible from these data to ascertain that they are indeed localized to the plasma membrane in the same topology expected for FER. I checked what is known about the subcellular localization of these proteins. In the case of FER, Escobar-Restrepo et al (2007) showed that FER is associated with the plasma membrane in onion cells and leaves (Figure 4 of Escobar-Restrepo et al 2007). Concerning the synergids, Escobar-Restrepo et al provide data similar to those in Figure 3A. These results do not ascertain that FER is indeed associated with the PM of synergids and they do not address at all that the FER protein is arranged with the expected topology. FER could be sticking to the PM of onion or leaf cells and yet not spanning it via the TM. Biochemical experiments are needed to address these questions. These experiments would indeed also confirm that the predicted ICD is indeed intracellular and the predicted ECD is indeed extracellular. Another point that is little discussed in Escobar-Restrepo et al 2007 and indeed in this manuscript concerns the signal peptide in the N terminal part of the protein (Figure 1 mentions the acronym "SP" but does not refer to it in the legend of the figure. Is it cleaved? What about the signal peptide of ANX1 and HERK1?

SP is now defined in the figure legend.

Depending on the answer one could imagine that the SP of ANX1 or HERK1 are not processed in the same manner as FER. This could change the topology of the protein,

particularly in its extracellular domain. In the case of FER, the amino acids 12-31 are predicted to contain a transmembrane region (http://smart.embl-heidelberg.de/smart/show_motifs.pl). Thus, the ECD of FER could be anchored to the PM in two places thus generating a bent ECD. The uncertainties concerning how FER, ANX1 and HERK1 are associated with the PM and what is their topology in this context need to be discussed in the text as they might change the interpretation.

Referee #3 makes some very good points regarding the localization and topolgy of FER in synergid cells. We currently do not have the technology to determine if the predicted ECDs are indeed extracellular in synergid cells. Given that there are only two cells per ovule and thus about 100 synergids per flower, we cannot do biochemical experiments in these tissues. However, we performed transient expression assays in onion epidermal cells with the pFER::ANX-GFP, pFER::HERK-GFP, and pFER::ANX[ECD]-FER[ICD]-GFP constructs and showed that they colocalize with membranes in plasmolyzed cells (Expanded View Figure E3). We now also discuss alternative scenarios of the effects of different topologies on *fer* complementation (page 9, last paragraph). However, microscopic evidence for FER and ANX1, as well as peptide evidence for FER and HERK from proteomics studies indicate that all three proteins are membrane localized. Furthermore, we discuss observations which indicate that, due to its pH sensitivity, GFP does not produce a detectable signal if it is extracellular in plants. Given that the GFP fusion proteins showed a clear GFP signal in the region of the filiform apparatus, it is thus likely that their topology is as expeted, i.e. the ICDs of these fusion proteins are indeed intracellular.

The concern regarding the exact subcellular localization and association with the PM of FER, ANX1 and HERK1 is further corroborated concerning the subcellular localization of ANX1, which does not appear to be strictly membrane associated. Indeed Miyazaki et al 2009 mentioned that they could detect ANX1 in the plasma membrane and cytoplasm. In Boisson-Dernier et al 2009 ANX1 seems to also be found in the cytoplasm (although they use the strong ACA9 pollen-specific promoter which could mask the genuine localization of the protein).

In conclusion, Kessler et al should discuss these issues and should at least make sure that their chimeric constructs are able to make proteins that are associated with the PM. This could be done as in figure 4A-4E of Escobar-Restrepo et al 2007.

FER and HERK1 have been identified in two membrane proteomics studies, indicating that their signal peptides direct them to the plasma membrane. ANX1-GFP, HERK1-GFP, ANX[ECD]-FER[ICD]-GFP, and HERK[ECD]-FER[ICD]-GFP fusion proteins are all associated with the plasma membrane and cell wall in transient infection experiments of onion epidermal cells (Figure E3), indicating that the fusion proteins are capable of PM localization.

Minor points

1) Figure 1D has a wrong or confusing color code. For example, the construct ANX1[ECD]-FER[ICD] is schematized with FER ECD (Green) and ANX1 ICD (red).

We thank Referee #3 for catching this. The color code has been corrected.

2) Same problem with Figure 3. For example, the text says "Primary transformants expressing the ANX1 ECD fused to the ICD of FER showed expression of the GFP fusion protein in the filiform apparatus of synergids in multiple transformants (Figure 3D)". Yet, Figure 3D is labeled "FER[ECD]-ANX1[ICD]". Shouldn't it be "ANX1[ECD]-FER[ICD]" ?

This labeling error has been corrected in the text.

3) I could not find where in the text it is specified that the acronym "ECD" stands for "extracellular domain".

The abbreviation has been defined in paragraph 3 of the introduction.

3rd Editorial Decision 31 October 2014

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now both positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

Referee 2 points out some minor modifications that would need to be incorporated in the ext before acceptance. In addition, this referee feels that the data at hand do not allow such strong conclusions about whether or not kinase activity is needed for FER function and recommends discussing this in a more careful manner. I think his/her suggestion of stating that the kinase-dead version of FER can complement the mutants is a very good way of interpreting the data and I would like to ask you to modify the abstract and manuscript text accordingly.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #2:

I recommend acceptance of this paper after a few minor edits are made. First, there are what must be two nagging 'typos' that generate confusion. In the section of Functional Analysis of the FER Intracellular Domain, they start with "The ICDs of CrRLK1 proteins contain a typical S/T kinase motif with a K at the active site (Figure 4A and Figure E4)." According to Figure E4, the lysine residue that was mutated to examine the effect of kinase mutation is K565 (K of KRG...). However, in Figure E2, the lysine residue of the current interest is shown at K561 (K of KVA...). I am assuming that this is a typing mistake and they did not mutate a wrong site in the actual mutagenetic experiment. Also in the Results section, they state that "Two additional non-conserved substitutions, K-A and K-E, could not complement fer-1 (Fig. 4B) indicating that kinase activity is not necessary for FER function in PT reception". Again, I believe it is a typing mistake and they are stating the opposite of what they intended, i.e., that the two additional susbstitutions COULD complement. Due to the above inconsistent information (I am assuming those are typing mistakes but cannot be certain), I am uncertain about which points should be considered to examine the effect of the lysine residue required for the ATP binding site of this receptor kinase. It might be a good idea to run RT-PCR with mRNA extracted from those transgenic plants that were transformed with point mutant constructs and sequence them to verify that what was used for phenotyping is indeed carrying the desired mutation.

Second, the whole question of whether kinase activity is required for Fer needs to be approached and discussed in the manuscript with more caution. While the in planta genetic data presented in this paper is important and somewhat compelling and suggests indeed that the kinase activity is not essential for some signaling to occur, I am worried that the logic is based on data that is not solid or based on analogy with related sequences that may NOT be conformationally similar. For example, the kinase activity measured in the original Science paper from this lab in 2007 shows autophosphophorylation with one substrate (autophosphorylation) and in one reaction, and isn't quantitative. Is the kinase 90% or 99% or 99.9 inactive? The reason this could be important is that in planta, we don't know for sure how much kinase activity is needed to get the signaling pathway

activated and there are instances in the literature where an in vitro result underestimates what is going on in vivo in this respect. Furthermore, we don't know how direct or indirect the effect of those mutations are. For example, if the lysine mutation causes the inability of the protein to fold properly in E. coli (or in planta too for that matter) rather than inability to perform catalysis, then the interpretation of the result needs to be different. As described in a recent paper using an ATP affinity label to identify lysine residues in the active site of plant protein kinases (Mol Cell Proteomics. Sep 2013; 12(9): 2481-2496.), there are four different lysine residues each of which may be operating under different conditions and without a clear crystal structure of the conformation of Feronia itself, some caution might be wise in terms of making conclusions with the data in hand. In the end, the mutant studies tell us more about what the mutant is doing, rather than what the wildtype enzyme is doing, and perhaps should be approached with some caution. At this point I would recommend being more conservative and stating only the observation that a "kinase-dead version of FERONIA can complement the fer mutant" and avoid the extended conclusion of saying that "kinase activity is not necessary (or not essential)", i.e., conclusively concluding that this protein only has a kinase-independent function.

In conclusion, apart from what must be two typos, and a little bit of overinterpretation, I believe this manuscript is acceptable for communication in this journal and don't wish to hold up publication. I believe the above considerations are minor glitches that can be easily handled by the editorial staff in consultation with the authors.

Referee #3:

I believe the authors have suitably addressed all the points I mentioned. I also went through their responses to the other reviewer's comments and I think they are acceptable as well.

2nd Revision - authors' response 10 November 2014

Response to Reviewer #2 Comments

I recommend acceptance of this paper after a few minor edits are made. First, there are what must be two nagging 'typos' that generate confusion. In the section of Functional Analysis of the FER Intracellular Domain, they start with "The ICDs of CrRLK1 proteins contain a typical S/T kinase motif with a K at the active site (Figure 4A and Figure E4)." According to Figure E4, the lysine residue that was mutated to examine the effect of kinase mutation is K565 (K of KRG...). However, in Figure E2, the lysine residue of the current interest is shown at K561 (K of KVA...). I am assuming that this is a typing mistake and they did not mutate a wrong site in the actual mutagenetic experiment.

The arrowhead in Figure E2 was mistakenly moved when the figure was modified, it now points to the correct K (K565 of the motif VAIK). The numbering on the sequence alignment was confusing since it referred to the alignment and not the FER protein. We removed these confusing numbers and added a 565 under the critical K.

Also in the Results section, they state that "Two additional non-conserved substitutions, K-A and K-E, could not complement fer-1 (Fig. 4B) indicating that kinase activity is not necessary for FER function in PT reception". Again, I believe it is a typing mistake and they are stating the opposite of what they intended, i.e., that the two additional substitutions COULD complement.

© European Molecular Biology Organization

This was indeed a typing mistake, the text on the bottom of page 6 has been changed to "Two additional non-conserved substitutions, K-A and K-E, could also complement *fer-1* (Fig. 4B) indicating that kinase activity is not necessary for *FER* function in PT reception."

Due to the above inconsistent information (I am assuming those are typing mistakes but cannot be certain), I am uncertain about which points should be considered to examine the effect of the lysine residue required for the ATP binding site of this receptor kinase. It might be a good idea to run RT-PCR with mRNA extracted from those transgenic plants that were transformed with point mutant constructs and sequence them to verify that what was used for phenotyping is indeed carrying the desired mutation.

We genotyped all of our transformants to check that the desired mutations/domain swaps were indeed present as is noted in the materials and methods. We don't feel it is necessary to show a figure with the sequencing results since this is routine molecular biology and the above typing mistake simply occurred when revising the manuscript.

Second, the whole question of whether kinase activity is required for Fer needs to be approached and discussed in the manuscript with more caution. While the in planta genetic data presented in this paper is important and somewhat compelling and suggests indeed that the kinase activity is not essential for some signaling to occur, I am worried that the logic is based on data that is not solid or based on analogy with related sequences that may NOT be conformationally similar. For example, the kinase activity measured in the original Science paper from this lab in 2007 shows autophosphophorylation with one substrate (autophosphorylation) and in one reaction, and isn't quantitative. Is the kinase 90% or 99% or 99.9 inactive? The reason this could be important is that in planta, we don't know for sure how much kinase activity is needed to get the signaling pathway activated and there are instances in the literature where an in vitro result underestimates what is going on in vivo in this respect. Furthermore, we don't know how direct or indirect the effect of those mutations are. For example, if the lysine mutation causes the inability of the protein to fold properly in E. coli (or in planta too for that matter) rather than inability to perform catalysis, then the interpretation of the result needs to be different. As described in a recent paper using an ATP affinity label to identify lysine residues in the active site of plant protein kinases (Mol Cell Proteomics. Sep 2013; 12(9): 2481-2496.), there are four different lysine residues each of which may be operating under different conditions and without a clear crystal structure of the conformation of Feronia itself, some caution might be wise in terms of making conclusions with the data in hand. In the end, the mutant studies tell us more about what the mutant is doing, rather than what the wildtype enzyme is doing, and perhaps should be approached with some caution. At this point I would recommend being more conservative and stating only the observation that a "kinase-dead version of FERONIA can complement the fer mutant" and avoid the extended conclusion of saying that "kinase activity is not necessary (or not essential)", i.e., conclusively concluding that this protein only has a kinase-independent function.

We toned down our interpretation of the dead kinase results in the abstract and discussion.

On Page 10, we changed the heading of the section and also added a sentence about how we can't know for sure if we have really knocked out FER kinase activity in vivo with the amino acid changes.

FER Mutants with Non-Conserved Changes in the Active Site Complement FER Function.

RLKs are defined by the presence of an ECD potentially involved in ligand binding, a transmembrane domain, and an intracellular kinase domain. Most RLKs are presumed to sense a ligand, and phosphorylate another protein to initiate a signal transduction cascade [25]. While FER has kinase activity *in vitro* that can be abolished by a K-R change in the active site [7], the dead

kinase version of FER was able to complement *fer-1*, indicating that kinase activity is not necessary for FER function in PT reception. The K565R dead kinase was also able to partially complement the reduced response to root mechanostimulation in *fer* mutants [35]. These results indicate that either a K565R change retains partial kinase function, or that another kinase in the complex is able to substitute for FER's role in signal transduction. Confirmation of the ability of K565 changes to completely abolish kinase activity awaits the identification of endogenous FER targets.

We also changed the last sentence of the paper to:

Furthermore, the kinase activity of FER may not be essential to execute its function, indicating that FER might act as an important co-receptor recruiting other co-factors or downstream targets to mediate signal transduction during PT reception.

4th Editorial Decision 11 November 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

© European Molecular Biology Organization