THE EMBO JOURNAL

Cell division protein FtsK coordinates bacterial chromosome segregation and daughter cell separation in Staphylococcus aureus

Helena Veiga, Ambre Jousselin, Simon Schäper, Bruno Saraiva, Leonor Marques, Patricia Reed, Joana Wilton, Pedro Pereira, Sergio Filipe, and Mariana Pinho
DOI: 10.15252/embj.2022112140

Corresponding author(s): Mariana Pinho (mgpinho@itqb.unl.pt) , Helena Veiga (hveiga@itqb.unl.pt)

Review Timeline:

Submission Date:	17th Jul 22
Editorial Decision:	9th Aug 22
Appeal:	16th Aug 22
Editorial Decision:	18th Aug 22
Revision Received:	3rd Feb 23
Editorial Decision:	2nd Mar 23
Revision Received:	15th Mar 23
Accepted:	21st Mar 23

Editor: Ieva Gailite

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Mariana,

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received three reviewer reports on your manuscript, which are included below for your information. Based on these comments, we unfortunately had to conclude that the study is not a sufficiently strong candidate for publication in The EMBO Journal, but I would recommend a transfer of the manuscript to EMBO Reports, where the responsible editor would like to invite a revision.

As you can see, all reviewers find the proposed model of FtsK-mediated regulation of cell wall degradation upon S. aureus cell division novel and of interest. However, while reviewer #2 is more positive in their assessment, reviewers #1 and #3 find that the proposed model of FtsK interaction with TF to regulate Sle1 stability and recruitment to the septum is not sufficiently supported by the current data, and they highlight alternative explanations that would require experimental testing and likely re-interpretation of the findings. Given these opinions from good experts in the research field, and since substantial experimental work with uncertain outcome would be needed to address these issues, I am afraid that we cannot offer further proceedings towards publication in The EMBO Journal.

That being said, based on the interest expressed in the reviewer reports, I have discussed your manuscript and referee comments with my colleague Martina Rembold at our sister journal EMBO Reports. I am glad to say that Martina is interested in receiving a revised version. She requests to either provide more data to substantiate the proposed role of TF in protecting Sle1 from degradation or tone down all relevant conclusions on this aspect and discuss alternative mechanisms. It will however be essential to further substantiate the proposed role of FtsK in the translocation of Sle1 or to test alternative models (see referee 3, major point 2 and additional point 3). If you are interested in this option, please contact Martina directly (martina.rembold@embo.org) to discuss the revisions further.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, and I hope you will view the possibility of a transfer favourably. If this is the case, you can use the link below to transfer the manuscript directly:

Link Not Available

With kind regards,

Ieva

Ieva Gailite, PhD
Senior Scientific Editor
The EMBO Journal
Meyerhofstrasse 1
D-69117 Heidelberg
Tel: +4962218891309
i.gailite@embojournal.org

****

Referee #1:

Cells of the bacterial pathogen Staphylococcus aureus are spherical and divide by building a flat peptidoglycan (PG) septum at midcell that separates the daughter compartments. The septum must then be processed by PG cleaving enzyme to promote septal splitting and the separation of the daughters. Sle1 is a PG hydrolase that is important for this splitting process. However, it has remained unclear how Sle1 is directed to the septal region or how it is regulated so that it does not prematurely initiate separation before other essential cell cycle events like chromosome segregation are complete. The paper from Viega and co-workers sought to address this mystery using an elegant screen for factors required for proper Sle1 export. Surprisingly, mutants inactivating the cell division DNA translocase FtsK were isolated that not only blocked Sle1 export but also prevented Sle1 accumulation. Accordingly, both delta-sle1 and delta-ftsK mutants were shown to have similar morphological defects indicative of a delay in cell separation. Notably, this phenotype was suppressed in both cases by the addition of purified Sle1 to cells, indicating that a lack of exported Sle1 was the cause of the problem. Additionally, suppressors of the delta-ftsK phenotype that restored cell separations and Sle1 accumulation were found in clpX, suggesting that Sle1 is degraded by the ClpXP protease in the absence of FtsK. A pull-down of tagged Sle1 identified a number of potential interaction partners of the PG hydrolase. One of these was the chaperone trigger factor (TF). Further analysis found that the subcellular localization of TF was altered from

being enriched at the septum to more diffuse in the cell following ftsK deletion or treatment with a DNA damaging agent. Two-hybrid analysis suggested an interaction between TF and FtsK. TF inactivation was also shown to delay cell cycle progression. Based on all of the data, the authors propose a model for Sle1 regulation in which the protein is protected from ClpXP degradation by TF and brought to the septum for export by the TF-FtsK interaction.

I think the authors have a very interesting discovery on their hands. The connection between Sle1 accumulation and FtsK is solid. However, I do not think the rest of the results definitively support a role for TF in this process or clearly indicate how the sensing of DNA damage is involved. It therefore remains unclear how Sle1 degradation by ClpXP is regulated during normal growth or upon DNA damage or how export of the protein is targeted to the septum. These issues need to be addressed to build on the initial exciting discovery and understand the underlying mechanism.

Major Comments:

1) A key prediction of the model is that the targeting of Sle1 to the septum via the TF-FtsK interaction is critical for blocking Sle1 degradation by ClpXP and promoting its export at the septum. If true, then inactivation of TF should prevent this targeting and lead to Sle1 degradation. However, Sle1 levels appear to be unaffected by TF inactivation. This discrepancy essentially invalidates the model and raises questions as to whether TF is involved in the process at all, especially since TF was of many Sle1 interactors chosen for investigation into its role in Sle1 targeting/regulation. Other potentially relevant interactors higher on the list thatn TF like the chaperone DnaK and the division protein FtsZ were not studied and seem equally likely to be involved.

2) The proposed model also relies critically on the detected interaction between TF and FtsK being real. However, the interaction was only detected by two-hybrid analysis and not validated by additional assays. Since TF is a chaperone capable of interacting with a variety of proteins, controls showing that TF doesn't interact with any protein partner in the two-hybrid assay are also needed. Thus, the TF-FtsK interaction remains suspect until it is validated further.

3) The localization patterns of TF-GFP are also a major pillar of the proposed model. They are interpreted as showing that TF-GFP forms a gradient with a bias towards the septum in WT cells and that this pattern is disrupted in FtsK mutants or cells treated with nalidixic acid. Some quantification of the localization patterns is needed here as I do not see a strong difference in pattern. In both WT and mutant/treated cells, it looks like TF-GFP is asymmetrically distributed to me.

4) The addition of DNA damaging agents appears to reduce the level of Sle1 that accumulates in/on cells. However, how this connects with FtsK and the potential checkpoint model is not clear at all and needs to be addressed if this line of investigation is to be included in the report. For example, are FtsK levels also altered upon DNA damage? Or is FtsK localization changed?

5) Another major prediction of the model is that blocking cell division should prevent Sle1 stabilization and export. Does inhibition of cell division by disrupting Z-ring formation or via other treatments that would prevent FtsK recruitment to the division site block Sle1 stabilization?

6) Figure 3A: A control using a deletion of sle1 is needed to support the identity of the band designated as Sle1 is indeed Sle1.

7) Figure 3C and 4B: If the cell cycle progression is a problem with Sle1 export, then it should be corrected by the addition of purified Sle1 as in the case of delta-sle1 and delta-ftsK. It would also be useful to include data for delta-sle1 in these panels for comparison.

Minor Points:

Line 45: comma after "SOS response"

Line 47: "which assembles at the..."

Line 68: Some discussion of the work from Frees and co-workers on Sle1 control by ClpX should be included in the intro.

Line 73: Is checkpoint really the appropriate term here?

Since both FtsK and Sle1 can be deleted and Sle1 can be added externally to complement, there must be additional layers of Sle1 regulation post-export that are at play. This concept should be discussed in the Discussion.

Line 108: I am not sure what it means to be "adjacent to the inner side of the membrane"

Line 290-294: Some sort of molecular explanation for how the completion of DNA translocation by FtsK would be "sensed" to affect Sle1 levels is needed here. Otherwise, there is little value in this speculative discussion.

Referee #2:

Veiga et al have studied the role of the N-terminus of Staphylococcus aureus FtsK during cell division/cell splitting. It is shown that in the absence of FtsK, cell wall hydrolase Sle1 becomes depleted from cells, in a ClpXP (proteasome) dependent manner. The authors further show that FtsK established a gradient of ribosomal chaperone trigger factor (TF) towards the division septum, while TF interacts with FtsK on the one hand, and with Sle1 on the other hand. Intriguingly, when chromosome segregation is perturbed, affecting FtzsK activity, Sle1 also becomes depleted from cells, or shows lowered expression, leading to a delay in cell splitting. The authors propose a model in which FtsK senses problems in chromosome segregation, and transduces them, via TF, to control Sle1 levels, which in turn affect cell splitting, thus leading to delayed splitting when segregation is itself delayed.

The manuscript is very well written, data are well presented. I enjoyed reading the work, it is very succinctly written, and a really intriguing mechanism, quite unanticipated. I have a few minor comments:

Line 32: "TF binds unfolded Sle1 ...." This is not shown, just binding.

Many control experiments are shown in the supplement. I wonder why the authors chose to show more supplementary figures than figures in the main manuscript. I would strongly encourage showing most control experiments in the manuscript, and leave only non-important information in the supplement.

Line 250: "suggesting that in these conditions, FtsK may undergo a conformational change that leads to loss of interaction with TF." I wonder why the authors suggest this, in line of the finding that motor activity of FtsK does influence Sle1 levels. Formally, it is always conformational changes that transfer information, or change enzyme activities, but in this case, I would think it is more likely that the activity of FtsK (increasing as segregation is perturbed) affects Sle1 levels. However, this point is really at the discretion of the authors to deal with.

Line 265 "On the contrary, S. aureus,..." I can not find a contradiction between the two sentences, please rephrase

Line 269 "possibly exposing an immature cell surface" .... I do not see how the cell surface could be immature. My best guess would be that S. aureus cells want to prevent complete separation despite unfinished segregation of chromosomes. If the authors see a link to the cell surface (i.e. a specific activity of Sle1 changing the structure of the peptidoglycan other than splitting activity) they should better explain this.

Line 272, more than semantics: "In this work, we have identified the mechanism for this newly recognized level of regulation..." This sentence implies that the mechanism is pretty much fully understood, but it is not. The authors provide very good evidence suggesting the mechanism they propose, but, for example, line 279: "Therefore, the concentration of TF´s cargo, Sle1, will be 279 higher near the Sec machineries present in the septal region and lower near...." this is what may be the case, but it is not shown. Therefore, the strongest claim the authors can make is "we have identified a mechanism....." or, to be most accurate "found data suggesting a mechanism .....". I would be ok with the prior sentence, but not with "the mechanism".

Referee #3:

This paper reports a novel role for FtsK in protein stability and secretion. The authors start with the surprising and very interesting finding that loss of FtsK leads to loss of Sle1. What ensues is a genetic and biochemical analysis to try and understand why. In the absence of FtsK, Sle1 is degraded in a ClpXP dependent fashion. They find that Trigger factor (TF) interacts with FtsK and Sle1 and is asymmetrically distributed in the cell in a FtsK dependent manner. This leads to a model in which TF acts as a bridge between FtsK and Sle1 so that Sle1 is secreted at the septum where FtsK is localized (model figure). This is quite novel if true. However, there appears to more going on, and there appears to be something missing - why is Sle1 degraded in the absence of FtsK but not degraded in the absence of TF? Why does loss of ClpX suppress the morphologic defects due to loss of FtsK?
Major points
1) A major point is the presence of Sle1 when TF is absent but its complete absence when FtsK is missing because it is degraded by ClpXP. So, TF does not protect Sle1 from degradation. If anything, it is more like TF interacts with Sle1 and brings it to FtsK but if FtsK is absent TF delivers it to ClpXP.
2) The most important point. The authors say that a ClpX mutant was isolated as a suppressor of ftsK (around line 180) and restores Sle1 levels. However, no information is given about how this was obtained (pure chance?). This should be explained more clearly and should be included. More importantly they show that the ClpX mutant suppresses the morphological defect of ftsK. If this is due to Sle1 getting secreted, then FtsK has nothing to do with secretion of Sle1. This seems to be ignored.
3) The introduction talks about the SOS response inhibiting division in E. coli but does not mention the more relevant situation that occurs in Caulobacter, Bacillus and even in Staphylococcus. In these latter cases the SOS response leads to the presence of small inner membrane peptides that interact with the division machinery (FtsWI) to block septal PG synthesis. This should be

discussed.
4) I am not sure why the authors want to call their translocase FtsK instead of the Bacillus name. SpoIIIE looks more like the Gram negative FtsK, in fact the transmembrane domains of the two proteins overlap (alphafold prediction models).
Additional points
1) The pull down of proteins with Sle1 is not very specific (Table S3). TF was only selected for additional study because it was a chaperone (and not a ribosomal protein).
2) The morphological defects of ftsK is more severe than that of sle1 in both strain backgrounds suggesting a more prominent role for FtsK (Fig. 1 and Fig. S2)
3) The observation that deletion of sle1 can be compensated by adding exogenous Sle1 suggests that Sle1 can localize effectively from the environment and that targeted secretion is not essential (Fig. S2). It suggests that Sle1 mature form is sufficient for localization. Have the authors checked to see if exogenously added HisTag-Sle1 is localized similarly to the endogenously produced Sle1?

Dear Ieva
First of all, I would really like to thank you for the highly efficient way in which you handled this manuscript and also the reviewers for completing the reviews in such a timely manner! This is uncommon nowadays and highly appreciated.

Reading the comments from the reviewers I think that all of them found the work novel and interesting. You mention that "since substantial experimental work with uncertain outcome would be needed to address these issues" you can not accept the manuscript.
However, we are convinced that we can do all required experiments to address reviewers´comments within 3 months (please see below detailed explanation, in a reply to major points of reviewer 1 and all points of reviewer 3, as reviewer 2 did not raise major concerns). We have all the required strains made (except one that we are currently constructing to try to delocalize FtsK and see if the TF pattern follows), and all the techniques optimized, so we are very confident we can address all issues raised. It took the first author 6 years to figure out this story, so she is more than glad to invest another 3 months

Therefore, we would like to ask you for the opportunity to make these experiments in the next 3 months. If they are informative, you would allow a resubmission to EMBO Journal. If they are not informative/do not contribute to answer the reviewers´ questions, we would submit an improved version to EMBO Reports.

Specifically, we would like to know if you would be willing to revise the manuscript if we address the following points:

- Strengthen data on TF-FtsK and/or TF/ClpX interactions by doing new pull down experiments and doing the requested negative controls for the bacterial two-hybris experiments
- Strengthen the role of FtsK on Sle1 secretion by analysing secreted levels of Sle1 in the autolytic extracts of various mutants (currently we show total cellular levels)
- Quantify the changes in TF localization pattern by doing heatmaps and quantifying the total signal in the septal region vs the rest of the cell
- Tone down the role of FtsK in the presence of DNA damaging agents in case we can not strengthen this role.

We also propose additional experiments (see below), that we can easily do, to address all other comments from the reviewers.

We look forward to hearing from you.

Kind regards
Mariana Pinho

Referee #1:

Cells of the bacterial pathogen Staphylococcus aureus are spherical and divide by building a flat peptidoglycan (PG) septum at midcell that separates the daughter compartments. The septum must then be processed by PG cleaving enzyme to promote septal splitting and the separation of the daughters. Sle1 is a PG hydrolase that is important for this splitting process. However, it has remained unclear how Sle1 is directed to the septal region or how it is regulated so that it does not prematurely initiate separation before other essential cell cycle events like chromosome segregation are complete. The paper from Viega and co-workers sought to address this mystery using an elegant screen for factors required for proper Sle1 export. Surprisingly, mutants inactivating the cell division DNA translocase FtsK were isolated that not only blocked Sle1 export but also prevented Sle1 accumulation. Accordingly, both delta-sle1 and delta-ftsK mutants were shown to have similar morphological defects indicative of a delay in cell separation. Notably, this phenotype was suppressed in both cases by the addition of purified Sle1 to cells, indicating that a lack of exported Sle1 was the cause of the problem. Additionally, suppressors of the delta-ftsK phenotype that restored cell separations and Sle1 accumulation were found in clpX, suggesting that Sle1 is degraded by the ClpXP protease in the absence of FtsK. A pull-down of tagged Sle1 identified a number of potential interaction partners of the PG hydrolase. One of these was the chaperone trigger factor (TF). Further analysis found that the subcellular localization of TF was altered from being enriched at the septum to more diffuse in the cell following ftsK deletion or treatment with a DNA damaging agent. Two-hybrid analysis suggested an interaction between TF and FtsK. TF inactivation was also shown to delay cell cycle progression. Based on all of the data, the authors propose a model for Sle1 regulation in which the protein is protected from ClpXP degradation by TF and brought to the septum for export by the TF-FtsK interaction.

I think the authors have a very interesting discovery on their hands. The connection between Sle1 accumulation and FtsK is solid. However, I do not think the rest of the results definitively support a role for TF in this process or clearly indicate how the sensing of DNA damage is involved. It therefore remains unclear how Sle1 degradation

by ClpXP is regulated during normal growth or upon DNA damage or how export of the protein is targeted to the septum. These issues need to be addressed to build on the initial exciting discovery and understand the underlying mechanism.

Major Comments:

1) A key prediction of the model is that the targeting of Sle1 to the septum via the TF-FtsK interaction is critical for blocking Sle1 degradation by ClpXP and promoting its export at the septum. If true, then inactivation of TF should prevent this targeting and lead to Sle1 degradation. However, Sle1 levels appear to be unaffected by TF inactivation. This discrepancy essentially invalidates the model and raises questions as to whether TF is involved in the process at all, especially since TF was of many Sle1 interactors chosen for investigation into its role in Sle1 targeting/regulation. Other potentially relevant interactors higher on the list than TF like the chaperone DnaK and the division protein FtsZ were not studied and seem equally likely to be involved.
Sle1 levels are indeed unaffected by TF inactivation. We think this is because Sle1 has a signal peptide for Sec export, so in the absence of other cues, it is still exported. However, this process is not as efficient/not properly localized, as a TF mutant is affected in cell cycle progression (delayed in phase 3, whose completion is dependent on Sle1 activity). We can make clearer in the manuscript that TF is not essential for Sle1, it optimizes the process for normal cell cycle progression to take place.

Importantly, in the manuscript we show that total levels of Sle1 are unaffected in the TF mutant. It is however possible that a TF mutant has decreased levels of secreted Sle1. To assess this, we can test the amount of secreted Sle1 in the TF mutant (as well as in the ClpX mutant to address comment 2 from reviewer 3 below) by analysing the autolytic extracts instead of total cellular extracts.

Additionally, we can try to delocalize FtsK, and test if this also leads to a change in the localization pattern of TF and a consequent delay in cell cycle phase 3 completion. We were previously unsuccessful in our attempts to delocalize FtsK. However, while we were writing this manuscript, a paper from the Hamoen group was published, showing that FtsK (B. subtilis SftA) localization requires an N-terminal amphipathic helix. We will express an FtsK variant lacking this helix and test if it delocalizes, and if it impacts TF localization. This would further strengthen our model.

2) The proposed model also relies critically on the detected interaction between TF and FtsK being real. However, the interaction was only detected by two-hybrid analysis and not validated by additional assays. Since TF is a chaperone capable of interacting with a variety of proteins, controls showing that TF doesn't interact with any protein partner in the two-hybrid assay are also needed. Thus, the TF-FtsK interaction remains suspect until it is validated further.
We can perform negative controls for the bacterial two hybrid experiments, using other proteins, as suggested by the reviewer.
We would like to point out that besides the Bacterial two hybrid data, we show that TF localization in a gradient with higher concentration at the septum is dependent of FtsK, which suggests a direct or indirect interaction between the two proteins. But we can also do pull-down assays to further test the interaction between TF and FtsK.

3) The localization patterns of TF-GFP are also a major pillar of the proposed model. They are interpreted as showing that TF-GFP forms a gradient with a bias towards the septum in WT cells and that this pattern is disrupted in FtsK mutants or cells treated with nalidixic acid. Some quantification of the localization patterns is needed here as I do not see a strong difference in pattern. In both WT and mutant/treated cells, it looks like TF-GFP is asymmetrically distributed to me.
We are completely sure that the changes in TF localization pattern are real and reproducible, as they are very clear in whole fields of cells and we see them consistently. But we agree that quantification will be helpful. We can do heatmaps of TF overall localization in multiple cells and then quantify total intensity of the signal in the septal region versus the rest of the cell.

4) The addition of DNA damaging agents appears to reduce the level of Sle1 that accumulates in/on cells. However, how this connects with FtsK and the potential checkpoint model is not clear at all and needs to be addressed if this line of investigation is to be included in the report. For example, are FtsK levels also altered upon DNA damage? Or is FtsK localization changed?
We think that the presence of DNA damaging agents is more likely to affect FtsK activity than its levels or localization (we never saw delocalization of FtsK in multiple conditions). Nevertheless, we can easily assess FtsK levels and localization in the presence of DNA damaging agents, as suggested by the reviewer. If these experiments are not informative (i.e., no changes are observed), we can tone down the role of FtsK in the response to DNA damaging agents.

5) Another major prediction of the model is that blocking cell division should prevent Sle1 stabilization and export.

Does inhibition of cell division by disrupting Z-ring formation or via other treatments that would prevent FtsK recruitment to the division site block Sle1 stabilization?
We can test the effect of blocking cell division by addition of an FtsZ targeting agent (PC190723) or by blocking septum synthesis using peptidoglycan synthesis inhibiting compounds.

6) Figure 3A: A control using a deletion of sle1 is needed to support the identity of the band designated as Sle1 is indeed Sle1.
This can be easily done

7) Figure 3C and 4B: If the cell cycle progression is a problem with Sle1 export, then it should be corrected by the addition of purified Sle1 as in the case of delta-sle1 and delta-ftsK. It would also be useful to include data for delta-sle1 in these panels for comparison.
This can be easily done

Referee #3:

This paper reports a novel role for FtsK in protein stability and secretion. The authors start with the surprising and very interesting finding that loss of FtsK leads to loss of Sle1. What ensues is a genetic and biochemical analysis to try and understand why. In the absence of FtsK, Sle1 is degraded in a ClpXP dependent fashion. They find that Trigger factor (TF) interacts with FtsK and Sle1 and is asymmetrically distributed in the cell in a FtsK dependent manner. This leads to a model in which TF acts as a bridge between FtsK and Sle1 so that Sle1 is secreted at the septum where FtsK is localized (model figure). This is quite novel if true. However, there appears to more going on, and there appears to be something missing - why is Sle1 degraded in the absence of FtsK but not degraded in the absence of TF? Why does loss of ClpX suppress the morphologic defects due to loss of FtsK?
Major points
1) A major point is the presence of Sle1 when TF is absent but its complete absence when FtsK is missing because it is degraded by ClpXP. So, TF does not protect Sle1 from degradation. If anything, it is more like TF interacts with Sle1 and brings it to FtsK but if FtsK is absent TF delivers it to ClpXP.
The reviewer suggested a model we also considered – that TF can bring Sle1 to FtsK or to ClpXP. We thought about this because in *E. coli,* TF interacts with ClpXP and does bring substrates to the proteolytic machinery. We did not include this model in the manuscript because we did not have evidence that this interaction also happens in *S. aureus*. However, we can test if a TF-ClpX interaction occurs in *S. aureus* by doing a pull-down experiment. And we can definitely discuss this model as a possible one.

2) The most important point. The authors say that a ClpX mutant was isolated as a suppressor of ΔftsK (around line 180) and restores Sle1 levels. However, no information is given about how this was obtained (pure chance?). This should be explained more clearly and should be included. More importantly they show that the ClpX mutant suppresses the morphological defect of ΔftsK. If this is due to Sle1 getting secreted, then FtsK has nothing to do with secretion of Sle1. This seems to be ignored.
Yes, the ClpX suppressor mutant was isolated by pure chance, while constructing the FtsK mutant (some colonies did not show the FtsK phenotype). We thought this was clear in our text, but we can state it in a clearer way.
We don´t think FtsK is essential for Sle1 secretion. Sle1 has a signal peptide for secretion by the Sec pathway and can be secreted in the absence of FtsK/TF, if present in the cell (eg in the ClpX mutant). But in those conditions, it is not secreted at the right place/time/amounts and leads to impaired cell cycle progression.
In the manuscript we show that total levels of Sle1 in the ClpX suppressor mutant are higher than in the wild type strain. However, it is possible that Sle1 secreted levels are not higher. In fact, immunofluorescence data on Sle1 localization in the suppressor mutant suggests exactly that, as the signal was lower than in the wild type strain. As immunofluorescence is not the best technique for quantification, we did not include this in the manuscript. But we can easily clarify this by testing Sle1 levels in autolytic extracts (which include only secreted Sle1 and not cytoplasmic Sle1) of the ClpX suppressor mutant.

3) The introduction talks about the SOS response inhibiting division in E. coli but does not mention the more relevant situation that occurs in Caulobacter, Bacillus and even in Staphylococcus. In these latter cases the SOS response leads to the presence of small inner membrane peptides that interact with the division machinery (FtsWI) to block septal PG synthesis. This should be discussed.
We can do this

4) I am not sure why the authors want to call their translocase FtsK instead of the Bacillus name. SpoIIIE looks more like the Gram negative FtsK, in fact the transmembrane domains of the two proteins overlap (alphafold prediction models).
Naming this protein FtsK was a requirement from a reviewer of our first paper on this protein, but we currently agree it was a poor choice. However, changing it at this point would create more confusion in the literature.

Additional points

1) The pull down of proteins with Sle1 is not very specific (Table S3). TF was only selected for additional study because it was a chaperone (and not a ribosomal protein).
We confirmed the TF-Sle1 interaction obtained from the proteomic data with additional pull down experiments, shown in the manuscript.

2) The morphological defects of ΔftsK is more severe than that of Δsle1 in both strain backgrounds suggesting a more prominent role for FtsK (Fig. 1 and Fig. S2)
This is not unexpected, as FtsK is a core divisome protein and it is likely to have other roles in cell division.

3) The observation that deletion of sle1 can be compensated by adding exogenous Sle1 suggests that Sle1 can localize effectively from the environment and that targeted secretion is not essential (Fig. S2). It suggests that Sle1 mature form is sufficient for localization. Have the authors checked to see if exogenously added HisTag-Sle1 is localized similarly to the endogenously produced Sle1?
The cell splitting defect of the Sle1 mutant can indeed be compensated by adding exogenous Sle1. That does not mean that normal cell cycle progression is ensured.
We think the key role of FtsK is ensuring secretion of Sle1 at the right place and at the right time. In the absence of Sle1, cells still divide and complete the cell cycle, by are delayed in phase 3.
As for the localization of exogenously added Sle1, we can easily check it by immunofluorescence or by using a purified GFP-Sle1 derivative.

Dear Mariana,

Thank you for sending me the preliminary revision plan for your manuscript. I have now gone through it, and I am glad to see that you are prepared to tackle the issues raised by referees #1 and #3 in a major revision. I would therefore like to invite you to submit a revised manuscript in response to the reviewers' comments as indicated in your preliminary point-by-point response.

If the revision experiments do not succeed in clarifying the major concerns raised by the referees, I would recommend, as also indicated in your letter, to submit the revised study to our sister journal EMBO Reports

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action. Should you foresee a problem in meeting this three-month deadline, please contact us to arrange an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if you have any further questions regarding the revision. Thank you again for the opportunity to consider your work for publication, and I look forward to receiving your revised manuscript!

With best regards,

Ieva

Ieva Gailite, PhD
Senior Scientific Editor
The EMBO Journal
Meyerhofstrasse 1
D-69117 Heidelberg
Tel: +4962218891309
i.gailite@embojournal.org

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. Please note that the Data Availability Section is restricted to new primary data that are part of this study. If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories.

*** Note - All links should resolve to a page where the data can be accessed. ***

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (16th Nov 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Point by point reply to the reviewers' comments (in blue)

Reading the reviewers´s comments, we realized that our initial version of the manuscript was misleading regarding the phenotype of cells lacking FtsK but having Sle1 (the suppressor mutant 8325-4Δ*ftsK* ClpX^{R95C}) or cells lacking Sle1 and having externally added Sle1. In both cases we focused on the fact that the presence of Sle1 was sufficient to disperse the tetrads that are observed in FtsK/Sle1 mutants, but we did not emphasize that the cell cycle defect, namely the delay in phase 3 cells (cells with a complete septum, before septum splitting occurs) was still present. This led the reviewers to (correctly) doubt the importance of the role of FtsK in Sle1 regulation. We have now made clear that in every condition where the preferential export of Sle1 at the septum is lost (due to lack of FtsK, lack of TF or upon addition of external Sle1) cell cycle progression is impaired due to a delay in phase 3. We hope this clarifies the importance of regulating Sle1 export, in time and space, for normal progression of the cell cycle.

We would also like to thank the reviewers for their constructive comments. As a result, the manuscript now includes stronger evidence of the TF-FtsK interaction, a key point in our model, and experimental evidence that in the absence of TF, Sle1 localization changes and is more dispersed over the cell surface, as initially predicted by our model (details below in point-by-point answer).

Referee #1:

Cells of the bacterial pathogen Staphylococcus aureus are spherical and divide by building a flat peptidoglycan (PG) septum at midcell that separates the daughter compartments. The septum must then be processed by PG cleaving enzyme to promote septal splitting and the separation of the daughters. Sle1 is a PG hydrolase that is important for this splitting process. However, it has remained unclear how Sle1 is directed to the septal region or how it is regulated so that it does not prematurely initiate separation before other essential cell cycle events like chromosome segregation are complete. The paper from Viega and co-workers sought to address this mystery using an elegant screen for factors required for proper Sle1 export. Surprisingly, mutants inactivating the cell division DNA translocase FtsK were isolated that not only blocked Sle1 export but also prevented Sle1 accumulation. Accordingly, both delta-sle1 and delta-ftsK mutants were shown to have similar morphological defects indicative of a delay in cell separation. Notably, this phenotype was suppressed in both cases by the addition of purified Sle1 to cells, indicating that a lack of exported Sle1 was the cause of the problem. Additionally, suppressors of the delta-ftsK phenotype that restored cell separations and Sle1 accumulation were found in clpX, suggesting that Sle1 is degraded by the ClpXP protease in the absence of FtsK. A pull-down of tagged Sle1 identified a number of potential interaction partners of the PG hydrolase. One of these was the chaperone trigger factor (TF). Further analysis found that the subcellular localization of TF was altered from being enriched at the septum to more diffuse in the cell following ftsK deletion or treatment with a DNA damaging agent. Two-hybrid analysis suggested an interaction between TF and FtsK. TF inactivation was also shown to delay cell cycle progression. Based on all of the data, the authors propose a model for Sle1 regulation in which the protein is protected from ClpXP degradation by TF and brought to the septum for export by the TF-FtsK interaction.

I think the authors have a very interesting discovery on their hands. The connection between Sle1 accumulation and FtsK is solid. However, I do not think the rest of the results definitively support a role for TF in this process or clearly indicate how the sensing of DNA damage is involved. It therefore remains unclear how Sle1 degradation by ClpXP is regulated during normal growth or upon DNA damage or how export of the protein is targeted to the septum. These issues need to be addressed to build on the initial exciting discovery and understand the underlying mechanism.

Major Comments:

1) A key prediction of the model is that the targeting of Sle1 to the septum via the TF-FtsK interaction is critical for blocking Sle1 degradation by ClpXP and promoting its export at the septum. If true, then inactivation of TF should prevent this targeting and lead to Sle1 degradation. However, Sle1 levels appear to be unaffected by TF inactivation. This discrepancy essentially invalidates the model and raises questions as to whether TF is involved in the process at all, especially since TF was of many Sle1 interactors chosen for investigation into its role in Sle1 targeting/regulation. Other potentially relevant interactors higher on the list than TF like the chaperone DnaK and the division protein FtsZ were not studied and seem equally likely to be involved.

Similar to the reviewer, when we initially tested Sle1 levels in the TF mutant we were also expecting to see a decrease, which was not what we observed.
However, Sle1 has a signal peptide for Sec export, so even in the absence of a chaperone, it may still exported. We have now quantified the levels of secreted Sle1 in the TF mutant (in the original manuscript we had quantified the levels of total Sle1) and confirmed that there is no significant change in the amount of Sle1 present in

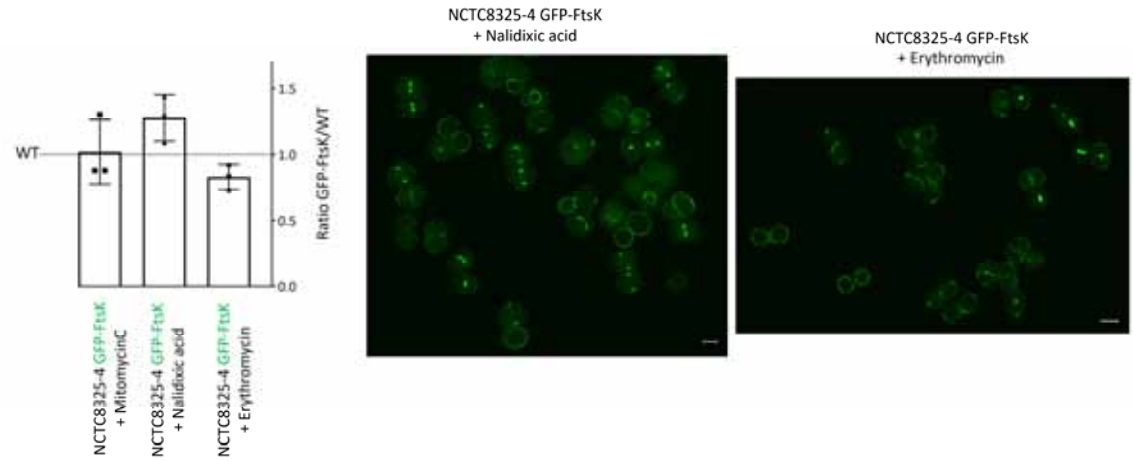
autolytic extracts in the TF mutant versus the wild type (new Fig 5A, last bar in graph). We think that TF has a double role of bringing Sle1 to the septum (via TF-FtsK interaction) or bringing Sle1 to ClpXP (in the absence of TF-FtsK interaction), as also suggested by reviewer #3. This hypothesis is now discussed in lines 300-310. Therefore, in the absence of TF, Sle1 is not directed to the septum but it is also not directed to ClpXP, so it is present in the cell.
Furthermore, in the initial manuscript we proposed that in the absence of TF, Sle1 was no longer directed specifically to the septal Sec machineries (via the Sle1-TF-FtsK interactions) but instead drifted to any Sec machinery present in the entire cell surface. We had predicted (but not shown) that this would result in a more dispersed localization of Sle1 at the cell surface in the TF mutant instead of an enrichment at the septum seen in wild type cells. We have now localized Sle1 in a TF mutant, by immunofluorescence, and shown that this is exactly what is observed (new Fig 5B). This mislocalization causes an impairment in cell cycle progression, namely a delay in phase 3 (stage before septum splitting) (Fig 5D).

2) The proposed model also relies critically on the detected interaction between TF and FtsK being real. However, the interaction was only detected by two-hybrid analysis and not validated by additional assays. Since TF is a chaperone capable of interacting with a variety of proteins, controls showing that TF doesn't interact with any protein partner in the two-hybrid assay are also needed. Thus, the TF-FtsK interaction remains suspect until it is validated further.
We have added 3 additional septal proteins as negative controls to the bacterial two hybrid assays, namely DivIC, EzrA and PBP2 (Fig S5). No interaction with TF was observed.

We agree with the reviewer that the TF-FtsK interaction is central to the manuscript, so we performed Co-IP assays for these two proteins, which confirm that TF interacts with FtsK (new fig 3B).
Therefore, the TF-FtsK interaction is now validated by (i) data from bacterial two hybrid, (ii) Co-IP assays and (iii) the fact that TF localization changes in the absence of FtsK.

3) The localization patterns of TF-GFP are also a major pillar of the proposed model. They are interpreted as showing that TF-GFP forms a gradient with a bias towards the septum in WT cells and that this pattern is disrupted in FtsK mutants or cells treated with nalidixic acid. Some quantification of the localization patterns is needed here as I do not see a strong difference in pattern. In both WT and mutant/treated cells, it looks like TF-GFP is asymmetrically distributed to me.
Having looked at thousands of cells, we did not have any doubt that there was a very reproducible difference in the localization pattern of TF in the presence and absence of FtsK. But we obviously understand and accept the reviewer´s comment. We thought the best way to quantify the TF gradients was to generate heatmaps of TF-GFP fluorescence in wildtype and FtsK mutant cells (Fig 4B). Visual inspection of the heatmaps shows that TF is more enriched in the septal region in the wildtype than in the FtsK mutant. This can be further quantified by determining the fraction of the area of the heat map where the fluorescence signal is above 70% of the maximum signal. This area corresponds to 0.47 in wild type cells and 0.68 in cells from the ftsK mutant, confirming that TF is more dispersed over the cytoplasm in the absence of FtsK. This information is now included in the legend of Fig 4. Similar quantification was also done for the changes in TF localization pattern in the presence of nalidixic acid (Fig 6C).

4) The addition of DNA damaging agents appears to reduce the level of Sle1 that accumulates in/on cells. However, how this connects with FtsK and the potential checkpoint model is not clear at all and needs to be addressed if this line of investigation is to be included in the report. For example, are FtsK levels also altered upon DNA damage? Or is FtsK localization changed?
We tested the FtsK levels upon DNA damage (in the presence of mitomycin C and nalidixic acid) and found no significant change (see image below). We also looked at FtsK localization in these conditions and although we do see some cells where FtsK appears delocalized (see image below), the phenotype is not strong enough for us to make a bold claim in the manuscript.
We think it is more likely that DNA damage affects the activity of FtsK, rather than its levels or localization. However, we do not have a mechanistic explanation for this (although the fact that DNA damage causes delocalization of TF suggests to us that the mechanism is mediated by FtsK) so we toned down the role of FtsK in the response to DNA damage in the discussion and we added a last paragraph in the results section (lines 263-268) to specifically say that we don´t have a mechanistic explanation for FtsK role upon DNA damage.



Graph shows ratio of GFP-FtsK levels in cells incubated with different antibiotics versus cells in the absence of antibiotics. Figure shown localization of FtsK in the presence of nalidixic acid or erythromycin.

5) Another major prediction of the model is that blocking cell division should prevent Sle1 stabilization and export. Does inhibition of cell division by disrupting Z-ring formation or via other treatments that would prevent FtsK recruitment to the division site block Sle1 stabilization?
We have used PC190723 (FtsZ inhibitor) to block cell division and indeed we observe a reduction in Sle1 levels, as predicted. However, we would prefer not to include this result in the manuscript without further investigation, as inhibition of septum formation could have various pleiotropic effects.

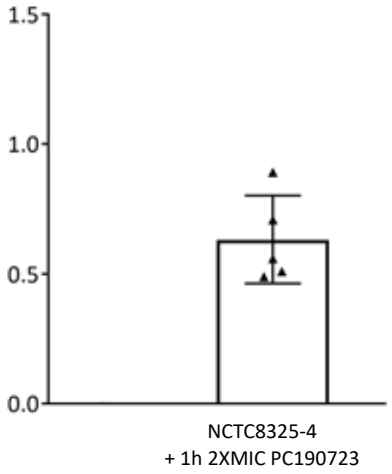


Figure shows ratio of Sle1 levels in cells incubated with an FtsZ inhibitor versus cells in the absence of the inhibitor.

6) Figure 3A: A control using a deletion of sle1 is needed to support the identity of the band designated as Sle1 is indeed Sle1.
The Co-IP experiment was repeated including a sle1 deletion mutant as suggested. We have replaced the previous Fig 3A image by the new data (Fig 3A, last lane)

7) Figure 3C and 4B: If the cell cycle progression is a problem with Sle1 export, then it should be corrected by the addition of purified Sle1 as in the case of delta-sle1 and delta-ftsK. It would also be useful to include data for delta-sle1 in these panels for comparison.
Addition of purified Sle1 corrects the “tetrads” phenotype, as the externally added enzyme is able to cut through septum and separate the tetrads. However, it does not fully correct the cell cycle progression defect in the Sle1 mutant, as can be seen in Panel C from Fig1- the length of phase 3 in the third column (Sle1 mutant plus

externally added Sle1) is similar to that of the second column (Sle1 mutant) and not to the first column (parental strain NCTC8325-4). We think this is because normal progression required coordinated activity of Sle1, at the right place (septum) and at the right time of the cell cycle, which is not provided by external addition of Sle1. We now make this clear in lines 132-137

Minor Points:

Line 45: comma after "SOS response"
Done

Line 47: "which assembles at the..."
“Assemble” referred to “divisome members (plural)”, which was not clear in the original sentence. We´ve removed the comma and replaced “which” by “that”.

Line 68: Some discussion of the work from Frees and co-workers on Sle1 control by ClpX should be included in the intro.
We have cited the work by D. Frees showing that ClpXP regulate Sle1 levels (lines 182-184). We have not included that reference in the introduction, as we only introduce ClpXP later on in the manuscript (section starting in line 172).

Line 73: Is checkpoint really the appropriate term here?
We removed the word checkpoint (new line 75).

Since both FtsK and Sle1 can be deleted and Sle1 can be added externally to complement, there must be additional layers of Sle1 regulation post-export that are at play. This concept should be discussed in the Discussion.
As we mentioned above, our initial text was indeed misleading, as it did not clearly state that complementation of the FtsK mutant with external Sle1 did not correct impairment in cell cycle progression, namely the delay in phase 3. This is now clearly mentioned in lines 132-137.

Line 108: I am not sure what it means to be "adjacent to the inner side of the membrane"
We replaced by “localizes as a ring at the leading edge of the division septum, similarly to other divisome proteins” (line 112)

Line 290-294: Some sort of molecular explanation for how the completion of DNA translocation by FtsK would be "sensed" to affect Sle1 levels is needed here. Otherwise, there is little value in this speculative discussion.
We do not have a molecular explanation for this, as we focused this manuscript in conditions which lead to decreased Sle1 levels, not to the increased Sle1 levels seen when FtsK DNA translocase activity is inhibited. However, we would still like to be allowed to speculate (and we clearly mention this is speculation only) about a role for FtsK during normal division, as we think it may be useful to prompt readers to think about experiments to address this question.

Referee #2:

Veiga et al have studied the role of the N-terminus of Staphylococcus aureus FtsK during cell division/cell splitting. It is shown that in the absence of FtsK, cell wall hydrolase Sle1 becomes depleted from cells, in a ClpXP (proteasome) dependent manner. The authors further show that FtsK established a gradient of ribosomal chaperone trigger factor (TF) towards the division septum, while TF interacts with FtsK on the one hand, and with Sle1 on the other hand. Intriguingly, when chromosome segregation is perturbed, affecting FtzsK activity, Sle1 also becomes depleted from cells, or shows lowered expression, leading to a delay in cell splitting. The authors propose a model in which FtsK senses problems in chromosome segregation, and transduces them, via TF, to control Sle1 levels, which in turn affect cell splitting, thus leading to delayed splitting when segregation is itself delayed.

The manuscript is very well written, data are well presented. I enjoyed reading the work, it is very succinctly written, and a really intriguing mechanism, quite unanticipated. I have a few minor comments:

Line 32: "TF binds unfolded Sle1 ...." This is not shown, just binding.
We removed the word “unfolded”

Many control experiments are shown in the supplement. I wonder why the authors chose to show more supplementary figures than figures in the main manuscript. I would strongly encourage showing most control experiments in the manuscript, and leave only non-important information in the supplement.

We moved previous figures S5B, S6B and S6C to the main text.

Line 250: "suggesting that in these conditions, FtsK may undergo a conformational change that leads to loss of interaction with TF." I wonder why the authors suggest this, in line of the finding that motor activity of FtsK does influence Sle1 levels. Formally, it is always conformational changes that transfer information, or change enzyme activities, but in this case, I would think it is more likely that the activity of FtsK (increasing as segregation is perturbed) affects Sle1 levels. However, this point is really at the discretion of the authors to deal with.
We agree that it is likely that there is a change in the activity of FtsK. But, as the reviewer says, that should generate some conformational change, so that the information is transferred to another protein, in this case TF. In any case, we have completely rephrased that section (lines 263-268) and no longer mention a possible conformational change.

Line 265 "On the contrary, S. aureus,..." I can not find a contradiction between the two sentences, please rephrase
We removed the words “on the contrary”

Line 269 "possibly exposing an immature cell surface" .... I do not see how the cell surface could be immature. My best guess would be that S. aureus cells want to prevent complete separation despite unfinished segregation of chromosomes. If the authors see a link to the cell surface (i.e. a specific activity of Sle1 changing the structure of the peptidoglycan other than splitting activity) they should better explain this.
We know cells spend one fourth of the cell cycle between completion of septum synthesis and initiation of septum splitting. We also know that addition of wall teichoic acids, for example, is relatively late in the process of septum assembly (Proc Natl Acad Sci U S A. 2010 Nov 2;107(44):18991-6. doi: 10.1073/pnas.1004304107). So we think that splitting of the septum before its completion may result in exposure of a cell surface that may be missing some surface polymers/proteins. However, as showing this was not an aim of this work, we have removed the mention to “immature cell surface” from the abstract and from the results section, leaving it however in the discussion section.

Line 272, more than semantics: "In this work, we have identified the mechanism for this newly recognized level of regulation..." This sentence implies that the mechanism is pretty much fully understood, but it is not. The authors provide very good evidence suggesting the mechanism they propose, but, for example, line 279: "Therefore, the concentration of TF´s cargo, Sle1, will be 279 higher near the Sec machineries present in the septal region and lower near...." this is what may be the case, but it is not shown. Therefore, the strongest claim the authors can make is "we have identified a mechanism....." or, to be most accurate "found data suggesting a mechanism .....". I would be ok with the prior sentence, but not with "the mechanism".
We replaced the sentence by “we have identified a mechanism” as suggested.
As for the localization of TF´s cargo, Sle1, the reviewer correctly pointed out that we had suggested, but not demonstrated that it was enriched at the septum in a manner that was dependent on TF. However, we have now looked at the localization of Sle1 in the presence and absence of TF by immunofluorescence, showing that, as predicted, it is more enriched in the septum in the presence of TF, but more dispersed over the cell surface in the absence of TF (new Fig 5B).

Referee #3:

This paper reports a novel role for FtsK in protein stability and secretion. The authors start with the surprising and very interesting finding that loss of FtsK leads to loss of Sle1. What ensues is a genetic and biochemical analysis to try and understand why. In the absence of FtsK, Sle1 is degraded in a ClpXP dependent fashion. They find that Trigger factor (TF) interacts with FtsK and Sle1 and is asymmetrically distributed in the cell in a FtsK dependent manner. This leads to a model in which TF acts as a bridge between FtsK and Sle1 so that Sle1 is secreted at the septum where FtsK is localized (model figure). This is quite novel if true. However, there appears to more going on, and there appears to be something missing - why is Sle1 degraded in the absence of FtsK but not degraded in the absence of TF? Why does loss of ClpX suppress the morphologic defects due to loss of FtsK?
Major points
1) A major point is the presence of Sle1 when TF is absent but its complete absence when FtsK is missing because it is degraded by ClpXP. So, TF does not protect Sle1 from degradation. If anything, it is more like TF interacts with Sle1 and brings it to FtsK but if FtsK is absent TF delivers it to ClpXP.
We think the reviewer is absolutely correct in saying that TF interacts with Sle1 and brings it to FtsK, but in the absence of FtsK it brings it to ClpXP. However, we did not present that model in the original manuscript because we did not have proof of an interaction between TF and ClpX by bacterial two hybrid. Following the reviewer´s comment, we tried to detect that interaction by Co-IP, but again we were unable to detect such interaction. It is possible that TF does not directly interact with ClpX, but does so via a third protein. STRING analysis (despite its obvious limitations) suggests that TF-ClpX interaction maybe mediated by GroS or GroL.

In *E coli* TF interacts with ClpX and promotes degradation of some substrates (*Nat Commun* **12**, 281, doi:10.1038/s41467-020-20553-x (2021). The fact that tig is located upstream of clpX in most bacterial genomes (*Nat Commun* **12**, 281, doi:10.1038/s41467-020-20553-x (2021), including S. aureus, may suggests that a functional association between TF and ClpXP extends beyond *E.coli*
We have now discussed these ideas in lines 300-310).

Please see also reply to the first comment of reviewer 1, for further explanation on why Sle1 is not degraded in the absence of TF.

2) The most important point. The authors say that a ClpX mutant was isolated as a suppressor of ΔftsK (around line 180) and restores Sle1 levels. However, no information is given about how this was obtained (pure chance?). This should be explained more clearly and should be included. More importantly they show that the ClpX mutant suppresses the morphological defect of ΔftsK. If this is due to Sle1 getting secreted, then FtsK has nothing to do with secretion of Sle1. This seems to be ignored.
Yes, the ClpX mutant was obtained by pure chance during the construction of the ftsK mutant. We always analyse more than one clone when constructing mutants, and we noticed that some clones lacking FtsK did not have the characteristic phenotype of impaired daughter cell separation, suggesting the existence of a suppressor mutation, so we sequenced the genome of one of these clones. We now clearly say that we inspected various mutants under the microscope and noticed that some lacked daughter cell separation defects (line 175-177).

We don´t think FtsK is essential for Sle1 secretion. We think it is essential for preferential secretion at the septum, which in turn is essential for normal progression of the cell cycle.
Sle1 has a signal peptide for secretion by the Sec pathway and can be secreted in the absence of FtsK, if present in the cell (eg in the ClpX mutant, see graph below), or in the absence of TF (new Fig 5A). But in those conditions, it is probably not secreted at the right place/time and leads to impaired cell cycle progression. We have now analysed cell cycle progression of the 8325-4Δ*ftsK* ClpX^{R95C} mutant and confirmed that it is delayed in phase 3 (New Fig S4C).

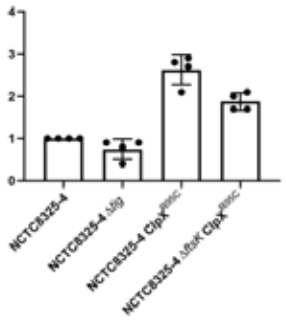


Figure shows ratio of of Sle1 levels in autolytic extracts of TF and ClpX mutants versus the parental strain NCTC8325-4.

3) The introduction talks about the SOS response inhibiting division in E. coli but does not mention the more relevant situation that occurs in Caulobacter, Bacillus and even in Staphylococcus. In these latter cases the SOS response leads to the presence of small inner membrane peptides that interact with the division machinery (FtsWI) to block septal PG synthesis. This should be discussed.
We have added a brief mention to inhibition of FtsW or later divisome proteins as part of the SOS response (line 45 in introduction) but did not discuss it in detail as the exact mechanism of cell division inhibition in *S. aureus* (mediated by SosA) is not yet clear.

4) I am not sure why the authors want to call their translocase FtsK instead of the Bacillus name. SpoIIIE looks more like the Gram negative FtsK, in fact the transmembrane domains of the two proteins overlap (alphafold prediction models).
When we published the first paper from our lab about the DNA translocases in S. aureus (Veiga H, Pinho MG. Mol Microbiol. 2017; 103:504-517. doi: 10.1111/mmi.13572.), we had given this protein a different name. However, one of the reviewers stated that we should call it FtsK and at the time we accepted that suggestion. Despite now regretting that decision (because in fact *E. coli* FtsK and *S. aureus* FtsK are quite different), we think that changing the name now would create even more confusion in the literature, as we would have two names for the same protein in papers from the same lab.

Additional points
1) The pull down of proteins with Sle1 is not very specific (Table S3). TF was only selected for additional study because it was a chaperone (and not a ribosomal protein).
That is correct, but we confirmed the interaction in by Co-IP (Fig 3A)

2) The morphological defects of ΔftsK is more severe than that of Δsle1 in both strain backgrounds suggesting a more prominent role for FtsK (Fig. 1 and Fig. S2)
That is correct, but we think this is expected, because FtsK is a bifunctional protein that has a DNA motor domain, which is not involved in Sle1 regulation and a N-terminal domain plus long linker, which is involved in Sle1 regulation and possibly also in other functions, as FtsK is a core member of the divisome.

3) The observation that deletion of sle1 can be compensated by adding exogenous Sle1 suggests that Sle1 can localize effectively from the environment and that targeted secretion is not essential (Fig. S2). It suggests that Sle1 mature form is sufficient for localization. Have the authors checked to see if exogenously added HisTag-Sle1 is localized similarly to the endogenously produced Sle1?

Our initial text was misleading as we emphasized the fact that addition of exogenous Sle1 resolves the “tetrads” phenotype in the null sle1 mutant, but not the fact that it does not revert the delay in cell cycle progression (please see answer to comment 7 of reviewer 1), indicating that exogenous Sle1 is not sufficient to ensure normal cell cycle progression. We have now made this clear in lines 132-137)

We tried to localize the exogenously added Sle1. We have antibodies against Sle1, which work for western blots, but are not sufficiently specific for immunofluorescence experiments. We also used an Anti-His antibody for immunofluorescence, but again the antibody was not sufficiently specific. Therefore, we were unable to localize exogenously added Sle1.

2nd Revision - Editorial Decision **2nd Mar 2023**

Dear Mariana,

Thank you for submitting a revised version of your manuscript. Your study has now been seen by all original referees, who find that most of their previous concerns have been addressed and now recommend publication of the manuscript. There remain only a few editorial points that have to be addressed before I can extend formal acceptance of the manuscript:

1. Please address the remaining points from referee #1.
2. Our publisher has done their pre-publication check on your manuscript. I have attached the file here. Please take a look at the word file and the comments regarding the figure legends and respond to the issues. Please also use this version when you resubmit the revised version.
3. Please make sure that the funding information is complete and identical in both manuscript text and our online submission system.
4. In the Author Checklist, please add information on the statistical tests in the line 84.
5. Figure panels 4A-B are not mentioned in the manuscript text, please add callouts.
6. Please move the Data Availability section to the end of Material and Methods.
7. CRediT has replaced the traditional author contributions section because it offers a systematic, machine-readable author contributions format that allows for more effective research assessment. Please remove the Authors Contributions from the manuscript and use the free text boxes beneath each contributing author's name in our online submission system to add specific details on the author's contribution. More information is available in our guide to authors.
8. Please rename "Conflict of interest" section into "Disclosure and competing interests statement" (further info: https://www.embopress.org/page/journal/14602075/authorguide#conflictsofinterest).
9. Please rename Supplemental information file into Appendix, add a short table of contents with page numbers to the first page, and update the nomenclature throughout the manuscript to "Appendix Table S1" etc and "Appendix Figure S1" etc. Please upload the Appendix file as a pdf.
10. We generally encourage publication of source data for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need one file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labeled with the appropriate figure/panel number and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as supplementary "Source Data". Please let me know if you have any questions about this policy.
11. Papers published in The EMBO Journal are accompanied online by a 'Synopsis' to enhance discoverability of the manuscript. It consists of A) a short (1-2 sentences) summary of the findings and their significance, B) 3-4 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height, jpeg or png format). You can either show a model or key data in the synopsis image. Please note that the image size is rather small and that text needs to be readable at the final size. Please send us this information together with the revised manuscript.

Please let me know if you have any further questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

With best regards,

Ieva

Ieva Gailite, PhD
Senior Scientific Editor
The EMBO Journal
Meyerhofstrasse 1
D-69117 Heidelberg
Tel: +4962218891309
i.gailite@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (31st May 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Referee #1:

The authors have done a nice job addressing my concerns with the initial submission. I do not have any remaining items to be addressed.

Referee #2:

the authors have dealt with all my comments in a satisfactory manner.

Referee #3:

This is a revised version of the manuscript about Sle1. The authors went at lengths to answer the previous comments of the reviewers and cleared up some confusion.

Sle1 contributes to septal splitting but is not an essential gene. In its absence septum splitting is slowed down leading to the formation of some tetrads due to slower splitting of the septum as well as more cells in phase 3. This suggests that some other enzyme can do the job and it is why the phenotype is rather mild.

The model proposed by the authors is that Sle1 interacts with TF, which is enriched at midcell, and delivers Sle1 to FtsK at the septum where it is secreted and where it acts to split newly divided cells. In the absence of FtsK, Sle1 is degraded by ClpXP and one has tetrads. In the absence of FtsK and ClpX there is a partial suppression of the phenotype as tetrads are no longer present. In the TF, Sle1 makes it to the surface but is no longer enriched at the septum, although the distribution is quite spotty. This suggests TF acts as a chaperone to deliver Sle1 to FtsK, but in the absence of FtsK it delivers it to ClpXP.

Exogenous Sle1 can eliminate tetrads but does not have any effect on the cell-it does not cause lysis for example. To me this suggests that Sle1 is waiting for a substrate to appear-one that is only present at the septum once its there, it acts on it. When DNA synthesis is blocked then septal PG synthesis stops (due to SosA induction), then there is no substrate for Sle1 to act on. Thus, its not clear that the ~2 fold decrease in Sle1 levels is significant, and if it is, that it is physiologically relevant.

A few comments below

Major comment

Fig. 5B. This is the comparison of Sle surface localization in WT vs TF. Two things; first the Sle1 signal appears so much stronger in the tig mutant than the control; second, it is dispersed but still spotty and not as evenly distributed as the sec machinery (Panel c).

Minor comments

It is interesting that a FtsK ClpX-R95C does not make tetrads. This argues that nonseptal secreted Sle1 is functional.

Fig. 3. What is the band in the bottom half of Panel A (lanes 1, 3 and 4; Anti Sle1 Ab). If it is some nonspecific band why isn't it in lane 2 and possibly 3?

The authors state that the Ab to SleI is not sufficient to localize exogenously added Sle1. However in Fig. 1C they use His-tagged suggesting that an anti-His Ab could be used or Anti-Flag if they have purified the Sle1-Flag version.

Point by point reply to referees (in black)

Referee #1:

The authors have done a nice job addressing my concerns with the initial submission. I do not have any remaining items to be addressed.

Referee #2:

the authors have dealt with all my comments in a satisfactory manner.

Referee #3:

This is a revised version of the manuscript about Sle1. The authors went at lengths to answer the previous comments of the reviewers and cleared up some confusion.

Sle1 contributes to septal splitting but is not an essential gene. In its absence septum splitting is slowed down leading to the formation of some tetrads due to slower splitting of the septum as well as more cells in phase 3. This suggests that some other enzyme can do the job and it is why the phenotype is rather mild.

The model proposed by the authors is that Sle1 interacts with TF, which is enriched at midcell, and delivers Sle1 to FtsK at the septum where it is secreted and where it acts to split newly divided cells. In the absence of FtsK, Sle1 is degraded by ClpXP and one has tetrads. In the absence of FtsK and ClpX there is a partial suppression of the phenotype as tetrads are no longer present. In the ΔTF, Sle1 makes it to the surface but is no longer enriched at the septum, although the distribution is quite spotty. This suggests TF acts as a chaperone to deliver Sle1 to FtsK, but in the absence of FtsK it delivers it to ClpXP.

Exogenous Sle1 can eliminate tetrads but does not have any effect on the cell-it does not cause lysis for example. To me this suggests that Sle1 is waiting for a substrate to appear-one that is only present at the septum once its there, it acts on it. When DNA synthesis is blocked then septal PG synthesis stops (due to SosA induction), then there is no substrate for Sle1 to act on.
Thus, its not clear that the ~2 fold decrease in Sle1 levels is significant, and if it is, that it is physiologically relevant.

A few comments below

Major comment

Fig. 5B. This is the comparison of Sle surface localization in WT vs ΔTF. Two things; first the Sle1 signal appears so much stronger in the tig mutant than the control; second, it is dispersed but still spotty and not as evenly distributed as the sec machinery (Panel c).
The signal of Sle1 is indeed stronger in the TF mutant that in the parental strain. However, this is an immunofluorescence experiment, which should not be used for quantitative analysis (there are no available internal controls). Fig 5A shows proper quantification, by western blot, in triplicate, of total and surface Sle1 levels in the TF mutant when compared to the parental strain, indicating that Sle1 levels are not higher in the TF mutant.

As for the localization pattern of Sle1, it is indeed more spotty that the sec machinery. Given that Sle1 is not anchored to the membrane, and the sec machinery includes membrane proteins, we do not necessarily expect the localizations to be identical.

Minor comments

It is interesting that a ΔFtsK ClpX-R95C does not make tetrads. This argues that nonseptal secreted Sle1 is functional.

We think non septal secreted Sle1 is indeed active and capable to peptidoglycan hydrolysis. However, only septal export of Sle1 can confer proper cell cycle progression (see Fig S4C which shows that ΔFtsK ClpX-R95C has a delayed phase 3)

Fig. 3. What is the band in the bottom half of Panel A (lanes 1, 3 and 4; Anti Sle1 Ab). If it is some nonspecific band why isn't it in lane 2 and possibly 3?

We do not know the nature of that non-specific band.

The authors state that the Ab to SleI is not sufficient to localize exogenously added Sle1. However in Fig. 1C they use His-tagged suggesting that an anti-His Ab could be used or Anti-Flag if they have purified the Sle1-Flag version.

We don´t have purified Sle1-FLAG. The purified Sle1 protein is a His tagged version. We tried to localize externally added Sle1 protein using both Anti-Sle1 and the Anti-His tag antibodies, but we observed non-specific signal in the negative controls.

Dear Mariana,

Thank you for addressing the final editorial points. I am now pleased to inform you that your manuscript has been accepted for publication.

Before we forward your manuscript to our publishers, I would like to propose a couple of minor changes in the article title, synopsis and abstract. I have also written a short blurb that will accompany the title of your manuscript in our online table of contents. Please take a look at the text below and in the attached manuscript text file and let me know if any corrections are necessary.

Title:
Cell division protein FtsK coordinates bacterial chromosome segregation and daughter cell separation in Staphylococcus aureus

Blurb:
FtsK interacts with the chaperone Trigger factor and establishes its gradient towards the septum to promote stability and export of the peptidoglycan hydrolase Sle1.

Synopsis:
Bacteria complete cell division by splitting the division septum, a process that requires the regulated activity of peptidoglycan hydrolases. Here, Staphylococcus aureus divisome protein FtsK is shown to regulate daughter cell splitting via stabilization of the peptidoglycan hydrolase Sle1.
• The chaperone Trigger factor (TF) interacts with septal FtsK, establishing a TF concentration gradient towards the septal region.
• TF also interacts with newly synthesized Sle1 and promotes its preferential export at the septal region, where its hydrolytic activity is required to catalyze daughter cell splitting.
• In the absence of FtsK, the TF gradient is dissipated and Sle1 is degraded by ClpXP proteolytic machinery.
• Sle1 levels are reduced and septum splitting is delayed when chromosome replication and segregation are impaired.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

You will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/tej_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

Best wishes,

Ieva

Ieva Gailite, PhD
Senior Scientific Editor
The EMBO Journal
Meyerhofstrasse 1

D-69117 Heidelberg
Tel: +4962218891309
i.gailite@embojournal.org

EMBO Press Author Checklist

Corresponding Author Name: Mariana Pinho	**USEFUL LINKS FOR COMPLETING THIS FORM**
Journal Submitted to: EMBO J	The EMBO Journal - Author Guidelines
Manuscript Number: EMBOJ-2022-112140R1	EMBO Reports - Author Guidelines
	Molecular Systems Biology - Author Guidelines
	EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Data availability

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Supplementay tables

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Supplementary tables and methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Methods and acknowledments

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)

If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	Statistical tests for proteome analysis,RNAseq analysis and bacteria two-hybrid assays are mentioned in the methods section. Statistical test for Fig 6 is mentioned in the legend. Notice that for Fig 6 sample size is very small (4 data points, as western blots were done in quadruplicate)

Sample definition and in-laboratory replication	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Figure legends

Ethics

Ethics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	**Yes**	Data availability
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	