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Direct Interaction of FtsZ with MreB is Required for Septum Synthesis and Cell Division in Escherichia coli

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: David del Alamo

1st Editorial Decision 06 March 2013

Thank you for the submission of your manuscript entitled "Direct interaction of bacterial FtsZ with MreB is required for septum synthesis and cell division". We have now received the full set of reports from the referees, which are copied below. As all three referees agree on the high interest of your manuscript and their comments are in general positive, I would like to invite you to revise it.

Without going into details that you will find below, all referees consider that your manuscript should be considered for publication in The EMBO Journal provided that a number of points are addressed in order to improve your message before final acceptance can be granted. As you will also see, the points raised by the referees are essentially rather specific technical concerns, some of which will need further experimental work.

Please be aware that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next version of the manuscript. Do not hesitate to contact me by e-mail or on the phone in case you have any questions, you need further input or you anticipate any problem during the revision process.

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 how to proceed. Should you foresee a problem in meeting this three-month deadline, please let me know in advance and I may be able to grant 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 iniciative, please visit our website:

http://www.nature.com/emboj/about/process.html

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

REFEREE REPORTS

Referee #1

In this paper the authors present evidence that MreB localizes to the Z ring in E. coli similar to what was observed in Caulobacter. Very strong genetic and biochemical evidence is presented that FtsZ and MreB interact and that this has functional significance. The authors argue that MreB is required for cells to divide. Furthermore, the authors show that overexpression of an MreB mutant blocks cell division. In the filaments that form the authors argue that PBP3 is present but that PBP2 and PBP1B are not. The evidence for this latter finding is weak due to the poor localization of these proteins in the WT conditions.
Mutations in both FtsZ and MreB that interfere with the interaction are apparently lethal so complex strain constructions are required to test the system, i.e. the alleles can not be put on the chromosome (or at least it was not tested). It may be possible under slow growth conditions where the interaction is not essential and cells grow as spheres.

Page 4, last full paragraph - the authors point out that divisome maturation occurs in 2 steps. It would be helpful to indicate that these two steps correlate with two phases of divisome assembly-the first being the Z ring and the second being the additional proteins that complete the divisome (even though this may be over simplified).

Page 4, three lines from bottom - rings were reported from one lab under special conditions. These rings were not reported in the 3 high resolution studies that rules out the continuous spiral of MreB.

Fig. 2D. These experiments were done in MC1000 which is MreB+. I do not understand why a band is not seen when His-MreB-D285A is used. The WT MreB that is present could still crosslink to FtsZ.

Fig. 3A. Mild overexpression of MreB-D285A. The expression system used was arabinose inducible which can vary a lot from cell to cell. In the figure it appears the increase in cell length is due to a few long cells - this is consistent with possible high level expression in these few cells and low level in others. Can one do a spot test at different inducer concentrations to show how toxic the mutant is compared to WT.

Page 10, middle paragraph - it is surprising that MreB can disappear in 1 hour in the depletion experiments unless MreB is very unstable. In 1 hour (~2 generations, it would be diluted to 25% of the original, however, as the plasmid is still present in some cells it should not be diluted that much.

Page 11, bottom of page. I am surprised by this reduction in localization of MreB-D285A to the Z ring. First the authors show that this mutant colocalizes with WT MreB. Then the mutant is expressed in cells where it only represents 6% of the total MreB and yet it goes poorly to the Z ring. One would not expect to see a decrease from 75 to 28% since the mutant should colocalize with the WT MreB at the Z ring. Perhaps it is the monomer of MreB that goes to the Z ring and the mutant cannot do this. In the supplement the authors show the position of D285 in the monomer but it looks like it could be buried in the MreB filament. Have the authors checked that? The authors show that MreB-D85A interacts with WT MreB but does it interact with itself, especially if it lacks the membrane binding sequence?

Fig. 4A what does the one and two pluses mean in terns of complementation. IN Fig. S4 it appears that FtsZ-P103Q interacts very poorly if at all (hard to tell) with MreB-D285A. It also interacts poorly with FtsZ - this seems very odd. This may be why it does not complement an FtsZ depletion strain (whereas the authors imply it is because it does not interact with WT MreB). If the authors are right then FtsZ-P203Q should support the growth of an MreB deletion strain.

Page 12 - using Pfu as an error prone polymerase - I thought it was a high fidelity enzyme.

Fig. 5C and D. The localization of PBP1B and PBP2 to the septum is not very convincing. The increased brightness at the septum could be due to double membranes at the septum. For example, the cells that lay side by side in panel C and where they abut the signal is very bright.

Page 17 and 18. Although the authors show that PBP3 is at the locked Z rings it is not clear if FtsN is there as the authors say in the discussion. Thus, the authors should not refer to the Z ring as mature.

Page 19, the authors say that MreB is essential in rapid growth. However, it would seem that MreB is essential when cells are rod shaped regardless of the growth rate. Presumably it is required when cells transition from cylindrical growth to septal growth. Since MreB is not essential when cells grow as spheres. Does the MreB mutant have an effect at slower growth rates?

Referee #2

This paper presents convincing evidence for a direct interaction of FtsZ and MreB. Previous co- or near-localization experiments had hinted at a possible interaction, but this paper provides much more definitive evidence. The experiments show that the FtsZ-MreB interaction is important for cell division under the standard growth conditions (although previous studies have already shown that MreB depletion can be tolerated with overexpression of FtsZ or slower growth conditions). The mechanisms are not yet clear - some of them are likely indirect as discussed below. Nevertheless this is an important step in showing that these two cytoskeletal components, originally thought to be independent, are cooperating in the mechanism of cytokinesis. I recommend publication following attention to the points below.

1. The Results refer the reader to Methods for the construction of mYPet-MreB. This is actually in SI Methods. But the insert site is not precisely specified. The paper cites a linker GPGP, but I could not find such a site in MreB. If that was an added linker between GFP and MreB, please put it in full context, i.e., give the MreB sequence before and after, show the aa's added as linker, and indicate the YPet insert. Also, I question calling it "functional" (first line of the paragraph). In Fig. S1B it looks like it is expressed at more than the stated 6%, but in any case the YPet-MreB was apparently used only as a dilute label. What was the arabinose level for this western, and was this level used for all experiments?

2. In Fig. 2A I would not say the signal was enhanced for ΔN in 2Aii. What is FtsZ (N)/(C)?

3. The D285A mutant was shown to lose FtsZ binding in the BTH, but this was only done on the full length MreB. It would seem important to test this with ΔN, since that was essential to get meaningful readout in 2A.

4. To follow up the effects of the mutant MreB losing FtsZ-binding activity, it was tested in vivo. It seems to have been tested first in an over-expression assay, where it was more toxic than wt MreB. This is surprising because one might expect that if you knocked out a binding activity, you would render the protein less toxic. Logistically, I find that this distracts from the much more important replacement experiment in 3B. I see that the overexpression system is used for many of the later assays, so it needs to be introduced. But I suggest to move the present 3A to the bottom of the figure, and present the depletion/replacement results first.

5. 3C and 3D also seem to be important in understanding the mutant, but here I am confused. My understanding is that mYpet-MreB is expressed as a dilute label in the presence of wt MreB (3C) or MreB-RFP (3D). In this case its localization should be dominated by the wt MreB, and this does seem to be the case in 3D. So how could YPet-MreB be dispersed as foci in 3C? Does it disrupt the polymers of wt MreB but not those of MreB-RFP?

6. For Fig. 3 and S3 arabinose is given as 33 μM. Please give the concentration as %, which is the usual expression for the pBAD, and used in Fig. 1. Also, we should know the arabinose level (and

hopefully the percent YFP-MreB) in all experiments.

7. "Mutations in FtsZ restore the FtsZ-MreB interaction." I think this is misleading. That would be a good title if one could test a strain where FtsZ-P203R were the sole FtsZ, and MreB-D285 A the sole MreB. But here one only tests for the effect in the over-expression assay, where D285A is more toxic than wt. A more conservative wording would seem appropriate.

8. The superscripts in the tables in Fig. 4 are too small to read without a magnifying glass. In Fig. S4 we are shown the data for P203Q suppressing the over-expression of MreB D285A. However this was the least successful of the FtsZ mutations. I think data should be shown for all of the FtsZ mutants.

9a. It is very instructive to consider the locations of the mutated aa's, using x-ray structures. I suggest a couple of improvements to this presentation. First, rather than showing a single subunit of MreB as in Fig. S2C, I think one should show two subunits in the context of the filament. The crystal structure actually has the TmMreB polymerized in a filament. One can extract this in PyMol with the command

PyMOL>symexp fil_=MreB,MreB, 3.0 (then hide the molecules except for a filament dimer).

9b. For FtsZ there are now two crystal structures of Streptococcus assembled in a straight protofilament. (The older "dimer" of MjFtsZ is no longer considered valid. Jan Löwe comments "however, we suspect that this FtsZ dimer may be distorted by crystal contacts" PNAS 107:19766) I prefer pdb 3VOA for the SaFtsZ protofilaments. Again one can generate a protofilament dimer with the symexp command. Here it would be useful to highlight all four of the compensating mutation positions, since they form a line just above the subunit interface.

9c. From looking at these models, I question the idea of direct contact of the aa's identified by the mutanta. It seems that D275 is exposed on the TmMreB filament surface, but it is actually down in a pocket, where it could not contact any aa's of FtsZ unless they were very protruding. It does seem to be making an ionic bond with K54 of the subunit below, so TmD275A might be disrupting Mreb's ability to polymerize. Some of the compensating aa's on FtsZ are likewise in cavities, probably not available for direct interactions with another protein. These mutations may be causing small changes in the conformation and polymerization abilities of FtsZ. The present mss seems to suggest that the MreB and FtsZ are interacting as polymers, and that mutations have been identified that block and restore the contacts. A more thorough analysis suggests that the mutations are likely acting indirectly by affecting polymerization and/or conformation of monomers and polymers.

10. The experiments in Fig. 5 would be much more compelling if they were done on cells where the D285A were the sole source of MreB, as in Fig. 3B, rather than an over-expression. I don't present this as necessary for publication, just a strong suggestion.

Referee #3

This important study reports that the bacterial actin MreB relocalizes from sidewall to the cell division septum and interacts directly with the bacterial tubulin FtsZ to recruit peptidoglycan synthetic enzymes to the septum. Previous studies have implicated an interaction between MreB and the cell division apparatus-showing MreB localization to bands adjacent to the Z ring, a yeast two-hybrid assay showing MreB-FtsZ interactions, and an interactome study suggesting a possible interaction between MreB and FtsZ as well as many other proteins. Progress in understanding the biochemistry of E. coli MreB has been slow because of difficulty in obtaining soluble active MreB protein.

In the present study, MreB is shown to interact with FtsZ in a bacterial two-hybrid system and by in vivo formaldehyde crosslinking. The key to the paper, though, is a remarkable mutant of MreB, D285A, that the authors obtained by mutating conserved residues outside the nucleotide binding pocket. The D285A mutant MreB is stable and seems to interact with other PG synthesis proteins, but fails to interact with FtsZ. The mutant MreB corrects the cell wall elongation defect of an MreB deletion, but these cells fail to divide because the mutant MreB fails to be recruited to the FtsZ ring.

As a result, the FtsZ rings, though able to recruit later division proteins such as PBP3, do not constrict, nor do they synthesize preseptal peptidoglycan. Evidence for a direct interaction also comes from mutations in FtsZ that specifically suppress the cell division defects caused by the mutant MreB. The model is that when MreB switches its position from the "elongasome" to the "divisome" by following FtsZ, the key peptidoglycan synthesis enzymes PBP1a, PBP1b and PBP2 are recruited by MreB to do the same, and it is this group of proteins that are required to drive septation under normal conditions.

Overall, the evidence that MreB binds directly to FtsZ is quite convincing. Although there is no direct biochemical proof, the evidence from the bacterial two-hybrid assays with MreB lacking its N-terminal membrane targeting sequence, the immunoblots from the crosslinking experiment, and the mutations in FtsZ that suppress the negative effects of the MreB mutant all make a fairly compelling case. Nevertheless, the authors need to be a bit more cautious about concluding that FtsZ and MreB interact directly. The negative effects on cell division by moderate overproduction of the D285A mutant are impressive and nicely support their model. The idea that MreB recruits part of the elongasome to the divisome is probably correct, although the localization data for the PBPs were not that convincing. The paper is well written, although there are some areas where the presentation could be more clear (see below).

Specific comments:

1) Introduction lined 10-11: the statement that actin-tubulin interactions in prokaryotes have not been described is not true. FtsA is a diverged actin homolog, and FtsZ-FtsA interactions have been clearly demonstrated genetically, biochemically and structurally.
2) Intro, bottom of page 3: An omitted reference for RodZ is Shiomi et al. EMBO J. 27:3081-91, 2008.
3) Page 5, top: in the discussion of pre-septal PG synthesis here and at the top of page 19, the authors should also include the recent paper from Kevin Young's lab (Potluri et al. J. Bact. 194:5334-5342, 2012) showing that ZipA is required for this step.
4) Page 9, lines 1-2: It should be noted here that the Butland et al. study identified many other protein partners for MreB other than FtsZ.
5) Page 10, lines 4-5: It should be pointed out that MreB,C and D were all coexpressed in these experiments.
6) Page 10, 3 lines from the bottom: The "D285A"should go after "mreB", not "CD".
7) Page 12, line 1: Did the authors ever check the localization of MreBD285A in the MreB depletion strain, when cells are filamenting? This would be a better and more definitive experiment to show that the mutant MreB protein cannot be recruited by FtsZ.
8) Page 12, line 8: should be "variants"
9) Page 12, line 10: should be "Methanococcus"
10) Fig. 2A: do the authors have an explanation for why the T25 FtsZ (N) does not interact with MreB or MreB delta N?
11) Fig. 2A: It would make sense that removing the N-terminal membrane targeting sequence might discourage BTH interactions between MreB and membrane septal ring proteins, except that the T25 domain of MreB is still attached, so it is harder to see how this would make a difference (but it clearly does).
12) Fig. 2B: Is the FtsZ shown in this panel in both cases (in combination with wt and mutant MreB) fused C-terminally to the T18 domain or N-terminally? Are the MreB and MreBD285A mutant proteins full length or N-terminally truncated?
13) Is the MreBA285A mutant defective in self-interaction? This is possible, given its proximity to the dimer interface. Did the authors try to measure this by BTH?
14) Fig. 2D: The pull downs were not explained very clearly in the legend, other than that anti-FtsZ or anti-MreB were used as primary antibodies. Was anti-MreB used for the pull down shown in the figure? If so, why is the second lane with MreBD285A + crosslinker overloaded? This would seem to make the results more convincing, which is fine, but it should be explained. Were the lanes normalized for total protein? Why do the non-crosslinked lanes have so much less stained protein?
15) Fig. 2D: If anti-FtsZ was used as a primary antibody for pull down, is that data not shown?
16) Fig. 4C: in the 3rd column of the table, rows 2 and 4 should list FtsZP230Q, not FtsZP230R as shown.
17) Fig. 5C-E: could some of the midcell staining, especially in panels C and D, be simply a result of increased signal because of the double septal membrane?

18) Fig. 5E: does HADA label these rings? As the authors have this tool in hand, it would help to determine whether preseptal PG synthesis was occurring.
19) Page 16, 1st paragraph of Discussion: The prior independence of FtsZ and MreB is somewhat exaggerated. Tan et al. 2011 showed a strong relationship, and Kruse et al. 2005 (from the Gerdes lab) showed that overproduction of MreB could inhibit cell division without necessarily perturbing nucleoid segregation.
20) Page 16, 2nd paragraph of Discussion: the patterns reported by Vats and Rothfield are likely artifacts due to the N-terminal EYFP fusion.
21) Page 18, line 6: Corbin et al. J. Bact. 186:7736-7744, 2004 was an earlier paper, possibly the first, which proposed FtsN as a trigger for septation, possibly acting through FtsA.
22) Page 18, 4th line from bottom: replace "a slow" with "during slow"

1st Revision - authors' response 23 April 2013

Referee #1

In this paper the authors present evidence that MreB localizes to the Z ring in E. coli similar to what was observed in Caulobacter. Very strong genetic and biochemical evidence is presented that FtsZ and MreB interact and that this has functional significance. The authors argue that MreB is required for cells to divide. Furthermore, the authors show that overexpression of an MreB mutant blocks cell division. In the filaments that form the authors argue that PBP3 is present but that PBP2 and PBP1B are not. The evidence for this latter finding is weak due to the poor localization of these proteins in the WT conditions.
Mutations in both FtsZ and MreB that interfere with the interaction are apparently lethal so complex strain constructions are required to test the system, i.e. the alleles can not be put on the chromosome (or at least it was not tested). It may be possible under slow growth conditions where the interaction is not essential and cells grow as spheres.

We would like to thank the reviewer for their constructive and helpful comments. To address your concern regarding the wt PBP localisation patterns we have added a new figure to the supplementary data (Figure S8). This figure better demonstrates 'wt' localisation patterns of the PBPs. Responses to your more specific points are below:

Page 4, last full paragraph - the authors point out that divisome maturation occurs in 2 steps. It would be helpful to indicate that these two steps correlate with two phases of divisome assembly-the first being the Z ring and the second being the additional proteins that complete the divisome (even though this may be over simplified).

Thank you for the helpful comment; we have inserted a sentence into this paragraph which specifically makes the 'two stage divisome' point clear.

Page 4, three lines from bottom - rings were reported from one lab under special conditions. These rings were not reported in the 3 high resolution studies that rules out the continuous spiral of MreB.

Indeed, the three high resolution studies to which you refer (we assume you mean: Domínguez-Escobar et al 2011, Garner el al 2011 and van Teeffelen et al 2011) all report a lack of overall MreB structure, each concluding that MreB moves in localised foci, 'spots' or 'patches'. This manuscript does not comment on the overall MreB structure, only shows that MreB co-localises with FtsZ through a variety of methods. The other studies, to which we refer, were the first to describe the MreB-Zring co-localisation pattern which we go on to both verify and show is a result of a direct interaction.

It's worth noting that two of the high resolution studies are based on TIRF microscopy. This technique maximises the contrast of images by refracting the light such that it effectively forms a standing wave at the interphase between the sample and the coverslip. This would effectively make the microscope 'blind' to any large MreB structure. The third high-resolution study suggests MreB moves laterally around the membrane which is, if anything, consistent with our observations.

All of these studies are properly referenced as part of this work.

Fig. 2D. These experiments were done in MC1000 which is MreB+. I do not understand why a band is not seen when His-MreB-D285A is used. The WT MreB that is present could still crosslink to FtsZ.

As we only retain His-tagged proteins as part of the protocol, any untagged MreB-FtsZ cross link products will be lost. In theory crosslink products consisting of three proteins, with MreB acting as a bridge between $MreB^{D285A}$ and FtsZ could occur. This may account for the very low detection of FtsZ in $MreB^{D285A}$ pull-downs. The relative 'cleanness' of this result is probably due to the very low level expression of the His-MreB proteins, the short cross linking activity of formaldehyde and the short pulse of crosslinking.

We've added a short discussion point on this to page 10.

Fig. 3A. Mild overexpression of MreB-D285A. The expression system used was arabinose inducible which can vary a lot from cell to cell. In the figure it appears the increase in cell length is due to a few long cells - this is consistent with possible high level expression in these few cells and low level in others. Can one do a spot test at different inducer concentrations to show how toxic the mutant is compared to WT.

Indeed, arabinose uptake does vary from cell to cell. However, over half of the $MreB^{D285A}$ expressing cells measured in this study were greater in size than the control+SD (446 of 741 cells were greater than 3.97 - this is illustrated in Figure 3A(ii)), therefore this result was not the result of a few elongated cells, but a huge increase in average cell lengths of the whole population. A P_{lac}-IPTG inducible system was not appropriate in this case, as even basal levels of expression were enough to perturb growth rate and give cell elongation.

We have added a spot test of diluted cultures on M9 media with a wide range of arabinose concentrations in Figure S3D to show the lethality expression and fully justify the arabinose concentration we used throughout this work.

Page 10, middle paragraph - it is surprising that MreB can disappear in 1 hour in the depletion experiments unless MreB is very unstable. In 1 hour (~2 generations, it would be diluted to 25% of the original, however, as the plasmid is still present in some cells it should not be diluted that much.

It is perhaps surprising that protein depletion can happen this quickly. Of course protein turnover, as MreB has a half-life of between 30-40 min, reduces that '25%' you mention down still further to somewhere less than 12.5%.

We've added a western blot showing time-points in the MreB depletion strain to Figure S4B.

Page 11, bottom of page. I am surprised by this reduction in localization of MreB-D285A to the Z ring. First the authors show that this mutant colocalizes with WT MreB. Then the mutant is expressed in cells where it only represents 6% of the total MreB and yet it goes poorly to the Z ring. One would not expect to see a decrease from 75 to 28% since the mutant should colocalize with the WT MreB at the Z ring. Perhaps it is the monomer of MreB that goes to the Z ring and the mutant cannot do this. In the supplement the authors show the position of D285 in the monomer but it looks like it could be buried in the MreB filament. Have the authors checked that? The authors show that MreB-D85A interacts with WT MreB but does it interact with itself, especially if it lacks the membrane binding sequence?

It seems strange a 75-28% drop seems somehow disproportionate to you, I'm afraid this is the result we observe. The fact that <u>any</u> co-localisation occurs despite the BTH and crosslinking results indicating no interaction is perhaps surprising. The residual 28% co-localisation activity is probably due to indirect interaction through MreB at the Z-ring as you say.

To address your points, we have checked the position of the D285 residue very carefully and contacted Jan Lowe about the position of the residue in regards the MreB dimer interphase. Also, we have added MreB^{D285A} 'self' interacting BTH data into Figure S3A.

Fig. 4A what does the one and two pluses mean in terms of complementation. IN Fig. S4 it appears that FtsZ-P103Q interacts very poorly if at all (hard to tell) with MreB-D285A. It also interacts poorly with FtsZ - this seems very odd. This may be why it does not complement an FtsZ depletion strain (whereas the authors imply it is because it does not interact with WT MreB). If the authors are right then FtsZ-P203Q should support the growth of an MreB deletion strain.

In terms of complementation, and we are assaying the complementation ability against the VIP205 P_{tac}::ftsZ strain. ++ = full-complementation (comparable to ftsZ depletion complemented by ftsZ^{WT} +ve control), + = partial (100 drop in CFU compared to ftsZ^{WT} control) and - = no complementation (same CFU as empty vector –ve control).

We have re-enforced the meanings of the ++, + and – scores in the text on page 13. Also we have added extra detail into the figure legend of Figure 4.

Page 12 - using Pfu as an error prone polymerase - I thought it was a high fidelity enzyme.

Ordinarily yes, Pfu is a high fidelity enzyme. In this study however we used a Pfu variant with the D473G substitution and lacking the exonuclease proof reading activity (D215A). These mutations render it error prone and very useful for mutagenesis work.

We have added additional detail to Page 12 to make it clearer to the reader that we are using an error-prone Pfu variant in this study.

Fig. 5C and D. The localization of PBP1B and PBP2 to the septum is not very convincing. The increased brightness at the septum could be due to double membranes at the septum. For example, the cells that lay side by side in panel C and where they about the signal is very bright.

The detection of the PBP1B- and PBPB2-mCherry fusions is very challenging. We are very aware the potential artefact of membrane-associated proteins forming 'bright' foci at division sites and are satisfied that this is not the case with these protein fusions, as we can observe recruitment before any visible cell division. We sought to demonstrate that this was not the case through time-lapse microscopy (Figure S10) and wt controls (Figure S6 and S7).

However, to make the microscopy data more robust and our conclusions clearer we have added Figure S8. In this figure, we have used FtsI inhibitors to block fully formed septa and inhibit cell division. In this figure, PBP1B and PBP2 form bright bands as expected, which are free of the potential double membrane bright foci artefact. This serves as a control for the wt division factor localisation patterns and shows readers contrasting localisation patterns in elongated cells to those seen in Figure 5, which increases the robustness of the observations as a whole.

Page 17 and 18. Although the authors show that PBP3 is at the locked Z rings it is not clear if FtsN is there as the authors say in the discussion. Thus, the authors should not refer to the Z ring as mature.

We agree and have included new FtsN localisation study to Figure 5, which shows FtsN is present at the locked Z rings. We've also added wt localisation pattern controls to Figure S7 and FtsI-inhibited control in Figure S8.

Page 19, the authors say that MreB is essential in rapid growth. However, it would seem that MreB is essential when cells are rod shaped regardless of the growth rate. Presumably it is required when cells transition from cylindrical growth to septal growth. Since MreB is not essential when cells grow as spheres. Does the MreB mutant have an effect at slower growth rates?

We tested this using our MreB depletion system to remove the complementing R1 (P_{lac}::mreBCD) plasmid from a Δmre strain (see Figure S4D for schematic) to create strains complemented by only P_{BAD}::mreBCD with and without the D285A mutation.

Although both strains grow in the supportive media described in Bendezú et al 2007, they fail to grow in rich media. Induction of the P_{BAD}::mreBCD construct failed to restore viability at any arabinose concentration, or alter the morphology in a reproducible manner. Thus, this approach tells us that mreBCD is essential in 'fast' growth conditions.

Referee #2

This paper presents convincing evidence for a direct interaction of FtsZ and MreB. Previous co- or near-localization experiments had hinted at a possible interaction, but this paper provides much more definitive evidence. The experiments show that the FtsZ-MreB interaction is important for cell division under the standard growth conditions (although previous studies have already shown that MreB depletion can be tolerated with overexpression of FtsZ or slower growth conditions). The mechanisms are not yet clear - some of them are likely indirect as discussed below. Nevertheless this is an important step in showing that these two cytoskeletal components, originally thought to be independent, are cooperating in the mechanism of cytokinesis. I recommend publication following attention to the points below.

We would like to thank the reviewer for their constructive and helpful comments. We have addressed your concerns regarding the crystal structure interpretations and made a number of modifications to Figures 2,3,4,S3,S4 as you suggest. We have made the microscopy in the manuscript more compelling by adding additional data to Figure S2 and S8. Responses to your more specific points given below:

1. The Results refer the reader to Methods for the construction of mYPet-MreB. This is actually in SI Methods. But the insert site is not precisely specified. The paper cites a linker GPGP, but I could not find such a site in MreB. If that was an added linker between GFP and MreB, please put it in full context, i.e., give the MreB sequence before and after, show the aa's added as linker, and indicate the YPet insert. Also, I question calling it "functional" (first line of the paragraph). In Fig. S1B it looks like it is expressed at more than the stated 6%, but in any case the YPet-MreB was apparently used only as a dilute label. What was the arabinose level for this western, and was this level used for all experiments?

The GPGP 'linker' region was added between full-length mYpet ORF and the mreB ORF. We have added an in-depth description of how this construct was generated to the Supplementary Methods.

2. In Fig. 2A I would not say the signal was enhanced for ΔN in 2Aii. What is FtsZ (N)/(C)?

We have removed 'and even enhanced' from the sentence on page 9 as suggested. To make it clearer that we mean FtsZ labelled at its N-terminus '(N)' or C-terminus '(C)' in Figure 2 we have edited the Figure 2 legend on Page 31, and included this in the text on Page 8.

3. The D285A mutant was shown to lose FtsZ binding in the BTH, but this was only done on the full length MreB. It would seem important to test this with ΔN, since that was essential to get meaningful readout in 2A.

The full-length MreB constructs were selected as it was considered less artificial than the ΔN construct. In addition using the full length protein is consistent with previous studies, the microscopy data and the crosslinking assay in Figure 2D. We have included additional BTH data in Figure S3A.

4. To follow up the effects of the mutant MreB losing FtsZ-binding activity, it was tested in vivo. It seems to have been tested first in an over-expression assay, where it was more toxic than wt MreB. This is surprising because one might expect that if you knocked out a binding activity, you would

render the protein less toxic. Logistically, I find that this distracts from the much more important replacement experiment in 3B. I see that the overexpression system is used for many of the later assays, so it needs to be introduced. But I suggest to move the present 3A to the bottom of the figure, and present the depletion/replacement results first.

The MreB^{D285A} mutant is 'toxic' to the cells as it effectively retains the PBP enzymes to MreB assemblies causing elongation; a property the wt does not have. Aside from the direct microscopic observations (Figure 3B) this can be measured through the reduction in long-term growth rates and CFU counts in M9 media + 0.000,05% arabinose from the P_{BAD}:mreBCD constructs with and without the D285A mutation (pAKF128 and pAKF129) in liquid media (Compare lines 1 and 3 in Figure 4C, see also and S5C). On plates, the loss of viability between the wt and mutant are similar.

To address your comments we have re-ordered the manuscript to bring the 'depletion' result before the 'overexpression' result on page 10 and 11, and re-arranged Figure 3 and Figure S4 to match. We have also included M9 arabinose viability plates in Figure S3 to show MreB^{D285A} toxicity and support the arabinose concentration used for this study.

5. 3C and 3D also seem to be important in understanding the mutant, but here I am confused. My understanding is that mYpet-MreB is expressed as a dilute label in the presence of wt MreB (3C) or MreB-RFP (3D). In this case its localization should be dominated by the wt MreB, and this does seem to be the case in 3D. So how could YPet-MreB be dispersed as foci in 3C? Does it disrupt the polymers of wt MreB but not those of MreB-RFP?

The experiment in Figure 3C concentrates on the ability of mYpet-MreB^{D285A} to enter MreB assemblies, a point on which you seem to agree with us that it does. The experiment in Figure 3D verifies the result of Figure 3C as now all MreB in these cells is labelled, the MreB-RFP^{SW} from the genome and the dilute mYpet-MreB expressed from the plasmid. Here again we would expect co-localisation, and this is what we observe.

In essence both localisation patterns are the same, there is no disruption of the MreB distribution pattern, but the conclusion is more compelling when taken together. Perhaps a point of confusion here is the usage of the term 'foci' and 'structures' on Page 12. To address this confusion we have re-phrased this paragraph and used the term 'assemblies' when referring to the MreB distribution pattern.

6. For Fig. 3 and S3 arabinose is given as 33 µM. Please give the concentration as %, which is the usual expression for the pBAD, and used in Fig. 1. Also, we should know the arabinose level (and hopefully the percent YFP-MreB) in all experiments.

The reason for giving arabinose concentration in µM is to make it consistent with the work of Guzmen et al 1995, who originally characterised the P_{BAD} promoter.

We have changed all arabinose concentrations to 0.000,05% (33.3 µM) in the manuscript, figure legends and supplementary material.

7. "Mutations in FtsZ restore the FtsZ-MreB interaction." I think this is misleading. That would be a good title if one could test a strain where FtsZ-P203R were the sole FtsZ, and MreB-D285 A the sole MreB. But here one only tests for the effect in the over-expression assay, where D285A is more toxic than wt. A more conservative wording would seem appropriate.

We have changed the title of this section to the more descriptive: 'Mutations in ftsZ restore the FtsZ – MreB BTH interaction signal'.

8. The superscripts in the tables in Fig. 4 are too small to read without a magnifying glass. In Fig. S4 we are shown the data for P203Q suppressing the over-expression of MreB D285A. However this was the least successful of the FtsZ mutations. I think data should be shown for all of the FtsZ mutants.

We have increased the font size of all elements in the Tables on Figure 4 and in Figure S5 (previously S4) so they are all easier to read.

We agree that it would be good to show data for the additional ftsZ mutants, and have included the suppression of the MreB D285A over-expression data for all ftsZ mutants identified in this study in Figure S5C.

9a. It is very instructive to consider the locations of the mutated aa's, using x-ray structures. I suggest a couple of improvements to this presentation. First, rather than showing a single subunit of MreB as in Fig. S2C, I think one should show two subunits in the context of the filament. The crystal structure actually has the TmMreB polymerized in a filament. One can extract this in PyMol with the command

PyMOL>symexp fil_=MreB,MreB, 3.0 (then hide the molecules except for a filament dimer).

9b. For FtsZ there are now two crystal structures of Streptococcus assembled in a straight protofilament. (The older "dimer" of MjFtsZ is no longer considered valid. Jan Löwe comments "however, we suspect that this FtsZ dimer may be distorted by crystal contacts" PNAS 107:19766) I prefer pdb 3VOA for the SaFtsZ protofilaments. Again one can generate a protofilament dimer with the symexp command. Here it would be useful to highlight all four of the compensating mutation positions, since they form a line just above the subunit interface.

9c. From looking at these models, I question the idea of direct contact of the aa's identified by the mutanta. It seems that D275 is exposed on the TmMreB filament surface, but it is actually down in a pocket, where it could not contact any aa's of FtsZ unless they were very protruding. It does seem to be making an ionic bond with K54 of the subunit below, so TmD275A might be disrupting Mreb's ability to polymerize. Some of the compensating aa's on FtsZ are likewise in cavities, probably not available for direct interactions with another protein. These mutations may be causing small changes in the conformation and polymerization abilities of FtsZ. The present mss seems to suggest that the MreB and FtsZ are interacting as polymers, and that mutations have been identified that block and restore the contacts. A more thorough analysis suggests that the mutations are likely acting indirectly by affecting polymerization and/or conformation of monomers and polymers.

Thank you for all the helpful advice on the use of crystal structures and PyMOL.

We have included the Streptococcus FtsZ crystal structure '3VOA.pdb', modelled into a dimer with the location of the ftsZ mutations highlighted as you suggested. This has now been added to Figure 4B.

To address your point about the location of the D285 residue, we have checked the position very carefully and contacted Jan Lowe about the position of the residue in regards the MreB dimer interphase. Also, we have added MreB^{D285A} 'self' interacting BTH data into Figure S3A. We find that using the 'symexp' function on the TmMreB '2WUS.pdb' file bears little resemblance to the MreB polymer figure shown in van den Ent et al 2010.

To address your point regarding possible indirect effects of our ftsZ or mreB mutants through the loss of self-interaction or the impact on polymerisation properties, we have re-phrased some areas of the discussion. Of course we tried to show that each ftsZ mutant and the mreB^{D285A} mutants can still bind its partner through BTH assays (see modified Figure S3A) and complementation assays (Figure S5) to control for these kinds of effects. However we acknowledge any modification to polymer forming proteins makes indirect effects a possibility.

10. The experiments in Fig. 5 would be much more compelling if they were done on cells where the D285A were the sole source of MreB, as in Fig. 3B, rather than an over-expression. I don't present this as necessary for publication, just a strong suggestion.

We used our MreB depletion system to remove the complementing R1 (P_{lac}::mreBCD) plasmid from a Δmre strain (see Figure S4D for schematic) to create strains complemented by only P_{BAD}::mreBCD with and without the D285A mutation. Although both strains grow in the supportive media described in Bendezú et al 2007, they fail to grow in rich media. Induction of the

P_{BAD}::mreBCD construct failed to restore any viability at any single arabinose concentration, or alter the morphology in a reproducible manner making this approach intractable.

To make the microscopy in the manuscript more compelling we have added an FtsI-inhibitor control (Figure S8). In this figure we show elongated cells treated with the inhibitor locking 'mature' Z-rings in an inactive form. This acts as a good imaging control for all the microscopy we show in Figure 5 of the manuscript. Critically PBP1B and PBP2 form clear bands/rings in FtsI-inhibited elongated cells, but not in $MreB^{D285A}$ elongated cells. This supports the main thrust of the mis-localisation argument in this manuscript.

Referee #3

This important study reports that the bacterial actin MreB relocalizes from sidewall to the cell division septum and interacts directly with the bacterial tubulin FtsZ to recruit peptidoglycan synthetic enzymes to the septum. Previous studies have implicated an interaction between MreB and the cell division apparatus-showing MreB localization to bands adjacent to the Z ring, a yeast two-hybrid assay showing MreB-FtsZ interactions, and an interactome study suggesting a possible interaction between MreB and FtsZ as well as many other proteins. Progress in understanding the biochemistry of E. coli MreB has been slow because of difficulty in obtaining soluble active MreB protein.

In the present study, MreB is shown to interact with FtsZ in a bacterial two-hybrid system and by in vivo formaldehyde crosslinking. The key to the paper, though, is a remarkable mutant of MreB, D285A, that the authors obtained by mutating conserved residues outside the nucleotide binding pocket. The D285A mutant MreB is stable and seems to interact with other PG synthesis proteins, but fails to interact with FtsZ. The mutant MreB corrects the cell wall elongation defect of an MreB deletion, but these cells fail to divide because the mutant MreB fails to be recruited to the FtsZ ring. As a result, the FtsZ rings, though able to recruit later division proteins such as PBP3, do not constrict, nor do they synthesize preseptal peptidoglycan. Evidence for a direct interaction also comes from mutations in FtsZ that specifically suppress the cell division defects caused by the mutant MreB. The model is that when MreB switches its position from the "elongasome" to the "divisome" by
following FtsZ, the key peptidoglycan synthesis enzymes PBP1a, PBP1b and PBP2 are recruited by MreB to do the same, and it is this group of proteins that are required to drive septation under normal conditions.

Overall, the evidence that MreB binds directly to FtsZ is quite convincing. Although there is no direct biochemical proof, the evidence from the bacterial two-hybrid assays with MreB lacking its N-terminal membrane targeting sequence, the immunoblots from the crosslinking experiment, and the mutations in FtsZ that suppress the negative effects of the MreB mutant all make a fairly compelling case. Nevertheless, the authors need to be a bit more cautious about concluding that FtsZ and MreB interact directly. The negative effects on cell division by moderate overproduction of the D285A mutant are impressive and nicely support their model. The idea that MreB recruits part of the elongasome to the divisome is probably correct, although the localization data for the PBPs were not that convincing. The paper is well written, although there are some areas where the presentation could be more clear (see below).

We would like to thank the reviewer for their supportive and helpful comments. We have revised the manuscript to be a little more cautious about the FtsZ and MreB direct interaction, as you suggest, and have made the microscopy in the manuscript more compelling by adding additional data to Figure S2 and S8. Responses to your more specific points given below:

Specific comments:

1) Introduction lined 10-11: the statement that actin-tubulin interactions in prokaryotes have not been described is not true. FtsA is a diverged actin homolog, and FtsZ-FtsA interactions have been clearly demonstrated genetically, biochemically and structurally.

Indeed, we have acknowledged the work on FtsZ-FtsA interaction multiple times in the manuscript.

There are no papers describing the direct exchange of protein factors between tubulin homologues (such as FtsZ) and actin homologues (such as MreB or FtsA) in prokaryotes. In eukaryotes, proteins are exchanged between tubulin and actin structures as part of cellular trafficking processes; perhaps analogous to the exchange of PBP proteins we study as part of this work.

We have made this point clearer in the manuscript

2) Intro, bottom of page 3: An omitted reference for RodZ is Shiomi et al. EMBO J. 27:3081-91, 2008.

We have added this reference to the manuscript

3) Page 5, top: in the discussion of pre-septal PG synthesis here and at the top of page 19, the authors should also include the recent paper from Kevin Young's lab (Potluri et al. J. Bact. 194:5334-5342, 2012) showing that ZipA is required for this step.

This reference was already in the manuscript a little further on; we have added it here to make the point that ZipA is required for pre-septal PG synthesis 'or PIPS'. This forms a supportive point for this manuscript, as without ZipA there is no Z-ring which means no MreB recruitment, so no PBP2 and thus no 'PIPS'.

4) Page 9, lines 1-2: It should be noted here that the Butland et al. study identified many other protein partners for MreB other than FtsZ.

Indeed, we have modified this sentence to include this point.

5) Page 10, lines 4-5: It should be pointed out that MreB,C and D were all coexpressed in these experiments.

We have added this to the first line in the paragraph.

6) Page 10, 3 lines from the bottom: The "D285A"should go after "mreB", not "CD".

Agreed; thank you for spotting this error.

7) Page 12, line 1: Did the authors ever check the localization of MreBD285A in the MreB depletion strain, when cells are filamenting? This would be a better and more definitive experiment to show that the mutant MreB protein cannot be recruited by FtsZ.

In this depletion strain the mYpet-MreB^{D285A} signal forms spotty foci along the cell length, however using the mYpet-MreB variant as the sole source of MreB in the depletion system leads to highly dimorphic cells which are unfit for further study.

8) Page 12, line 8: should be "variants"

We have made this change

9) Page 12, line 10: should be "Methanococcus"

We have made this change, thank you for spotting this.

10) Fig. 2A: do the authors have an explanation for why the T25 FtsZ (N) does not interact with MreB or MreB delta N?

In the bacterial two hybrid screen used in this study no FtsZ-MreB interaction could be detected when FtsZ is tagged at its N-terminus with either T18 or T25 domains (Figure 2A). The FtsZ N-terminal fusions are able to bind the C-terminal fusions (and each other) in every combination and are therefore presumably forming stable fusion proteins (Figure S3). As for the reason, it would be far too speculative to say.

11) Fig. 2A: It would make sense that removing the N-terminal membrane targeting sequence might discourage BTH interactions between MreB and membrane septal ring proteins, except that the T25 domain of MreB is still attached, so it is harder to see how this would make a difference (but it clearly does).

Yes, it is surprising that this deletion makes a difference - but it does.

12) Fig. 2B: Is the FtsZ shown in this panel in both cases (in combination with wt and mutant MreB) fused C-terminally to the T18 domain or N-terminally? Are the MreB and MreBD285A mutant proteins full length or N-terminally truncated?

We used the C-terminal FtsZ T25 fusion for Figure 2B, to make this clearer we have added this to the Figure and revised the figure legend. The MreB and MreBD285A are full-length protein fusions; this information has also been added to the figure legend.

13) Is the MreBA285A mutant defective in self-interaction? This is possible, given its proximity to the dimer interface. Did the authors try to measure this by BTH?

We did, and have included MreB^{D285A} self-interaction data in Figure S3A.

14) Fig. 2D: The pull downs were not explained very clearly in the legend, other than that anti-FtsZ or anti-MreB were used as primary antibodies. Was anti-MreB used for the pull down shown in the figure? If so, why is the second lane with MreBD285A + crosslinker overloaded? This would seem to make the results more convincing, which is fine, but it should be explained. Were the lanes normalized for total protein? Why do the non-crosslinked lanes have so much less stained protein?

We have re-phrased this part of the figure 2 legend and added extra detail to the supplementary materials and methods.

15) Fig. 2D: If anti-FtsZ was used as a primary antibody for pull down, is that data not shown?

Anti-FtsZ was not used as a primary antibody for the pull down assays. We have re-phrased this part of the figure 2 legend and added extra detail to the supplementary materials and methods to make it clear what protocol we used.

16) Fig. 4C: in the 3rd column of the table, rows 2 and 4 should list FtsZP230Q, not FtsZP230R as shown.

Thank you very much for spotting this, we have made the changes to the Figure 4C.

17) Fig. 5C-E: could some of the midcell staining, especially in panels C and D, be simply a result of increased signal because of the double septal membrane?

This was a concern echoed by the reviewer above, and we have copied our response again below:

The detection of the PBP1B- and PBPB2-mCherry fusions is very challenging. We are very aware the potential artefact of membrane-associated proteins forming 'bright' foci at division sites and are satisfied that this is not the case with these protein fusions, as we can observe recruitment before any visible cell division. We sought to demonstrate that this was not the case through time-lapse microscopy (Figure S10) and wt controls (Figure S6 and S7).

However, to make the microscopy data more robust and our conclusions clearer we have added Figure S8. In this figure we have used FtsI inhibitors to block fully formed septa and inhibit cell division. In this figure PBP1B and PBP2 form bright bands as expected, which are free of the potential double membrane bright foci artefact. This serves as a control for the wt division factor localisation patters and shows readers contrasting localisation patterns in elongated cells to those seen in Figure 5, which increases the robustness of these observations as a whole.

18) Fig. 5E: does HADA label these rings? As the authors have this tool in hand, it would help to determine whether preseptal PG synthesis was occurring.

The HADA is not incorporated into locked Z rings in $MreB^{D285A}$ elongated cells, suggesting that pre-septal synthesis is inhibited (compare cells in Figure 5E with the wt control).

To make this result more robust, we have added an additional control; where cells are elongated through exposure to the FtsI-inhibitor Aztreonam, inhibited Z-rings in these cells are known to still carry out pre-septal PG synthesis, therefore these Z rings (tagged using the FtsZ-mCherry tag) incorporate the HADA (Figure S6C).

19) Page 16, 1st paragraph of Discussion: The prior independence of FtsZ and MreB is somewhat exaggerated. Tan et al. 2011 showed a strong relationship, and Kruse et al. 2005 (from the Gerdes lab) showed that overproduction of MreB could inhibit cell division without necessarily perturbing nucleoid segregation.

To our knowledge the Tan et al 2011 manuscript is the only paper to suggest an interaction between FtsZ and MreB, but do not show what this may mean for the cells. In the literature a relationship between FtsZ and MreB (especially in the context of mre operon) has been inferred through functional overlap between FtsZ- and MreB-complexes for a long time.

We have softened this sentence in the discussion as suggested.

20) Page 16, 2nd paragraph of Discussion: the patterns reported by Vats and Rothfield are likely artifacts due to the N-terminal EYFP fusion.

Perhaps this is true, however it is still important to reference them as a part of this study as they did report similar MreB structures to the ones we identify and study further here.

We have added further information to this sentence to make it clear the contrast between the structures observed in this study and those reported in Vats and Rothfield, 2007.

21) Page 18, line 6: Corbin et al. J. Bact. 186:7736-7744, 2004 was an earlier paper, possibly the first, which proposed FtsN as a trigger for septation, possibly acting through FtsA.

We have added this to the manuscript.

22) Page 18, 4th line from bottom: replace "a slow" with "during slow"

We have made the replacement as suggested.

