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Holliday junction affinity of the base excision repair factor Endo III contributes to cholera toxin phage integration

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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.)

1st Editorial Decision 18 May 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal, and please accept my apologies for the unusual delay in its evaluation, which was owed to a very late referee report. We have now finally received all three sets of comments, which you will find copied below. As you will see, while all referees indicate potential interest in the novel implication of EndoIII as a host factor in CTX phage integration, they also raise a number of substantive concerns with the study at the current stage. In particular, referees 2 and 3 stress the need for a better understanding of the mechanism of EndoIII action on XerCD. Should you be able to extend the analysis to substantially deepen the mechanistic insights (by following the reviewers' constructive suggestions to this effect), as well as to adequately address the various other specific concerns raised in the reports, then we should be able to consider a revised version of the manuscript further for publication. Please be reminded however that it is our policy to allow a single round of major revision only, and that it will therefore be important to diligently and comprehensively answer to all the specific points raised at this stage in the process. In addition, there are also several editorial issues that I need to bring to your attention at this stage:

- please carefully edit the manuscript text to remove systematic spelling mistakes (e.g. 'reasonned') and inappropriate word usage, ideally having the final version proof-read by a native speaker of English
- please also carefully revise the reference list - it is currently not only in a very heterogeneous citation format, but also lacks at least some references cited in the text (e.g. I noticed Kono et al 2011, cited on page 3)
- we will need a brief Conflict of Interest statement at the end of the manuscript text (next to the Acknowledgements/Author Contributions)
- finally, I am wondering whether the manuscript title could be changed, to more explicitly make

clear that we are looking at an unexpected novel molecular role of EndoIII? E.g. something like 'A novel function of the BER (or: repair) enzyme Endo III in stabilizing Holliday junction intermediates stimulates CTX phage integration'

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

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript describes the finding that Endonuclease III (Nth) acts as an accessory factor in vivo the site-specific recombination of CTXphi into the Vibrio dif site. The in vivo and in vitro analyses are thorough and show that Nth binds specifically to the pseudo-HJ substrates and block the XerC-mediated cleavage of the site, probably by competing with XerC binding. This is actually quite surprising, suggesting that the likely tetramer of (XerC)2 (XerD)2 is disrupted by Nth on the HJ - does Nth "kick" off the whole tetramer, or just the XerC dimer? It wasn't 100% clear, though the XerD footprint is weaker in the presence of Nth (Fig 6 panel C lane 8 vs lane 4). If the latter, this would be a first in the "world" of tyrosine recombinases.

This activity as an accessory factor does not require the catalytic activity of Nth, and cannot be substituted by similar enzymes. Further analysis of the mechanism and interactions of Nth with this and other site-specific recombination reactions will be very interesting. The experiments are clean and the conclusions well-supported, and the analysis solves the mystery of the inefficiency of XerC-driven HJ formation and resolution. Is XerD catalytic activity actually required for CTXphi integration?

The manuscript was well-written. I did however find a few typos/spelling errors, which would be easier to point out if the lines were numbered:

p 7, 2nd line from bottom: reasoned
p 8, 5th line from top: restored
p 8, 2nd line from bottom: transferred; substitute "idea" with "possibility"
p 12, 3rd line from bottom: reasoned
p 14, 6th line from top: led
p 16, 3rd line from bottom: exchanges
p 17 , middle paragraph: replace "So far" with "To date"; later in the paragraph, discriminate; next line, scenarios

Referee #2 (Remarks to the Author):

Bischerour et al have identified a factor, Endo III, that promotes the integration of a CTX phage analog into dif. Integration is catalyzed by XerC/D, and involves the formation of a Holliday junction intermediate. The authors propose that Endo III modulates the relative rates of forward and backward reactions catalyzed by XerCD by inhibiting new strand exchange once a Holiday junction has formed. They demonstrate that Endo III can bind to attP and dif sequences in the context of a Holliday junction, but not as simple ds DNA, even when XerCD are not present. Overall their data support a role for Endo III in enhancing CTX phage integration; however, the underlying mechanism and conclusions regarding the effect of Endo III are not clearly demonstrated.

Major comments:

P5, also p14. Although there is evidence that the catalytic activity of Endo III is not absolutely required for enhancing CTXphage integration, catalytic activity clearly has an effect. The statement that "the catalytic activity was not implicated" is misleading.

P7 (also Fig. 3 legend). The authors should clarify that although most exconjugants were at least partially blue (based on Fig. 2), the frequency of integration was calculated based solely on the percentage of white colonies. Thus although colonies may have been scored at 14 hrs, they are not really monitoring the 'frequency of integration events 14 hr after conjugation' but rather the frequency with which integration had already occurred at the time at which selection began. The percentages shown in Fig. 3 don't match all that well with the images in Fig. 2 (ie white colonies are rare); were they monitored under different conditions?

P7. There is clearly a lower average copy # of integrated RS1 in the endo III mutant; however, it is not at all clear how the figure of 36% single RS1 was arrived at. It would be preferable to acknowledge that there is extensive heterogeneity in array size for an individual nth enconjugant colony (shown in Fig. S2).

P8. As above, the effect of the K120Q mutation is somewhat ambiguous. Complementation with the K120Q plasmid is rather poor (Fig. 3b), and its utility in in vitro assays is far from wt (Fig. S2; claims for "the same in vitro activities" on p9 are misleading). It would be good to assess integration frequency in this background.

P10, Fig. 5A. The meaning of Fig. 5a is not clear; 5b is sufficient to show binding of HJ by Endo III. Given that Endo III alone can shift HJs to the well, the shift seen in 5a does not necessarily reflect simultaneous binding by Endo III and XerCD, as is implied by the "HJ-intermediate covered...XerC "(also claimed on p11; "even when they are covered"). To visualize competition between XerCD and Endo III, it would be better to add increasing amts of XerCD and see if they redirect the HJ from the well to a faster migrating complex, as seen with XerCD alone bound. If 5a is maintained, the concentration gradient of Endo III should be defined.
Overall, p10/11 seem to imply simultaneous binding of XerCD and Endo III, which is confusing and conflicts with data presented later in the paper, eg. 5e, 6d).

Can the authors more clearly reconcile the data presented in Fig. 4D and 6B? It is not clear why a reduction in cleavage frequency is not also observed in Fig. 4D in the presence of Endo III, if this is the process inhibited by this enz. If Endo III competes with XerC for binding at HJ, then why is less cleavage not see in the presence of EndoIII, regardless of whether or not religation of the sequences can occur?

P13. Is it thought that XerD can remain bound in the absence of XerC, or is their binding thought to be interdependent, and if so, can this be related to the effect of Endo III binding? Although the authors conclude that Endo III "specifically dislodges" XerC, this does not seem to be the case in Fig. 6C (right) in which loss of protection by XerD is also apparent in the presence of Endo III. XerC site hypersensitivity (6d) is significantly greater than at the XerD site; this may account for its detection (and not that of the XerD site) even in the presence of XerCD in 6D.

P18. The conclusion that Endo III binds to HJ in general should be backed up by looking at binding to HJs with sequences that are unrelated to either dif or attP; the two HJ tested are too similar to

warrant such a broad conclusion. Demonstration of HJ binding that is not sequence specific could also make the paper of broader interest.

Minor comments:
Abstract: re [preventing Xer recombination cycles] relies on the ability of Endo II to bind...even in the absence of the recombinases. While Endo III can bind w/o XerCD, I'm not sure the authors have shown this capacity is required for its effect.

'reasoned' has only a single n; it is misspelled throughout. Also, 'appearance' might be a better word choice than 'apparition'.

Figure legends could benefit from inclusion of more descriptive detail, particularly the molecular assays. Alternatively it might help to show the putative stages of the XerCD mediated reaction in greater detail within the figures, in order to clarify how various products are generated. This might also help avoid the suggestion that oligo complexes (e.g., HJ) remain intact within the gels, which might be inferred from the depiction of such complexes adjacent to particular bands. It should also be noted that dotted lines represent unlabeled oligos that are included within the assays.

P7. The authors note that xerD mutants were not obtained in their screen; can they supply a likely explanation?

Although it is possible that the reduced array size in the nth mutant reflects the reduced integration rate, it is also possible that smaller arrays reflect increased repression of replicative RS1 after a single integration event. The authors have a nice control, using non-replicative attP (Fig 4b), to demonstate that nth deletion alters integration even in the absence of replication, so this caveat does not apply to their overall conclusion; however, they should be cautious wrt their conclusions regarding array size. Fig 4a is probably not necessary.

P9. A reference regarding El Tor and classical phage variants should be supplied, and "data not shown" noted; it is not clear what the authors mean here when they state that 'different processes' govern rolling circle replication in El Tor and classical CTX phage.

Fig 4. Does 1st, 2nd refer to order of addition to the reaction? Should be noted in legend.

Would it be feasible/useful to define the Endo III binding site using footprinting and DNA strands that are cleaved by XerC, to get a better sense of what part of the HJ structure is bound? In Fig. 6c, using non-cleaved probe strands, the Endo III footprint is not all that distinct.

Referee #3 (Remarks to the Author):

Xer is a ubiquitous and functionally versatile site-specific recombination system that is primarily involved in the resolution of bacterial chromosome and plasmid dimers to ensure their proper segregation at the time of cell division. The XerC/D tyrosine recombinases and the chromosome resolution site dif have more recently been found to be embezzled by a variety of mobile genetic elements termed IMEXs (integrative mobile elements exploiting Xer) to promote their integration into the host genome. The different functions of Xer appear to be controlled by specific cellular factors that act on the activation the recombination reaction and its directionality. Controlling the recombination complex activity is further facilitated by the heteromeric structure of the XerCD recombination complex, each of the two recombinases being responsible for the exchange of a specific pair of DNA strands. In the case of the filamentous bacteriophage CTXphi, which encodes the cholera toxin genes of Vibrio cholerae, integrative recombination takes place between the ssDNA form of the phage genome and the dsDNA dif sites of one or the other Vibrio chromosome. The reaction stops after the first DNA strands exchange catalysed by the XerC protomers in the complex, generating a pseudo-Holliday junction intermediate. This intermediate must be stable enough for not being resolved by a second round of recombinase-catalysed strands exchange prior to be processed by replication. Using an elegant genetic screen, Bischerour et al. have identified the host-encoded base excision repair (BER) enzyme Endo III as being a key cellular factor for the stabilization of the pseudo-HJ intermediate. They show that this function of Endo III is independent

from its catalytic activity and its contribution to BER, unveiling a new and possibly more generalized role for the enzyme. They provide in vitro evidence that Endo III specifically binds to four-way DNA junctions and propose a mechanism whereby the protein would stabilize the pseudo-HJ intermediate by inducing a conformational change to displace the recombination complex from its substrate.
Thus, the study establishes a new type of functional link between mobile genetic elements and their host and proposes a novel mechanism to control DNA site specific recombination. This represents a valuable contribution that should interest a broad audience. However specific aspects regarding the way the data are presented and/or discussed in the paper deserve some clarification to make it more convincing.

During CTXphi integration, it is also crucial that the pseudo-HJ product made by XerC would not be processed further by exchanging the second pair of DNA strands either by XerD or by any other cellular HJ resolving process, as this would generate potentially lethal DNA rearrangements. Thus, another obvious function for Endo III would be to protect the cell from such DNA damaging recombination reactions, which seems not to be discussed in the paper. Is there any evidence that CTXphi infection affects the viability or the fitness of the Endo III-minus (nth) mutant of V. cholera more than the wild type?

It is not totally clear whether the authors consider that Endo III binding to the pseudo-HJ DNA competes with the recombinases, promoting their dissociation from the substrate (as suggested by the footprint and protection analyses shown in Fig5 and 6) or whether it 'associates' with the recombination complex (e.g.; P10L18, P13 bottom, P15L20; also discussed P16) to induce a conformational change ('displacement'?) that inactivates the recombinases. How does the dynamics of Endo III binding to the free pseudo-HJ compare to that shown for the XerCD-bound substrate in Fig.5A? Another experiment to determine whether Endo III promotes the complete dissociation of the recombination complex would be to co-incubate the XerCD-bound pseudo-HJ with linear substrates and see whether addition of Endo III induces the relocation of the recombinase proteins from one substrate to the other. Caution should be taken with the interpretation of KMnO4 protection patterns shown in Fig. 6D. It could be well that both XerC and XerD are displaced from the substrate, the thymine in the XerC arm of the junction being just more sensitive to KMnO4 than the thymine in the XerD binding site. The same conclusion holds for the footprints shown in Fig. 6C. Thus the statement that Endo III 'can penetrate the XerCD/pseudo-HJ complex and specifically dislodge XerC'(P13) should be softened (also P5L13 and P15L17).

The statement found in different parts of the paper that 'Endo III inhibits the first step of the strand exchange reaction catalysed by XerC' (e.g., P13L6, P14 bottom and P15L19) is somewhat confusing as it seems to contradict the proposed mechanism in which Endo III would act after formation of the Pseudo-HJ intermediate by XerC to deactivate the recombinases. This mechanism is clearly distinct from that previously proposed for other systems in which auxiliary factors play an architectural role in the assembly and/or activation of the recombination complex. Direct comparison with those systems as discussed P15 is thus misleading.

Additional comments:

Figure 1A and B. According to current models for the tyrosine recombinases pathway, it is difficult to figure out how similar arrangements of the synapse could give alternative orders of strand exchange. Figure 1D. The proposed architecture of the synaptic complex at the cer and psi sites suggesting a parallel inter-wrapping of the accessory sequences is inconsistent with well-established models based on the topology of recombination reaction.

P4. References for the 'propensity of Xer to recycle HJs back to substrate' are from studies on the E. coli Xer/dif system. A more specific reference asserting that this is also true for CTXphi integration should be given as well.

How to conciliate the proposed mechanism of action of Endo III with the observation that its presence appears to inhibit the strand exchange reaction catalysed by XerC without affecting cleavage (P10L12, Fig. 4D)? This observation seems to be contradicted by the experiment performed with the suicide pseudo-HJ substrate shown in Fig. 6B.

P10 bottom. It is disturbing that reference to Fig S5 comes before reference to Fig. S3 (P18) and Fig. S4 (P20).

P11L17. Fig. 6C shows a protection pattern for the Pseudo-HJ, not for the dif1/dif1 HJ.

P12L9. 'When the corresponding oligonucleotide is labelled at its 3'extremity'. The diagrams of Fig. 4D and Fig. 6B suggest that the substrates were labelled at the 5' side of the XerC arm. The author should specify that in this case, cleavage by XerC is expected to give a tyrosyl adduct at the 3' end of the DNA fragment after protease K treatment, which may explain the the detection of 2 (or more) product bands. What are the multiple bands seen in Fig. 4C (bottom gel), since in this case the substrate was labelled at the 3' end of XerC binding site?

P13L11. Refer to Fig. 6C (instead of 6D). P13L16. Refer to Fig. 6A (instead of Fig. 4C).

P15L7. 'Resolution (no 's') sites'

P15. What experimental evidence (reference?) supports the assertion that the HJ intermediates which are formed during Xer recombination of ColEI dimers are resolved by replication? Such a mechanism is expected to leave an unresolved copy of the plasmid dimer after each round of recombination/replication, which is obviously not the case.

P15 bottom. There is no evidence in Fig. 5 that shows that Endo III does not associate with the XerCD/attP(+)/dif synaptic complex.

Fig. S1. It is not clear why the legend given for the triple insertion (lacZ::dif ... LacZ) differs from that of the single and triple insertions.

Legend of Fig. S2. 'The 3'end labelled strand' (no 's').

Legend of Fig. S3. Refer to Fig. 4C (instead of 3C) and to Fig. 4D (instead of 3D). There is no Fig. 7b in the examined version of the MS.

1st Revision - authors' response 26 June 2012

We were pleased that all three referees commented on the general interest of our discovery that the Base Excision Repair enzyme Endo III stimulates *Vibrio cholerae* toxigenic conversion by binding to and stabilizing the pseudo-Holliday junction integration intermediate of phage CTX (referee #1 "*[This activity] would be a first in the "world" of tyrosine recombinases. […] the analysis solves the mystery of the inefficiency of XerC-driven HJ formation and resolution.*"; referee #2 "*[The authors] have identified a factor, Endo III, that promotes the integration of a CTX phage analog into dif*"; referee #3 "*[the results] unveil a new and possibly more generalized role for [Endo III]. [They] establish a novel mechanism to control DNA site-speific recombination. This represents a valuable contribution that should interest a broad audience.*").

We were also pleased that they appreciated the overall quality of our work (referee #1 "*The in vivo and in vitro analyses are thorough […] The experiments are clean and the conclusions well-supported. […] The manuscript was well-written.*"; referee #2 "*[the authors] demonstrate that Endo III can bind to attP and dif sequences in the context of a Holliday junction, but not as simple dsDNA, even when XerCD are not present.*"; referee #3 "*Using an elegant genetic screen, [the authors] have identified […] Endo III as being a key cellular factor for the stabilization of the pseudo-HJ intermediate [of the phage]. […] they provide in vitro evidence […] that establishes a new type of functional link between mobile genetic elements and their host.*").

We found the detailed comments of the referees and of the editor to be very constructive. Much of their remarks dealt with the presentation of the manuscript (referee #3 "*[…] specific aspects regarding the way the data are presented and/or discussed in the paper deserve some clarification to make it more convincing*"**).** We would like to thank the referees for their for these numerous remarks, which helped improve the manuscript. Other than that, three issues were mainly raised, which we summarize below along with our answers.
(i) referee #2 (see referee #2 point 7 and point 12 in the detailed rebuttal) and referee 3 (see referee #3 point 11 in the detailed rebuttal) found difficult to reconcile the results obtained on a suicide pseudo-HJ (fig 6B of the previous manuscript) and these obtained on the normal pseudo-HJ (fig 4D of the previous manuscript).
These data are not contradictory! As stated in the initial version of the manuscript: "*Cleavage of each of the two recombining strands of the pseudo-HJs can be monitored […] (Figure 4D). However, the amount of cleaved DNA detected […] only reflects the <u>equilibrium</u> between cleavage and subsequent re-ligation, whether strands are exchanged or not. To gain information on the efficiency of cleavage of a particular recombining strand, it is therefore necessary to use synthetic suicide pseudo-HJs that abolish any possibility for self re-ligation and/or ligation to the partner recombining strand [Figure 6B].*" However, we now realize that our explanation of the experiments and of their rational was not sufficiently clear to the non-specialist. Indeed, referee #2 felt that "*it might help to show the putative*

stages of the XerCD mediated reaction in greater details within the figures" (see referee #2 point 12a and 12b).
In the present version of the manuscript, we have added a schematic (new Figure 4C), depicting the different stages of the XerC-mediated reaction, which should help non-expert readers follow the experiments. Using Figure 4C as a support, we now clearly formulate why results of Figure 4D (Figure 4E in the new manuscript) cannot be used to estimate the stage at which Endo III inhibits XerC-strand exchanges. We explain why a definitive proof can only be obtained using a suicide pseudo-HJ. In addition, we added a kinetic experiment (Figure S6), in which we observe XerC-cleavage inhibition at early time points of the reaction on a normal pseudo-HJ.
(ii) referee #1 (point 1), referee #2 (point 6-8) and referee #3 (point 4) found the discussion on the mechanism of inhibition of XerC-catalysis by Endo III unclear. In particular, they wanted clarifications on whether Endo III competed with the recombinases for binding to the pseudo-HJ or whether it associated with the complex and induced a small displacement of the recombinases. We have added a schematic (Figure 7), in which we depict these two modes of action and recapitulate the results that sustain them in the discussion. We also state why we favour the second mode of action: first, the order of addition of Endo III and the recombinases would be expected to affect *in vitro* results in the case of competitive binding, which was not the case in the experiments we present. Note also that we found that Endo III-binding kinetics were similar on free pseudo-HJ and on pseudo-HJ preincubated with XerCD (with time points as shirt as 1'). Second, we have added gel shift experiments (Figure S3) demonstrating that *V. cholerae* XerC-binding to DNA is inefficient and that binding relies on cooperative interactions with XerD, as previously observed with the *E. coli* Xer recombinases (see Sherratt's lab results for *dif*, Val *et al*. 2005 for *att*P(+)). Therefore, any conformational change of the XerCD/pseudo-HJ complex is likely to lead to the dissociation of the XerC recombinases before any dissociation of XerD, which fits with the stronger effect of the addition of Endo III on the XerC-footprint and protection results compared to the XerD-footprint and protection results.
(iii) referee #1 (point 2) and referee #3 (point 1) asked for a clarification of the role of the catalytic activity of XerD in the integration of the phage. This point was answered by previous work from our team (Val et al. 2005; Das et al. 2010 and 2011): XerD-catalytic activity is not required. Moreover, XerD cleavage wouldn't lead to any strand exchanges because of the lack of homology on the XerD-side of the central region of *att*P and *dif*. This is why we didn't discuss any possible effect of Endo III on protecting the pseudo-HJ from a resolution event by XerD (which would lead to a chromosome lethal rearrangement, as rightly indicated by referee #3). In the new version of the manuscript, we now clearly state in the introduction that the pseudo-HJ cannot be processed by the catalytic activity of XerD.

We would like to thank again the referees and the editor for their careful and in-depth comments, which allowed us to considerably improve our work. We sincerely believe that the revised version of our manuscript is now suitable for publication in the EMBO Journal.

Detailed point by point rebuttal

Answer to the Editor comments:

Point 1. "*please crefully edit the manuscript text to remove [...] spelling mistakes and inappropriate word usage [...].* "

We have taken into consideration all the points raised by the editor and the 3 referees. The final manuscript has been checked by a native speaker of english.

Point 2. "*please also carefully revise the reference lis [... and] citation format*"

A bug in the automatic formatting software was found and fixed. The reference list and citation format have been carefully revised manually.

Point 3. "*we will need a brief Conflict of Interest statement*. "

A conflict of interest statement has been added at the end of the manuscript text (p23, below the Authors Contributions statement):

> "**Conflicts of Interest**
> The authors declare that there are no competing commercial interests in relation to the submitted work. "

Point 4. "*the manuscript title coul be changed to more explicitly make clear [...] the novel molecular role of EndoIII*"

Following the editor advice, the manuscript title has been changed to:

> "The affinity of Endo III for Holliday junctions contributes to cholera toxin phage integration".

Answer to the Referee #1 comments:

Point 1. "***[Nth blocks XerC-mediated cleavage], probably by competing with XerC binding. This is actually quite surprising, suggesting that the likely tetramer of (XerC)2(XerD)2 is disrupted by Nth on the HJ - does Nth "kick" off the whole tetramer, or just the XerC dimer? It wasn't 100% clear, though the XerD footprint is weaker in the presence of Nth (Fig 6 panel C lane 8 vs lane 4).***"

We have added a model figure (Figure 7) and now explicitly discuss the mode of action of Endo III in the manuscript on p17:

> " How Endo III achieves XerC-cleavage inhibition also remains to be more extensively explored. Enzymatic DNA footprints suggest displacement of the XerC recombinases from their specific DNA binding sequences on the pseudo-HJ (Figure 5 and 6). However, pre-incubation of pseudo-HJs with Endo III before the addition of the Xer recombinases did not potentiate resolution inhibition (Figure 4D, lanes 3 and 4), indicating that Endo III does not directly compete with the recombinases for DNA binding (Figure 7, arrow 1). Therefore, we favour the idea (Figure 7, arrow 2) that Endo III can bind to pseudo-HJs covered by the Xer recombinases (as suggested by Figure 5A), induce DNA structures changes in the central region of the pseudo-HJs (as suggested by Figure 5E and 6D), which eventually inhibits the catalysis of the recombinases and promotes their dissociation. As XerC displays a much weaker affinity for *dif* and *att*P than XerD (Figure S3), the primary effect of Endo III binding would be the loss of XerC-catalysis (as suggested by Figure 4) and its subsequent dissociation (as suggested by Figure 5 and 6)"

Point 2. "***Is XerD catalytic activity actually required for CTXphi integration?***"

XerD catalytic activity is not required for CTXphi integration {Val, 2008 #591}. It only plays a structural role, to form the $(XerC)_2(XerD)_2$/*dif*/*att*P nucleoprotein complex. More importantly, the lack of sequence homology in the overlap regions of *att*P(+) and *dif* at the bases close to the XerD cleavage site compromises any potential strand exchange (see {Das, 2011 #772; Das, 2010 #680}, for a demonstration of the importance of pairing homology in the central regions of the recombining sites in

the case of CTX integration).
The role of XerD in the integration of CTX has been clarified in the introduction of the manuscript on p4:

> "Lysogenic conversion results from the exchange of a single pair of strands between attP(+) and the dif site of one or the other of the two circular chromosomes of V. cholerae, which is catalysed by the XerC recombinases {Val, 2008 #591}. Resolution of the resulting pseudo-HJ by a second pair of strand exchanges would lead to the formation of a lethal linear covalently-closed dsDNA chromosome {Bouvier, 2005 #467}. However, the lack of homology in the overlap regions of attP(+) and dif next to the XerD cleavage sites prevents any potential XerD-meditated strand exchange {Das, 2011 #772; Das, 2010 #680}. Correspondingly, CTXϕ-integration does not depend on the catalytic activity of the XerD recombinases, which only play a structural role in the formation of the synaptic complex {Val, 2008 #591}. The pseudo-HJ is thought to be converted into product by replication (Figure 1C; {Das, 2010 #680;Val, 2005 #426}). "

Point 3. "***p 7, 2nd line from bottom: reasoned; p 8, 5th line from top: restored; p 8, 2nd line from bottom: transferred; substitute "idea" with "possibility" p 12; 3rd line from bottom: reasoned. p 14, 6th line from top: led; p16, 3rd line from bottom: exchanges; p 17, middle paragraph: replace "So far" with "To date"; later in the paragraph, discriminate; next line, scenarios.***"
These different typos/spelling errors have all been dealt with.

<u>Answer to the Referee #2 comments:</u>

Point 1. "***P5, also p14. Although there is evidence that the catalytic activity of Endo III is not absolutely required for enhancing CTXphage integration, catalytic activity clearly has an effect. The statement that "the catalytic activity was not implicated" is misleading".***
The K120Q mutation we introduced into *V. cholerae* Endo III completely abolishes the DNA glycosylase and AP lyase activities of the enzyme {Thayer, 1995 #792}. As production of the K120Q mutant can still stimulate CTXϕ integration *in vivo* (Figure 3B) and as the mutated protein can still stabilize the pseudo-HJ intermediate of the phage *in vitro* (Figure S2), our conclusion that the catalytic activity of Endo III is dispensable for the role it plays in CTXϕ integration is fully justified. In addition, if the catalytic activity of Endo III participated in the enhancement in CTXϕ integration, we would have expected mutations in the *xthA* and *nfo* to diminish the efficiency of integration of the phage *in vivo,* since these enzymes are required to process the 3' unsaturated aldehydic ends left by the enzyme. This was clearly not the case (Figure 3A). Furthermore, we did not detect any DNA glycosylase and AP lyase activity of Endo III on *att*P, *dif* or the pseudo-HJ integration intermediate in our *in vitro* sequencing gels (Figure 4, 5 and 6). Therefore, we are confident that the catalytic activity of Endo III does not participate in the stimulation of CTXphage integration. Note that the other two referees fully agreed with our conclusions (referee #1 "*This activity [...] does not require the catalytic activity of Nth*"; referee #3 "*They show that this function of Endo III is independent from its catalytic activity*").
As stated by referee #2, we did observe that the introduction of the K120Q mutation decreased the extent of the stimulation of CTXϕ integration *in vivo* (~3 fold reduction in the stimulation) and the extent of the stabilization of the pseudo-HJ *in vitro*. However, it is frequent for mutations in the catalytic domain of enzymes to diminish their affinity for the substrate. Therefore, one should always be very cautious when comparing the efficiency of different forms of proteins. In the case of Endo III, a lower affinity for DNA would be expected to affect the interaction with the XerCD/pseudo-HJ complex, which would explain a loss in activity. In agreement with this explanation, we observed that that the K120Q form *V. cholerae* Endo III was eluted at a much lower salt concentration from heparin (which is used as a DNA mimick in affinity chromatography purification).
Nevertheless, we agree with referee #2 that the term "implicated" was too vague to be scientifically appropriate. In consequence, we have edited the manuscript and replaced the "*the catalytic activity*

was not implicated" by "the catalytic activity was not required", according to the formulation of referee #1.

Point 2a. "***P7 (also Fig. 3 legend). The authors should clarify that although most exconjugants were at least partially blue (based on Fig. 2), the frequency of integration was calculated based solely on the percentage of white colonies. Thus although colonies may have been scored at 14 hrs, they are not really monitoring the 'frequency of integration events 14 hr after conjugation' but rather the frequency with which integration had already occurred at the time at which selection began.***"
We now clearly state in the legend of Figure 3 (p28) that

> "Conjugants were grown overnight (14hours) at 37°C in rich media supplemented with chloramphenicol. The proportion of fully white colonies (lysogenics) over the total number of chloramphenicol resistant clones was used to calculate the frequency of integrants. Mean and standard deviation of at least 3 independent experiments."

To further clarify this point, we now refer to "Relative frequency of RS1 integrants after 14h (%)" in Figure 3A and "Frequency of RS1 integrants after 14h (%)" in Figure 3B.

Point 2b. "***The percentages shown in Fig. 3 don't match all that well with the images in Fig. 2 (ie white colonies are rare); were they monitored under different conditions?***"
Different conditions were used in Figure 2 and Figure 3. The pictures presented in the case of Figure 2 correspond to cells plated directly after conjugation. This is the condition that we used for our screening. The purpose of the pictures was to show (i) the 'fully blue' and 'almost fully blue' phenotypes that were selected during the screen and (ii) the reproducibility of the phenotype.
This is now precised in the Figure 2 legend (928):

> "Cells were plated immediately after conjugation to mimick the condition of the screen."

We also precise on the figure itself that the pictures illustrate the "Phenotype after direct plating".
In contrast, results of Figure 3 correspond to the number of integrants obtained after 14h of liquid growth. This is now clearly stated in the figure legend and in the figure itself (see referee #2 point 2a). The purpose of this second method was to give quantitative results.

Point 3. "***P7. There is clearly a lower average copy # of integrated RS1 in the endo III mutant; however, it is not at all clear how the figure of 36% single RS1 was arrived at. It would be preferable to acknowledge that there is extensive heterogeneity in array size for an individual nth enconjugant colony (shown in Fig. S2).***"
We now precise in the figure legend how the proportion of single RS1 integration events was calculated:

> "The signals corresponding to a single integrated copy in the 8 colonies that were analysed were added. The signals corresponding to double integrated copies in the 8 colonies that were analysed were added. The signals corresponding to triple integrated copies in the 8 colonies that were analysed were added. The fraction of the genomes containing 1, 2 and 3 copies were obtained by dividing these numbers by the total signal detected. Fractions are indicated at the bottom of the gels."

Point 4a. "***P8. As above, the effect of the K120Q mutation is somewhat ambiguous. Complementation with the K120Q plasmid is rather poor (Fig. 3b), and its utility in in vitro assays is far from wt (Fig. S2; claims for "the same in vitro activities" on p9 are misleading).***"
The formulation"*the same in vitro activities*" referred to the comparison of *V. cholerae* Endo III and *E. coli* Endo III. We agree that adding a reference to K120Q in the same sentence could be misleading. In the new version of the text, we now separate the two points into two separate sentences and precise that K120Q is less efficient than the wild-type version of the protein (p11):

> "The same *in vitro* activities were observed with purified *V. cholerae* Endo III (Figure S2). Similarly, the K120Q mutant form of the *V. cholerae* protein was able to stabilize the pseudo-HJ integration intermediate of the phage *in vitro*, albeit with a lower efficiency

(Figure S2). Taken together, these results suggest that Endo III facilitates toxigenic conversion by stabilizing the pseudo-HJ integration intermediate of CTXϕ."

As explained above (referee #2 point1), our conclusion that the catalytic activity of Endo III is dispensable for the role it plays in CTXϕ integration is fully justified despite the lower stabilization efficiency of the K120Q mutant.

Point 4b. "***It would be good to assess integration frequency in this background.***"
We did test the action of the K120Q mutant in RS1 integration. See Figure 3B and our answer to referee #1 point 1.

Point 5. "***P10, Fig. 5A. The meaning of Fig. 5a is not clear; 5b is sufficient to show binding of HJ by Endo III. Given that Endo III alone can shift HJs to the well, the shift seen in 5a does not necessarily reflect simultaneous binding by Endo III and XerCD, as is implied by the "HJ-intermediate covered...XerC "(also claimed on p11; "even when they are covered"). To visualize competition between XerCD and Endo III, it would be better to add increasing amts of XerCD and see if they redirect the HJ from the well to a faster migrating complex, as seen with XerCD alone bound. If 5a is maintained, the concentration gradient of Endo III should be defined.***"
The aim of Figure 5A was neither to show the simultaneous binding of Endo III and XerC/XerD to the pseudo-HJ nor to visualize their potential competition for binding to it. Its purpose was to show that Endo III can interact with the pseudo-HJ/XerCD complex and/or a pseudo-HJ pre-covered by XerC and XerD. To further clarify this point, the description of the results has been changed to (p11):

" Stabilization of the attP/dif1 pseudo-HJ by Endo III suggested that the protein could either specifically interact with the Xer recombinases when engaged on a pseudo-HJ or with the pseudo-HJ itself, even if it should be partially masked by the recombinases. In both cases, we expected Endo III to be able to target the XerCD/pseudo-HJ complex. To test this possibility, we reconstituted XerCD/pseudo-HJ complexes by preincubating radioactively labelled pseudo-HJs with an excess amount of XerC and XerD. Conditions for full coverage of the pseudo-HJs were determined based on the conditions required for full coverage of attP and dif1 dsDNA substrates (Figure S3). The XerCD/pseudo-HJ complex was visualized as a single retarded band in a gel shift assay (Figure 5A, 2nd lane). Addition of Endo III led to the loss of the XerCD/pseudo-HJ band and the progressive accumulation of a very slow migrating complex (Figure 5A, lanes 3-4). In contrast, Endo III did not affect the migration of XerCD/attP(+) or XerCD/dif1 nucleoprotein complexes (Figure S4). Taken together, these results indicate that Endo III can specifically interact with the XerCD/pseudo-HJ complex."

Even though we show in Figure 5B that Endo III binds to 4-way DNA junctions, it is important to present Figure 5A because the presence of XerCD on the pseudo-HJ could have prevented Endo III from binding to it. To clarify this point, we now stress at the end of this result section (p13) that:

"Taken together, these results demonstrate that Endo III can bind 4-way DNA junctions and modify the structure around their branch point, even if these junctions are initially covered by XerC and XerD."

Note that neither referee #1 nor #3 disputed the interest of Figure 5A.

Point 6. "***Overall, p10/11 seem to imply simultaneous binding of XerCD and Endo III, which is confusing and conflicts with data presented later in the paper, eg. 5e, 6d).***"
The point of Figure 5E was to confirm that Endo III can bind 4-way DNA junctions even when they are covered by XerC and XerD. To clarify the aim of the figure, the corresponding paragraph was cut in two. In part 1, we describe the T7 endo I protection result that further demonstrate Endo III binding to 4-way junctions. Part 1 directly follows the previous paragraph on the gel shift experiments (p12):

"[...] DNA forks did not compete against pseudo-HJs (Figure 5B, 6th lane), suggesting preferential association to 4-way DNA junctions in general. Indeed, Endo III retarded the migration of a HJ with arms unrelated to *dif*1 or *attP*(+) (Figure S5).

Binding of Endo III to 4-way DNA junctions was confirmed by a protection experiment against T7 Endo I. Incubation of *att*P(+)/*dif*1 pseudo-HJs with T7 Endo I resulted in

specific cleavages at their branch point (Figure 5D, 2nd lane), which was repressed in the presence of Endo III (Figure 5D, 3rd lane)".

In part 2, we describe the KMnO4 protection results obtained in the presence of XerC and XerD. To clarify the aim of the experiment, we introduce the experiments as follows (p12):

"Because the bases at the branch point of *dif*1/*dif*1 HJs or *att*P(+)/*dif*1 pseudo-HJs are protected from nucleases by the binding of XerC and XerD (see {Arciszewska, 1997 #6;Arciszewska, 1995 #654} and Figure 6C, respectively), we wondered if the capacity of Endo III to bind 4-way DNA junctions was sufficient to explain its ability to bind pseudo-HJs covered by excess amounts of XerC and XerD (Figure 5A). Addition of Endo III increased the exposure of the bases at the branch point of *att*P(+)/*dif*1 pseudo-HJs to $KMnO_4$, a chemical agent that preferentially attacks unstacked thymines (Figure 5E, 5th lane). In contrast, binding of XerC and XerD to pseudo-HJs did not alter the chemical modification pattern of $KMnO_4$ (Figure 5E, 2nd lane). Even though the samples were incubated at 4°C, XerC-catalysis was observed (Figure 5E, #), demonstrating efficient binding of the recombinases. Nevertheless, XerCD-binding did not prevent Endo III from unstacking the bases at the junction of the four arms of the pseudo-HJ (Figure 5E, 3rd lane)."

Finally, at the end of this section, we stress that (See referee #2 point 5):

"Taken together, these results demonstrate that Endo III can bind 4-way DNA junctions and modify the structure around their branch point, even if these junctions are initially covered by XerC and XerD."

Point 7. "***Can the authors more clearly reconcile the data presented in Fig. 4D and 6B? It is not clear why a reduction in cleavage frequency is not also observed in Fig. 4D in the presence of Endo III, if this is the process inhibited by this enz. If Endo III competes with XerC for binding at HJ, then why is less cleavage not see in the presence of EndoIII, regardless of whether or not re-ligation of the sequences can occur?***"

The amount of cleaved oligonucleotide in the wild-type pseudo-HJ resolution assay cannot be used as measure of the efficiency of the cleavage reaction because it only reflects the equilibrium between cleavage and re-ligation. Therefore, any decrease in the efficiency of cleavage could be masked by a similar decrease in the efficiency of re-ligation.

This is now clearly stated in the text, with reference to the new Figure 4C, in which we present a scheme of the 3 steps of the XerC-mediated reaction (p13):

"**Endo III-binding blocks XerC-catalysis after HJ formation.**

We next investigated which step of the strand exchange reaction catalysed by XerC was prevented by the binding of Endo III to the XerCD/pseudo-HJ complex (Figure 4C, from right to left): (i) XerC-mediated cleavage of the recombining strands of the pseudo-HJ (strand I and strand III of Figure 5C); (ii) exchange of the liberated 5'-hydroxyl extremities; (iii) religation to the partner recombining strand. By labelling one of the two recombining strands at its 5' extremity, we can detect the amount of cleaved strand that is reached after a given time (Figure 4E). However, it only reflects the equilibrium between cleavage and re-ligation, whether strand exchange has occurred or not. Therefore, any decrease in the efficiency of cleavage (Figure 4C, (ii)) could be masked by a similar decrease in the efficiency of re-ligation (Figure 4C, (iii))."

We then explain how the use of a suicide pseudo-HJ allowed us to correctly address the issue (p14):

"We circumvented this difficulty by using synthetic suicide pseudo-HJs that abolish any possibility for self re-ligation and/or ligation to the partner recombining strand."

The notion that results with wild-type substrates only inform us about the equilibrium between cleavage and re-ligation is stressed in the legend of the new Figure 4D (p29).

"**(D)** *In vitro* equilibrium reached after 4h of incubation of a short radioactively labelled *dif*1 substrate and a longer cold *att*P(+) substrate with the Xer recombinases."

Another possibility would have been to ensure that the cleavage/re-ligation reactions were not at an equilibrium. To this end, we have now repeated the wild-type and suicide pseudo-HJ resolution assays in a kinetic experiment (Figure S6). This is now referred to as follows (p14):

"Addition of Endo III led to a further diminution in the amount of detected strand exchanges, as expected (Figure 6B, top gel, 3rd lane). Moreover, it almost completely blocked the accumulation of cleaved fragments (Figure 6B, bottom gel, 2nd lane), indicating that Endo III inhibits the first step of the strand exchange catalysed by XerC. In agreement with this result, a lower amount of cleaved product was also detected in the presence of Endo III at early time points, i.e. at a time when the equilibrium between cleavage and re-ligation had less chances of being reach, with the non-suicide version of the *attP*(+)/*dif* pseudo-HJ (Figure S6)."

Point 8. "***P13. Is it thought that XerD can remain bound in the absence of XerC, or is their binding thought to be interdependent, and if so, can this be related to the effect of Endo III binding? Although the authors conclude that Endo III "specifically dislodges" XerC, this does not seem to be the case in Fig. 6C (right) in which loss of protection by XerD is also apparent in the presence of Endo III. XerC site hypersensitivity (6d) is significantly greater than at the XerD site; this may account for its detection (and not that of the XerD site) even in the presence of XerCD in 6D.***"
In the conditions of the assay (10nM substrate, 100nM XerD and 150nM XerC), XerD binds independently of XerC, but the binding of XerC is highly dependent of XerD (Figure S3). Therefore, we do expect primary dissociation of XerC compared to XerD. Although the XerD site hypersensitivity is weaker than the XerC site, we are confident that there is little or no effect of Endo III on the binding of XerD (Figure 6D). We have added DNAseI footprint on strand III (FigureS7), which along the footprint on strand IV is particularly explicit. However, we do agree that the adverb "specifically" in "*Endo III specifically dislodges XerC*" was misleading. It has been removed (p15):

"Taken together, these results suggest that Endo III can penetrate the XerCD/pseudo-HJ complex and can dislodge XerC."

In addition, we now discuss explicitly the mechanism of action of Endo III, with the addition of a model figure (Figure 7). See referee #1 point 1.

Point 9. "***P18. The conclusion that Endo III binds to HJ in general should be backed up by looking at binding to HJs with sequences that are unrelated to either dif or attP; the two HJ tested are too similar to warrant such a broad conclusion. Demonstration of HJ binding that is not sequence specific could also make the paper of broader interest.***"
We now have repeated HJ-binding experiments with sequences unrelated to *dif* and *att*P (Figure S5). This is now referred to in the text as (p12):

"[...] DNA forks did not compete against pseudo-HJs (Figure 5B, 6th lane), suggesting preferential association to 4-way DNA junctions in general. Indeed, Endo III retarded the migration of a HJ with arms unrelated to *dif*1 or *attP*(+) (Figure S5)."

Point 10: "***Abstract: [preventing Xer recombination cycles] relies on the ability of Endo III to bind...even in the absence of the recombinases. While Endo III can bind w/o XerCD, I'm not sure the authors have shown this capacity is required for its effect.***"
We agree with the referee #2. The awkward "even in the absence of the recombinases" has been deleted from the abstract:

"*Vibrio cholerae* toxigenic conversion results from the integration of a filamentous phage, CTXϕ. Integration is driven by the Xer recombinases of the bacterium, which catalyse the exchange of a single pair of strands between the phage single stranded DNA and the host double stranded DNA genomes. The resulting pseudo-Holliday junction is thought to be converted into product by replication. The natural tendency of the Xer recombinases to recycle back Holliday junction intermediates into substrate should thwart this integration strategy, which prompted us to search for missing co-factors. Here, we show that Endo III, a ubiquitous Base Excision Repair enzyme, promotes CTXϕ-integration *in vivo*. *In vitro*, we show that it prevents futile Xer recombination cycles by impeding new rounds of strand exchanges once the pseudo-Holliday junction is formed. We further demonstrate that this activity relies on the ability of Endo III to bind to Holliday junctions, which was so far

unsuspected. Our results explain how tandem copies of the phage genome are created, which is crucial for subsequent virion production."

Point 11. ***Typos. "'reasoned' has only a single n; it is misspelled throughout. Also, 'appearance' might be a better word choice than 'apparition'. "***
See Editor point 1.

Point 12a. "***Figure legends could benefit from inclusion of more descriptive detail, particularly the molecular assays. Alternatively it might help to show the putative stages of the XerCD mediated reaction in greater detail within the figures, in order to clarify how various products are generated. This might also help avoid the suggestion that oligo complexes (e.g., HJ) remain intact within the gels, which might be inferred from the depiction of such complexes adjacent to particular bands.***"
Adding a scheme depicting the reactions for each assay would have considerably increased the size of figures. However, we agree with referee #2 that a more descriptive detail if the reaction was necessary for the broad redership of the EMBO J. To this end, we now have added a scheme of the reaction catalysed by XerC in new Figure 4C and describe the reaction in greater detail in the result section (p9):

"The Xer recombination reaction leading to the formation of *att*P(+)/*dif* pseudo-HJs can be reconstituted *in vitro* using purified *V. cholerae* XerC and XerD recombinases and annealed oligonucleotides mimicking the attachment site of the different CTXϕ variants and their cognate chromosomal target {Das, 2010 #680}. Three steps can be defined in the process (Figure 4C): (i) a single strand in each of the two recombining sites is cleaved by the XerC recombinases engaged in the synaptic complex, which generates two 3'-phosphorotyrosyl recombinase/DNA covalent intermediates; (ii) the liberated 5'-hydroxyl extremities are exchanged; (iii) they attack the phosphorotyrosyl bond of the partner site to form phosphodiester bonds. Each of these steps is reversible, leading to an equilibrium (Figure 4C)."

Point 12b. "***It should also be noted that dotted lines represent unlabeled oligos that are included within the assays.***"
This is a very good suggestion. We now explain in the legend of the new Figure 4D and 4E that dotted lines represent non observable strands. We also explain why samples were treated with proteinase K and why we used denaturing gels (p29):

"**(D)** *In vitro* equilibrium reached after 4h of incubation of a short radioactively labelled *dif*1 substrate and a longer cold *att*P(+) substrate with the Xer recombinases. The shorter migration product reveals cleavage of the recombining *dif*1 strand. The longer migration product reveals formation of the *att*P/*dif* pseudo-HJ formation. Unlabelled oligonucleotides cannot be directly visualize and do not interfere with the migration of the labelled fragments in the denaturing gel. Schemes of substrate and products are drawn on the left of the gels. A star indicates the position of the radioactive label. Unlabelled oligonucleotides are represented as dashed lines. **(E)** Cleaved and resolved products obtained after 1h of incubation of labelled *att*P(+)/*dif* pseudo-HJs with the Xer recombinases. Samples were treated with proteinase K to avoid any interference of the covalent 3'-phosphotyrosyl bonds between XerC and the cleaved fragments in the oligonucleotide migration. 1st and 2nd indicate the order of addition of Endo III and of the Xer recombinases. Legend as in Figure 4D."

Point 13. "***P7. The authors note that xerD mutants were not obtained in their screen; can they supply a likely explanation?***"
We have no explanation other than that the screen was not extensive enough: we have made a XerD null mutant. There is no integration of the phage in it!

Point 14. "***Although it is possible that the reduced array size in the nth mutant reflects the reduced integration rate, it is also possible that smaller arrays reflect increased repression of replicative RS1 after a single integration event. The authors have a nice control, using non-replicative attP (Fig 4b), to demonstrate that nth deletion alters integration even in the absence of replication, so this caveat does not apply to their overall conclusion; however, they should be cautious with their conclusions regarding array size.***"
We now use the most cautious formulation (p18):

> " To date, it was unknown how these tandem copies were generated. They could result from a single integration event, the phage DNA being duplicated by an uncharacterized DNA repair and/or replication event, or from multiple successive integration events (Figure 1C). Our observation that the number of integrated copies was reduced in Δ*nth* cells compared to *nth*+ cells (Figure S1) is in favour of the latter scenario since the average number of integrated copies should have been independent of the efficiency of integration if the former hypothesis had been correct."

Point 15. "***Fig 4a is probably not necessary.***"
We disagree. This figure is important for the broad readership of EMBO J.

Pont 16. "***P9. A reference regarding El Tor and classical phage variants should be supplied, and "data not shown" noted; it is not clear what the authors mean here when they state that 'different processes' govern rolling circle replication in El Tor and classical CTX phage.*** "
Dealt with. The corrected sentence now reads (p9):

> "Finally, deletion of *nth* also reduced the integration of the replication and integration region of two CTXϕ variants, El Tor and Classical, in which production of the RstA relaxase is governed by different regulators (data not shown; {Safa, 2009 #845})."

Point 17. "***Fig 4. Does 1st, 2nd refer to order of addition to the reaction? Should be noted in legend***"
Dealt with. The legend no reads (p29):

> "**(E)** Cleaved and resolved products obtained after 1h of incubation of labelled *att*P(+)/*dif* pseudo-HJs with the Xer recombinases. 1st and 2nd indicate the order of addition of EndoIII and of the Xer recombinases. Legend as in Figure 4D."

Point 18. "***Would it be feasible/useful to define the Endo III binding site using footprinting and DNA strands that are cleaved by XerC, to get a better sense of what part of the HJ structure is bound? In Fig. 6c, using non-cleaved probe strands, the Endo III footprint is not all that distinct.***"
We spent time and energy to map more precisely the EndoIII binding on the pseudo-HJ but did not observe any specific footprint. We believe that the general DNA binding properties of Endo III probably hide more specific DNA binding properties. However, T7 endonuclease I experiment demonstrated that Endo III protects the central region of the pseudo-HJ. In the new version, we added a footprint study on strand III of the pseudo-HJ, the strand that is cleaved by XerC. It gave similar results than the footprints on strand II and IV. This experiment is now added as (Figure S7).

<u>Answer to the Referee #3 comments:</u>

Point 1. "***During CTXphi integration, it is also crucial that the pseudo-HJ product made by XerC would not be processed further by exchanging the second pair of DNA strands either by XerD or by any other cellular HJ resolving process, as this would generate potentially lethal DNA rearrangements. Thus, another obvious function for Endo III would be to protect the cell from such DNA damaging recombination reactions, which seems not to be discussed in the paper.***"
Referee #3 is right to say that a second pair of strand exchange would generate lethal DNA rearrangements, as extensively discussed for the integration of integron cassettes - a process which is

also catalysed by a tyrosine recombinase {Bouvier, 2005 #467}. In the case of CTXϕ-integration, however, no second air of strand exchange can be catalysed by the XerD recombinases engaged in the synaptic complex because of the lack of homologies in the overlap regions of *att*P(+) and *dif* next to the XerD cleavage sites. This is why we didn't discuss the possibility that Endo III might protect the cell from deadly resolution of the pseudo-HJ by XerD-catalysis. We agree that this might not be clear to the non specialist and now more explicitly detail in the introduction why the recombination reaction cannot proceed further than pseudo-HJ formation (p4):

> "Lysogenic conversion results from the exchange of a single pair of strands between *att*P(+) and the *dif* site of one or the other of the two circular chromosomes of *V. cholerae,* which is catalysed by the XerC recombinases {Val, 2008 #591}. Resolution of the resulting pseudo-HJ by a second pair of strand exchanges would lead to the formation of dead-end linear covalently closed molecules, as in the case of integron cassette insertions {Bouvier, 2005 #467}. In the case of CTXϕ-integration, the lack of homology in the overlap regions of *att*P(+) and *dif* next to the XerD cleavage sites prevents any potential XerD-meditated strand exchange {Das, 2011 #772; Das, 2010 #680}. Correspondingly, CTXϕ-integration does not depend on the catalytic activity of the XerD recombinases, which only play a structural role for the formation of the synaptic complex {Val, 2008 #591}. The pseudo-HJ is thought to be converted into product by replication (Figure 1C; {Das, 2010 #680;Val, 2005 #426})."

A role for Endo III in preventing the resolution of the pseudo-HJ integration intermediate by other cellular resolvases is far fetched as it would imply (i) the removal of XerCD by the cellular resolvases, and (ii) blocking of the resolvase activity by Endo III. Our in vitro experiments demonstrate that Endo III is able to stabilize the pseudo-HJ by itself. Therefore, the most parcimonious explanation for the effect of Endo III in CTXϕ-integration *in vivo* is direct interference with the XerCD/pseudo-HJ complex. However, we agree with referee # 3 that future work should explore the possibility that Endo III serves to control the resolution of other 4-way junctions in the cell. This is alluded to at the end of the discussion (p20):

> "Finally, our observation that Endo III, which is shared by most organisms, including humans {Aspinwall, 1997 #777;Denver, 2003 #793}, binds to HJs in general (Figure S5) provides a direct connection between BER and recombinational repair. Such a connection might not be fortuitous since homologous recombination and BER are both required for fork progression through alkylated DNA {Vazquez, 2008 #822}. Thus, it is tempting to propose that Endo III might participate in the balance between the different processes that take care of HJs in the cell {Wu, 2003 #808;Chen, 2001 #810;Taylor, 2008 #821;Svendsen, 2009 #811;Fekairi, 2009 #812;Andersen, 2009 #813;Ip, 2008 #809;Wechsler, 2012 #807}."

Point 2. "*Is there any evidence that CTXphi infection affects the viability or the fitness of the Endo III-minus (nth) mutant of V. cholera more than the wild type?*"

When monitoring the efficiency of integration of RS1 (Figure 3A), we plate cells after 14h of growth in liquid and also right after conjugation. This allows us to calculate a number of generations. We did not observe any difference in the number of generations reached by a nth- strain compared to a wild type strain. When performing the complementation assay (Figure 3B), we conjugate the cells in the absence of arabinose. The same conjugation is then diluted in two, one is grown with arabinose and the other without (to induce Endo III production). We did not observe any change in the number of divisions that could be reached when Endo III was produced or not. Taken together, these results indicate that RS1 propagation (and integration) does not affect the viability of nth- cells.

Finally, our experiments with the non replicative plasmid carrying only attP(+) demonstrate that Endo III plays a direct role in the integration process itself. Therefore, we do not think that it is necessary to extensively discuss the possibility. Nevertheless, we now mention in the results section (p7) that:

> "**Endo III facilitates RS1-integration.**
>
> We isolated 5 independent clones in which transposition disrupted the *nth* ORF, the gene encoding for Endo III (Table S1). To confirm this observation, we engineered a complete deletion of the *nth* ORF in the *lacZ::dif*1 reporter strain. We did not observe any defect of proliferation of the Δ*nth* cells after conjugation of the R6K suicide vector harbouring the RS1 replication and integration region. However, the conjugants yielded colonies with small

white sectors at the periphery of a large blue heart (Figure 2B). In addition, the number of integrated RS1 copies was smaller in the absence of Endo III: 36% of Δ*nth* lysogens carried a single integrated RS1 copy whereas wild-type lysogens always harboured 2 or more copies of it (Figure S1). In order to gain a quantitative measure of the integration defect of Δ*nth* cells, we compared the number of integrants 14 hours after conjugation (Figure 3). Deletion of *nth* led to a 5-fold reduction in the integration efficiency of RS1 at this time (Figure 3A). Integration was fully restored when Endo III was produced from an ectopic plasmid under the control of the arabinose promoter, excluding any polar effect of the deletion (Figure 3B)."

Point 3. "***It is not totally clear whether the authors consider that Endo III binding to the pseudo-HJ DNA competes with the recombinases, promoting their dissociation from the substrate (as suggested by the footprint and protection analyses shown in Fig5 and 6) or whether it 'associates' with the recombination complex (e.g.; P10L18, P13 bottom, P15L20; also discussed P16) to induce a conformational change ('displacement'?) that inactivates the recombinases.*** "
We have added a model figure (Figure 7) and now more explicitly detail the mechanism of action of Endo III. See referee #1 point 1.

Point 4a. "***How does the dynamics of Endo III binding to the free pseudo-HJ compare to that shown for the XerCD-bound substrate in Fig.5A?***"
In both cases, kinetics of binding of Endo III are in the order of a few seconds (<1').

Point 4b. "***Another experiment to determine whether Endo III promotes the complete dissociation of the recombination complex would be to co-incubate the XerCD-bound pseudo-HJ with linear substrates and see whether addition of Endo III induces the relocation of the recombinase proteins from one substrate to the other.***"
This is a very smart experiment but practically difficult: we perform the experiments in the presence of an excess of XerC and XerD (to ensure full coverage of the pseudo-HJ). Consequently, any additional *dif* or *attP* sites should be covered with the recombinases. In addition, XerC depends on XerD for binding to *dif* or *att*P (see Figure S3). Therefore, we wouldn't be able to detect XerC dissociation before the dissociation of its partner XerD recombinase.

Point 5. "***Caution should be taken with the interpretation of KMnO4 protection patterns shown in Fig. 6D. It could be well that both XerC and XerD are displaced from the substrate, the thymine in the XerC arm of the junction being just more sensitive to KMnO4 than the thymine in the XerD binding site. The same conclusion holds for the footprints shown in Fig. 6C. Thus the statement that Endo III 'can penetrate the XerCD/pseudo-HJ complex and specifically dislodge XerC'(P13) should be softened (also P5L13 and P15L17).***"
We do believe the signals are clear. We have added a new Dnase I footprint assay on Strand III (Figure S7). However, we do agree that the adverb "specifically" was an overstatement. It has been removed. See referee #2 point 8.

Point 6. "***The statement found in different parts of the paper that 'Endo III inhibits the first step of the strand exchange reaction catalysed by XerC' (e.g., P13L6, P14 bottom and P15L19) is somewhat confusing as it seems to contradict the proposed mechanism in which Endo III would act after formation of the Pseudo-HJ intermediate by XerC to deactivate the recombinases.***"
Thanks for making us realize that this might have been unclear. This has been corrected in the new version of the manuscript, notably with the addition of a scheme of the XerC-mediated strand exchange reaction (Figure 4C) and with a model figure (Figure 7). In the discussion, we now write (p16):

"[...] Endo III didn't promote formation of the pseudo-HJ integration intermediate but rather stabilized it by impeding new rounds of strand exchanges by XerC-catalysis once it had been formed (Figure 4). Using a suicide pseudo-HJ DNA substrate, we further showed that Endo III inhibited pseudo-HJ resolution by blocking its cleavage by XerC (Figure 6). We observed

that it exclusively associated with the XerCD/pseudo-HJ nucleoprotein complex and not with the XerCD/*att*P(+) or XerCD/*dif* complex (Figure 5 and Figure S4), which explains why the XerC-strand exchange leading to the formation of the pseudo-HJ was left unaffected".

Point 7. "***This mechanism is clearly distinct from that previously proposed for other systems in which auxiliary factors play an architectural role in the assembly and/or activation of the recombination complex. Direct comparison with those systems as discussed P15 is thus misleading.***"

We agree. We are sorry that the discussion was misleading. The new text is now straightforward (p16):

"**A new mechanism to control site-specific recombination**

The catalytic activity of Endo III was not required for CTXϕ-integration (Figure 2 and Figure S2), which suggested that it could help assemble a synapse with a specific architecture by binding to accessory sequences flanking the core recombination sites, similarly to Fis and IHF during phage λ integration and to PepA and ArgR/ArcA during plasmid dimer resolution. However, Endo III does not bind to specific DNA motifs and the DNA molecules employed in our *in vitro* reactions lacked any homologies with the phage (+) ssDNA and the genomic DNA of *V. cholerae* apart from the *att*P(+) and *dif*1 sites. Indeed, further investigations revealed that Endo III didn't promote formation of the pseudo-HJ integration intermediate but rather stabilized it by impeding new rounds of strand exchanges by XerC-catalysis once it had been formed, unveiling a novel mechanism for the control of DNA site-specific recombination. (Figure 4). "

Point 8a. "***Figure 1A and B. According to current models for the tyrosine recombinases pathway, it is difficult to figure out how similar arrangements of the synapse could give alternative orders of strand exchange.***

Based on the Cre/loxP synaptic complex structures, it is expected that different arrangements of the recombinases are necessary in the XerC-first or XerD-first pathway of recombination. However, this is not important for the message of the present paper, and we believe that it would lead to unnecessary complication sin the introduction and in the figure for the non expert reader. Nevertheless, we have changed the angle in the 4 arms of the synaptic complex depicted in Figure 1B to satisfy the more expert-reader and we now indicate in the figure legend (p27) that:

"According to current models for the tyrosine recombinases pathway, alternative orders of strand exchange are dictated by different arrangements of the synapse. This is indicated in the figure by a different angle between the XerC- and XerD-binding sites in the two recombination substrates."

Point 8b. "***Figure 1D. The proposed architecture of the synaptic complex at the cer and psi sites suggesting a parallel inter-wrapping of the accessory sequences is inconsistent with well-established models based on the topology of recombination reaction.***"

Referee #3 is right. We are sorry for the mistake, which was introduced while copy-pasting schemes and deforming them to fit in Figure 1. The figure has been corrected!

Point 9. "***P4. References for the 'propensity of Xer to recycle HJs back to substrate' are from studies on the E. coli Xer/dif system. A more specific reference asserting that this is also true for CTXphi integration should be given as well.*** "

These data are not published: they are part of our results!

We now precise in the introduction (p5):

" In addition, it was intriguing to observe that XerC catalysed the formation of the pseudo-HJ integration intermediate of CTXϕ (Das et al, 2010; Val et al, 2005) since its propensity to recycle back HJs to substrate (see (Arciszewska et al, 2000; Barre et al, 2000; Hallet et al, 1999) for *dif/dif* HJs and Figure 4E for *att*P(+)/*dif* pseudo-HJs)) should severely decrease the chances for replication to finalize the integration process (Figure 1A and 1C)."

Point 10. "***How to conciliate the proposed mechanism of action of Endo III with the observation that its presence appears to inhibit the strand exchange reaction catalysed by XerC without affecting cleavage (P10L12, Fig. 4D)? This observation seems to be contradicted by the experiment performed with the suicide pseudo-HJ substrate shown in Fig. 6B.***"
See referee #2 point 7.

Point 11. "***It is disturbing that reference to Fig S5 comes before reference to Fig. S3 (P18) and Fig. S4 (P20) Fixed, supplemental figure numbers were re-attributed.***"
Dealt with.

Point 12. "***P11L17. Fig. 6C shows a protection pattern for the Pseudo-HJ, not for the dif1/dif1 HJ.***"
Protection for dif/dif HJs was previously published in {Arciszewska, 1997 #6;Arciszewska, 1995 #654}. To make this more explicit, we changed the text to (p12):

> "Because the bases at the branch point of *dif*/*dif* HJs or *att*P(+)/*dif* pseudo-HJs are protected from nucleases by the binding of XerC and XerD (see {Arciszewska, 1997 #6;Arciszewska, 1995 #654} and Figure 6C, respectively), [...]. "

Point 13a. "***P12L9. 'When the corresponding oligonucleotide is labelled at its 3'extremity'. The diagrams of Fig. 4D and Fig. 6B suggest that the substrates were labelled at the 5' side of the XerC arm.***"
HJ substrates are labelled in 5' not in 3'. We have corrected the diagram to make this more clear.

Point 13b. "***The author should specify that in this case, cleavage by XerC is expected to give a tyrosyl adduct at the 3' end of the DNA fragment after protease K treatment, which may explain the detection of 2 (or more) product bands.*** "
In Figure 4C, we now clearly depict the tyrosyl adduct. In addition, we specify that proteinase K treatment is used in the text (see referee # 2 point 12b). We believe that 2 product bands are detected because of conatminating shorter oligos in the mix used to reconstitute the substrates: although these oligonucleotides are purified on acrylamide gels, we cannot avoid shorter oligos (it is not possible to separate 69 and 70 nt). As we have to force the contrast to observe the cleaved products, these contaminants are detected.

Point 13c. "***What are the multiple bands seen in Fig. 4C (bottom gel), since in this case the substrate was labelled at the 3' end of XerC binding site?***"
We often observe low level of degradation of the oligos substrates after long incubations. This pattern of degradation is also observed in the absence of recombinases. This degradation is very limited (less than 1% of the total DNA substrate). However, we have to force the contrast to observe the cleaved intermediate. See Figure S2 for the complete degradatauon pattern.

Point 14. "***P13L11. Refer to Fig. 6C (instead of 6D); P13L16. Refer to Fig. 6A (instead of Fig. 4C); P15L7. 'Resolution (no 's') sites***"
Dealt with.

Point 15. "***P15. What experimental evidence (reference?) supports the assertion that the HJ intermediates which are formed during Xer recombination of ColEI dimers are resolved by replication? Such a mechanism is expected to leave an unresolved copy of the plasmid dimer after each round of recombination/replication, which is obviously not the case.***"
{McCulloch, 1994 #133} showed that "Xer-mediated recombination [on ColE1 plasmids] generates Holliday junction-containing DNA molecules in which a specific pair of strands has been exchanged in addition to complete recombinant products. Generation of Holliday junctions and recombinant products is equally efficient in RuvC- and RuvC+ cells, and in cells containing a multicopy RuvC+ plasmid". This left replication as the simplest explanation for the 'resolution' of the HJ intermediate However, we agree with referee # 3 that no direct evidence for the resolution of the HJ intermediates

which are formed during Xer recombination of ColEI by replication and the corresponding sentences have been removed from the discussion. See referee #3 point 7.

Point 16. "***P15 bottom. There is no evidence in Fig. 5 that shows that Endo III does not associate with the XerCD/attP(+)/dif synaptic complex.*** "
The referee is right. This is shown in Figure S4. The sentence has been corrected to (p16):

> "We observed that it exclusively associated with the XerCD/pseudo-HJ nucleoprotein complex and not with the XerCD/*att*P(+) or XerCD/*dif* complex (Figure 5 and Figure S4), which explains why the XerC-strand exchange leading to the formation of the pseudo-HJ was left unaffected."

Point 17. Supplementary figures mispelling/typos. Fig. S1. "***It is not clear why the legend given for the triple insertion (lacZ::dif ... LacZ) differs from that of the single and triple insertions.***"
Legend of Fig. S2. "***The 3'end labelled strand' (no 's')***"**. Legend of Fig. S3. "Refer to Fig. 4C (instead of 3C) and to Fig. 4D (instead of 3D)." "There is no Fig. 7b in the examined version of the MS."**
Dealt with.

Acceptance letter 11 July 2012

Your revised manuscript has now been seen once more by the original referee 3 (see comments below), and I am happy to inform you that there are no more objections against publication of the study in The EMBO Journal.

Before formal acceptance, there are only a few minor modifications I need to ask you for, which in my opinion do not require another revision round. First, please consider the comments of referee 3, and accordingly introduce a clarification or caveat in the manuscript as requested. Second, with the general readership of our journal in mind, I have made some small edits to the manuscript title and abstract aimed at improving clarity and appeal - please see the proposal below. If you are happy with these modifications, I would simply introduce them into the manuscript from our side. Finally, when checking the figures I noticed that two panels (Fig 5B & D) are, probably due to exaggerated contrast/brightness adjustments, too pale to allow visualization of the complete background or even gel boundaries; please therefore provide us with a modified Figure 5 in which these panels more closely represent the original scans (e.g. as in Fig 6).

The easiest procedure for this would be if you simply sent us a modified text file, and a modified Figure file (for Figure 5) by email, and we would then replace them in our manuscript tracking system. After that, we should be able to swiftly proceed with acceptance and production of the article.

Yours sincerely,
Editor
The EMBO Journal

Referee #3

(Remarks to the Author)
In this revised version of the manuscript, the authors have thoroughly and satisfactorily answered to most of the reviewers concerns and criticisms. Additional material was added to the paper, making it more solid on some aspects. One common issue that was raised by all three referees was on the mechanism whereby EndoIII acts on the XerCD/Pseudo-HJ complex to inactivate it. This mechanism is now discussed more thoroughly and more cautiously, considering different alternatives. Models are clearly summarised in a new figure (Fig. 7) at the end of the manuscript. The suggestion that both XerC and XerD dissociate from the complex, but that the observed effect is greater for XerC due to its lower DNA binding affinity sounds like a reasonable one. The authors also recognise that more work has to be done to fully clarify this mechanism.

I think that both the referee #2 and #3 perfectly understood why the authors made the use of suicide substrates to specifically detect the cleavage step. The question was about the relative inhibition of cleavage versus strand exchange by EndoIII. The new supplemental figure S6 clearly shows that both the cleavage and strand exchange are inhibited. Thus the statement p11 that 'addition of Endo II blocked conversion of the pseudo-HJ back into substrate without apparently affecting the formation of cleaved substrate' remains confusing. This is of course a very minor concern that should be easily addressed without requiring an additional turn of revision.

Additional correspondence 12 July 2012

We were pleased to learn that our mansucript is now suitable for publication in the EMBO journal. The title you proposed is a good title. You also did a good work with the abstract. We are happy with both changes.
We checked Figure 5B and 5D. The pale background is linked to the conversion from the ADOBE illustrator format to PDF: as a rule, we avoid playing with brighthness/contrast adjustments. Therefore, I have attached to this message the original illustrator figure. Can you work with it? I have also attached a PDF figure in which Julien forced the background signal. The change is noticeable for Figure 5B, but less evident for Figure 5D. There was little or no background in this experiment and it is difficult to make one appear!
Finally, we understand the point of referee #3. Correspondingly, we have corrected the offending sentence by simply deleting its end. The text now reads "addition of Endo III blocked conversion of the pseudo-HJ back into substrate".

Thanks again for your prompt decision and your efforts to improve our mansucript.

