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Tbf1 and Vid22 promote resection and non-homologous end joining of DNA double-strand break ends

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Additional correspondence (editor) 29 August 2012

Thank you for submitting your manuscript EMBOJ-2012-82862, "The Tbf1 and Vid22 proteins promote double-strand break repair by influencing chromatin structure" for consideration by The EMBO Journal. We have now received all the reports from three experts, which I am enclosing copied below. As you will see, the reviewers express some interest in your study, yet they also raise a number of substantive concerns. Before taking a final decision, I would like to give you the opportunity to consider and respond to these comments, detailing whether and how you might be able to address the concerns if offered the possibility to revise the manuscript. In particular, we would like to hear whether (and how) you could imagine addressing the concerns regarding mechanisms of Tbf1 recruitment and function (as raised by referee 3 but also referee 2). These tentative response (parts of which we may choose to share and discuss with some of the referees) would be taken into account when making our final decision on this manuscript. I would therefore appreciate if you could send us such a response at your earliest convenience.

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The Longhese lab has uncovered two proteins that promote double-strand break repair in yeast, apparently by influencing chromatin structure. Overall the data strongly support the idea that Vid22 and Tbf1 are important in controlling both 5' to 3' resection and NHEJ, possibly in both cases by promoting the recruitment of the Mre11 complex to DSB ends. Mutation of Vid22 and Tbf1, but not their associated partner, Env11, cause an increased sensitivity to DNA damaging agents and show genetic interactions with other chromatin modifiers. There are some aspects of the results that should be cleared up before the paper is accepted.

1. In Fig. 1 and in other instances, it is hard to evaluate the degree of sensitivity in the dilution assays because the colony sizes from tbf1 mutants are so tiny compared to WT that it is hard to see if there are fewer cells surviving. Perhaps an acute exposure to MMS, followed by plating and counting colonies of comparable size would clear this up.

2. There is clearly a defect in resection that markedly reduces success of even a "short" SSA assay. This is reflected in a very low activation of the DNA damage checkpoint protein Rad53. Do cells show a lack of checkpoint-mediated arrest?

3. tbf1-1 and vid22 mutants are surprisingly synthetically sensitive with rad51 or rad52 mutants to phleomycin. What does this mean? A rad52 deletion should eliminate all HR repair, so the vid22/tbf1 mutants must affect another aspect. One possibility is that NHEJ is also eliminated. It would therefore be useful to compare the vid22 rad52 mutant to dnl4 rad22. Are they comparable or is there another problem revealed by knocking out these factors (for example a problem specific to telomeres)?

4. Fig. 4 the small colony issue comes up here again, as I can't easily evaluate the effect of the mutants on surviving and HO cut. See also the top of Fig. 6, though there's no doubt that the double mutant combinations in Fig. 6 are quite distinct.

5. How is the repair efficiency normalized? The figure legend says the uncut band was monitored, but relative to what?

6. Another condition that blocks resection - exo1 sgs1 - also reduces checkpoint activation but has an unexpected phenotype of promting robust telomere addition to DSBs. It it possible that the lower NHEJ efficiency is actually the result of creating new telomeres? Does overexpression of Exo1 suppress the defects of vid22/tbf1-1?

7. Fig. 5. The data seem clear that both proteins are recruited to the vicinity of a DSB and the tbf1-1 mutant impairs Mre11 binding. As the authors note, this could explain lower resection and poor end-joining. It might be interesting to know if mre11 vid22 is more sensitive or not to phleomycin, etc.

8. Fig. 5. Can Vid22 be recruited to the DSB in the trf1-1 mutant or vice versa?

9. Fig. 6. There seems to be a clear defect in nucleosome eviction for histones H2A and H3 in the tbf1-1 mutant, which again might be a reflection of resection defects (or the resection defects are a reflection of a lack of nucleosome repositioning). The authors should comment, however, on why their results are so different from those of Shroff et al. 2004, who stated that 60 min after the DSB, "DSB Formation is Not Accompanied by Histone Depletion." This is a key point, whether there is a marked loss of nucleosomes accompanying resection and needs to be discussed.

Referee #2 (Remarks to the Author):

This is an interesting and promising paper that reports an unexpected link between the TTAGG binding factor Tbf1 in budding yeast, and its cofactor Vid22, in chromatin remodelling and resection at DSBs. The authors set out to discover the essential role of Tbf1 by isolating ts alleles. Instead of shedding light on Tbf1's essential function, they discovered and characterized the role of Tbf1 in DSB repair. This is a novel and important finding. However, there are two major problems with the data as presented. First they characterize an allele (tbf1-1) that does not efficiently separate Tbf1's role in repair from its essential role (presumably in transcription) - which another allele they isolate (tbf1-3) does do. It is not clear why the experiments presented exclusively present tbf1-1, which at nonpermissive temperature is both phleomycin sensitive and defective for the essential (transcription?) function. It is unclear why they did not use the separation of function mutant that they isolated (tbf1-3) ! This leaves the lingering doubt whether the phenotypes observed (eg lethality in double mutants with various cofactors of repair) stem from the "essential" or the "nonessential repair" function of the protein.

One major recommendation, therefore, is to include data on tbf1-3 (or at the very least to explain why it is not included) and to discuss the fact that tbf1-1 inactivates both functions. A second major point concerns the CHIP data at the DSB. A number of essential controls must be done to prove that Tbf1 and Vid22 actually are recruited to the site of damage. As described below, precipitation with anti-Myc at DSBs generally yields high background due its ability to bind ssDNA. A "non-tagged control" in which anti-Myc is used on an identical extract from a non-tagged strain, is essential. If Tbf1 and Vid22 are not at the break, the interpretation of their data may be very different.

Major problems:
1)As stated above - it is not clear why the authors used the tbf1-1 allele rather than the more interesting tbf1-3 allele, which is only phleomycin sensitive (and not ts). While it would be too much to redo everything with the tbf1-3 allele, a few key experiments should definitely be included (e.g. the H2A ChIP in G1 and G2; the resection assay, and some double mutant studies).
2)The ChiP data in Figure 5 is inconclusive as presented. As stated above, a number of anti-Myc monoclonal antibodies precipitate ssDNA with high selectivity. As ss DNA forms at the resected DSB over time - anti-Myc precipitates the DNA flanking the cut with increasing efficiency, independent of any protein being bound. Thus the only convincing control is a parallel precipitation with anti-Myc, using a strain that does not carry the Myc-tagged allele of Tbf1. It is not sufficient to do a no antibody or "beads background" control. The authors must find conditions under which anti-Myc does not precipitate the resected break, or else prove that the presence of Tbf1-Myc or Vid22-Myc increases the amount of break site precipitated over anti-Myc in a strain lacking the epitope-tagged proteins. One can avoid the sticky-ssDNA problem by washing with higher salt, including tRNA or non-yeast ssDNA in the precipitation or pre-incubating the anti-Myc beads with sheared ssDNA prior to using them in the CHIP assay- but the non-tagged control still has to be done ! Without this, the increase in "Tbf1" or "Vid22" over time could simply reflect the increase in ssDNA.
The second experiment that must be done in this context is to determine whether the two proteins bind in a manner dependent on each other. In other words: Tbf1-Myc CHIP in a vid22 delta strain, and Vid22-Myc CHP in a tbf1-1 or tbf1-3 strain. Of course, many other mutants could be used for testing what recruits Tbf1. Most important would be to test whether Tbf1 recruitment occurs in the absence of resection or in a mre11 delta background.
3)The third experiment that should be done is to directly quantify the generation of ssDNA in the two mutants (or three) with the Lydall QAOS method of quantitative ssDNA PCR (Nucleic Acids Res. 2001 Nov 1;29(21):4414-22); alternatively strand specific ssDNA Southern (e.g. Osley in Nature. 2005 Nov 17;438(7066):379-83) . The data presented "indirectly suggest" that resection is impaired, but it is quite easy to check ssDNA directly and the paper would be greatly enriched by including such results.

Less major problems:
1)The authors show synthetic sickness/lethality between the tbf1-1 allele and mms4, mre11, and sgs1 and conclude that it has a functional interaction with HR genes, but that is also affects NHEJ. Then, depending on the background, the tbf1-1 allele either is almost lethal with rad52 delta or else viable (Fig. 2E and 3; text page 8 bottom). To what extent are all the additive effects dependent on strain background ? This is also relevant for the drop assays of double mutants with remodelers. It should at least be mentioned to what extent the effects (which are sometimes relatively minor) are

background dependent. Finally, double mutants should also be tested between tbf1-3 and vid22 with yKu and lig4. The interaction between tbf1-1/-3 and NHEJ would be strengthen by testing yku/tbf1-1 and lig4/tbf1-1 double mutants in the assays shown in Fig. 4.
2)On p. 11 they show that Mre11 association with DSBs is low in G2 phase in the tbf1-1 mutant. They concluded that "this decreased binding might account for resection and the NHEJ defects displayed by tbf1-1 and possibly vid22". Are the NHEJ defects also explained by lower Mre11 levels at DSBs in G1 ? This can be easily tested. They should also prove that Mre11 levels (and its cofactors) are not affected by tbf1 mutations (by Western blot).
3)Their data put Tbf1 upstream of Mre11, which is very surprising. The authors should comment on this. Tbf1 may simply promote Mre11 association to DSBs, or may affect (indirectly) its transcription, stability or its access to the break. Again, repeating the Tbf1 ChIP (with proper controls) in an mre11 mutant would be informative.
4)The "search for Tbf1 mutants" section could be somewhat more clearly written with a schematic of the construct used to transform yeast, and a schematic of Tbf1 in which the mutations are mapped.
5)An excellent addition (possibly beyond the scope of the paper if they complete the "major additions" requested above) would be to perform ChIP for the remodelers (eg RSC, Arp8 for INO80 and SWR1 - all of which exist in epitope tagged forms) at the DSB in the vid22 or tbf1 mutants. This would show whether remodelers recruit Tbf1 or vice versa.

Referee #3 (Remarks to the Author):

TBF1 encodes an essential telobox-containing DNA binding protein that is known to bind TTAGGG repeats within subtelomeric regions. Tbf1 also regulates expression of snoRNAs and is believed to function as general transcriptional regulator. In this report, the authors have investigated a possible role for Tbf1 in DNA repair. Alleles of TBF1 are isolated that show hypersensitivity to DNA damaging agents, and mutants show synthetic interactions with genes encoding components of both the HR and NHEJ pathways. Consistent with genetics, the authors show that Tbf1 is required for SSA repair of an HO-induced DSB, and Tbf1 is required for a simple plasmid re-ligation NHEJ event. The authors present solid evidence demonstrating recruitment of Tbf1 to HO-induced DSBs, and Tbf1 appears to play a key role in DSB resection, histone eviction, and recruitment of Mre11. In general the data are technical quite sound.

Although the data are solid, and the conclusions justified, the reader is left wondering how Tbf1 actually functions. Thus, the work seems incomplete, especially for a high tier journal such as EMBO J. Tbf1 is presumably a site-specific DNA binding protein, so why is it recruited to DSBs? What are the frequency of TTAGGG sequences in the yeast genome? Furthermore, how do the authors envision this protein interacting with ssDNA during resection? The ChIP data implies spreading of Tbf1 during the resection process. Does Tbf1 show high affinity for DNA ends in vitro? or does it directly interact with the MRX complex? I believe that much, if not all, of the genetic data are consistent with a role in facilitating MRX function. In this reviewers opinion, this work needs additional mechanistic experiments to shed light on how Tbf1 is both recruited and functions at DSBs.

Additional correspondence (author) 02 September 2012

Thank you very much for the opportunity you gave us to respond to the reviewers' comments on our manuscript EMBOJ-2012-82862. Please find enclosed a file with our response that I hope can help you in reaching a final decision.

We thank the reviewers for the suggestions provided.

Response to referees:

Referee #1

1. In Fig. 1 and in other instances, it is hard to evaluate the degree of sensitivity in the dilution assays because the colony sizes from tbf1 mutants are so tiny compared to WT that it is hard to see if there are fewer cells surviving. Perhaps an acute exposure to MMS, followed by plating and counting colonies of comparable size would clear this up.

We will incubate cells with phleomycin or MMS and plate them at different time points to count colony forming units.

2. There is clearly a defect in resection that markedly reduces success of even a "short" SSA assay. This is reflected in a very low activation of the DNA damage checkpoint protein Rad53. Do cells show a lack of checkpoint-mediated arrest?

Yes, they do. This information can be added.

3. tbf1-1 and vid22 mutants are surprisingly synthetically sensitive with rad51 or rad52 mutants to phleomycin. What does this mean? A rad52 deletion should eliminate all HR repair, so the vid22/tbf1 mutants must affect another aspect. One possibility is that NHEJ is also eliminated. It would therefore be useful to compare the vid22 rad52 mutant to dnl4 rad22. Are they comparable or is there another problem revealed by knocking out these factors (for example a problem specific to telomeres)?

Indeed, our data in Fig. 4 indicate that *tbf1-1* and *vid22* mutants are impaired also in NHEJ. Anyhow, we will compare the sensitivity to phleomycin of *dnl4 rad52, vid22 rad52* and *tbf1 rad52* mutants, as suggested, to assess whether the phleomycin sensitivity can be entirely explained by defective HR and NHEJ.

4. Fig. 4 the small colony issue comes up here again, as I can't easily evaluate the effect of the mutants on surviving and HO cut. See also the top of Fig. 6, though there's no doubt that the double mutant combinations in Fig. 6 are quite distinct.

We will repeat the analysis by plating for colony forming units in liquid cultures at different time points after galactose addition.

5. How is the repair efficiency normalized? The figure legend says the uncut band was monitored, but relative to what?

The repair efficiency has been calculated by evaluating the increase of the uncut band intensity at 60, 90 and 120 minutes after galactose addition relative to the same band at 30 minutes after HO induction. For each time point the uncut band has been normalized relative to another DNA fragment that acts as a loading control. We will include this information.

6. Another condition that blocks resection - exo1 sgs1 - also reduces checkpoint activation but has an unexpected phenotype of promting robust telomere addition to DSBs. It it possible that the lower NHEJ efficiency is actually the result of creating new telomeres? Does overexpression of Exo1 suppress the defects of vid22/tbf1-1?

This is an interesting possibility that we can easily test by looking at the recruitment of telomerase at the HO-induced DSB in wild type, *tbf1* and *vid22* mutants. Also the effect of *EXO1* overexpression can be easily tested.

7. Fig. 5. The data seem clear that both proteins are recruited to the vicinity of a DSB and the tbf1-1 mutant impairs Mre11 binding. As the authors note, this could explain lower resection and poor end-joining. It might be interesting to know if mre11 vid22 is more sensitive or not to phleomycin, etc.

The *mre11 vid22* double mutant seems to be more sensitive to phleomycin than each single mutants. However, this analysis is biased by the fact that *mre11 vid22* double mutant cells grow more poorly than each single mutant even in the absence of phleomycin. That's why we did not include this data in the manuscript.

8. Fig. 5. Can Vid22 be recruited to the DSB in the trf1-1 mutant or vice versa?

We are in the process to address this very interesting point (see also responses to ref. 2 and 3).

9. Fig. 6. There seems to be a clear defect in nucleosome eviction for histones H2A and H3 in the tbf1-1 mutant, which again might be a reflection of resection defects (or the resection defects are a reflection of a lack of nucleosome repositioning). The authors should comment, however, on why their results are so different from those of Shroff et al. 2004, who stated that 60 min after the DSB, "DSB Formation is Not Accompanied by Histone Depletion." This is a key point, whether there is a marked loss of nucleosomes accompanying resection and needs to be discussed.

Shroff et al., 2004 analyze histone evinction in G1 at the HO-induced DSB made at the *MAT* locus, while we analyze histone depletion in G1 at the HO-induced DSB made at the *LEU2* locus. This difference can explain the different results obtained. In any case, I have to mention that histone loss at DSBs has been observed in G1 also in Shim et al., Mol Cell Biol. 2007.

Referee #2

One major recommendation, therefore, is to include data on tbf1-3 (or at the very least to explain why it is not included) and to discuss the fact that tbf1-1 inactivates both functions. A second major point concerns the CHIP data at the DSB. A number of essential controls must be done to prove that Tbf1 and Vid22 actually are recruited to the site of damage. As described below, precipitation with anti-Myc at DSBs generally yields high background due its ability to bind ssDNA. A "non-tagged control" in which anti-Myc is used on an identical extract from a non-tagged strain, is essential. If Tbf1 and Vid22 are not at the break, the interpretation of their data may be very different.

Major problems:

1)As stated above - it is not clear why the authors used the tbf1-1 allele rather than the more interesting tbf1-3 allele, which is only phleomycin sensitive (and not ts). While it would be too much to redo everything with the tbf1-3 allele, a few key experiments should definitely be included (e.g. the H2A ChIP in G1 and G2; the resection assay, and some double mutant studies).

We agree with the reviewer. The reason why we used mainly the *tbf1-1* mutant instead of the *tbf1-3* mutant is trivial: the search for *tbf1* mutants was repeated three times and *tbf1-1* was identified during the first screening, while *tbf1-3* was found only later, when most of the experiments had already been done on the *tbf1-1* mutant. Now we are repeating most of the analysis also in the *tbf1-3* mutant. We have already analyzed the genetic interactions of *tbf1-3* with the *mre11, sgs1, mms4, rad52, rad51* mutations and the results are very similar to those obtained with *tbf1-1*. Moreover, we have constructed *tbf1-3* strains suitable to test SSA and to perform CHIP analysis on histones. We can forsee that most experiments done in *tbf1-1* will be repeated also in *tbf1-3* cells in less than one month.

2)The ChiP data in Figure 5 is inconclusive as presented. As stated above, a number of anti-Myc monoclonal antibodies precipitate ssDNA with high selectivity. As ss DNA forms at the resected DSB over time - anti-Myc precipitates the DNA flanking the cut with increasing efficiency, independent of any protein being bound. Thus the only convincing control is a parallel precipitation with anti-Myc, using a strain that does not carry the Myc-tagged allele of Tbf1. It is not sufficient to do a no antibody or "beads background" control. The authors must find conditions under which anti-Myc does not precipitate the resected break, or else prove that the presence of Tbf1-Myc or Vid22-Myc increases the amount of break site precipitated over anti-Myc in a strain lacking the epitope-tagged proteins. One can avoid the sticky-ssDNA problem by washing with higher salt, including tRNA or non-yeast ssDNA in the precipitation or pre-incubating the anti-Myc beads with sheared ssDNA prior to using them in the CHIP assay- but the non-tagged control still has to be done ! Without this, the increase in "Tbf1" or "Vid22" over time could simply reflect the increase in ssDNA.

We used a CHIP protocol from Zakian lab, which was specifically developed to detect association of Myc-tagged proteins to DNA, because washes are with high salt. All our ChIP experiments with Myc-tagged protein shown in figure 5 have been performed together with a strain that does not carry

Myc-tagged proteins. Since the enrichment with the untagged strain was very low (and much lower than the enrichment we got with the tagged strains), we did not include this data in figure 5. We apologize to have underestimated the importance of this control strain, and we are ready to provide the ChIP data on the untagged strain for all th experiments of figure 5.

The second experiment that must be done in this context is to determine whether the two proteins bind in a manner dependent on each other. In other words: Tbf1- Myc CHIP in a vid22 delta strain, and Vid22-Myc CHP in a tbf1-1 or tbf1-3 strain.

We are currently trying to address this very interesting point (see response to ref. 1 and 3)

Of course, many other mutants could be used for testing what recruits Tbf1. Most important would be to test whether Tbf1 recruitment occurs in the absence of resection or in a mre11 delta background.

We have preliminary data showing that Tbf1 binds the DSB ends in G1 (see Fig. 1), suggesting that Tbf1 recruitment occurs in the absence of resection. To confirm this data, we will analyze Vid22 recruitment in G1 and Tbf1/Vid22 recruitment in *mre11* mutant cells (see also response to ref. 3, point 2).

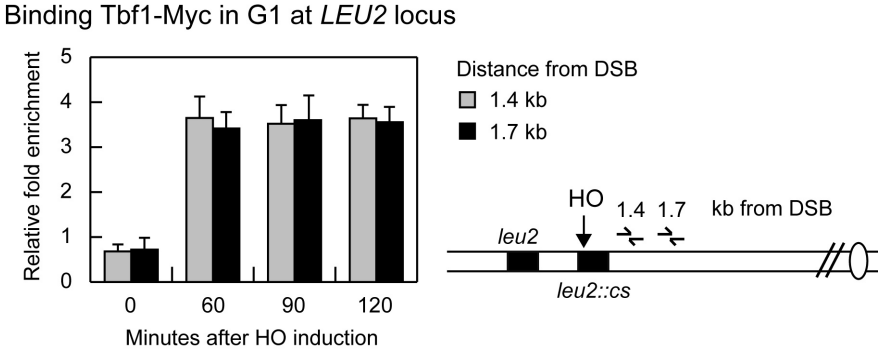

Fig. 1. Relative fold enrichment of Tbf1-Myc at the indicated distance from the HO cleavage site at the *LEU2* locus in G1-arrested wt cells after ChIP with an anti-Myc antibody. Cells were kept arrested in G1 during HO induction.

3)The third experiment that should be done is to directly quantify the generation of ssDNA in the two mutants (or three) with the Lydall QAOS method of quantitative ssDNA PCR (Nucleic Acids Res. 2001 Nov 1;29(21):4414-22); alternatively strand specific ssDNA Southern (e.g. Osley in Nature. 2005 Nov 17;438(7066):379-83) . The data presented "indirectly suggest" that resection is impaired, but it is quite easy to check ssDNA directly and the paper would be greatly enriched by including such results.

We can easily detect directly ssDNA by performing a denatured Southern blot and probe with a ssDNA sequence that is complementary to the unresected strand (Clerici et al., EMBO rep 2006) in the strains used to detect SSA. In order to independently confirm a resection defect, we will perform this analysis also in strains where an irreparable HO-induced DSB is made at the *MAT* locus (JKM139 background).

Less major problems:
1)The authors show synthetic sickness/lethality between the tbf1-1 allele and mms4, mre11, and sgs1 and conclude that it has a functional interaction with HR genes, but that is also affects NHEJ. Then, depending on the background, the tbf1-1 allele either is almost lethal with rad52 delta or else viable (Fig. 2E and 3; text page 8 bottom). To what extent are all the additive effects dependent on strain background ? This is also relevant for the drop assays of double mutants with remodelers. It should at least be mentioned to what extent the effects (which are sometimes relatively minor) are background dependent.

As we show that the *tbf1-1 rad52* combination is viable in both the YMV45 (Fig. 2E and Fig. 7A) and the W303 (Fig 3, bottom) backgrounds, we believe the reviewer refers to the data on the *vid22 rad52* combination, which we show to be viable in the W303 background (Fig. 3, bottom), and we assess to be lethal in the YMV45 background (text page 8 bottom, and text page 13, middle). We agree that this point was confusing in the manuscript. Moreover, we can now say that our statement that deletion of *RAD52* was lethal in the YMV45 *vid22* background was wrong. Briefly, we had no problems in obtaining both *tbf1-1 rad52* and *vid22 rad52* double mutants in the W303 background by simply crossing suitable isogenic strains and dissecting tetrads. Because YMV45 strains cannot be crossed to each other, we directly deleted *RAD52* by transforming the *RAD52* disruption cassette into YMV45 *vid22* and YMV45 *tbf1-1* strains. As we failed to obtain *vid22 rad52* transformants at the first attempts, we concluded that this double mutant combination was inviable. We recently repeated this gene-disruption and we obtained trustable and viable *vid22 rad52* double mutant transformants in the YMV45 background. Therefore, there is no a background-dependent effect of *RAD52* deletion in either *tbf1-1* or *vid22delta* strains and we have now performed all the missing experiments with *vid22 rad52* YMV45 strains.

Finally, double mutants should also be tested between tbf1-3 and vid22 with yKu and lig4. The interaction between tbf1-1/-3 and NHEJ would be strengthen by testing yku/tbf1-1 and lig4/tbf1-1 double mutants in the assays shown in Fig. 4.

This can be easily tested and we have already generated the suitable strains.

2)On p. 11 they show that Mre11 association with DSBs is low in G2 phase in the tbf1-1 mutant. They concluded that "this decreased binding might account for resection and the NHEJ defects displayed by tbf1-1 and possibly vid22". Are the NHEJ defects also explained by lower Mre11 levels at DSBs in G1 ? This can be easily tested.

We will test Mre11 and Lig4 association to a DSB in *tbf1* and *vid22* mutants in G1.

They should also prove that Mre11 levels (and its cofactors) are not affected by tbf1 mutations (by Western blot).

We have already measured the amount of Mre11 in the total protein extracts of *vid22* and *tbf1* cells by western blot and we found that its level is similar to that we found in wild type cells, indicating that *tbf1* and *vid22* mutations do not affect Mre11 level. We have the strains to repeat this analysis on Mre11 cofactors like Sae2, Xrs2 etc.

3)Their data put Tbf1 upstream of Mre11, which is very surprising. The authors should comment on this. Tbf1 may simply promote Mre11 association to DSBs, or may affect (indirectly) its transcription, stability or its access to the break. Again, repeating the Tbf1 ChIP (with proper controls) in an mre11 mutant would be informative.

See above and response to ref. 3, point 3.

4)The "search for Tbf1 mutants" section could be somewhat more clearly written with a schematic of the construct used to transform yeast, and a schematic of Tbf1 in which the mutations are mapped.

We will clarify this section and introduce a scheme.

5)An excellent addition (possibly beyond the scope of the paper if they complete the "major additions" requested above) would be to perform ChIP for the remodelers (eg RSC, Arp8 for INO80 and SWR1 - all of which exist in epitope tagged forms) at the DSB in the vid22 or tbf1 mutants. This would show whether remodelers recruit Tbf1 or vice versa.

We agree, although it is a lot of work and the answers might not be not clearcut, because the different subunits of the complexes above perform different functions. Nonetheless, we could start by performing this analysis by inactivating just one subunit for each complex, as suggested.

Referee #3

Although the data are solid, and the conclusions justified, the reader is left wondering how Tbf1 actually functions. Thus, the work seems incomplete, especially for a high tier journal such as EMBO J. Tbf1 is presumably a site-specific DNA binding protein, so why is it recruited to DSBs? What are the frequency of TTAGGG sequences in the yeast genome? Furthermore, how do the authors envision this protein interacting with ssDNA during resection? The ChIP data implies spreading of Tbf1 during the resection process. Does Tbf1 show high affinity for DNA ends in vitro? or does it directly interact with the MRX complex? I believe that much, if not all, of the genetic data are consistent with a role in facilitating MRX function. In this reviewers opinion, this work needs additional mechanistic experiments to shed light on how Tbf1 is both recruited and functions at DSBs.

This reviewer raises concerns about the lack of mechanistic details on how Tbf1 and Vid22 interact and act at DSBs.

Specific points:

"Tbf1 is presumably a site-specific DNA binding protein, so why is it recruited to DSBs? What are the frequency of TTAGGG sequences in the yeast genome?"

Tbf1 was found to bind the TTAGGG sequence because a radiolabelled TTAGGG oligonucleotide was used during the screening. Subsequent work (Preti et al., Mol Cell. 2010) has shown by CHIPseq that Tbf1 is present at snoRNA promoters, where it binds an aRCCCTaa sequence with high flexibility for the first and the last two "A" bases. Furthermore, Tbf1 is found at the promoters of 136 genes, where it binds a similar aRCCCTaa sequence, but with an higher bias toward the first and the two last "A" bases) (Preti et al., Mol Cell. 2010). Therefore, the consensus site for Tbf1 binding appears quite flexible in terms of sequence and orientation. As the conserved core sequence "RCCCT" is present at 23.954 sites scattered into the entire *S. cerevisiae* genome, it is not difficult to find this sequence near the DSBs we generated and near DSBs randomly occurring into the genome.

Importantly, although there are 23.954 "RCCCT" sequences into the genome, Tbf1 is found by CHIPseq only at 197 of these sites (Preti et al., 2010). Furthermore, Vid22 colocalizes with Tbf1 only at the promoters of non-snoRNA genes, but not at snoRNA promoters that contain the same target sequence. Altogether, these findings indicate that Tbf1/Vid22 association to DNA is driven by other proteins and/or histone modifications (Tbf1 was found to interact with histones H2A and H2B, Gavin et al., 2006 Nature). We think that a similar mechanism occurs also at the DSB, where Tbf1/Vid22 recruitment can be driven by histone modifications (such as gamma-H2A, see below).

"how do the authors envision this protein interacting with ssDNA during resection? The ChIP data implies spreading of Tbf1 during the resection process. Does Tbf1 show high affinity for DNA ends in vitro?"

We found that Tbf1 and Vid22 are bound to the DSB at the *LEU2* locus already at 60 minutes from HO induction (Fig. 5C of the manuscript), when resection is quite limited, suggesting that these two proteins are bound to the double-stranded DNA. At the DSB made at the *MAT* locus, Tbf1/Vid22 recruitment increases over time (Fig. 5E of the manuscript), but this does not necessarily mean that Tbf1 interacts with ssDNA, because DSB resection is an highly asynchronous process, so it is not possible to assess whether Tbf1 is bound at the DSBs thatr have been already resected or to the DSBs that have not been resected yet. In any case, our finding that Tbf1 is bound at DSB in G1 (see enclosed new Fig. 1), where resection is inhibited due to the low Cdk1 activity, indicates that Tbf1 binds double-strand DNA. This is in agreement with its role in promoting resection. Because MRX is required to resect the break, we will confirm this data by analysing Tbf1 recruitment in *mre11* mutant cells (see point 3 below). Whether Tbf1 and Vid22 remain bound to ssDNA during resection is difficult to assess and this information is not known for most (if not all) of the protein complexes involved in resection (MRX, Sae2, Sgs1, Exo1). An in vitro assay can help, but we think that it will provide limited mechanistic insights into the role of these proteins in vivo.

"or does it directly interact with the MRX complex?"

See point 5 below.

In conclusion, besides addressing the specific points raised by ref. 1 and 2 as described above, we propose the following mechanistic experiments, which can be performed in a reasonable time:

1. Assessing whether Tbf1 and Vid22 recruitment to DSBs depends on H2A phosphorylation (gamma-H2A). As Tbf1 physically interacts with histone H2A and H2B (Gavin et al., 2006 Nature), one interesting possibility is that Tbf1 and Vid22 recruitment at DSBs can be induced by gamma-H2A. Our preliminary data indicate that this may be the case, because Tbf1 association at the break site appears to decrease when H2A phosphorylation is impaired (*hta2-S129A* mutant in Fig. 2). We are in the process to repeat the same experiment with Vid22.

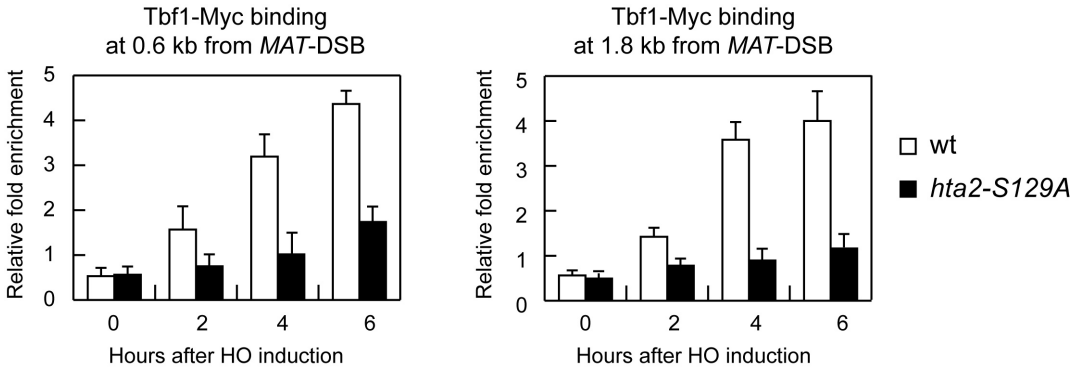

Fig. 2. Relative fold enrichment of Tbf1-Myc at the indicated distance from the HO cleavage site at the *MAT* locus in wt and *hta2-S12A* cells after ChIP with an anti-Myc antibody. The DSB was induced by galactose addition in exponentially growing cells.

2. Evaluating whether Tbf1 binding to DSB depends on Vid22 and viceversa (see also response to ref. 1 and 2). The finding that Vid22 is not present together with Tbf1 at telomeres and at some promoters raises the interesting possibility that the recruitment of Tbf1 to DSB can be promoted by the presence of Vid22. We will therefore analyze Tbf1-DSB association in *vid22* mutant cells and viceversa.
3. Assessing Tbf1 and Vid22 association to DSB in G1 and in *mre11* mutants (see preliminare data shown in Fig. 1 and response to ref. 2).
4. Epistasis analysis for the resection defect of *tbf1*, *vid22* and *mre11* mutants. Our data strongly indicate that Tbf1 and Vid22 promote MRX function and the proposed epistasis analysis should provide additional evidence that MRX does not work properly in *tbf1* and *vid22* mutants. A similar analysis was successfully performed in the past in order to dissect the interrelationships among factors involved in the resection machinery.
5. Unravelling MRX-Tbf1/Vid22 association. We will test whether Tbf1 and/or Vid22 physically interact with MRX. It is difficult to imagine whether this experiment will work or not, because other proteins (i.e. Sae2) that are known to regulate MRX do not coimmunoprecipitate with MRX, possibly because the interaction among them is labile and occurs onto DNA. In any case, it is worth trying.

We agree with the reviewer that some mechanistic details are lacking (not only for Tbf1/Vid22, but also for the entire machinery acting at DSBs). On the other hand, as these Tbf1 and Vid22 are novel regulators of DSB repair and this area is very competitive, we think that our findings, together with the information from the experiments suggested by the other reviewers and those proposed above, are sufficient to envisage a reliable model.

We estimate that performing all the experiments (including those suggested by referees 1 and 2) will require about 2-2.5 months.

1st Editorial Decision 06 September 2012

Thank you for response to the comments of our three referees on your recent submission, EMBOJ-2012-82862. I have now had a chance to consider your plans for revision, and I am pleased to say that we shall be happy to consider a manuscript incorporating such revisions further for publication in The EMBO Journal. Regarding the analysis of chromatin modifying complexes proposed by

referee 2's final point, I agree that it may not be straightforward to obtain conclusive results within a reasonable time frame. Therefore, while any such results you may be able to get could certainly strengthen the mechanistic aspects of the study, they may not strictly be required, depending on the outcome of the mechanistic analyses you proposed at the end of your letter.

I would therefore like to invite you to prepare and submit a new version of the manuscript along the proposed lines, together with a comprehensive response letter, preferentially within our standard revision period of three months. As per our EMBO Journal editorial policy, competing manuscripts published elsewhere 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. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

1st Revision - authors' response 31 October 2012

Response to referees:

Referee #1

1. In Fig. 1 and in other instances, it is hard to evaluate the degree of sensitivity in the dilution assays because the colony sizes from tbf1 mutants are so tiny compared to WT that it is hard to see if there are fewer cells surviving. Perhaps an acute exposure to MMS, followed by plating and counting colonies of comparable size would clear this up.

We have incubated cells with different amount of phleomycin or MMS for two hours and then plated them on YEPD plates to count colony-forming units. We have added this data as new Figure 1E.

2. There is clearly a defect in resection that markedly reduces success of even a "short" SSA assay. This is reflected in a very low activation of the DNA damage checkpoint protein Rad53. Do cells show a lack of checkpoint-mediated arrest?

It is difficult to detect checkpoint activation in wild type YMV45 strain after generation of the HO-induced break, because the break is rapidly repaired by SSA. We have performed a similar analysis in the JKM139 strain, where checkpoint activation is clearly detectable because the HO break at the *MAT* locus is irreparable due to the absence of the homologous donor sequences. Under these conditions, we found that *tbf1-1, tbf1-3* and *vid22*Δ mutants were defective in checkpoint-mediated cell cycle arrest. We have added this information as new Figure 4E.

3. tbf1-1 and vid22 mutants are surprisingly synthetically sensitive with rad51 or rad52 mutants to phleomycin. What does this mean? A rad52 deletion should eliminate all HR repair, so the vid22/tbf1 mutants must affect another aspect. One possibility is that NHEJ is also eliminated. It would therefore be useful to compare the vid22 rad52 mutant to dnl4 rad52. Are they comparable or is there another problem revealed by knocking out these factors (for example a problem specific to telomeres)?

We deleted *DNL4* in *tbf1-1* and *vid22*Δ cells and tested the sensitivity to phleomycin of the resulting double mutants. Deletion of *DNL4* did not exacerbate the sensitivity to phleomycin of *tbf1-1* or *vid22*Δ cells, which is consistent with the possibility that Dnl4, Tbf1 and Vid22 act in the same genetic pathway. We also found that *tbf1-1 rad52*Δ *dnl4*Δ and *vid22*Δ *rad52*Δ *dnl4*Δ triple mutants were as sensitive to phleomycin as *tbf1-1 rad52*Δ and *vid22*Δ *rad52*Δ double mutants, respectively,

indicating that the enhanced DSB sensitivity of *tbf1-1 rad52*Δ and *vid22*Δ *rad52*Δ cells compared to *rad52*Δ cells can be explained by the incapacity of the double mutants cells to perform NHEJ. Finally, we found that the sensitivity to phleomycin of *tbf1-1 rad52*Δ *dnl4*Δ and *vid22*Δ *rad52*Δ *dnl4*Δ triple mutants was even higher than that of *dnl4*Δ *rad52*Δ cells, suggesting that the roles of Tbf1 and Vid22 in genome maintenance do not rely exclusively on facilitating resection and NHEJ. We have included all these data as new Figure 5A and B.

4. Fig. 4 the small colony issue comes up here again, as I can't easily evaluate the effect of the mutants on surviving and HO cut. See also the top of Fig. 6, though there's no doubt that the double mutant combinations in Fig. 6 are quite distinct.

We have repeated the drop assay on galactose including the *tbf1-3* mutant (new Figure 6B). We do not think that an assay in liquid media is necessary, because the difference between wild type and mutant cells on galactose-containing plates is quite dramatic.

5. How is the repair efficiency normalized? The figure legend says the uncut band was monitored, but relative to what?

The repair efficiency has been calculated by evaluating the increase of the uncut band intensity at 60, 90 and 120 minutes after galactose addition relative to the same band at 30 minutes after HO induction (maximum efficiency of DSB formation). For each time point the uncut band has been normalized relative to another DNA fragment that acts as a loading control. This information was reported in Materials and methods, as we now mention in the legend of Figure 6 (see materials and methods for details).

6. Another condition that blocks resection - exo1 sgs1 - also reduces checkpoint activation but has an unexpected phenotype of promting robust telomere addition to DSBs. It it possible that the lower NHEJ efficiency is actually the result of creating new telomeres? Does overexpression of Exo1 suppress the defects of vid22/tbf1-1?

This is an interesting possibility. We have analyzed the recruitment of telomerase at the HO-induced DSB in wild type, *tbf1* and *vid22* mutants, but we did not detect any differences between wild type and mutant cells, suggesting that the lower NHEJ efficiency in *tbf1* and *vid22* is not due to telomere addition.
EXO1 overexpression was unable to suppress the sensitivity to phleomycin of *tbf1* and *vid22* mutants. This is consistent with the finding that the sensitivity to DSB-inducing agents in *tbf1* and *vid22* mutants is not exclusively due to a defective HR. We did not mention the above results in the manuscript, because they do not provide additional information to understand the role of Tbf1 and Vid22 in DSB repair.

7. Fig. 5. The data seem clear that both proteins are recruited to the vicinity of a DSB and the tbf1-1 mutant impairs Mre11 binding. As the authors note, this could explain lower resection and poor end-joining. It might be interesting to know if mre11 vid22 is more sensitive or not to phleomycin, etc.

The *mre11 vid22* double mutant seems to be more sensitive to phleomycin than each single mutant. However, this analysis is biased by the fact that *mre11 vid22* double mutant cells grow more poorly than each single mutant even in the absence of phleomycin. That's why we did not include these data in the manuscript.

8. Fig. 5. Can Vid22 be recruited to the DSB in the trf1-1 mutant or vice versa?

We found that Tbf1 and Vid22 associate to DSBs independently of each other, as recruitment of Tbf1 in *vid22*Δ cells or Vid22 in *tbf1-1* cells was as efficient as in wild type cells. We have included this data as new figure 7F and G.

9. Fig. 6. There seems to be a clear defect in nucleosome eviction for histones H2A and H3 in the tbf1-1 mutant, which again might be a reflection of resection defects (or the resection defects are a reflection of a lack of nucleosome repositioning). The authors should comment, however, on why their results are so different from those of Shroff et al. 2004, who stated that 60 min after the DSB,

"DSB Formation is Not Accompanied by Histone Depletion." This is a key point, whether there is a marked loss of nucleosomes accompanying resection and needs to be discussed.

Histone loss at DSBs has been observed in G1 also in Shim et al. (Mol Cell Biol. 2007). Shroff et al. (2004) analyzed histone eviction in G1 at the HO-induced DSB 60 minutes after DSB induction. Our experiments analyze histone removal 60, 120 and 240 minutes after HO induction and show histone removal only at later time points (120 and 240 minutes in Figure 9C). Furthermore, we analysed histone removal at the *LEU2* locus, while Shroff et al. did it at the *MAT* locus. So, it is quite difficult to compare the results obtained. In any case, as stated in the manuscript, histone removal is much more efficient in G2 than in G1. We point out in the discussion section that we cannot exclude that the slower DSB resection in *tbf1* and *vid22* mutants might contribute to the observed histone occupancy changes.

Referee #2

Major problems:
1)As stated above - it is not clear why the authors used the tbf1-1 allele rather than the more interesting tbf1-3 allele, which is only phleomycin sensitive (and not ts). While it would be too much to redo everything with the tbf1-3 allele, a few key experiments should definitely be included (e.g. the H2A ChIP in G1 and G2; the resection assay, and some double mutant studies).

We agree with the reviewer. The reason why we used mainly the *tbf1-1* mutant instead of the *tbf1-3* mutant is trivial: the search for *tbf1* mutants was repeated three times and *tbf1-1* was identified during the first screening, while *tbf1-3* was found only later, when most of the experiments had already been done with the *tbf1-1* mutant. As requested, we have repeated most of the experiments (genetic interactions, SSA, resection, NHEJ, histone removal) also in the *tbf1-3* mutant and the results (which are similar to those obtained for *tbf1-1* mutant) are shown in the following figures:
Genetic interactions with *MMS4*, *MRE11* and *SGS1* deletions: new Figure 2B
Genetic interactions with *RAD51* and *RAD52* deletions: new Figure 5
SSA: new Figure 3
Resection and checkpoint: new Figure 4A-E
NHEJ: new Figure 6A, B
Histone removal: new Figure 9A.

2)The ChiP data in Figure 5 is inconclusive as presented. As stated above, a number of anti-Myc monoclonal antibodies precipitate ssDNA with high selectivity. As ss DNA forms at the resected DSB over time - anti-Myc precipitates the DNA flanking the cut with increasing efficiency, independent of any protein being bound. Thus the only convincing control is a parallel precipitation with anti-Myc, using a strain that does not carry the Myc-tagged allele of Tbf1. It is not sufficient to do a no antibody or "beads background" control. The authors must find conditions under which anti-Myc does not precipitate the resected break, or else prove that the presence of Tbf1-Myc or Vid22-Myc increases the amount of break site precipitated over anti-Myc in a strain lacking the epitope-tagged proteins. One can avoid the sticky-ssDNA problem by washing with higher salt, including tRNA or non-yeast ssDNA in the precipitation or pre-incubating the anti-Myc beads with sheared ssDNA prior to using them in the CHIP assay- but the non-tagged control still has to be done ! Without this, the increase in "Tbf1" or "Vid22" over time could simply reflect the increase in ssDNA.

We used a CHIP protocol from Zakian lab, which was specifically developed to detect association of Myc-tagged proteins to DNA, because washes are with high salt. Nonetheless, most of our ChIP experiments with Myc-tagged protein were performed using also a strain that did not carry Myc-tagged proteins. As the background was lower than the enrichment we got with the tagged strains, we did not include these data in the previous version of the manuscript. We apologize for having underestimated the importance of this control strain, and now the ChIP data on the untagged strain are shown in new Figure 7B, C and E.

The second experiment that must be done in this context is to determine whether the two proteins bind in a manner dependent on each other. In other words: Tbf1- Myc CHIP in a vid22 delta strain, and Vid22-Myc CHP in a tbf1-1 or tbf1-3 strain.

We found that Tbf1 and Vid22 associate to DSBs independently of each other, as recruitment of Tbf1 in *vid22*Δ cells or Vid22 in *tbf1-1* cells was as efficient as in wild type cells. We have included this data as new Figure 7F and G.

Of course, many other mutants could be used for testing what recruits Tbf1. Most important would be to test whether Tbf1 recruitment occurs in the absence of resection or in a mre11 delta background.

We have analyzed Tbf1 and Vid22 recruitment in *mre11* mutant cells and we found that the lack of Mre11 does not impair Tbf1 and Vid22 loading on DSBs. We have included this data as new Figure 7F and G.
We also show that Tbf1 association at DSBs is promoted by gamma-H2A (new Figure 7F).

3)The third experiment that should be done is to directly quantify the generation of ssDNA in the two mutants (or three) with the Lydall QAOS method of quantitative ssDNA PCR (Nucleic Acids Res. 2001 Nov 1;29(21):4414-22); alternatively strand specific ssDNA Southern (e.g. Osley in Nature. 2005 Nov 17;438(7066):379-83) . The data presented "indirectly suggest" that resection is impaired, but it is quite easy to check ssDNA directly and the paper would be greatly enriched by including such results.

We used *tbf1-3* and *vid22*Δ strains where an irreparable HO-induced DSB is made at the *MAT* locus (JKM139 background) to probe a denatured Southern blot with a ssDNA sequence that is complementary to the unresected strand. This experiment showed that *tbf1-3* and *vid22*Δ cells are defective in DSB resection. We have included this data in Figure 4A-C.

Less major problems:
1)The authors show synthetic sickness/lethality between the tbf1-1 allele and mms4, mre11, and sgs1 and conclude that it has a functional interaction with HR genes, but that is also affects NHEJ. Then, depending on the background, the tbf1-1 allele either is almost lethal with rad52 delta or else viable (Fig. 2E and 3; text page 8 bottom). To what extent are all the additive effects dependent on strain background ? This is also relevant for the drop assays of double mutants with remodelers. It should at least be mentioned to what extent the effects (which are sometimes relatively minor) are background dependent.

We agree that this point was confusing in the manuscript. Moreover, we can now say that our statement that deletion of *RAD52* was lethal in the YMV45 *vid22*Δ background was wrong. Briefly, we had no problems in obtaining both *tbf1-1 rad52* and *vid22*Δ *rad52* double mutants in the W303 background by simply crossing suitable isogenic strains and dissecting tetrads. Because YMV45 strains cannot be crossed to each other, we directly deleted *RAD52* by transforming the *RAD52* disruption cassette into YMV45 *vid22*Δ and YMV45 *tbf1-1* strains. As we failed to obtain *vid22*Δ *rad52* transformants at the first attempts, we concluded that this double mutant combination was unviable. We recently repeated this gene-disruption and we obtained trustable and viable *vid22*Δ *rad52* double mutant transformants in the YMV45 background. Therefore, there is no a background-dependent effect of *RAD52* deletion in either *tbf1-1* or *vid22*Δ strains.

Finally, double mutants should also be tested between tbf1-3 and vid22 with yKu and lig4. The interaction between tbf1-1/-3 and NHEJ would be strengthen by testing yku/tbf1-1 and lig4/tbf1-1 double mutants in the assays shown in Fig. 4.

We deleted *DNL4* in both *tbf1-1* and *vid22*Δ cells and tested the sensitivity of the resulting double mutants to phleomycin. Deletion of *DNL4* did not exacerbate the sensitivity to phleomycin of either *tbf1-1* or *vid22*Δ cells (new Figure 5B), which is consistent with the possibility that Dnl4, Tbf1 and Vid22 act in the same genetic pathway. Yet, *tbf1-1 rad52*Δ *dnl4*Δ and *vid22*Δ *rad52*Δ *dnl4*Δ triple mutants were as sensitive to phleomycin as *tbf1-1 rad52*Δ and *vid22*Δ *rad52*Δ double mutants, respectively (new Figure 5B), indicating that the enhanced DSB sensitivity of *tbf1-1 rad52*Δ and *vid22*Δ *rad52*Δ double mutants compared to that of *rad52*Δ single mutant can be explained by the incapacity of the double mutants to perform NHEJ.

2)On p. 11 they show that Mre11 association with DSBs is low in G2 phase in the tbf1-1 mutant. They concluded that "this decreased binding might account for resection and the NHEJ defects

displayed by tbf1-1 and possibly vid22". Are the NHEJ defects also explained by lower Mre11 levels at DSBs in G1? This can be easily tested.

To investigate the molecular mechanism of the NHEJ defect, we have measured the association of the NHEJ factor Dnl4 at the DSB site. We found that *tbf1-1* and *vid22*Δ mutants are defective in Dnl4 binding (new Figure 6F). Since Dnl4 is required to ligate the DSB ends, its decreased binding can account for the NHEJ defect of *tbf1* and *vid22*Δ mutants.

They should also prove that Mre11 levels (and its cofactors) are not affected by tbf1 mutations (by Western blot).

We measured by western blot the levels of Mre11, Sae2, Exo1, Sgs1 and Dnl4 in *tbf1* and *vid22*Δ mutants and we found that they were similar to that found in wild type cells. Since we showed that Mre11 and Dnl4 recruitment at DSBs was defective in the *tbf1* and *vid22* mutants, we show the western blot analysis only of Mre11 (new Figure 4H) and Dnl4 (new Figure 6G).

3)Their data put Tbf1 upstream of Mre11, which is very surprising. The authors should comment on this. Tbf1 may simply promote Mre11 association to DSBs, or may affect (indirectly) its transcription, stability or its access to the break. Again, repeating the Tbf1 ChIP (with proper controls) in an mre11 mutant would be informative.

We measured the Mre11 protein level and we found that its amount is not affected in *tbf1* mutants (new Figure 4H). We think that Tbf1 and, possibly, Vid22 promote the persistence of MRX at the site of damage by creating a nucleosome-free region at the DSB ends. We have discussed this point. For Tbf1 ChIP (with proper controls) in an *mre11* mutant see response to point 2.

4)The "search for Tbf1 mutants" section could be somewhat more clearly written with a schematic of the construct used to transform yeast, and a schematic of Tbf1 in which the mutations are mapped.

We have introduced the requested schemes in new Figure 1A and B.

5)An excellent addition (possibly beyond the scope of the paper if they complete the "major additions" requested above) would be to perform ChIP for the remodelers (eg RSC, Arp8 for INO80 and SWR1 - all of which exist in epitope tagged forms) at the DSB in the vid22 or tbf1 mutants. This would show whether remodelers recruit Tbf1 or vice versa.

We started to analyse the binding of the chromatin remodelers at the DSBs in *tbf1* and *vid22* mutants. However, given that the subunits of the chromatin remodeler complexes are many and some of them perform distinct functions, this analysis will require long time and will not be included in the manuscript.

Referee #3

This reviewer raises concerns about the lack of mechanistic details on how Tbf1 and Vid22 interact and act at DSBs.

We agree with the reviewer that some mechanistic details are lacking (not only for Tbf1/Vid22, but also for the entire machinery acting at DSBs). On the other hand, as Tbf1 and Vid22 are novel regulators of DSB repair and this area is very competitive, we think that our findings, together with the information from the experiments suggested by the other reviewers, will be sufficient to envisage a reliable model.

Specific points:
"Tbf1 is presumably a site-specific DNA binding protein, so why is it recruited to DSBs? What are the frequency of TTAGGG sequences in the yeast genome?"
Tbf1 was found to bind the TTAGGG sequence because a radiolabelled TTAGGG oligonucleotide was used during the screening. Subsequent work (Preti et al., Mol Cell. 2010) has shown by CHIPseq that Tbf1 is present at snoRNA promoters, where it binds an aRCCCTaa sequence with high flexibility for the first and the last two "A" bases. Furthermore, Tbf1 is found at the promoters

of 136 genes, where it binds a similar aRCCCTaa sequence, but with an higher bias toward the first and the two last "A" bases) (Preti et al., Mol Cell. 2010). Therefore, the consensus site for Tbf1 binding appears quite flexible in terms of sequence and orientation. As the conserved core sequence "RCCCT" is present at 23.954 sites scattered into the entire *S. cerevisiae* genome, it is not difficult to find this sequence near the DSBs we generated and near DSBs randomly occurring into the genome. Importantly, although there are 23.954 "RCCCT" sequences into the genome, Tbf1 is found by CHIPseq only at 197 of these sites (Preti et al., 2010). Furthermore, Vid22 colocalizes with Tbf1 only at the promoters of non-snoRNA genes, but not at snoRNA promoters that contain the same target sequence. Altogether, these findings indicate that Tbf1/Vid22 association to DNA is driven by other proteins and/or histone modifications (Tbf1 was found to interact with histones H2A and H2B, Gavin et al., Nature, 2006). We think that a similar mechanism occurs also at the DSB, where Tbf1 recruitment is driven by histone modifications such as gamma-H2A (new Figure 7F). We have discussed in the manuscript the sequence binding specificity of Tbf1.

"how do the authors envision this protein interacting with ssDNA during resection? The ChIP data implies spreading of Tbf1 during the resection process. Does Tbf1 show high affinity for DNA ends in vitro? Or does it directly interact with the MRX complex?"
We investigated whether Tbf1 and/or Vid22 can interact with MRX by coimmunoprecipitation. Unfortunately, we failed to detect interactions between Tbf1/Vid22 with MRX.
The revised version of the manuscript now includes the following additional results regarding the mechanistic details of Tbf1 action at DSBs:

1. we show that Tbf1 and Vid22 associate to DSBs independently of each other (new Figure 7F and G).
2. we show that Tbf1 and Vid22 association to DSBs does not require the MRX complex, suggesting that Tbf1 and Vid22 proteins are recruited to DSBs independently of resection (new Figure 7F and G). Consistent with this hypothesis, we also show that Tbf1 and Vid22 association at the DSB occurs in G1, where DSB resection is inhibited (new Figure 7C).
3. we show that Tbf1 association to DSBs is facilitated by H2A phosphorylation (gamma-H2A) (new Figure 7F).
4. We show that tbf1 and *vid22*Δ mutants are defective in the loading of the NHEJ protein Dnl4 at the site of DSB (new Figure 6F).

Acceptance letter 12 November 2012

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

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

Yours sincerely,
Editor
The EMBO Journal

Referee #1

(Remarks to the Author)
The authors have carefully responded to all the reviewers' comments with a series of convincing additional experiments. I believe they have done everything necessary to assure the paper should be published.

Referee #2

(Remarks to the Author)
The revised version of the manuscript by Bonetti et al is now much improved. All of my concerns have been addressed and I therefore recommend publication.

I also believe that the authors addressed Reviewer #1's concerns appropriately. While Reviewer #3's point about the lack of biochemical data to support the extensive genetic results presented in this manuscript is a good one, I tend to agree with Bonetti et al in that these experiments would go beyond the scope of the current manuscript.

