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Lac Operator repeats generate a traceable fragile site in mammalian cells

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Transaction Report:

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

1st Editorial Decision 05 April 2011

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees acknowledge that the system you present may be a potentially useful tool to study fragile sites. However, they also point out that better evidence needs to be provided to demonstrate that the lacO array does indeed behave like a fragile site and that novel insight into the biology/dynamics of fragile sites gained with this technology should be presented as well. Both referees 1 and 3 indicate that it needs to be demonstrated that the lacO array is resulting in DSB and both also mention that for publication in EMBO reports, the manuscript should be more than a pure description of a technology. EMBO reports methods papers usually contain novel biological insight as well and given that both referees mention this point it should be addressed (either by providing novel data on the biology/dynamics of fragile sites, or by making very clear which novel insight has been gained already, if applicable).

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

This paper shows that a bacterial lacO array bound by fluorescently tagged lac repressor protein causes anaphase bridges that can be easily visualized. Consequences are deficient segregation of the lacO locus and increased presence in micronuclei. The authors make the point that this system will be a useful tool in understanding the dynamics of fragile sites in mammalian cells.
The system is simple yet elegant, and will be useful for the study of fragile sites and anaphase bridges. However, the following issues should be addressed before publication.
Main issue: The authors claim that the array behaves as a fragile site, i.e. that it breaks. This claim is overstated, as the only evidence for breakage is H2AX staining. H2AX also localizes to stalled forks, anaphase bridges were shown by Chan et al to be induced by replication stress, and the lacO array is known to stall forks. The authors should do another experiment or two to make the distinction. For example, they could try to observe chromatid breakage microscopically, or analyze for deletions of the lacO array in cells that exhibited the bridges, or stain for other proteins that localize to breaks but not stalled forks or vice versa. FANCD2 might be a good one for marking replication stress but not breakage.
Other Issues:
1. Figure 1: need to show quantification for Brac1 and Bard1. One example is not scientifically convincing.
2. Since this is not purely a methods paper, make an observation about what was learned from tracking the fragile site in living cells.
Writing Issues:
1. The rationale as presented in the last paragraph of the introduction should be improved. The link between a protein bound array and natural fragile sites is not clear as written. Also, is the size of the lacO array comparable to the size of the FRA3B region lacking an origin? It is not clear to me how the Letessier et al. study mentioned provided a rationale for these experiments (especially since it was just published).
2. aphidicolin is spelled incorrectly.
3. Sentence at end of first paragraph and the one starting with "Interestingly,..." in the second paragraph do not make sense as written.

Referee #2:

Jacome and colleagues present the interesting observation that lacO repeats in mammalian cells behave like fragile sites. This observation is important as it will provide a very useful tool for the community.

The paper is very well crafted and the conclusions reached are based on solid data. I only have minor comments.

1- The paper would be stronger if the authors could determine if the anaphase bridges observed represent ultrafine bridges (UFBs). Those usually co-localize with PICH and BLM.

2-In the introduction, the authors should also mention that telomere are now also considered to be fragile sites.

Referee #3:

The manuscript by Jacome and Fernandez-Capetillo describes the use of a pre-existing cell line containing multiple integrated repeats of the lac generator sequence, coupled with fluorescently tagged lac repressor, to follow the fate of what is described as a fragile site during mitosis. The system is without doubt very neat, and the applications of the technology are several. However, the manuscript has some weaknesses that diminish my enthusiasm for it.

Specifically:

1. There are 2 fundamental problems as I see it:

a) The manuscript is essential a description of a technology. There is no real biology added to it. While the technology is very nice, it is doubtful if this represents a sufficient enough advance for publication in EMBO Reports.
b) Throughout the manuscript the system is described as being a 'fragile site'. While this is probably what the authors set out to create, no serious evidence is presented that the locus is fragile in the way in which fragile sites are classically defined. Such analysis is important to show. Seeing g-H2AX foci at the locus does not indicate fragility necessarily. Furthermore, some fragile loci don't show g-H2AX foci in interphase or metaphase.

2. The manuscript is very light on data and feels more like the start of a project than the conclusion.

3. I find it quite curious that aphidicolin treatment generates only a relatively modest increase in 'expression' of their fragile site. Generally, fragility is hard to detect in unstressed cells and increases several fold following aphidicolin treatment. I realize that it is assumed that this construction generates constitutive problems, but it cannot be too severe or the cells wouldn't proliferate. Why doesn't aphidicolin make the situation much worse? Do the authors have an explanation for this?

4. The data on anaphase bridging and micronucleus formation are rather superficial. First, Figure 3 shows an anaphase bridge, but this stains with DAPI. The fragile sites associated anaphase bridges reports previously were of the type not stained by DAPI. Have the authors quantitated all bridges as being DAPI-staining or not?

For the micronuclei, there is an indication what the level of micronucleus formation is in the lacO containing cells versus those lacking lacO. Most micronuclei clearly do not form from the lacO region (95% don't in the absence of aphidicolin, and nor does the lagging chromosome shown in Fig. 3). Because micronuclei can contain several chromosomes, it is not surprising, therefore, that lacR foci are seen in some micronuclei. That would be expected by chance.

5. The Fanconi anemia proteins FANCI and D2 were shown previously to define the location of fragile sites. Have the authors looked for these proteins? Why was BRCA1 analyzed instead?

1st Revision - authors' response 21 June 2011

To the referees of our MS:

We thank our colleagues for their overall positive comments on our manuscript. We believe that the inclusion of the new experiments suggested in your review has significantly improved the MS. The detailed responses follow.

Referee #1

> This paper shows that a bacterial lacO array bound by fluorescently tagged lac repressor protein causes anaphase bridges that can be easily visualized. Consequences are deficient segregation of the lacO locus and increased presence in micronuclei. The authors make the point that this system will be a useful tool in understanding the dynamics of fragile sites in mammalian cells. The system is simple yet elegant, and will be useful for the study of fragile sites and anaphase bridges. However, the following issues should be addressed before publication.

Thanks for your words in the system.

> Main issue: The authors claim that the array behaves as a fragile site, i.e. that it breaks. This claim is overstated, as the only evidence for breakage is H2AX staining. H2AX also localizes to stalled forks, anaphase bridges were shown by Chan et al to be induced by replication stress, and the lacO array is known to stall forks. The authors should do another experiment or two to make the distinction. For example, they could try to observe chromatid breakage microscopically, or analyze for deletions of the lacO array in cells that exhibited the bridges, or stain for other proteins that localize to breaks but not stalled forks or vice versa. FANCD2 might be a good one for marking replication stress but not breakage.

Agreed, and this is why we also tried other markers like Brca1 and Bard1. We now also show FANCD2 foci as requested, which also mark the array. This was an important point since, as pointed by the reviewer, FANCD2 can mark the fragile sites previous to the breakage. In what relates to the actual breakage, we have now also performed FISH analyses that revealed the presence of frequent breaks involving the lacO insertion.

> Other Issues:1. Figure 1: need to show quantification for Brac1 and Bard1. One example is not scientifically convincing.

Done. This and all other foci data are now quantified and explicitly mentioned in the text.

> 2. Since this is not purely a methods paper, make an observation about what was learned from tracking the fragile site in living cells.

We introduced this video to illustrate the fact that the anaphase bridges concur with significant movements/stretching that could create tensions at the fragile site DNA. This, I believe, is interesting since it illustrates the mechanical forces that can impinge on fragile site sequences in mitosis. In addition, we thought it would be nice to provide an example that illustrates that the system is amenable for live cell imaging of anaphase bridges containing the lacO. For all of the above, which is now better explained in the text, we decided to leave the video as part of our submission and to share it with the readers. Nevertheless, if the reviewer does not find it particularly informative, we would be happy to leave it out of the MS.

> Writing Issues:1. The rationale as presented in the last paragraph of the introduction should be improved. The link between a protein bound array and natural fragile sites is not clear as written. Also, is the size of the lacO array comparable to the size of the FRA3B region lacking an origin? It is not clear to me how the Letessier et al. study mentioned provided a rationale for these experiments (especially since it was just published). 2. aphidicolin is spelled incorrectly.3. Sentence at end of first paragraph and the one starting with "Interestingly,..." in the second paragraph do not make sense as written.

The MS has now been rewritten to address these and other comments on the writing.

Referee #2

> Jacome and colleagues present the interesting observation that lacO repeats in mammalian cells behave like fragile sites. This observation is important as it will provide a very useful tool for the community.The paper is very well crafted and the conclusions reached are based on solid data. I only have minor comments.

We thank this reviewer for his very generous words on our work.

> 1. The paper would be stronger if the authors could determine if the anaphase bridges observed represent ultrafine bridges (UFBs). Those usually co-localize with PICH and BLM.

The anaphase bridges that contain lacO sequences frequently show DAPI within the bridge. However, we are actually not sure whether DAPI positive bridges and UFBs are completely independent entities. For instance, BLM was shown to limit both DAPI positive and DAPI negative bridges (Chan et al, 2007). Therefore, I don´t know to what extent these events can be considered fully distinct. In agreement with this idea, and even though lacO bridges contain DAPI, we now show that they also collocalize with PICH. Given the fragility of the locus that we now formally see with FISH, I believe that this phenomenon is not fundamentally distinct to that reported for fragile sites. We now explicitly discuss this in the MS.

Importantly, this part of the work also led us to discover the presence of PICH foci at the lacO locus in interfase cells, which I believe is another interesting observation that can be learned from our MS.

> 2-In the introduction, the authors should also mention that telomere are now also considered to be fragile sites.

Done.

Referee #3

> The manuscript by Jacome and Fernandez-Capetillo describes the use of a pre-existing cell line containing multiple integrated repeats of the lac generator sequence, coupled with fluorescently tagged lac repressor, to follow the fate of what is described as a fragile site during mitosis. The system is without doubt very neat, and the applications of the technology are several. However, the manuscript has some weaknesses that diminish my enthusiasm for it.

We thank this reviewer for his words on the system. I hope that our current version addresses his concerns.

> Specifically:1. There are 2 fundamental problems as I see it: a) The manuscript is essential a description of a technology. There is no real biology added to it. While the technology is very nice, it is doubtful if this represents a sufficient enough advance for publication in EMBO Reports.

I acknowledge that the previous version of the MS was written with a methodological focus. With the current enthusiasm on anaphase bridges as the origin of fragile site breakage my original idea was indeed to provide a powerful tool for further studies in the field. In addition, many researchers are now using lacO/lacR-based systems to bring factors to chromatin, and I believed that it was important to reveal that this sequence has inherent fragility. With that being said, we have now also added two new pieces of information that I hope have made the MS a stronger piece of work for *EMBO Reports*.

> (a) First, we have used this system to show that the PICH helicase localizes to the lacO array in interphase cells. Given the wide use of PICH antibodies as a marker of anaphase bridges, we believe these data support that PICH might have additional roles on the biology of fragile sites beyond mitosis.

> (b) Several oncogenes have been shown to induce RS. We hypothesized that the presence of an oncogene could therefore increase the instability of the lacO locus. We now show that this is the case, and we provide evidence to show that the Myc oncogene increases the number of anaphase bridges and micronuclei containing the lacO locus. To our knowledge, this is the first time to be shown that oncogenes can promote the formation of anaphase bridges or micronuclei containing fragile site sequences.

We hope that these data, plus the description of the system itself as a new tool for the study of fragile sites in mammalian cells, make now our report strong enough for its publication.

> b) Throughout the manuscript the system is described as being a 'fragile site'. While this is probably what the authors set out to create, no serious evidence is presented that the locus is fragile in the way in which fragile sites are classically defined. Such analysis is important to show. Seeing g-H2AX foci at the locus does not indicate fragility necessarily. Furthermore, some fragile loci don't show g-H2AX foci in interphase or metaphase.

Agreed, and this is why we also tried other markers like Brca1 and Bard1. In what relates to the actual breakage, we have now also performed FISH analyses that revealed the presence of frequent breaks involving the lacO insertion.

> 2. The manuscript is very light on data and feels more like the start of a project than the conclusion.

I hope our previous answers address this concern.

> 3. I find it quite curious that aphidicolin treatment generates only a relatively modest increase in 'expression' of their fragile site. Generally, fragility is hard to detect in unstressed cells and increases several fold following aphidicolin treatment. I realize that it is assumed that this construction generates constitutive problems, but it cannot be too severe or the cells wouldn't proliferate. Why doesn't aphidicolin make the situation much worse? Do the authors have an explanation for this?

Our interpretation of this is that this site is indeed very fragile. In this context, the fact that the constitutive fragility is that big, allows for little enhancing with other reagents. I guess this is somewhat similar to what can be observed in BLM cells, for instance. Levels of fragility in BLM cells are already constitutively very large, so that a treatment with aphidicolin does not increase this as much as it does in a wild type cells.

> 4. The data on anaphase bridging and micronucleus formation are rather superficial. First, Figure 3 shows an anaphase bridge, but this stains with DAPI. The fragile sites associated anaphase bridges reports previously were of the type not stained by DAPI. Have the authors quantitated all bridges as being DAPI-staining or not? For the micronuclei, there is an indication what the level of micronucleus formation is in the lacO containing cells versus those lacking lacO. Most micronuclei clearly do not form from the lacO region (95% don't in the absence of aphidicolin, and nor does the lagging chromosome shown in Fig. 3). Because micronuclei can contain several chromosomes, it is not surprising, therefore, that lacR foci are seen in some micronuclei. That would be expected by chance.

The anaphase bridges that contain lacO sequences frequently show DAPI within the bridge. However, we are actually not sure whether DAPI positive bridges and UFBs are completely independent entities. For instance, and whereas much emphasis has now been placed on DAPI-less DNA bridges, the

reality is that BLM also localizes to and suppresses DAPI-containing anaphase bridges (see Chan, KL *et al* EMBO J 2007). Thus, it is possible that those events are not necessarily unlinked. In agreement with this idea, and even though lacO bridges contain DAPI, we now show that they also collocalize with PICH. Given the fragility of the locus that we now formally see with FISH, I believe that this phenomenon is not fundamentally distinct to that reported for fragile sites. We now explicitly discuss this in the MS. Importantly, this part of the work also led us to discover the presence of PICH foci at the lacO locus in interfase cells, which I believe is another interesting observation that can be learned from our MS.

In what regards to the MN, the reality is that this is perhaps the most greatly enhanced phenotype in response to aphidicolin, and the increase in the % of MN that contain lacO can hardly be explained by chance. Actually, in our hands this is the easiest way to look at “fragility”, since in contrast to the very transient nature of anaphase bridges, MN accumulate with time. We are not making this point anyway, and just share it with this reviewer within the context of this letter.

> 5. The Fanconi anemia proteins FANCI and D2 were shown previously to define the location of fragile sites. Have the authors looked for these proteins? Why was BRCA1 analyzed instead?

The reason to look at BRCA1/Bard1 was only to show the distribution of other markers of DNA breakage, not necessarily linked to fragile sites. However, the FANCD2 foci are indeed very informative to put our work in context with the recent works on fragile sites. We have now included the fact that FANCD2 foci are also present at the lacO locus. We thank this reviewer for this suggestion.

2nd Editorial Decision 08 July 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

You can continue with the process of acceptance. I include important information about how to proceed at the end of this email. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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

Yours sincerely,

Editor
EMBO Reports