THE
EMBO
JOURNAL

Manuscript EMBO-2017-97630

Whole chromosome loss and associated breakage-fusion-bridge cycles transform mouse tetraploid cells

Rozario Thomas, Daniel H Marks, Yvette Chin & Robert Benezra

Review timeline:

Submission date:	20 June 2017
Editorial Decision:	02 August 2017
Revision received:	02 October 2017
Editorial Decision:	19 October 2017
Revision received:	25 October 2017
Accepted:	07 November 2017

Editor: Hartmut Vodermaier

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 02 August 2017

Thank you for submitting your manuscript on the consequences of whole-chromosome loss in tetraploid cells to The EMBO Journal. Three expert referees have now assessed and discussed the manuscript, with their reviews copied below for your information. As you will see, the referees consider the topic of the study important and the results and conclusions interesting and potentially significant as well. At the same time, they do however raise a number of substantial concerns regarding essentially two aspects, (a) decisive validation of the system and (b) understanding of the underlying mechanisms by which whole chromosome loss is tumorigenic. In their further pre-decision discussions, the referees noted that a good start towards the latter could be a comparative transcriptome analysis combined with tumor suppressor and oncogene mapping according to Davoli et al 2013, focussing further on factors encoded on lost chromosomes. However, the referees also agreed that while such analyses would add considerable value to the study, the overriding key issue to address would be validating whether the observed phenotypes in the MEFs are directly due to clonal chromosome losses (and not other driving events), as detailed specifically in the reports by reviewers 1 and 2.

In this light, should you be able to adequately validate the system according to the concerns and suggestions of these referees, we shall be happy to consider a revised manuscript further for publication. Any additional insights into the underlying mechanisms would be highly valuable, but shall not be essential within the scope of this revision. I should nevertheless point out that it is our policy to allow only a single round of major revision, making it important to diligently respond to all the raised points during this round. When revising the manuscript, please also reorganize the current 4-figure presentation in order to capitalize on the more extended format of an EMBO Journal article (additional guidelines on preparing revisions and figure presentation can be found below).

Should you have any additional questions/comments regarding the referee reports or the revision requirements, please do not hesitate to get in touch with me ahead of resubmission. If needed, we might also arrange for an extended revision period, during which time the publication of any competing work elsewhere would have no negative impact on our final assessment of your own study.

Thank you again for the opportunity to consider this work for The EMBO Journal. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In this manuscript, Thomas et. al. investigated the effect of whole chromosome loss on tumorigenesis in the context of a tetraploid state. The authors made use of a Cre-recombinase-mediated system to induce targeted chromosome loss in murine tetraploid MEFS. The obtained cell lines were further characterized (chromosome complement, growth properties, DNA damage, metabolic changes and genomic stability) and tested for their tumorigenic potential in vivo in an allograft mouse model. Importantly, whole genome sequencing data showed the early effects of their strategy, confirming a copy number variation of at least part of the targeted chromosome (Chromosome 9, 10, 12 or 14). Importantly, 3 out of 4 cell lines showed the ability to form tumors in immunocompromised mice. Based on the differential effects between the cell lines on the phenotypes following the induced-aneuploidy the authors conclude that both general chromosome loss effects, as well as chromosome specific effects likely contribute to tumorigenic potential in this model system.

Although aneuploidy has been implicated in tumor formation for decades, much debate still surrounds its precise role. This paper implies a causative link between of the loss of a chromosome in a tetraploid background and tumor formation in mice. This is an important finding which could help us to understand how an abnormal karyotype could contribute to tumorigenesis. However, there are several problems with the experimental data reported in the current form of the manuscript. Firstly, the authors do not provide a robust proof of whole chromosome loss in the later stages of their cell lines, thereby complicating the interpretation of their results. Furthermore, although the authors attempted to understand the mechanism behind the increased tumorigenic potential caused by chromosome loss, the provided explanations are unsatisfactory and do not provide a clear insight on the mechanisms involved in the acquired tumorigenic ability. Thus, in its current format, I do not support publication in EMBO Journal.

Major issues:
1) Validation of their system:
The authors perform WGS to determine the copy number variation only on the early stage cells (short after Cre induction). After these short time points, the authors could only show a full loss for Ch 12 but only loss of the distal part for Ch9, Ch10 and Ch14. To determine the CNV of later time points (importantly the time of injection in the allograft experiments), the authors only perform karyotyping. With karyotyping it is easy to overlook chromosome fragments that fused to other chromosomes. Something that can occur after BFB-cycles instead of losing the chromosome. The only way to proof loss of the whole chromosome over time is by performing WGS/CNV at later time points and I don't understand why the authors did not do this experiment in the first place. Importantly, after some short culturing, it seems that the effects on Ch 9 and 10 are reverted at least in part of the cells (Figure 1D versus S1A). The authors do not comment on this. Possibly, there is a small contamination of 'parental' cells in the population that starts to take over so by the time of injection this effect could even be more prominent. Again, emphasizing the importance of performing the CNV analysis just prior to injection. Alternatively, FISH probes of both distal and proximal parts of the targeted chromosomes would at least confirm a complete loss of the targeted chromosome although this would not provide information on the residual chromosomes.
2) The authors need to provide more detailed and quantitative data on the evolution of the karyotypes: The authors determined increased levels of genomic instability at late passages of the

cell lines, by quantification of average chromosome loss, structural rearrangements and marker chromosomes per metaphase. They suggest that these ongoing changes could facilitate adaptation to stresses of anchorage independent growth and contribute to the so called mutator phenotype (although it is not sufficient). The data in figure S2 suggests that besides random events, also some recurrent events are occurring in the cells. However, they only show the examples for ch9. Is this data also available for the other lines? It would be important to show this data for the other cell lines too as well as showing the other chromosomes in the tumor explant examples in Figure S3A to obtain a more complete view on karyotype evolution. In both cases, CNV analysis would already give an idea of the reccurent, penetrant changes.
3) Despite their attempts to understand the mechanism behind the increased tumorigenic potential upon chromosome loss, they fail to find a solid explanation. Only in specific cases the chromosome loss per se is sufficient, suggesting that chromosome specific events are determining the outcome. Their data is further complicated by the fact that loss of Ch12, which does result in increased CIN and DNA damage, does not display increased tumorigenic potential. Although the authors acknowledge this, they still spent a large part of their discussion on the phenotypic changes involving CIN and DNA damage as a possible drivers. Unfortunately, the lack of a solid mechanistic explanation remains a big caveat in the story.

Specific points:
- Further explanation of the system and the constructs used is required. Are the cells immortalized before or after targeting of the recombination system? Thus, do the cells bear 2 copies of the targeted chromosome? If so, Fig.1B does not faithfully model the recombination situation and it would also explain in part the relative inefficient success of the desired recombination event.
- Important information about the process of making the cell line is lacking. How long are the cells in culture before the experiments were done? Where are the exact locations of the lox P sites? Were they located on the break site reflected in the CNV analysis?
- Why did some cell lines require 14 days of Cre induction whereas others only 18h (is the Tamoxifen system less efficient?)? During these 14 days there could already be some karyotype evolution so this is an important difference.
- Why does the cell size increase of the recombined population?
- Why did the authors not sort GFP- on top of hCD2-? If right after recombination there is not such a population yet, could this GFP minus have been sorted out on the early stage population already sorted out for hCD2 minus?
- Which mechanism could explain how dicentric chromosomes that undergo BFB are eventually completely lost?
- The authors describe the cell populations over time as "early" and "late". These vague terms should be specified for a better understanding of the evolution occurred in each cell line.
- WGS/CNV of the early passages compared to the ones sequenced right after exposure to Cre-recombinase show a reduction of the aneuploid subpopulation. If this is explained a selective pressure and the targeted cells overgrown by parental ones, how can this population evolve to a more severe effect on copy number variation?
- By the results in mice after injecting early passage cell lines, the authors conclude that the targeted chromosome loss is largely sufficient (in 2 out of 4) to promote tumor formation, although the sequenced population shows for both cases only a distal part of the chromosome is lost. This conclusion should be reformulated.
- Also, the CNV in Fig S1 indicates that other events are happening although they do not seem to be fully penetrant (yet?). For example, Ch7 in the Ch14 and Ch9 cells is clearly lost in a subset of cells. This should not be ignored.
- In Fig. S2, the authors claim that the most penetrant events involve the targeted chromosome. However, this seems not to be the case regarding chromosome 4, which displays (by counting) an abnormal copy number in 2 control cells (10%) vs. 18 ICL cells (90%). This should be emphasized. It also raises the question whether this is a general event after chromosome loss (co-loss of chromosome 4) or specific to chromosome 9 or even specific to this specific clone (also see point below).
- An additional strong point to support the loss of chromosome 9, 10 or 14 as a driver of tumorigenesis in tetraploid murine cells, would be the data (tumorigenic potential alongside their evolved karoytypes) showing similar results obtained by the use of different MEFs (biological replicates). Although the text includes such description, the authors do not show data (mistaken reference to figure 2B?).
- A table summarizing the detailed quantification of the karyotypes of all cell lines would strongly

support and nicely complement the hypothesis of increased CIN in these late populations but would also uncover recurrent events that might follow after chromosome loss, also on other chromosomes (for example the loss of ch 4 in the ch 9 line).
- In Fig S3B, the karyotype of the cells derived from the tumor explants had of course undergone many more cell divisions as their parental controls, as they did not expand in vivo. Would the karyotype of the parentals also change upon longer times of proliferation?
- 3 out of 4 cell lines with chromosome losses display increased tumorigenic potential. Curiously, this is specific to the cell lines that do not seem to lose the full chromosome (at least at early time points). The one cell line (Ch 12) that seems to lose the full chromosome at early stages, is the one that does not have increased tumorigenic potential. So possibly, the BFB-cycle that occurs after the recombination event is the actual oncogenic driver. Could the authors comment on that?

Referee #3:

Chromosomal instability (CIN) and the resulting aneuploidy are hallmark features of cancer cells. While many mouse models have been generated to investigate the contribution of CIN to tumorigenesis, one important limitation is that these models all relied on (partial) inactivation or overexpression of genes involved in the regulation of faithful chromosome segregation that might also have other roles in the cell. In this study, Thomas et al made use of an elegant in vitro system to generate aneuploid cells in which one particular chromosome loss event can be selected for. They conclude that while all forced chromosome losses result in reduced proliferation potential in vitro, that some chromosome losses do confer a malignant fate upon the MEFs when xenografted. Furthermore, the authors show evidence that the aneuploid MEFs exhibit CIN and increased DNA damage which in part contributes to the adoption of the malignant fate in some lines, but not others. Overall, the data in this paper is convincing and the model is certainly elegant, but there are, in my opinion, a few concerns that the authors need to address before the study is suitable for publication.

The authors explain their system well. The only aspect that is not clear to me is how the MEFs become tetraploid. MEFs have an intrinsic tendency to become tertraploid, but typically this does not affect the whole culture. Is this because of the LargeT immortalization? Do the authors in their later FACS sorting experiments specifically select for cells with a tetraploid DNA content? Did the authors attempt to grow the diploid fraction as well and then select for loss of chromosomes in these or is this too toxic? The authors should explain this (and substantiate this with data) in a revised manuscript.

Analogous to this point: the authors state that no tumors arise from a p19-/- immortal MEF line when chrs. 10 or 14 are deleted, but do not show this data: this data should be presented in the manuscript, especially as this involves diploid MEFs, which would make this data an important addition to the paper as it would provide further evidence that cells would need to go through tetraploidization before they become transformed.

The authors show that some chromosome losses lead to transformation, while others do not. How many individual MEF lines were generated per individual chromosome loss? This is not completely clear to me from the main text or description of the methods and should therefore be better described either in the main text or methodology section. If the authors derived one MEF line per chromosome and injected this in multiple mice, the authors should generate at least two more biological replicates (i.e. individual MEF lines) to show that these chromosome loss events specifically promote tumorigenesis.

Related to this: the authors report that in their system chromosome losses coincide with breakage-fusion-bridge (BFB) events, both of which could contribute to the observed transformation. If the authors want to solely attribute this to chromosome loss events, they should show that these the BFB events are indeed lost in their cell lines and not only refer to earlier published work (Zhu et al, 2010). This is especially relevant, as the CGH data does suggest partial losses (and thus BFB), while the karyotypes show indeed aneuploidy, suggesting that both BFB and aneuploidy coincide. Alternatively, the authors could conclude that both BFB and aneuploidy contribute to the phenotype.

One page 8, the authors state: "There are multiple reasons that we attribute the tumor phenotype we

observe to the loss of the targeted chromosome and not to other sporadic basal chromosome variations in these cells." If I understand correctly, all the karyotypes presented in Figure 2 are karyotypes from the MEF cultures prior to injections. It would be very helpful to also display the karyotypes from the tumor cells, to compare the CIN rate between the primary, the ICL and the transformed ICL cells. These CIN rates can then also be reported in Fig. 5.

In addition to the MEF data, the authors also provoke chromosome loss in the hematopoietic system and show selective depletion of the CD2- population. In this case, information on the karyotype distribution of cells early after Cre activation (30 days) and late after Cre activation (80 days) is missing. Authors should add some karyotyping data for these experiments (for instance interphase FISH) to show that Chr.10 monosomic cells are present 30 days post-tamoxifen (70% or so) and selectively depleted at 80 days post-tamoxifen.

Referee #4:

The article of Rosarion Thomas and colleagues addresses the tumourigenic potential of aneuploid cells. Gain or loss of whole chromosomes have been associated with tumourigenic potential for many years, however work performed in many labs has also put in evidence that certain aneuploid cells can have decreased proliferation capacity.
In this article, the authors generated chromosome losses and addressed their consequences by injecting these cells in nude mice. Out of 4 independent chromosome losses, 3 appear to be able to lead to large tumours in vivo..

This is an interesting work that fuels the field in showing once more the tumourigenic capacity of aneuploid cells, but the originality of this paper is related with its focusing on analysing chromosome losses specifically. However, as stated by the authors in the title and in the text, this happens in a background where tetraploidy was previously induced. So in the end, we do not know whether it is a combination of tetraploidy and specific chromosome losses that generate these phenotypes or whether chromosome loss in a diploid background would lead to the same outcome. This is particularly important in light of the considerable chromosome instability already noticed in controls.

Nevertheless I consider that the work is well done and generates novel concepts worth to be considered and so I would recommend publication. I just have a suggestion related with Figure 4, where I cannot see the signal for g-H2av.
Can the authors provide images of better quality?

Also the authors desctibe the presence of caspase 3 positive cells, but in the end I did not understand whether this is related with cell cell death, or any other function. Can they conclude it is cell death and not other function as it has been already described for other caspase activation?

1st Revision - authors' response 02 October 2017

Thank you for submitting your manuscript on the consequences of whole-chromosome loss in tetraploid cells to The EMBO Journal. Three expert referees have now assessed and discussed the manuscript, with their reviews copied below for your information. As you will see, the referees consider the topic of the study important and the results and conclusions interesting and potentially significant as well. At the same time, they do however raise a number of substantial concerns regarding essentially two aspects, (a) decisive validation of the system and (b) understanding of the underlying mechanisms by which whole chromosome loss is tumorigenic. In their further pre-decision discussions, the referees noted that a good start towards the latter could be a comparative transcriptome analysis combined with tumor suppressor and oncogene mapping according to Davoli et al 2013, focussing further on factors encoded on lost chromosomes. However, the referees also agreed that while such analyses would add considerable value to the study, the overriding key issue to address would be validating whether the observed phenotypes in the MEFs are directly due to clonal chromosome losses (and not other driving events), as detailed specifically in the reports by reviewers 1 and 2.

In this light, should you be able to adequately validate the system according to the concerns and suggestions of these referees, we shall be happy to consider a revised manuscript further for publication. Any additional insights into the underlying mechanisms would be highly valuable, but shall not be essential within the scope of this revision. I should nevertheless point out that it is our policy to allow only a single round of major revision, making it important to diligently respond to all the raised points during this round. When revising the manuscript, please also reorganize the current 4-figure presentation in order to capitalize on the more extended format of an EMBO Journal article (additional guidelines on preparing revisions and figure presentation can be found below).

We would like to thank the editor for an encouraging appraisal of our work. To further validate our chromosome loss system, we have now provided additional data showcasing the loss of the targeted chromosomes in two biological replicates for all four chromosome lines (**Figures 3, 4 and EV1 and Table 1**). These data clearly show that, taken together, the only consistently significant event in all the ICL lines (including all the replicates of all the 4 different chromosome lines) is the respective targeted chromosome loss. Importantly, the copy number change of the targeted chromosome, at the earliest possible time point, segregates faithfully with the tumor forming phenotype, indicating a causative role for the targeted chromosome loss in this process.

To further strengthen the validation of our model, we have now included the shallow pass whole genome sequencing data (WGS) of the late passage ICL and control MEFs (**Figure EV3**), similar to the WGS data of the no-passage and early passage MEFs shown in the original manuscript.
Also, we have now included the complete karyotypes of the tumor explants from the mice (**Figure EV2**), to show that they these explants harbor additional chromosomal aberrations, on top of the targeted loss.

Regarding the other main concern, we agree with the reviewers and the editor that the mechanism by which the initial chromosome loss is transforming the MEFs is an extremely important unanswered question. While this is an important aspect, and we definitely plan on pursuing this mechanistic question in the future, we feel that it is beyond the scope of the manuscript at this stage. In light of us having addressed almost all of the other concerns that the reviewers raised, we hope that the editor and the reviewers would view our revised manuscript favorably and as being substantially improved over the original submission.

Referee #1:

In this manuscript, Thomas et. al. investigated the effect of whole chromosome loss on tumorigenesis in the context of a tetraploid state. The authors made use of a Cre-recombinase-mediated system to induce targeted chromosome loss in murine tetraploid MEFS. The obtained cell lines were further characterized (chromosome complement, growth properties, DNA damage, metabolic changes and genomic stability) and tested for their tumorigenic potential in vivo in an allograft mouse model. Importantly, whole genome sequencing data showed the early effects of their strategy, confirming a copy number variation of at least part of the targeted chromosome (Chromosome 9, 10, 12 or 14). Importantly, 3 out of 4 cell lines showed the ability to form tumors in immunocompromised mice. Based on the differential effects between the cell lines on the phenotypes following the induced-aneuploidy the authors conclude that both general chromosome loss effects, as well as chromosome specific effects likely contribute to tumorigenic potential in this model system.

Although aneuploidy has been implicated in tumor formation for decades, much debate still surrounds its precise role. This paper implies a causative link between of the loss of a chromosome in a tetraploid background and tumor formation in mice. This is an important finding which could help us to understand how an abnormal karyotype could contribute to tumorigenesis. However, there are several problems with the experimental data reported in the current form of the manuscript. Firstly, the authors do not provide a robust proof of whole chromosome loss in the later stages of their cell lines, thereby complicating the interpretation of their results. Furthermore, although the authors attempted to understand the mechanism behind the increased tumorigenic potential caused by chromosome loss, the provided explanations are unsatisfactory and do not provide a clear insight on the mechanisms involved in the acquired tumorigenic ability. Thus, in its current format, I do not support publication in EMBO Journal.

Major issues:

1) Validation of their system:
The authors perform WGS to determine the copy number variation only on the early stage cells (short after Cre induction). After these short time points, the authors could only show a full loss for Ch 12 but only loss of the distal part for Ch9, Ch10 and Ch14. To determine the CNV of later time points (importantly the time of injection in the allograft experiments), the authors only perform karyotyping. With karyotyping it is easy to overlook chromosome fragments that fused to other chromosomes. Something that can occur after BFB-cycles instead of losing the chromosome. The only way to proof loss of the whole chromosome over time is by performing WGS/CNV at later time points and I don't understand why the authors did not do this experiment in the first place.

We thank the reviewer for this valuable suggestion, which will solidify our findings. Accordingly, we have now performed shallow whole genome sequencing on the late passage MEFs and added the results to the manuscript **(Page 9, Line 7 and Figure EV3)**. While only distal portions are lost in the early passage MEFs, in the late passage MEFs, portions along the entire length of chromosomes 9 and 14 appear to have been lost. Ch12 ICLs, similar to the earlier WGS results, displayed a copy number loss along the entire length of chromosome 12. In late passage Ch10 ICL however, only the central portion of chromosome 10 is lost. The most likely explanation is, after initial distal chromosome 10 loss, during subsequent passaging, chromosome 10 may be subjected to a series of chromosomal rearrangements and copy number changes leading to specific depletion of the central region. In addition, we observed aberrant copy number profiles of other non-targeted chromosomes like chromosomes 4,6,12 etc.

Irrespective of these downstream aberrations at later passage, we are confident that the targeted chromosome loss was the first event and the additional genomic redistributions are secondary to the original event. Importantly, these subsequent events likely contribute to the acquisition of transforming potential of induced chromosome 10 loss.

Importantly, after some short culturing, it seems that the effects on Ch 9 and 10 are reverted at least in part of the cells (Figure 1D versus S1A). The authors do not comment on this. Possibly, there is a small contamination of 'parental' cells in the population that starts to take over so by the time of injection this effect could even be more prominent. Again, emphasizing the importance of performing the CNV analysis just prior to injection. Alternatively, FISH probes of both distal and proximal parts of the targeted chromosomes would at least confirm a complete loss of the targeted chromosome although this would not provide information on the residual chromosomes.

Figure 1D and S1A (in the old manuscript) or **Figure 1D and 2A** (in the revised manuscript) are two separate experiments – independent Cre treatment regimens and FACS sorting setups. The difference in the copy number variation between these two figures can be explained by stochastic nature of the inverted Cre recombination and the variation in efficiency of the Cre recombinase itself. It must be noted that both of these cells would eventually lead to whole chromosome losses upon additional mitoses. Also, we have performed additional WGS analyses also, as recommended by the reviewer (see previous point).

2) The authors need to provide more detailed and quantitative data on the evolution of the karyotypes: The authors determined increased levels of genomic instability at late passages of the cell lines, by quantification of average chromosome loss, structural rearrangements and marker chromosomes per metaphase. They suggest that these ongoing changes could facilitate adaptation to stresses of anchorage independent growth and contribute to the so called mutator phenotype (although it is not sufficient). The data in figure S2 suggests that besides random events, also some recurrent events are occurring in the cells. However, they only show the examples for ch9. Is this data also available for the other lines? It would be important to show this data for the other cell lines too as well as showing the other chromosomes in the tumor explant examples in Figure S3A to obtain a more complete view on karyotype evolution. In both cases, CNV analysis would already give an idea of the reccurent, penetrant changes.

The reviewer raises a very important point and we have now provided the complete karyotypes for two independent biological replicates each, for all the 4 chromosome lines (late passages) and a

summary table for one replicate each **(Figures 3,4 and EV1 and Table 1).** Based on these replicate analyses, we conclude that, at later passages, in addition to harboring the targeted chromosome losses, there are additional non-targeted chromosomal copy number variations, as a result of the extended passaging. However, from our early passage WGS data, it is clear that the copy number loss of the targeted chromosome is the initial event.

As per the reviewer's suggestion, we have also performed shallow whole genome sequencing on the late passage MEFs (similar to the WGS already performed in early stage MEFs) and are including that data (**Figure EV3**, see earlier point).

Also, we have included the tumor karyotypes of all the chromosomes in each ICL line (and the controls), to give a global snapshot of chromosome copy numbers **(Figure EV2)**. It should be noted that Ch9 control cells did not yield tumors and therefore for the Ch9 line, the tumor karyotype is provided only for the Ch9 ICL. These tumor karyotypes show, that as the ICL cells grow *in vivo*, their karyotypes evolve to harbor both the targeted chromosome loss and other non-targeted aberrations. While, in the tumor karyotypes of the Ch14 Control, chromosome 14 appears to be triploid, prior to injection in mice, chromosome 14 (in the Ch14 control) is largely tetraploid. This suggests that the karyotype of the Ch14 controls is also evolving *in vivo*. However, this was not sufficient to robustly transform the Ch14 control cells. Thus, the initial targeted chromosome 14 loss is necessary along with the subsequent chromosomal instability to transform.

3) Despite their attempts to understand the mechanism behind the increased tumorigenic potential upon chromosome loss, they fail to find a solid explanation. Only in specific cases the chromosome loss per se is sufficient, suggesting that chromosome specific events are determining the outcome. Their data is further complicated by the fact that loss of Ch12, which does result in increased CIN and DNA damage, does not display increased tumorigenic potential. Although the authors acknowledge this, they still spent a large part of their discussion on the phenotypic changes involving CIN and DNA damage as a possible drivers. Unfortunately, the lack of a solid mechanistic explanation remains a big caveat in the story.

We acknowledge the caveat that the reviewer raises and agree that the complete mechanism tying the chromosome loss to increased tumorigenesis is lacking in this manuscript. We wish to state that the observation that a targeted chromosome loss leads to enhanced tumorigenic potential is an important finding and we intend to continue to conduct further research to more fully describe a comprehensive mechanism to explain this result. We are not proposing that increased chromosome instability and DNA damage are sufficient to induce tumorigenesis (as evidenced by the Ch12 result), but that they may contribute in the context of specific induced chromosome losses.

Specific points:
- Further explanation of the system and the constructs used is required. Are the cells immortalized before or after targeting of the recombination system? Thus, do the cells bear 2 copies of the targeted chromosome? If so, Fig.1B does not faithfully model the recombination situation and it would also explain in part the relative inefficient success of the desired recombination event.

Prior to any experimentation, the primary MEFs are first immortalized, rendering them tetraploid. These tetraploid MEFs are then subjected to Cre recombination to yield the ICL cells. We used a diploid system in Figure 1B so as to provide a simplified version of the recombination procedure and the system can be expanded accordingly to reflect a tetraploid scenario, where two homologs of a chromosome have the loxP site and the other two homologs are wildtype.

- Important information about the process of making the cell line is lacking. How long are the cells in culture before the experiments were done? Where are the exact locations of the lox P sites? Were they located on the break site reflected in the CNV analysis?

We thank the reviewer for bringing this important detail to our attention. Accordingly we have now made this addition to the methods section of the manuscript (**Page 19, Line 10 and Page 20, Line 2**). Briefly, immediately after the primary MEFs were isolated and cultured from the embryos, they were transfected with a plasmid containing SV40 Large T antigen (as described in the Materials and Methods). Once the transfected MEFs attained 90% confluence, they were split from one well of a 6

well plate entirely into a 10cm dish. After they attained 90% confluence in the 10 cm dish, they were split at a ratio of 1:10 into another 10cm dish (passage dilution factor 10). This procedure was serially repeated until the passage dilution factor became 10^6. At this point, the primary MEFs successfully transfected with the SV40 Large T antigen were immortalized, while the primary cells were out competed (as they senesced) (**Page 19, Line 10**). 'Early passage' MEFs denotes 2 weeks post sort and 'Late passage' denotes 4 weeks post sort (**Page 20, Line 2**).

Also, we are attaching the locations of the iLoxP construct on all the 4 chromosomes and the break point in the WGS data (distal loss of the chromosomes in WGS) corresponds accurately with the location of the loxP site on all four chromosomes. (**Page 6, Line 11 and Appendix Figure S1B**).

- Why did some cell lines require 14 days of Cre induction whereas others only 18h (is the Tamoxifen system less efficient?)? During these 14 days there could already be some karyotype evolution so this is an important difference.

The Ch10 and Ch14 mice, in addition to the inverted loxP sites, have the tamoxifen responsive Cre recombinase allele - *RERT*^{n}) whereas the Ch9 and Ch12 do not have this allele. This dictated the use of either 4 hydroxy tamoxifen or an Adeno-Cre. We have performed experiments using Adeno-Cre on Ch10 and Ch14 MEFs and found consistent results as previously generated using 4OHT. This is now clarified in the methods section (**Page 19, Line 20**).

- Why does the cell size increase of the recombined population?

The reviewer is correct in that the side scatter is higher for the MEFs treated with 4OHT in Figure 1C. This phenomenon is not observed uniformly in all the FACS experiments and we therefore attribute it to batch variations in the FACS setup. We have now included this explanation in the text (**Page 5, Line 11**). Accordingly we are attaching a FACS plot where the side scatter does not vary between the ethanol treated and the 4OHT treated **(Appendix Figure S1A)**

- Why did the authors not sort GFP- on top of hCD2-? If right after recombination there is not such a population yet, could this GFP minus have been sorted out on the early stage population already sorted out for hCD2 minus?

While we acknowledge that this is a very important point, there is a technical difficulty that prevented us from using the GFP for any sorting purposes. There is a transcriptional repression of the GFP gene from the adjacent hCD2 promoter, rendering the GFP unreliable (Zhu, Y et al., (2010) J Biol Chem. 2010 Aug 20;285(34):26005-12). Thus hCD2 was utilized for all the sorting experiments.

- Which mechanism could explain how dicentric chromosomes that undergo BFB are eventually completely lost?

The dicentric fragment can (1) be lost at the metaphase plate itself (without being segregated into either daughter cell), (2) segregate to one daughter cell (where it undergoes these same fate options in the next round of mitosis) or (3) get attached to both the spindle poles and break randomly into the daughter cells. In the third fate, these broken fragments would have exposed telomere free ends which leads to fusion (between the exposed ends of the chromatids) and then breakage again as the two centromeres are then pulled apart from the opposite poles (Breakage-Fusion-Bridge (BFB) cycle). Successive BFB cycles would lead to a stepwise reduction in the size of the broken chromosome. We are not attributing the loss of the fragment to any one particular mechanism.

- The authors describe the cell populations over time as "early" and "late". These vague terms should be specified for a better understanding of the evolution occurred in each cell line.

We thank the reviewer for making this important suggestion and accordingly we have now included this detail in the methods section of the manuscript. Briefly, 'Early passage' denoted 2 weeks post sort and 'Late passage' denotes 4 weeks post sort. (**Page 20, Line 2**)

- WGS/CNV of the early passages compared to the ones sequenced right after exposure to Cre-

© European Molecular Biology Organization

recombinase show a reduction of the aneuploid subpopulation. If this is explained a selective pressure and the targeted cells overgrown by parental ones, how can this population evolve to a more severe effect on copy number variation?

Figure 1D and S1A (in the old manuscript) or **Figure 1D and 2A** (in the revised manuscript) are two separate experiments – independent Cre treatments and FACS sorting setups. The difference in the copy number variation between these two figures can be explained by stochastic nature of the inverted Cre recombination and the variation in efficiency of the Cre recombinase itself. It must be noted that both of these cells would eventually lead to whole chromosome losses upon additional mitoses.

- By the results in mice after injecting early passage cell lines, the authors conclude that the targeted chromosome loss is largely sufficient (in 2 out of 4) to promote tumor formation, although the sequenced population shows for both cases only a distal part of the chromosome is lost. This conclusion should be reformulated.

To address this concern, for these early passages, we have now stated explicitly that the loss of distal portion of the chromosomes is sufficient to initiate tumors. (**Page 7, Line 10**). It should be noted that the loss of distal portion eventually leads to whole chromosome loss as evidenced by the karyotyping of the late passage MEFs.

- Also, the CNV in Fig S1 indicates that other events are happening although they do not seem to be fully penetrant (yet?). For example, Ch7 in the Ch14 and Ch9 cells is clearly lost in a subset of cells. This should not be ignored.

We have now made changes in the text to reflect this observation (**Page 6, Line 18**)

- In Fig. S2, the authors claim that the most penetrant events involve the targeted chromosome. However, this seems not to be the case regarding chromosome 4, which displays (by counting) an abnormal copy number in 2 control cells (10%) vs. 18 ICL cells (90%). This should be emphasized. It also raises the question whether this is a general event after chromosome loss (co-loss of chromosome 4) or specific to chromosome 9 or even specific to this specific clone (also see point below).

The reviewer is correct in that this Ch9 ICL line harbored a high percentage of cells with losses of chromosome 4. We have since edited the text of the manuscript to accurately reflect this observation (**Page 8, Line 4**). However, this change appears to be specific to this clone as in another independent clone that was karyotyped, chromosome 4 levels in the Ch9 ICLs did not change (**Figure EV 1**). Also, we have now provided karyotypes of two replicates of each of the late passage ICL lines (**Figures 3,4 and EV1 and Table 1**). Taken together, the only event that is consistently significant in all the replicates of all the different ICL lines is the copy number loss of the respective targeted chromosome.

- An additional strong point to support the loss of chromosome 9, 10 or 14 as a driver of tumorigenesis in tetraploid murine cells, would be the data (tumorigenic potential alongside their evolved karoytypes) showing similar results obtained by the use of different MEFs (biological replicates). Although the text includes such description, the authors do not show data (mistaken reference to figure 2B?).

We have now shown karyotypes of two biological replicates of each of the late passage ICL lines (**Figures 3,4 and EV1 and Table 1)**. Also, we have since updated the methods section to reflect the use of biological replicates (**Page 19, Line 17**). Briefly, 2 independent biological replicates (completely separate MEF lines followed by independent immortalization and FACS sorting procedures) were used for the Ch12 ICL lines and similarly 3 biological replicates were used for Ch10, Ch14, and Ch9 lines.

- A table summarizing the detailed quantification of the karyotypes of all cell lines would strongly support and nicely complement the hypothesis of increased CIN in these late populations but would

also uncover recurrent events that might follow after chromosome loss, also on other chromosomes (for example the loss of ch 4 in the ch 9 line).

We thank the reviewer for making this insightful observation and accordingly we have provided a table with the copy number profiles of each chromosome in all the four late passage lines (please refer to point 2 earlier for discussion and **Table 1**)

- In Fig S3B, the karyotype of the cells derived from the tumor explants had of course undergone many more cell divisions as their parental controls, as they did not expand in vivo. Would the karyotype of the parentals also change upon longer times of proliferation?

While the reviewer raises a very interesting experiment, we feel that this is beyond the scope of this manuscript, as it would warrant doing the tumor injection experiments again and allowing the control tumors to undergo more cell divisions followed by karyotyping. This suggestion is a long-term experiment, which we would take into account for future studies in our lab.

- 3 out of 4 cell lines with chromosome losses display increased tumorigenic potential. Curiously, this is specific to the cell lines that do not seem to lose the full chromosome (at least at early time points). The one cell line (Ch 12) that seems to lose the full chromosome at early stages, is the one that does not have increased tumorigenic potential. So possibly, the BFB-cycle that occurs after the recombination event is the actual oncogenic driver. Could the authors comment on that?

We thank the reviewer for raising this important point. We want to reiterate that we do not attribute the tumor phenotype solely to the chromosome losses. On the contrary, we hypothesize that it is in fact a combination of chromosome losses **and** BFB induced instability that drives the observed tumorigenesis in our model. It is difficult to parse out the contribution of these two processes – whole chromosome losses and BFB mediated instability – to the transforming potential.
Also, our model is clinically relevant since BFB cycles are a common cause of genomic redistributions and genomic instability in human tumors.

Referee #3:

Chromosomal instability (CIN) and the resulting aneuploidy are hallmark features of cancer cells. While many mouse models have been generated to investigate the contribution of CIN to tumorigenesis, one important limitation is that these models all relied on (partial) inactivation or overexpression of genes involved in the regulation of faithful chromosome segregation that might also have other roles in the cell. In this study, Thomas et al made use of an elegant in vitro system to generate aneuploid cells in which one particular chromosome loss event can be selected for. They conclude that while all forced chromosome losses result in reduced proliferation potential in vitro, that some chromosome losses do confer a malignant fate upon the MEFs when xenografted. Furthermore, the authors show evidence that the aneuploid MEFs exhibit CIN and increased DNA damage which in part contributes to the adoption of the malignant fate in some lines, but not others. Overall, the data in this paper is convincing and the model is certainly elegant, but there are, in my opinion, a few concerns that the authors need to address before the study is suitable for publication.

The authors explain their system well. The only aspect that is not clear to me is how the MEFs become tetraploid. MEFs have an intrinsic tendency to become tertraploid, but typically this does not affect the whole culture. Is this because of the LargeT immortalization? Do the authors in their later FACS sorting experiments specifically select for cells with a tetraploid DNA content? Did the authors attempt to grow the diploid fraction as well and then select for loss of chromosomes in these or is this too toxic? The authors should explain this (and substantiate this with data) in a revised manuscript.

We thank the reviewer for bringing up this critical point. Immediately after the primary MEFs were isolated and cultured from the embryos, they were transfected with a plasmid containing SV40 Large T antigen (as described in the Materials and Methods). Once the transfected MEFs attained 90% confluence, they were split from one 6 well entirely into a 10cm dish. After they attained 90% confluence in the 10 cm dish, they were split at a ratio of 1:10 into another 10cm dish (passage dilution factor 10). This procedure was serially repeated till the passage dilution factor becomes 10^6,

by when the primary MEFs that have successfully been transfected with the SV40 Large T antigen, were immortalized, while the primary cells were out competed (as they senesced). Primary MEFs that are immortalized as a result of this procedure have been shown to become tetraploid (Hein J et al.,J Virol. 2009 Jan;83(1):117-27; Lionnet, T et al.,Nat Methods. 2011 Feb;8(2):165-70) (as described in the text in **Page 4, Line 22**). We have also clarified the immortalization procedure in detail in the methods section (**Page 19, Line 8**).

The entire population is tetraploid as a result of this culturing protocol and we did not use FACS to sort for the tetraploid content alone. Karyotyping analysis of these MEFs showed that all the cells are indeed tetraploid. As mentioned in the manuscript, we have performed experiments to test the consequences of chromosomes losses in diploid MEFs. MEFs immortalized using short hairpins against p19 are known to be diploid in early passages (Randle DH et al., Proc Natl Acad Sci U S A. 2001 Aug 14;98(17):9654-9; Kamijo T et al., Cell. 1997 Nov 28;91(5):649-59; Zindy F et al., Oncogene. 1997 Jul 10;15(2):203-11). These references have now been added to the manuscript. (**Page 12, Line 8).** Loss of chromosomes 10 and 14 in this diploid context, while not being toxic, did not yield tumors in the diploid MEFs suggesting that tetraploidy is necessary. This tumor data is now included in the manuscript (**Page 12, Line 9 and Appendix Figure S4).**

Analogous to this point: the authors state that no tumors arise from a p19-/- immortal MEF line when chrs. 10 or 14 are deleted, but do not show this data: this data should be presented in the manuscript, especially as this involves diploid MEFs, which would make this data an important addition to the paper as it would provide further evidence that cells would need to go through tetraploidization before they become transformed.

As mentioned in the previous point, loss of chromosomes 10 and 14 in this diploid context did not yield tumors in the diploid MEFs. This tumor data is now included in the manuscript, showing that both the controls and ICLs (for both Ch10 and Ch14) do not form tumors (**Page 12, Line 9 and Appendix Figure S4).**

Regarding the karyotypes of the sh-p19 immortalized MEFs, established literature exists that these sh-p19 MEFs are diploid (Randle DH et al., Proc Natl Acad Sci U S A. 2001 Aug 14;98(17):9654-9; Kamijo T et al., Cell. 1997 Nov 28;91(5):649-59; Zindy F et al., Oncogene. 1997 Jul 10;15(2):203-11). We have now included these references in the manuscript (**Page 12, Line 8**). The chromosome loss in this case was confirmed by hCD2 marker loss in FACS analysis.

Additionally, we have other data in the manuscript that highlights the importance of the tetraploid context for losing chromosomes in tumorigenesis:

1) The diploid ICLs being outcompeted by the control cells in the peripheral blood cells **(Figure 7A).**
2) The increased rate of chromosome losses in a tetraploid context in human solid tumors based on analysis of human tumor data in the Mitelman Database (**Figures 7B and C).**

The authors show that some chromosome losses lead to transformation, while others do not. How many individual MEF lines were generated per individual chromosome loss? This is not completely clear to me from the main text or description of the methods and should therefore be better described either in the main text or methodology section. If the authors derived one MEF line per chromosome and injected this in multiple mice, the authors should generate at least two more biological replicates (i.e. individual MEF lines) to show that these chromosome loss events specifically promote tumorigenesis.

We have since updated the methods section to reflect the use of biological replicates. Briefly, 2 independent biological replicates (completely separate MEF lines followed by independent immortalization and FACS sorting procedures) were used for the Ch12 ICL lines and similarly 3 biological replicates were used for Ch10, Ch14, and Ch9 lines. Representative results are provided in the manuscript from these replicates. (**Page 19, Line 17**)

Related to this: the authors report that in their system chromosome losses coincide with breakage-fusion-bridge (BFB) events, both of which could contribute to the observed transformation. If the

authors want to solely attribute this to chromosome loss events, they should show that these the BFB events are indeed lost in their cell lines and not only refer to earlier published work (Zhu et al, 2010). This is especially relevant, as the CGH data does suggest partial losses (and thus BFB), while the karyotypes show indeed aneuploidy, suggesting that both BFB and aneuploidy coincide. Alternatively, the authors could conclude that both BFB and aneuploidy contribute to the phenotype.

We thank the reviewer for raising this important point. We want to reiterate that we do not attribute the tumor phenotype solely to the chromosome losses. On the contrary, we hypothesize that it is in fact a combination of chromosome losses **and** BFB induced instability, which often precedes chromosome losses, that drives the observed tumorigenesis in our model. It is difficult to parse out the contribution of these two processes – whole chromosome losses and BFB mediated instability – to the transforming potential. Also, our model is clinically relevant since BFB cycles are common cause of genomic redistributions and genomic instability in human tumors.

One page 8, the authors state: "There are multiple reasons that we attribute the tumor phenotype we observe to the loss of the targeted chromosome and not to other sporadic basal chromosome variations in these cells." If I understand correctly, all the karyotypes presented in Figure 2 are karyotypes from the MEF cultures prior to injections. It would be very helpful to also display the karyotypes from the tumor cells, to compare the CIN rate between the primary, the ICL and the transformed ICL cells. These CIN rates can then also be reported in Fig. 5.

We have now included the karyotypes from the tumor cells of the Ch10 and Ch14 controls and ICLs (**Figure EV2**). Since the Ch9 controls did not form any tumors, we do not have those karyotypes. These tumor karyotypes show, that as the ICL cells grow *in vivo*, their karyotypes evolve to harbor both the targeted chromosome loss and other non-targeted aberrations. While, in the tumor karyotypes of the Ch14 Control, chromosome 14 appears to be triploid, prior to injection in mice, chromosome 14 (in the Ch14 control) is largely tetraploid. This suggests that the karyotype of the Ch14 controls is also evolving. However, this was not sufficient to robustly transform the Ch14 control cells. Thus, the initial targeted chromosome 14 loss is necessary along with the subsequent chromosomal instability to transform.

Also, as per the reviewer's suggestion, we have done analyses on these tumor karyotypes to obtain the chromosomal instability readouts for the Ch10 and Ch14 control and ICLs. This chromosomal instability analysis was not possible for the Ch9 tumors as there were no Ch9 control tumors to compare. These changes are also added in the text (**Page 15, Line 3 and Figure EV 4**).

In addition to the MEF data, the authors also provoke chromosome loss in the hematopoietic system and show selective depletion of the CD2- population. In this case, information on the karyotype distribution of cells early after Cre activation (30 days) and late after Cre activation (80 days) is missing. Authors should add some karyotyping data for these experiments (for instance interphase FISH) to show that Chr.10 monosomic cells are present 30 days post-tamoxifen (70% or so) and selectively depleted at 80 days post-tamoxifen.

We had previously unsuccessfully attempted to obtain this karyotype data from the peripheral blood cells due to technical challenges (difficulties to get the blood cells to cycle *in vitro* to obtain metaphases). However, we are confident that the hCD2 marker losses observed in our system translates to chromosome losses based on

1) The MEFs data where the marker losses lead to targeted chromosome losses.
2) Data from the study by Zhu, Y et al., (2010) J Biol Chem. 2010 Aug 20;285(34):26005-12, showing chromosomes losses occurring as a result of inverted Cre recombination.
3)

Referee #4:

The article of Rosarion Thomas and colleagues addresses the tumourigenic potential of aneuploid cells. Gain or loss of whole chromosomes have been associated with tumourigenic potential for many years, however work performed in many labs has also put in evidence that certain aneuploid cells can have decreased proliferation capacity.

In this article, the authors generated chromosome losses and addressed their consequences by injecting these cells in nude mice. Out of 4 independent chromosome losses, 3 appear to be able to lead to large tumours in vivo.

This is an interesting work that fuels the field in showing once more the tumourigenic capacity of aneuploid cells, but the originality of this paper is related with its focusing on analysing chromosome losses specifically. However, as stated by the authors in the title and in the text, this happens in a background where tetraploidy was previously induced. So in the end, we do not know whether it is a combination of tetraploidy and specific chromosome losses that generate these phenotypes or whether chromosome loss in a diploid background would lead to the same outcome. This is particularly important in light of the considerable chromosome instability already noticed in controls.

We thank the reviewer for the positive appraisal of our work. Regarding the reviewer's concern about the tetraploidy context, as mentioned in the manuscript (**Page 18, Line 8**), we feel that tetraploidy is in fact a clinically relevant system to model chromosome losses. The chromosome losses were tumorigenic only in the context of tetraploidy as the same chromosome losses in a diploid background did not yield tumors. We also note that the chromosome instability observed in the controls (in the absence of targeted chromosome loss) was not sufficient to transform the MEFs, suggesting a critical role for the induced chromosome losses.

Nevertheless I consider that the work is well done and generates novel concepts worth to be considered and so I would recommend publication. I just have a suggestion related with Figure 4, where I cannot see the signal for g-H2av.
Can the authors provide images of better quality?

We are grateful to the reviewer for recommending our work for publication. Accordingly, we have provided better quality figures to detect H2AX in **Figure 4**

Also the authors describe the presence of caspase 3 positive cells, but in the end I did not understand whether this is related with cell cell death, or any other function. Can they conclude it is cell death and not other function as it has been already described for other caspase activation?

The increase in cleaved caspase 3 is not associated with apoptosis as no cell death was observed in culture. This sub-lethal caspase 3 activation has been known to cause additional DNA damage and contributes to the overall genomic instability of the ICL cells as described by Liu X et al., 2015 (Mol Cell. 2015 Apr 16;58(2):284-96). Thus in our system, this partial caspase 3 activation is another path to generating chromosomal instability, endowing the ICL cells with tumorigenic properties.

2nd Editorial Decision 19 October 2017

Thank you for submitting your revised manuscript to The EMBO Journal. Referees 1 and 3 have now looked at it once more, and I am pleased to say that both of them consider the key experimental and conceptual concerns they initially raised well-addressed. However, both of them feel that the in light of the additional clarifications, the original title of the paper is not fully appropriate, and that changes to the text (in particular abstract and conclusions) would also be warranted before eventual acceptance. Given these highly unanimous opinions detailed below, I am therefore returning the manuscript to you once more, with the invitation to briefly respond to the remaining comments and to incorporate them into the title, abstract and text as appropriate.

Once we will have received your final version adequately addressing these remaining issues, we should hopefully be able to swiftly proceed with formal acceptance and production of the paper!

REFEREE REPORTS

Referee #1:

The authors have now successfully addressed most of my major and minor concerns facilitating the interpretation of the obtained results. They have now included WGS/CNV, karyotypes and biological replicates, as well as text clarifications and summary data tables to illustrate their working system.

Overall, the data in this manuscript shows interesting and valuable findings helping to understand the tumorigenic capacity of aneuploid cells in a tetraploid background. However, I still have one concern related to the message of the paper.

CNV sequencing of the late passages suggests that the targeted chromosomes (except for Ch12) are not lost as a whole chromosome and are likely to have undergone considerable rearrangements. Importantly, such rearrangements or translocations are not always detectable by karyotyping of the chromosome spreads and may be interpreted as losses although not all the genetic material is lost (compare CNV data to spread karyotyping of the late passage samples). Therefore, the current title is not covering the data and requires adjustment (there is no evidence presented that the loss of whole chromosomes drives the tumorigenic potential, but rather the introduction of chromosome rearrangements and BFB-cycles, resulting in loss of genetic material). Although the authors acknowledge this in the revised version in the discussion, it requires more attention and adjustment of the title and the text.

If this issue can be fixed I recommend publication.

Referee #3:

The authors have added a substantial number of experiments to solidify the data reported in the first version of this manuscript. While most of my concerns have been addressed, there is one important remaining issue.

While the authors clearly agree with reviewer 1 and 3 that BFB events (and the resulting structural abnormalities observed for chromosomes 9, 10 and 14) are likely to contribute to the transformation phenotype, the title and abstract and conclusion only mention whole chromosome loss from a tetraploid cell as a newly identified driver for transformation. While this might be true for chromosome 12 loss, I am not fully convinced whether this is true for the other 3 tested chromosomes. For these chromosomes, the BFB events are most likely the driving events. The authors argue that the substantial karyotyping they performed argues that whole chromosome loss has taken place. Indeed, the targeted chromosomes appear to be lost in the metaphase spreads, but many new aneuploidies arose in the cancer cells as well, albeit most significantly for the targeted chromosomes. However, as structural abnormalities are virtually impossible to detect with metaphase spreads (bits and pieces of chromosomes might have gotten lost in the spreads), and as the late passage WGS is arguing structural abnormalities instead of whole chromosome loss, I find the conclusion that whole chromosome loss of chrs. 9, 10 and 14 are driving tumorigenesis still not fully supported, or at least not the key driver.

In fact, the authors very nicely phrase this in their rebuttal letter: "We want to reiterate that we do not attribute the tumor phenotype solely to the chromosome losses. On the contrary, we hypothesize that it is in fact a combination of chromosome losses and BFB induced instability that drives the observed tumorigenesis in our model. It is difficult to parse out the contribution of these two processes - whole chromosome losses and BFB mediated instability - to the transforming potential. Also, our model is clinically relevant since BFB cycles are a common cause of genomic redistributions and genomic instability in human tumors."

I therefore believe that this more balanced view of their findings should also come back in the manuscript, which might warrant rephrasing of the title, abstract and conclusion, but also in other parts of the text: for instance at the end of page 6 they argue: "Accordingly, when the ICL (and the control cells) are expanded further in culture for subsequent experiments (see next section), we observe the loss of entire chromosomes in all four lines." Unless I misunderstand the new

© European Molecular Biology Organization

sequencing data, this is not what the sequencing data is suggesting (rather structural abnormalities instead).

Overall, I still do think this is a relevant and well-performed study, and I do favour publication in EMBO journal, but I also strongly recommend to rephrase the title, abstract and some sentences in the main text to provide a more balanced view of the data.

2nd Revision - authors' response 25 October 2017

Thank you for submitting your revised manuscript to The EMBO Journal. Referees 1 and 3 have now looked at it once more, and I am pleased to say that both of them consider the key experimental and conceptual concerns they initially raised well-addressed. However, both of them feel that the in light of the additional clarifications, the original title of the paper is not fully appropriate, and that changes to the text (in particular abstract and conclusions) would also be warranted before eventual acceptance. Given these highly unanimous opinions detailed below, I am therefore returning the manuscript to you once more, with the invitation to briefly respond to the remaining comments and to incorporate them into the title, abstract and text as appropriate.

We would like to thank the editor for a positive assessment of our manuscript. We have accordingly changed the title of the manuscript to read **"Whole chromosome loss and associated breakage-fusion-bridge cycles transform mouse tetraploid cells"** and also made the recommended changes in the abstract and in the main text of the manuscript (see below)

Referee #1:

The authors have now successfully addressed most of my major and minor concerns facilitating the interpretation of the obtained results. They have now included WGS/CNV, karyotypes and biological replicates, as well as text clarifications and summary data tables to illustrate their working system.
We thank the reviewer for all the insightful comments on the earlier version of our manuscript and for approving our responses to their concerns.

Overall, the data in this manuscript shows interesting and valuable findings helping to understand the tumorigenic capacity of aneuploid cells in a tetraploid background. However, I still have one concern related to the message of the paper.

CNV sequencing of the late passages suggests that the targeted chromosomes (except for Ch12) are not lost as a whole chromosome and are likely to have undergone considerable rearrangements. Importantly, such rearrangements or translocations are not always detectable by karyotyping of the chromosome spreads and may be interpreted as losses although not all the genetic material is lost (compare CNV data to spread karyotyping of the late passage samples). Therefore, the current title is not covering the data and requires adjustment (there is no evidence presented that the loss of whole chromosomes drives the tumorigenic potential, but rather the introduction of chromosome rearrangements and BFB-cycles, resulting in loss of genetic material). Although the authors acknowledge this in the revised version in the discussion, it requires more attention and adjustment of the title and the text.

If this issue can be fixed I recommend publication.

We thank the reviewer for recommending our manuscript for publication and for raising an important point. Accordingly we have now changed the title of the paper to read **"Whole chromosome loss and associated breakage-fusion-bridge cycles transform mouse tetraploid cells".**

We have also made other changes throughout the manuscript to stress the importance of the breakage-fusion-bridge mediated instability in tumorigenesis. These changes are summarized in italics below:

© European Molecular Biology Organization

1) Abstract: “Here we used a Cre recombinase-mediated chromosome loss strategy to individually delete mouse chromosomes 9, 10, 12 or 14 in tetraploid immortalized murine embryonic fibroblasts. *This methodology also involves the generation of a dicentric chromosome intermediate which subsequently undergoes a series of breakage-fusion-bridge (BFB) cycles”*

2) Abstract: “Thus, these studies demonstrate a causative role for whole chromosome loss *and the associated BFB mediated instability* in tumorigenesis and may shed light on the early consequences of aneuploidy in mammalian cells.”

3) Page 4: “Here, we show that *a combination of losing individual chromosomes and a series of breakage-fusion-bridge (BFB) cycles* in a tetraploid background induces further genetic instability and drives tumorigenesis in a mouse allograft model.”

4) Page 6: “This can be explained by the fact that the hCD2 marker (used to FACS sort), which is present on the distal portion on the chromosome (Figure 1A), is lost initially, followed by *additional rounds of BFB cycles in subsequent cell cycles*. Accordingly, when the ICL (and the control cells) are expanded further in culture for subsequent experiments (see next section), we observe *that portions along the entire length of chromosomes 14 and 9 appear to have been lost in Ch14 and Ch9 ICLs respectively (Figure EV3). Ch10 ICL cells, upon further expansion, displayed a loss only in the central portion of chromosome 10 (Figure EV3) (discussed in next section).”*

5) Page 7: Deleted “At later time points as expected, a higher proportion of the ICL cells exhibit chromosome 10 losses (see next section)”

6)Page 7: “Indeed, as shown in Figure 2A, compared to the control cells, *complete loss (Ch12) or distal loss (Ch9, Ch10 and Ch14) of the targeted chromosome* was the only prominent event in the ICL cells.”

7) Page 7: Deleted “by the targeted chromosome loss” in “Importantly, neither of these ICL lines showed enhanced growth potential in vitro, suggesting that the advantages conferred on the ICL cells by the targeted chromosome loss are manifested only in certain contexts, such as the stressed environment in the mice (Appendix Figure S2).”

8) Page 7: “It should be noted that the loss of distal portion *of chromosomes 9 and 14 eventually leads to losses across the entire lengths of these chromosomes* in late passage MEFs (see next section).”

9) Page 9: “Irrespective of these downstream aberrations at later passage, we are confident that the *copy number changes in the* targeted chromosome loss was the first event and the additional genomic redistributions are secondary to the original event.”

10) Page 10: There are multiple reasons that we attribute the tumor phenotype we observe to the *copy number variations in* the targeted chromosome and not to other sporadic basal chromosome variations in these cells.

11) Page 11: “Taken together, given that the tumor phenotypes of at least two ICL lines (9 and 14) were observed prior to the accrual of any non-targeted chromosomal anomalies, argues strongly that these targeted chromosomal *aberrations* were the driving event in the acquisition of tumorigenicity. We cannot rule out the possibility that Ch10 ICL, which acquired tumor potential only after longer passaging, had suffered other events that facilitated their transformation. We suspect that the initial *distal* loss of Chr10 must at least be contributing to the tumor phenotype since, in the 4 different Ch10 lines examined, only the ICLs (and never the control cell lines), after extensive passaging, ever acquire tumorigenic potential.

12) Page 14: “For these reasons we sought to determine if the induced aneuploid cells had evidence of further genomic instability *beyond changes in the targeted chromosomes.”*

13) Page 15: “In this study, we have successfully adapted the iLoxP inverted recombination system to generate an aneuploidy model, inducing targeted chromosome losses *and generating dicentric*

fragments, in the context of a tetraploid state, without perturbing the expression of a specific protein."

14) Page 16: "Taken together, these results demonstrate that the initial loss of the targeted chromosome *along with the genomic instability associated with the BFB cycles of the dicentric fragments (see later section for the discussion on BFB mediated genomic rearrangements)* is critical for these tumor phenotypes."

15) Page 16: "We also observed that the initial *copy number variation in the targeted* chromosome, irrespective of the identity of the *targeted* chromosome, induced a significant increase in chromosomal instability, DNA damage and secondary chromosome losses."

16) Page 18: "This benefit of chromosome *copy number reductions* from a tetraploid genome is consistent with previous data from other groups."

17) Conclusion Page 19: "In summary, our ICL model demonstrates that individual chromosome loss events, *along with BFB mediated chromosomal instability*, in a tetraploid background can be a potent driver of tumorigenesis in mouse cells and provides a new platform to further our understanding of the consequences of whole chromosome loss events in cancer."

Referee #3:

The authors have added a substantial number of experiments to solidify the data reported in the first version of this manuscript.
We are thankful to the reviewer for a favorable evaluation of the additional experiments reported in the revised manuscript.

While most of my concerns have been addressed, there is one important remaining issue.

While the authors clearly agree with reviewer 1 and 3 that BFB events (and the resulting structural abnormalities observed for chromosomes 9, 10 and 14) are likely to contribute to the transformation phenotype, the title and abstract and conclusion only mention whole chromosome loss from a tetraploid cell as a newly identified driver for transformation. While this might be true for chromosome 12 loss, I am not fully convinced whether this is true for the other 3 tested chromosomes. For these chromosomes, the BFB events are most likely the driving events. The authors argue that the substantial karyotyping they performed argues that whole chromosome loss has taken place. Indeed, the targeted chromosomes appear to be lost in the metaphase spreads, but many new aneuploidies arose in the cancer cells as well, albeit most significantly for the targeted chromosomes. However, as structural abnormalities are virtually impossible to detect with metaphase spreads (bits and pieces of chromosomes might have gotten lost in the spreads), and as the late passage WGS is arguing structural abnormalities instead of whole chromosome loss, I find the conclusion that whole chromosome loss of chrs. 9, 10 and 14 are driving tumorigenesis still not fully supported, or at least not the key driver.

In fact, the authors very nicely phrase this in their rebuttal letter: "We want to reiterate that we do not attribute the tumor phenotype solely to the chromosome losses. On the contrary, we hypothesize that it is in fact a combination of chromosome losses and BFB induced instability that drives the observed tumorigenesis in our model. It is difficult to parse out the contribution of these two processes - whole chromosome losses and BFB mediated instability - to the transforming potential. Also, our model is clinically relevant since BFB cycles are a common cause of genomic redistributions and genomic instability in human tumors."

We thank the reviewer for bringing up this critical point in our manuscript and we have now addressed this issue by making various changes to the title, abstract and the text of the manuscript. (Please see response to Referee 1)

I therefore believe that this more balanced view of their findings should also come back in the manuscript, which might warrant rephrasing of the title, abstract and conclusion, but also in other parts of the text: for instance at the end of page 6 they argue: "Accordingly, when the ICL (and the control cells) are expanded further in culture for subsequent experiments (see next section), we

© European Molecular Biology Organization

observe the loss of entire chromosomes in all four lines." Unless I misunderstand the new sequencing data, this is not what the sequencing data is suggesting (rather structural abnormalities instead).

This sentence has now been edited to read," Accordingly, when the ICL (and the control cells) are expanded further in culture for subsequent experiments (see next section), we observe that portions along the entire length of chromosomes 14 and 9 appear to have been lost in Ch14 and Ch9 ICLs respectively (Figure EV3). Ch10 ICL cells, upon further expansion, displayed a loss only in the central portion of chromosome 10 (Figure EV3) (discussed in next section)."

Overall, I still do think this is a relevant and well-performed study, and I do favour publication in EMBO journal, but I also strongly recommend to rephrase the title, abstract and some sentences in the main text to provide a more balanced view of the data.

We thank the reviewer for recommending our work to be published in EMBO Journal.

© European Molecular Biology Organization

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dr. Robert Benezra
Journal Submitted to: EMBO Journal (Hartmut Vodermaier, Senior Editor)
Manuscript Number: EMBOJ-2017-97630

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo
http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun
http://datadryad.org
http://figshare.com
http://www.ncbi.nlm.nih.gov/gap
http://www.ebi.ac.uk/ega
http://biomodels.net/
http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Three replicates were generally used for each experimental condition for in vitro experiments and 5 mice per group were typically used in each mouse experiment. The sample sizes were determined based on an expected large effect size. With 3 replicates per condition, an effect size as small as 3 can be detected with 80% power at a two-sided significance level of 0.05 using a two-sample t-test. With 5 mice per group, an effect size as small as 2 can be detected with 80% power at a two-sided significance level of 0.05 using a two-sample t-test. We have mentioned the sample sizes in all the relevant Figure Legends, Expanded View Figure Legends and Appendix Figure Legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Three replicates were generally used for each experimental condition for in vitro experiments and 5 mice per group were typically used in each mouse experiment. The sample sizes were determined based on an expected large effect size. With 3 replicates per condition, an effect size as small as 3 can be detected with 80% power at a two-sided significance level of 0.05 using a two-sample t-test. With 5 mice per group, an effect size as small as 2 can be detected with 80% power at a two-sided significance level of 0.05 using a two-sample t-test. We have mentioned the sample sizes in all the relevant Figure Legends, Expanded View Figure Legends and Appendix Figure Legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	All the corresponding statistical tests are mentioned in the Figure Legends and Materials and Methods

Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	N/A
Is there an estimate of variation within each group of data?	Statistical analyses are reported in the materials and methods section. For each experiment involving replicates, we have mentioned the corresponding center (mean) and the measures of dispersion (whether the error bars denote standard deviation or standard error of mean) in the corresponding figure legends. Exact p values are also mentioned in the figure legends.
Is the variance similar between the groups that are being statistically compared?	Statistical analyses are reported in the materials and methods section. For each experiment involving replicates, we have mentioned the corresponding center (mean) and the measures of dispersion (whether the error bars denote standard deviation or standard error of mean) in the corresponding figure legends. Exact p values are also mentioned in the figure legends.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All the relevant antibody details are mentioned in the Materials and Methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All the MEFs were generated in our lab and were tested to be free of Mycoplasma contamination by at the MSKCC Antibody & Bioresource Core Facility.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	These details have been mentioned in the Materials and Methods section
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	These details have been mentioned in the Materials and Methods section
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A