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A kinesin-based approach for inducing chromosome-specific mis-segregation in human cells

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Thank you for submitting your manuscript on motor-induced specific chromosome missegregation to The EMBO Journal. I have now received comments from three expert referees, copied below for your information. I am happy to say that all reviewers appreciate the described strategy and its potential value for studying aneuploidy, and that we would therefore be interested in pursuing the work further for publication. As you will see, the reports do still bring up a number of specific queries and constructive criticisms, which I would invite you to answer/address in a revised version of the manuscript.

Referee #1:

In this manuscript, Truong et al develop a highly innovative strategy to generate specific whole or arm chromosome aneuploidies by employing microtubule minus-end directed transport derived from kinesin14VIb protein of earth moss. The kinesin14VIb protein counteracts the mitotic force and consequently forces a specific chromosome or chromosome arm to mis-segregate. In order to achieve specific targeting of the whole chromosome or chromosome arm, the team made use of Tet-O repeats or dead Cas9 protein fused to FKBP protein in which the latter could be activated upon addition of Rapalog. Using these two strategies, Truong et al are able to induce specific mis-segregation of chromosome 1p and chromosome 9q.

This is a widely relevant and very elegant study that takes a creative and innovative approach to address an important need in the field, the ability to induce specific whole chromosome or chromosome arm gain or loss without the reliance on clonal expansion. We are very enthusiastic about the potential this toolbox can bring and have listed a few points herein to help improve this work:

1. In this manuscript, Truong et al demonstrate ample evidence on successfully mis-segregating chromosome arm 1p or 9q. However, very little is shown on these cells upon exiting mitosis (interphase). Given the goal is to follow the fate of these cells upon specific mis-segregation, we would suggest that the authors follow these mis-segregating cells also in G1 and in addition look at whether this mis-segregation results in micronucleus formation containing that specific chromosome. The unexplained lack of corresponding gains is a major weakness and should be explained. Could this be due to the selection of replicating cells? Moreover, the gain of chromosome 19 is unexplained. Is this an off-target effect of Chr9sgRNA?
2. Figure 3b and 3e: Only Ctrl 1-1 and Kinesin 2-0 data are displayed. It might be interesting to display data for Kinesin 1-1 as an intermediate point
3. Figure 5: The authors establish specific mis-segregation by performing FISH against chromosome 1. As the system is designed to specifically mis-segregate chromosome 1p, FISH against chromosome 1p arm might be more informative
4. Figure 8d: Most of the chromosome 9q are lost instead of gained. Is this observation specific to Chromosome 9 due to its submetacentric nature or is it due to the Kinesin14VIb system that opts for chromosome loss instead of gain? Since the model is meant for generating loss and gain of specific chromosomes, the authors should explore and report on chromosome gain events if possible.
5. It would be helpful to expand on the additional benefit gained from this compared to prior methods which use CRISPR based techniques to induce chromosome arm losses (ie Leibowitz, M.L., Papathanasiou, S., Doerfler, P.A. et al. Chromothripsis as an on-target consequence of CRISPR-Cas9 genome editing. Nat Genet 53, 895-905 (2021)

6. Minor comments:
a) For Figure 1b-d, please put arrows for the TetR foci on the merge image, it is very difficult to see. In addition, please provide an in enlarged image. Please include examples of 2-1 missegregations. How well are you able to distinguish between 1-0 and 2-0? It is possible that two overlapping TetR foci are misscalled as 1-0, do you confirm that 1-0 cells remain monosomic after mitosis?
b) Figure 1d: Distribution of signal in daughter nuclei shows 1-0 and 3-1 conformation as well. Author should elaborate what it means in the legend/main text
b) Figure 2: Immunofluorescence staining is difficult to follow. Splitting the channels might clarify the staining.
c) Please correct legend in Fig 4a (perhaps instead of "CEN" you meant "lagging"?)
d) Can you please elaborate the origin 11% calculus (page 7 line 11)

Referee #2:

Reviewer overview:
This is a captivating resource article by Truong, Cane-Gasull, and colleagues that presents a highly innovative approach to induce chromosome-specific mis-segregations in human cells. To induce chromosome-specific mis-segregations the authors came up with an pioneering idea to oppose plus-end directed forces acting upon chromosomes during congression by tethering arm of specific chromosome to microtubule minus-end directed kinesin-14 (Kin14Vib) from a Physcomitrella patens. In that way, the specific chromosome would be pulled toward the spindle poles, which could presumably induce chromosome mispositioning and subsequent mis-segregation. To execute this idea the authors employed either Tet system based on chromosome 1-integrated Tet operon and Tet repressor in aneuploid U-2 OS cells or chromosome-specific endogenous repeats of chromosomes 1 or chromosome 9 combined with nuclease dead Cas9 system in near-diploid RPE-1 cells. In U-2 OS cells, they attached Kin14Vib to the subtelomeric TetO locus while in RPE-1 cells they attached Kin14Vib to endogenous highly repetitive subtelomeric repeats of chromosome 1 or subcentromeric repeats of chromosome 9. In U-2 OS cells, they found that upon binding of Kin14Vib to the arm of one copy of chromosome 1, the chromosome tended to misalign outside the metaphase plate, resulting in an unequal distribution of both chromatids to one of the daughter cells. The authors also observed that misplaced chromosomes were frequently pulled out of the metaphase plate with telomere region approaching spindle pole, whereas both centromeres were able to congress efficiently despite constant pulling of associated chromosome arm. Interestingly, the authors did not observe any increase in the number of unattached kinetochores in this system. During anaphase, the authors observed the characteristic phenotype in which a chromatid arm is strongly stretched in the spindle midzone during anaphase, leading to an increase in H3S10 phosphorylation on the stretched chromosome arm during subsequent telophase, but without signs of ongoing DNA damage during late mitotic stages. Tethering of the telomere of chromosome 1 close to the spindle pole resulted in an unequal distribution of chromosome 1 in 40% of cells, which led to a subtle increase in the aneuploidy specifically associated with p-arm of chromosome 1. The binding of both copies of chromosome 1 in RPE1 cells to dimerized Kin14b in the subtelomeric region led to rather similar mis-segregation phenotypes as those observed in U-2 OS cells. Binding of Kin14b to the subcentromeric region of both copies of chromosome 9 also led to chromosome misalignment and chromosome mis-segregation. Intriguingly, even in this harsh tug-of-war where Kin14b opposes plus-end directed forces generated on near-centromere region, centromeres were not displaced from the metaphase plate. Instead, the pericentromeric chromatin was highly stretched toward the spindle pole, presumably inducing breakage of the chromosome arm during subsequent cytokinesis or G1. Finally, motor-induced pulling on the pericentromeric region resulted in a subtle but specific increase in aneuploidy of the q arm of chromosome 9.

With that said, I think that this is the study of high quality that will be of interest to researchers working on basic mechanisms of mitosis, causes and consequences of chromosome mis-segregation, aneuploidy and chromosomal instability. Likewise, different cell lines developed in this study will be of usage to researchers studying these hot-topic questions. Prior studies that attempted to resolve immediate effects of chromosome mis-segregation have been hampered by the complexity of the experimental systems used to induce chromosome mis-segregation. It has proved difficult to experimentally control both chromosome identity and degree of chromosome mis-segregation that one induces in cells, and both could be important for the cellular response to aneuploidy. In addition, many groundbreaking approaches have relied on different cell lines that have already adapted to some specific aneuploidy, which hinders the study of immediate effects. In that regard, I congratulate the authors on this original idea that I see as a first and enormous step toward inducing chromosome-specific mis-segregations in human cells. However, as often in cell biology studies, the nice ideas and their implementations are complicated by the intricacy of the cellular systems studied. In that regard, the presented approach is limited to aneuploidies of chromosome arms, and although the microscopy approaches showed a rather high throughput of the method, the single cell sequencing data were much less convincing, especially in RPE-1 cells. Moreover, chromosome breakage events and segmental aneuploidies are known to induce highly different cellular response when compared to whole chromosome aneuploidies (Santaguida et al., 2017, Dev Cell; Hintzen et al., 2021, bioRxiv, Soto et al., 2017, Cell Rep).

The manuscript is well-written, and Results, Materials and methods sections are very clean and clear for the most part except for a few instances emphasized in the comments below. Although I think that the manuscript presents highly interesting approach that could allow for more detailed follow-up studies on cellular consequences of inducing aneuploidy that is specific for the arm of certain chromosomes, my view is that authors should revise manuscript to better reflect certain results, as per comments below, and introduce one paragraph explaining limitation of the presented approach. If authors would succeed in presenting the clearer picture of main implications and limitations of the approach, my opinion is that the manuscript would be quite at the EMBO Journal level.

Major comments:
1) I encourage the authors to include the 'Limitation of the Study' paragraph and to discuss more the limitation of the presented approach.

Minor comments:

2) Data on Figure 3 A-B is pretty confusing. The authors should explain the logic between the central axis approach and why they combined both 'errors along the TetO-TetO axis and pole-pole mid axes' to define anaphase and telophase error involving TetO chromosome. Moreover, I did not understand what defines the categories 'stretched arms' versus 'bridge/lagger'. For example, in the third row of Figure 3A the kinetochore is seen in the CENP-A channel as laggard in the spindle midzone, while in the fourth row the CENP-A signal is absent and only some chromosome signal is present within the midzone. Do the presence or absence of the CENP-A signal define these categories?
3) Regarding the data in Figure 4, the authors should at least discuss the differences between the 'intact' and 'fragmented/split' phenotype of the chr1-CEN signal, as it is currently not clear how these similar phenotypes were distinguished. Furthermore, the authors should discuss the mitotic mechanisms that could resolve the lagging chromosome in the right daughter cell (Orr et al., 2021, Curr Biol; Sen et al., 2021, Dev Cell), otherwise it is unfair to include anaphases with Chr1-CEN foci that appear lagging or fragmented, as such structures could resolve in the right daughter cell and could not be apparent in increased aneuploidy scores. Furthermore, the authors comment that the distribution was unequal between the daughter nuclei, but they do not have markers for the nuclear envelope to distinguish if such a chromosome eventually incorporates into the main nuclei or became trapped within the micronuclei. Authors should at least discuss such possibilities and maybe redefine categories presented in Figure 4.
4) If 'bridge/lagger on the mid axis' implies mis-segregation of the target chromosome, would that mean that the percentage of ctrl cells with mis-segregation of chromosome 1 is already higher than expected if mis-segregations are completely random? This would mean that around 10% of cells had mis-segregated chromosome 1 in ctrl cells (Figure 3B, and similarly Figure 3E). Do authors think this is natural or induced by the usage of the TetO system?
5) It would be nice if the authors could discuss their expectations if Cas9 technology would be suitable in a similar way for every chromosome in human cells, due to the variable number of chromosome-specific endogenous repeats that harbor different numbers of sgRNA binding sites.
6) Are chromosome breaks observed on Figure8B enriched on sites close to the position where Kin14VIb was pulling the chromosome?
7) The authors should discuss why aneuploidy is observed only in a subset of cells on Figure 8B in +sgRNA, +rapalog condition, and why losses are much more prevalent than gains.
8) I would encourage the authors to draw small schemes of phenotypes 'in and out', '1-1, 2-0', 'chr out, stretched/frag, arms out', and the phenotypes presented in Figures 3 and 4, as it would really ease the reading of the figures, and the distinction between different categories.

Additional comments
9) Figure S2D is currently not cited in the text.
10) The haplotype genome is not usual, at least not in somatic human cells, as stated by the authors on page 3, line 2.
11) On page 4, line 21 please cite also Figure 1A.
12) I think Figure S1B is currently surplus unless backed up by some IF images showing the connection of the kinetochore with the microtubule in this particular system. Otherwise, citation of a review paper detailing types of kinetochore attachments to microtubules would suffice in my opinion. Also, the whole paragraph starting from line 27 on page 5 could go into the Discussion part of the manuscript because it is mostly speculative.
13) I presume that '11% of cell divisions with complete Chr1 mis-segregation and subsequent aneuploidy' are implied by the fact that 11% of cells had an 'intact' unequal distribution of Chr1-CEN during anaphase/telophase? Authors should discuss this more clearly (see also comment 6).
14) In non-transduced U-2 OS cells, gains of certain chromosomes appear to predominate the karyotype of the population, while in rTetR-GFP-Kin14VIb population chromosome losses appear to be more prevalent. The authors should discuss the reasons. The authors gave a one sentence explanation on page 9, line 36, but I think this does not suffice, and could be explained in more detail.
15) On page 8, line 10, the authors should cite the Figure where similar U-2 OS data is presented.
16) Is there some threshold value for 'GFP focus observed out of metaphase plate', as currently it is unclear how this was assigned?
17) On page 18, line 35, the authors mention the 'dotted line' but I do not see it in the Figure 2C.
18) Are the images presented in Figure 3A-D maximal projections or single z-planes?
19) On page 19, line 10, Figure 3D should be cited within parenthesis instead of figure 3B.
20) On page 23, line 10, 'images' is written twice in a row.

21) Movies cannot be opened easily, I managed to open them only in Fiji/Image J. Also, movies could be cited at more appropriate places through the text, and not just as a bulk at one place.
22) Figure 2C should be labeled ctrl or Kin14VIb, and one surplus vertical line appear between the top and bottom rows of the figure. In the same figure, I would add some arrowheads to guide the reader where to look (GFP foci), as currently it is not clear.
23) In Figure 4A, the authors should label the categories as in 4B.
24) Is the percentage of cells with the 'lagging' phenotype of Chr1-CEN seen in Figure 4B similar to the percentage of CENP-A foci observed on the TetO-TetO axis in fixed cells seen in Figure 3A.
25) Please label treatments on Figure S2 and S4.

Referee #3:

Comments to the authors

Recurrent aneuploidy patterns have been observed in various cancer types. The origin of these aneuploidy patterns and how do they evolve remains unknown. The challenge has been the lack of strategy to induce specific chromosome aneuploidies in specific cell types so that the immediate cellular responses can be evaluated and eventually the later adaptive responses during clonal expansion examined. The approaches available so far are limited because either rely on clonal expansion upon an initial karyotype change or generate karyotype changes randomly. In this study, Truong et al. describe a novel strategy to mis-segregate specific chromosomes in different human cell types. The strategy consists on targeting an exogenous microtubule minus-end-directed kinesin 14VIb to Tet operon repeats or chromosome-specific endogenous repeats. This induces poleward movement of the targeted loci during prometaphase and results in chromosomal arm aneuploidies after a single cell division. The strategy still faces limitations but provides an exciting basis for further improvement. Whereas the Tet operon repeats' strategy allows the targeting of one homologue but would imply chromosome-specific genome editing for Tet operon inclusion, the endogenous repeats are limited by their chromosome-specific localization and the targeting of both homologues. Also, both strategies primarily induce segmental vs. whole chromosomal aneuploidies, which might induce different cellular responses. Nevertheless, an important step towards the generation of specific aneuploidies was given, using elegant challenging methodologies. The authors carefully interpreted the results, and discussed potential methods to study immediate single-cell responses. I am supportive of the publication provided the authors address the concerns below.

Major comments

1. The title should refer to 'chromosome arm-specific mis-segregations' rather than 'chromosome-specific mis-segregations' as the strategy results in segmental vs. whole chromosomal aneuploidies. Nevertheless, I consider that the authors have carefully acknowledged that the kinesin-induced targeted mis-segregations predominantly result in chromosomal arm aneuploidies in the abstract, introduction and discussion sections.

2. The authors should provide a comparative analysis or discuss the efficacy of the strategies targeting TetO and endogenous repeats in subtelomeric region Chr1p36. In the discussion section, page 11, lines 19-22, the authors suggest that GCN-induced Kin14VIb tetramers are more efficient than Kin14VIb dimers. Are they comparing endogenous vs. TetO repeats? While targeting both homologues, endogenous repeats should work more efficiently. Also, aren't the sgRNA bindings sites in the endogenous repeats (~1.400) higher than the TetR-binding sites (200)?

3. In page 9, lines 6-7, the authors mention there were striking differences in the consequences of Kin14VIb binding near telomeres or near centromeres, referring to the distinct chromatin stretching. But which localization appears to work better? Also, which repeats' size is better? It appears that the pericentromeric and large Chr9-cen region had side effects even in the absence of rapalog, likely due to replication stress in the repeats, but also affecting Chr19 segregation.

4. It would be useful to mention in the discussion section, which other chromosomes exhibit targetable endogenous repeats (localization and size) to infer the extrapolation of the strategy to other chromosomes.

5. The clonal expansion to derive the dCas9-Kin14VIb cell line ended up generating an X trisomy. This somehow limits the efficacy of the strategy to study immediate effects of induced chromosome-specific aneuploidies. Did the authors check any other clones?
Also, could the authors clarify whether the use of STLC, as described in page 14 for the cell line generation procedure, was only to select the RPE1 dCas9-Kin14VIb clone that better responded to rapalog?

Minor comments

6. In Fig. 1 the authors show that ~90% of the dividing cells expressing rTetR-GFP-KinVIb exhibit the TetO locus outside the metaphase plate. Then 45% result in 2-0 distribution of the TetO locus and 40% end up in 1-1 distribution.
6.1. Do the later exhibit the TetO locus inside the metaphase plate later on (transient release from KinVIb-driven poleward movement)?

6.2. On the transition from Fig.1 to Fig.2, and considering that the 1-1 distribution is causing the mitotic delay in 40% of the cells, is the percentage of Mad1+ cells correlating to the 1-1 distribution?

7. In Fig. 1A replace 'p36 200x96x TetO' by 'p36 200x96mer TetO'.

8. Could the authors zoom Fig.2C and better highlight the KT pairs' orientation?

9. In Fig. 8 replace 'gRNA' by 'sgRNA'.

10. In page 9, line 31, replace 'rapolog' by 'rapalog'.

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Truong, Cané-Gasull et al.

Point-by-point reply to the comments of the referees.

Response to all referees:
We thank the three referees for their constructive critical feedback. We would like to point out that to meet the EMBO J figure format and data representation requirements, we had to change the display of most of the figures significantly as, for instance stacked bars did not work to show the datapoints of individual experiments. We apologize to the referees if this makes the re-reviewing process more cumbersome. The major textual changes are highlighted in a different text color in the revised version of the manuscript.

Referee #1:

In this manuscript, Truong et al develop a highly innovative strategy to generate specific whole or arm chromosome aneuploidies by employing microtubule minus-end directed transport derived from kinesin14VIb protein of earth moss. The kinesin14VIb protein counteracts the mitotic force and consequently forces a specific chromosome or chromosome arm to mis-segregate. In order to achieve specific targeting of the whole chromosome or chromosome arm, the team made use of Tet-O repeats or dead Cas9 protein fused to FKBP protein in which the latter could be activated upon addition of Rapalog. Using these two strategies, Truong et al are able to induce specific mis-segregation of chromosome 1p and chromosome 9q.

This is a widely relevant and very elegant study that takes a creative and innovative approach to address an important need in the field, the ability to induce specific whole chromosome or chromosome arm gain or loss without the reliance on clonal expansion. We are very enthusiastic about the potential this toolbox can bring and have listed a few points herein to help improve this work:
1. In this manuscript, Truong et al demonstrate ample evidence on successfully mis-segregating chromosome arm 1p or 9q.

1a. However, very little is shown on these cells upon exiting mitosis (interphase). Given the goal is to follow the fate of these cells upon specific mis-segregation, we would suggest that the authors follow these mis-segregating cells also in G1 and in addition look at whether this mis-segregation results in micronucleus formation containing that specific chromosome.

Response: We thank the reviewer for their constructive feedback and suggestions. To look whether the on-target mis-segregations would result in micronuclei (MN) formation in G1, we re-analyzed the live cell imaging data presented in Figs 1 and 6 (data shown for Chr1 in Fig EV1 and for Chr9 in Fig EV4), as we could follow some of the cells for longer timepoints after anaphase. In addition, we performed another live cell imaging experiment for Chr9-cen in RPE1 cells, to collect more cells for MN analysis and quantification. Note that we included cells that were already in mitosis at the start of imaging to increase the numbers; these cells were not included in the quantifications shown in Fig 6. We find that in all Chr9-cen distribution categories (3-1, 4-0, 2-2 same sister, bridge/fragm), the mis-segregating 9q locus and arm occasionally formed a micronucleus in at least one of the daughter cells. Overall, in 29% of the daughter cells, a GFP-positive MN was observed, versus 14% of the daughter cells in which Kin14VIb was not bound to the Chr9-cen locus. In case of a 2-2ss, or 4-0 distribution of the 9q locus, GFP-positive micronuclei mostly originated from Kin14VIb-bound Chr9q arms that were misaligned in metaphase and that remained polar during anaphase (Fig EV4A). In

contrast, in the cases of a 3-1 locus distribution, or when the locus appeared as bridge/fragmented during anaphase (i.e. the latter also seen in ctrl), MN predominantly originated from lagging chromatin.

For Chr1 in U-2 OS TetO cells, we observed MN that included the locus (GFP+ MN) only in the very small 1-0 category, both in the control and Kin14VIb-expressing cells (Fig 1 and EV1). In fact, the 1-0 category was called this way because one of the TetO loci was not incorporated into one of the main daughter nuclei, and instead formed a MN. However, in the rTetR-Kin14VIb expressing cells displaying a 2-0 distribution of the TetO locus, we observed an increase in the formation of MN that do not include the TetO locus (GFP- MN). These MN formed from chromatin 'spikes' or chromatin bridge remnants facing the midzone, in line with the idea that these MN originate from a broken stretched 1p arm.
Fig EV1 is discussed on page 8, and Fig EV4 on page 12 of the revised manuscript.

1b. The unexplained lack of corresponding gains is a major weakness and should be explained. Could this be due to the selection of replicating cells?

Response: We agree with the reviewer that this was unexpected. We sorted EdU+ 2n cells, 24 hrs after treatment with doxycyclin (to induce Kin14VIb expression). Cells were treated with rapalog (to tether Kin14VIb onto the chromosome), and exposed to Edu during the final 16 hrs. This way, we selected for cells that had gone through S and M phase during the 16 hr period, and avoided sorting cells that had not gone through S/M phase during that period (i.e. the vast majority of the asynchronously growing RPE1 cell population). We expected that at least a subset of these EdU+ cells should have gone through mitosis with Kin14VIb attached to the chromosome of interest.
Moreover, from the longer live cell imaging analyses (see point 1a), we learned that the Chr9q locus and most likely the 9q arm occasionally ends up in a micronucleus (MN) after being mis-segregated by Kin14VIb. Particularly in case of a 3-1 distribution these MN more often originate from lagging chromatin. Variations in locus distribution (i.e. 3-1 versus 4-0 and 2-2ss), and subsequent 9q fate most likely reflect differences in Kin14VIb chromosome pulling efficiency caused by variations in sgRNA expression levels per cell and per experiment after lentiviral transduction (compare Fig 6 and Fig 7 for experimental variation in the percentage of cells with 3-1 locus distribution. Note that some of the cells that fall into the category '2ss-1 + lag' in Fig 7C-F, may eventually divide with a 3-1 Chr9q distribution in case the laggard is correctly resolved). Apparently, in the scKaryo-seq experiment more divisions resulting in a 3-1 than a 4-0 locus distribution had taken place. We argue that, despite sorting whole single G1 cells, DNA in MN may get lost during cell suspension, sorting and subsequent library preparation (Worrall et al., Cell Rep. 2018), particularly when these MN originated from lagging chromatin. The nuclear envelopes of these type of micronuclei are considered to be more prone to rupture (Liu et al. Nature, 2018). We propose on page 12 of the revised manuscript that this may explain the bias for 9q monosomies in our scKaryo-seq results.

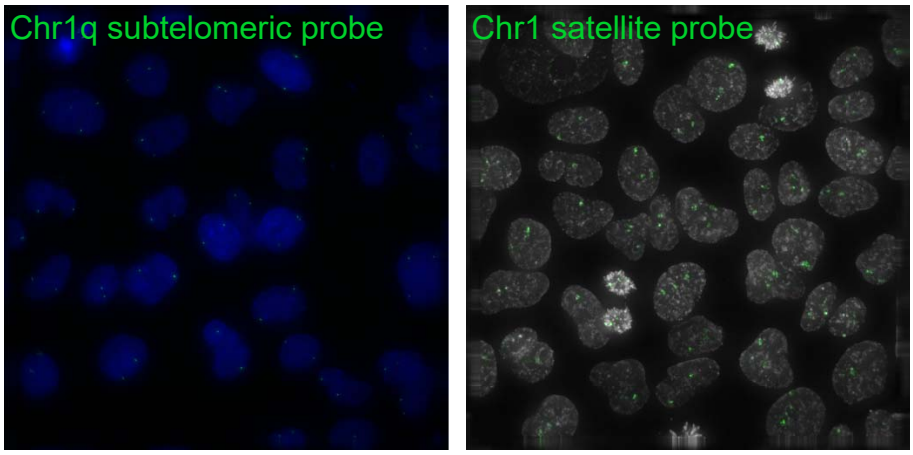
1c. Moreover, the gain of chromosome 19 is unexplained. Is this an off-target effect of Chr9sgRNA?
Response: We made an overview of potential off-target effects of the sgRNA for Chr9 using the command-line Cas-OFFinder program (accessible at http://www.rgenome.net/cas-offinder) and the T2T-CHM13v2.0 genome. Chr19 is predicted to only have 5 potential binding sites for the Chr9 cen sgRNA when 0 mismatches are allowed and 19 binding sites when 3 mismatches are allowed. The highest number of off-targets for this sgRNA are found in Chr 22 (20,309; 0 mismatches), a chromosome we do not see mis-segregating in Fig 8A and B. We therefore consider an off-target effect of the Chr9 sgRNA an unlikely explanation for the Chr19 alternations. As some Chr19 deviations are also seen in the cells not expressing the sgRNA, we think that this is a deviation caused by clonal selection of the RPE1 cCas9-Kin14VIb clone.

2. Figure 3b and 3e: Only Ctrl 1-1 and Kinesin 2-0 data are displayed. It might be interesting to display data for Kinesin 1-1 as an intermediate point

Response: We thank the reviewer for this suggestion. We included the data in a new Fig EV2. It is important to mention is that in the fixed cell experiments that we showed in the original figure, we observed a 1-1 TetO distribution only in a small number of the Kin14VIb expressing cells (Experiments 1 and 2 in Fig EV2A). Apparently, cells in these experiments expressed higher levels of the rTetR-GFP-Kin14VIb, which enhances the TetO 2-0 mis-segregation frequency (see also new Fig EV2B showing quantifications of GFP intensity levels in the rTetR-GFP-Kin14VIb expressing cells displaying a 1-1 or 2-0 distribution of the TetO locus). We performed an additional experiment, in which the frequencies of 1-1 and 2-0 distribution of the TetO locus in rTetR-GFP-Kin14VIb expressing cells were similar to the live cell imaging experiment shown in Fig 1 (Exp 3, Fig EV2A). We find that the 1-1 distribution in Kin14VIb expressing cells is not associated with an increase in lagging or bridging of the TetO chromosome. We show the data in Fig EV2C and combined the quantifications of the ctrl 1-1 and Kin14VIb 2-0 groups of this extra experiment with the quantifications of the other two experiments in a new version of Fig 3.

3. Figure 5: The authors establish specific mis-segregation by performing FISH against chromosome 1. As the system is designed to specifically mis-segregate chromosome 1p, FISH against chromosome 1p arm might be more informative

Response: We would like to emphasize that the system was not designed to specifically mis-segregate chromosomal arms, but after testing, it appeared to work this way. In fact, we used FISH to figure out if the entire chromosome would be mis-segregated or only the p arm. Therefore, we deliberately did not choose a FISH probe that detects the p arm (since we could follow the p arm via its TetO locus with rTetR-GFP or rTetR-GFP-Kin14VIb), but instead we used a probe that labelled another part of the chromosome. It turned out that one of the three Chr1 homologs in U-2 OS cells was not detected by a FISH probe directed against the (sub) telomere of 1q (for unknown reasons we did not further explore), but the three Chr1 copies were detected using a FISH-CEN (alpha-satellite) probe (see images below). We therefore used the latter probe for our analysis.



4. Figure 8d: Most of the chromosome 9q are lost instead of gained. Is this observation specific to Chromosome 9 due to its submetacentric nature or is it due to the Kinesin14VIb system that opts for chromosome loss instead of gain?

Response: We do not think that the losses are caused by Kinesin14VIb system itself. We argue that due to the FACS sorting and library preparation procedures prior to scWGS, we may lose DNA from micronuclei originating from lagging chromatin (a type of MN we see more often associated with a 3-1 distribution of the Chr9-cen locus), and that therefore gains are underrepresented. Please see also our comments to point 1b.

Since the model is meant for generating loss and gain of specific chromosomes, the authors should explore and report on chromosome gain events if possible.

Response: Although after scWGS sequencing we more frequently observed chromosomal losses than gains (for potential reasons explained above), we would like to point out that we do detect typical gains after scWGS sequencing of the Kin14VIb expressing cells: e.g. tetrasomies for 9q (Fig 8).

5. It would be helpful to expand on the additional benefit gained from this compared to prior methods which use CRISPR based techniques to induce chromosome arm losses (ie Leibowitz, M.L., Papathanasiou, S., Doerfler, P.A. et al. Chromothripsis as an on-target consequence of CRISPR-Cas9 genome editing. Nat Genet 53, 895-905 (2021)

Response: We thank the referee for this suggestion. Because one of the other referees, suggested to discuss the limitations of our approach, we now discuss both the opportunities and limitations of the presented approach in a separate paragraph of the discussion (pages 15-18 of the revised manuscript).

6. Minor comments:
a) For Figure 1b-d, please put arrows for the TetR foci on the merge image, it is very difficult to see.

Response: We thank the reviewer for pointing this out to us, we have now included arrowheads for the foci on the merge images.

b) In addition, please provide an in enlarged image. Please include examples of 2-1 missegregations.

Response: We have removed two time points from the stills shown in Fig 1B, allowing us to enlarge all the remaining frames. In addition, we included schematic representations of the behavior of the TetO locus during mitosis next to the stills. Two examples of a 2-1 distribution are now included as Appendix Fig S1. It shows that it is caused by splitting of one of the two loci either prior to or during anaphase for unknown reasons. We have now called this category 1-1split in Fig 1 and Appendix Fig S1. Please note that this was only observed in 3/59 (ctrl) and 2/57 (Kin14VIb) cells.

c) How well are you able to distinguish between 1-0 and 2-0? It is possible that two overlapping TetR foci are misscalled as 1-0, do you confirm that 1-0 cells remain monosomic after mitosis?

Response: We have now included examples of the 1-0 distribution in Fig EV1. It is an initial 2-0 (Kin14VIb) or 1-1 (ctrl) distribution of the locus during anaphase, but with one of the loci eventually forming a (GFP+) micronucleus. For Fig 1D we assessed the distribution of the TetO locus over the two **main** daughter nuclei, and therefore refer to this as 1-0, instead of 1-1 or 2-0. Please note that this was only observed in a very small number of cells.

d) Figure 1d: Distribution of signal in daughter nuclei shows 1-0 and 2-1 conformation as well. Author should elaborate what it means in the legend/main text.

Response: In the legend of Fig 1 we now refer to Fig EV1 and Appendix Fig S1 in which we show examples of the 1-0 and 2-1 distribution, respectively.

e) Figure 2: Immunofluorescence staining is difficult to follow. Splitting the channels might clarify the staining.

Response: We thank the reviewer for pointing this out to us. For clarity, we now highlight the TetO foci with arrowheads in Fig 2A and included a magnification of the regions with the TetO foci, in the corners of the images shown in Fig 2C.

f) Please correct legend in Fig 4a (perhaps instead of "CEN" you meant "lagging"?)

Response: We thank the reviewer for pointing this out to us.

g) Can you please elaborate the origin 11% calculus (page 7 line 11)

Response: This number represents the category where we see the CEN FISH foci being unequally distributed over the two main masses of segregating chromosomes during anaphase. We assume that when the centromeres of chromosome 1 are unequally distributed, this most likely results into a whole chromosome mis-segregation in the daughter cells. We now explain this in the text (page 8).

Referee #2:

Reviewer overview:
This is a captivating resource article by Truong, Cane-Gasull, and colleagues that presents a highly innovative approach to induce chromosome-specific mis-segregations in human cells. To induce chromosome-specific mis-segregations the authors came up with an pioneering idea to oppose plus-end directed forces acting upon chromosomes during congression by tethering arm of specific chromosome to microtubule minus-end directed kinesin-14 (Kin14Vib) from a Physcomitrella patens. In that way, the specific chromosome would be pulled toward the spindle poles, which could presumably induce chromosome mispositioning and subsequent mis-segregation. To execute this idea the authors employed either Tet system based on chromosome 1-integrated Tet operon and Tet repressor in aneuploid U-2 OS cells or chromosome-specific endogenous repeats of chromosomes 1 or chromosome 9 combined with nuclease dead Cas9 system in near-diploid RPE-1 cells. In U-2 OS cells, they attached Kin14Vib to the subtelomeric TetO locus while in RPE-1 cells they attached Kin14Vib to endogenous highly repetitive subtelomeric repeats of chromosome 1 or subcentromeric repeats of chromosome 9. In U-2 OS cells, they found that upon binding of Kin14Vib to the arm of one copy of chromosome 1, the chromosome tended to misalign outside the metaphase plate, resulting in an unequal distribution of both chromatids to one of the daughter cells. The authors also observed that misplaced chromosomes were frequently pulled out of the metaphase plate with telomere region approaching spindle pole, whereas both centromeres were able to congress efficiently despite constant pulling of associated chromosome arm. Interestingly, the authors did not observe any increase in the number of unattached kinetochores in this system. During anaphase, the authors observed the characteristic phenotype in which a chromatid arm is strongly stretched in the spindle midzone during anaphase, leading to an increase in H3S10 phosphorylation on the stretched chromosome arm during subsequent telophase, but without signs of ongoing DNA damage during late mitotic stages. Tethering of the telomere of chromosome 1 close to the spindle pole resulted in an unequal distribution of chromosome 1 in 40% of cells, which led to a subtle increase in the aneuploidy specifically associated with p-arm of chromosome 1. The binding of both copies of chromosome 1 in RPE1 cells to dimerized Kin14b in the subtelomeric region led to rather similar mis-segregation phenotypes as those observed in U-2 OS cells. Binding of Kin14b to the subcentromeric region of both copies of chromosome 9 also led to chromosome misalignment and chromosome mis-segregation. Intriguingly, even in this harsh tug-of-war where Kin14b opposes plus-end directed forces generated on near-centromere region, centromeres were not displaced from the metaphase plate. Instead, the pericentromeric chromatin was highly stretched toward the spindle pole, presumably inducing breakage of the chromosome arm during subsequent cytokinesis or G1. Finally, motor-induced pulling on the pericentromeric region resulted in a subtle but specific increase in aneuploidy of the q arm of chromosome 9.

With that said, I think that this is the study of high quality that will be of interest to researchers working on basic mechanisms of mitosis, causes and consequences of chromosome mis-segregation, aneuploidy and chromosomal instability. Likewise, different cell lines developed in this study will be of usage to researchers studying these hot-topic questions. Prior studies that attempted to resolve immediate effects of chromosome mis-segregation have been hampered by the complexity of the experimental systems used to induce chromosome mis-segregation. It has proved difficult to experimentally control both chromosome identity and degree of chromosome mis-segregation that one induces in cells, and both could be important for the cellular response to aneuploidy. In addition, many groundbreaking approaches have relied on different cell lines that have already adapted to some specific aneuploidy, which hinders the study of immediate effects. In that regard, I congratulate the authors on this original idea that I see as a first and enormous step toward inducing chromosome-specific mis-segregations in human cells.

However, as often in cell biology studies, the nice ideas and their implementations are complicated by the intricacy of the cellular systems studied. In that regard, the presented approach is limited to aneuploidies of chromosome arms, and although the microscopy approaches showed a rather high throughput of the method, the single cell sequencing data were much less convincing, especially in RPE-1 cells. Moreover, chromosome breakage events and segmental aneuploidies are known to induce highly different cellular response when compared to whole chromosome aneuploidies (Santaguida et al., 2017, Dev Cell; Hintzen et al., 2021, bioRxiv, Soto et al., 2017, Cell Rep).

The manuscript is well-written, and Results, Materials and methods sections are very clean and clear for the most part except for a few instances emphasized in the comments below. Although I think that the manuscript presents highly interesting approach that could allow for more detailed follow-up studies on cellular consequences of inducing aneuploidy that is specific for the arm of certain chromosomes, my view is that authors should revise manuscript to better reflect certain results, as per comments below,

and introduce one paragraph explaining limitation of the presented approach. If authors would succeed in presenting the clearer picture of main implications and limitations of the approach, my opinion is that the manuscript would be quite at the EMBOJournal level.

Major comments:
1) I encourage the authors to include the 'Limitation of the Study' paragraph and to discuss more the limitation of the presented approach.

Response: We thank the reviewer for this suggestion and their constructive feedback on the manuscript in general. Since referee 1 asked about the benefits of the approach, we wrote an extensive paragraph that discusses both opportunities and limitations of the approach. Please see pages 15-18 of the revised manuscript.

Minor comments:
2) Data on Figure 3 A-B is pretty confusing. The authors should explain the logic between the central axis approach and why they combined both 'errors along the TetO-TetO axis and pole-pole mid axes' to define anaphase and telophase error involving TetO chromosome. Moreover, I did not understand what defines the categories 'stretched arms' versus 'bridge/lagger'. For example, in the third row of Figure 3A the kinetochore is seen in the CENP-A channel as laggard in the spindle midzone, while in the fourth row the CENP-A signal is absent and only some chromosome signal is present within the midzone. Do the presence or absence of the CENP-A signal define these categories?

Response: We agree with the referee that the original Fig 3A-B was confusing. We have now tried to simplify the various categories by omitting the different axes, and by including cartoons explaining the different categories (see revised Fig 3A). The stretched arm category is typical for the Kin14VIb-expressing cells and is indeed the situation where we can connect CENP-C to the TetO loci located near one of the spindle poles. In some cells, however, we cannot identify the centromere belonging to the bridging chromosome, but we can draw a line along the bridge to the TetO loci. We now call this ‘bridge in line’ and combined this with the stretched arm category. When observing lagging, bridging or misaligned chromosomes *in addition* to a stretched arm or a bridge in line with the TetO locus, we scored it as a separate group and indicate it with a (+). The latter reflects the background CIN level of the U-2 OS TetO cells that exists next to the Kin14VIb-induced effect on Chr1p. Note, that for ctrl U-2-OS TetO cells, the bridges and laggards are mostly not in line with the separated TetO locus.

For the late anaphases/telophases (old Figure 3D), we realized that due to furrow ingression (Fig EV2F), the axis approach did not make much sense and we therefore cumulated the spike and bridge/lag categories as one single category in the revised Fig 3B.

3) Regarding the data in Figure 4, the authors should at least discuss the differences between the 'intact' and 'fragmented/split' phenotype of the chr1-CEN signal, as it is currently not clear how these similar phenotypes were distinguished. Furthermore, the authors should discuss the mitotic mechanisms that could resolve the lagging chromosome in the right daughter cell (Orr et al., 2021, Curr Biol; Sen et al., 2021, Dev Cell), otherwise it is unfair to include anaphases with Chr1-CEN foci that appear lagging or fragmented, as such structures could resolve in the right daughter cell and could not be apparent in increased aneuploidy scores. Furthermore, the authors comment that the distribution was unequal between the daughter nuclei, but they do not have markers for the nuclear envelope to distinguish if such a chromosome eventually incorporates into the main nuclei or became trapped within the micronuclei. Authors should at least discuss such possibilities and maybe redefine categories presented in Figure 4.

Response: The referee raises important points regarding Figure 4. First, the referee is right that categorizing the different CEN-FISH signals in the Kin14VIb condition as 'unequal' is not correct, particularly in case of a lagging CEN for which we do not know whether it would eventually be unequally distributed or not. In addition, we are thankful to the reviewer for pointing out unclarities regarding the "fragmented/split" CEN phenotype. To obtain clarity, we re-analyzed all images of each experiment, and found that the deconvolution processing step, which was applied to all images acquired on our Deltavision microscope, appeared to sometimes enhance background signals that we scored as a split/fragmented FISH signal. However, when examining the corresponding non-deconvolved image we were no longer confident in assigning it as such (*please see comparison of deconvolved and non-deconvolved image below)*. We therefore analyzed all the unprocessed, non-deconvolved images and deconvolved images side by side, allowing us to confidently assign the following categories:
-Unequal: when FISH CEN foci are unequally distributed over the two main masses of segregating chromosomes.
-Lagging: when a FISH CEN focus is lagging between the two main masses of segregating chromosomes. Note that there is a slight increase in this category compared to our previous analysis, as based on the referee's feedback we now categorize two cells in early anaphase as having a 'lagging CEN', instead of a previously annotated 'unequal, intact CEN'.
-Late-equal: when the FISH CEN foci are equally distributed over the over the two main masses of segregating chromosomes but with one of the CEN foci appearing on the edge of the segregating chromosome mass, and with chr1-CEN-negative bridging/lagging chromatin in the vicinity of this focus. We consider this a late chr1-CEN arrival, likely resulting from a previous lagging CEN, due to Kin14VIb pulling at the other end of the p arm.
-Bridging: when part of the CEN FISH signal is detected splitted or fragmented on a chromatin bridge. This category was previously scored as "fragmented/split CEN". Note that we previously also included cells in the so-called split/fragmented category in which we observed small fluorescent signals in one or both of the main masses of segregated chromosomes, in addition to the main CEN FISH foci. However, we now consider these deconvolution artefacts (*see example below*). These cells are now part of the equal or late-equal category.

For the anaphases where we see a clear unequal distribution of the CEN-FISH loci, we consider it likely that this could give rise to a whole chromosome aneuploidy in the daughter cells.

Please see page 8 of the revised manuscript where we describe the revised figure.

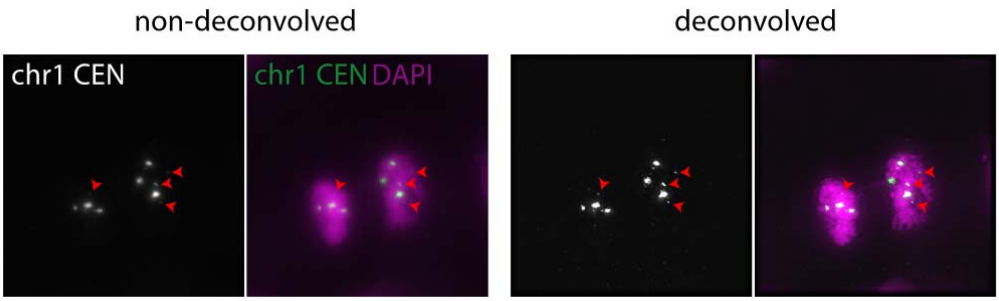


Figure *comparing non-deconvolved an deconvolved images. Red arrowheads indicate signals that were previously scored as fragmented FISH CEN signals, but we now consider background. Maximum projections of 10/64 identical z stacks are shown.*

4) If 'bridge/lagger on the mid axis' implies mis-segregation of the target chromosome, would that mean that the percentage of ctrl cells with mis-segregation of chromosome 1 is already higher than expected if mis-segregations are completely random? This would mean that around 10% of cells had mis-segregated chromosome 1 in ctrl cells (Figure 3B, and similarly Figure 3E). Do authors think this is natural or induced by the usage of the TetO system?

Response: We realize that our mid-axis and TetO-TetO axis (which were collectively called (mid-) axis) quantification was not clear and we now display it differently (see also response to point 2). Furthermore, only in 5 ± 1% (n= 3 exp, 217 cells analyzed in total) of the ctrl cells we observed a bridge formed by the TetO locus, which we consider a consequence of the TetO system. However, these cells were not included in the analysis as it did not result into a 1-1 distribution of the locus. In the revised Fig 3A, we observe in 3 ± 3% of the Ctrl 1-1 cells a bridge between the two TetO foci, most likely reflecting a mis-segregation of Chr1 in the control.

5) It would be nice if the authors could discuss their expectations if Cas9 technology would be suitable in a similar way for every chromosome in human cells, due to the variable number of chromosome-specific endogenous repeats that harbor different numbers of sgRNA binding sites.

Response: On page 17 of the revised manuscript we now propose for which chromosomes our method could be suitable. Throughout the revision process we have been in close contact with dr. McClelland. Since she could run a repeat and sgRNA binding site prediction analysis of the T2T genome assembly, we based our predictions on their data and on the analyses presented in the recent BioRxiv submission by the Davoli lab (https://doi.org/10.1101/2022.09.27.509580).

6) Are chromosome breaks observed on Figure8B enriched on sites close to the position where Kin14VIb was pulling the chromosome?

Response: Yes, they are. We applied different methods to analyze breakpoint positions for chromosome 9 which are shown in the new Fig EV5. The initial results of the AneuFinder breakpoint refinement analysis suggested that for most of the cells the breakpoints are located roughly 10 Mb upstream from the pericentromeric repeat targeted by Chr9-cen sgRNAs (Fig EV5C, left panel). However, based on visual inspection of the results of individual cells, these estimates appeared to be off (Fig EV5C, right), and we therefore applied two different methods to obtain a better breakpoint estimate. First, a shifting breakpoint approach was implemented where the residual sum of squares (RSS) was calculated for different transitions (i.e. possible breakpoints) across the entire

chromosome (Fig EV5B and methods). This analysis predicted that for most cells, the breakpoint is just after the pericentromeric repeat (Fig EV5C). However, two bins overlapping the pericentromeric repeat were excluded from this analysis. These bins showed aberrant read counts, which have a substantial influence on the calculation and can result in incorrect breakpoint estimates. Because of this limitation, we next implemented a non-integer copy number calculation, which includes these bins, and estimates the average breakpoint location of all cells with a Chr9q gain or loss (see methods). The first bin that showed a significant difference in copy number between cells without and with a 9q gain or loss was the first bin (Bin 1) after the centromere bin (Bin 0). This bin has a large overlap with the pericentromeric repeat. In addition, Bin 0 already showed some difference, although not statistically significant (p=0.05833). These results suggest that the breakpoint (on average) is located close to the centromere, and within the pericentromeric repeat region of 9q, in line with our predictions based on the fixed and live cell imaging results.

7) The authors should discuss why aneuploidy is observed only in a subset of cells on Figure 8B in +sgRNA, +rapalog condition, and why losses are much more prevalent than gains.

Response: The reason why we only observe Chr9-specific aneuploidies in a subset of cells, is because the Kin14VIb-mediated pulling efficiency is not identical in all the cells, and it also varies per experiment, most likely due to variations in sgRNA expression levels per cell and per experiment. Although we reach sgRNA transduction efficiencies of ±90% based on dCas9-GFP localization at intended loci (Appendix FigS4G), when we consider Chr9q locus localization near the spindle pole during metaphase as a proxy for kinesin pulling efficiency, we observe this in~ 65% of the cells (based on Fig 7A, ~35% of the Kin14VIb expressing cells Chr9-cen locus is still aligned). Moreover, a fraction of this population shows a 2-2ss distribution which will not be detected as a copy number alteration after scWGS. Furthermore, we sorted EdU+ G1 cells, to select for cells that had gone through S and M phase, and to avoid sorting G1 cells that had not gone through S and M phase during the 16 hr Edu labeling period (i.e. the vast majority of the cell population). We expected that at least a subset of these Edu+ cells should have gone through mitosis with sufficient amounts of Kin14VIb attached to the chromosome of interest to induce its mis-segregation, which appeared to be the case given the increase in Chr9q aneuploidies.

In addition, from the later timepoints of the live cell imaging experiments (Fig EV4), we learned that the Chr9q locus and most likely the 9q arm occasionally end up in a micronucleus (MN) after being mis-segregated by Kin14VIb. Particularly in the case of a 3-1 distribution, these MN more often originate from lagging chromatin. Variations in locus distribution (i.e. 3-1 versus 4-0 and 2-2ss), and subsequent 9q fate most likely reflect differences in Kin14VIb chromosome pulling efficiency caused by variations in sgRNA expression levels per cell and per experiment after lentiviral transduction (compare Fig 6 and Fig 7 for experimental variation in the percentage of cells with 3-1 locus distribution). Apparently, in the scKaryo-seq experiment, more divisions resulting in a 3-1 than a 4-0 locus distribution had taken place. We argue that, despite sorting whole single G1 cells, DNA in MN may get lost during cell suspension and sorting and subsequent library preparation (Worall et al., Cell Rep., 2018), particularly when these MN originated from lagging chromatin. The nuclear envelopes of these type of micronuclei are considered to be more prone to rupture (Liu et al. Nature 561, 2018). We discuss on page 11 of the revised manuscript that this may explain the bias for 9q monosomies in our scKaryo-seq results.

8) I would encourage the authors to draw small schemes of phenotypes 'in and out', '1-1, 2-0', 'chr out, stretched/frag, arms out', and the phenotypes presented in Figures 3 and 4, as it would really ease the reading of the figures, and the distinction between different categories.

Response: We thank the reviewer for this feedback. We have now included explanatory schemes of various phenotypes in Fig 1, 3, 7, 8, EV1, EV2, EV4 and Appendix Fig S1.

Additional comments
9) Figure S2D is currently not cited in the text.
Response: We thank the reviewer for pointing this out. We have corrected this.

10) The haplotype genome is not usual, at least not in somatic human cells, as stated by the authors on page 3, line 2.

Response: We thank the reviewer for pointing this out. We have corrected this.

11) On page 4, line 21 please cite also Figure 1A.
Response: We thank the reviewer for pointing this out. We have corrected this.

12) I think Figure S1B is currently surplus unless backed up by some IF images showing the connection of the kinetochore with the microtubule in this particular system. Otherwise, citation of a review paper detailing types of kinetochore attachments to microtubules would suffice in my opinion.
Response: We thank the reviewer for this suggestion. We omitted the schemes from the supplemental figure (now Appendix Fig S2) and instead refer to Godek et al. (Nat. Rev. Mol. Cell Biol., 2015), who reviewed these types of erroneous kinetochore-microtubule attachments.

Also, the whole paragraph starting from line 27 on page 5 could go into the Discussion part of the manuscript because it is mostly speculative.
Response: We thank the reviewer for this suggestion. We have now omitted this part from the results.

13) I presume that '11% of cell divisions with complete Chr1 mis-segregation and subsequent aneuploidy' are implied by the fact that 11% of cells had an 'intact' unequal distribution of Chr1-CEN during anaphase/telophase? Authors should discuss this more clearly (see also comment 6).

Response: The reviewer is right that this number refers to the 'intact' unequal distribution of the Chr1-CEN during anaphase/telophase. We now mention the range (9-11%) and explain it more clearly on page 7.

14) In non-transduced U-2 OS cells, gains of certain chromosomes appear to predominate the karyotype of the population, while in rTetR-GFP-Kin14VIb population chromosome losses appear to be more prevalent. The authors should discuss the reasons. The authors gave a one sentence explanation on page 9, line 36, but I think this does not suffice, and could be explained in more detail.

Response: We apologize to the reviewer, but we are not sure what the reviewer means by their comment that the non-transduced karyotypes are dominated by gains and the rTetR-GFP-Kin14VIb transduced ones by losses. The graphs in Fig 4C show that more chromosomes have a higher aneuploidy score in the non-transduced cells (note that the aneuploidy score takes both gains and losses into account). Furthermore, we assume the reviewer refers to line 12/13 on page 7 (i.e line 22/23 p8 of revised manuscript) where we wrote the following: "Note, all chromosomes displayed variations due to the overall heterogenous nature of U-2 OS karyotypes." We agree with the reviewer that this is a generic remark to explain the higher aneuploidy scores in the non-transduced

cells. However, given the high levels of CIN in this cell line (Fig 3), we consider it a valid argument. We do realize that to fully substantiate this, it requires multiple independent single cell sorting and sequencing experiments of non-transduced U-2 OS TetO cells to compare karyotypes and aneuploidy scores over time. Yet, we considered this type of additional scWGS analyses (and associated costs) of U-2 OS cells to be outside the scope of this manuscript.

15) On page 8, line 10, the authors should cite the Figure where similar U-2 OS data is presented.
Response: We thank the reviewer for pointing this out. We have corrected this.

16) Is there some threshold value for 'GFP focus observed out of metaphase plate', as currently it is unclear how this was assigned?
Response: We did not apply a threshold value for GFP focus observed out of the metaphase plate. But simply scored if the focus was apart from the metaphase plate.

17) On page 18, line 35, the authors mention the 'dotted line' but I do not see it in the Figure 2C.
Response: We thank the reviewer for pointing this out. We have corrected this.

18) Are the images presented in Figure 3A-D maximal projections or single z-planes?

Response: The presented images in Fig 3 are maximal projections of z-planes. In other figures we often show maximal projections of a subset of z-planes. We have indicated in the corresponding legends the number of z-planes that are shown.

19) On page 19, line 10, Figure 3D should be cited within parenthesis instead of figure 3B.
Response: We thank the reviewer for pointing this out. We have corrected this.

20) On page 23, line 10, 'images' is written twice in a row.
Response: We thank the reviewer for pointing this out. We have corrected this.

21) Movies cannot be opened easily, I managed to open them only in Fiji/Image J. Also, movies could be cited at more appropriate places through the text, and not just as a bulk at one place.
Response: We apologize for the inconvenience. We have fixed this. Also, we refer to the movies in the text at the appropriate places.

22) Figure 2C should be labeled ctrl or Kin14VIb, and one surplus vertical line appear between the top and bottom rows of the figure. In the same figure, I would add some arrowheads to guide the reader where to look (GFP foci), as currently it is not clear.

Response: We thank the reviewer for pointing this out. We have adapted the figure accordingly.

23) In Figure 4A, the authors should label the categories as in 4B.
Response: We thank the reviewer for pointing this out. We have corrected this.

24) Is the percentage of cells with the 'lagging' phenotype of Chr1-CEN seen in Figure 4B similar to the percentage of CENP-A foci observed on the TetO-TetO axis in fixed cells seen in Figure 3A.
Response: the percentage of 'lagging' Chr1-CEN (11-13%) and 'late-equal' (7-11%), which together represents 18-24% of the cells in Figure 4B, is lower than the percentage of stretched arms (78 ± 16%), i.e. the category where we can connect CENP-C along the chromosomal arm to the TetO locus at the pole) in Fig 3A. This is due to the fact that we were unable to combine FISH with IF and hence did not select for cells expressing rTetR-GFP-Kin14VIb, nor for cells with a 2-0 distribution of the

TetO locus,i.e. the ones that we score in Fig 3. The fraction of Kin14VIb cells with a 2-0 distribution of the locus varies per experiment (see Fig EV2A). Moreover, we also scored more cells in late anaphase/telophase for Fig 4, and therefore for some of the cells that we categorize as either equal or unequal, the Chr1-CEN may have appeared as lagging in early anaphase.

25) Please label treatments on Figure S2 and S4.
Response: We thank the reviewer for pointing this out. We have included labels in the revised figures (now Appendix Fig S2 and S3, respectively).

Referee #3:

Comments to the authors

Recurrent aneuploidy patterns have been observed in various cancer types. The origin of these aneuploidy patterns and how do they evolve remains unknown. The challenge has been the lack of strategy to induce specific chromosome aneuploidies in specific cell types so that the immediate cellular responses can be evaluated and eventually the later adaptive responses during clonal expansion examined. The approaches available so far are limited because either rely on clonal expansion upon an initial karyotype change or generate karyotype changes randomly. In this study, Truong et al. describe a novel strategy to mis-segregate specific chromosomes in different human cell types. The strategy consists on targeting an exogenous microtubule minus-end-directed kinesin 14VIb to Tet operon repeats or chromosome-specific endogenous repeats. This induces poleward movement of the targeted loci during prometaphase and results in chromosomal arm aneuploidies after a single cell division. The strategy still faces limitations but provides an exciting basis for further improvement. Whereas the Tet operon repeats' strategy allows the targeting of one homologue but would imply chromosome-specific genome editing for Tet operon inclusion, the endogenous repeats are limited by their chromosome-specific localization and the targeting of both homologues. Also, both strategies primarily induce segmental vs. whole chromosomal aneuploidies, which might induce different cellular responses. Nevertheless, an important step towards the generation of specific aneuploidies was given, using elegant challenging methodologies. The authors carefully interpreted the results, and discussed potential methods to study immediate single-cell responses. I am supportive of the publication provided the authors address the concerns below.

Major comments

1. The title should refer to 'chromosome arm-specific mis-segregations' rather than 'chromosome-specific mis-segregations' as the strategy results in segmental vs. whole chromosomal aneuploidies. Nevertheless, I consider that the authors have carefully acknowledged that the kinesin-induced targeted mis-segregations predominantly result in chromosomal arm aneuploidies in the abstract, introduction and discussion sections.

Response: We understand the reviewer's consideration. However, we consider it valid to refer to the observed segregation phenotypes as chromosome mis-segregation and CIN when we indicate that the resulting aneuploidies are arm-level/segmental.

2. The authors should provide a comparative analysis or discuss the efficacy of the strategies targeting TetO and endogenous repeats in subtelomeric region Chr1p36. In the discussion section, page 11, lines 19-22, the authors suggest that GCN-induced Kin14VIb tetramers are more efficient than Kin14VIb dimers.

Are they comparing endogenous vs. TetO repeats? While targeting both homologues, endogenous repeats should work more efficiently. Also, aren't the sgRNA bindings sites in the endogenous repeats (~1.400) higher than the TetR-binding sites (200)?

Response: We thank the reviewer for their useful comments and suggestions. We have now included a systematic comparison of the efficacy of the strategies targeting TetO in U-2 OS or the different endogenous repeats in RPE1 cells. In addition, we included data supporting our conclusion that GCN4-induced Kin14VIb tetramers are more efficient than Kin14VIb dimers in RPE1 cells. The data are shown in a new Figure (Appendix Fig S4). Using the localization of the targeted loci near the

centrosomes of monopolar spindles as a proxy for Kin14VIb pulling/transport efficiency, we show that:

1) The GCN4-induced Kin14VIb tetramers are more efficient than the Kin14VIb dimer in driving poleward movement of the Chr1p36 telo locus in RPE1 cells (Appendix Fig S4A and C).
2) The monoclonal RPE1 cell line expressing GCN4-Kin14VIb displays superior Kin14VIb function compared to the polyclonal cell line. This difference is not explained by selection of a clone expressing the highest levels of Kin14VIb or reduced levels of endogenous FKBP12 (Appendix Fig S4C, D and F; see also point 3, below).
3) 200 x 96mer = 18,600 TetO binding sites are more efficient than ~1,400 endogenous sgRNA binding sites in inducing poleward recruitment of Chr1p36 (Appendix Fig S4B and C). Please note that it is important to realize that apart from the number of binding sites, the manner of Kin14VIb tethering is different between U-2 OS and RPE1. In U-2 OS cells, Kin14VIb is directly fused to rTetR, whilst in RPE1 cells Kin14VIb is expressed separately from dCas9 and interacts with dCas9 through rapalog-induced FKBP12-FRB dimerization. We choose the latter strategy because it allowed live cell imaging of the loci with or without Kin14VIb binding and we considered a comparison of the +/- rapalog conditions the best way to assess the contribution of Kin14VIb binding to the induction of CIN and aneuploidy of the targeted chromosome. However, we do realize that the indirect binding makes it more difficult to predict and control the number of motors that will be recruited to the locus per cell, as endogenous FKBP12 can interfere and may fluctuate per cell. Direct fusion of Kin14VIb to dCas9 could in principle solve this. However, this generates such a large cDNA construct that transfection and transduction becomes an issue.
4) In the same RPE1 monoclonal cell line, expression of the sgRNA for Chr9-cen results in more efficient Kin14VIb mediated poleward transport than expression of the sgRNA for Chr1-telo, most likely explained by the larger number of sgRNA binding sites (~550,000) in the Chr9 pericentromeric repeat (Appendix Fig S4H).

Based on this we conclude that in our RPE1 cell system, 1,400 sgRNA binding sites are the bare minimum to accumulate a sufficient number of motors to transport (part of) a chromosome of interest towards the spindle pole. We now discuss this in the opportunities and limitations paragraph of the discussion (pages 15-18).

3. In page 9, lines 6-7, the authors mention there were striking differences in the consequences of Kin14VIb binding near telomeres or near centromeres, referring to the distinct chromatin stretching. But which localization appears to work better? Also, which repeats' size is better? It appears that the pericentromeric and large Chr9-cen region had side effects even in the absence of rapalog, likely due to replication stress in the repeats, but also affecting Chr19 segregation.

Response: We thank the reviewer for this feedback. Which localization appears to work "better" depends on the definition of better. More sgRNA bindings sites as for Chr9q, are better to accumulate more Kin14VIb and thus to enhance mis-segregation efficiency. However, the reviewer is right that this comes with a price, as the replication of larger repeats is more prone to be affected by mere dCas9 binding during S phase. Yet, this issue may be solved by engineering and expressing a dCas9 that is specifically degraded during S phase (e.g. dCas9 fused to Cdt1 1-100 (Cy-), Sakaue-Sawano et al. Mol Cell, 2017), a strategy we are currently developing. Based on our observations, localization of Kin14VIb on pericentromeres most likely allows a better prediction of where the chromosome would eventually break (see breakpoint analysis for Chr9 in Fig EV5), something that is more difficult to predict for the stretched Chr1p arm. We now discuss these considerations, limitations and potential improvements more extensively in the Discussion on page 15-18 and

compare our approach to another dCas9-based method recently posted on BioRxive by the Davoli lab (https://doi.org/10.1101/2022.09.27.509580).

4. It would be useful to mention in the discussion section, which other chromosomes exhibit targetable endogenous repeats (localization and size) to infer the extrapolation of the strategy to other chromosomes.

Response: On page 16 of the revised manuscript, we now propose for which chromosomes our method could be suitable. Throughout the revision process we have been in close contact with dr. McClelland. Since she could run a repeat and sgRNA binding site prediction analysis of the T2T genome assembly we based our estimations on their data as well as on the analyses presented in the recent BioRxive submission by the Davoli lab (https://doi.org/10.1101/2022.09.27.509580).

5. The clonal expansion to derive the dCas9-Kin14VIb cell line ended up generating an X trisomy. This somehow limits the efficacy of the strategy to study immediate effects of induced chromosome-specific aneuploidies. Did the authors check any other clones?

Response: We low pass whole genome bulk sequenced the FACS-sorted polyclonal cell line expressing dCas9-GFP-3xFKBP and FRB-mCherry-GCN4-Kin14VIb and two other dCas9-Kin14VIb expressing clones we derived from this polyclonal cell line. The partial gain of Chr X is also observed in these two clones, but not in the main karyotype of the polyclonal line. Remarkably, the polyclonal cell line harbored a gain of Chr12 which is also a known clonal karyotypic variation of RPE1 (Ippolito et al. Dev Cell, 2021; Zhang et al Nature, 2015). The Chr12 gain, however, was not observed in the single cell derived clones from this cell line. RPE1 cells are apparently not as chromosomally stable as originally thought. We consider it feasible to isolate RPE1 clones that do not harbor the ChrX karyotype change, as the vast majority of the cells of the polyclonal cell line express dCas9-GFP-3xFKBP and FRB-mCherry-GCN4-Kin14VIb but do not display this gain. We therefore do not consider this a limitation, but an unlucky selection of RPE1 clones.

6. Also, could the authors clarify whether the use of STLC, as described in page 14 for the cell line generation procedure, was only to select the RPE1 dCas9-Kin14VIb clone that better responded to rapalog?

Response: Please also see our response to point 2. The STLC assay was used as a simple way to screen cell lines for efficient locus pulling by Kin14VIb after sgRNA transduction. The reviewer could be right that in the end we may have selected for a cell line that responded better to rapalog, although we cannot explain this improved response by having selected for cells with lower levels of endogenous FKBP12 (see western blot of FKBP12 in Appendix Fig S4F).

Minor comments

6. In Fig. 1 the authors show that ~90% of the dividing cells expressing rTetR-GFP-KinVIb exhibit the TetO locus outside the metaphase plate. Then 45% result in 2-0 distribution of the TetO locus and 40% end up in 1-1 distribution.
6.1. Do the later exhibit the TetO locus inside the metaphase plate later on (transient release from KinVIb-driven poleward movement)?

Response: We occasionally observed that in the last frame before anaphase, the TetO locus was still outside the metaphase plate, but in others it was inside. In some cases we captured an early anaphase, where we observed splitting of the locus during early anaphase, followed by complete segregation of the locus in the next frame, suggesting that the motor detached during early anaphase. However, in most of the cases, we were unable to assess this because during the delay, the metaphase plate started to rotate and we could not determine whether the locus was inside or outside the metaphase plate in the frame before anaphase onset.

6.2. On the transition from Fig.1 to Fig.2, and considering that the 1-1 distribution is causing the mitotic delay in 40% of the cells, is the percentage of Mad1+ cells correlating to the 1-1 distribution?

Response: The reviewer raises an interesting point, however since the percentage of Mad1-positive kinetochores in proximity of the TetO locus is not significantly different between Kin14VIb-expressing cells and the control, we are unable to make this correlation. However, on page 15 of the discussion we now postulate a hypothesis for the observed delay in Kin14VIb expression cells that eventually display a 1-1 distribution of the TetO locus.

7. In Fig. 1A replace 'p36 200x96x TetO' by 'p36 200x96mer TetO'.
Response: We thank the reviewer for pointing this out. This has been done

8. Could the authors zoom Fig.2C and better highlight the KT pairs' orientation?
Response: We thank the reviewer for pointing this out. This has been done.

9. In Fig. 8 replace 'gRNA' by 'sgRNA'.
Response: We thank the reviewer for pointing this out. This has been fixed.

10. In page 9, line 31, replace 'rapolog' by 'rapalog'.
Response: We thank the reviewer for pointing this out. This has been fixed.

Thank you again for submitting your revised manuscript, and my sincere apologies for its delayed re-evaluation, linked to my repeated absence from the office. I am now sending you the re-reviews by the three original referees, who all consider the study significantly improved and their original comments well-addressed. Following modification of some remaining presentational issue noted by referees 2 in their comments below, we shall therefore be happy to accept the study for The EMBO Journal!

At this stage, please also address the following still open editorial points:

Referee #1:

I would like to congratulate the authors on the revised manuscript. They have addressed our comments fully and we happily support publication of this work.

Referee #2:

The revised manuscript "A motor-based approach to induce chromosome-specific mis-segregations in human cells" by Truong, Cané-Gasull et al. is, in my opinion, a significant improvement over the first version of the manuscript originally submitted to the journal. The authors have done a great job in responding to the reviews' comments, resulting in the expansion of conclusions by introducing a set of new experimental data, reanalyzing existing data, reorganizing the figure panels, and especially significantly improving the discussion of pros and cons of the presented method. The authors have also done a nice job in improving the clarity of complex figures by introducing schematic representations of different subcategories. Together, the new data and improved figure and text presentations strengthen the main conclusions of the manuscript.

In my opinion, the manuscript presented in this way would be a valuable contribution to the field and therefore I strongly advocate the publication of the manuscript. I have only few minor comments to the authors on improving the final clarity of the manuscript:

Minor comments:
1) In the sentence "To understand how simultaneous pulling by KT-attached MTs and Kin14VIb would affect chromosome orientation and segregation during anaphase, we analyzed fixed anaphases of control and Kin14VIb5 expressing cells with a 1-1, or a 2-0 distribution of the TetO locus (Fig EV2A)." the authors cite only figure EV2A. I would recommend to cite here, in addition to EV2A, also some associated figures with microscopy images that were analyzed like Fig. 3A.
2) The order of the EV figures confused me a bit. Why is Fig. EV1 the first figure among EVs when most of its data is cited later in the text? I do not understand why EV1 is cited earlier than EV2? Similarly, I do not understand why Fig. EV4 is cited before Fig. EV5? It could be beneficial to the authors for better clarity of the manuscript to reorder these figures in a more logical way that follows the text.
3) The authors state that "For ~21% of cell divisions with a 2-0 distribution of the TetO locus, we detected a GFP negative MN in at least one of the two daughter cells, compared to 4-9% of divisions with 1-1 distribution of the locus (Fig EV1A)." It would be nice to comment on why GFP+ micronuclei do not increase after Kin14VIb induction? Having the benefit of reading the comments to the reviewers, I personally understand why, but I am not sure if the readers would understand it only from this sentence.

Referee #3:

In this study, Truong et al. describe an innovative strategy to mis-segregate specific chromosomes in different human cell types. In the revised version of the manuscript, the authors have included in the Discussion section the advantages, limitations and potential improvements of their strategy in comparison to previous approaches and to another dCas9-based method meanwhile reported (Davoli lab). This has significantly improved the manuscript and helped to clarify concerns raised by all the referees. Although the strategy faces limitations, there is basis for further improvement that the authors now carefully address. To evaluate immediate cellular responses to aneuploidy, one important step will be to generate dCas9-Kin14VIb cell lines that remain diploid upon clonal expansion. I consider that the authors have greatly addressed the major points raised by the referees. In particular, the authors have now included a systematic comparison of the efficacy of the strategies targeting TetO in U-2 OS or the different endogenous repeats in RPE1 cells, as well as detailed description on issues during the establishment and selection of dCas9-Kin14VIb cell clones. Also, I consider that issues regarding micronucleation and bias to monosomy in the scKaryoSeq have been adequately discussed. Figures and schemes have been substantially improved, allowing the manuscript to read better. Additional control experiments have been included. I congratulate the authors for the revision, and I am supportive of the manuscript's publication in the present format.

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We are pleased to hear that the reviewers considered the manuscript improved after our revisions and that they support publication in EMBO J.

Please find below our response to the minor comments of reviewer 2 and an overview of the other minor textual and presentational edits we made.

Point-by-point response to reviewer #2:

In my opinion, the manuscript presented in this way would be a valuable contribution to the field and therefore I strongly advocate the publication of the manuscript. I have only few minor comments to the authors on improving the final clarity of the manuscript:

Minor comments:
1) In the sentence "To understand how simultaneous pulling by KT-attached MTs and Kin14VIb would affect chromosome orientation and segregation during anaphase, we analyzed fixed anaphases of control and Kin14VIb5 expressing cells with a 1-1, or a 2-0 distribution of the TetO locus (Fig EV2A)." the authors cite only figure EV2A. I would recommend to cite here, in addition to EV2A, also some associated figures with microscopy images that were analyzed like Fig. 3A.

We thank the reviewer for pointing this out, we now refer to both Fig 3A, B and Fig EV2A…note that the latter is now named Fig EV1A (page 7, line 4)

2) The order of the EV figures confused me a bit. Why is Fig. EV1 the first figure among EVs when most of its data is cited later in the text? I do not understand why EV1 is cited earlier than EV2? Similarly, I do not understand why Fig. EV4 is cited before Fig. EV5? It could be beneficial to the authors for better clarity of the manuscript to reorder these figures in a more logical way that follows the text.

We understand the reviewer's confusion. This is because we matched the order of the EV numbers to the order of the main Figures to which to EV's belong. However, to improve readability we now have re-numbered the EVs as follows:

Fig EV1 (old) >> now Fig EV3 (an expansion for Fig 1)
Fig EV2 (old) >> now Fig EV1 (an expansion for Fig 3)
Fig EV3 (old) >> now Fig EV2 (an expansion for Fig 4)
Fig EV4 (old) >> now Fig EV5 (an expansion for Fig 6)

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The Netherlands

Fig EV5 (old) >> now Fig EV4 (an expansion for Fig 8)

3) The authors state that "For ~21% of cell divisions with a 2-0 distribution of the TetO locus, we detected a GFP negative MN in at least one of the two daughter cells, compared to 4-9% of divisions with 1-1 distribution of the locus (Fig EV1A)." It would be nice to comment on why GFP+ micronuclei do not increase after Kin14VIb induction? Having the benefit of reading the comments to the reviewers, I personally understand why, but I am not sure if the readers would understand it only from this sentence.

We have included the following sentence to make it more understandable for the reader:
"The observation that these MN were GFP-negative implied that resolution of the stretched Chr1p arm did not involve the subtelomeric TetO locus."

EMBO Press Author Checklist

Corresponding Author Name: S.M.A. Lens
Journal Submitted to: EMBO Journal
Manuscript Number: 2022_111559

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The data shown in figures should satisfy the following conditions:
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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- 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.).
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  - are tests one-sided or two-sided?
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Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data availability
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
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