Author's response to reviews

Title: Simultaneous Aurora-A/STK15 overexpression and centrosome amplification induce chromosomal instability in tumour cells with a MIN phenotype

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Author's response to reviews: see over
Dear Sirs,

thank you for your response and the helpful suggestions of the Reviewers on our manuscript:

“Simultaneous Aurora-A/STK15 overexpression and centrosome amplification are required to induce chromosomal instability in cells with a MIN phenotype” by Laura Lentini, Angela Amato, Tiziana Schillaci and Aldo Di Leonardo (MS: 7269406251453814)

As requested, we have performed the changes suggested and corrected grammatical errors as well as typos present in the text.

RESPONSE TO REVIEWERS’ COMMENTS

Reviewer: Jonathan JJ Li

General comments.

1. The referee asks for the characterization of the HCT-116 cells as well as for the frequency of MIN in this cell line. Indeed HCT116 cells (a chromosomally stable colorectal carcinoma cell line) and their isogenic derivatives p53−/− and p21−/− are well known and previously characterized cells by the Vogelstein group (Bunz F. et al.; Science 1998; 282 (5393):1497-501). We add this info both in the Results and MM sections.

HCT116 cells are known to have a homozygous mutation in the mismatch repair gene hMLH1 on human chromosome 3, and have high MIN frequency (61%) [Koi et al. 1994; Fishel et al. 1998]. The chromosome number of this cell line is near diploid with a modal chromosome number of 45.

2. The full-length cDNA encoding for Aurora-A/STK15 that we used to transfect HCT116 cells was a gift from Dr. Bischoff JR, and it was showed to be functional and transform immortalized rodent fibroblasts in culture (Bischoff JR, Embo J 1998;17(11):3052-65) (we add this reference both in Results and in MM sections). Based on this published data we did not check for Aurora-A activity.

Regarding the in-vitro evidence in HeLa cells that both active and inactive Aurora-A (Meraldi P., et al. Embo J 2002;21(4):483-92) induced supernumerary centrosomes and CIN, these results might depend on the specific cells used (already ployploid) and likely does not reflect a general feature of Aurora-A ectopic overexpression.

Furthermore Anand et al. demonstrated that the kinase dead form of Aurora-A did not induce polyploidy in MEFs.

Minor essential revisions

3. The difference in the data obtained in these two groups might be due to the different p53
status as we discussed in the text (please see the discussion). Also these differences might reflect the fact that once p53-/- cells acquire a new chromosomal asset (aneuploid) these cells do not necessitate Aurora–A overexpression to maintain it, so that depletion of this kinase does not affect the frequency of aneuploid cells. (We comment these in the results section).

4. We did not understand fully the change requested. Please could the Referee explain what changes we have to incorporate in the Conclusions.

5. The suggested reference was added and the Reference list was updated accordingly.

Reviewer: Prigent Claude

The Referee states that “experiments are well performed but all the data have been previously reported”. We are sorry but we cannot agree in toto with this statement.

We believe that our manuscript presents some new findings, as well as it is consistent with and extends previous observations. We demonstrate that HU-induced centrosome amplification in HCT116 cells is only transient and not sufficient to stimulate aneuploidy. In contrast, overexpression of Aurora-A led to centrosome amplification and aneuploidy. The general conclusion is that centrosome amplification has to be associated with altered expression of critical proteins regulating mitosis in order to trigger aneuploidy.

Also, we observed that supernumerary centrosomes coalesce and form pseudo-bipolar spindles in most HCTp53 KO cells. Whether amplified centrosomes form supernumerary spindle poles, or coalesce in two main poles, has long been debated and is considered an important point. Under our experimental conditions, it would explain why genomic instability does generally not increase after release from the HU block, despite of the transient deregulation of the centrosome cycle induced by HU.

Another interesting finding is that Aurora-A depletion is unable to decrease aneuploidy once it is generated in p53KO cells.

Specific comments

-The Referee suggests that “centrosome amplification” must be used with care.

Even though this term is widely used to indicate increase in centrosome number in many printed papers, we agree with the Referee that it should be used more appropriately. Accordingly, we limited its use to the paragraph describing HU effects. Furthermore, we have changed “centrosome amplification” with the terms “supernumerary/extra centrosomes” throughout the remaining part of the manuscript.

-The Referee recalls the effects of STK15 overexpression in HeLa cells (Meraldi P., et al. Embo J 2002;21(4):483-92). In this paper it is shown that HeLa cells overexpressing Aurora-A undergo centrosome amplification through cytokinesis failure that results in tetraploidization in p53-/— cells. It might be possible that these results are specific for the HeLa cells used. We have to note that HeLa cells are human epithelial cells from a cervical carcinoma transformed by human papillomavirus 18 (HPV18). Horizontal gene transfer from human HPV18 to human cervical cells created the HeLa genome which is different from the parent genome in various ways including its number of chromosomes. HeLa cells have a modal chromosome number of 82, with four copies of chromosome 12 and three copies of chromosomes 6, 8, and 17.

For our experiments we used instead chromosomally stable HCT116 tumor cells. The chromosome number of this cell line is near diploid with a modal chromosome number of 45.
We observed that in these cells after STK15 ectopic overexpression the presence of supernumerary centrosomes is not associated with multinucleate cells (this could be considered a new finding).

- The Referee states that pictures showing γ-tubulin detection are not sufficient to assess “centrosome amplification”.

   In principle we agree with this statement. Nevertheless, γ-tubulin detection is a widely used and accepted mean to look at the presence of supernumerary (extra) centrosomes in mammalian cells. In the attempt to overcome this possible problem we stained centrosomes with an antibody against centrin (a centrosomal protein). Unfortunately this antibody in HCT116 showed high background making very difficult to detect supernumerary centrosomes.

   Anyway, in figure 4 –A pictures show the presence of multipolar spindles suggesting the presence of extra centrosomes, as revealed by γ-tubulin detection, which resulted in the generation of altered mitotic spindles.

- We found that p53 dysfunction is associated with higher Aurora-A expression also in HeLa, and in both SW480 and H1299 cells.

We corrected mistakes in the text:

- Page 7 lane 11: We changed in the text the term “immunocytochemistry” with immunofluorescence as suggested.

- The Referee asks for statistical analysis of results shown in fig.6C. We imagine that the Referee meant fig.5C.

   To this regard we have indicated in the text (page 8 line 18) how many cells with mitotic abnormalities we recorded by live cells imaging. For clarity we have added the total number of cells observed and have indicated the number of STK15 overexpressing mitotic cells showing chromosomes abnormalities (page 8 last line and page 9 line 3)

- We cannot exclude a cytokinesis defect in these cells. However, we observed neither an increase in multinucleate cells nor supernumerary centrosomes associated with multinucleate cells.

- In this manuscript we do not want to investigate the mechanism by which Aurora-A/STK15 overexpression induces centrosome amplification. The point we want to make is that in MIN tumor cells supernumerary centrosomes (caused by HU) alone are not sufficient to induce and maintain CIN. Aurora-A/STK15 overexpression collaborates to induce CIN by altering both centrosome number and mitosis progression also in the presence of functional p53 (at least in HCT116 cells), in addition p53 lack exacerbates this response.

- We did not check if the mitotic checkpoint is on or off. However, cells overexpressing Aurora-A/STK15 progressed through mitosis and this result suggests that the mitotic checkpoint could not be engaged under this conditions.

- The point we want to make by using siRNA strategy is that Aurora-A/STK15 overexpression could directly induce aneuploidy and centrosome alterations. Then its reduction in cells over expressing the kinase should reduce these effects. In fact reduction of Aurora-A/STK15 in HCT-wt stably overexpressing the kinase, has been able to reduce the effects of its overexpression. On the contrary in cells p53−/− depletion of...
Aurora-A/STK15 by RNAi did not affect CIN and reduced slightly cells with supernumerary centrosomes. This finding suggests that its overexpression is not anymore necessary once the chromosomal instability has been induced in p53−/− cells.

-By western blot analysis the Aurora-A/STK15 level left after RNAi seems less that the one present in cells that do not overexpress Aurora-A/STK15.

Reviewer: Stenoein David

1) We revised the manuscript and fixed the grammatical and spelling errors as requested.

2) As suggested we slightly changed the title of the manuscript.

Discretionary Revisions

- The referee suggests that the increase of Aurora-A in the p53−/− cells might be due to the ability of these cells of dividing more rapidly. We have done a FACScan analysis and the distribution of the cells in the G1, S and G2/M cell cycle phases are similar for both the HCT wt and HCT p53−/− cells. In addition, we have evaluated the mitotic index (MI) of these cells to estimate the percentage of cells in mitosis. We found that the Mitotic Index is 20% for HCT-wt and 22 % for HCT-p53KO ruling out a possible difference at the level of cell division rate.