Author's response to reviews

Title: Effects of Delta40p53, an isoform of p53 lacking the N-terminus, on transactivation capacity of the tumor suppressor protein p53.

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Effects of Delta40p53, an isoform of p53 lacking the N-terminus, on transactivation capacity of the tumor suppressor protein p53.

Hind Hafsi, Daniela Santos-Silva, Stéphanie Courtois-Cox and Pierre Hainaut.

Dear Editor,

Please find below a point-by-point letter explaining how we have dealt with each of the reviewer’s comments. The letter also explains what changes we have made in the text.

We would like to thank the reviewers for their positive approach and their constructive comments.

With my best wishes

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Answers to Reviewer’s comments

Reviewer 1:
Major Compulsory Revisions
None

Minor Essential Revisions

1) In M&M section, Cell Culture, paragraph 1, line 3: "10 mg/ml Epidermal Growth Factor" shall be changed to the real concentration, that should be more in the range of "10 ng/ml Epidermal Growth Factor", authors shall check the numbers.

Answer: We thank the reviewer for spotting this mistake. All numbers have been checked and corrections made.

2) In M&M section, Expression vectors, transfections and reporter gene assays, lane 5, (ATG TTG) shall be changed to (ATG>TTG)

Answer: correction made.
Discretionary Revisions

1) Western blot in Figure 6 would improve by adding an additional treatment with a proteasome inhibitor (e.g. MG132), to make clear that p53 degradation or stabilization is through the Ub-proteasome pathway and differences are not due to slight changes in expression or transfection efficiency.

Answer: we have not performed experiments with MG132 in this context. We have used proteasome inhibitors (MG132, lactacystin) or inhibitors of p53:Hdm2 interactions (e.g. Nutlin3A) in contexts of endogenously expressed p53 and we are familiar with their effects. However, we have not used them in a context of co-transfection of p53 together with Hdm2. We are confident that the differences presented in Figure 6 cannot be solely attributed to small variations in transfection (these experiments have been repeated at least 3 times with similar results). However, we agree that additional experiments with proteasome inhibitors would help to clarify whether proteosomal degradation is the main mechanism responsible for p53 “disappearance” in cells co-transfected with Hdm2.

2) Figure 4 can be improved by analyzing the transactivation efficiency of p53 with an additional p53-response element.

In this figure we have selected to work with a beta-Galactosidase reporter driven by a generic (RGC) response element. The reason for choosing Beta-gal rather than luciferase is that, while being less sensitive, beta-Gal gives more stable and reproducible measures than luciferase reporters. RGC was selected because it has the same p53 consensus sequence as the oligomer used in DNA-binding experiments (this information was added in the methods section). We are aware that there is a large diversity of potential p53RE and that effects may vary according to the exact sequence and position of the sequence within gene regulatory regions and this limitation of our study is discussed in the “Discussion” section.

Reviewer 2

Major

1. Figs 4 and 5 are key figures. From the text and figure legends one can conclude that the data points in Fig. 5 (triangles and circles) correspond to the reporter assays in Fig. 4. However, the authors cannot claim that “In Saos-2 cells, expression of #40p53 relative to TAp53 at ratios below or equal to 1:1 led to a small, but reproducible increase of 20-25% in TAp53 transcriptional activity”, because ratios below 1:1 were not tested (if they were, it should be shown in Fig. 4). If the smallest tested ratio was indeed 1:1 (0.5ug each plasmid), the first row of data points in Fig. 5 (corresponding to the activity in the absence of #40p53) is inappropriately placed between ratios “0” and “1” (should be aligned with “0” on the x-axis and should correspond to “100” on the y-axis). Overall, adding more data points which clearly show increased p53 activity would improve the paper.

Answer: The reviewer is right in pointing that experiments in Figure 4 and 5 are of the same type (same cells, same vectors). However, they have been performed
independently, and data in Figure 4 are not from the same experimental series as those used to compile Figure 5. In Figure 5, the TA/Delta40p53 ratios have been selected to correspond to the proportions shown in panel A. Beta-galactosidase activity observed with only TAp53 was adjusted to 100%. The first data point thus corresponds to a ratio of 3TAp53/1Delta40p53. The graph in Figure 5 is therefore correct. To avoid confusion, we have added in “Results” that in Figure 5 the “expression of the TAp53-dependent beta-galactosidase reporter in H1299 and in Saos-2 cells (was tested) at three different TAp53/Δ40p53 ratios. We have also added this information in the legend to Figure 5.

2. Fig. 4 legend says that for both H1299 and Saos-2 cells, the data come from three independent experiments, each performed in triplicate. Then what exactly does each data point represent in Fig. 5 (4-6 triangles for H1299 and only 3 circles for Saos-2) and why are these numbers different?

Answer: as explained above, Figures 4 and 5 come from distinct datasets. The values are therefore different even if the general trend of the results is comparable. In Figure 5, we have compiled data from 6 independent experiments (triangles) for H1299 and from three independent experiments (circles) in Saos2 cells. We have now clarified this in the legend to Figure 5 and we have used colors to better distinguish between the two data series.

3. With regard to (arguably) increased TAp53 transcriptional activity in the presence of low dose of #40p53 (0.5ug plasmid), the authors initially say that “the transcriptional activity was maintained or even slightly increased”. However, they later claim that there was “a small but reproducible increase of 20-25%”. Since the error bar is rather large in Fig 4B, it would be helpful to know the exact p value, as it was done for the other bars.

Answer: we are now giving p-values for these comparisons. The p-value for the comparison between TAp53 0.5 and TAp53 0.5/Delta40p53 0.5 is 0.55 (N.S). In this revised version, we have described these results by stating: “the transcriptional activity was maintained or even slightly increased”. In fact, an increase of between 7 and 25% observed in each independent experiment. However, this increase was not statistically significant when compared to the values used to generate the 100% reference. Thus, we cannot substantiate that there is a significant increase of p53 transcriptional activity at low doses of Delta40p53. The main significant difference lies in the effect of low doses of Delta40p53 in Saos2 cells as compared to H1299 cells. The p-values given in Figure 5 refer to this difference.

4. p21 Western blot signal should be discussed as a p53 activity read-out. In fact, for both H1299 and Saos-2 cells, p21 levels seem to go down as #40p53 is increased. Thus, the slight spike under question might not be real.

Answer: again, the reviewer is right with this point. The data show that p21 expression levels are strongly reduced with Delta40p53 in H1299 cells but are less strongly reduced in Saos2 cells. We have added a sentence in the Result section to explain this.

5. Finally, how do the authors reconcile the strongest inhibition of TAp53 transcriptional activity by #Np53 at 0.5:1.5 ratio (Fig. 4) with their data in Fig. 2
where there is almost no protein interaction at 0.5:1.5 ratio?

Answer: The conditions and TAp53/Delta40p53 ratios are not the same in these two experiments. In Figure 2, the “almost no protein interaction” referred to by the reviewer is seen with 1.5/0.5 TA/Delta40 ratio, whereas the strongest inhibition in Figure 4 is seen with 0.5/1.5 TA/Delta40 ratio.

Minor
Fig. 1A. What are mTA, MTA, asterisk and black bar?

Answer: these acronyms stand for minor transactivation domain (mTA), major transactivation domain (MTA). The black bar highlighted by an asterisk shows the location of the sequence binding Hdm2. These points have been clarified in the Figure legend.

Fig. 1B. The black triangle (“TAp53”) in the 1801 blot does not match up with the DO7 blot. How do you know that this is TAp53, given at least two unspecific bands (the top one visible in the 1st lane) of the same intensity running nearby? Also, please indicate that this experiment analyzed endogenous proteins (unclear).

Answer: We have clarified the size of the bands by showing the position of the molecular weight markers. Note that the two antibodies DO7 and PAb1801 do not react with p53 in a similar way. While DO7 generally reacts with a single band, PAb1801 consistently reacts with several bands. One of them, running with an approximate size of 80 kDa, has been shown to be unrelated to p53 and to be constitutively expressed in many p53-null cells (Bonsing et al., Cytometry 28:11-24, 1997). A recent study has identified that PAb1801 reacts with components of P-Bodies, which plays a role in the regulation of mRNA processing in the cytoplasm (Thomas et al., PLoS One, 7:e36447, 2012). Nevertheless, this antibody generates a clear signal at 53 kDa corresponding to p53. In the case of HCT116 cells, the identification of the 53 kDa and 40 kDa species has been made easier by comparing with HCT116-p53/- cells developed by Bunz et al., Science, 282:1497-1501, 1998. In these cells, the endogenous p53 locus has been interrupted by introduction of a cassette into intron 2, which prevents the assembly of TAp53 but leaves intact the coding sequence of Delta40p53. These cells express levels of Delta40p53 comparable to those observed in parental HCT116 cells, but no TAp53.

Fig. 3. How do you explain that TAp53 appears to bind DNA less in the presence of 1.5ug #40p53 than with 0.5ug #40p53 (judged by super-shift with PAb421, lanes 8 and 12), even though 1ug of #40p53 alone binds DNA better than 0.5ug?

Answer: we do not draw conclusions from these differences in the intensity of bandshifts. This method is not appropriate to derive quantitative information (unless using a range of protein concentrations and demonstrating a dose-dependent relation over several points). The difference pointed by the reviewer did not appear to be consistent from one experiment to the other. In the present case we discuss qualitative observations based on changes in the size and profiles of super-super shifts but our data are too fragmentary to draw conclusions on this.

In the text, “super-super-shift” term should be used to be consistent with Fig. 3
leged.

Answer: This has been made in the revised version

In the first paragraph under the title "Effects of #40p53 on p53 transcriptional activity", references and other p53 targets ("others") are missing.

Answer: The relevant references have been introduced.

Fig. 5B: Clarify what the p values are referring to (difference between the two cell lines?)

Answer: We have modified the Figure legend to clarify what the p-values refer to.

Discussion. Unclear why "Our results suggest that, in order to be efficiently degraded, a complex of p53 isoforms should contain at least two Hdm2-binding domains".

Answer: this is a suggestion based on confronting our results with those of Kubbutat et al., Nature 15: 299-303, 1997, who have shown that dimerization, but not tetramerization, is required for degradation of p53 by Hdm2. In the revised version, we have removed the sentence, which we agree is highly speculative at this point.

Period, not comma, should be used to separate decimals in Figures.

Answer: This has been corrected

Symbols # and # should be used in “Delta40p53” and “beta-galactosidase” throughout the text and figures.

Answer: we have modified the manuscript to use Greek letters as requested.

EMSA stands for electrophoretic mobility (not electro-mobility) shift assay.

Answer: this has been corrected.