Author's response to reviews

Title: The anticancer effect of saffron in two p53 isogenic colorectal cancer cell lines

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Author's response to reviews: see over
POINT-BY-POINT RESPONSE TO REVIEWERS’ COMMENTS

First of all, we would like to sincerely thank the reviewers for their helpful and critical feedback! We have spent the given time adjusting the data to address the points raised and have very carefully modified the manuscript. As a result, the revised version is much stronger and clearer and for this we are truly thankful to yours and the reviewers’ efforts. All changes are marked in red in the attached revised version of our manuscript.

Kindly find below, our detailed ‘point-by-point’ response to all the reviewers’ comments:

Referee 1
1. Include SDS-PAGE conditions (How long was the gel run? Voltage?

   Authors: ...
   We included: Voltage 30mA, running time 1.5-2h into Material and Methods

2. The authors state in the Results section “Saffron has an anti-proliferative effect in p53 isogenic HCT116 cell lines” that “There was a tendency for HCT116 p53 wildtype cells to die more than HCT116 p53 +/-...”. Is the reduction in cell viability between the two cell lines statistically significant? If this is the case, then it should be mentioned in the text. If this is not the case, then the sentence is misleading.

   Authors:
   The significance of the statistical difference between the two cell lines has been added to the text.

3. FACS analysis: The authors show a representative analysis at each time point in HCT116 wildtype and HCT116 +/-, but this is n=1. In order to draw any valid conclusion, the authors should have performed n=4 experiments, determine the mean and standard deviation of the percentage of cells in each fraction and then see whether the differences between the two cell lines are statistically significant.

   Authors:
   We have performed n=3 experiments; s.d. values were always below 10% of the average of the three single values.

4. Fig. 3B: Again, are the differences between the two cell lines at the different time-points statistically significant? If this is not the case, then the sentence on page 11, lines 1-3 “... although especially the early apoptotic fraction ... was higher in HCT116 wildtype cells at 48h (12.8% versus 5.4%, respectively).” Is misleading.

   Authors:
   There were significant differences between the two cell lines at the different time-points. The statistical significance as well as the mean and the standard deviation (n=3) have been added to the corresponding result part.

5. Fig. 4 A-D: The differences between the cell lines regarding the cleaved caspase 3 as well as the expression of PARP, gammaH2AX, LC3-I/II and beclin 1 is not dramatic, so that the critical question is how reproducible these effects are. If one repeats the incubations in three independent experiments, does one always see the same effects?

   Authors:
   The experiments have been repeated 3 times, showing the same effect of saffron on PARP, gammaH2AX, LC3-I/II and beclin 1 protein levels.

6. English needs to be strongly improved throughout the manuscript.

   Authors:
   The manuscript has been improved in English language.

Referee 2
1. The Saffron induced DNA-damage and apoptosis in both cell lines. The role of Saffron in +/- cells is clear. However, it appears that the effect, as noted by authors, is mild in HCT +/- cells. Perhaps, they need to address this with caution as the therapeutic end point in HCT +/- is not as clear and needs to be documented in future studies using long term assays and in vivo models. The Discussion and conclusion need to be tuned accordingly.

   Authors:
   We have added to the Abstract:
   “... further research is needed to elucidate the long-term effects of saffron in p53 +/- tumors.”
   We have added to the Discussion:
“Although saffron demonstrates potential as an anti-cancer drug, further research is needed to elucidate the mechanisms and effects of saffron in p53 -/- tumors. The therapeutic endpoint in p53 -/- tumor cells has to be clarified using long term assays and in vivo models.”

2. *Is p53 induced by Saffron? Can the authors speculate on possibly other mechanisms that lead to induction of apoptosis in both p53 +/- and p53 -/-? Could p73 play any role in HCT -/-?*

Authors:
Reanalyzing p53 protein levels in Western Bloting we showed an increase after saffron treatment. This increase was associated with higher apoptosis induction in HCT116 p53 wildtype cells compared to the HCT116 p53-/- cells. We have added the p53 Western Blot into Figure 2B.

We have further added to the discussion:
Indeed, the role of autophagy in cancer development is complex, as it has been implicated in both tumor survival and tumor cell death [36, 37]. At the present time, we still do not know the genes responsible for inducing autophagy in the p53 -/- cells after saffron treatment. We could however speculate that a p53 homolog such as p73 may have been activated in p53-/- cells and modulated autophagy [38, Crighton et al. 2007] or mild apoptosis. In this regard, it has been shown that p73 interacts with p53-responsive elements and induces transcription of p53-inducible genes [39, Vilgelm et al. 2008].

3. *The authors need to add the p value in the figures.*

Authors:
p value has been added in the figures.