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HSF1 deficiency and impaired HSP90-dependent protein folding are hallmarks of aneuploid human cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 08 May 2014

Thank you again for submitting your manuscript on impaired HSF1 activity and heat shock responses in aneuploid human cells. We have now received reports from three expert referees, which are copied below for your information. While referee 3 is not favorable, both referees 1 and 2 find this work interesting in principle, and we would therefore be happy to consider a revised version of the manuscript further for publication in The EMBO Journal. For such a revision, it will be important to satisfactorily respond to the various concerns raised especially by referee 1. Whereas most of these points can probably be addressed in the text, further experimental data would be required to address points 1 and 2. On the other hand, we would consider it beyond the scope of this revision to elucidate the mechanistic link between aneuploidy and HSF1 impairment (pt. 9), or to repeat the experiments in complex aneuploid cells (pt. 10). With regard to referee 1's point 4, any further data to address this question would clearly be helpful, but we also realize that conclusive results may be difficult to achieve here within the time frame of a revision, and would therefore not consider them essential for publication.

When preparing your response letter, please keep in mind that it will be important to carefully answer to all points at this stage, since it is our policy to allow only a single round of major revision. Please also remember that this letter will form part of the Review Process File, and will therefore be available online to the community upon eventual publication. We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study; should you foresee a problem in meeting this three-month deadline, please let me know in advance and we could discuss the possibility of an extension.

Thank you again for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward

to your revision!

Referee #1:

In this manuscript, Donnelly et al. report that overall protein folding is impaired in trisomic and tetrasomic human cell lines. They find that these cell lines are more sensitive to the HSP90 inhibitor 17-AAG than their euploid controls. They observe this defect to be specific to HSP90 inhibition but not to inhibitors of other protein chaperones. They also study the levels and activity of the heat shock response transcription factor (HSF1) and find both to be lower in the trisomic and tetrasomic cell lines. They demonstrate the rescue of the protein folding defect by overexpressing HSF1. They also report the levels of the HSP90 client proteins decreasing, to show that the defective HSF machinery directly affects the client protein abundance. Finally, they compare the proteome and transcriptome signature of these anueploid cell lines to cells harboring lower HSP90 and HSF1 levels and observe a resemblance.

While the experiments described are of interest to the field, there are a number of significant concerns that reduce the overall impact of the paper.

1. The authors claim that their findings might help understand how protein folding inhibitors can function in cancer therapy. But they also report that gain of long arm of chromosome 8(which harbors HSF1) and gene amplification of HSF1 are frequent occurrences in cancer. If the cancer cells have already adapted to the proteotoxic stress, by upregulating HSF1, the authors should comment on why cancer cells would still be sensitive to protein folding inhibitors. They should test cancer cells harboring increased HSF1 levels or HCT116 cell lines overexpressing comparable levels of HSF1 to test if they are sensitive to 17-AAG.

2. The authors show that when they overexpress HSF1 to very high levels, they are able to rescue the protein folding defect of the aneuploid cells when they are exposed to 17-AAG. They should determine whether overexpression of HSF1 also makes the aneuploid cells resistant to 17-AAG treatment by doing cell viability and colony forming assays (as done in Fig 2A and 2B).

3. The authors claim that the protein folding defect is due to the increased proteotoxic stress that arises because of the extra chromosome(s). It is not clear then why there is no dosage dependent protein folding defect in Fig 1B vs Fig 1A (4 copies of chromosome 5 vs 3 copies of chromosome 5).

4. The authors show that the aneuploid cell lines have decreased levels of HSF1. They should knockdown HSF1 levels in the parental cell lines to approximately the levels observed in the aneuploid lines to determine if the HSF1 decrease observed is sufficient for the protein folding and sensitivity to 17-AAG phenotypes.

5. In Fig S2A, the authors show that the aneuploid cells are not sensitive to AZC, a drug that causes misfolding of all newly synthesized proteins. In fact, the parental cell lines are relatively more sensitive (although not significant). They should comment on why this is the case, given the fact that the aneuploid cells translate more proteins due to their supernumerary chromosomes and thus should be more sensitive to a drug that misfolds all proteins.

6. In Fig S5D, they should elaborate on why the transcriptome signature of the HeLa c-Myc knockdown cells has barely shifted at all from the y axis.

7. In Fig 3A and 3B, the authors show that the aneuploid cells have decreased levels of HSP70 (more pronounced than the decrease in HSP90). Yet these cells are not sensitive to VER 155008, the HSP70 inhibitor (Fig 2C).

8. In trisomic mouse embryonic fibroblasts, Tang et al. observed that the aneuploid MEFs are sensitive to 17-AAG. But they observed an increase in the expression of the chaperone HSP72, contrary to the decrease in levels of HSP90 and HSP70 observed by the authors (Fig 3A and 3B).

They should comment on why they see the opposite effect while using a similar model of aneuploidy.

9. The authors do not present any mechanistic insight into how aneuploidy triggers a decrease in HSF1.

10. The experiments performed are all on simple chromosome gain aneuploid models and not on cells harboring complex karyotypes characteristic of tumor cells.

Minor points:

1. The color schemes on the bar graphs make it difficult to interpret the results.

2. There is a typo in the sentence,"...slightly reduced in all four HCT116 8/3 cell lines(figure S4C,d), in line with our previous funding that the abundance..."

Referee #2:

This manuscript investigates the impact of aneuploidy on protein quality control specifically the Hsp90 chaperone in human cells. The authors very nicely show that in aneuploid cells created by various different means Hsp90 activity is impaired, indicating that the folding reservoir of aneuploid cells is either limiting or impaired. The authors go on to further show why cells not simply turn on the heat shock response in response to depleted chaperone activity. It appears that the heat shock response is impaired and the result of lower levels of the transcription factor HSF1 in aneuploid cells. This is a remarkable finding. The effects are subtle but nevertheless convincing. Importantly, subtle overexpression of HSF1 suppresses the decreased folding capacity of Hsp90 in aneuploid cells and also improves proliferation of aneuploid cells. This finding shows that limited protein folding is at the heart of the aneuploidy-associated defects.

In all, this is a very nice and interesting paper. The experiments are well executed and support the conclusions, all of which are interesting. I support publication with enthusiasm.

Referee #3:

The investigators find that HSF1 and the Hsp90 molecular chaperone function seem mildly decreased in aneuploid cells. However, the studies are extremely limited and make no efforts to determine the mechanistic link, if any between aneuploidy and HSF1 expression / function. The work is thus largely phenomenological in nature and not suitable for publication in a high impact journal. Most of the claims for significance in the Abstract are not valid.

1st Revision - authors' response 28 July 2014

Point-by-point response is presented below. All the reviewers' comments are shown in full.

Referee #1:

In this manuscript, Donnelly et al. report that overall protein folding is impaired in trisomic and tetrasomic human cell lines. They find that these cell lines are more sensitive to the HSP90 inhibitor 17-AAG than their euploid controls. They observe this defect to be specific to HSP90 inhibition but not to inhibitors of other protein chaperones. They also study the levels and activity of the heat shock response transcription factor (HSF1) and find both to be lower in the trisomic and tetrasomic cell lines. They demonstrate the rescue of the protein folding defect by overexpressing HSF1. They also report the levels of the HSP90 client proteins decreasing, to show that the defective HSF

machinery directly affects the client protein abundance. Finally, they compare the proteome and transcriptome signature of these anueploid cell lines to cells harboring lower HSP90 and HSF1 levels and observe a resemblance.

While the experiments described are of interest to the field, there are a number of significant concerns that reduce the overall impact of the paper.

>We appreciate that the reviewer considers the experiments of interest to the field. We note the concerns raised and in response have provided new experimental data to strengthen the claims we advance in our manuscript. We have also amended the text in several instances in order to address the points raised by the reviewer. We believe that our manuscript is now stronger and more complete and we hope that we have adequately addressed all the concerns raised. Please see our point-by-point response below.

1. The authors claim that their findings might help understand how protein folding inhibitors can function in cancer therapy. But they also report that gain of long arm of chromosome 8 (which harbors HSF1) and gene amplification of HSF1 are frequent occurrences in cancer. If the cancer cells have already adapted to the proteotoxic stress, by upregulating HSF1, the authors should comment on why cancer cells would still be sensitive to protein folding inhibitors. They should test cancer cells harboring increased HSF1 levels or HCT116 cell lines overexpressing comparable levels of HSF1 to test if they are sensitive to 17-AAG.

> We thank the reviewer for this remark. In fact, not all cancers are sensitive to protein folding inhibitors, and not all cancers upregulate HSF1. To our knowledge, there is no study that directly analyzed whether protein folding capacity or HSF1 levels in cancer cells in general correlate with their sensitivity to 17-AAG. Moreover, we do not seek to claim that aneuploid karyotypes are the only explanation for the sensitivity of cancer cells to protein folding inhibitors, rather that they represent one of several underlying reasons. We apologize for the overly general nature of our previous statement that we have now corrected as follows (page 14, rows 17-21):

" Our findings together with previous work {Tang, 2011 #7} may provide an additional rationale for why inhibitors of protein degradation and protein folding emerge as a potentially effective cancer therapy and suggest that levels of HSF1 protein and/or activity may be important determinants of sensitivity to 17-AAG."
Additionally, to test whether, in general, the levels of HSF1 correlate with sensitivity to 17-AAG, we have employed transient overexpression of ca-HSF1 in both WT HCT116 and RPE-1 cells and tested the effects on sensitivity to 17-AAG. These results confirmed that indeed the cellular sensitivity to 17-AAG is highly responsive to the levels of HSF1: whereas low levels of HSF1 lead to sensitivity, high levels confer increased resistance to 17-AAG. These new results were now added to a figure 4H, I and the text was amended as follows (page 9, line 14):

"Importantly, the transient ca-HSF1 expression significantly improved the survival of aneuploid cells in the presence of 17-AAG as well as protected the folding of the FlucDM sensor against its effects (Figure 4F, G). Our observations suggest that cellular sensitivity to 17-AAG is finely tuned to the levels of HSF1. To test the generality of this conclusion, we transfected the control HCT116 and RPE1 cells with ca-HSF1 and analyzed their sensitivity to 17-AAG. In agreement with our hypothesis transient overexpression of ca-HSF1 also significantly protected control diploid cells against 17-AAG-associated toxicity (Figure 4H, I)."

2. The authors show that when they overexpress HSF1 to very high levels, they are able to rescue the protein folding defect of the aneuploid cells when they are exposed to 17-AAG. They should determine whether overexpression of HSF1 also makes the aneuploid cells resistant to 17-AAG treatment by doing cell viability and colony forming assays (as done in Fig 2A and 2B).

>The suggested experiment is a logical extrapolation of our findings and indeed, it would be expected that cell viability and colony forming ability upon treatment with 17-AAG will be rescued in aneuploid cells to a similar extent as the protein folding defect was. One pitfall to these experiments, however, is that aneuploid cells are difficult to transfect. We can negotiate this challenge in the protein folding assay, where, because of co-transfection with FlucDM

followed by luciferase assay we score only the transfected cells. Unfortunately, it is rather difficult to normalize the results of cell viability assays to the percentage of transfected cells; therefore, we did not include these experiments in the original submission. Using electroporation, in order to ensure a high transfection efficiency in aneuploids we observed a significantly enhanced resistance to 17-AAG in RPE1 21/3 cells transiently transfected with ca-HSF1 (new Figure 4G, for text see above). Due to the fact that the timeframe required for colony forming assays far exceeds the time when transiently transfected cells express the transgene, we have not performed these experiments. Neverthelless, we are convinced that the above describe result obtained by cell viability assay together with our other findings sufficiently supports the claim that the levels of HSF1 correlate with the cellular protein folding capacity.

3. The authors claim that the protein folding defect is due to the increased proteotoxic stress that arises because of the extra chromosome(s). It is not clear then why there is no dosage dependent protein folding defect in Fig 1B vs Fig 1A (4 copies of chromosome 5 vs 3 copies of chromosome 5).

>We believe that our results do point to a dosage-dependent protein folding defect which can be most clearly discerned in the difference between trisomy and tetrasomy of chromosome 5 in nearly all instances (compare eg. Figure 1C and 1D, Figure 1E, 2A, 3C and 3D). In fact, figures 1A and 1B represent the only experiment in which there is no clear dosage-dependent difference in protein folding between trisomy and tetrasomy of chromosome 5. We speculate that the reason for this may lie in the relatively lower dependency of the wild type firefly luciferase (FlucWT) on the protein folding machinery. The use of double mutated Fluc (FlucDM) reveals a marked difference between the trisomy and tetrasomy of chromosome 5 (Figure 1C and D, please note the different scale of the Y axes). Therefore, we believe that our claim that aneuploidy exerts dosage-dependent effects on protein folding is justified.

4. The authors show that the aneuploid cell lines have decreased levels of HSF1. They should knockdown HSF1 levels in the parental cell lines to approximately the levels observed in the aneuploid lines to determine if the HSF1 decrease observed is sufficient for the protein folding and sensitivity to 17-AAG phenotypes.

>We thank the reviewer for suggesting this important control which helps to strengthen the claims we advance in our manuscript. To address this question, we have depleted HSF1 in HCT116 and RPE1 to 75 % and 50 %, respectively, using a previously published siRNA sequence. In line with our hypothesis, and in concordance with previous reports (e.g. Chen et al. 2013 Oncotarget), we observed that indeed the sensitivity to 17-AAG significantly increases in both instances by approximately 10 – 20 %. These new results are now a part of the Figure 3E, F. We have amended the text as follows (page 8, top):
"The decrease in HSF1 expression observed in aneuploid cells is relatively small and therefore we asked whether it is sufficient to cause the observed impairment in maintenance of proteostasis and protein folding. To address this concern, we transfected the control cell lines with siRNA to partially deplete HSF1 to 75 % and 50 %, respectively (Figure 3E). Indeed, consistent with previous results (Chen et al. 2013), this partial and transient depletion of HSF1 rendered cells sensitive to treatment with 17-AAG, thus suggesting a striking dosage sensitivity of the cellular response to proteotoxic stress (Figure 3F)."

5. In Fig S2A, the authors show that the aneuploid cells are not sensitive to AZC, a drug that causes misfolding of all newly synthesized proteins. In fact, the parental cell lines are relatively more sensitive (although not significant). They should comment on why this is the case, given the fact that the aneuploid cells translate more proteins due to their supernumerary chromosomes and thus should be more sensitive to a drug that misfolds all proteins.

>We agree that these results may appear difficult to reconcile with the fact that aneuploid cells synthesize more proteins and at the same time that HSF1 activity is impaired. We explain these observations based on following facts. First, treatment with AZC affects protein folding at an early stage and leads to the massive and irreversible misfolding of all newly synthesized proteins. These misfolded proteins need to be rapidly ubiquitinated by protein quality control machinery at the ribosome and degraded by the proteasome or by autophagy. Thus, the

machineries of protein degradation likely represent a limiting factor in how well cells can cope with AZC. Since protein degradation, both through the proteasome (Figure S1 B,C) and via autophagy (Tang et al. 2011; Stingele et al. 2012) is enhanced in aneuploid cells, this may explain their moderately enhanced resistance to AZC.
In contrast, HSP90 activity is required later in the protein folding cascade, when proteins are already substantially folded (Taipale, Jarosz, Lindquist, Nature Reviews MCB, 2010). We propose that the HSP90 sensitivity of aneuploid cells is likely a reflection of the impaired function of HSP90-dependent clients and pathways. This reasoning is now explained in the text as follows (page 12, lines 25-31 and page 13, lines 1-7).

"This is mainly due to a defect in HSP90-dependent protein folding, whereas targeting the early steps in protein folding immediately after release from the ribosome, through AZC or HSP70 inhibition, does not preferentially impair the viability of human aneuploid cells (Figure 1, 2, S2). We hypothesize that the toxicity associated with impairment of proper protein folding at an early stage is determined by how efficiently and quickly cells can dispose of terminally misfolded proteins. Since both proteasome activity (Figure S1 B, C) and autophagic degradation (Tang et al. 2011; Stingele et al. 2012) are elevated in mammalian aneuploid cells, this may explain why they are not more sensitive or even slightly more resistant to such impairment. In contrast, we propose that the sensitivity to HSP90 inhibition observed in all the aneuploids analyzed in this study, regardless of the identity of the supernumerary chromosome(s) or the cell line rather reflects the loss-of-function of HSP90 clients and of HSP90-dependent processes."

6. In Fig S5D, they should elaborate on why the transcriptome signature of the HeLa c-Myc knockdown cells has barely shifted at all from the y axis.

> We understand that this result might be surprising. There are two reasons that may explain the results. First, the Microarray data of c-MYC knock down in HeLa cells indeed lead to only a small number of deregulated proteins: the authors report that the highest number of deregulated transcripts in at least one of the four tested cell lines was 741, which is less than 5 % of coding sequences (Capellen et al, EMBO Report 2007). This rather small number of affected transcripts might explain the small quantitative changes of enriched pathways; in other words, the small number of deregulated genes cannot lead to a great change in up or down regulation of a whole pathway. Additionally, the result is influenced by the algorithm used for the analysis (the 2-D pathway enrichment analysis, Cox et al 2012). In this algorithm, not qualitative change of a gene expression, but a quantitative change of a whole pathway normalized to total transcription levels is calculated (calculated as median expression changes of all pathway members divided by median expression changes of all transcripts in analyzed cell lines). Thus, a few deregulated genes would not necessarily affect the whole pathway in a quantitative manner. Nevertheless, we believe that our key claim in this regard, i.e. that the transcriptional consequences of altered HSF1 expression levels and not of c-myc expression levels correlate with aneuploidy, is well illustrated by the plot. We have now specified this point in the text as follows (page 11, bottom lines):

"This analysis suggests that the transcriptional activity of c-Myc does not affect the pathways that are deregulated by aneuploidy".

7. In Fig 3A and 3B, the authors show that the aneuploid cells have decreased levels of HSP70 (more pronounced than the decrease in HSP90). Yet these cells are not sensitive to VER 155008, the HSP70 inhibitor (Fig 2C).

>There are at least two explanations for why aneuploid cells are not more sensitive to this inhibitor. First, the inhibitor VER155008 also binds HSC70 and GRP78 (with IC_{50} values of approximately 2.5 µM, Massey et al., 2010, Cancer Chemother Pharmacol); thus, the toxic effects of VER 155008 may not solely be due to inhibition of HSP70. Alternatively, and similarly to AZC, treatment with VER 155008 likely leads to the misfolding of all newly synthesized peptides. Since protein degradation pathways are activated in aneuploid cells, the removal of these proteins might proceed highly efficiently. In contrast, as stated above, we propose that the sensitivity to HSP90 inhibitors is due to the deficiency in specific HSP90-dependent clients in aneuploid cells (please see our response to point 5).

8. In trisomic mouse embryonic fibroblasts, Tang et al. observed that the aneuploid MEFs are sensitive to 17-AAG. But they observed an increase in the expression of the chaperone HSP72, contrary to the decrease in levels of HSP90 and HSP70 observed by the authors (Fig 3A and 3B). They should comment on why they see the opposite effect while using a similar model of aneuploidy.

> Our observations do indeed differ in some respects from the previous report of Tang et al and we agree with the reviewer that it is necessary to address this particular discrepancy with earlier results. We believe that it is important to note that while levels of inducible HSP72 are elevated in the trisomic MEFs characterized in Tang et al., levels of HSP27, HSP90 and other HSF1-responsive factors (as published in Williams at al, 2008, by the same laboratory) remain unchanged in these cells. If the Heat Shock Response were to be generally activated in trisomic MEFs, then it would be expected that HSP90 levels would also be elevated at least in a proportion of these trisomies. Thus, the higher levels of inducible HSP72 in trisomic MEFs may not be related to activated HSF1-dependent transcription, but rather reflect a specific effect on HSP72, e.g. due to post-transcriptional stabilization of the HSP72 transcript or protein. We have now added comment on this issue as follows (page 13, row 16-19):

"Interestingly, elevated levels of HSP72, but not HSP90 or other factors, were identified in aneuploid murine fibroblasts compared to diploid controls (Tang et al, 2011). The different regulation of HSP72 and HSP90 suggests the possibility that the activation of HSP72 in murine aneuploids is not due to elevated HSF1-dependent transcription, but rather modulated by other means that are specific to HSP72. Despite this difference aneuploidy renders the murine fibroblasts sensitive to HSP90 inhibitors 17-AAG similarly as observed in human aneuploids, thus further strengthening the notion that the HSP90 mediated protein folding is specifically limiting for aneuploid cells."

9. The authors do not present any mechanistic insight into how aneuploidy triggers a decrease in HSF1.

> This is a key focus of our ongoing experiments and while we have some intriguing data which suggest a potential posttranscriptional mechanism by which HSF1 activity is impaired in aneuploids, these results are still preliminary and not suitable for addition to the current manuscript. Nevertheless, we agree that this is an important point and therefore we have briefly expanded our discussion and present new hypotheses for how HSF1 may be impaired in aneuploid cells (Page 13, lines 22-26):
"We propose two hypothetical mechanisms for how HSF1 function might be impaired by aneuploidy. First, the metastable protein HSF1 may be incorporated into the ubiquitin-positive cytoplasmic deposits in aneuploid cells, and thereby rendered inactive. Alternatively, the HSF1 protein may be subject to posttranslational inhibitory regulation that is elevated in aneuploids."

10. The experiments performed are all on simple chromosome gain aneuploid models and not on cells harboring complex karyotypes characteristic of tumor cells.

> The reviewer raises an important point. Indeed, we have recently established a model system of human aneuploid cell lines with a complex karyotypes more similar to the karyotypes observed in tumor cells (Durrbaum et al, 2014). However, we have so far only partially characterized these cell lines. Among their salient features is a high variability and chromosomal instability (CIN). These CIN phenotypes of complex aneuploids make it technically difficult to obtain consistent results across a panel of complex aneuploids. Since we are only slowly untangling which phenotypes arise as a consequence of aneuploidy and which as a consequence of CIN, we decided to focus in this submitted manuscript on the relatively chromosomally stable aneuploid cell lines with trisomies and tetrasomies.

Minor points:

1. The color schemes on the bar graphs make it difficult to interpret the results.

>We agree that our previous color scheme may have led to some confusion and have now changed the color schemes of the graphs to simplify interpretation.

2. There is a typo in the sentence,"...slightly reduced in all four HCT116 8/3 cell lines (figure S4C,d), in line with our previous funding that the abundance..."

We thank the reviewer for bringing this mistake to our attention. "Funding" has now been corrected to "finding".

Referee #2:

This manuscript investigates the impact of aneuploidy on protein quality control specifically the Hsp90 chaperone in human cells. The authors very nicely show that in aneuploid cells created by various different means Hsp90 activity is impaired, indicating that the folding reservoir of aneuploid cells is either limiting or impaired. The authors go on to further show why cells not simply turn on the heat shock response in response to depleted chaperone activity. It appears that the heat shock response is impaired and the result of lower levels of the transcription factor HSF1 in aneuploid cells. This is a remarkable finding. The effects are subtle but nevertheless convincing. Importantly, subtle overexpression of HSF1 suppresses the decreased folding capacity of Hsp90 in aneuploid cells and also improves proliferation of aneuploid cells. This finding shows that limited protein folding is at the heart of the aneuploidy-associated defects.

In all, this is a very nice and interesting paper. The experiments are well executed and support the conclusions, all of which are interesting. I support publication with enthusiasm.

>We are grateful to the reviewer for the enthusiastic support of our research.

Referee #3:

The investigators find that HSF1 and the Hsp90 molecular chaperone function seem mildly decreased in aneuploid cells. However, the studies are extremely limited and make no efforts to determine the mechanistic link, if any between aneuploidy and HSF1 expression / function. The work is thus largely phenomenological in nature and not suitable for publication in a high impact journal. Most of the claims for significance in the Abstract are not valid.

>**We respectfully disagree with the reviewer's evaluation of our manuscript. We provide for the first time evidence that the protein folding environment of human cells is impaired by the addition of a single normal chromosome. Although the decrease in HSF1 and HSP90 activity in response to aneuploidy might be eventually described as mild (for example from 50 to 400 % difference in refolding capacity upon heat shock, Figure 1E, F), this shows that even minor changes in expression levels of heat shock factors might significantly affect the cellular heat shock response and protein folding capacity. Indeed, the impairments which we describe have profound consequences for the transcriptome of human cells and for their proteome composition. We agree that understanding the mechanistic link between aneuploidy and HSF1 activity is crucial and we already have preliminary data suggesting the mechanisms which may be involved. The presented work lays down the basis for future experiments on the effects of aneuploidy on proteostasis in human cells.**

2nd Editorial Decision 06 August 2014

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referee 1 (see comments below), and I am happy to inform you that they are happy with the revisions and have no further objections towards publication in The EMBO Journal.

Before formal acceptance of the paper, I would like to ask you to consider the remaining suggestions of referee 1 and incorporate them into the manuscript text where applicable. You could then simply

send us a modified text document file via email.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

_____

Referee #1

The authors have done an excellent job of responding to the majority of my concerns and I believe this has improved the manuscript. I would like to suggest that they further clarify the discussion of HSF1 levels and sensitivity to 17-AAG, so the readers get a clearer sense of the main issues. Perhaps the focus should not be on absolute levels of HSF1, and how they relate to sensitivity, but on whether the levels of HSF1 are sufficient to compensate for whatever levels of protein folding stress are ongoing in the cell. For example, in figure 4, HCT116 8/3 c3 have substantially unchanged HSF1 levels, but increased sensitivity to 17-AAG. In this case, compared to WT controls, HSF1 levels do not strongly correlate with sensitivity to 17-AAG. These cells, like the other aneuploid lines studied, do have protein folding defects, as measured by the FlucDM assay. Thus, it is perhaps not the absolute values of HSF1 that are important, but how well they are compensating for any protein folding stress. Thus, while we agree with the new statement "...and suggest that levels of HSF1 protein and/or activity may be important determinants of sensitivity to 17-AAG", perhaps there could be a greater discussion of how these two factors would be related. Conceivably, the FlucDM assay is a better indicator of the protein folding state of the cell (and sensitivity to 17-AAG), which integrates both HSF1 activity with the amount of protein folding stress.

Additional correpsondence 11 August 2014

Referee #1

The authors have done an excellent job of responding to the majority of my concerns and I believe this has improved the manuscript. I would like to suggest that they further clarify the discussion of HSF1 levels and sensitivity to 17-AAG, so the readers get a clearer sense of the main issues. Perhaps the focus should not be on absolute levels of HSF1, and how they relate to sensitivity, but on whether the levels of HSF1 are sufficient to compensate for whatever levels of protein folding stress are ongoing in the cell. For example, in figure 4, HCT116 8/3 c3 have substantially unchanged HSF1 levels, but increased sensitivity to 17-AAG. In this case, compared to WT controls, HSF1 levels do not strongly correlate with sensitivity to 17-AAG. These cells, like the other aneuploid lines studied, do have protein folding defects, as measured by the FlucDM assay. Thus, it is perhaps not the absolute values of HSF1 that are important, but how well they are compensating for any protein folding stress. Thus, while we agree with the new statement "...and suggest that levels of HSF1 protein and/or activity may be important determinants of sensitivity to 17-AAG", perhaps there could be a greater discussion of how these two factors would be related. Conceivably, the FlucDM assay is a better indicator of the protein folding state of the cell (and sensitivity to 17-AAG), which integrates both HSF1 activity with the amount of protein folding stress.

>We are gratified that the reviewer is satisfied with our response to the issues raised concerning our manuscript. In addition, we appreciate the opportunity to further discuss and clarify the role of HSF1 in protecting cells from the toxic effects of 17-AAG. This is an important point that deserves careful discussion. We agree that the absolute levels of HSF1 protein per se are probably not the critical determinant of how resistant a cell is to HSP90 inhibition. Rather, it is the magnitude of the Heat Shock Response which cells can mount in response to 17-AAG, which is the key factor. Further, our data demonstrate that increased levels of HSF1 can alleviate chronic and less severe effects on protein folding capacity, such as is the case in aneuploid cells. We believe that our data are consistent with these assertions and we have endeavoured to bring these points across even stronger in the text (page 14):

"However, our results suggest that an additional increase in copy numbers of HSF1 is necessary to rescue the defects arising in response to another whole chromosomal aneuploidy in de novo created trisomic cells so that the activity of HSF1 is sufficient to override the negative effects of aneuploidy on the protein folding machinery. More generally, our results suggest that in the context of the cellular response to severe proteotoxic stress (e.g., HSP90 inhibition), augmentation of HSF1 levels and/or activity fulfils a powerful cytoprotective function."

