Global dissociation of HuR-mRNA complexes promotes cell survival after ionizing radiation

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1st Editorial Decision 23 September 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, referees 1 and 2 express significant interest in your work, and are broadly in favour of publication pending satisfactory revision. Referee 3, on the other hand, is significantly less positive - primarily due to a large number of concerns with the experimental set-up and the interpretation of the data. Most critical here is that of timing: while you conduct your RIP-chip and validation analysis shortly after IR treatment, all the subsequent follow up studies are done several hours after IR. Thus, the effects of HuR dissociation can not be directly linked to any changes in mRNA or protein levels, or polysome association. This links directly to the second major set of concerns of this referee - also reflected in the comments of referee 1, namely that the effects of IR on mRNA stability and translation are rather weak and inconsistent. It would therefore be important firstly to conduct these experiments at a more appropriate time point, and ideally also to extend the analysis to more targets, such that consistent patterns can be more clearly seen.

Given the positive assessment of referees 1 and 2, we are willing to over-rule referee 3’s negative recommendation and invite you to submit a revised version of the manuscript. However, I would stress that it will be essential that you adequately respond to the serious concerns of this critical reviewer. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html
We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This manuscript presents a global analysis (with a detailed follow up 7 selected transcripts) of mRNA-HuR complexes during a Chk2-mediated response of HCT116 cells to ionizing radiation. Interestingly, the authors describe a Chk2-phosphorylation-mediated dissociation of HuR-mRNA complexes that is associated with increased cell survival. Overall this study is a well-designed and generally well-executed demonstration of the contribution of a post-transcriptional regulatory pathway on the cell's response to DNA damage. I think that it will be of high interest to the broad EMBO readership. I do, however, have a few points that should be addressed to polish the study:

1. I'm curious if there is anything in common (e.g. sequence elements, common targets of another RBP, etc) among the subset of mRNAs that fail to dissociate from HuR after IR.
2. Fig. 4: several of the interpretations of these polysome profiles are based on rather subtle effects with no clear indication of their reproducibility. To give full confidence in the interpretations, would it be feasible to provide additional confirmatory data to clearly demonstrate IR modulation of translational status of select HuR targets?
3. Fig. 5A: These data should be quantified to clearly support the claim of more extensive PARP cleavage in Chk2-/− cells. The variations in GAPDH levels between the wild type and null samples make the data a little hard to evaluate qualitatively.
4. Fig. 6C: Since the data presented in this panel are negative, it would be great if the authors can demonstrate that the HuR 3D protein retains some basal activity. The data in Fig. 7B might suffice, but I am a bit concerned that these were done at a different IR dose than the RNA binding data. Perhaps they can demonstrate that it still interacts with one of the constitutively associated mRNAs identified in Fig. 1D?
5. Fig. 7: I note that the significant cytotoxicity assays to assess the role of HuR-mRNA dissociation in cell survival are done at 1 Gy while all of the RNA interaction assays are done at a dose of 10 Gy. Does HuR still dissociate from its mRNA targets at this lower dose of DNA damage?

Referee #2 (Remarks to the Author):

In this manuscript, the authors establish a nice example of how a 3' UTR-RNA-binding protein, HuR, exerts its effect on enhancing cell survival after IR treatment. They show that following IR treatment HuR dissociates with its mRNA targets as a result of phosphorylation by Chk2 kinase, which in turn alters the stability and translation of its mRNA targets identified through co-IP and microarray profiling. As significant number of HuR mRNA targets encode proteins that are directly involved in cell proliferation and apoptosis, the dissociation of HuR from these mRNAs nicely account for the observed increase in cell survival. All the data go into making a very interesting story to general readers.
This reviewer has the following minor points for the authors:

1. Page 3 bottom and page 4 top paragraphs: The authors need to make it clear that increased association of HuR with cytoplasmic mRNA targets via PKC, p38 or cdk1 signaling is a result of HuR translocation to the cytoplasm and thus is a case of HuR abundance increase in the cytoplasm. This is different from increased association (binding) of HuR with its mRNA targets per se, which appears to be the case for Chk2 signaling.

2. Page 12, perspective: It is unclear to this reviewer how the implications fit their proposed model. The authors may consider to rephrase this paragraph. In their model, after IR treatment HuR becomes phosphorylated, leading to its dissociation from mRNA targets. This in turn helps clonogenic survival of the irradiated cells. Then, how "targeting HuR may afford an alternative way of sensitizing cancer cells to therapies like IR"? The authors need to be more explicit about what they mean by "targeting HuR". Similarly, they also need to be more explicit about what they meant by "modulating the phosphatases" in the sentence stating "identifying and modulating the phosphatases that act on phosphorylated residues ...". Do they imply enhancing the phosphatase activity?

Referee #3 (Remarks to the Author):

In this manuscript, the authors present a follow up study to their previous papers in which they report the ability of Chk2 to phosphorylate and subsequently alter the binding of HuR to its mRNA targets (Abdelmohsen et al., 2007; Kim et al., 2008; Kim and Gorospe, 2008). In this manuscript the authors attempt to establish a link between IR treatment, Chk2-mediated phosphorylation of HuR and how this event leads to the dissociation of some HuR mRNA targets and consequently to cell survival. cDNA microarray analysis was performed on mRNAs that were immunoprecipitated from HCT116 colorectal carcinoma cells (WT) or Chk2-/- IR-treated or not, with the anti-HuR antibody (RIP-CHIP experiment). From the list of affected mRNAs the authors focused on validating the association of HuR with seven messages. TJP1, Sox4, Sox9, Myt-1, TP53BP2, Bax that are pro-apoptotic and the KRAS mRNA that is anti-apoptotic. Unfortunately, many of the conclusions made in this manuscript are not supported by the data presented. The authors performed the RIP-CHIP and the subsequent validation experiments on cells treated for 30 min with 10Gy. However, they performed all the functional studies: expression levels, polysome fractionation, survival assay and rescue experiments, on cells treated with IR for 8h or few days (Figures 3-7). There is no experiment showing that upon 8h or few days of IR treatments HuR is still bound to its mRNA targets in Chk2-/- cells. Without this information, it will be difficult to conclude that the effects seen in Figures 3-7 are due to differential association between HuR and these mRNAs. While, the data shown in this paper indicate that IR-treatment does trigger the dissociation from HuR of only some of these mRNAs, yet the authors concluded in the abstract and the discussion sections "that all these messages" were affected the same way in response to IR, by loosing association in IR-treated WT cells, while this association is maintained in IR-treated Chk2-/- cells (see explanation below).

Additionally, the fact that the authors did not provide any experiment assessing whether HuR is involved in the expression of these messages, questions why the authors assessed only the translational effect and not stability as well. The authors are well aware that the absence of an effect on the steady state level of an mRNA does not rule out an effect on stability. Therefore, the fact that IR-treatment does not affect the steady state levels of some of these mRNAs (Figure 3), does not exclude an effect on their half-life and also that this effect could depend on HuR expression. This is an important issue that needs to be addressed, before any conclusion could be made after of the rescue experiments shown in Figure 7 could. If the expression levels of these mRNAs is affected by HuR, and if the authors want to conclude that their association with HuR mediates the survival of cells exposed to IR, they should assess the effect of the phosphomutants of HuR on their expression. These issues and the fact that the interpretation of many other experiments presented in the result sections are not supported by the figures (see below) lowered my enthusiasm for this paper.

It is well-established that apoptosis is induced in cells exposed to IR treatment. The author show that few days are needed for this to happen (Figure 5). My understanding is that exposure to IR-treatment for 24 to 48h is sufficient to induce the death of a variety of cell lines. The HCT116 cells used by the authors are already transformed and have been shown to resist a variety of apoptotic
inducers such as IR. Therefore, the authors should also test the effect HuR have on the survival of normal cells. This is important, since HuR is involved in the regulation of a variety of processes.

Below are examples of the issues outlined above:

1) Figure 2A: The description and the interpretation outlined in the result section do not match the results shown in the figure. The data show that in WT cells, IR treatment reduced the association of HuR with TJP1, Sox4, Sox9 and Myt1 in WT cells. In IR-treated Chk2-/- cells, however, the association of HuR with TJP1, Sox4 and Sox9 mRNAs increased significantly while the association with Myt-1 mRNA did not change in both cell lines. Despite this the authors stated "In agreement with the microarray data.....HuR binding to TJP1, SOX4, SOX9, MYT1 and BAX mRNAs decreased by 30 min after IR treatment in WT cells, but increased in IR-treated Chk2-/- cells (Figure 2A)" (p6, second paragraph, lanes 3-6). This same criticism goes for the association between BAX mRNA and HuR. The figure clearly shows that the association of BAX mRNA with HuR significantly decreased in both WT and Chk2-/- cells exposed to IR. Yet in the same statement the authors considered that BAX-HuR association increased in Chk2-/- cells exposed to IR when compared to WT. In fact, the association of BAX with HuR increased in Chk2-/- cells in the absence of IR-treatment. Since in Figure 3C, the expression of BAX protein increases in these cells only 2h after IR-treatment, it is likely that BAX expression in these cells has nothing, or very little, to with the association of its mRNA with HuR. The fact that BAX is a very potent apoptotic activator, these cells could be already engaged in the apoptotic pathway. This make it difficult to understand how with an increased BAX expression, these cells promote survival pathway as claimed by the authors.

2) Figure 3: Since in this figure the cells are also exposed to IR for 8h and this time is 16 fold longer then the 30min that the authors used to perform the IP experiments in Figures 1 and 2, assessing the association between these messages and HuR upon 8h treatment is absolutely necessarily.

3) Figure 3B: The authors measure the half-life of BAX mRNA in both wt and Chk2 IR-treated cells. They observed that BAX mRNA is stabilized by IR treatment in both cells and its half-life is even longer in Chk2-/- cells. There are several problems with this experiment. 1) Since this experiment entails treating the cells with ActD and IR, the authors should assess the viability of these cells exposed to these two lethal treatments at the same time. Additionally, the figure legend and the Materials and Methods do not indicate for how long these cells were exposed to IR, nor whether ActD was administered at the same than IR or afterwards. I will be very surprised if after IR and ActD treatments (whether together or one after another) the cells remain viable. Moreover, as explained above, stability should also be determined for all the other mRNAs. These are important issues that should be addressed before any conclusion could be made.

4) Figure 3C: In p7, second paragraph, lanes 2-4, the authors state that the protein levels of TJP1, Myt-1, TP53BP2 and BAX were constitutively elevated in Chk2-/- cells with and without IR treatment. The westerns shown in Figure 3C do not support this statement. Indeed, the levels of TJP1, TP53BP2 and BAX proteins significantly increase in Chk2-/- cells only upon 2h of IR treatment (compare lane 0h to 2h in the Chk2-/- cells) while in untreated cells (time 0 of the Chk2-/- side) it remains as low as in wt cells. This indicates that in the absence of Chk2 the expression of these proteins is inducible by IR and is not constitutively high as per the authors' conclusion. Also, no experiment is provided to show that HuR remains associated with these mRNAs during the time course of IR treatment.

5) Figure 4: The main conclusion of this figure is that in Chk2-/- cells treated with IR for 8h, Myt1, TJP1, BAX and TP53BP2 mRNAs are recruited to heavy polysomes due in part to maintenance of their association with HuR. The same criticism outlined above applies here too. The authors do not provide any proof that upon 8h of IR treatment, HuR is still expressed in in Chk2-/- cells and that these mRNAs are still associated with it. Therefore, the statement in p8, second paragraph, is premature and is not supported by the experiments.

6) Figure 5: Since the role of Chk2-/- in cell death and survival is controversial, rescuing the expression of Chk2 in these cells and repeat the same experiments described in Figure 5, is needed before any conclusion could be made regarding the role of Chk2 in cell survival.

7) Figure 6: The association between the phospho mutant HuR isoforms and its mRNA targets was
assessed upon 30 IR treatment. This is not consistent with the 8h treatments used for the experiments described in Figures 3-4. Therefore, before any conclusion could be made regarding the link between the chk2-mediated HuR phosphorylation and the association with target mRNAs, these experiments should be repeated upon 8h of IR-treatment.

Minor points:
- p3, lane 12: the word in "....protein substrates which effect many...." Should be replaced by affect.
- p4, lanes 1-2: The statement " HuR cytoplasmic translocation is mediated by transport proteins like CRM1, transportins 1 and 2, and importin-a1" is misleading. Since transportins, 1 and 2 and importin-a1 regulates mainly the import of HuR, this sentence should read "HuR nucleocytoplasmic movement..."
- p5, lane 6: Since the paper is based on the fact that IR trigger, via Chk2 activation, the phosphorylation of HuR, this data should be shown.

Response to the comments of Reviewer 1:

We appreciate this Reviewer’s positive evaluation of our manuscript and his/her suggestions to improve it.

1. The Reviewer brings up an important question. At this time, we have not been able to identify shared features among the 246 mRNAs that remain bound to HuR after IR. It is possible that specific mRNA sequences do not dissociate upon HuR phosphorylation. As the Reviewer suggests, mRNAs which remain bound may be the targets of another RNA-binding protein (RBP) that preserves HuR binding to these mRNAs, maybe as a multi-RBP complex. In addition, it is possible that some microRNAs help to retain HuR binding to a subset of target mRNAs. These interesting possibilities are too complex to be examined in depth in this manuscript. We will study them in detail as our work progresses.

2. We agree that the effects on polysome distribution profiles, as examined 8 hr after IR in the original Figure 4, were relatively subtle. We appreciate the Reviewer’s request that we obtain additional data supporting a role of HuR in regulating the translation of these mRNAs. In response to this comment, we have done two sets of experiments. First, using larger amounts of cells (which improved the profile analysis, Figure 4A, as there was more RNA available for analysis), we tested polysome distribution profiles at earlier time points. At 3 hr (revised Figure 4), the shifts in polysome distribution were more apparent than at 8 hr (original Figure 4). For example, in WT untreated cells the TJP1 mRNA polysomes peaked at fractions 6 and 11, shifting leftward (consistent with reduced translation) to fractions 3 and 8 after IR; in the CHK2-/- population, TJP1 mRNA polysomes peaked at fractions 6 and 9, shifting rightward to fractions 7 and 10 (suggesting more active translation) in IR-treated cells. Similar trends were seen for SOX4, SOX9, and MDM2 mRNAs. In the case of BAX mRNA, instead of distinct shifts to the right or left, the size of the polysomes (at fractions 6 and 11) decreased after IR. For TP53BP2 mRNA, there was a combination of reductions and shifts in polysome peaks after IR in WT cells, with gains in polysome sizes after IR. For TP53BP2 mRNA, there was a combination of reductions and shifts in polysome peaks after IR in WT cells, with gains in polysome sizes after IR. KRA$ mRNA showed overlapping distribution in WT cells, with apparent increases in polysome sizes after IR in CHK2-/- cells. By 3 hr after IR, GAPDH mRNA overlapped in both cell types regardless of treatment. At least three separate sets of polysomes were prepared and analyzed, showing similar trends (some mRNAs were analyzed more than 3 times).

The second set of experiments is still preliminary and therefore is presented in the Supplementary Figure 1 for Reviewers. Here, we hypothesized that HuR would bind the 3'UTR of targets TJP1, MDM2, KRA$, TP53BP2, and BAX mRNAs and prepared reporter constructs derived from the parent vector psiCHECK-2, which expresses reporter protein RL (renilla luciferase) and internal control protein FL (firefly luciferase) from different constitutive promoters on the same plasmid backbone. As the KRA$ 3'UTR is ~4 kb long, we split it into two overlapping 3'UTR segments. By 24 hr after transient transfection of each reporter construct into HCT116 cells (WT and
CHK2−/−), cells were either left untreated or treated with IR and collected 2 hr later. RL activity was normalized to FL activity in each transfection group and represented as change relative to the empty vector (pLuc). As shown, expression of the reporter constructs was unchanged or lower in IR-treated WT cells, while in CHK2−/− cells, the reporters showed modest but consistent increases in reporter activity. These results support the view that IR selectively enhances expression of these reporter constructs in CHK2-deficient cells, but causes little change or slight reductions in reporter expression in WT cells. While in keeping with the main conclusions of our study, we feel that these data are too preliminary for the main manuscript, since we first would need to 1) map where HuR binds, 2) optimize the transfection conditions (plasmid concentration, times after transfection, times after IR, etc) for each of the 6 reporters, 3) establish whether the changes in luciferase activity arise from altered stability or translation of the reporter transcripts, 4) prove that changes in reporter expression are attributed to HuR, etc... We explain in the revised Results section that detailed analyses of each individual target will be the subject of future work.

3. The Reviewer makes another excellent suggestion. In response to his/her request, we have tested PARP cleavage more extensively, assessing it daily for 5 days after treatment with a range of doses. As shown in the revised Figure 5A, exposure to 1, 2.5 or 10 Gy led to more extensive and persistent cleavage of PARP over the following 5 days in CHK2−/− cells than in WT cells (with the exception of day 3, 10Gy, WT cells). These data further strengthen the view that IR treatment is preferentially toxic to CHK2-deficient cells.

4. The Reviewer brings up a good question. We were unable to identify strong binding of HuR(3D) to any target mRNA. For more systematic identification of possible targets, we planned to repeat the microarray analysis using the HuR(3D) and HuR(3A) mutants. However, the minimum 64 microarrays (likely many more) needed to perform this analysis was too costly to perform at present. We will carry out this investigation as soon as possible.

5. To address this important concern, we assayed binding to HuR target mRNAs in a dose- and time-dependent manner. This extensive analysis is shown in the revised Figure 2. As can be observed, in WT cells HuR dissociated rapidly from all of the target mRNAs tested and remained dissociated for the ensuing 24 hr. By contrast, in CHK2−/− cells HuR remained bound to these mRNAs regardless of time and IR dose tested.

Response to the comments of Reviewer 2:

We thank this Reviewer for his/her positive comments on our manuscript.

1. We appreciate the Reviewer’s important request for clarification. The revised text on pages 3 and 4 explicitly distinguishes more clearly among kinases that influence HuR localization and kinases that influence HuR binding to target mRNAs. The Nebreda and Eberhardt laboratories reported that HuR phosphorylation by p38 and PKC directly affected both the subcellular localization of HuR and HuR binding to target mRNAs. Thus we included these kinases in both types of HuR regulatory mechanisms – HuR translocation and HuR binding to target mRNAs.

2. We appreciate the Reviewer’s additional requests for clarification. In the revised Perspective we address the Reviewer’s questions on HuR and the efficacy of radiotherapy.

Response to the comments of Reviewer 3:

We appreciate this Reviewer’s thorough evaluation of our manuscript and his/her suggestions to improve the work.

We shared this Reviewer’s concern (also raised by Reviewer 1) regarding HuR binding to target mRNAs at later time points after IR. In the revised Figure 2, we have examined HuR binding to target mRNAs over a range of IR doses and times after IR. This extensive set of experiments, shown in the revised Figure 2, revealed that in WT cells HuR dissociated rapidly from the target mRNAs tested, and remained dissociated for the ensuing 24 hr; all doses studied (1, 2.5, and 10 Gy) triggered a comparable dissociation of HuR from target mRNAs. By contrast, in CHK2−/− cells HuR remained
essentially bound to these mRNAs regardless of time and dose tested. The Reviewer’s additional point is well taken. It will be important to extend the analysis to more HuR target mRNAs in order to see how universally HuR affects its target transcripts after IR. Although a molecular characterization of dozens of HuR target mRNAs is beyond the scope of this investigation, we have added the analysis of one more target (MDM2 mRNA). To avoid misleading the reader, we have carefully revised the text and have removed inappropriate generalizations.

The Reviewer is correct that a lack of changes in steady-state mRNA levels does not necessarily mean that the stability of the mRNA is unchanged. Theoretically, increases or decreases in mRNA stability could be compensated by changes in mRNA transcription, having a net effect of unchanged steady-state mRNA abundance. However, when the changes in the levels of a protein are more potent than the changes in the mRNA that encodes it, efforts are not typically directed at dissecting the relative contribution of mRNA transcription versus mRNA stability. Instead, they are aimed at understanding what mechanisms directly affect protein expression from the available pool of mRNA (e.g., changes in translation rates). Nonetheless, we did examine the stability of several of the mRNAs studied in detail in the manuscript – besides BAX mRNA and the housekeeping transcript GAPDH mRNA, we looked at TJP1, MDM2, KRAS, and P53BP2 mRNAs. As shown in the Figure 2 for Reviewers, most of these mRNAs are relatively long-lived and IR does not dramatically change their half-lives over untreated cells. Given that there was toxicity after >4 hr in Actinomycin D, their half-lives could not be compared accurately. Moreover, the changes in protein abundance occurred earlier than changes attributable to altered mRNA stability. We have revised the Results section to incorporate these observations.

As the Reviewer points out, IR can trigger apoptotic death, but the magnitude of this response is highly dependent on the cell type. For HCT116 cells, we carefully titrated the dose of IR which killed only a fraction of the cells, so that we could ascertain survival differences between WT and CHK2-/- cells. In the revised manuscript, we have examined the toxic response to IR in greater detail by monitoring PARP cleavage over a broader range of doses and a more complete time course (revised Figure 5, panels A and B). As shown, CHK2-/- HCT116 cells showed more PARP cleavage at almost all IR doses and times. Testing the response of untransformed cells (e.g., human diploid fibroblasts) relative to that of HCT116 cells would have been interesting. However, there is extensive literature that compares the differential response to IR by transformed relative to untransformed cells, so it would not be particularly informative to repeat this comparison here (nor would it offer additional insight into the role of HuR). Besides these general considerations, there are technical limitations that make such analysis impossible: for example 1) there are no untransformed cells bearing somatic CHK2 deletions, and 2) transfection rates are typically very low for untransformed cells, making it extremely difficult to interpret experiments in which HuR or Chk2 levels change in only a small fraction of cells.

Specific Comments

1. Following up on his/her comments above, the Reviewer notes that binding of HuR to some mRNAs increases in the CHK2-/- cells. To have a more complete understanding of this process, we tested binding of HuR to a subset of mRNAs over time (up to 24 hr) and over a range of IR doses. These results, presented in the revised Fig. 2, indicate that in CHK2-/- cells the mRNAs remain bound, with moderate fluctuations (higher or lower by up to ~twofold). By contrast, in WT cells, these mRNAs are markedly reduced at all IR doses and remain dissociated from HuR during the time period analyzed. We did not attempt to elucidate the specific contribution to apoptosis by each of these many targets; in fact, as the reviewer appreciates, engagement in apoptosis depends on the balance among Bcl-2 family members (including Bcl-2, Bcl-xL, Bcl-w, Bcl-b, Bad, Bcl2a1, Bid, Bax, Bak, Bok, Bim, McI1, Hrk...). Establishing the relative levels of all members of this large family is beyond the aims of this study.

2. We fully agree, it is essential that we examine the association between HuR and the target transcripts at later time points. These data are presented in the revised Figure 2.

3. As discussed above, the Reviewer is correct in saying that the absence of changes in steady-state mRNA levels does not necessarily mean that mRNA stability is unchanged. In principle, increases or decreases in mRNA stability could be compensated by changes in mRNA transcription, having a net effect of unchanged steady-state mRNA abundance. However, when the changes in the levels of a protein are more pronounced than changes in the mRNA that encodes it, it is generally considered more important to examine what mechanisms directly affect protein expression on the available pool of mRNA (e.g., changes in translation rates) than to study the relative contribution of mRNA...
transcription versus mRNA stability. As mentioned earlier, we examined the stability of several of the mRNAs studied in the manuscript – besides BAX mRNA and the control transcript GAPDH mRNA, we looked at TJP1, MDM2, KRAS, and P53BP2 mRNAs. Figure 2 for Reviewers shows that most of these mRNAs are relatively long-lived and IR does not dramatically change their half-lives relative to untreated cells. This fact is mentioned in the revised Results section. The Reviewer is also correct in noting that both IR and actinomycin D are toxic treatments, so their combined effect might be especially damaging; this fact is now explicitly noted in the Results section. In response to his/her specific question, we added actinomycin D immediately after cells were subjected to IR. By 4 hr of actinomycin D treatment, the toxicity in the IR group was comparable to the toxicity seen in the no-IR group; we saw substantial toxicities at later time points, so the relative half-lives of this group of stable transcripts (TJP1, MDM2, KRAS, and P53BP2 mRNAs) could not be compared.

4. The concern regarding the persistence of dissociation from HuR has been addressed above (general critique and comment 2) and in the revised Figure 2. We appreciate the more precise description of Figure 3C offered by the Reviewer and have modified the text as advised.

5. The Reviewer brings up two valuable questions. In response to the first question, HuR does remain dissociated from target mRNAs in WT cells, but remains bound in CHK2−/− cells (Figure 2A). In response to the second question, HuR is indeed expressed by 8 hr after IR (‘480 minutes’, according to the time scale used in Figure 1C).

6. The Reviewer makes the important request that we further test whether the influence on survival is truly due to Chk2. As he/she points out, the role of Chk2 on cell survival is controversial. In fact, when we began the studies described here, we hypothesized that the CHK2−/− cells would be selectively resistant to IR. Re-expressing Chk2 in the CHK2−/− cells at the right concentration, time, etc, would have been exceedingly challenging and we were unable to obtain an expression plasmid to attempt this. Therefore, to answer the Reviewer’s question, we silenced Chk2 transiently in WT cells by using CHK2 mRNA-directed siRNA. As shown in the revised Figure 5 (new panel B), silencing Chk2 selectively increased PARP cleavage. These results support the view that in this colon cancer model (HCT116 cells), Chk2 is protective against cell death induced by IR and our further conclusions that HuR ribonucleoprotein dynamics critically influence the overall IR response.

7. Once again, these important data are shown in the revised Figure 2.

Minor Points

- The suggested the replacement of ‘effect’ with ‘affect’ (p3, lane 12) is appreciated. Both ‘affect’ and ‘effect’ might be used here, although with different meanings. In this case, ‘effect’ had been chosen deliberately, as the word was intended to mean ‘carry out’ or ‘implement’ (not ‘influence’, as the Reviewer read it). We have substituted the word ‘effect’ for ‘implement’ to avoid confusion in the revised Introduction.

- We appreciate the alternative wording on page 4 regarding nucleocytoplasmic movement. We have revised it as advised.

- We agree with the need to show data on HuR phosphorylation. These results are shown in the revised Figure 1 (new panel C).
Supplementary Figure 1 for Reviewers. Preliminary reporter analysis of HuT targets. (A) Schematic representation of the pGL3-ROCS plasmid (pLuc) and constructs containing the complete 3'UTR of the main HuT target mRNAs studied in the manuscript. All 3'UTR sequences (including the sequence shown on the right) were subcloned immediately after the FL stop codon. The long 3'UTR of KRAS-mRNA (4 kb) was subcloned as two partly overlapping segments (KRAS-A and KRAS-B). (B) Twenty-four hr after transient transfection of each reporter construct into HuT-118 cells (WT and CHK2-/-), cells were either left untreated or treated with IR (10 Gy) and collected 2 hr later. RL activity was normalized to FL in each transfection group and represented as change relative to the empty vector (pLuc).
Many thanks for submitting the revised version of your manuscript EMBOJ-2010-75804R to the EMBO Journal. It has now been seen again by referee 1, whose (brief) comments are enclosed below: as you will see, he/she is satisfied with your revision and now fully supports publication. I am therefore pleased to be able to tell you that we will be able to accept your manuscript for publication here. However, before we can do so, I need to ask you for an author contributions statement: this is something that we are now routinely requesting. Please could you send me a revised version of the manuscript text including this statement that we can upload in place of the previous version? Once we have this, we should then be able to accept your manuscript without further delay.

Referee #1
The authors have carefully considered all of the criticisms raised in the previous review and in my opinion have effectively addressed them.