THE EMBO JOURNAL

Manuscript EMBO-2011-80045

The p57 CDKi integrates stress signals into cell cycle progression to promote cell survival upon stress

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Review timeline:

Submission date:	04 November 2011
Editorial Decision:	21 December 2011
Revision received:	17 March 2012
Accepted:	04 April 2012

Transaction Report:

(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 21 December 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal, and once more apologies for the delay in its evaluation. We have now received all three sets of referee comments, which you will find copied below. All referees acknowledge the interest and potential importance of your identification of a new link between p38 and p57Kip2 in the cellular stress response. At the same time, they raise a number of concerns that would have to be adequately addressed before eventual acceptance. As you will see, most of these points and suggestions correspond to relatively specific issues and further clarification/explanation/discussion; however, there are also a few more substantive concerns, in particular referee 3's question (point 1) about the relative importance of p57 compared to other p38 targets, and referee 2's request for stronger demonstration of physiological significance in figure 4.

Should you be able to satisfactorily address these major as well as the various more specific concerns, we should be able to consider a revised version further for publication. In this respect, please be reminded that it is our policy to allow a single round of major revision only, and that it will therefore be important to diligently and comprehensively answer to all the specific points raised at this stage in the process. When preparing your letter of response to the referees' comments, please also 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, please visit our website: http://www.nature.com/emboj/about/process.html

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. 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 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.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript sheds light in the regulation of p57KIP2 after various forms of stress providing in vitro and in vivo evidences of a new molecular link between p38 and p57KIP2 with a significant role in cell control. It is a well-written manuscript, providing in a constructive manner the regulation of p57KIP2 after the different types of stress, examining also the impact on the others members of CIP/KIP family. Taking into consideration the importance of both p38 and p57KIP2 in the developmental processes as well as its anti-oncogenic effects with the frequent down-regulation of p57KIP2 in several types of cancer, this link seems to have an important impact in cancer biology. However, the authors should clarify a few points in order to reinforce the present work.

Major comments
1. Could the authors clarify the type of death after the various forms of stress in the p38-/- and p57KIP2-/- MEFS providing in addition an assay determining in a direct way the cell viability (like MTT assay)?

Minor Comments
1. In Figure S4 you show that in p57KIP2-/- MEFS upon osmostress p21WAF1 is up-regulated. Could you comment on this?
2. In the discussion it is worthwhile to refer to the work of Chang TS et al (2003), mentioning that p57KIP2 interacts with and inhibits the JNK/SAPK.
3. In Figure 1A please specify the cell line used.
4. In Figure 5B you could provide within the figure what the open and solid circles represent.

Referee #2 (Remarks to the Author):

Review to The p57 CDKi integrates stress signals into cell cycle progression to promote cell survival upon stress, M. Joaquin et. Al

In their paper the authors uncover a new function the cyclin kinase inhibitor p57 in the regulation of osmo-stess.

Fig.1: The authors show that bacterially expressed p57 is phosphorylated by p38 in vitro. The kinase assay and coomassie gels show two fragments where p57 runs. What is the identity of the lower band? Is it a cleavage fragment of p57?
I am also confused by the labeling and corresponding figure legends of figure 1E. In Fig. 1c a band which is labeled MKK6DD runs below the p57 bands. In fig. 1e it runs above the p57 band even though p57 is flag-tagged in this experiment. None of the additional bands is described in the figure legend. This needs to be clarified.

Fig. 2: Have the authors tried to co-precipitate endogenous p38 with endogenous p57. Are both endogenous proteins expressed in the same sub-cellular compartment. Where do the overexpressed proteins localize?

Fig. 3: Even though I find figure three convincing in so far as that it shows that overexpressed p57 will be phosphorylated at T143A I do not understand why the authors show the results gained with the unspecific antibody in the main text and the result using their own antibody in the supplement. Is it necessary to show both?
Moreover the interpretation that the observed p57 is caused by p38 stems from the use of a pharmacological inhibitor. Have the authors used other ways to inactivate p38 in vivo i.e. siRNA or dominant negative versions of p38? What happens if the osmo stress signalling cascade is inhibited further upstream? What is Birb 0976? I could not find any reference for this inhibitor, nor evidence that it is specifically inhibiting p38 at the concentrations used.
Does the T143 antibody recognize endogenous p57?

Fig. 4: The experiments in fig. 4 suggest that phosphorylated p57 will inhibit cdk2 associated kinase activity better then non-phosphorylated p57. All of these experiments are done with overexpressed proteins. It will be necessary to show that endogenous p57 will get phosphorylated at T143 after osmo-stress, that this is induced by p38 and that endogenous cdk2 is inhibited under these conditions. Otherwise I fear that these results might not hold much relevance for the in vivo situation.

Fig. 6: Fig. 6 a is a central experiment of this work. Particularly important ist he question whether the re-constitution of G1 arrest in response to osmostress can be achieved by expression of the T143A mutant. The figure makes it difficult to jundge this as it displays the results in separate sub-figures. It seems as if at 8 hours after osmostress the wt and T143 re-constituted cells seem to behave very similar while the pure nulls have already progressed in the cell cycle. Only at later time points does the T143 mutant seem to show a difference in maintaining the G1 arrest. Do the authors have an explanation for this?

Fig. 7: The differences in cell survival upon osmostress seem to start at concentrations of 200mmol NaCl. Before that I do not see a significant difference in the survival plot shown in fig. 7a (significance levels are also not shown). In figure 6 the authors used 100mmol NaCl to investigate the effects of p57 on osmostress induced cell cycle arrest. Would the results obtained in fig. 6 be identical if 200 or 300mmol NaCl would have been used? Have the authors tested this?

General remark:
Even though I believe that the the authors might be up to something interesting here, I feel that the evidence presented at this stage is not convincing enough to recommend publication.

Referee #3 (Remarks to the Author):

This is an interesting manuscript that demonstrates a role for p38 MAPK in the regulation of p57Kip2. Specifically, the authors show that p38 phosphorylates p57 on Thr 143 and that this causes increased cdk2 binding and inhibition. This is a strength of the manuscript. However, there are a number of questions that the authors do not address:

1) Previous studies have demonstrated that other p38-targeted proteins regulate cell cycle in response to osmotic (and other) stress by a p38 MAPK pathway, including Cdt1 and MK2. The relative importance of these p38 targets is unclear. Why is p57 an important p38 target with biological consequences when there are other targets that also affect the cell cycle ? This question needs to be directly addressed.

2) p27kip1 is also a p38 target - what is the relevance of the p57kip2 findings in the context of previous knowledge concerning p27?

3) How does p57 phosphorylation affect Cdk2 binding - can insight be obtained from molecular modeling ?

4) p57 has been reported to regulate cyclin D sub-cellular localization. Is this role of p57 affected by p38 MAPK ?

1st Revision - authors' response 17 March 2012

Thank you for your interest and suggestions on our work. Attached is a revised version of our manuscript (EMBOJ-2011-80045) entitled **"The p57 CDKi integrates stress signals into cell cycle progression to promote cell survival upon stress"**. The reviewers were very positive about the interest and potential importance of the link between the p38 SAPK and the p57 CDKi. They also raised a number of questions on some of the aspects presented in the previous manuscript. We have addressed all the specific and general issues raised by the reviewers which have been very helpful to improve the manuscript.

New major experiments included in the revised manuscript are the detection of endogenous p57 phosphorylation, the interaction of endogenous p57 and p38, the study of cell death induced upon stress in $p38^{-/-}$ and $p57^{-/-}$ deficient cells and the critical biological role of p57 in cell survival upon stress when compared to other cell cycle regulators targeted by p38 such as p27, Cdt1 or Mk2. In addition, all the changes and modifications by the reviewers have been included in the revised version of the manuscript.

We believe that the revised manuscript is therefore improved over the initial submission and we hope that its general interest, novelty and conclusions make it suitable for *The EMBO Journal*.

Reviewer 1

The reviewer stated the interest and quality of the work presented as well as the relevance of the regulation of p57KIP2 after different types of stress. The reviewer also pointed out the significance of the data for other areas of knowledge based on the role of both p38 and p57KIP2 in cellular processes such as cancer. He/She asked to clarify a few points to reinforce the manuscript that are discussed below point by point.

1.The reviewer suggested to clarify the type of cell death caused by stress in the $p38^{-/-}$ and $p57^{-/-}$ MEFS providing an assay determining in a direct way cell viability. He/She proposed to assess cell viability by using the MTT assay. As suggested by the reviewer we have performed an MTT assay in wild type, $p38^{-/-}$ and $p57^{-/-}$ deficient cells upon several stress treatments. Correspondingly to previous data, the assay showed that $p57^{-/-}$ and $p38^{-/-}$ cells suffered a strong reduction in viability when subjected to NaCl, H_2O_2 and Ionomycin when compared to wild type. In contrast, $p57^{-/-}$ cells were as resistant as wild type cells to UV as shown before. We have included the new data as Supplemental Figure S6.

Furthemore, we have also monitored nuclear condensation, a hallmark of programmed cell death. Consistent with what we observed in the MTT assay, $p38^{-/-}$ and $p57^{-/-}$ cells displayed strong nuclear condensation when subjected to NaCl, H_2O_2 and Ionomycin. In contrast, UV induced nuclear condensation only in $p38^{-/-}$ cells but not in $p57^{-/-}$ cells, as expected from cell viability assays. Therefore, the reduction on cell viability caused by stress is due to apoptosis in both $p38^{-/-}$ and $p57^{-/-}$ cells. We have included the new data as Figure S7 and modified the manuscript accordingly.

2.The reviewer asked to comment on the data presented in Figure S4, in which we showed that, in $p57KIP2^{-/-}$ MEFS upon osmostress p21WAF1 was up-regulated. As the reviewer pointed out, this is indeed a very interesting observation as it seems that expression of p21 increases to compensate for the loss of p57, independently of stress (Figure S5C). We have extended this initial observation by analysing the levels of p21 in $p57^{-/-}$ cells transfected with an empty plasmid or a plasmid carrying wild type p57. The expression of p57 in $p57^{-/-}$ cells resulted in

the reduction of p21 levels, confirming that the lack of p57 results in the upregulation of p21. We have included the new data as Figure S5B and C and discussed the observation in the Discussion section as suggested by the reviewer.

3.The reviewer suggested to refer in the discussion the work of Chang TS et al (2003), mentioning that p57KIP2 interacts with and inhibits the JNK/SAPK. Indeed, this is a good suggestion and we have modified the discussion to include the relationship of JNK with p57.

4.The reviewer suggested to specify in Figure 1 the cell line used. We have done as suggested and modified Figure 1 legend accordingly.

5.The reviewer asked to provide within Figure 5B what the open and solid circles represent. We have modified Figure 5B and included the symbol legends as suggested.

Reviewer 2

This reviewer also pointed out the interest of uncover a new function for the p57 CDKi in osmostress. He/She felt that additional work was required to make it more convincing. We have followed all the suggestions of the reviewer to improve the manuscript that are discussed below point by point.

1.The reviewer raised some questions on the labeling of the bands present in Figure 1. Precisely, he/she asked about the identity of the lower band present in the kinase assay and coomassie gels where p57 run. This is, as noticed by the reviewer, a cleavage fragment of p57 (a little bit shorter) that also contains GST. We have modified figure 1 legend to clarify the nature of the band. He/She also asked to clarify the reason of the different size of the bands of Mkk6 and p57 in figures 1C and 1E. The differences on the two panels are due to that p57 is tagged with GST in Figure 1C and with Flag in Figure 1E. However, we completely agree with the reviewer that this was not sufficiently clear in the figure and we have now modified the figure and the figure legend to improve the clarity of the data presented.

2. The reviewer asked to clarify several points on the interaction of p38 with p57. First he/she asked to co-precipitate endogenous p38 with endogenous p57; second, whether both endogenous proteins were expressed in the same sub-cellular compartment and, third, the localisation of the overexpressed proteins. We agree with the reviewer that these are relevant points and we have addressed all experimentally.

We have been able to optimize the pull-down experiments with p57 and we are able to detect the interaction between endogenous p38 and endogenous p57. We have done it on both directions and using as a control either IgG, or $p38^{-/-}$ and $p57^{-/-}$ deficient cells. The data obtained support our initial observation that pointed out towards the interaction of both proteins. We have included the new data as part of Figure 2 (Figure 2C and 2D) and modified the text accordingly.

As for the localisation; we have seen that p57 fused to DsRed, is mainly nuclear and this localization is not altered upon stress. The localisation of p38 fused to GFP is throughout the cell, displaying also a clear nuclear accumulation. Under the microscope, it seems as there is an increase on the nuclear accumulation of p38 upon stress (Figure S3B). To monitor endogenous p38 and p57 we have made use of cellular fractioning since the antibodies against p57 were not sufficiently good for immunofluorescence detection. Here, we have seen that p57 is mainly nuclear and that p38 is present in both cytoplasm and nuclei. We do not observe major changes in cellular distribution upon stress. Therefore, the data from the tagged proteins reflect quite well the distribution of endogenous proteins and clearly show that both p38 and p57 co-localise when p38 is activated upon stress. The new data have been included as part of Figures 2 and S3.

3.The reviewer found the data from figure 3 convincing but asked whether it was necessary to show the data presented in Figure S1 with our phospho-p57 antibody for being redundant with the results presented in Figure 1. Indeed, the data presented in both figures are redundant but

served to demonstrate that it is specifically T143 in p57 the target for p38. We have now used the phospho-p57 antibody also to visualize *in vivo* p57 phosphorylation (new Figure 3D) and thus, we believe that showing its specificity in a supplementary figure it is now justified.
The reviewer also mentioned that specificity of the phosphorylation on p57 by p38 was mainly demonstrated by the use of a pharmacological inhibitor for p38. He/She asked to assess the phosphorylation of p57 by using alternative ways of p38 inhibition. Following the reviewer suggestion we have assessed the phosphorylation of p57 in response to stress (NaCl) in $p38^{-/-}$ deficient cells and found that, consistent with our previous data, in $p38^{-/-}$ cells p57 is not phosphorylated upon osmostress. The new data have been included as the new Figure S1D.
The reviewer asked whether p57 phosphorylation was affected by inhibition of upstream components of the signaling cascade. Unfortunately, there are no good inhibitors or siRNAs that completely block all the MAPKKs or MAPK3Ks upstream of p38 and thus this experiment is very difficult to be performed. However, the data presented in the manuscript clearly points out that the MAPKKs upstream of p38 are regulating the p57 upon stress via p38. For instance, the phosphorylation of p57 by p38 can not only be induced by stress in a p38 manner but also by overexpression of a constitutive active MKK6 (the MAPKK upstream of p38). The phosphorylation of p57 upon $MKK6^{DD}$ induction is also p38 dependent (Figure 3). Therefore, taken together all our data, it is reasonable to assume that activation of the p38 signaling pathway is who controls p57 phosphorylation.
The reviewer asked for the nature of the Birb 0976 inhibitor because he/she did not find references for it. The reviewer was absolutely right about this point. We used in our experiments the BIRB 0796 inhibitor from AxonMedChem at standard concentrations (known to inhibit p38 alpha and beta isoforms) and not the BIRB 0976. In the previous manuscript, we wrote the wrong number BIRB 0976 by mistake. We have now corrected the number of the inhibitor throughout the manuscript.
The reviewer also asked whether the phospho-p57 antibody was able to recognize endogenous p57. For some reason, it is very difficult to assess the phosphorylation of endogenous p57 by western blot. However, we have been able to use the antibody in direct immunofluorescence detection. To assess p57 phosphorylation we subjected wild type MEFs and $p38^{-/-}$ cells to several stresses. We found a clear induction of p57 phosphorylation when wild type cells were subjected to stress that was not observed in $p38^{-/-}$cells. These data support our initial observations and show that endogenous p57 is phosphorylated at T143 in response to p38 activation. The new data have been included as Figure 3D.

4.The reviewer made some comments on the experiments presented in Figure 4. As pointed out by the reviewer, phosphorylated p57 associates and inhibits Cdk2 activity more efficiently than non-phosphorylated p57. He pointed out that this was assessed using overexpressed proteins and that we should show, in addition, that endogenous p57 was phosphorylated at T143 after osmostress, that this was induced by p38 and that endogenous cdk2 was inhibited under these conditions to demonstrate its biological relevance *in vivo*. We completely agree with the reviewer that this is a very important point and we have seen that there are a few points that might need of further clarification. Figure 4A is a binding assay using purified proteins from *E. coli* as it is the *in vitro* kinase assay performed in Figure 4B. We have labeled the figures adequately to make it clear. These two panels basically demonstrate a different affinity and inhibitory activity of phosphorylated p57 towards Cdk2. This might have been not sufficiently clear from the text and to clarify this point we have modified the manuscript and figure legend accordingly. In contrast, and as it has been suggested by the reviewer, Figures 4C and 4D showed the assessment of endogenous Cdk2 activity. As for Figure 4C, we assessed endogenous Cdk2 activity precipitated from wild type, $p38^{-/-}$ or $p57^{-/-}$ deficient cells. In the revised manuscript (see comment#3; new Figure 3D), we have been able to show that endogenous p57 is phosphorylated by p38 in response to the same stress conditions used here and thus, we can conclude now, that when p57 is phosphorylated by p38 upon stress, p57 is able to inhibit more efficiently endogenous Cdk2 (Figure 4C). Correspondingly, cells deficient in p57 ($p57^{-/-}$) or cells expressing the mutant $p57^{T143A}$ did not alter endogenous Cdk2 activity upon stress when compared to wild type (Figure 4D). Taken together, our data show that phosphorylation of p57 *in vivo* by p38 is a key determinant to alter endogenous Cdk2 activity. We have now modified the text and included the new Figure 3D to clarify this point that supports our previous observations.

5.The reviewer asked to comment on the cell cycle delay observed in $p57^{-/-}$ null mutants or those that express wild type p57 or the mutant $p57^{T143A}$. As pointed out by the reviewer the greater effect in cell cycle progression is observed in the null mutant whereas at 8 hours there is not a significant difference on the p57 or $p57^{T143A}$ cells. In clear contrast, from 8 to 14 hours cells containing wild type p57 are still able to arrest cell cycle whereas cells expressing the mutant $p57^{T143A}$ are deficient in cell cycle arrest. Our data showed that the phosphorylation of p57 increases its affinity for Cdk2 and thus, it seems reasonable that the solely presence of p57 might be sufficient to delay cell cycle progression but clearly not to the same extent that when p57 is phosphorylated. We have included this point in the revised manuscript.

6. The reviewer pointed out that the differences in cell survival upon osmostress seem to start at concentrations of 200mM NaCl (Figure 7A) since at lower concentrations (100 mM) the differences observed are less important. He/She asked what would be seen in cell cycle analysis (cell cytometry analysis) if we were to analyse it at 200 or 300 mM of NaCl. We have assessed cell cycle progression at 200 mM NaCl as suggested (Figure S9). However, whereas it is possible to monitor cell cycle progression in wild type cells and they show a longer delay than in 100 mM NaCl, the reduced viability of $p38^{-/-}$ and $p57^{-/-}$ deficient cells makes the analysis impossible when subjected to stress longer than 8 hours. This is consistent with the data on nuclei condensation and the decrease on cell viability reported in Figure 7 and the new Figures S6 and S7.

Reviewer 3

The reviewer also found that this was an interesting manuscript that demonstrated a role for p38 in the regulation of p57. He/She also suggested addressing a number of interesting questions that we have followed point by point and that are described below.

1.The reviewer mentioned that previous studies have demonstrated that other p38targeted proteins regulate cell cycle in response to osmotic (and other) stresses by the p38 MAPK pathway, including Cdt1 and MK2. He/She mentioned that the relative importance of these p38 targets was unclear and pointed out on the biological relevance of p57 when there were other targets that also affected the cell cycle. The reviewer asked to address directly this question. Cdt1 and Mk2 have been involved in S and G_2/M phases of cell cycle whereas our data clearly indicates that p57 is mainly involved in G1 and thus, it is unlikely that they could be redundant. However, this was a very interesting point raised by the reviewer and we have now addressed the relevance of Cdt1 and Mk2 to stress as suggested. We have down-regulated MK2 activity by the use of the widely used MK2 inhibitor III (Calbiochem) at a concentration that is able to reduce Hsp27 phosphorylation as described previously (e.g. Chandrasekaran et al., 2011). The inhibition of MK2 did not alter the viability of cells to NaCl in contrast to $p38^{-/-}$ or $p57^{-/-}$ cells. The data have now been included as Figure S10. We have also addressed the relevance of Cdt1 in stress survival. Cdt1 was down-regulated by the use of a specific siRNA (Sigma EMU044861) that reduces Cdt1 levels. The down-regulation of Cdt1 did not alter cell viability upon NaCl in contrast to $p38^{-/-}$ or $p57^{-/-}$ cells (New Figure S11). These results indicate that albeit p38 can target several proteins involved in cell cycle, the regulation of p57 plays a critical role in cell survival to osmostress. We have included the new data as Figures S10 and S11 and modified the manuscript accordingly.

2. The reviewer pointed out that p27kip1 has been described as a possible target for p38 target. The reviewer asked on the relevance of p27 when compared to p57. In contrast to p57, the published data indicates that p27 is an indirect target for p38 and that it is important to respond to DNA damage (Cuadrado et al., 2009). However, this is a very interesting point raised by the reviewer and thus, we have followed cell viability in response to different stresses in wild type, $p57^{-/-}$ and $p27^{-/-}$ deficient cells. In contrast to $p57^{-/-}$, the lack of p27 did not alter cell viability to NaCl, H_2O_2 or Inomycin. Therefore, p57 is a cell cycle regulator more relevant for cell survival to stress than p27. The new data obtained have been included as Figure S8.

3. The reviewer asked whether insights from molecular modeling could be obtained of the role of p57 phosphorylation by p38. This is indeed an interesting suggestion, unfortunately, the crystal structure of p57 has not been determined. The structure of some N-terminal CDKis

together with cyclins and Cdk binding domains have been deciphered (Rosso et al., 1996; Kriwacki et al., 1996; Lacy et al., 2004). However, albeit some regions in CDKis are conserved among them, the region that comprises p38 phosphorylation is unique in p57. Thus, the data available are not sufficient to perform this analysis.

4. The reviewer pointed out that in some circumstances p57 has been reported to regulate cyclin D sub-cellular localization (e.g. Tesioi and Trumpp, 2011). He/She asked whether this function of p57 was affected by p38 MAPK. As pointed out by the reviewer cyclin D localisation was regulated in hematopoietic stem cells (Zou et al., 2011). We have seen that p57 is mainly nuclear in the cells analysed here. To assess the localisation of Cyclin D, we took advantage of cell fractioning done for p57 localisation (Figure S3A). We have observed that cyclin D is mainly nuclear, as it is p57, in the cells analysed in this study. Therefore, the role of cyclin D by p57 might be restricted to particular cell types. We have included the new data on Figure S3A.

Acceptance letter 04 April 2012

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

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor
The EMBO Journal

Referee #2

(Remarks to the Author)
I think the authors have now greatly improved their manuscript. I think it should be published now.

Referee #3

(Remarks to the Author)
The authors have revised the manuscript - it is greatly improved. I strongly support the publication of this manuscript in EMBO J.

