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Phosphorylation of Mcl-1 by CDK1-cyclin B1 initiates its Cdc20-dependent destruction during mitotic arrest

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1st Editorial Decision 21 September 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first of all apologise for the long time taken to get back to you with a decision, which was due to a delay in receiving the third referee's report. However, we have now received the comments of all three referees, which are appended below. As you will see, all three express interest in your work, but also raise significant concerns that would need to be addressed by substantial revision of your manuscript, before we could consider it for publication in the EMBO Journal. In particular, I would like to highlight one major concern, raised by all three reviewers: namely that the relationship between phosphorylation and degradation of Mcl-1 remains incompletely understood. Clearly, this is a critical issue that needs to be addressed in greater detail.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I realise that the referees (and #2 in particular) suggest many additional experiments, and recognise that you may not be able to follow all these suggestions in the normal 3 months allowed for revision; while it may not be necessary to tackle every individual comment experimentally, it will be important to address the broad concerns raised. In this context, should you wish to discuss your plans for revision further, I would be happy to do so. In addition, if you need an extension beyond the three-month period, please just let me know - we can extend this up to a maximum of six months. 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.

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):

In this study, the authors address the important issue of coordination between the cell cycle machinery and the regulation of apoptosis in response to microtubule poisons. Harley et al. demonstrate that phosphorylation of Mcl-1 on residue Thr92 by CDK1-cyclin B1 mediates Mcl-1 destruction during mitosis, and they show that a non-phosphorylatable mutant of Mcl-1 (T92A) protects from nocodazole-induced cell death. Though the data presented are generally convincing and interesting, a few issues still need to be resolved.

1. The data supporting APC/CCdc20-mediated degradation of Mcl-1 are suggestive, but further characterization is necessary. The data shown in Figure 6A (knockdown of Cdc20) demonstrate that Mcl-1 is stabilized in the absence of Cdc20. This result is consistent with the authors' hypothesis, but it does not exclude the possibility that this effect is indirect. Also, it is very unusual that a phosphorylation event would confer degradation by the APC. In the case of the APC/C substrate Skp2, its phosphorylation actually impairs its degradation by the APC/C (Gao, Nat. Cell Biol., 2009). Phosphorylation does confer degradation by the SCF, another E3 complex important for cell cycle progression. Additional experiments in support of APC/CCdc20-mediated Mcl-1 degradation might include:
1) The identification and mutation of motifs in Mcl-1 that confer degradation by APC/CCdc20 (such as the canonical DBox, found in most APC substrates). Expression of a non-degradable mutant should protect cells from nocodazole-induced death.
2) In vitro Mcl-1 ubiquitylation assays with purified APC/C components (such as those shown in Bashir et al. and Wei et al., Nature 2004).
3) In addition, the expression of a phospho-mimetic mutant (T92D/E) should destabilize Mcl-1 in the absence of CDK1-cyclin B1 activity. Testing the stability of such a mutant during interphase (when APC/CCdc20 is not active) could be informative.

2. These and previous data from the Clarke lab suggest that Cdk1/CyclinB1 plays a somewhat contradictory role in simultaneously promoting survival via caspase-9 suppression (based on authors' previous Mol. Cell paper) and apoptosis via phosphorylation of Mcl-1. As the authors imply, the rapid degradation of Mcl-1 (2 hours post-arrest) may eliminate Mcl-1 prior to the "mitotic slippage"-induced loss of caspase-9 phosphorylation, thereby preparing the way for mitotic catastrophe. Whether or not this actually occurs is critical to the relevance of the manuscript. The authors could begin to address this issue by blotting for caspase-9 phospho-Thr125 and Mcl-1 phospho-Thr92 within the same experiment, over an extended time course of nocodazole arrest.

3. Marash et al. (2008, Molecular Cell) showed that mitotic translation of Bcl-2 promotes survival during extended mitotic arrest. Since Mcl-1 and Bcl-2 appear to be functionally redundant (with respect to apoptosis), it seems likely that steadily increasing the expression of Bcl-2 might counteract the degradation of Mcl-1. Given the precedent, the authors should monitor Bcl-2 expression in parallel with Mcl-1 during extended mitotic arrest. This issue should also be addressed in the discussion.

Referee #2 (Remarks to the Author):

In the first part of the submitted manuscript on Mcl-1 regulation in mitosis, Harley et al. describe threonine 92 and serine 64 to be phosphorylated by CDK1 in mitosis. This part of the manuscript is nicely done and the identification of CDK1 as the kinase responsible for T92 phosphorylation (and in part of S64) is convincingly shown.

The second part concentrates on the functional significance of T92 phosphorylation in mitosis. The authors show that a non-phosphorylatable mutant, T92A, is stabilised in mitotically arrested cells and that expression of this mutant is associated with a lower incidence of apoptosis in nocodazole-arrested cells. That higher levels of anti-apoptotic molecules, like Mcl-1, reduce the number of apoptotic cells is, however, not surprising per se, unless it is a mechanism that cells use if they face problems in progression through mitosis. Although this important issue is addressed in the manuscript, the evidence is not compelling and many questions concerning the mechanism of Mcl-1 destabilisation by phosphorylation as well as well its role in controlling survival are left unanswered (which is reflected in the Discussion section of the manuscript).

Thus, to strengthen the link between T92 phosphorylation by CDK1 and the control of cell survival in mitosis more direct evidence is required. Because the authors speculate on a direct role of CDC20 in the destabilisation of Mcl-1 after phosphorylation by CDK1 (which also features in the title) the authors should provide more direct evidence how CDC20 is involved in the destabilisation of Mcl-1.

Major points:

Mcl-1 phosphorylation:

1. The phosphorylation of Mcl-1 reduces its motility in SDS-PAGE. Why is this retardation only detectable in cells treated with microtubule interfering agents (Fig.1A) but not in mitosis of unperturbed cells (Fig.1B)? Is it visible on blots after longer exposure or does it reflect poor synchronisation of the cells? Can it be detected in cells arrested at metaphase by inhibition of the proteasome?

2. When exactly do Mcl-1 levels start to decline? Have the signals been quantified in order to relate them to pS10-H3 and cyclin levels? Looking at Fig.1B suggests that Mcl-1 levels are already low at beginning of mitosis (before cyclin A becomes unstable in prometaphase). In Suppl.Fig.3, however, a decline of (overexpressed) Mcl-1 levels in mitosis is not visible and Mcl-1 phosphorylation takes place only after the onset of mitosis as judged by pS10-H3 signals. It would be very informative to know when endogenous Mcl-1 becomes phosphorylated during the cell cycle. The authors should repeat immunoblotting for pT92 and pS64 using extracts prepared from synchronised cells.

3. In Fig.4A it is shown that chemical inhibition of CDK activity abolishes the phosphorylation of recombinant Mcl-1 on both T92 and S64. Immunodepletion/add-back experiments shown in Fig4B show that both sites can be phosphorylated by recombinant CDK1. In contrast, recombinant CDK1 only phosphorylates T92 but not S64 to detectable levels. It would be interesting to know whether Mcl-1 requires an additional factor to become phosphorylated or whether the kinase requires an additional factor to be able to phosphorylate Mcl-1 at S64. Why did the authors not include a pS64 blot in Fig.4C?

Mcl-1 stability:

Several issues concerning the regulation of Mcl-1 stability are left unanswered in the current manuscript. The manuscript would be strengthened by a molecular explanation for the destabilisation of Mcl-1 in mitosis.

4. As shown in Fig.5A, mutation of T92 to A prevents the turnover of Mcl-1 in mitosis but not in interphase. What is the t1/2 of the different mutants and the wild-type protein in mitosis and interphase? The blots shown Fig.5B indicate a time-dependent decay and a protein half-life of of less than 1h. In mitosis however, the wild-type protein declines after 2h but seems to increase at 6h, The S64A mutant, seems to have a much smaller half-life than the wild-type. Have these signals been quantified? A metabolic labelling experiment might be useful to determine the t1/2 of the different proteins in mitosis.

5. What is the effect of phosphomimetic mutations on Mcl-1 stability? Have the authors tested the effects of T92D or E and S64D or E on Mcl-1 stability and cellular survival?

6. The levels of Cyclin A are stabilised by expression of both T92A and S64A, although only T92A

is stabilised. The authors conclude that because overexpression of the mutants causes stabilisation of cyclin A both proteins compete for the same degradation machinery. But why, if the T92A mutant is stable, should it compete with cyclin A for degradation? Is the T92A mutant still turning over in nocodazole-arrested cells? Does overexpression of the wild-type also interfere with degradation of cyclin?

7. If the degradation of Mcl-1 competitively blocks the destabilisation of other proteins that have to be degraded during mitosis, overexpression of Mcl-1, or it's stabilised T92A mutant, should interfere with progression through mitosis. Is the mitotic index higher in cells expressing Mcl-1-T92A?

8. CDC20 knockdown by transient transfection of siRNA has a strong effect on Mcl-1 levels in nocodazole-arrested cells. As in Fig.5A, the Mcl-1 levels are stable until 2h, then drop and appear stable again until 6h. Cells with partial knockdown of CDC20, exhibit higher Mcl-1 levels that also migrate in multiple forms in SDS-PAGE. At 2h, however, CDC20 levels in controls are high and so are Mcl-1 levels, while in CDC20 knockdown, CDC20 is reduced while there is no effect on Mcl-1. Thus, the effect of CDC20 knockdown does not seem to have an immediate effect on Mcl-1 levels.

What happens if this experiment is performed in the absence of nocodazole? Cdc20 RNAi should arrest cells in mitosis or at least significantly delay progression through mitosis.

Is the APC/C involved in the destabilisation of Mcl-1? RNAi against a core subunit of the APC/C might be more effective than targeting Cdc20.

The authors show that overexpressed FLAG-tagged Mcl-1 interacts with CDC20. Does this IP also contain cyclin A and the APC/C?

Can the interaction between Mcl-1 and CDC20 also be confirmed for the endogenous proteins?

If CDC20 is directly responsible for the degradation of Mcl-1 in mitosis, then the mechanism should be defined. So far, the only CDC20 targets carry a D-box type of degron and no phospho-degron has been defined for any APC/C substrate yet. Thus, the effect seen in CDC20 RNAi could be indirect. To distinguish between these possibilities, the authors should define the degron motif required for destabilisation of Mcl-1. Is the N-terminal domain sufficient? Can it be grafted onto a heterologous protein for destabilisation in mitosis?

It would be very informative if the authors could show that APC/C-CDC20 is able to ubiquitylate Mcl-1, e.g. by reconstituted in vitro assays.

Mcl-1 levels and apoptosis:

The correlation between Mcl-1 levels and the onset of apoptosis in nocodazole treated cells is not direct. If the above raised questions can be addressed this issue can be left open for follow up studies. For completeness sake, I just include the remaining open issues.

9. The authors claim that CDK1 activity has two opposing effects on the control on cell survival in mitosis. In a previous paper CDK1 was found to inhibit Caspase 9, thereby providing a pro-survival signal in mitotically arrested cells (Allan and Clarke, 2007). In the current manuscript, the authors show that CDK1 activity promotes cell death signal by destabilisation of anti-apoptotic Mcl-1. This model suggests that the onset of cell death is defined by a CDK1 activity threshold. It also predicts that mitochondrial changes and the release of cytochrome C, should be readily detectable in mitotically arrested cells.

The correlation between Mcl-1, cyclin B1 levels and apoptosis can only be addressed properly on a single cell level, e.g. by immunostaining or GFP-labelling approaches. The authors should combine active Caspase3, Mcl-1 and cyclin B1 stainings.

10. A large set of data is derived from cells arrested in mitosis by nocodazole treatment. Disruption of the mitotic spindle activates the mitotic checkpoint and this signalling pathway might not only inhibit APC/C function but might also activate survival pathways. Have the authors compared cellular survival in cells arrested by inhibition of the proteasome? How do cells with reduced APC/C

function survive compared to nocodazole treated cells? Some reports (Motwani et al., 1999) claim that taxol treatment followed by inhibition of CDK activity induces higher percentage of apoptosis than either treatment alone. What is the effect of inhibiting CDK activity on cellular survival in the presence or absence of nocodazole and or MG132?

11. A clearer correlation between Mcl-1 levels and apoptosis susceptibility could be obtained by RNAi and RNAi rescue experiments. Do Mcl-1 RNAi cells have a cell death index in mitosis? Are they more susceptible to apoptosis if arrested by nocodazole? Can these effects be rescued by RNAi resistant versions of the wild-type or phospho-site mutants?

Minor points:

1. Fig.3 D: Was the input control run on the same gel (PhosTag)? If so, why does the Mcl-1 antibody recognise fewer species than in the IP-blot shown above? How do the authors know that 50% of Mcl-1 is phosphorylated on T92 as stated on page 8 of their manuscript? How did they compare the two antibodies?

2. The quality of the manuscript and the interpretation of the data would be much improved by a quantification of some of the data by quantitative immunoblotting (e.g. Odyssey technology) or at least by densitometry scanning of the films. This would be particularly helpful in Fig.1A, 2B, 4B, 5A and B. Quantification of Fig.5A and B would also allow determination of the proteins's half-life.

3. Figure 7A lacks errors bars and a statistical test should be included for the data shown in Fig.7B.

4. The description of the experiment shown in Fig.6 lack indication of the time of RNAi treatment before addition of nocodazole. This should be included.

Referee #3 (Remarks to the Author):

This manuscript builds on earlier findings from the Clarke lab that, for the first time, had linked mitotic events to control of apoptosis.

Recently it has become clear that cells respond to a sustained mitotic arrest either by 'mitotic slippage' or 'mitotic death'. Finding molecular targets that could activate death patwhays specifically in mitosis is clearly needed to better understand how cells control these different biological outcomes. Such understanding is of biological and potential clinical relevance.

Most data are supportive of the conclusions. The finding that Mcl-1 phosphorylation might be key to its stability in mitosis is an important novelty. However, additional controls are necessary to rule out alternative explanations, and to shed more light on the mechanisms that control of Mcl-1 destruction as well as its effects on mitotic death.

In particular, many questions remain on how Mcl-1 could be detected by the APC/C-Cdc20 in mitosis.

How do the authors suggest that Thr 92 phosphorylation contributes to APC/C-dependent Mcl-1 ubiquitination and destruction?

Does Mcl-1 have a functional destruction box? This Reviewer found two minimal consensus D-box consensus sequences (RXXL) downstream of Thr92. Does point-mutation within any of these stabilise Mcl1, too? Can Mcl-1 domains be defined, containing the Thr92 residue, that in itself are functional as a degron?

Is a phospho-mimicking mutant less stable in interphase?

Does the spindle checkpoint control Mcl1 destruction? This could be tested by following Mcl1 levels in cells expressing non-degradable cyclin B1. These also arrest in mitosis, but do so independently of the mitotic checkpoint.

Figure 5:
One concern is the proposed competition between Mcl1 and cyclin A for APC/C-Cdc20. Could the authors provide more direct evidence that cyclin A stabilisation, apparently associated with expression of a more stable, non-phosphorylated Mcl1, is not in itself protective against apoptosis?

Here, overexpression of a D-box mutant of Mcl1, or a non-Cdc20 binding mutant in case this is different, could be particularly interesting. Testing the effects of a mutant Mcl1 that does not compete for APC/C-Cdc20, yet protects against apoptosis, would be most illumainating.

Can further APC/C substrates, like Securin be shown? Do cyclin A and B1 levels go down at later time-points in mitosis or only after mitotic slippage?

Figure 6: Does APC/C depletion by RNAi (other than Cdc20 RNAi) stabilise Mcl1 in mitosis? Does Cdh1 depletion stabilise Mcl1 in interphase?

Does Mcl1 (and do its mutants) bind to the APC/C more prominently in mitosis, as compared to in G2 phase? Can binding of Mcl1 to core APC/C subunits such as APC2 or APC3 be found in Mcl1 IPs?

Does depletion of Mcl1 by RNAi make cells more sensitive to mitotic drugs?

What is the effect of over-expressing stabilised Mcl1 on normal mitotic progression? Serious competition with cyclin A and B1 destruction would predict induction of a mitotic delay. How does stabilised Mcl1 influence the ability of cells to undergo mitotic slippage?

Other points:

Figure 1, 2: Can FACS profiles be shown to reveal that the mitotic shake-offs are indeed pure mitotic fractions?

Figure 7/ Figure S4: can a more complete time-course be shown, including a >24h timepoint? Can changes in caspase 9 activity be measured?

What is the intracellular localisation of Mcl1 in mitosis, as compared to G2 phase? Where do the phospho-mutants localise as compared to the total pool of Mcl1?

1st Revision - Authors' Response 29 March 2010

Referee 1

1. (i) We have identified a D-box motif in Mcl-1 which when mutated stabilises the protein during mitotic arrest. This mutant, like the T92A mutant makes cells resistant to apoptosis induced by nocodazole. The identification of this motif is a strong indication of a direct role for Cdc20 in the destruction of Mcl-1. We have also shown that knockdown of APC3 stabilises Mcl-1 in mitotic arrest, whereas knockdown of Cdh1 and Mule have no inhibitory effect, showing the specificity of the effect Cdc20 knockdown. (ii) Although we have observed ubiquitination of Mcl-1 in mitotically arrested cells, we have been unable so far to reconstitute the process in vitro using purified components. This suggests that an additional protein is required, which if limiting would explain the stabilisation of strongly over-expressed Mcl-1. Obvious candidates are BH3 domain proteins (Bim and Noxa) that bind to Mcl-1 in cells and which have been previously suggested to alter Mcl-1 stability. Alternatively, a previously unidentified component may be required that could be involved in the recognition of the phosphorylated form of the protein. This will be a focus of our future work.
2. We think that the dual role of CDK1-cyclin B1 in both inhibiting caspase-9 activation and in initiating Mcl-1 destruction is key to understanding the temporal control of apoptosis during a prolonged mitotic arrest. We have provided additional data indicating that Mcl-1 destruction occurs prior to caspase-9 dephosphorylation (Supp Fig 11), which is consistent with this hypothesis.

3. The relative roles of Mcl-1, Bcl-2 and Bcl-x_L during mitotic arrest are interesting and may be relevant to understanding the particular importance of Mcl-1. Several studies have suggested that the biological functions of the proteins are not identical, for instance they generate a different tumours when over-expressed in the mouse haematopoetic system, and they have different affinities for partner proteins. We have now included data to show that Bcl-2 and Bcl-x_L are stable but highly phosphorylated during mitotic arrest. We did not observe any increase in the levels of the proteins during synchronised mitotic arrest. It is possible that both are inactivated during mitosis, as suggested by recent work by Terrano et al (2010), leaving cells dependent on Mcl-1 to control apoptosis, which has implications for anti-cancer drug therapy. We have included a discussion of this point as requested.

Referee 2.

Major points

1. We now show that Mcl-1 is phosphorylated during a normal mitosis as well as a mitotic arrest (Supp Fig 7). In common with most mitotic substrates, the phosphorylation is more pronounced in the arrested cells. The lower level of phosphorylation in normal mitotic cells is likely to be one of the key elements to understanding the difference between a normal mitosis and a prolonged arrest, and this is addressed in the discussion.
2. It is of great interest to us to know exactly when Mcl-1 levels decline, particularly in a normal mitosis. However, this cannot be done with any great resolution by western blotting. We are currently developing a Mcl-1-fluorescent protein construct for imaging in live cells, but this will require some further work due to the problems with artificial stabilisation. Supp Fig 6 shows the phosphorylation of S64 and T92 in cells synchronised in the cell cycle. The best one can say from this sort of experiment is that their phosphorylation peaks around mitosis, and we show that both are phosphorylated in collected mitotic cells. Unfortunately, we have been unable to get a specific immunofluorescence signal with either antibody that would allow us to be more precise about which stage of mitosis the phosphorylation of either site peaks. We find this is a common problem with mitotic phosphoepitopes.
3. Indeed, it seems that an additional factor is required for S64 phosphorylation, but since we can find no role for this site as yet, we have not pursued this point further. The S64 phosphoblot was not included in Fig 4C because it was blank and not informative.
4. The $t_{1/2}$ for the destruction of wild-type and mutant Mcl-1 proteins can be estimated from the blots shown, but a more detailed quantification is probably unwarranted, because of the samples are only synchronised +/- 2 hours in the arrest. A much better and more precise assay would be the loss of a fluorescent protein-Mcl-1 fusion in live cells (see above) and we are working on this at present.
5. We have derived a new stably-transfected cell line that expresses the phosphomimetic T92D mutant of Mcl-1. This mutant is unstable in mitotic arrest, like phosphorylated wild-type Mcl-1, in contrast to the stabilised, non-phosphorylatable mutant T92A. This provides additional evidence that it is phosphorylation of this site is necessary for the destruction of the protein.
6. We think that stabilised Mcl-1 competes for some component that is also required for the degradation of cyclin A and cyclin B. We have observed a similar effect with strong over-expression of the wild-type protein. An obvious candidate for a binding protein that is sequestered by stabilised Mcl-1 is Cdc20. This may explain the cyclin stabilisation effect in part, but we suspect that another mechanism is also involved, since the D-box mutant of Mcl-1 still at least partially delays cyclin destruction.
7. The possible effect of stabilised or over-expressed Mcl-1 on mitotic progression is an intriguing one. We have not observed a significant increase in mitotic index, but the cyclins are presumably still degraded once the spindle assembly checkpoint is switched off in a normal mitosis. We will investigate further if there are more subtle mitotic defects by microscopy, but we think this is beyond the subject of the present manuscript.

8. The regulation of Cdc20 itself in mitotic arrest is clearly complex, and I agree that it is surprising that its levels drop around the time when we see Mcl-1 destruction. This is also true for cyclin A destruction. Loss of Cdc20 is thought to be part of the mechanism to maintain the checkpoint., but it clearly can still work even when reduced to lower levels. In the absence of nocodazole we are not able arrest cells synchronously in the arrest solely by Cdc20 knockdown. We now show that knockdown of the APC3 subunit of the APC/C also stabilises Mcl-1 during mitotic arrest (Fig. 6B). The role of Cdc20 in the mitotic destruction of Mcl-1 is strongly supported by new data showing that a D-box mutant of Mcl-1 is also stabilised (Fig. 7).

We also thank this referee for making a number of additional suggestions for further work. Of the suggested experiments, we have found that knockdown of Mcl-1 does indeed increased apoptosis in response to nocodazole (Supp Fig. 1), whereas stabilisation of Mcl-1 by the T92A or D-box mutation reduces apoptosis (Supp figs 8 & 9, respectively).

Minor points

1. In figure 3D, the input is run on a normal gel, the IP samples on a Phos-tag gel. The figure has now been labelled to make this clearer. As further evidence of the high level of stoichiometry we now show another experiment in which the WT, T92A and S64A proteins are analysed on a Phos-tag gel (Fig. 3). This shows unambiguously that both sites are highly phosphorylated in mitotic arrested cells.
2. Although it is possible for us to quantify the blots by scanning, we think that the data are clear and this approach is not sufficiently accurate to provide additional information that cannot be obtained from the images.
3. We have included errors bars for the data now shown in Figure 8A which is derived from three separate experiments.
4. The protocol for RNAi treatment is described in the materials and methods.

Referee 3

The precise mechanism by which T92A phosphorylation is required for the destruction of Mcl-1 is not certain, since it does not appear to be required for the association of Cdc20 (Fig. 6D). One possibility is that phosphorylation induces a conformational change that allows the interaction of the Cdc20-Mcl-1 complex with the APC/C. We had suspected that the C-terminal IR motif might be involved, but we have found no evidence for this as yet. Another possibility is that phosphorylation removes an inhibitor from Mcl-1.

As the reviewer indicated, we have found that there is a potential D-box in Mcl-1 that is similar to that in securin (Figure 7A). We have now shown that mutation of the RxxL minimal consensus within this motif stabilises Mcl-1 in stably-transfected cells (Figure 7C).

We now also show that the T92D mutant, which is predicted to mimic phosphorylated Mcl-1, is unstable in both mitotic arrest and in interphase cells, like the wild-type (Fig. 5C). The T92D mutant doesn't appear to be less stable than the wild-type in interphase (Fig. 5D). This indicates that phosphorylation affects the stability of Mcl-1 specifically during mitosis.

The data indicate that Mcl-1 destruction is not inhibited by the spindle assembly checkpoint, much like cyclin A destruction. Unfortunately, the suggested experiment in which cells would be arrested in mitosis by expression of a stable cyclin B does not permit the temporal regulation of Mcl-1 destruction during the arrest to be determined by western blotting, since the population would not be synchronous in the arrest. With regard to the question about whether Mcl-1 stabilisation could affect

apoptosis through the stabilisation of cyclin A, we are not aware of any reason to think that cyclin A would inhibit apoptosis and this stabilisation is a delay rather than a complete block. By contrast, the anti-apoptotic effect of Mcl-1 is known, so it seems reasonable to assume that it inhibits apoptosis by this well-characterised mechanism.

We now show that as well as Cdc20 depletion (Fig. 6A), APC3 depletion (Fig. 6B), but not Cdh1 depletion (Fig. 6C) inhibits the mitotic destruction of Mcl-1.

At present we have been unable to see stable association of Mcl-1 with the APC/C. This may be due to the rapid destruction of the protein once it does interact.

We now show that confirms that Mcl-1 depletion sensitises cells to cell killing by nocodazole (Supp Fig. 1). This is in agreement with published work by others that shows that loss of Mcl-1 sensitises cells to taxanes and other microtubule poisons.

Over-expression of Mcl-1 does not appear to arrest cells in mitosis, although it may produce a more subtle delay that we have not yet quantified. This suggests that Mcl-1 does not compete with cyclins for the destruction machinery when the spindle assembly checkpoint is switched off.

We now show the flow cytometry data to accompany figure 2A etc as Supplementary figure 2. More than 75% of the cells collected by our protocol are mitotic as judged by histone H3 serine 10 phosphorylation.

We have unfortunately been unable to localise the phosphorylated endogenous or transfected wild-type protein in mitotic cells by immunofluorescence. We will attempt to localise GFP-fusions of the phosphorylation site mutants, although there are potential problems with tagging Mcl-1, such as mislocalisation and artificial stabilisation.

2nd Editorial Decision 19 April 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-72237R. It has now been seen again by all three referees, whose comments are enclosed below. As you will see, all three referees find the manuscript much improved and are supportive of publication in the EMBO Journal. However, referee 3 still has a number of concerns that he/she feels need to be addressed before eventual acceptance. Of these, point 2 is the most critical, and will require additional experimental work (or rather, the repetition of experiments to allow accurate comparison between the effects of the T92A vs. the D-box mutations). In addition, he/she criticises the quality of a couple of the other blots (detailed in the "minor points"), and if you can provide better quality blots, this would be beneficial. I think that all other points can be addressed by modification to the text.

I would therefore like to invite you to revise your manuscript according to the comments of referee 3 (as well as providing the statistical test requested by referee 2). I hope that these final revisions should not be too time-consuming - I do recognise that you have already put a huge amount of work into this version of the paper! Please just let me know if you have any questions or comments regarding this final round of revision.

I look forward to receiving your revision.

Yours sincerely,

Editor
The EMBO Journal
REFEREE REPORTS

Referee #1 (Remarks to the Author):

The work is now suitable for publication.

Referee #2 (Remarks to the Author):

The re-submitted version of the manuscript is greatly improved and contains several interesting data that characterise the destabilisation of Mcl-1 during mitosis, in particular in mitotically arrested cells. Although many mechanistic questions how degradation occurs are still left open and the functional significance is, in my opinion still not very strong, these data warrant publication. My only request is that the authors should carry out a statistical test on the data shown in Fig.8A and B and mention them in the Results section.

Referee #3 (Remarks to the Author):

The revised version of the paper by Harley et al, describing the identification of a potential timing mechanism that could signal a defective mitosis to the apoptotic machinery, has much improved as compared to the initially submitted version. Most importantly the authors have added more compelling evidence to the model that Mcl1 becomes a phospho-dependent APC/C-Cdc20 substrate in mitosis. The authors show a clear effect of Cdc20 RNAi and have identified a D-box required for destruction of Mcl1 in mitosis, but not interphase.

It seems that even upon the destabilising, cycB-Cdk1 dependent, phosphorylation of Mcl1 (robustly demonstrated in Figs 3 and 4), Mcl1 is a rather poor, yet potentially spindle checkpoint-independent APC/C substrate. It is still detected after an arrest of 2-4 hours, when cyclin A is lost from checkpoint-arrested mitotic cells. These characteristics of Mcl1 as an APC/C substrate may thus encompass a potentially crucial timing effect, decreasing cellular fitness only after a mitotic arrest exceeds several hours. Such a delay would likely be beyond a stochastically long mitosis and reflective of a significant problem in chromosome alignment. This is, to me, most clear from the data presented in the last Supplemental Fig (Sup. Fig. 11). Surprisingly, this crucial figure was not described in the Results section, whereas I think it is clearer than many other blots of Mcl1 in this paper (that are sometimes faint or seem to reveal unequal protein transfer, e.g. Fig 2B, 6B, Luc Control, and 7C).

Nevertheless I feel most of the conclusions are now supported by the data, which are novel and significant. I do have some remaining questions though that I hope the authors could answer.

Major points:
1) The authors only briefly discuss that their findings are in potential conflict with observations by the Mitchison lab, showing that Cdc20-depleted cells are prone to mitotic cells death. I recommend they add to the Discussion (p. 15) that, likely, alternative apoptotic or cell death inducing signaling pathways exist, independent of Mcl1 destruction, that could accumulate during a prolonged mitotic arrest. Further, could they indicate whether they think a role for Mcl1 loss in mitosis could be cell-type specific? Even the level to which Mcl1 is destabilised seems variable, as in Fig 3C,D and E, when HeLa cells are used, no obvious decrease of flag-Mcl1 levels in mitosis is seen.

2) I think it is crucial to show that the D-box mutated Mcl1 protects against apoptosis to a similar extent as the T92A mutant does. However, Supplemental Figs 8 and 9 cannot be compared now, due to different levels of cell death in the controls. Could the authors show a FACS experiment in which Mcl1-WT, Mcl1-T92A and the Mcl1 D-box mutant are directly comparable, together with a Western blot showing protein levels? Further, have the cells collected by mitotic shake-off as shown in Sup Fig 2/3/11 also been assayed for subG1 cells?

3) Could the authors indicate whether stabilised Mcl1, or Mcl1 depletion, similarly affects survival of taxol-arrested cells as compared to nocodazole-arrested cells?

4) Other APC/C substrates are known that might be degraded in a phosphorylation dependent

manner (Aurora A, see Kitajima S, et al. PLoS One. 2007) and Cdc6, see: Mailand and Diffley 2006; or Duursma et al., 2006). Can the authors include this in their discussion (p15/16)?

Minor points:
- Supplementary Fig 4 is of insufficient quality to support the conclusion and should be replaced by a clearer Mcl1 blot, or the point of ZVAD-independency should be omitted;
- Could the authors mention, like they comment in their reply, that the Mcl1 T92A mutant does not delay normal mitotic progression (in relation to any competition effect with other APC/C substrates)?
- The Mcl1 blot of Fig 6B is unclear and in current form adds little to the results of Fig 6A. Can another Mcl1 blot be shown of the same samples?
- The text to Fig 6 D should indicate that Flag-Mcl1 is shown in the blot;
- In its present form, Figure 7E, right panels, shows that the delta IR mutant is more stable as compared to the wild-type Mcl1. Could the authors comment on this? Could this mutant be intermediately stable?

Additional Author Correspondence 26 April 2010

Thank you for your response to our revised manuscript. Having had time to consider the comments, we realise that we should have included the statistical analysis requested by referee 2 before and we will now do this. We will also modify the text where necessary. However, we do not see the value of repeating the separate experiments shown in figure 8 (the stabilizing and anti-apoptotic effect of the T92A mutation) and figure 9 (the stabilizing and anti-apoptotic effect of the D-box mutation) together in another series of experiments. Each experiment is properly controlled and there is nothing new to be learned from a direct comparison. It is certainly not at all contentious that stabilized Mcl-1 inhibits apoptosis. It is unimportant whether one is more or less quantitatively effective than the other-either way does not affect the interpretation of the results in the slightest. Within the error of the experiment I doubt very much that this could be established and I would not wish to make such a claim in any case.

Additional Editorial Correspondence 26 April 2010

Thanks for your message, and for your comments regarding the request of the referee that you directly compare the anti-apoptotic effects of the T92A and the D-box mutations. Firstly, I would just say that I do share your desire to get the paper published after such a long period, and I do recognise the large amount of work you put into the revision, which both we and all the referees appreciate has improved the paper greatly. I can also see your point that the requested experiment would not likely change the conclusions of the study in any way. I guess the one possibility I do see is that, if - for example - the anti-apoptotic effects of T92A were significantly greater than those of the D-box mutation, that might imply that phosphorylation has another effect, beyond promoting degradation of Mcl1. I presume that this is what referee 3 has in mind when he/she requested this experiment.

The referee does state in his confidential remarks to the editor that this point would be important for eventual acceptance, and I don't want to disregard his/her concerns, especially since I could envisage outcomes that might have an implication on your conclusions. I also assume that this should be a relatively straight-forward and quick experiment for you to do. I would therefore encourage you to perform this experiment if possible. However, I don't want to make acceptance of the paper absolutely contingent upon this experiment and we - like you - do not want to delay publication too much longer. Therefore, if there are good reasons why you can't do these experiments within a relatively rapid time-frame, please let me know and we can discuss further.

I look forward to hearing from you.

Yours sincerely,

Editor
The EMBO Journal

Additional Author Correspondence 27 April 2010

Thanks for your considered response. We will carry out the additional experiments and we will send you the revised manuscript as soon as possible.

2nd Revision - Authors' Response 10 May 2010

Thank you for the opportunity to revise our manuscript again in response to the referees' comments.

We have added the statistical analysis requested by referee 2 for fig 8A,B in the figure legend. In response to the comments by referee 3, we have carried out the suggestion to move the data showing the relative timing of Mcl-1 destruction and caspase-9 dephosphorylation to the main body of the results and this data is now presented as figure 9 rather than as a supplementary figure. We have addressed the other points raised by this referee as follows:-

Major points

1. We have expanded the discussion of the effects of Cdc20 depletion and the Mitchison lab paper (p15) as suggested. The referee's comments that Mcl-1 is stable in HeLa cells are incorrect. The experiment presented in Figure 3C,D shows endogenous Mcl-1 immunoprecipitated from HeLa cells, whereas Figure 3E shows Flag-Mcl1 from stable U2OS clones. In each case they have been treated with nocodazole for only 2 hours so that Mcl-1 is not yet degraded in order that it can be analysed. These conditions are clearly stated in the figure legend.
2. With regard to the most important point of the comparison of the effects of the T92A and D-box mutants of Mcl-1, we now show data from an additional series of flow cytometry experiments in which the amount of apoptosis in cells stably expressing T92A and D-box mutants is compared directly (Supplementary figure 10). The two mutants inhibit apoptosis equally well. Therefore there is no evidence for any additional effect of either mutant on Mcl-1 activity and they work equally well simply because they both prevent Mcl-1 degradation.
3. We have not repeated the analysis using taxol. This would require a whole new series of lengthy experiments and we do not expect this to provide any additional insight into the mechanism of regulation of Mcl-1. We note that this was not an issue previously.
4. It is incorrect to state that other APC/C substrates are known to be degraded in a phosphorylation dependent manner, if that means that degradation requires their phosphorylation. Rather, the mitotic degradation of Cdc6 and Aurora A is *inhibited* by their phosphorylation. The mechanism of inhibition is not clear, but at least in the case of Cdc6 it involves resistance to Cdh1-APC/C mediated degradation. Since there is no reason to think that this is related to the contrasting effect of phosphorylation on Cdc20-APC/C mediated degradation of Mcl-1, we have not modified the relevant part of the text.

Minor points

- We think that the data shown in Supplementary figure 4 are clear and this figure must be retained because it demonstrates that mitotic Mcl-1 degradation is not simply a consequence of apoptosis.
- The effect of the Mcl-1 mutants on normal mitosis would require a much more detailed analysis before we could make any further assertions. It is possible that Mcl-1 does not compete with the degradation of other APC/C substrates under conditions where the spindle assembly checkpoint is released, as happens a normal mitosis.
- We have replaced figure 6B with data obtained from another experiment. The Mcl-1 blot shows the loss in control cells more clearly and it is apparent that Mcl-1 is stabilised and

phosphorylated in APC3 knockdown cells. This data is not repetitive of Figure 6A and shows the requirement for the APC/C for Mcl-1 destruction.

- The legend to figure 6D does make it clear that it is Flag-Mcl1 that is being analysed.
- The data shown in Figure 7 clearly show that the IR mutant is not stabilised like the D-box mutant. It remains possible that the degradation of the IR mutant is slightly slower than the WT, but we cannot assert this confidently from the data. We have amended the text on p13 slightly to reflect the possibility that it is not exactly the same as WT.

In summary we believe that we have fully addressed all of the points raised by the referees. We hope that our manuscript will now be suitable for publication.

