NEDDylation regulates E2F-1 dependent transcription

Sarah J. Loftus, Geng Liu, Simon M. Carr, Shonagh Munro and Nicholas B. La Thangue

Corresponding author: Nicholas B. La Thangue, Oxford University

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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 31 January 2012

Thank you for the submission of your research manuscript to our editorial offices. Please accept my apologies for not being able to return to you with a decision on your manuscript sooner. We have now received the three enclosed reports on it. As you will see, while appreciating the potential interest of the findings, the referees also raise a substantial number of concerns about the conclusiveness of the data and suggest a number of additional experiments to strengthen the manuscript.

Referee 1 remarks that the contribution of single lysine residues in E2F-1 remain unclear and that it should be explained why some lysine mutants are more efficiently neddylated than wild-type E2F-1. S/he also points out that some effects could be indirect, for example by involving different Nedd8 substrates. Along similar lines, referee 3 states that there is no indication that the phenotypes observed are indeed mediated via altered E2F-1 neddylation; in other words, this reviewer feels that there might be indirect effects of Nedd8 overexpression on E2F-1, for example by interfering with ubiquitin-dependent protein degradation. Both referees 1 and 2 point out that the statistical analysis and significance would need to be strengthened. In addition, referee 1 also points out that in some instances, protein loading is not even, which could bias the interpretation of the results. One of the main points made by reviewer 3 is that the physiological relevance of the proposed neddylation of E2F-1 is unclear and would need to be strengthened. S/he also states that it should be verified that the same Nedd8 conjugation pathway occurs during overexpression and in the endogenous condition. Finally, this referee states that the discrepancies between the effects of E2F-1 lysine
From the analysis of these comments it is clear that publication of your manuscript in our journal cannot be considered at this stage and that a significant amount of further work would be required before the study could be published here. On the other hand, given the potential interest of your study, I would like to give you the opportunity to address the reviewers concerns and submit a revised manuscript with the understanding that the referee concerns must be fully addressed and that acceptance of the manuscript would entail a second round of review. I do realize that referee #2 is somewhat more positive than the other two referees, but we agree that the concerns raised by both referee 1 and 3 are crucial and need to be addressed.

I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. I realize that addressing the referees’ comments in full would involve a lot of additional experimental work and I am uncertain whether you will be able to return a revised manuscript within our 3 months deadline or whether you want to seek rapid publication elsewhere at this stage.

I look forward to seeing a revised form of your manuscript when it is ready. Should you choose to submit your paper elsewhere, I would welcome a message to this effect.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In this manuscript by Loftus et al. the authors demonstrate that E2F1 can be NEDDylated. Furthermore, the authors suggest that its NEDDylation(s) decreases the stability of E2F1 and diminishes its transcriptional activity as well as its growth inhibitory properties. The possibility that E2F1 is NEDDylated resulting in changes in its activities is a novel and interesting observation. However, the data presented in this manuscript are preliminary and additional experiments are required to properly demonstrate that NEDDylation indeed occurs and affects E2F1 activity. Of note, for some of the experiments included in this manuscript it is not clear whether the changes suggested by them are reproducible and statistically significant. This must be addressed prior to publication.

My specific concerns are as follows:

1) Regarding the data in figure 1- It is not entirely clear why the authors state that "we identified lysines 182, 183, 185, 117, 120 and 125 to be required for efficient NEDDylation in cells (Fig 1E)." In figure 1E the authors show that a mutated E2F1, K117,120,125R is NEDDylated less efficiently than wt. However, since single mutants (K120R; K117R etc) were not studied, it is difficult to assess which of these three lysines is indeed NEDDylated and/or are required for NEDDylation. Also, the mutants K182R and K160,164R seem to be NEDDylated more efficiently than WT. Why is that? The authors should discuss and explain these data.

In addition, NEDDylation of K160,164R, which shows a two-fold increase in the bar graph, seems to be more than two fold higher than WT in the original data. The authors should explain, in the figure legend, how was the bar graph generated (is it normalized to total E2F1 levels?).

2) Regarding the data presented in figure 2 - One should keep in mind that the data presented in figure 2 A, B and D could represent the effect of NEDDylation of a different player and not necessarily of E2F1. For example the NEDDylation substrate could be DP1, p300 etc. It is surprising that this possibility is not discussed.

Regarding the experiments with the E2F1 mutant K185R presented in figure 2C and E - the authors should monitor more carefully the levels of E2F1 in panel C and of NEDP1 in panel E. In C it seems that the level of mutant K185R is higher than WT (especially when normalized to actin levels) and this may explain some of the enhanced luciferase activity. In E-ii the levels of NEDP1 in the K185R mutants and NEDP1 overexpression should be clarified.
sample are significantly lower than its level in the WT sample and this may explain the lack of increased binding of E2F1 to the Cdc6 promoter. Once the authors correct for these differences in amounts their data will be more convincing.

3) The bars that are shown in figure 2F-i and 2F-ii do not have SDs. It is difficult to understand how many times these experiments were done and whether the observed differences are statistically significant.

4) Figure 3A,B: In figure 3B it seems that half-life of WT-E2F1 is about 40 minutes while based on figure 3A the authors claim that half-life is "2.4 hours for NEDDylated E2F-1 compared to 7.6 hours for total E2F-1". The reason(s) for this discrepancy is not clear and should be discussed.

Also, regarding figure 3A - It is not clear from the text how did the authors assess the levels of unmodified E2F1. Is this unmodified E2F1 from the same cells that express the NEDDylated E2F1? This should be explained.

5) Figure 3C: In the text the authors refer to the experiment presented in figure 3C as a "colony forming assay". However, the experiment shown in this figure 3C-iii is a bit different and it shows changes in cell number. Figure 3C-i indeed shows colonies on plates, however, there is no information on the number of colonies in each plate, the number of experiments done and the statistical significance. Also, in figure 3C-iii there are no SDs and no analysis of the statistical significance of the differences. These should be added.

Referee #2:

1. Do the contents of this manuscript report a single key finding? YES

The manuscript's single key finding is that the ability of the transcription factor E2F-1 to control transcription, cell growth and cell proliferation is regulated through covalent modification by NEDD8. Additional findings include the demonstration that NEDDylation of E2F-1 enhances its turnover and that NEDDylation is affected by E2F-1 methylation.

2. Is the main message supported by compelling experimental evidence? YES

Overall, the data are of high quality and compelling. The main conclusions are supported by multiple approaches, including expression of wild type and mutant proteins and siRNA knockdown. The findings are also supported through multiple readouts of E2F-1 activity, including stability, transcription activation and cell proliferation.

Several minor issues should be addressed:

1) Statistical analysis/significance of results in Figures 2C, 2D(iii) and 2F(i),(ii) should be reported. Results in 2F should be representative of at least three independent assays.

2) Results in reported in Figure 3C should be representative of at least 3 assays.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? NO

4. Is the main finding of general interest to molecular biologists? YES

E2F-1 represents a crucial regulator of the cell cycle and the finding that its activities are controlled through NEDDylation is significant. Moreover, relatively few substrates for NEDD8 have been characterized to date, thus the addition of E2F-1 to the list is an important advance.
5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

in EMBO reports

6. Please add any further comments you consider relevant:

This is a solid manuscript describing an important new insight into E2F-1 regulation. As with many valuable studies, the findings raise important new questions about NEDD8 function that are likely to inspire new studies.

Referee #3:

Transcription factors of the E2F family function downstream of the tumor suppressor pRB and play a key role in controlling cell-cycle progression. In this manuscript, the authors demonstrate that the best characterized member of this family, E2F-1, can be modified by the ubiquitin-like protein NEDD8 when both proteins are overexpressed in the cell. Using overexpression experiments, they present data suggesting that NEDD8 conjugation to E2F-1 (or neddylation) may impair its transcription activity. Finally, they suggest an interesting interplay between E2F-1 neddylation and methylation. These data are potentially of broad interest since they extend the functional repertoire of protein neddylation and propose a new mode of regulation for a crucial cell cycle regulator. However, the authors have made little efforts to analyze the relevance of E2F-1 neddylation under endogenous conditions.

Major comments:

1) In Figure 1, the authors demonstrate that overexpressed E2F-1 can be neddylated when NEDD8 is overexpressed in the cell. However, they do not provide any evidence for E2F-1 neddylation under physiological conditions. In panel 1B, the authors attempted to demonstrate that endogenous E2F-1 can be neddylated upon NEDD8 overexpression. However, this experiment has several shortcomings. First, according to the legend, the authors have performed their purification under native conditions. They can therefore not conclude whether E2F-1 is directly modified. Second, the authors do not provide a control to demonstrate that endogenous E2F-1 does not bind nonspecifically to the Ni containing resin. Finally, it would be much more relevant to determine whether E2F-1 can be neddylated under fully endogenous conditions (i.e. without NEDD8 overexpression).

2) Since the authors have not demonstrated that E2F-1 can be neddylated in cells that are not overexpressing NEDD8, I have difficulties to understand how they can confidently interpret the results of the experiments shown in Figures 2A, 2B, 2C, 2E, 3B, 3C, 4E and 4F.

3) NEDD8 overexpression has been shown to trigger an atypical conjugation pathway, which is independent of the classical NEDD8 E1 and E2 enzymes (Hjerpe R et al, Biochem J. (2012) 441:927-36; see also Kim W et al, Mol Cell. (2011) 44:325-40). Consequently, to relate experiments performed with either endogenous or overexpressed NEDD8, the authors should verify that the same pathway is responsible for E2F-1 neddylation in both conditions (if E2F-1 is indeed neddylated in endogenous conditions). This can easily be done using siRNAs or inhibitors targeting the NEDD8 E1 and Ube1 (which is responsible for NEDD8 activation in the atypical pathway). However, this cannot be done using dominant negative forms of NEDD8 E2s, which deplete free NEDD8 (see for instance Wada H et al, J Biol Chem. (2000) 275:17008-170152000) and thus inhibit both classical and atypical neddylation pathways.

4) Irrespective of the previous comments, I have difficulties to interpret the experiments performed with NEDP1 and the E2F-1 lysine mutants. According to the authors, both NEDP1 and the E2F-1 lysine mutants should impair E2F-1 neddylation and thus produce similar a phenotype. Furthermore, since neither NEDP1 nor the lysine mutants fully impair E2F-1 neddylation, their combination should produce a stronger (or at least as strong) phenotype. This is not what the authors observed.
when they have done this experiment, neither in Figure 2E, nor in Figure 4F. For example, Figure 2E shows that NEDP1 expression increases the recruitment of E2F-1 to Cdc6 and E2F-1 promoters, while the K185R mutation has no effect on its own and canceled NEDP1 induced E2F-1 chromatin recruitment. A similar antagonist effect between NEDP1 expression and inhibition of E2F-1 neddylation is observed in Fig 4E when the authors analyzed the effect of Set7/9 depletion (which appears to strongly inhibit E2F-1 neddylation in NEDD8 overexpressing cells). These results are not supporting the hypothesis that the phenotypes of NEDP1 overexpression observed in panels 2A, 2B, 2E, 4E and 4F are linked to E2F-1 deneddylation. Along the same line, there is no indication that the phenotypes of NEDD8 overexpression shown in panels 2D, 2F are mediated by E2F-1 neddylation. The authors should thus provide more evidences indicating that the phenotypes they observe are indeed mediated via impaired or increased E2F-1 neddylation.

Minor comments:

1) Page 3, second paragraph: "post translational modifications" should read "post translational modifiers" and "(PTMs)" should be deleted.

2) Page 4, first paragraph: The description of the mechanism of cullin activation is not clear.

3) Page 3, 4, 5, and 21: "E2 ligase" should be replaced by "E2"

4) Page 7, last paragraph: It is not correct to state that "K185 of E2F-1 is required for its neddylation" since the data presented in Figure 1E indicate the K185R mutant is not even twice as less neddylated as wild type E2F-1.

5) Page 8: The authors write "depletion of Set7/9 reduced the level of NEDD8 apparent on the wild-type E2-F1, to a level comparable with the K185R mutant". However, in the immunoblot provided in Figure 4D, Set7/9 depletion seems to have a much stronger effect than the K185R mutation.

6) Supplementary Methods - Generation of Nedd8-inducible cell line: Since the pTRE2hyg vector contains a Hygromycin resistance cassette, the authors probably did not use G418 to select their stable cell line.

Response to Referee 1

In response to the referee’s concerns:

1) The referee commented on our interpretation of the data in Fig 1G, questioning which residues in E2F-1 are modified by NEDDylation. The data show that K185 influences NEDDylation efficiency, which is further impacted by K182 and K183. The question of which residue out of K117, K120 and K125 is NEDDylated has been partly resolved by the additional data included in the revised manuscript, which show that the new mutant K117/120R is NEDDylated as efficiently as wild-type E2F-1, suggesting that K125 is the NEDDylated residue in the K117/120/125R mutant (SI Fig1B). Unfortunately technical difficulties encountered prevented us from generating point mutants in the K117 to K125 region in the time allowed for revision of the manuscript.

In addition, the referee questioned why some mutants were more efficiently NEDDylated than wild-type E2F-1. In response, this is an important question but one where we cannot provide a
clear cut scientific explanation. It is clear however that the increased NEDDylation that occurs on K182R and K160/164R is entirely reproducible (Fig 1G; SI 1B; n = 4). Moreover, studies on other NEDD substrates, including cullin 3 (Zhou et al, 2001), have described an analogous mutant strategy where the level of NEDD modification is similarly at variance in some mutants, and the challenges in identifying a single residue that is the target for NEDDylation has led many groups, like us, to use mutants with several residue changes (Xirodimas et al., 2004, Gao et al., 2006, Oved et al., 2006, Abida et al., 2007). Equally, however, many other NEDD8 substrate studies have not taken a mutagenesis approach (Watson et al., 2006, Xirodimas et al., 2008). The most important point in our study is that the K185R and K117/120/125R mutants exhibit a decreased level of NEDDylation, which provided the rationale for using these mutants in further experiments to understand the role of NEDDylation in E2F-1.

In addition, we emphasise to the referee that the identification of residues that are modified by NEDD8 has proved to be a challenging exercise in most other studies. It has been frequently found that mutation results in a quantitative rather than qualitative change in NEDDylation (for example Zhou et al, 2001; Gao et al 2004; Xirodimas et al 2006). Our results obtained with E2F-1 mutants are thus similar to other studies.

2) The referee commented we cannot rule out that other proteins which influence E2F-1 activity (DP-1, p300) could be modulated through NEDDylation. This is a very good point and certainly one that we agree is not ruled out by our study. However, we suggest our data show that E2F-1 is NEDDylated in cells and define the effects on E2F-1 activity. We have modified the text to incorporate the referee’s important comment (p 8).

The referee pointed out some inconsistencies in the level of ectopic E2F-1 in the previous figures, suggesting that this may account for the observed differences in the luciferase assays. In response, we have provided new data for Fig 2G (previous Fig 2E), and similarly for Fig 2E (previous Fig 2C).

3) The referee commented on the statistical analysis in the previous Fig 2F (Fig 2H in the revision). In the revision, we have supplied new results and included standard error bars together with details for how many times (n = 3) each experiment was performed (detailed in the revised Fig 2 legend).

4) The referee pointed out the difference in half-life observed between the previous Fig 3A and 3B. We have re-quantitated the results and present improved data which we believe more accurately reflects the observed half-lives in the two experiments (detailed in the figure legend to Fig 3A). Further, the technical issue raised by the referee on how we assessed the levels of E2F-1 has now been detailed in the legend.
5) The referee is correct to point out the inaccurate description of the “colony forming assay” in Fig 3E (previous Fig 3C). In the revision, we have corrected the text, and described Fig 3E (i) as the colony assay (stained with crystal violet), and clarified the technical details for the proliferation (cell number) assay in Fig 3E (iii). In addition, we have indicated how many times in the experiment was performed (n = 3), and added the standard error for each time point.

Thanks to the referee for the helpful comments, which we believe have strengthened the study.

Relevant Literature


Leidecker O, Matic I, Mahata B, Pion E, Xirodimas DP (2012) The ubiquitin E1 enzyme Ube1 mediates NEDD8 activation under diverse stress conditions. Cell Cycle 11: 1142-50
Response to Referee 2

The referee made a number of comments. Our revisions and modifications are detailed below.

1) The referee asked for statistical analysis which we have added as requested in the revised manuscript to the results in Fig 2 E, F, G and H (previous C, D and F) including the additional data provided in the revised Fig 2. The number of times the experiment in Fig 2H was performed is now indicated in the legend (n = 3).

2) Similarly, the number of times the results in Fig 3E (previous 3C) were performed is detailed in the legend (n = 3), and standard error bars have been added to each data point.

Thanks to the referee for the helpful comments, which we believe have strengthened the study.
Response to Referee 3

The referee raised a number of important points. Our modifications to the manuscript and responses to his/her comments are detailed below.

Major comments:

1) The referee commented on the relevance of demonstrating NEDDylation of E2F-1 under endogenous conditions. We agree wholeheartedly with this request and have put considerable effort into addressing the point, including both mass spectrometry and immunoprecipitation under diverse conditions. It has however proved extraordinarily difficult, with limited success (see for example the two sets of preliminary results; Fig 1 for Referee 3). We note however the dearth of published data for any NEDD8 substrate that documents endogenous NEDDylation under physiological conditions (in fact to our knowledge most NEDD8 substrate studies use similar experimental procedures as ourselves) and thus we conclude that current technical approaches do not facilitate resolution of endogenous NEDDylated substrates. We have however included in the revision a variety of new results which further strengthen the identification of E2F-1 as a NEDD8 substrate. For example, NEDDylation of ectopic E2F-1 by NEDD8 is inhibited in a dose-dependent fashion by the small molecule inhibitor MLN4924 of the NEDD8-activating enzyme (Fig 1C). Moreover, treating cells with MLN4924 enhances endogenous E2F-1 levels (Fig 1D), which is precisely the effect predicted given other results presented in the manuscript showing that NEDD8 confers a shorter half-life on E2F-1 (Fig 3A and 3B) and E2F-1 mutants deficient in NEDDylation (for example K185R and K117/120/125R) have an extended half-life (Fig 3B). Thus, although we have not been able to completely address the particular point raised by the referee, we would hope that the combined conclusion of the new and existing data is enough to persuade the referee that E2F-1 is NEDDylated.

The referee raised a number of other technical points. The ectopic NEDD8/endogenous E2F-1 was purified under native conditions, and the referee is technically correct that we cannot exclude that E2F-1 was indirectly purified through association with an unrelated NEDDylated protein. Given the large amount of data in the paper supporting E2F-1 NEDDylation, this possibility seems unlikely. We have, further, provided the control requested by the referee, namely that E2F-1 does not bind non-specifically to the Ni-containing resin, but rather requires conjugation to His-NEDD8 (revised Fig 1C (lane 4)).

2) The referee made the comment that because we had not shown endogenous NEDDylation of E2F-1, it was unclear how we could “confidently interpret” other results in the manuscript.

We agree entirely with this important point. Equally, however, we have employed techniques and an experimental strategy used in other studies that established NEDDylation of a
new substrate (for example Zhou et al, 2001; Xirodimas et al 2004; Gao et al 2006). Thus, although the referee is technically correct, we suggest that the level of scientific scrutiny applied to our study is perhaps harsh, given our experimental strategy and approach is similar to other studies in the field, and the current technical limitations that prevent us from performing the requested experiment.

In addition, the new data on E2F-1 derived from chemical inhibition of the NEDD8-activating enzyme with MLN4924 are consistent with endogenous NEDDylation of E2F-1 (Fig 1C and D, Fig 2I and Fig 3D).

3) The referee raised a very important point namely how do we know that the same conjugation pathway is active in our experimental conditions. We have taken up the referee’s suggestion, namely a chemical biology approach, and show a variety of new results derived from using MLN4924 to inhibit the NEDD8-activating enzyme (by blocking UBA3). Thus, under ectopic expression of E2F-1 (Fig 1C, 2C and 2D) or endogenous E2F activity (Fig 1D, 2H and 2I and 3D), a similar trend of increased E2F activity was apparent, thus suggesting that NEDD8-activating enzyme is responsible for the regulation of E2F-1 and E2F activity in our experimental conditions, whether the read out reflects ectopic E2F-1 or endogenous E2F.

4) The referee commented that we should provide more evidence that the phenotypes we observe are mediated by impaired or increased E2F-1 NEDDylation, focussing on Fig 2 and Fig 4. In response, we hope that the new results in the revision, together with our explanation below, help to clarify this important point.

Thus, new results (Fig 1C) show that E2F-1 is directly NEDDylated, and further that NEDD8-activating enzyme is responsible for the effect. Moreover, the effect of NEDP1 (namely deNEDDylation) and MLN4924 (inhibiting NEDDylation) are similar as both treatments enhance E2F-1 luciferase activity (Fig 2A to D). Moreover, mutants which have compromised levels of NEDDylation, such as K185R and K117/120/125R, have increased levels of E2F-1 luciferase activity (Fig 2E). These biochemical and functional results are entirely compatible with the idea that E2F-1 is modified by NEDD8 which impacts on its functional activity.

The referee commented on the ChIP assay, questioning the effect of NEDP1 on K185R. In response, we have repeatedly observed the different effect of NEDP1 on K185R relative to wild-type E2F-1 ChIP binding activity (Fig 2G). We agree that this is surprising but it is, nevertheless, the result that has been repeatedly observed (n = 4). It is possible therefore that NEDDylation of E2F-1 has pleiotropic effects on E2F-1 activity, hence our choice of the title for the manuscript. Also note that Fig 2G, (i) (single time point ChIP) and Fig 2G, (iii) (real time PCR ChIP) represent different experiments.
Similarly, the referee commented on Fig 4E and 4F, suggesting that the effect of NEDP1 was not as expected. In response, the results show that E2F-1 luciferase activity is enhanced by NEDP1 (Fig 4E and 4F), which is consistent with other results presented in the manuscript (Fig 2A and B). The important point is that in conditions of Set7/9 depletion, the effect of NEDP1 is lost (Fig 4E) and, when a similar experiment was performed on the K185R mutant (namely, compromised in NEDDylation), this effect of NEDP1 was absent (Fig 4F). We interpret this as evidence that methylation of E2F-1 is required for subsequent NEDDylation.

The referee raised a number of minor comments:

1) “Post-translational modifications” is grammatically correct.
2) We have improved the description of cullin activation.
3) E2 ligase has been replaced by E2.
4) We have reworded the description of the role of K185, and in the revision described it as necessary for efficient NEDDylation of E2F-1.
5) We have modified accordingly.
6) The NEDD8-inducible cell line was made using G418 and hygromycin selection, as detailed in Supplementary Information.

Thanks to the referee for the helpful comments, which we believe have strengthened the study.

Relevant Literature


Leidecker O, Matic I, Mahata B, Pion E, Xirodimas DP (2012) The ubiquitin E1 enzyme Ube1 mediates NEDD8 activation under diverse stress conditions. Cell Cycle 11: 1142-50

Figure 1 for Referee 3

A) U2OS

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NEDD8-E2F-1

E2F-1

actin

B) HeLa

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NEDD8-E2F-1

E2F-1

actin

Cells were treated with MLN4924 (1µM) (where indicated) and endogenous NEDD8 substrates purified from U2OS (A) and HeLa (B) cell lysates by immunoprecipitation with anti-NEDD8 (Nd8) antibody. Immunoprecipitation with non-specific antibody (NS) was used as a control. NEDD8-E2F-1 was identified by immoblot analysis using an antibody against E2F-1. Cells were treated with 20µM MG132 4 hours prior to harvesting. The position of NEDD8-E2F-1 is indicated by an arrow.

Many thanks for the submission of your revised study to EMBO reports. The manuscript was seen by two of the original referees and while referee #1 (an expert on E2F transcription factors) is satisfied with the revision, referee #3, an expert on protein neddylation, still has concerns about the study. What becomes clear from his/her report is that s/he is not yet fully convinced that the data is strong enough to support the idea of neddylation of endogenous E2F-1. The referee, for example, maintains that the neddylation assay should be performed under denaturing conditions. S/he also states that in order to exclude the possibility that the neddylation is mediated by the non-classical UBE1 pathway, the experiments would have to be performed in cells depleted of UBE1.

In order to make sure that this referee is not overly critical, I have taken the liberty of contacting an external advisor who is also an expert in the neddylation field and I am pasting excerpts of her/his advice below. S/he agrees with the criticism of reviewer #3 and states that "...the authors cannot
exclude an artificial activation of the Ub-machinery and thus non-specific neddylation, among other
of E2F-1'. S/he further states that '...they need to provide convincing evidence that E2F-1 is
neddylated under endogenous conditions, or at least exclude the possibility that E2F-1 neddylation
under their experimental conditions is mediated by the ub-machinery (see paper by Kunz laboratory
for suitable approaches). I would put the emphasis on trying to reproduce E2F-1 neddylation in
Ube1-depleted cells.'

Given the overall interest the referees expressed in your study, I would like to give you the
exceptional opportunity to revise the study a second time, with the understanding that referee 3’s
concerns must be fully addressed before the study can be published in EMBO reports. Formally,
papers in EMBO reports have to be accepted within 6 months of the initial decision, which in your
case would be July 31st. Given that referee 3 still needs to look at the final version, I would
recommend a submission date for the final version around the end of June. If you submit your study
at a later time we are, of course, still interested in publishing it if the remaining concerns have been
adequately addressed, but we would need to assess its novelty afresh at this point. Another formal
point: in Fig 3E, you state that standard errors are representative of three repeats. I assume you mean
that the experiment has been repeated three times independently, rather than three replicates of one
experiment? If the former is the case, please indicate that the repeats were independent. If they were
mere replicates, they should not be used to calculate standard errors, as in this case n=1.

Please do let me know if you anticipate problems with this time-frame, as I am sure we can find a
solution.

Yours sincerely,

Editor
EMBO Reports

REFeree REPORTS:

Referee #1:

The new data and new statistical analysis that the authors added to the revised version address the
concerns I have raised regarding the original version. The observation that E2F1 is NEDDylated
resulting in changes in its activities is novel and interesting and, therefore, this manuscript can and
should be published in EMBO Reports.

Referee #3:

In their revised manuscript, Loftus et al. have added a number of experiments in response to the
reviewers' suggestions. Here are some comments regarding experiments that were performed (or
not) in response to my initial concerns.

1) In my comments, I pointed out that the data presented in the original manuscript did not
demonstrate that endogenous E2F-1 is a direct NEDD8 target, neither under fully endogenous
conditions nor upon NEDD8 overexpression. This is still not demonstrated in the revised
manuscript. I acknowledge that the identification of neddylated proteins under fully endogenous
conditions is a very difficult challenge, which has not been properly addressed in many other
manuscripts. I however expected that the authors would manage to properly demonstrate a direct
neddylation of endogenous E2F-1 in NEDD8 overexpressing cells. This could have been done by
replacing the panel 1B (which, as previously explained, is in my opinion not conclusive) with a
similar experiment performed under denaturing conditions and including uninduced cells as a
negative control.

2) To overcome this weakness of their manuscript, the authors have included new data that
demonstrate [1] that neddylation of overexpressed E2F-1 is inhibited by MLN4924 (new panel 1C)
and [2] that MLN4924 treatment increases endogenous E2F-1 levels (new panel 1D). I first would
like to comment on the results shown in the new panel 1D. I agree that these results are consistent
with the results presented in Figure 3, which indicate that neddylation destabilizes E2F-1. However, E2F-1 has previously been shown to be ubiquitylated by several cullin-RING ubiquitin ligases (Marti et al., Nat Cell Biol. 1999 vol 1:14-9; Ohta and Xiong, Cancer Res. 2001 vol 61:1347-53).

Since the activity of cullin-RING ubiquitin ligases tightly depends on the neddylation of their cullin subunit (which is rapidly inhibited upon MLN4924 treatment) I am not fully convinced that the results shown in the new panel 1D can be interpreted as an evidence for E2F-1 neddylation under endogenous conditions.

3) The experiment shown in the new panel 1C is very important. It addresses whether E2F-1 neddylation depends on the classical neddylation pathway. This pathway is active both in normal and NEDD8 overexpressing cells. In contrast, UBE1 dependent protein neddylation is induced in cells presenting an increased ratio of free NEDD8 to Ubiquitin, such as NEDD8 overexpressing cells. If E2F-1 modification in NEDD8 overexpressing cells would depend on the classical neddylation pathway, it would suggest that E2F-1 is also neddylated under physiological conditions, which would strongly support the authors’ interpretations. In the new panel 1C the authors show that ectopic E2F-1 neddylation is drastically reduced in NEDD8 overexpressing cells treated for 16h with MLN4924. Since MLN4924 is a very specific inhibitor of the classical NEDD8 activating enzyme this result may indicate that E2F-1 is indeed a target of the classical neddylation pathway. I however have 2 major concerns with this experiment. First, the authors did not control whether E2F-1 neddylation is reduced upon UBE1 inactivation (for instance in UBE1 knockdown cells). Second, the NEDD8 immunoblot shows that, in the conditions used here, MLN4924 treatment does not only reduce NEDD8 conjugation to E2F-1, but also to a wide smear of proteins. This is a typical sign of UBE1 dependent neddylation (in normal conditions, cullins are the main NEDD8 targets in the cell and appear as discrete bands in a NEDD8 immunoblot). I am therefore not convinced that the experiment presented in the new panel 1C definitely demonstrates that E2F-1 neddylation in NEDD8 overexpressing cells occurs by the classical neddylation pathway. I would therefore urge the authors to include a control with UBE1 knockdown cells.

4) To further strengthen their conclusions, the authors have added experiments using MLN4924 to inhibit protein neddylation. I agree that the results presented in the new figures 1D, 2C, 2D, 2I and 3D are consistent with a possible neddylation of endogenous E2F-1. Nevertheless, none of these experiments enable to conclude whether the observed effects are directly due an impaired neddylation of E2F-1. I would have expected the authors to provide more conclusive evidences for a direct function of E2F-1 neddylation. For instance, if the transcriptional effects of MLN4924 treatment and of the K117/120/125R E2F-1 mutant (which seems better than the K185R since it has a stronger effect on neddylation) were both simply due to an impaired neddylation of E2F-1, one would expect [1] that a treatment with 5uM MLN4924 (which drastically inhibits E2F-1 neddylation) has an effect at least as strong as the K117/120/125R mutant and [2] that MLN4924 treatment of K117/120/125R expressing cells has a similar effect as in cells expressing wt E2F-1. In contrast, if the effects of MLN4924 and the K117/120/125R mutant are independent of E2F-1 neddylation and differentially affect transcription, the K117/120/125R mutant should potentiate the effect of MLN4924 treatment. In the same vein, if the effect of NEDD8 overexpression on transcription is mediated by E2F-1 neddylation, it should be considerably reduced in cells expressing the K117/120/125R mutant. When the authors performed this type of experiments with the K185R mutant and NEDP1 (Figures 2G (previously 2E), 4E and 4F), the results were, in my opinion (see previous comments), not fully consistent with a simple and direct effect of E2F-1 neddylation.

Minor comments:
1) Figure 2C and 2D: the legend and the figure annotations do not clearly indicate that panel 2C was performed in U2OS cells and 2D in SAOS2 cells
2) Figure 2I: The increased transcription of E2F-1 target genes in MLN4924 treated cells is most likely (at least partly) due to an increase in E2F-1 levels (shown in Figure 1D). This must be explicitly stated in the text.
EMBOR-2011-35696V2: Response to Referee 3

The referee raised a number of points on our manuscript, which we have carefully considered and as a consequence incorporated new results into the revised manuscript.

Our specific modifications are detailed below:

1) The referee asked us to demonstrate endogenous E2F-1 NEDDylation in the NEDD8 overexpressing cells under denaturing conditions. This new experiment is shown in SI Figure 1A, where the NEDDylation assay was performed under denaturing conditions. NEDDylated E2F-1 is present under these conditions indicating that E2F-1 is covalently modified by NEDD8.

2) The existing data in the revised manuscript, derived from MLN4924 treatment were provided in response to the referee’s previous request (point 3), requesting that we “use siRNAs or inhibitors targeting NEDD8 E1”. Treating cells with MLN4924 results in the induction of E2F-1 in different cell types (Figure 1D), which concurs and is compatible with the overall conclusion of the study on the role of NEDD8 in the control of E2F-1.

3) The referee asked for the E2F-1 NEDDylation experiment to be performed in cells depleted of UBE1. The new data shown in SI Figure 1Bi address this point, and demonstrate that E2F-1 is NEDDylated under conditions of reduced UBE1. In addition, we have provided further examples of this type of experimental design and result (Figure 1 for Referee 3), in order to emphasise that the results are entirely reproducible. Further, we show in SI Figure 1Bii that UBE1-depleted cells have compromised E2F-1 ubiquitination, in contrast to the NEDDylation of E2F-1 (quantified underneath Bii with respect to the input level of E2F-1).

4) The point the referee made in point 4 is a little unclear. MLN4924 was used in the reporter assays in Figure 2C and 2D, and had the predicted effects, namely activation of E2F-1 activity. Similarly, the NEDD8 protease NEDP1 has the expected effect, again activating E2F-1 activity (Figure 2A and 2B). The effect of NEDP1 was, as expected, significantly less on K185R mutant derivative (Figure 4F). We conclude therefore that inhibitors of NEDDylation (MLN4924 and NEDP1) behave as predicted if E2F-1 is a NEDDylated substrate.

The minor comments have been corrected.
Figure 1 (A-B) NEDD8 substrates were isolated by His-purification from U2OS cells co-expressing E2F-1 (1µg) and His-NEDD8 (3µg) under conditions of either control (C) or UBE1 (U) siRNA depletion. His-purification was performed under denaturing conditions and NEDD8-E2F-1 detected by immunoblotting.

Thank you for the submission of your revised study to EMBO reports. It has been seen again by referee 3, who is now supportive of publication and has no further comments. As the handling editor is currently out of the office, I have taken over the handling of your manuscript in order to prevent you from an unnecessary loss of time. I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal!

I have noted that the identity of the error bars shown in several panels of figures 2 and 4 is not specified in the corresponding figure legends. Are these standard errors (as in figure 3), standard deviations, other? Please let me know in reply to this email and I will include this information in the legends, as needed.

In addition, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary “Source Data” files. If you have any questions regarding this please contact me.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.
Yours sincerely,

Editor
EMBO Reports

Authors’ response 06 July 2012

Thank you for your email. We are very pleased that the manuscript has finally been accepted for publication.

In response to the issues that you identified:

All of the error bars are standard error.

We have provided appropriately annotated scans of the unmodified source data for “key” blots in the manuscript, included as an attachment.

Thanks again to the EMBO Reports Editorial Staff for handling this manuscript in a proper and professional manner.