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The RNA-binding E3 ubiquitin ligase MEX-3C links ubiquitination with MHC-I mRNA degradation

Florencia Cano, Helen Bye, Lidia M Duncan, Karine Buchet-Poyau, Marc Billaud, Mark R Wills and Paul J Lehner

Corresponding author: Paul Lehner, University of Cambridge-CIMR

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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 18 May 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. I apologize for the delay in getting to you with our decision.

As you will see from the comments, the referees express some interest in the findings reported in your manuscript, which would in principle make it possible for us to consider a revised version, but they also raise very serious concerns that need to be addressed.
Most importantly, referee #3 is worried that the reported mechanism may lack physiological relevance, since experimental data mostly rely on Mex-3C over-expression. This is a crucial point for the overall validity of the proposed mechanism, and it is will therefore be necessary for you to provide substantial new experimental data showing that endogenous Mex-3C suffices to regulate the stability of HLA-A2 mRNA, before we can consider further steps towards potential publication of the manuscript. In addition, you would need to address all remaining criticisms to the full satisfaction of the referees, especially regarding the specificity of Mex-3C in mRNA regulation and the presence of additional factors in the Mx3c/USP7 complex.

Should you be able to address these criticisms in full, we could consider a revised manuscript. I should remind you that it is EMBO Journal 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 in this revised version and a final assessment by the reviewers. I do realize that addressing the physiological relevance as well as all the remaining points raised by the referees will require a lot of additional time and effort. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere. If this would be the case, please let us know so we can withdraw it from our system.

If you decide to thoroughly revise the manuscript for the EMBO Journal, please include a detailed point-by-point response to the referees' comments. Please bear in mind that this will form part of the Peer 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. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

Although it is known that there are several E3 ubiquitin ligases that have putative RNA-binding activity, their role in the regulation of mRNA stability and the associated requirement for ligase activity has not been reported. The work presented in this manuscript demonstrates that the ligase activity of the novel RNA binding ubiquitin ligase Mex-3c is required for controlled expression of the HLA-A2 cell surface antigen, and that the activity of this protein in HLA-A2 stability is antagonised by the deubiquitinating enzyme USP7. The key findings of this study are: the I44A ubiquitin mutant causes a post-transcriptional decrease in the expression level of HLA-A2; depletion of Mex-3c caused an increase in HLA-A2 levels; a Mex-3 mutant lacking ligase activity had a strong effect on HLA-A2 protein levels but did not impact on the levels of the HLA-A2 transcript; USP7 was physically associated with Mex-3c and antagonised the effect of Mex-3c on HLA-A2 levels.

The experimental work shown is of a very high technical standard and the conclusions drawn are strongly supported by the data. The manuscript has been well written, the experimental work has been well planned and the findings represent a coherent set of data.

The authors should consider the following points whilst revising the manuscript:

1. The authors should explain briefly why only a subset of potential ubiqutin ligases (375 of the ~ 600, as stated) were screened.

2. It would be helpful to the reader to specify the precise nature of the RINGless and mutKH mutants.

3. It would be of interest to show as supplemental data the results obtained from the protein pull-down and mass spectrometric analyses that were aimed to identify Mex-3C interacting proteins. The main query is how many other proteins might be anticipated to be associated in the Mex-3c/USP7 complex.

4. In fig 4G/H it is shown in separate experiments that exogenously expressed USP7 increases the levels of HLA-A2 and that addition of the USP7 inhibitor P045204 causes a decrease in HLA-2A. To demonstrate that the inhibitor is actually blocking USP7 activity, additional data should be

included where it has been added to the UPS7 transfected cells.

Referee #2

In this manuscript, Cano et al describe the identification of MEX-3C as a novel regulator of HLA-A molecules. In a very convincing manner they show that this E3 ligase mediates decay of HLA-A2 via degradation of the corresponding mRNA in model 293 cells and activated NK cells. This process is facilitated by the necessary auto-ubiquitinating RING domain and inhibited by the DUB USP7. The study is well-performed and provides a complete and novel mechanism for regulation of HLA-A, a major component of a wide range of immune reactions. The paper is also timely and relevant from the mechanistic point of view, since the authors show for the first time direct E3 ligase involvement in the control of mRNA levels.

Minor concerns:
1) Can the abbreviation "NT" in Fig. 1B (no band in GAPDH?) and 4B be explained in the legends?
2) Fig. 4A shows that MEX-3C does not affect A2 promotor activity. CIITA is shown as control, while A2-siRNA would have been the appropriate control for this experiment.
3) The authors mention in Fig. S5 B*4501 as the most frequent B-allele, while B*0801 is actually the most frequent. Furthermore, the authors describe in the text that HLA-A1, A2, A3 and A11 are the most prevalent A-alleles in the caucasian population; adding A24 to this list would cover >90% of the caucasian population, and also adding A11 and A24 to the alignments in Fig. S4 would be interesting and highly supportive for their broad HLA-A allotype claim.

4) Can the authors discuss whether MEX-3C may also interact with other mRNAs in NK cells (as an alternative explanation for the results in Fig. 5G, but also interesting to know)?
5) Are any viral proteins known to be homologues of MEX-3C, and thus possibly mediating immune escape via mRNA decay?
6) In the discussion, the authors indicate a possible similar mechanistic role for Roquin in mRNA decay. However in Athanasopoulos et al (FEBS, 2010) showed that the RING domain of this protein is not involved in the mRNA breakdown.

Referee #3

Cano et al, MEX-3C
The manuscript by Cano and colleagues describes MEX-3C as an RNA-binding ubiquitin ligase responsible for the post-transcriptional regulation of allotype-specific regulation of MHC-I. Using a functional siRNA-based screen, the authors identified MEX-3C as a protein responsible for the post-transcripitional regulation of HLA-A2 mRNA. The majority of the work utilizes HEK293 cells in which the authors were unable to observe expression of endogenous MEX-3C. This is a serious concern as all of the critical experiments seeking to develop the mRNA decay/ubiquitin ligase activity relationship rely on over-expression. This is further compounded by the absence of data showing relative levels of expression for comparing these proteins. There is also notable over-interpretations and a lack of consideration of alternative explanations.
The authors state that their data provide a direct link between mRNA decay and a ubiquitin ligase. However, one is left with a confusing model with an unclear function for MEX-3C. The most interesting aspect of the work is the finding in NK cells that MEX-3C expression is regulated by stimulation and that there is an effect on target lysis (Fig 5). It seems that the significant limitations and distractions of the present manuscript could possibly be mitigated by focusing more of the functional studies in the biological context of MEX-3C in NK cells.

Specific Points
The authors utilize excessive jargon that is not defined. They should not include undefined jargon in the abstract (KIRs). They also need to define KIR, CTL, etc upon first usage in the manuscript. The term "KIR receptor" (page 3 line 2) is redundant.
The statement in the abstract that the authors "...provide a direct link between ubiquitination and mRNA degradation." is not supported by the data presented. This implies that the E3 activity of MEX-3C directly regulates mRNA degradation, which is clearly not shown.
page 3, line 7: run-on sentence of unclear meaning

page 3, line 15: "these processes" referred to by the authors are unclear.
Fig 1A: How does this experiment differ from recently published data by the same group?
Fig 1B: How does wild-type ubiquitin or ubiquitin that cannot be conjugated affect mRNA levels?
Fig 1B, 1C: It is important for the authors to show steady-state protein levels from the transfected constructs as they are making a direct comparison between their ability to activate gene expression.
Fig 2B, C: The authors need to show evidence for on-target depletion of MEX-3B, MEX-3D, and MEX-3C with endogenous proteins. Although the MEX-3C antibody used by the authors was apparently not sensitive, a variety of other antibodies are commercially available and should be tested. The authors need to provide evidence of on-target effects on endogenous protein/mRNA levels. Alternatively, the experiments should be done in cells in which MEX-3C can be detected (i.e., stimulated NK cells).
page 6, line 13: Related to the point above, there is no evidence presented to support the authors' claim "...depletion of endogenous MEX-3C increased HLA-A2 expression..."
page 7, line 1: The description of the evolutionary conservation of MEX-3 seems out of place in the results. Verbatim sentences are found in the discussion. (compare to page 11, line 9). The final sentence of the discussion paragraph can be found in similar form on page 8, last line.
Fig 3B, 3C: The authors need to show the steady-state protein levels from the transfected constructs as they are making a direct comparison between their effects on HLA-A2 expression. Moreover, the data in 3B, 4F should be quantified to allow for comparison.
page 7, line 18"...degradation of HLA-A2 mRNA by MEX-3C...implies a critical role for ubiquitin ligase activity in mRNA decay." is not supported by the data. To claim an "absolute requirement", one would need a sufficiency experiment.
page 7, line 21: "To confirm..." This statement is an over-interpretation of the presented data. Moreover, Fig 4A argues that the effect is not transcriptional, but does not support the contention that the effects of MEX-3C are due to mRNA decay. The authors need to directly measure the changes in abundance of HLA-A2 mRNAs through pulse-chase or similar analyses.
page 8, line "These results are consistent...(Figure 3B)...through the HLA-A2 3'UTR." This is not a correct representation of the data. The statement is apparently referencing the KH-dependency of HLA-A2 cell surface expression.
page 9, line 5: The authors should present a summary of the peptides/interactors (with counts) identified by mass spectrometry. The data shown (Fig 4E) relies on over-expression of both proteins (the figure legend should provide more clear details) and the efficiency of the interaction also cannot be judged due to the lack of an input western blot showing relative expression.
Fig 4F, G, H: The authors need to evaluate if the expression of USP7 alters the steady state levels or half-life of MEX-3C. Does the expression of USP7 alter the binding of MEX-3C to the HLA-A2 3'UTR?
page 9, line 22: The authors need to explain their statement regarding how an immunoblot analysis confirms a transcriptional analysis.
Fig 5B: Why is MEX-3C a doublet in NK-92 cells?
Fig 5: Do the RING and KH mutant forms of MEX-3C regulate HLA-A and affect the functional phenotype observed in NK cells? Does USP7 suppress the effects of MEX-3C in this biological context?

1st Revision - authors' response 20 June 2012

Point-by-point response to the referees' comments:

Referee #1

Although it is known that there are several E3 ubiquitin ligases that have putative RNA-binding activity, their role in the regulation of mRNA stability and the associated requirement for ligase activity has not been reported. The work presented in this manuscript demonstrates that the ligase activity of the novel RNA binding ubiquitin ligase Mex-3c is required for controlled expression of the HLA-A2 cell surface antigen, and that the activity of this protein in HLA-A2 stability is antagonised by the deubiquitinating enzyme USP7. The key findings of this study are: the I44A ubiquitin mutant causes a post-transcriptional increase in the expression level of HLA-A2; depletion of Mex-3c caused an increase in HLA-A2 levels; a Mex-3 mutant lacking ligase activity had a strong effect on HLA-A2 protein levels but did not impact on the levels of the HLA-A2 transcript; USP7 was physically associated with Mex-3c and antagonised the effect of Mex-3c on HLA-A2 levels.

The experimental work shown is of a very high technical standard and the conclusions drawn are strongly supported by the data. The manuscript has been well written, the experimental work has been well planned and the findings represent a coherent set of data.

The authors should consider the following points whilst revising the manuscript:

1. The authors should explain briefly why only a subset of potential ubiqutin ligases (375 of the ~ 600, as stated) were screened.

The figure quoted for the number of ubiquitin E3 ligases in the literature is often around 600. These include the RINGs (c.347), HECT ligases (c.28), F Box (c.61) and BTB (c.169). In our bespoke E3 ligase library which we made a few years ago, we compiled a list of active RINGs and HECTS. We did not include the BTBs as they are classed as E3s because they are thought to mediate substrate recognition in Cullin complexes. They do not however interact with E2s. At the time, we did not include the multisubunit ligases as they all share a common ring (Roc1 or 2). To clarify this point we have stated in the text (page 5) that our library covers only the known RING and HECT E3 ligases.
'We screened a ubiquitome library, specific for RING and Hect E3 ligases.'

2. It would be helpful to the reader to specify the precise nature of the RINGless and mutKH mutants.

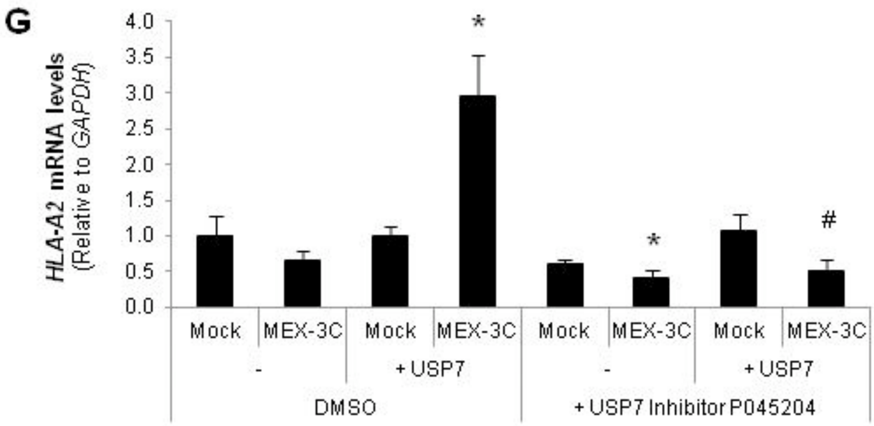
The suggested information about the MEX-3C mutants has been added to the manuscript (page 6), as indicated below:
"A similar decrease in surface HLA-A2 expression was seen in cells transfected with RINGless (aa 1-636) MEX-3C, which has no ubiquitination activity. In contrast, the MEX-3C KH mutant (G249D; G343), with single point mutations in each of the two RNA-binding domains, caused only a minor decrease in cell surface HLA-A2 (Figure 3B and S1C)."

3. It would be of interest to show as supplemental data the results obtained from the protein pull-down and mass spectrometric analyses that were aimed to identify Mex-3C interacting proteins. The main query is how many other proteins might be anticipated to be associated in the Mex-3c/USP7 complex.

A supplementary table (Table S1) with the identified MEX-3C interacting proteins has been included. We are in the process of trying to identify which other proteins are indeed members of the complex, but as can be seen from the table, validating these findings is beyond the scope of the present paper.

4. In fig 4G/H it is shown in separate experiments that exogenously expressed USP7 increases the levels of HLA-A2 and that addition of the USP7 inhibitor P045204 causes a decrease in HLA-2A. To demonstrate that the inhibitor is actually blocking USP7 activity, additional data should be included where it has been added to the UPS7 transfected cells.

As the reviewer suggested, we have performed additional experiments to demonstrate that the USP7 inhibitor is indeed blocking USP7 activity. We show that addition of the inhibitor to USP7 transfected cells abolishes the effect of USP7; i.e.: the observed increase in *HLA-A2* mRNA levels is lost.
Figure 4G has therefore been modified to include the addition of USP7 inhibitor P045204 to mock or USP7 transfected HEK293T cells, as also shown.



Referee #2

In this manuscript, Cano et al describe the identification of MEX-3C as a novel regulator of HLA-A molecules. In a very convincing manner they show that this E3 ligase mediates decay of HLA-A2 via degradation of the corresponding mRNA in model 293 cells and activated NK cells. This process is facilitated by the necessary auto-ubiquitinating RING domain and inhibited by the DUB USP7. The study is well-performed and provides a complete and novel mechanism for regulation of HLA-A, a major component of a wide range of immune reactions. The paper is also timely and relevant from the mechanistic point of view, since the authors show for the first time direct E3 ligase involvement in the control of mRNA levels.

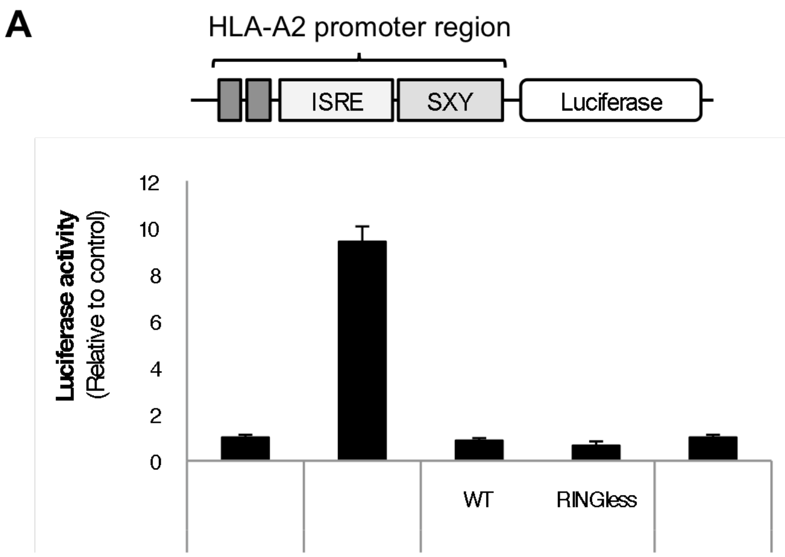
Minor concerns:

1) Can the abbreviation "NT" in Fig. 1B (no band in GAPDH?) and 4B be explained in the legends?

Many thanks for pointing this out. NT refers to No Template control, hence the lack of a PCR band in *GAPDH*. 'NT' explanation has been added to the legend for Figure 1.

2) Fig. 4A shows that MEX-3C does not affect A2 promotor activity. CIITA is shown as control, while A2-siRNA would have been the appropriate control for this experiment.

We found it difficult to understand the referee's comment here. Since this is a reporter assay, and we didn't see any effect of either wildtype or RINGless MEX-3C, we used CIITA as a positive control for luciferase activity, to ensure the system was working correctly. We can't quite see the relevance of using an siRNA against HLA-A2? We wondered if the reviewer was referring to a depletion of MEX-3C and have therefore shown that MEX-3C depletion has no effect on HLA-A2 promoter activity (Fig 4A, as shown below).



*3) The authors mention in Fig. S5 B*4501 as the most frequent B-allele, while B*0801 is actually the most frequent. Furthermore, the authors describe in the text that HLA-A1, A2, A3 and A11 are the most prevalent A-alleles in the caucasian population; adding A24 to this list would cover >90% of the caucasian population, and also adding A11 and A24 to the alignments in Fig. S4 would be interesting and highly supportive for their broad HLA-A allotype claim.*

Thank you for these helpful comments and suggestions. The manuscript (page 10) has been modified to:
"These two alleles (HLA-A2 and HLA-A3) along with HLA-A01, A11 and A24 are the most common HLA-A alleles and share identical 3'UTR sequences (Figure S4)."

Figures S4 and S5 have been altered to include the alignments of HLA-A11 and HLA-A24 as suggested by the reviewer.

4) Can the authors discuss whether MEX-3C may also interact with other mRNAs in NK cells (as an alternative explanation for the results in Fig. 5G, but also interesting to know)?

A new sentence has been added (page 14) in the discussion regarding other potential mRNA targets of MEX-3C in NK cells:

"It is likely that MEX-3C binds and regulates other mRNA species. Future work will focus on identifying these additional mRNA targets and should give further insight into MEX-3C's broader physiological relevance and role in NK cell function."

5) Are any viral proteins known to be homologues of MEX-3C, and thus possibly mediating immune escape via mRNA decay?

This is an interesting point. As the referee correctly points out, viral genes are known to intersect almost every aspect of the MHC class I presentation pathway. It is therefore very likely that a virus may appropriate MEX-3C to decrease HLA-A expression. However, a bioinformatic analysis (in the form of blast searches) has so far failed to find any viral homologue of MEX-3C. Of course, this does not exclude their presence as they may not share homology with the cellular gene.

6) In the discussion, the authors indicate a possible similar mechanistic role for Roquin in mRNA decay. However in Athanasopoulos et al (FEBS, 2010) showed that the RING domain of this protein is not involved in the mRNA breakdown.

We apologize if the discussion led to some confusion. What we were trying to point out was that Roquin and MEX-3C share some features, as they are both thought to be RNA binding proteins and E3 ligases. However, we also state that they differ in the mechanism of mRNA degradation as

"Roquin participates in the decapping pathway (Glasmacher et al, 2010) while MEX-3C is implicated in RISC-mediated degradation pathway."
We have modified the discussion to clarify this point as shown here:

From page 13: "The best characterized is Roquin which, like MEX-3C, contains an E3 ligase RING Finger domain, but has a CCCH RNA-binding domain, rather than the tandem repeat KH domains seen in MEX-3C. Roquin was first identified in a screen for autoimmune regulators in mice, in which a mutation in Roquin resulted in a lupus-like pathology. Roquin deficiency increased *Icos* mRNA and cell surface Icos expression on T cells, causing the accumulation of lymphocytes associated with a lupus-like autoimmune syndrome (Vinuesa et al, 2005). Like MEX-3C, Roquin localizes to cytosolic RNA granules implicated in mRNA stability, though they differ in the mechanism for mRNA decay. While Roquin participates in the decapping pathway (Glasmacher et al, 2010), MEX-3C is implicated in RISC-mediated decay."

Referee #3

Cano et al, MEX-3C
The manuscript by Cano and colleagues describes MEX-3C as an RNA-binding ubiquitin ligase responsible for the post-transcriptional regulation of allotype-specific regulation of MHC-I. Using a functional siRNA-based screen, the authors identified MEX-3C as a protein responsible for the post-transcripitional regulation of HLA-A2 mRNA. The majority of the work utilizes HEK293 cells in which the authors were unable to observe expression of endogenous MEX-3C. This is a serious concern as all of the critical experiments seeking to develop the mRNA decay/ubiquitin ligase activity relationship rely on over-expression. This is further compounded by the absence of data showing relative levels of expression for comparing these proteins.

There is also notable over-interpretations and a lack of consideration of alternative explanations. The authors state that their data provide a direct link between mRNA decay and a ubiquitin ligase. However, one is left with a confusing model with an unclear function for MEX-3C. The most interesting aspect of the work is the finding in NK cells that MEX-3C expression is regulated by stimulation and that there is an effect on target lysis (Fig 5). It seems that the significant limitations and distractions of the present manuscript could possibly be mitigated by focusing more of the functional studies in the biological context of MEX-3C in NK cells.

Specific Points

The authors utilize excessive jargon that is not defined. They should not include undefined jargon in the abstract (KIRs). They also need to define KIR, CTL, etc upon first usage in the manuscript. The term "KIR receptor" (page 3 line 2) is redundant.

We have defined the abbreviations as recommended.

The statement in the abstract that the authors "...provide a direct link between ubiquitination and mRNA degradation." is not supported by the data presented. This implies that the E3 activity of MEX-3C directly regulates mRNA degradation, which is clearly not shown.

We have modified the abstract to take into account the referees comments: "We have therefore uncovered a novel post-transcriptional pathway for regulation of HLA-A allotypes and provide a link between ubiquitination and mRNA degradation."

page 3, line 7: run-on sentence of unclear meaning.

We have modified this sentence to try and clarify the meaning:
"Post-translational modification of MHC-I molecules by ubiquitin provides a potent mechanism for regulating MHC-I turnover and several viral as well as cellular ubiquitin E3 ligases (Randow and Lehner, 2009) regulate MHC-I assembly in the endoplasmic reticulum and at the cell surface."

page 3, line 15: "these processes" referred to by the authors are unclear.

We have modified the sentence to clarify the referee's concerns:

"Although ubiquitin is best recognized for its role in post-translational protein regulation, a potential role in the regulation of RNA stability has emerged from the finding that at least 15 E3 ubiquitin ligases encode predicted RNA-binding domains (RBDs) (Cano et al, 2010). While there is some understanding of how these proteins regulate mRNA, their requirement for E3 ligase activity is unclear. Turnover of AU-rich cytokine mRNAs is dependent on ubiquitination (Laroia et al, 2002), though the link between mRNA turnover and ubiquitination is not defined."

Fig 1A: How does this experiment differ from recently published data by the same group?

This experiment does not differ significantly from our recent work. Since many of the readers may not be familiar with our previous work, we felt it helpful to show this figure at the start of the paper. Furthermore, it is a control for the expression levels of exogenous GFP-Ubiquitin in subsequent experiments in Figure 1 and is therefore important.

Fig 1B: How does wild-type ubiquitin or ubiquitin that cannot be conjugated affect mRNA levels?

We have added to Figure 1 (see text and as shown below) to show the wild-type ubiquitin (wt-UB) control. This indicates that wild-type ubiquitin has no effect over *HLA-A2* mRNA levels. We found that ubiquitin that cannot be conjugated was toxic under these conditions and have not included this data.

Fig 1B, 1C: It is important for the authors to show steady-state protein levels from the transfected constructs as they are making a direct comparison between their ability to activate gene expression.

We assume the referee is referring to Figure 1A and the transfected ubiquitin constructs. These are ubiquitin-GFP constructs where the GFP is co-translational cleaved as normally occurs with ubiquitin genes which are expressed as a series of tandem repeat genes. Thus GFP is expressed independently of ubiquitin and provides a very accurate marker of protein expression. The experiment shown in Figure 1C is an extension of Figure 1A, for which we now show the % of cells expressing ubiquitin-GFP as a measure of the amount of exogenous ubiquitin being expressed. See below:

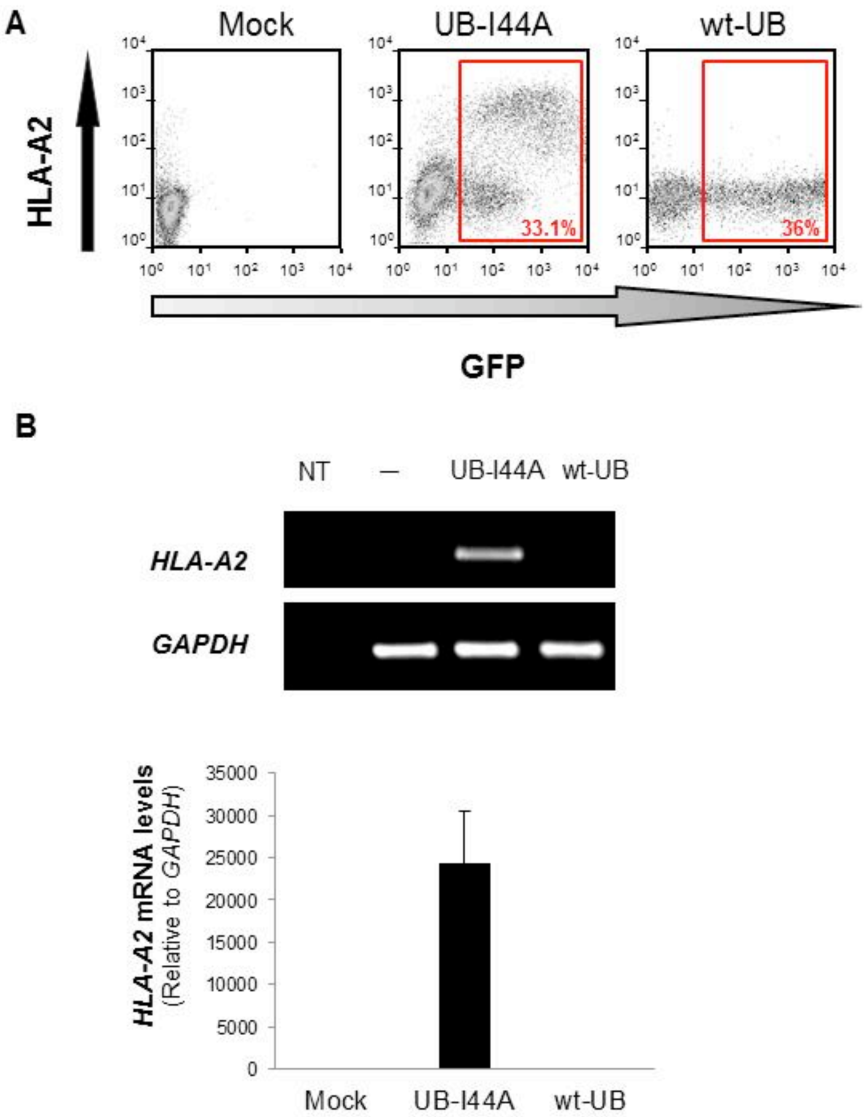
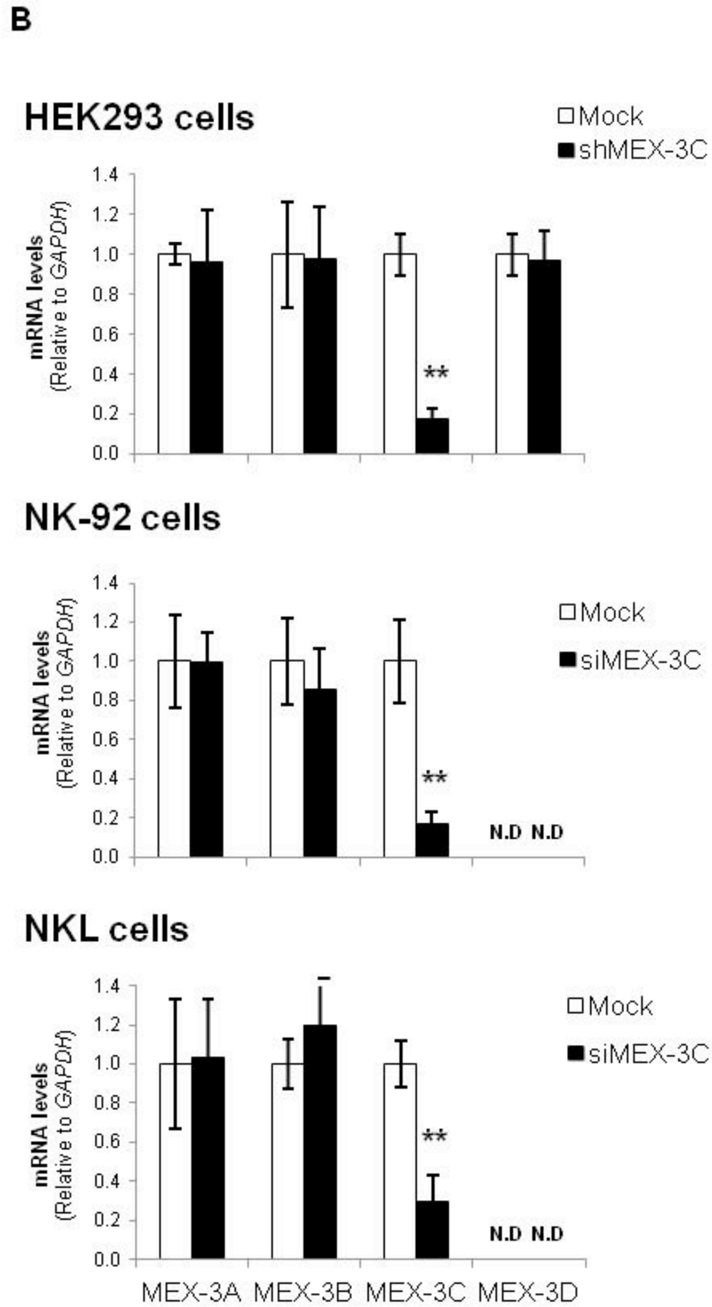


Fig 2B, C: The authors need to show evidence for on-target depletion of MEX-3B, MEX-3D, and MEX-3C with endogenous proteins. Although the MEX-3C antibody used by the authors was apparently not sensitive, a variety of other antibodies are commercially available and should be tested. The authors need to provide evidence of on-target effects on endogenous protein/mRNA levels. Alternatively, the experiments should be done in cells in which MEX-3C can be detected (i.e., stimulated NK cells).

As requested by the reviewer we have tested that the on-target depletion of MEX-3C in HEK293 cells as well as NK-92 and NKL cells has no effect on the levels of expression of MEX-3C's human homologues MEX-3A, -3B and -3D by qRT-PCR (as now shown in Supplementary Figure S1B and below).



In terms of the availability of commercial antibodies against MEX-3C, we have painfully tried many of the available antibodies and are using the best antibodies currently available. Unfortunately, almost all the antibodies, despite being commercialised by different companies, are actually manufactured from the same source (ProSci).

page 6, line 13: Related to the point above, there is no evidence presented to support the authors' claim "...depletion of endogenous MEX-3C increased HLA-A2 expression..."

We understand that the reviewer refers to the fact that as our MEX-3C antibody is not sufficiently sensitive to detect endogenous MEX-3C protein levels in HEK293s, we cannot claim that we are depleting it efficiently. We have therefore performed quantitative RT-PCR to show the efficient depletion of *MEX-3C* mRNA in these cells. These results are included in supplementary Figure S1B and as shown above.

We have also modified the text to include in page 5 the following statement:

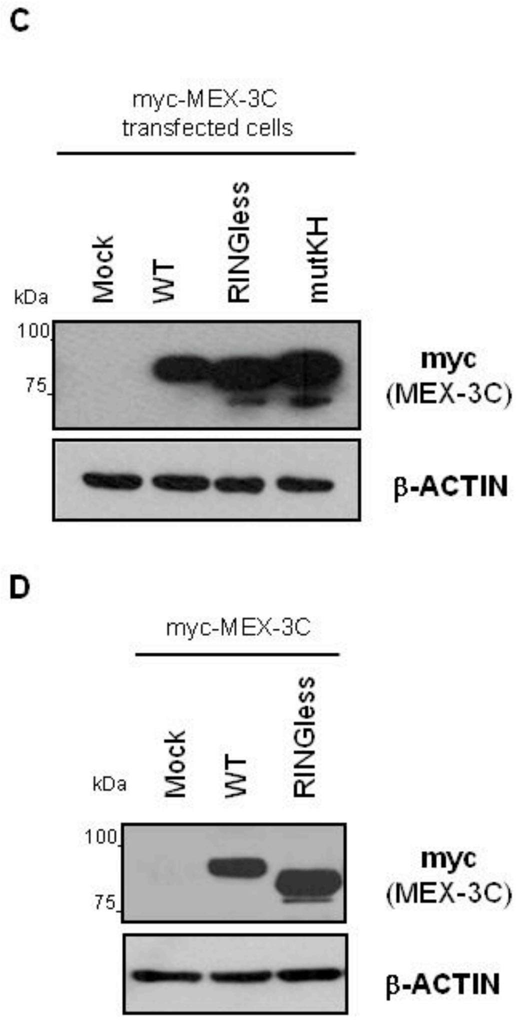
"Effective on-target depletion of endogenous *MEX-3C* mRNA was detected by qRT-PCR; with no effect on its homologues *MEX-3A*, *MEX-3B* and *MEX-3D* (Figure S1B)."

page 7, line 1: The description of the evolutionary conservation of MEX-3 seems out of place in the results. Verbatim sentences are found in the discussion. (compare to page 11, line 9). The final sentence of the discussion paragraph can be found in similar form on page 8, last line.

We thought long and hard about planning the results section. We think the evolutionary argument fits in well in the results section as it describes the acquisition of the RING as a result of evolutionary diversification, and have opted to keep it there. We agree that this is also a discussion point but emphasise that it enables the reader to follow the results section more easily. We apologise if the reviewer found the text repetitive, the other two reviewers thought the paper was well written. E.g.: Referee #1:'*The manuscript has been well written, the experimental work has been well planned and the findings represent a coherent set of data.'* We have in fact used the repetition as a means to emphasize important points throughout the text.

Fig 3B, 3C: The authors need to show the steady-state protein levels from the transfected constructs as they are making a direct comparison between their effects on HLA-A2 expression.

We show GFP levels as a surrogate marker for MEX-3C expression as this method depicts that increasing GFP (MEX-3C) levels are associated with a more marked phenotype; i.e.: decreased cell surface expression of HLA-A2. However, we have followed the reviewer's suggestion and included in the supplementary section (Figures S1C and S1D) the immunoblots corresponding to Figures 3B and 3C, as shown below:



Moreover, the data in 3B, 4F should be quantified to allow for comparison.

The quantification of HLA-A2 protein expression has already been shown as the geometric fluorescent means (GFM) in the legend to Figure 3. The quantification for Figure 4F was missing, and is now included in the figure legend. The new figure legend for Figure 4F reads:

"(F) Cytofluorometric analysis of cell surface HLA-A2 in HEK293Ts expressing wtMEX-3C and/or USP7 cDNAs. GFP expression is a surrogate marker for MEX-3C and USP7 expression. The HLA-A2 geometric fluorescent means in transfected (GFP^+) cells are: Mock= 177; MEX-3C only= 48; USP7 only= 164 and MEX-3C + USP7= 45.7."

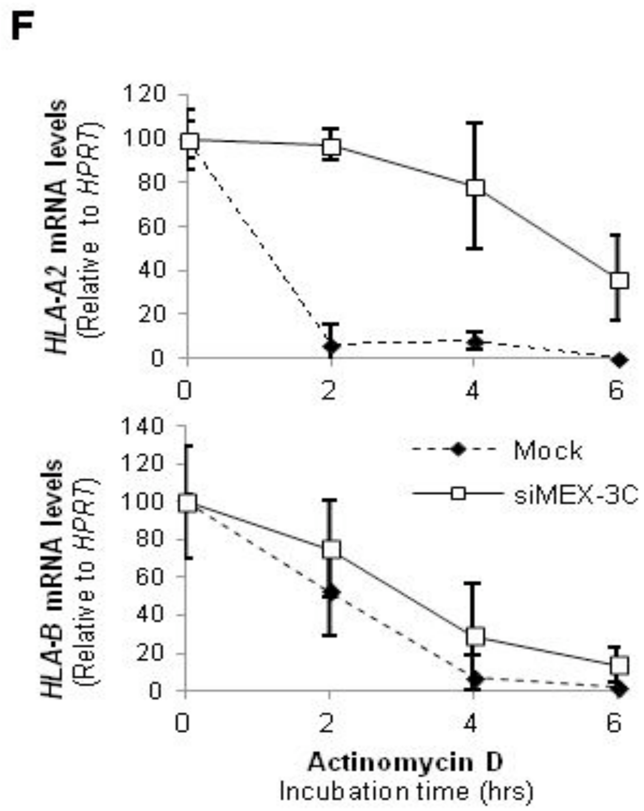
page 7, line 18"...degradation of HLA-A2 mRNA by MEX-3C...implies a critical role for ubiquitin ligase activity in mRNA decay." is not supported by the data. To claim an "absolute requirement", one would need a sufficiency experiment.

This is a valid point. The text has been modified: "Therefore, degradation of *HLA-A2* mRNA by MEX-3C is dependent on a functional RING domain and implies a critical role for ubiquitin ligase activity in mRNA decay."

page 7, line 21: "To confirm..." This statement is an over-interpretation of the presented data. Moreover, Fig 4A argues that the effect is not transcriptional, but does not support the contention that the effects of MEX-3C are due to mRNA decay. The authors need to directly measure the changes in abundance of HLA-A2 mRNAs through pulse-chase or similar analyses.

These were helpful comments. The suggestion to use Actinomycin D to measure the half-life of *HLA-A2* mRNA by MEX-3C is beneficial as this experiment confirms a role for MEX-3C in *HLA-A2* mRNA decay. We now use Actinomycin D to measure the HLA-A2 mRNA half-life in Mock or siMEX-3C depleted NKL cells and show that the MEX-3C depletion extends the HLA-A2 mRNA half-life 4-fold, while having no significant effect on the corresponding HLA-B mRNA. The results have been added as Figure 5F (and shown below) and to page 10 where the text now reads:

"Depletion of MEX-3C caused a 4-fold increase in *HLA-A2* mRNA half-life, from 1.48 hrs in mock cells to 5.68 hours in siMEX-3C cells, while having no significant effect on the corresponding HLA-B mRNA half-life. (Figure 5F)."



page 8, line "These results are consistent...(Figure 3B)...through the HLA-A2 3'UTR." This is not a correct representation of the data. The statement is apparently referencing the KH-dependency of HLA-A2 cell surface expression.

The statement the reviewer is referring to on page 8 is: "These results are consistent with our previous data (Figure 3B) showing that wt and RINGless MEX-3C reduce cell surface HLA-A2 through the *HLA-A2* 3'UTR."

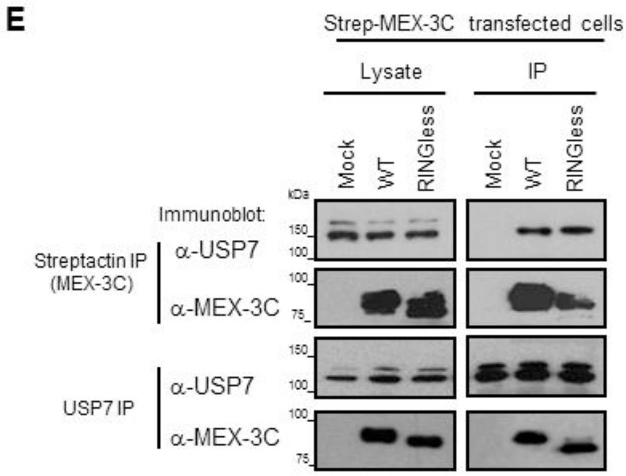
We apologize if this line is confusing as it does not refer to the "*KH-dependency of HLA-A2 cell surface expression"* but points out that the previously observed downregulation of HLA-A2 by wt and RINGless MEX-3C is mediated by MEX-3C's effect on *HLA-A2* mRNA 3'UTR (as depicted by the luciferase reporter assay).

page 9, line 5: The authors should present a summary of the peptides/interactors (with counts) identified by mass spectrometry.

A supplementary table (Table S1) with the identified MEX-3C interacting proteins has been included. We are trying to identify which other proteins are indeed members of the complex, but as can be seen from the table, validating these findings is beyond the scope of the present paper.

The data shown (Fig 4E) relies on over-expression of both proteins (the figure legend should provide more clear details) and the efficiency of the interaction also cannot be judged due to the lack of an input western blot showing relative expression.

Only tagged MEX-3C was over-expressed in this figure, USP7 expression is endogenous. We have modified the text and figure legend so this is clear. As suggested, we have also expanded the figure to include the input (lysate) protein levels.



The main text (page 9) now reads:

"To identify additional MEX-3C binding partners we performed a MEX-3C (Streptactin) pull-down followed by mass spectrometry analysis (Table S1). We found that both wt and RINGless MEX-3C bind the de-ubiquitinating (DUB) enzyme USP7, a finding readily confirmed by immunoprecipitation (Strep-MEX-3C or endogenous USP7) and immunoblotting in both directions (Figure 4E). The interaction was further confirmed in NK cells where immunoprecipitation of endogenous MEX-3C reveals an association with endogenous USP7 (see later and Figure S7A)."

The Figure 4E legend now reads: "(E) MEX-3C associates with the DUB USP7. HEK293Ts were co-transfected with Strep-His-wt or RINGless MEX-3C. Cells were lysed in 1% NP-40 and immunoprecipitated on Streptactin beads (MEX-3C IPs) or for endogenous USP7, and immunoblotted for USP7 and MEX-3C."

Fig 4F, G, H: The authors need to evaluate if the expression of USP7 alters the steady state levels or half-life of MEX-3C. Does the expression of USP7 alter the binding of MEX-3C to the HLA-A2 3'UTR?

The suggestion whether USP7 alters the binding of MEX-3C to the *HLA-A2* mRNA was helpful. A MEX-3C pull-down (myc-tagged) from HEK293Ts in the presence or absence of exogenously expressed USP7 showed a small increase in *HLA-A2* mRNA in the presence of USP7. These results are consistent with a model that when MEX-3C is released from USP7 DUB activity, its ubiquitin ligase activity allows the *HLA-A2* mRNA to be transferred to the RNA degradation machinery.

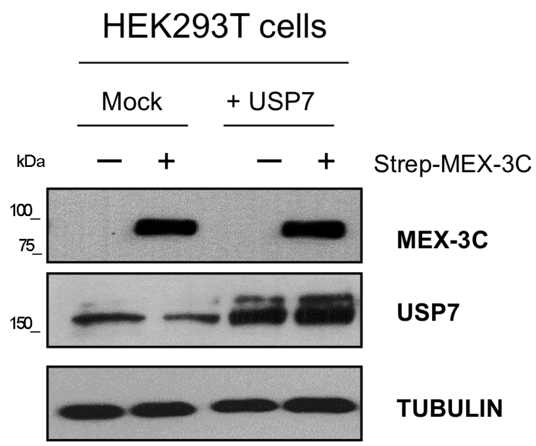
This experiment was added to Figure S7D and the main text now includes (page 9) the following line:

"In the presence of the USP7 DUB, more *HLA-A2* is bound to wtMEX-3C, as overexpression of USP7 increases the amount of *HLA-A2* mRNA co-immunopreciptated with MEX-3C (as measured by qRT-PCR) (Figure S7D)."

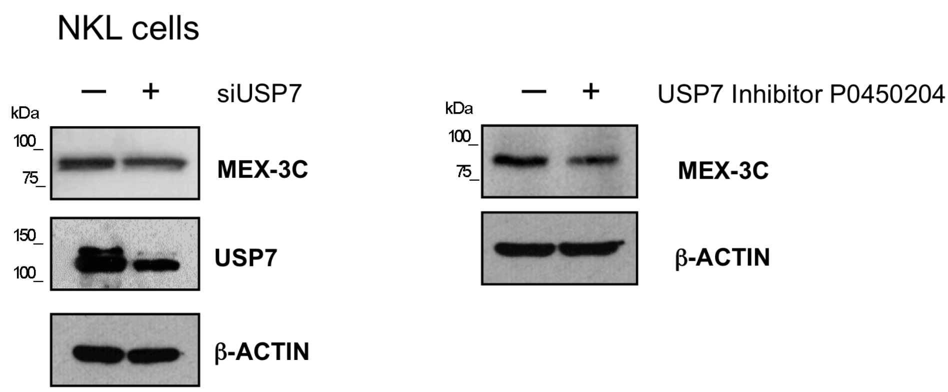
Immunoblot analysis showing that neither overexpression nor depletion of USP7 affected steady state levels of MEX-3C were added to Figures S7B and S7C. This information was also included in the results section (page 9):

"Neither overexpression nor depletion of USP7 affected MEX-3C protein levels (Figures S7B and S7C)."

B



C



page 9, line 22: The authors need to explain their statement regarding how an immunoblot analysis confirms a transcriptional analysis.

The text was changed to improve clarity. It now reads:

"Immunoblot analysis of MEX-3C in primary human NK cells and two NK cell lines (NK-92; NKL) was consistent with this transcriptional analysis (Figure 5)."

Fig 5B: Why is MEX-3C a doublet in NK-92 cells?

This is an important point, for which we do not at present have an answer. We have investigated the possibility of post-translational modifications such as phosphorylation (although the mass change is too large) or monoubiquitination (for which we have no positive evidence at present).

Fig 5: Do the RING and KH mutant forms of MEX-3C regulate HLA-A and affect the functional phenotype observed in NK cells?

Unfortunately, although we and others have tried, it is at present not possible to efficiently transduce NK cells. There are published protocols for transfection and transduction of human NK cells but we have found these unreliable and despite much effort have not achieved transfection above 3%. Therefore we are not able to address the effect of the MEX-3C RING and KH mutants in NK cells.

Does USP7 suppress the effects of MEX-3C in this biological context?

We show (Figure 4H) that treatment of the NK cell line (NKL) with the USP7 inhibitor increases *HLA-A2* mRNA levels. We infer that this is mediated by the DUB effect on MEX-3C's activity, as MEX-3C levels do not change upon USP7 depletion or inhibition (Figure 7C).

2nd Editorial Decision 10 July 2012

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below. As you will see there are still a few concerns that require you to rephrase or elaborate parts of the manuscript before we can accept it for publication.

Regarding the comments about repetitive sentences made by one of the referees, I understand that you want to emphasize the same issues in both the results and discussion section, but could you please rephrase the descriptions slightly to avoid highly similar sentences in different parts of the manuscript? The referee also mentions Fig 1A which has been included in another form in a previous paper; I do not object to you keeping it in the manuscript, but I need you to confirm that the actual data represented derives from a different experiment than the one included in your previous paper. If this is not the case, then you need to clearly state in both text and figure legend that this is a reprint from a previous paper with permission from the authors.

For all figures depicting error bars the figure legends need to state how many independent experiments form the basis for the statistical calculations (at least 3 needed). Furthermore, in fig 3A part of the mutation description is missing (currently says G249D, G343) and for cytometric flow analysis depicted in fig 3B and 4F the TfR control is not defined.

On a different note 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 files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

Yours sincerely

Editor
The EMBO Journal.

REFEREE REPORTS

Referee #1

I am satisfied with the amendments the authors have made during revision, but they should clarify whether the KH double point mutant is G249D, G343D.

Referee #3

This resubmission by Cano and colleagues reports that MEX-3C functions as a novel RNA-binding ubiquitin ligase involved in MHC-I mRNA degradation. My major concerns regarding the original manuscript were: (1) most of the experiments relied on over-expression to draw conclusions and (2) the data appear over-interpreted without considering alternative explanations. The revised manuscript has been improved. There are a few remaining issues to be addressed.
Specific Points
I still take exception to re-presenting data that has already been published by the authors in highly similar (identical) form. This is not appropriate and is not justified as a service for the audience of the manuscript who might not be familiar with previous published work.
I raised the issue that there are verbatim sentences in the result and discussion sections that appeared to be a result of "copy/paste". At the time, this was considered an inadvertent mistake by the authors, similar to other errors and ambiguities noted. In spite of the authors' explanation, this still needs to be corrected. Repetition to emphasize a point is one thing and is acceptable, but it is convention to paraphrase or re-state points in a different way rather than directly copying large portions of text.
There are still issues with poorly defined jargon (e.g., KIRs in the abstract) which need to be corrected.
Experimental details: how were IP mass spec experiments performed and controlled? These are poorly described in the manuscript.

2nd Revision - authors' response 12 July 2012

<u>Point-by point response to the referees' comments</u>

Referee #1

I am satisfied with the amendments the authors have made during revision, but they should clarify whether the KH double point mutant is G249D, G343D.
We have clarified in the manuscript, both main text and figure legend, that the double KH point mutant is G249D, G343D.

Referee #3

This resubmission by Cano and colleagues reports that MEX-3C functions as a novel RNA-binding ubiquitin ligase involved in MHC-I mRNA degradation. My major concerns regarding the original manuscript were: (1) most of the experiments relied on over-expression to draw conclusions and (2) the data appear over-interpreted without considering alternative explanations. The revised manuscript has been improved. There are a few remaining issues to be addressed.

Specific Points
I still take exception to re-presenting data that has already been published by the authors in highly similar (identical) form. This is not appropriate and is not justified as a service for the audience of the manuscript who might not be familiar with previous published work.
The data derives from a completely different dataset from that published previously, and the editor has agreed to keep Figure 1A.
I raised the issue that there are verbatim sentences in the result and discussion sections that appeared to be a result of "copy/paste". At the time, this was considered an inadvertent mistake by the authors, similar to other errors and ambiguities noted. In spite of the authors' explanation, this still needs to be corrected. Repetition to emphasize a point is one thing and is acceptable, but it is convention to paraphrase or re-state points in a different way rather than directly copying large portions of text.

The text has been modified to avoid repetitions as noted.
In particular, we have removed the repetitive sentence "The HLA-A restricted specificity of MEX-3C was unanticipated, and the high 3'UTR sequence conservation..." from the results section (page 10) and left it in the discussion, so this no longer repeats itself, as requested.
We have also modified the sentence commencing with "Evolutionary diversification from *C. elegans*…" from the discussion section (page 12) to read "The subsequent evolutionary diversification from *C. elegans* is associated with the acquisition of a C-terminal RING finger (eg as

seen in Drosophila) with serial duplication events from a single dMex-3 (RNA-binding + RING finger domain) gene, to four genes in higher eukaryotes".
We have rephrased the first paragraph of the discussion (page 12). It now reads "Here we show that the RING domain of MEX-3C, and hence its ubiquitin ligase activity, is absolutely required for the degradation of *HLA-A2* mRNA, providing a link between ubiquitination activity and mRNA decay."

Experimental details: how were IP mass spec experiments performed and controlled? These are poorly described in the manuscript.
A new section has been added to the 'Materials and Methods' section with a detailed description of the immunoprecipitation protocol and control (mock IP) used.