EMBO reports

Chemical and genetic control of IFNγ-induced MHCII expression

Ruud H. Wijdeven, Marvin M. van Luijn, Annet F. Wierenga-Wolf, Jimmy J. Akkermans, Peter J. van den Elsen, Rogier Q. Hintzen, Jacques Neefjes

Review timeline:

Submission date:	27 November 2017
Editorial Decision:	29 November 2017
Revision received:	29 March 2018
Editorial Decision:	27 April 2018
Revision received:	5 June 2018
Accepted:	24 June 2018

Editor: Achim Breiling

Transaction Report: This manuscript was transferred to *EMBO reports* following review at *The EMBO Journal*

1st Editorial Decision 29 November 2017

Thank you for the transfer of your research manuscript to EMBO reports. I now went through the referee reports from The EMBO Journal.

Both referees acknowledge the potential interest of the findings. Nevertheless, they have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here. As EMBO reports emphasizes novel functional over detailed mechanistic findings, we will not require further mechanistic insight, or the direct target of Keap1 in the context of the manuscript. However, both referees indicate that the two parts of the manuscript (i.e. Keap1- and oxidative-stress-mediated modulation of MHC class II regulation) are not well connected, in particular as inhibition of the enzymatic activity of Keap1 apparently has no effect on histone acetylation levels. I think it would be important to strengthen this, and link both parts better, maybe by following the suggestion of referee #2 (major concerns - Figure 3).

Further, we require that all technical and minor concerns are addressed, and the details and the results of the RNAi screen are shown (see 1st major concern of referee #2). Also the first 3 points of referee #1 (mentioned before the major concerns) should be addressed.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that the all referee concerns must be addressed in the revised manuscript and in a point-by-point response (as outlined above). Acceptance of your manuscript will depend on a positive outcome of a second round of review, based on the re-evaluation by both referees. Both will be informed that the manuscript is now under consideration for EMBO reports.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Please refer to our specific guidelines for preparing your revised manuscript:
http://embor.embopress.org/authorguide#manuscriptpreparation

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends. Please provide statistical testing where applicable.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of about 400 pixels) that can be used as part of a visual synopsis on our website.

Please note that we now mandate that all corresponding authors list an ORCID digital identifier that is link to the EMBO reports account!

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The expression of MHC class II molecules on non-hematopoietic cells has been implicated in exacerbated graft-host reactions, autoimmunity, and tumor immunity. Here Wijdeven et al explore the basis for interferon-gamma-mediated upregulation of class II in non-hematopoietic cells. Much

of the work investigates involvement of the Keap1 E3 ubiquitin ligase in interferon-gamma-mediated class II upregulation. Keap 1 scored at the top of an E3 ligase siRNA screen, and regulation of class II expression would represent a novel role for Keap1, which has been intensively studied in other settings. Based upon studies using siRNA knockdown or specific inhibitors with readouts of surface class II or mRNA expression, the authors construct a model in which Keap1, in concert with p62, downregulates HDAC1 and HDAC2 (possibly through direct degradation?), which repress interferon-gamma-induced class II expression through deacetylation of histones H3 and H4 at class II loci. Because Keap1 has been reported to be a sensor of oxidative stress, remaining studies focus on this condition as the initial trigger upstream of Keap1. Indeed, treatment of HeLa cells with AS(III) suppresses interferon-gamma-stimulated class II expression via an HDAC-dependent mechanism. However, Keap1 knockdown had no impact on the AS(III) effect. Rather, previously reported direct interaction of AS(III) with the H4K16-specific histone acetyltransferase MYST1 may be the mechanism, since MYST1 knockdown, independent of a Keap1 pathway, reduces class II upregulation in response to interferon-gamma. Finally, DMF and tBHQ, which both target Keap1, inhibit interferon-gamma-mediated class II upregulation. However, this also appears to be independent of Keap1 since the effect is independent of histone acetylation levels.

The authors provide many compelling results in this paper. Clearly, regulation of MHC class II expression is complex and varying depending upon cell type. This is a message that should resonate with both broad and focused audiences. There are some limitations of the studies that should be acknowledged:
1) Only impact on class II expression, not T cell activation, is examined. Thus, it is unknown whether the observed changes (2-fold decrease in many cases) are meaningful. What is the half-life of surface class II in the cells that were studied? Is there relatively little nascent class II in the loading compartments?
2) Results lead the authors to conclude that Keap1 regulates histone acetylation at a global level. This suggests that class II regulation is part of a larger Keap1-mediated program. Thus impact of oxidative stress on immune function may have nothing to do with altered class II expression in response to interferon-gamma.
3) Figure 4d suggests that there are many Keap1 interacting proteins besides p62 and BPTF whose knockdown impacts interferon-gamma-induced class II expression. So, the picture could be much more complicated.

Major concerns:
1) This paper is essentially two stories: Keap1- and oxidative stress-mediated modulation of class II upregulation by interferon-gamma. Because Keap1 has been reported to be a sensor for oxidative stress, the authors probably expected to observe that it is an input for the Keap1 pathway. However, other than control of class II expression, there is very little to tie the two parts together. Considering that there are many other cellular pathways involving Keap1 (point 2 above) and that there appear to be many other ways of regulating class II expression (point 3 above), putting these two stories together seems almost arbitrary. Adding to the confusion is the prolonged treatment of oxidative stress in the discussion even though much of the story revolves around Keap1. The authors might consider dropping the second part and adding depth to the Keap1 story. Examples: elucidating the relevant input signals to Keap1 or exploring how Keap1 and p62 interact and control HDAC1/2 activity.
2) The clinical significance of class II expression by non-hematopoetic cells is strongly emphasized. However, this paper exclusively relies on analysis of laboratory cell lines. Ideally some experiments would be corroborated in primary cells of non-hematopoetic origin. At the very least, some discussion of the cell types that express Keap1 would be useful.

Minor concerns:

1) A key point is that the Keap1 effect is specific to non-hematopoietic cells. The contrast is only THP-1 cells and this appears to be "data not shown". The case would be strengthened by showing the data not just for THP-1 cells but several others.
2) Several western blots, particularly in figure 3, could be of higher quality
3) Figure 4g is not referred to in the text.
4) Figure 5c: Quantification of the H4ac bands would be useful.
5) The same for Figure 6g.

6) While mentioned in the introduction, the discussion would be strengthened by discussing the possible implications for cancer that the oxidative stress results have.
7) Several times in the text "hereby" is used but "thereby" appears to be the appropriate word.
8) Figure legends use capital letters for the figure panels. Elsewhere they are lower case.

Referee #2

Wijdeven et al report identifying the Cullin3-ligase adaptor Keap1, best known for its regulatory function in the oxidative stress response, as a positive regulator of IFNγ-induced MHC II expression using an siRNA screen. Depletion of Keap1 leads to ~50% reduction in IFNγ-induced MHC II expression in HeLa cells due to reduced HLA-DRα and Ii transcription, and these effects are reversed by HDAC1/2 inhibition or depletion. Strikingly, Keap1 depletion leads to a substantial reduction in steady state levels of acetylated H3 and H4. Similar effects on IFNγ-induced MHC II expression and acetylated histone levels are seen following depletion of p62. In figure 5, it is shown that the oxidative stressor arsenite inhibits IFNγ-induced HLA-DRα and Ii expression. The effects are reversed with HDAC inhibitors but are proposed to be Keap1 independent as arsenite does not substantially affect acetylated H4 levels. Figure 6 identifies a role for dimethyl fumarate (DMF) in reducing IFNγ-induced expression of MHC II and proinflammatory chemokines, however, these effects were independent of histone acetylation.

This manuscript presents a series of interesting novel observations and insights, although not all clearly linked, concerning the regulation of IFNγ-induced MHC II regulation in non-haematopoietic cells that have relevance for autoimmune disease, anti-tumour immunity and organ transplant. On the whole, the data presented support the conclusions made. However, very little mechanistic insight is provided as to how the cytoplasmic protein Keap1 and p62 regulate acetylated histone levels and IFNγ-induced MHC II expression, and furthermore how the global changes in histone acetylation relate to histone acetylation/deacetylation at MHC II gene regulatory regions. As presented, figures 5 & 6 don't link particularly well with figures 1-4 and it is not really clear why these compounds that are reported to inhibit Keap1 activity don't have similar effects on acetylated histone levels as Keap1 depletion?

Major Concerns
It is stated that Keap1 was identified in an siRNA screen targeting ubiquitination pathway proteins, however the details and results of the screen are not presented. In order to evaluate the significance of Keap1 as a regulator of MHC II expression it is important that the screen is shown e.g. how many hits were identified in addition to Keap1, how the was screen performed e.g. cell type, time-course, and how many genes were targeted in the siRNA library are all very relevant. Also, were any other hits identified that would support the premise that the ubiquitination activity of Keap1 is important for sustaining MHC II expression e.g. Cullin-3 , E2 conjugating enzymes, CAND1?

Figure 3 - Keap1 depletion leads to reduced levels of acetylated H3 and H4, which can be partially restored following inhibition of HDAC1/2 suggesting that Keap1 may be acting via HDAC1/2; however Keap1 depletion did not lead to increased levels of HDAC1/2 or enhanced total HDAC activity. These observations could be extended and more directly linked to the observed effects of Keap1 depletion on MHC II expression by exploring whether histone acetylation and recruitment of HDAC1/2 (+/- other components of HDAC1/2 repressive complexes) at HLA-DRα/Ii gene regulatory elements are modulated by IFNγ-stimulation in a Keap1-dependent manner (e.g. using ChIP-PCR). The global changes in histone acetylation following Keap1 depletion are fairly remarkable and raise the possibility that HDAC activity is deregulated and perhaps indiscriminately targeted to chromatin, therefore it is important to understand what drives the specificity for HLA-DRα and Ii regulation e.g. compared to IRF1 and CIITA.

Figure 4a/b - The complementation experiments suggest that both the substrate binding motif and ubiquitin transfer are essential for Keap1 to promote IFNγ-induced MHC II expression. Are substrate binding and ubiquitination activity of Keap1 similarly essential for restoration of levels of acetylated histones? This would provide important confirmation that histone acetylation/deacetylation is regulated (albeit indirectly) by the ubiquitination activity of Keap1-Cullin3 (also see comments for figure 5) and help to support the model that this is the mechanism by which Keap1 modulates IFNγ-induced MHC II expression. Although identifying the direct target of

Keap1 activity is clearly key to understanding its function in this context, this may be difficult if not forthcoming from screening Keap1-interacting proteins and known substrates and I don't feel is necessarily essential if other mechanistic insight can be provided as outlined above .

Figure 5 - Arsenite has been shown to inhibit Keap1-Cullin3 ubiquitin ligase activity, at least in the context of NRF2 ubiquitination, so it is not clear what the proposed explanation for the lack of change in histone acetylation is here - that Keap1 ubiquitination activity is not being successfully inhibited by arsenite, or that histone deacetylation is differentially affected by inhibition of Keap1-Cullin3 ubiquitination activity compared to siRNA-mediated Keap1 depletion? Is there other evidence as to whether arsenite treatment blocks Keap1 ubiquitination activity in these experiments e.g stabilisation of NRF2 or other known Keap1 substrates or inhibition of auto-ubiquitination?

Other Comments

Figure 1a/1b - The manuscript centres around regulation of IFNγ-induced MHC class II regulation, however, does Keap1 regulates MHC II expression (with or without IFNγ treatment) in non-haematopoietic cancer cell lines that express MHC II constitutively (e.g. melanoma lines aberrantly expressing MHC II)? In Figure 3a/3b Keap1 depletion leads to global reduction in acetylated H3 and H4 in several cancer cell lines, regardless of the presence or absence of IFNγ-stimulation, suggesting a potential role for Keap1 in regulating constitutive as well as IFN-induced gene expression.

Figure 4f - Do HDAC inhibitors restore IFNγ-induced MHC II expression in p62 depleted cells?

1st Revision - authors' response 29 March 2018

Referee #1:

The expression of MHC class II molecules on non-hematopoietic cells has been implicated in exacerbated graft-host reactions, autoimmunity, and tumor immunity. Here Wijdeven et al explore the basis for interferon-gamma-mediated upregulation of class II in non-hematopoietic cells. Much of the work investigates involvement of the Keap1 E3 ubiquitin ligase in interferon-gamma-mediated class II upregulation. Keap 1 scored at the top of an E3 ligase siRNA screen, and regulation of class II expression would represent a novel role for Keap1, which has been intensively studied in other settings. Based upon studies using siRNA knockdown or specific inhibitors with readouts of surface class II or mRNA expression, the authors construct a model in which Keap1, in concert with p62, downregulates HDAC1 and HDAC2 (possibly through direct degradation?), which repress interferon-gamma-induced class II expression through deacetylation of histones H3 and H4 at class II loci. Because Keap1 has been reported to be a sensor of oxidative stress, remaining studies focus on this condition as the initial trigger upstream of Keap1. Indeed, treatment of HeLa cells with AS(III) suppresses interferon-gamma-stimulated class II expression via an HDAC-dependent mechanism. However, Keap1 knockdown had no impact on the AS(III) effect. Rather, previously reported direct interaction of AS(III) with the H4K16-specific histone acetyltransferase MYST1 may be the mechanism, since MYST1 knockdown, independent of a Keap1 pathway, reduces class II upregulation in response to interferon-gamma. Finally, DMF and tBHQ, which both target Keap1, inhibit interferon-gamma-mediated class II upregulation. However, this also appears to be independent of Keap1 since the effect is independent of histone acetylation levels.

The authors provide many compelling results in this paper. Clearly, regulation of MHC class II expression is complex and varying depending upon cell type. This is a message that should resonate with both broad and focused audiences. There are some limitations of the studies that should be acknowledged:
1) Only impact on class II expression, not T cell activation, is examined. Thus, it is unknown whether the observed changes (2-fold decrease in many cases) are meaningful. What is the half-life of surface class II in the cells that were studied? Is there relatively little nascent class II in the loading compartments?

We thank the reviewer for his/her suggestions and evaluation of our manuscript. Both tested cell lines contain significant amounts of MHCII in the loading compartment as determined by staining for total MHCII by confocal microscopy (included as figure EV1c). The half-life of surface MHCII is relatively long, since after 6 hours 90% of the surface MHCII was still present in our antibody internalization experiment, suggesting a half-life of about 24 hours. Regarding the T-cell assays: given the 50% overall reduction in MHCII levels we think that for some epitopes it might be relevant (low expressed ones), while for others the remaining 50% might still suffice to induce a response.

2) Results lead the authors to conclude that Keap1 regulates histone acetylation at a global level. This suggests that class II regulation is part of a larger Keap1-mediated program. Thus impact of oxidative stress on immune function may have nothing to do with altered class II expression in response to interferon-gamma.

We agree that oxidative stress has many other functions , which could mediate its impact on the immune system, and have included this in the discussion (line 277-285). Regarding Keap1 and histone acetylation, that part is removed from the manuscript, see point 4a/b of reviewer 2.

3) Figure 4d suggests that there are many Keap1 interacting proteins besides p62 and BPTF whose knockdown impacts interferon-gamma-induced class II expression. So, the picture could be much more complicated.

We agree that many more proteins are involved in the regulation of MHCII expression. Therefore we further analysed the top 10 hits from the screen for effects on mRNA levels and thus confirmed three candidates that control MHCII mRNA expression, BPTF, p62 and Cullin3. All three are included in the manuscript now and also more elaborately mentioned in the discussion (lines 286-305). However, for all three hits inhibition of HDAC activity failed to restore MHCII expression, suggesting additional pathways of MHCII regulation by these genes, and illustrating the point of the reviewer that MHCII regulation is very complex.

Major concerns:

1) This paper is essentially two stories: Keap1- and oxidative stress-mediated modulation of class II upregulation by interferon-gamma. Because Keap1 has been reported to be a sensor for oxidative stress, the authors probably expected to observe that it is an input for the Keap1 pathway. However, other than control of class II expression, there is very little to tie the two parts together. Considering that there are many other cellular pathways involving Keap1 (point 2 above) and that there appear to be many other ways of regulating class II expression (point 3 above), putting these two stories together seems almost arbitrary. Adding to the confusion is the prolonged treatment of oxidative stress in the discussion even though much of the story revolves around Keap1. The authors might consider dropping the second part and adding depth to the Keap1 story. Examples: elucidating the relevant input signals to Keap1 or exploring how Keap1 and p62 interact and control HDAC1/2 activity.

We thank the reviewer for suggestion simplification of the process by removing part of the data. However, that would at the same time ignore the complexity of this pathway. We have performed several assays to try to find a link between both pathways (Keap1 and HDACs) but were unsuccessful to demonstrate HDAC targeting to the MHCII locus, as well as physical interactions or co-localization between p62/Keap1 and HDACs. Given the new information about the effect of Keap1 on histone acetylation (see comment 4a/b of reviewer 2) we think that Keap1 regulates MHCII expression independenly of histone acetylation. In the effect of arsenite on MHCII is at least in part mediated via targeting of Keap1, linking both parts of the story better.

2) The clinical significance of class II expression by non-hematopoetic cells is strongly emphasized. However, this paper exclusively relies on analysis of laboratory cell lines. Ideally some experiments would be corroborated in primary cells of non-hematopoetic origin. At the very least, some discussion of the cell types that express Keap1 would be useful.

We again thank the reviewer for this helpful suggestion. The identified genes are all widely expressed according to the human protein atlas database (now included in discussion lines 301-303. However, we could not get access to primary cell material for testing and feel this is beyond the scope of our article.

Minor concerns:

1) A key point is that the Keap1 effect is specific to non-hematopoietic cells. The contrast is only THP-1 cells and this appears to be "data not shown". The case would be strengthened by showing the data not just for THP-1 cells but several others.

We have included the data of the THP-1 cells in the manuscript (EV1b) and did similar knockdowns in another hematopoietic cell line, U937. No effect was observed on THP-1 cells and only a small effect on U937, which have only marginal constitutive MHCII expression. We therefore removed the claim that it is specific for MHCII expression in non-hematopoietic cells, but we do show that it does not have an effect on constitutive MHCII expression.

2) Several western blots, particularly in figure 3, could be of higher quality

Given the new results (see figure 4a/4b point of reviewer 2) we have decided to remove the Western blots from figure 3 from the paper, as well as the ones from figure 5 and 6 (now figure 4 and 5).

3) Figure 4g is not referred to in the text.

Thanks for pointing this out and this is corrected.

4) Figure 5c: Quantification of the H4ac bands would be useful.

See minor concern 2

5) The same for Figure 6g.

See minor concern 2

6) While mentioned in the introduction, the discussion would be strengthened by discussing the possible implications for cancer that the oxidative stress results have.

Thanks and we have done this in the discussion (line 277-285)

7) Several times in the text "hereby" is used but "thereby" appears to be the appropriate word.

We have corrected this.

8) Figure legends use capital letters for the figure panels. Elsewhere they are lower case.

Is also corrected, thanks.

Referee #2:

Wijdeven et al report identifying the Cullin3-ligase adaptor Keap1, best known for its regulatory function in the oxidative stress response, as a positive regulator of IFNγ-induced MHC II expression using an siRNA screen. Depletion of Keap1 leads to ~50% reduction in IFNγ-induced MHC II expression in HeLa cells due to reduced HLA-DRα and Ii transcription, and these effects are reversed by HDAC1/2 inhibition or depletion. Strikingly, Keap1 depletion leads to a substantial reduction in steady state levels of acetylated H3 and H4. Similar effects on IFNγ-induced MHC II expression and acetylated histone levels are seen following depletion of p62. In figure 5, it is shown that the oxidative stressor arsenite inhibits IFNγ-induced HLA-DRα and Ii expression. The effects are reversed with HDAC inhibitors but are proposed to be Keap1 independent as arsenite does not substantially affect acetylated H4 levels. Figure 6 identifies a role for dimethyl fumarate (DMF) in reducing IFNγ-induced expression of MHC II and proinflammatory chemokines, however, these effects were independent of histone acetylation.

This manuscript presents a series of interesting novel observations and insights, although not all clearly linked, concerning the regulation of IFNγ-induced MHC II regulation in non-haematopoietic cells that have relevance for autoimmune disease, anti-tumour immunity and organ transplant. On the whole, the data presented support the conclusions made. However, very little mechanistic insight is provided as to how the cytoplasmic protein Keap1 and p62 regulate acetylated histone levels and IFNγ-induced MHC II expression, and furthermore how the global changes in histone acetylation relate to histone acetylation/deacetylation at MHC II gene regulatory regions. As presented, figures 5 & 6 don't link particularly well with figures 1-4 and it is not really clear why these compounds that are reported to inhibit Keap1 activity don't have similar effects on acetylated histone levels as Keap1 depletion?

Many thanks for this point. While doing other experiments (see comment Figure 4a/b) we realized that the effect of Keap1 on histone acetylation is separate from its action on MHCI. Using this information we could link arsenite to Keap1 in control of MHCII expression, providing a link between the chemical and genetic part of the story.

Major Concerns

It is stated that Keap1 was identified in an siRNA screen targeting ubiquitination pathway proteins, however the details and results of the screen are not presented. In order to evaluate the significance of Keap1 as a regulator of MHC II expression it is important that the screen is shown e.g. how many hits were identified in addition to Keap1, how the was screen performed e.g. cell type, time-course, and how many genes were targeted in the siRNA library are all very relevant. Also, were any other hits identified that would support the premise that the ubiquitination activity of Keap1 is important

for sustaining MHC II expression e.g. Cullin-3 , E2 conjugating enzymes, CAND1?
We thank the reviewer for his/her thorough and helpful review that helped improving the manuscript. The screen that we performed was actually one using siRNAs targeting de-ubiquitinating enzymes and E2-ligase for their effects of MHCII expression. Keap1 was identified after a secondary screening step for interactors of one of the DUBs (OTUD1), from which it came out as the strongest overall hit. The results of the DUB RNAi screen (that describe a completely different DUB) are included in a different paper, which is currently under review at Nature Communications. To clarify the situation we now describe this more transparently in the first paragraph of the results. From our Keap1-interactor screen Cullin-3 also appeared as a regulator of MHCII expression (it was actually the third best hit), which we have now included in the new manuscript (Figure 3c-g).

Figure 3 - Keap1 depletion leads to reduced levels of acetylated H3 and H4, which can be partially restored following inhibition of HDAC1/2 suggesting that Keap1 may be acting via HDAC1/2; however Keap1 depletion did not lead to increased levels of HDAC1/2 or enhanced total HDAC activity. These observations could be extended and more directly linked to the observed effects of Keap1 depletion on MHC II expression by exploring whether histone acetylation and recruitment of HDAC1/2 (+/- other components of HDAC1/2 repressive complexes) at HLA-DRα/Ii gene regulatory elements are modulated by IFNγ-stimulation in a Keap1-dependent manner (e.g. using ChIP-PCR). The global changes in histone acetylation following Keap1 depletion are fairly remarkable and raise the possibility that HDAC activity is deregulated and perhaps indiscriminately targeted to chromatin, therefore it is important to understand what drives the specificity for HLA-DRα and Ii regulation e.g. compared to IRF1 and CIITA.
We agree that these excellent suggestions would have clarified part of the mechanisms of MHCII regulation and set out to perform the ChIP-qPCR experiments. However, while performing additional experiments we observed that histone acetylation was differently regulated by Keap1 from MHCII expression (see next point) and moved on to other types of experiments instead.
Figure 4a/b - The complementation experiments suggest that both the substrate binding motif and ubiquitin transfer are essential for Keap1 to promote IFNγ-induced MHC II expression. Are substrate binding and ubiquitination activity of Keap1 similarly essential for restoration of levels of acetylated histones? This would provide important confirmation that histone acetylation/deacetylation is regulated (albeit indirectly) by the ubiquitination activity of Keap1-Cullin3 (also see comments for figure 5) and help to support the model that this is the mechanism by which Keap1 modulates IFNγ-induced MHC II expression. Although identifying the direct target of Keap1 activity is clearly key to understanding its function in this context, this may be difficult if not forthcoming from screening Keap1-interacting proteins and known substrates and I don't feel is necessarily essential if other mechanistic insight can be provided as outlined above .
We performed the rescue experiment for histone acetylation for the different stable cell lines but to our surprise not even wild-type Keap1 expression restored histone acetylation (see figure a below, Stable cell line #1). This was further confirmed in a newly generated stable cell line (figure a below, stable cell line #2), even though MHCII surface expression was again restored. To investigate potential off-target effects we tested 4 different siRNA sequences targeting Keap1 that all reduced histone acetylation levels (figure b). One option is that the GFP-tag on Keap1 affected the ability to restore histone acetylation. However, since GFP-tagged Keap1 did restore MHCII expression, this means that histone acetylation and MHCII expression regulation are independent processes. To reduce the complexity of MHCII transcriptional control, we removed the data related to the original figure 3 from our manuscript.

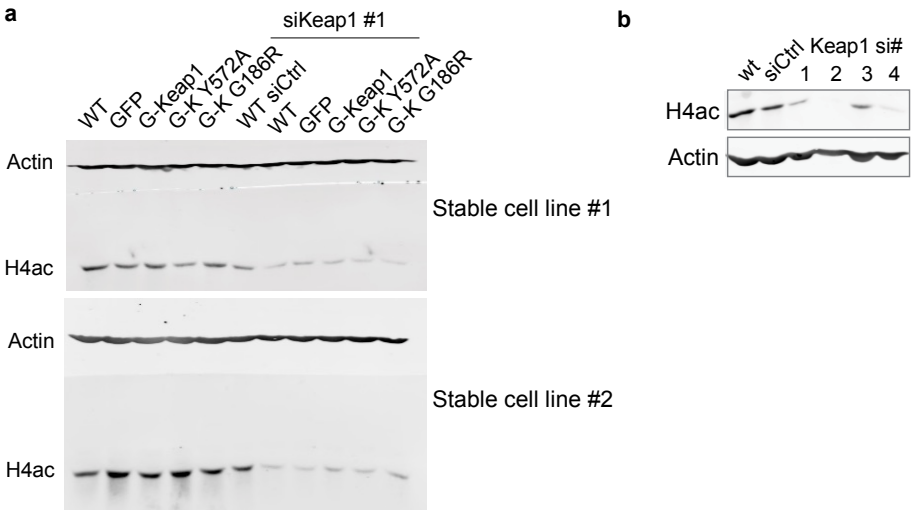


Figure 5 - Arsenite has been shown to inhibit Keap1-Cullin3 ubiquitin ligase activity, at least in the context of NRF2 ubiquitination, so it is not clear what the proposed explanation for the lack of change in histone acetylation is here - that Keap1 ubiquitination activity is not being successfully inhibited by arsenite, or that histone deacetylation is differentially affected by inhibition of Keap1-Cullin3 ubiquitination activity compared to siRNA-mediated Keap1 depletion? Is there other evidence as to whether arsenite treatment blocks Keap1 ubiquitination activity in these experiments e.g stabilisation of NRF2 or other known Keap1 substrates or inhibition of auto-ubiquitination?
Following the arguments above, we believe that the effect of Keap1 on histone acetylation is independent of Cullin-3 and arsenite. Arsenite does control MHCII expression at least in part via Keap1, which we now better described in the new manuscript. Furthermore, arsenite induces the increased expression of nrf2-target NQO1 (Fig 4b), further illustrating that in our system arsenite indeed targets Keap1.

Other Comments
Figure 1a/1b - The manuscript centres around regulation of IFNγ-induced MHC class II regulation, however, does Keap1 regulates MHC II expression (with or without IFNγ treatment) in non-haematopoietic cancer cell lines that express MHC II constitutively (e.g. melanoma lines aberrantly expressing MHC II)? In Figure 3a/3b Keap1 depletion leads to global reduction in acetylated H3 and H4 in several cancer cell lines, regardless of the presence or absence of IFNγ-stimulation, suggesting a potential role for Keap1 in regulating constitutive as well as IFN-induced gene expression.
We have analysed three melanoma cell lines that express MHCII constitutively but in none of these cells knockdown of Keap1 decreased MHCII expression (Fig EV1a). We had also performed a genome-wide screen for factors controlling MHCII expression and did not identify Keap1 (Paul et al., Cell 2011). Given the earlier results that uncouple the effect of Keap1 on histone acetylation and MHCII expression we now think that the effect is probably via an alternative signalling pathway.

Figure 4f - Do HDAC inhibitors restore IFNγ-induced MHC II expression in p62 depleted cells?
We performed this experiment for depletion of p62, Cullin-3 and BPTF but in none of the cases restoration of IFNy-induced MHCII expression was observed (Fig 4g). Depletion of p62 in combination with HDAC-inhibitors was somewhat toxic to the cells (50% loss of viability) but the lack of restoration is obvious and unrelated to this toxicity. Therefore we changed our model and included a more data on BPTF and Cullin-3, since it appears that they are a likely to work together with Keap1 as p62. We adjusted our discussion and model (Fig. 6) accordingly.

2nd Editorial Decision 27 April 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study (you will find

enclosed below).

As you will see, referee #2 now supports the publication of your manuscript in EMBO reports. However, referee #1 has still concerns and suggestions, we ask you to address in a final revised version of your manuscript. Regarding the points by referee #1, please address these in a point-by-point response, and revise the results section and discussion accordingly (first paragraph of the report of referee #1 and his/her point 1). Regarding point 2 of referee #1, we would ask you to add further data addressing the role of Keap1 in arsenite-induced MHC-II downregulation, if you already have these, or could obtain such data in a timely manner (there is still space for additional EV figures).

Further, I have the following editorial requests:

- Please restrict the number of keywords on the title page to five.

- J. Akkermans is missing from the author contributions. Please add him, and indicate his contribution.

- Please provide the source data for Fig. 1 and Fig. EV1 in separate files.

- Please provide also the source data for the Western blots in Fig. 3 (and of additional blots that might be added during the revision).

- In the legends of Figures 1E, 5F and EV1C it is stated that the data was obtained in 2 independent experiments. Thus, showing error bars and statistical testing does not make sense. Please modify the figure just showing the data for the two experiments.

- It seems Fig. 1B is not called out in the text. Please fix that.

- There is a reference to Fig. 6b-6e in the author contributions, but Fig. 6 is a graphic, with no panels. Please correct this.

Finally, please provide (as separate text file):
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

REFEREE REPORTS

Referee #1:

In this revised manuscript Wijdeven et al have undertaken additional experiments in response to the comments raised. They clearly identify Cullin-3, as well as Keap1, BPTF and p62 as positive regulators of IFNγ-induced MHC II expression via promotion of HLA-DRα and Ii, but not CIITA, transcription. HDAC1/2 inhibition/depletion reverses the reduction in MHC II expression in Keap1 depleted cells but not in Cullin-3, BPTF or p62 depleted cells. The authors further investigated the potential role of Keap1 in regulation of histone acetylation and, given that they were unable to clearly link this to regulation of MHC II expression, have amended the manuscript accordingly. As such, the revisions have resolved many of the discrepancies in the data present in the original manuscript. The strength of this study lies in the clear identification of several novel regulators of IFN-induced MHC II expression, which is important and of general interest. However, the link between the Keap1 story (figures 1-3) and figures 4&5 is still fairly tenuous and has not been substantially strengthened since the previous submission. Although the data in the latter two figures is not uninteresting and the authors' justify retaining these to demonstrate the complexity of MHC II regulation, figure 4 in particular does not add much to the story in its current form. In addition, the discussion in the text regarding some of the new results showing effects of HDAC inhibition in

different contexts is vague and occasionally contradictory making it difficult to grasp the central message, or in some sections to understand the authors' interpretation of their findings.

Major Concerns
1. In Figure 2 it is shown that HDAC inhibitors substantially augment IFNγ-induced MHC II expression, as has previously described, and overcome the reduction in IFNγ-induced MHC II expression observed following Keap1 depletion. Whilst it is shown that promotion of MHC II expression by Keap1 requires Cullin-3 binding and presumably ubiquitin conjugation activity (figure 3a/b), curiously, inhibition of MHC II expression in Cullin-3 depleted cells is not reversed by HDAC1/2 inhibition. The description of these contrasting results for Cullin-3 and Keap1 in the text is confusing. Whilst in lines 173-175 the authors suggest that these factors are working together, they then appear to contradict this statement in the subsequent sentence (lines 175-177). Furthermore, whilst the title of figure 2 and relevant results section (lines 125-148) suggest that Keap1 regulates MHC II via HDAC1/2, in the discussion lines 295-301 it is suggested that the lack of effect of Keap1 siRNA in the presence of HDAC inhibitors may be because HDAC inhibitors downregulate Keap1 expression anyway (despite substantially enhancing IFN-γ induced MHC II expression). If this is true, it would appear to negate most of the data presented in figure 2 and what then is the evidence that Keap1 regulates MHC II via HDAC1/2? What are Keap1 levels in HDAC inhibitor treated/HDAC1&2 depleted cells and are these levels further affected by Keap1 siRNA treatment? Although the authors present evidence that regulation of MHC II transcription via Keap1 is independent of Keap1's possible effects on global levels of histone acetylation, I am not entirely clear whether they are also suggesting that Keap1 does not regulate MHC II by modulating levels of histone acetylation locally at HLA-DRα and Ii gene regulatory regions? If so, this again questions the significance of the reversal of the effect with HDAC1/2 inhibition? e.g. 'We have performed several assays to try to find a link between both pathways (Keap1 and HDACs) but were unsuccessful to demonstrate HDAC targeting to the MHCII locus, as well as physical interactions or co-localization between p62/Keap1 and HDACs. Given the new information about the effect of Keap1 on histone acetylation (see comment 4a/b of reviewer 2) we think that Keap1 regulates MHCII expression independenly of histone acetylation.' Alternatively are the authors suggesting that Keap1 depletion may promote deacetylation of a non-histone target via HDAC1/2? If so this is not explicitly stated.

2. In figure 4 the authors demonstrate that arsenite treatment inhibits IFN-γ induced MHC II expression and the description of this section concludes that this 'probably' occurs via Keap1 and the histone acetytransferase MYST1 (lines 195-196), both of which are known to be inhibited by arsenite. If figure 4 is to be retained in the manuscript, this would be better linked to figure 1-3 by investigating the potential role of Keap1 in arsenite induced MHC-II downregulation e.g. Is the effect of arsenite on MHC-II also NRF2 independent (as observed for Keap1 depletion)? What is the effect of arsenite on IFNγ-induced MHC II levels in Keap1 and/or MYST1 depleted cells? Can forced Keap1 expression increase IFN-γ induced MHC II expression in arsenite treated cells? Is p62 involved?

Referee #2:

The authors have not substantially changed the manuscript from what was submitted to EMBO, opting for a broad rather than deep picture of interferon-gamma-mediated MHC class II expression in non-hematopoietic cells. The picture is, as the authors concede, complicated with many gaps to be filled. Nevertheless, this outline will likely be of general interest and provides a solid base upon which to build.

2nd Revision - authors' response 5 June 2018

Referee #1:

In this revised manuscript Wijdeven et al have undertaken additional experiments in response to the comments raised. They clearly identify Cullin-3, as well as Keap1, BPTF and p62 as positive regulators of IFNγ-induced MHC II expression via promotion of HLA-DRα and Ii, but not CIITA,

transcription. HDAC1/2 inhibition/depletion reverses the reduction in MHC II expression in Keap1 depleted cells but not in Cullin-3, BPTF or p62 depleted cells. The authors further investigated the potential role of Keap1 in regulation of histone acetylation and, given that they were unable to clearly link this to regulation of MHC II expression, have amended the manuscript accordingly. As such, the revisions have resolved many of the discrepancies in the data present in the original manuscript. The strength of this study lies in the clear identification of several novel regulators of IFN-induced MHC II expression, which is important and of general interest. However, the link between the Keap1 story (figures 1-3) and figures 4&5 is still fairly tenuous and has not been substantially strengthened since the previous submission. Although the data in the latter two figures is not uninteresting and the authors' justify retaining these to demonstrate the complexity of MHC II regulation, figure 4 in particular does not add much to the story in its current form. In addition, the discussion in the text regarding some of the new results showing effects of HDAC inhibition in different contexts is vague and occasionally contradictory making it difficult to grasp the central message, or in some sections to understand the authors' interpretation of their findings.

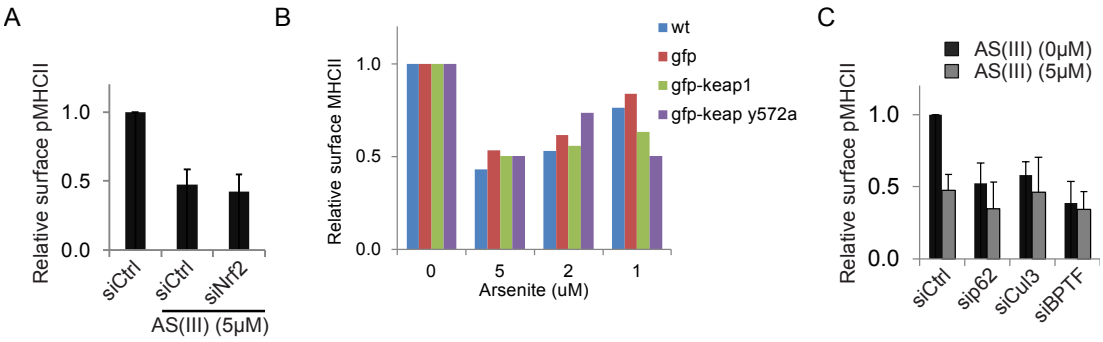
Major Concerns
1. In Figure 2 it is shown that HDAC inhibitors substantially augment IFNγ-induced MHC II expression, as has previously described, and overcome the reduction in IFNγ-induced MHC II expression observed following Keap1 depletion. Whilst it is shown that promotion of MHC II expression by Keap1 requires Cullin-3 binding and presumably ubiquitin conjugation activity (figure 3a/b), curiously, inhibition of MHC II expression in Cullin-3 depleted cells is not reversed by HDAC1/2 inhibition. The description of these contrasting results for Cullin-3 and Keap1 in the text is confusing. Whilst in lines 173-175 the authors suggest that these factors are working together, they then appear to contradict this statement in the subsequent sentence (lines 175-177). Furthermore, whilst the title of figure 2 and relevant results section (lines 125-148) suggest that Keap1 regulates MHC II via HDAC1/2, in the discussion lines 295-301 it is suggested that the lack of effect of Keap1 siRNA in the presence of HDAC inhibitors may be because HDAC inhibitors downregulate Keap1 expression anyway (despite substantially enhancing IFN-γ induced MHC II expression). If this is true, it would appear to negate most of the data presented in figure 2 and what then is the evidence that Keap1 regulates MHC II via HDAC1/2? What are Keap1 levels in HDAC inhibitor treated/HDAC1&2 depleted cells and are these levels further affected by Keap1 siRNA treatment?
Although the authors present evidence that regulation of MHC II transcription via Keap1 is independent of Keap1's possible effects on global levels of histone acetylation, I am not entirely clear whether they are also suggesting that Keap1 does not regulate MHC II by modulating levels of histone acetylation locally at HLA-DRα and Ii gene regulatory regions? If so, this again questions the significance of the reversal of the effect with HDAC1/2 inhibition? e.g. 'We have performed several assays to try to find a link between both pathways (Keap1 and HDACs) but were unsuccessful to demonstrate HDAC targeting to the MHCII locus, as well as physical interactions or co-localization between p62/Keap1 and HDACs. Given the new information about the effect of Keap1 on histone acetylation (see comment 4a/b of reviewer 2) we think that Keap1 regulates MHCII expression independenly of histone acetylation.' Alternatively are the authors suggesting that Keap1 depletion may promote deacetylation of a non-histone target via HDAC1/2? If so this is not explicitly stated.

We obviously have to concur with the reviewer that the message was not very clear and –in parts- contradictory. Regarding our initial hypothesis that HDACs regulate the expression of Keap1; we performed additional experiments and can exclude that this is the reason why HDAC inhibition restores MHCII expression after Keap1 depletion. Firstly, HDAC inhibitors only decreased Keap1 mRNA levels by 50% and siRNA mediated depletion still reduced Keap1 mRNA levels further, to a level comparable to Keap1 depletion in non-HDAC inhibitor treated cells (6 versus 8%). Secondly, GFP-Keap1 expressing cells (where GFP-Keap1 expression is not affected by HDAC-inhibitors as assessed by flow cytometry) do not respond better to HDAC-inhibitors than wild-type or GFP expressing cells, arguing that regulation of Keap1 mRNA expression by HDAC-inhibitors is not a relevant mechanism in our situation. This suggests then that Keap1 mediated control of acetylation is the correct order, as also depicted in the overview Figure.
Based on the reviewers comments and these additional data, we modified the text accordingly. The conclusions in the text for figure 3 (lines 174-176) have incorporated our new insights and state that the function of Keap1 and its interaction partners do not fully overlap. Furthermore, we improved the discussion and included a description of how Keap1 could possibly regulate HDAC activity.

Given that Cullin-3 depletion has a stronger effect on MHCII mRNA expression than Keap1 depletion, and that Cul3 is known to partner with multiple substrate adaptors, it is possible that Cul3 works in assembly with Keap1 in an acetylation dependent pathway, as well as in another, HDAC independent pathway to control MHCII expression. This could explain the discrepancy between Keap1 and Cul3 depletion in the context of HDAC inhibition.

2. In figure 4 the authors demonstrate that arsenite treatment inhibits IFN-γ induced MHC II expression and the description of this section concludes that this 'probably' occurs via Keap1 and the histone acetytransferase MYST1 (lines 195-196), both of which are known to be inhibited by arsenite. If figure 4 is to be retained in the manuscript, this would be better linked to figure 1-3 by investigating the potential role of Keap1 in arsenite induced MHC-II downregulation e.g. Is the effect of arsenite on MHC-II also NRF2 independent (as observed for Keap1 depletion)? What is the effect of arsenite on IFNγ-induced MHC II levels in Keap1 and/or MYST1 depleted cells? Can forced Keap1 expression increase IFN-γ induced MHC II expression in arsenite treated cells? Is p62 involved?

Thanks for these suggestions. We have performed the experiments proposed and included some of them as Figure 4D. Other data are shown below for the editors consideration. In cells already depleted for Keap1 or MYST1 the additional effect of Arsenite is negligible, arguing that they are the functional target or at least part of the same pathway (Figure 4D). Co-depletion of both molecules gave a similar result, but the individual effects of depletion are fairly minimal here, likely because of the dilution of the siRNAs. For these reasons, this experiment was not included in the new manuscript. The effect of arsenite is also independent of NRF2, because NRF2 depletion did not restore MHCII expression (see below, A), in line with the data for Keap1. Overexpression of GFP-Keap1 did not make cells more or less sensitive to AS(III), suggesting that at certain antioxidant levels also GFP-Keap1 was effectively inhibited (see below, B). Arsenite in the context of p62 depletion still had an additional effect (see below, C), albeit to a considerably lesser extent than in wild-type cells. These data do not add to an obvious conclusion and were also not included in the manuscript. For Cul3 and BPTF depletion the additional effect of arsenite was minimal at best, suggesting that they do operate in the same pathway. These data suggest that -while the function of p62 is still unclear- arsenite, Keap1 and MYST1 act in the same pathway of IFNg-induced MHCII control in non-immune cells.



Referee #2:

The authors have not substantially changed the manuscript from what was submitted to EMBO, opting for a broad rather than deep picture of interferon-gamma-mediated MHC class II expression in non-hematopoietic cells. The picture is, as the authors concede, complicated with many gaps to be filled. Nevertheless, this outline will likely be of general interest and provides a solid base upon which to build.

3rd Editorial Decision 224 June 2018

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. As you will see below, also referee #1 now supports the publication of your manuscript.

Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

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.

REFEREE REPORTS

Referee #1

The authors have responded to all my queries and the revisions to the manuscript have improved the clarity of the discussion. The authors have also presented additional supportive evidence that the effects of arsenite on IFNg-induced MHC II expression are partly mediated via inhibition of Keap1 activity but independently of NRF2 stabilisation. I do not have any additional concerns.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jacques Neefjes
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-45553-T

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	In principle experiments were performed three times, only when the variation was too high to mask an effect they were repeated. However, some experiments were performed much more frequently because they were part of other experiments as well
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	All results were included in the study
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Homogeneous populations of cells were used, and with Flow Cytometry a normal distribution of signal was observed, both for the wild-type and treated samples.
Is there an estimate of variation within each group of data?	The estimate is that the variation is based mostly on the person performing the experiment, as all other factors (reagents etc.) are kept constant. Therefore the variation within groups of data are estimated to be similar
Is the variance similar between the groups that are being statistically compared?	Yes, based on flow cytometry data it is.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers of all antibodies are provided in the materials and methods. All antibodies were chosen because they were cited by other people and yielded bands at the expected height.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cells were regularly checked for mycoplasm contamination. HeLa and MelJuSo cells were verified for identity by deep sequencing, and all cells were stained for MHCII, as well as other surface markers, to confirm their identity. FM3 and FM78 were a kind gift from Daniel Peeper (NKI, the Netherlands) and THP-1 and U937 from Linda Smit (VU, the Netherlands)

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA