nature portfolio

Peer Review File

PTPN23-dependent ESCRT machinery functions as a cell death checkpoint

Corresponding Author: Dr Nicholas Tonks

This manuscript has been previously reviewed at another journal. This document only contains reviewer comments, rebuttal and decision letters for versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this revised version, the authors have addressed several of my concerns, and the paper has improved. However, I still have a few comments, which need to be addressed.

In the rebuttal letter (comment 2), authors kindly explain the difference between the values obtained in a pulse-chase experiment with cycloheximide (without the ligand) with values obtained in an experiment without cycloheximide (also without ligand). I agree with the authors. However, what is the turnover rate of TNRF1 in PTPN23-depleted cells (in the presence of cycloheximide)?

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In comment 8 (Fig 7e), what I found (and still find) surprising is that the overall distribution of NAP1 is very different from that of RAB5Q79L and TNFR1, whether some molecules colocalize or not. In fact, TNFR1 seems to accumulate all over the cytoplasm and not only in the enlarged RAB5Q79L-endosomes. Finally, I believe that it is somewhat overstated to describe the function of NAP1 (linked to PTPN23 and TNFR1) as an adaptor in the absence of further evidence, and to conclude that therefore it shows a punctate distribution.

Minor
The model is unclear and ambiguous (Fig 8h). PTPN23 (associated with NAP1 on endosomes) seems to interact in trans with ESCRTs (on MVB), as if PTPN23 and ESCRTs were present on separate membranes. I do not think that this model is what the authors had in mind.

Reviewer #3

(Remarks to the Author)
The authors have provided thorough and thoughtful revisions based on reviewer comments. While they have not been able to conclusively address all comments, they now clearly present their findings and limitations. This work is an important advance worthy of publication.

Reviewer #4

(Remarks to the Author)
Review 28th May 2024

- You should be trying to help the work get published, not necessarily in this journal but ultimately.
- Don't criticize an experiment unless you can tell the authors how they could do it better:
My mentor would say, “If you just want to throw darts, go to the pub”.
- Keep in mind that no one ever built a statue to a critic.
- Try to act as a peer in the process of peer review.
Science Signaling 2009 Michael Yaffe

Title: PTPN23-dependent ESCRT machinery functions as a cell death checkpoint in restraining apoptosis, necroptosis and pyroptosis

Manuscript # NCOMMS-23-28868A

General Remarks

This study finds that loss of PTPN23 in an AML cell line results in activation of NF-κB and cell death pathways. Fig. 1 shows that loss of PTPN23 is deleterious to cells and the experiment with a dTAG version of PTPN23 on a knock out background to acutely delete PTPN23 are convincing. Fig. 2 shows nicely that loss of PTPN23 activates NF-κB and cell death pathways, cleaved caspase-3, cleaved caspase-8 pMLKL and cleaved GSDMD. Also ASC speckles and cells don't die on a Casp8-/-Ripk3-/- caspase-1 or GSDMD knock out. Fig. 3 shows that several DRs are upregulated in PTPN23 deficient cells. Fig. 4 shows that PTPN23 co-localises with some components of the ESCRT machinery. Fig. 5 shows that getting rid of any of several ESCRT components results in an increase in DR levels and a reduction in cell viability. The exception being STAMBP which didn't affect DR levels nor did it affect viability. Fig. 6 shows that PTPN23 interacts with NAP1 a TBK interacting protein. Fig. 7 shows that increased levels of NAP1 result in increased accumulation of TNFR1 and increased sensitivity to TNF induced death. Fig. 8 is data that was provided during the revision process and shows that reduced levels of PTPN23 in BMDMs results in increased levels of cell death in BMDMs. Overall this looks to be a well controlled study, with many well performed experiments and with findings that should be interesting to the field. I do have concerns (like another reviewer) about the "specificity" of the effect: there are a number of papers showing that several receptors are affected by loss of PTPN23, e.g. EGFR Singh et al doi: 10.1038/s41419-023-06201-4, Neurotropin receptors, Budzinska et al doi: 10.1242/jcs.242412. and I find it disappointing that papers that can be found with a simple ESCRT/ PTPN23 PubMed search are not discussed. Likewise I find it hard to understand how an enhanced cell death phenotype can be squared with an association of PTPN23 mutation with Microcephalic Complex Hereditary Spastic Paraplegia, doi: 10.3390/brainsci11050614 and doi: 10.1038/s41431-019-0487-1 and that this published data is not discussed or contextualised.

Specific Remarks

As I've been asked to step in at the first round of revision I've done my best to focus on key concerns.

Fig. 1d where is PRR in top full length PTPN23 schematic?
Fig. 1l Why were dead cells per area calculated? Very unusual for non-adherent cells, why not a normal percentage using a more accurate approach like flow cytometry?
Fig. 3c why aren't wild type values calculated?
Fig. 3e if the values are normalised to sgNEG how can sgNEG have values less (or more) than 1 (e.g. CD45).
Fig. 4 the title is
“V domain of PTPN23 is indispensable for the endocytic trafficking of death receptors mediated by the ESCRT machinery” but none of the data shows DR trafficking.
Fig. 5 Unless I missed it and the data are in the extended data figures they don't show that the gRNAs deplete their target.
The apoptotic data in Fig. 8e look convincing, however the necroptotic and pyroptotic data in Fig. 8f and g are far less so. The phospho-MLKL levels in Fig. 8f are the same in the knock out as in the control, while in 8g it seems disingenious (and far less convincing) to not show cleaved GSDME (or GSDMD) or caspase-1 as the authors did in Fig. 2. I can't understand because there are certainly good antibodies for mouse proteins.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
1) To illustrate the role PTPN23, the authors were asked to compare the turnover rate of TNRF1 with and without PTPN23 (in the presence of cycloheximide). However, I do not find the new data (Ex Data Fig 3C) very conclusive. The sgNEG and sgPTPN23 profiles are quite similar and the difference is mainly weighted by the 120min time-point. The bulk of TNRF1 degradation appears to occur rapidly, in a PTPN23-independent manner. This does not support the notion that PTPN23 plays an important role in sorting TNRF1 to degradation.

2) The second comment was that the overall distribution of NAP1 is different from that of RAB5Q79L and TNFR1. Do the authors mean that many (most?) TNFR1mCherry-positive structures are early endosomes containing endogenous RAB5 but not the Q79L mutant? Is there evidence for the presence of “wt” endosomes (containing Rab5) in cells expressing the Q79L mutant? If so, wouldn’t it be difficult to explain the mutant’s phenotype in endosome dynamics and transport? In my opinion,

it is not appropriate to compare the volume percentage of TNFR1 over RAB5-Q79L-eGFP in the presence and absence of NAP1, without introducing a bias into the analysis. Moreover, the overall distribution of NAP1 is different from that of RAB5Q79L and TNFR1, which is difficult to reconcile with the notion that NAP1 interacts with PTPN23 to promote TNFR1 sorting into RAB5-containing endosomes. I am afraid that these points are not consistent with the view that NAP1 interacts with PTPN23 in sorting TNRF1 to degradation, and I believe that authors should tone down their conclusions.

Reviewer #4

(Remarks to the Author)
The authors have addressed most of my concerns. However I personally think that the existence of a human syndrome with a mutation in PTPN23 is highly relevant and should be acknowledged/cited. The explanation given in the rebuttal seems reasonable for why it may not be relevant in myeloid cells and would be a pertinent point in the discussion, in my opinion. Nevertheless I leave this point to the editors discretion.

Reviewer's Comments:

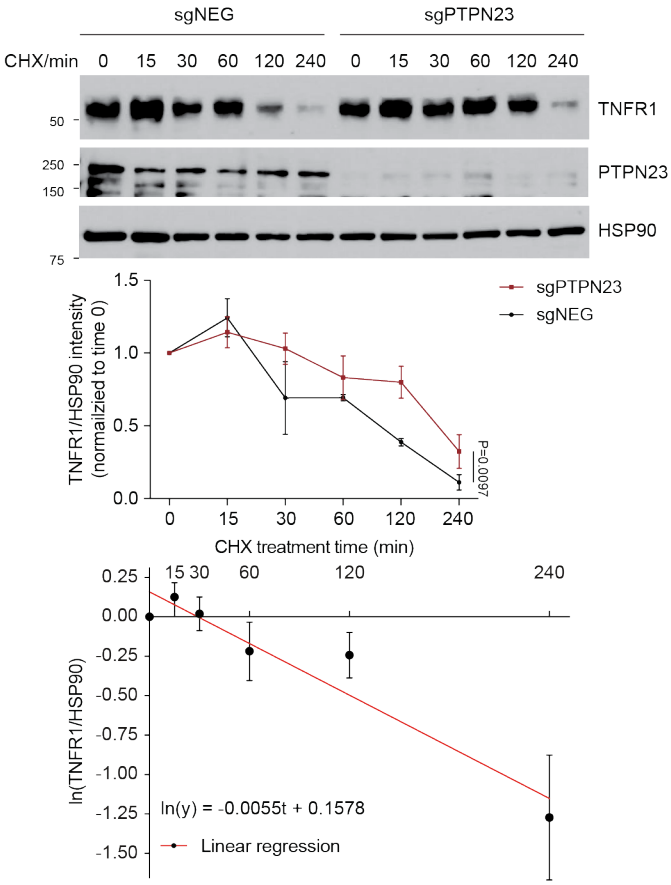
Reviewer #1 (Remarks to the Author)

In this revised version, the authors have addressed several of my concerns, and the paper has improved. However, I still have a few comments, which need to be addressed.

In the rebuttal letter (comment 2), authors kindly explain the difference between the values obtained in a pulse-chase experiment with cycloheximide (without the ligand) with values obtained in an experiment without cycloheximide (also without ligand). I agree with the authors. However, what is the turnover rate of TNRF1 in PTPN23-depleted cells (in the presence of cycloheximide)?

As requested, we determined the turnover rate of TNRF1 in PTPN23-depleted cells in the presence of cycloheximide; the $t_{1/2}$ was observed to be 154.7 ± 25.4 minutes. To determine this, we used PTPN23-dTAG NOMO-1 cells transduced with either sgNEG or sgPTPN23 to deplete endogenous PTPN23. Following treatment with 200 nM dTAG-13 for 24 hours to degrade the exogenously expressed PTPN23-dTAG recombinant protein, the cells were treated with CHX for the indicated time (Top panel, Revised Extended Data Fig. 3c). These results illustrate that the degradation of TNFR1 in the absence of PTPN23 is significantly slower than that measured in cells expressing PTPN23 (67 ± 5.7 minutes; Middle panel, Revised Extended Data Fig. 3c).

Revised Extended Data Fig. 3c



Revised Extended Data Fig. 3c. Top, representative immunoblotting analysis of endogenous TNFR1 following CHX treatment in PTPN23-dTAG NOMO-1 cells. Prior to CHX treatment, the cells were treated with 200 nM dTAG-13 for 24 hours. Middle, quantification of TNFR1/HSP90 intensity, based on three independent experiments (n = 3). Statistical

analysis was performed using two-way ANOVA, followed by Tukey's multiple comparisons test. Bottom, plot showing natural logarithm-transformed TNFR1/HSP90 ratio against CHX treatment time, used for calculating half-life ($t_{1/2}$). The equation for $t_{1/2}$ calculation: $t_{1/2} = (\ln(0.5) - 0.1578)/-0.0055$. The calculated half-life of TNFR1 in the absence of PTPN23 is 154.7 ± 25.4 minutes.

In general, authors could have been more careful in formulating the rebuttal comments. For example, in comment 4, I did not state that the level of TNFR1-mCherry in the control panel was lower. I wrote that the microscopy experiments in Fig 3c were not convincing, in part because I did not see TNFR1-mCherry in the control panel. The panel was black, and there was nothing to see. The revised Fig 3c is more convincing.

We are gratified that the referee finds the revised Fig 3c to be more convincing.

In comment 8 (Fig 7e), what I found (and still find) surprising is that the overall distribution of NAP1 is very different from that of RAB5Q79L and TNFR1, whether some molecules colocalize or not. In fact, TNFR1 seems to accumulate all over the cytoplasm and not only in the enlarged RAB5Q79L-endosomes. Finally, I believe that it is somewhat overstated to describe the function of NAP1 (linked to PTPN23 and TNFR1) as an adaptor in the absence of further evidence, and to conclude that therefore it shows a punctate distribution.

In the Fig. 7e, we show that TNFR1 distributed in both RAB5-Q79L-eGFP-positive, enlarged endosomes and RAB5-Q79L-eGFP-negative, cytoplasmic regions. In this experiment, we express RAB5Q79L-eGFP on top of the native RAB5 protein. Consequently, it is possible that not all the endosomes incorporate RAB5Q79L-eGFP and endosomes in which WT RAB5 may predominate would be eGFP-negative. With this in mind, we only compare the volume percentage of TNFR1 over RAB5-Q79L-eGFP in the presence and absence of NAP1 (Fig. 7e, right panel).

As we acknowledged in the manuscript, the localization of NAP1 does not coincide completely with that of RAB5Q79L and TNFR1. In addition, NAP1-knockout did not rescue cell death caused by PTPN23 depletion. We attributed this to the potential for contribution of other adaptors. Furthermore, I think it is important to note that we have provided data from a variety of functional assays, beyond localization studies, to demonstrate a role of NAP1 in linking PTPN23 and TNFR1. Firstly, overexpression of NAP1 sensitizes cells to TNF-α induced cell death (Fig. 7b). Secondly, knockout of NAP1 abolishes the effects of PTPN23 depletion on NF-κB activation and RIPK3 phosphorylation (extended data Figure 7a). Additionally, we showed that knockout of NAP1 significantly delays GFP depletion at early time points following PTPN23 depletion (see figure below). These findings collectively underscore the functional importance of NAP1 in the TNFR1-PTPN23 pathway.

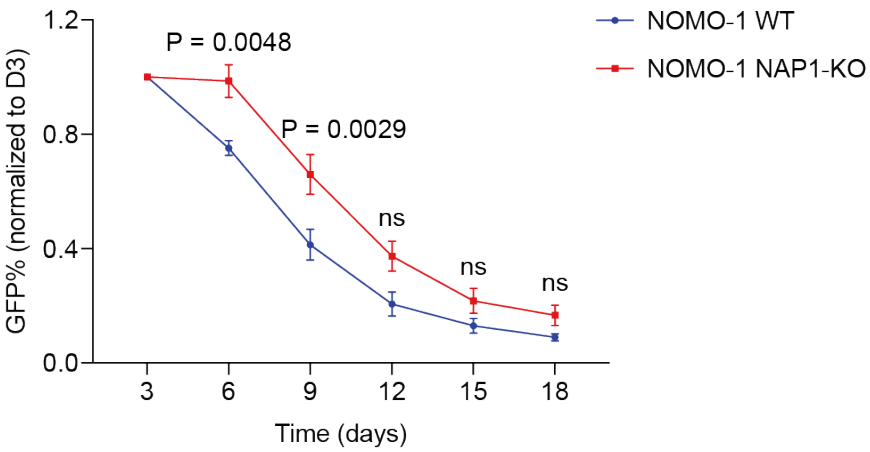


Figure: GFP competition growth assay performed in WT and NAP1 knockout NOMO-1 cells. NAP1 knockout cells were generated using two independent sgRNAs, and PTPN23 depletion was conducted using N23-sg1 and N23-sg2. Data are presented as mean ± SEM, statistical analysis by Two-way ANOVA, Sidak's multiple comparisons test, n = 3 independent experiments.

Minor

The model is unclear and ambiguous (Fig 8h). PTPN23 (associated with NAP1 on endosomes) seems to interact in trans with ESCRTs (on MVB), as if PTPN23 and ESCRTs were present on separate membranes. I do not think that this model is what the authors had in mind.

We revised Fig. 8h in an attempt to represent our findings more accurately and clearly summarize the interactions and mechanisms described in our study.

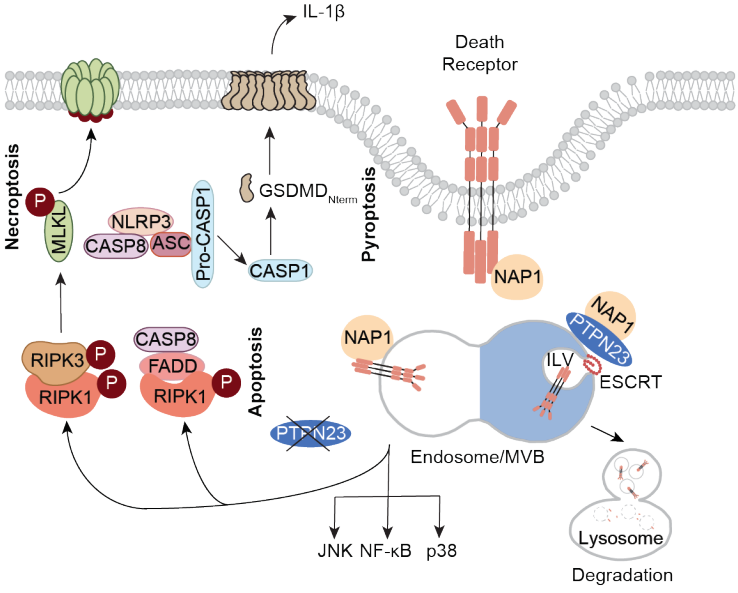


Figure: The proposed model for PTPN23 in regulating death receptor sorting and degradation. PTPN23 collaborates with NAP1 to facilitate receptor sorting. On the right-hand side, representing the normal condition, this process involves the engagement of ESCRT, leading to the formation of multivesicular bodies and subsequent lysosomal degradation of death receptors. On the left-hand side, loss of PTPN23 results in the prolonged accumulation of death receptors within the endosomal compartment, triggering the activation of multiple cell death pathways.

Reviewer #3 (Remarks to the Author)

The authors have provided thorough and thoughtful revisions based on reviewer comments. While they have not been able to conclusively address all comments, they now clearly present their findings and limitations. This work is an important advance worthy of publication.

We are delighted that the reviewer sees our work as “an important advance worthy of publication”.

Reviewer #4 (Remarks to the Author)

Review 28th May 2024

- You should be trying to help the work get published, not necessarily in this journal but ultimately.
- Don't criticize an experiment unless you can tell the authors how they could do it better: My mentor would say, “If you just want to throw darts, go to the pub”.
- Keep in mind that no one ever built a statue to a critic.
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Science Signaling 2009 Michael Yaffe

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This study finds that loss of PTPN23 in an AML cell line results in activation of NF-κB and cell death pathways. Fig. 1 shows that loss of PTPN23 is deleterious to cells and the experiment with a dTAG version of PTPN23 on a knock out background to acutely delete PTPN23 are convincing. Fig. 2 shows nicely that loss of PTPN23 activates NF-κB and cell death pathways, cleaved caspase-3, cleaved caspase-8 pMLKL and cleaved GSDMD. Also ASC speckles and cells don't die on a Casp8-/-Ripk3-/- caspase-1 or GSDMD knock out. Fig. 3 shows that several DRs are upregulated in PTPN23 deficient cells. Fig. 4 shows that PTPN23 co-localises with some components of the ESCRT machinery. Fig. 5 shows that getting rid of any of several ESCRT components results in an increase in DR levels and a reduction in cell viability. The exception being STAMBP which didn't affect DR levels nor did it affect viability. Fig. 6 shows that PTPN23 interacts with NAP1 a TBK interacting protein. Fig. 7 shows that increased levels of NAP1 result in increased accumulation of TNFR1 and increased sensitivity to TNF induced death. Fig. 8 is data that was provided during the revision process and shows that reduced levels of PTPN23 in BMDMs results in increased levels of cell death in BMDMs. Overall this looks to be a well controlled study, with many well performed experiments and with findings that should be interesting to the field. I do have concerns (like another reviewer) about the "specificity" of the effect: there are a number of papers showing that several receptors are affected by loss of PTPN23, e.g. EGFR Singh et al doi: 10.1038/s41419-023-06201-4, Neurotropin receptors, Budzinska et al doi: 10.1242/jcs.242412. and I find it disappointing that papers that can be found with a simple ESCRT/ PTPN23 PubMed search are not discussed.

We acknowledge that prior studies have shown that PTPN23 regulates several receptors, including EGFR, Neurotrophin receptors, and PDGFR. Unfortunately, space constraints prevent us from citing/discussing every paper that is retrieved from an ESCRT/ PTPN23 PubMed search. Indeed, our manuscript cites several relevant studies including Manteghi, S. et al. Cell Reports (2016); Kharitidi, D. et al. Cell Reports (2015); Ma, H. et al. Cellular Signalling (2015); Doyotte, A. et al. PNAS (2008); Ali, N. et al. Current Biology (2013); Gahloth, D. et al. Structure (2016); Gahloth, D. et al. Structure (2017). Nevertheless, it is important to note also that these receptors are either absent or expressed at very low levels in myeloid cells, specifically in NOMO-1 cells. This is supported by our RNA-seq data. The table below illustrates this point with the normalized expression levels of EGFR, NGFR, PDGFR, compared with TNFR1, FAS, DR4, DR5, and TLR4, from four of our NOMO-1 RNA-seq samples. This limits our ability to study such receptors in this context. We have summarized the known functions of PTPN23 in the Introduction of the revised manuscript.

	I	II	III	IV
EGFR	0	1.02	0.98	0
NGFR (neurotrophin receptor)	5.2	4.1	8.8	3.8
PDGFRA	5.2	3.1	4.9	4.8
PDGFRB	11.4	2.0	5.9	10.5
TNFRSF1A (TNFR1)	639.7	632.3	692.0	656.0
FAS	70	126	86	65
TNFRSF10A (DR4)	167	168	135	156
TNFRSF10B (DR5)	800.9	846.9	989.4	880.7
TLR3	0	0	1	0
TLR4	402.5	408.6	418.1	488.4

Table: Normalized expression levels of each protein calculated using DESeq2 from four NOMO-1 RNA-seq samples. These levels represent the counts of RNA reads for each gene, adjusted to account for differences in sequencing depth and library size across samples.

Likewise I find it hard to understand how an enhanced cell death phenotype can be squared with an association of PTPN23 mutation with Microcephalic Complex Hereditary Spastic Paraplegia, doi: 10.3390/brainsci11050614 and doi: 10.1038/s41431-019-0487-1 and that this published data is not discussed or contextualised.

It is well established that many proteins exhibit distinct roles depending on their cellular context. Our study specifically examines PTPN23 function in myeloid cells, whereas the pathogenesis of neurodegenerative diseases associated with PTPN23 mutations likely involves different mechanisms, which are beyond the scope of this work.

The reviewer raises a point regarding the association of mutations in *PTPN23* with microcephalic complex hereditary spastic paraplegia; however, the premise that these mutations necessarily lead to an enhanced cell death phenotype is not supported by our findings. In fact, we have demonstrated that the V domain of PTPN23 is crucial for cell survival due to its interaction with UBAP1. Specifically, the F678D mutation disrupts UBAP1 binding and impairs the ability of

PTPN23 to maintain cell survival (Extended Data Fig. 4c). Nevertheless, this was not the case with two PTPN23 V domain mutations associated with epileptic encephalopathy (N634K and P532L, which were reported by Smigiel et al. [Eur J Hum Genet. 2018] and Sowada et al. [Hum Genet. 2017]). Our experiments (see Figure below) revealed that these mutations still support cell survival and growth. These findings suggest that although these mutations do not compromise cell survival, they may have more subtle effects that extend beyond this function.

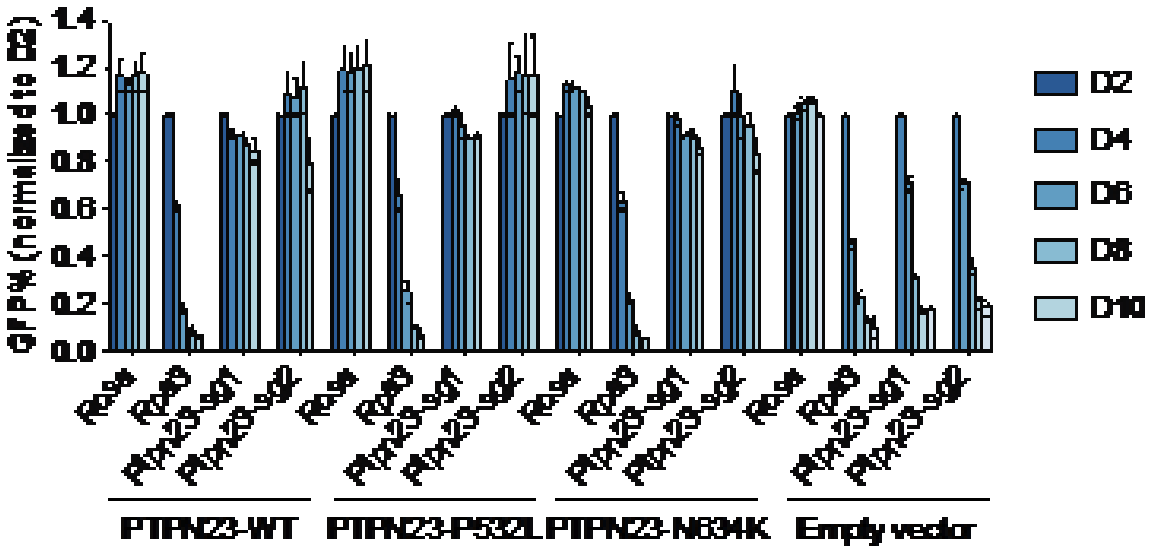


Figure: GFP competition growth assay in D2-5 cells. Stable cell lines were established in mouse acute myeloid leukemia D2-5 cells overexpressing PTPN23-WT, PTPN23-P532L, PTPN23-N634K, or an empty vector control. The GFP competition growth assay was performed using the indicated sgRNAs.

Specific Remarks
As I've been asked to step in at the first round of revision I've done my best to focus on key concerns.

Fig. 1d where is PRR in top full length PTPN23 schematic?
The PRR (proline-rich region) is part of the HIS domain. In the full-length PTPN23 schematic, we only labeled the HIS domain, but we have clarified this in the revised figure legend.

Fig. 1l Why were dead cells per area calculated? Very unusual for non-adherent cells, why not a normal percentage using a more accurate approach like flow cytometry?
Although NOMO-1 cells are non-adherent, they settle at the bottom of the ibidi µ-chamber, which allows for effective imaging. In addition, we performed several other assays to confirm cell death, including flow cytometry-based GFP competitive growth assay, CellTiter-Glo (CTG) assay, immunoblotting for cell death markers, and ELISA for IL-1β secretion.
The primary purpose of the SytoxGreen imaging experiment was to demonstrate the lytic form of cell death. To ensure a representative analysis, we quantified 60 fields per condition.
In the revised manuscript, we now present cell death quantification using CTG assay (Revised Fig. 8f, h), providing a more representative measure of the overall cell population.

Fig. 3c why aren't wild type values calculated?

In wild type cells, TNFR1 does not form punctate structures making it impossible to calculate the colocalization of a dispersed protein.

Fig. 3e if the values are normalised to sgNEG how can sgNEG have values less (or more) than 1 (e.g. CD45).
In the revised Fig. 3e, the values were normalized to sgNEG for the TLR4 group, which resulted in variations for sgNEG in the CD45, TNFR1, FAS, DR4, and DR5 groups. We updated the graph to normalize the values to sgNEG within each individual group. The updated figure is provided below.

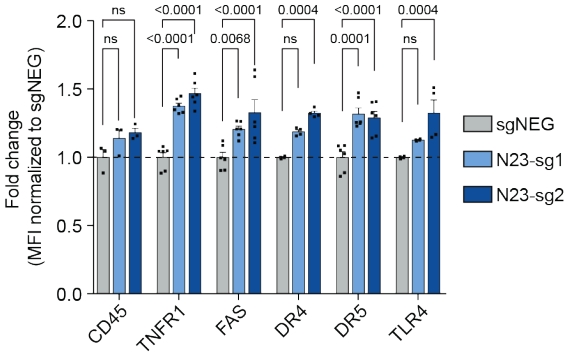
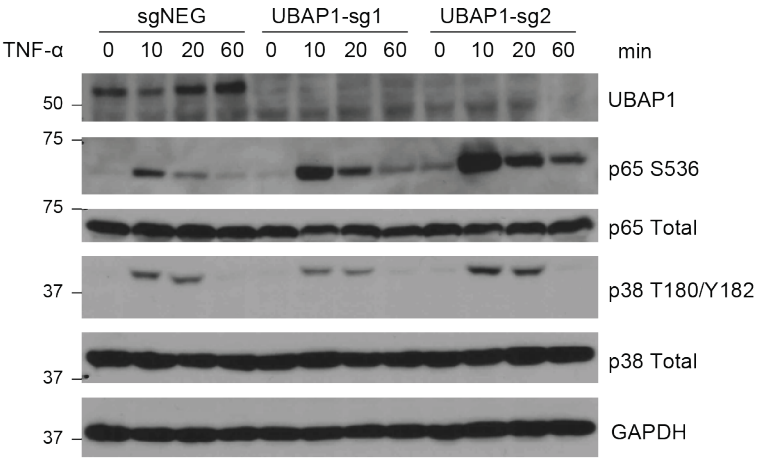


Figure: Fold changes of death receptors and TLRs in PTPN23-dTAG NOMO-1 cells after 3 days of 200 nM dTAG-13 treatment measured by flow cytometry.

Fig. 4 the title is “V domain of PTPN23 is indispensable for the endocytic trafficking of death receptors mediated by the ESCRT machinery” but none of the data shows DR trafficking.
We have revised the figure title to reflect the data presented more accurately. The new title is: “V domain of PTPN23 is indispensable for sustaining cell survival via interaction with UBAP1.”

Fig. 5 Unless I missed it and the data are in the extended data figures they don't show that the gRNAs deplete their target.
The knockout efficiency of UBAP1 sgRNAs can be found in Extended Data Fig. 4d.

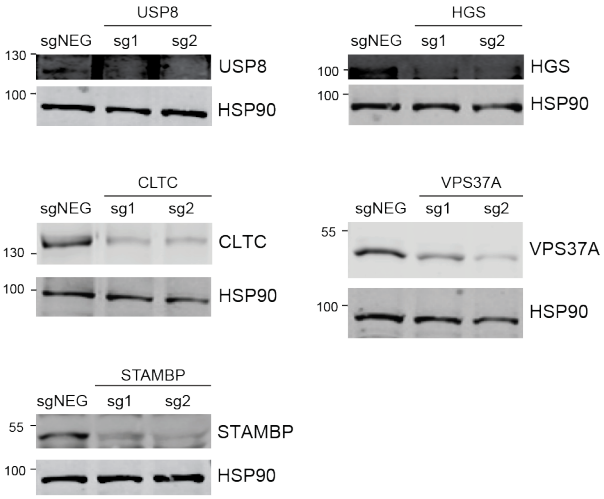
Extended Data Fig. 4d



Extended Data Fig. 4d, Immunoblotting analysis of p65 and p38 phosphorylation in response to UBAP1 depletion. NOMO-1 cells were stimulated with 20 ng/ml TNF-α for the indicated time at day 6 post sgRNA infection.

In the revised manuscript, we included immunoblots to demonstrate the depletion efficiency of gRNAs targeting USP8, HGS, CLTC, VPS37A, and STAMBP (Revised Extended Data Fig. 5b).

Revised Extended Data Fig. 5b



Revised Extended Data Fig. 5b, Immunoblotting analysis of NOMO-1 cells infected with sgNEG or sgRNAs targeting the indicated genes. Cells were harvested 5 days post-infection.

In addition, we noted from our RNA-seq analysis that the expression of TLR3 expression is also minimal in NOMO-1 cells. In light of this low expression, we suspected that the TLR3 antibody staining observed was nonspecific. To test this, we generated TLR3 knockout (KO) cell lines using five distinct sgRNAs. As shown in the figure below, no intensity shift of antibody staining was detected in TLR3-KO cells compared to NOMO-1 WT cells, suggesting that the antibody used previously was not specific. Consequently, we have removed all TLR3-related data from the manuscript.

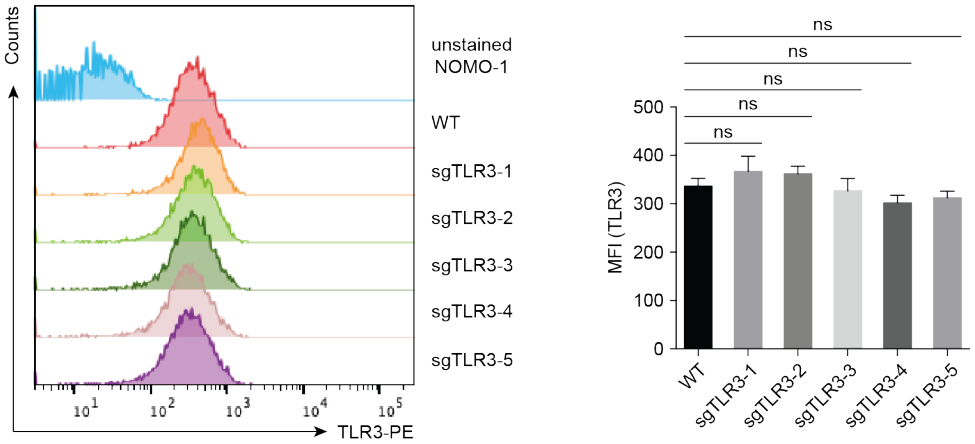


Figure: The flow cytometry analysis of NOMO-1 WT cells and TLR3-KO cells stained with anti-TLR3 antibody.

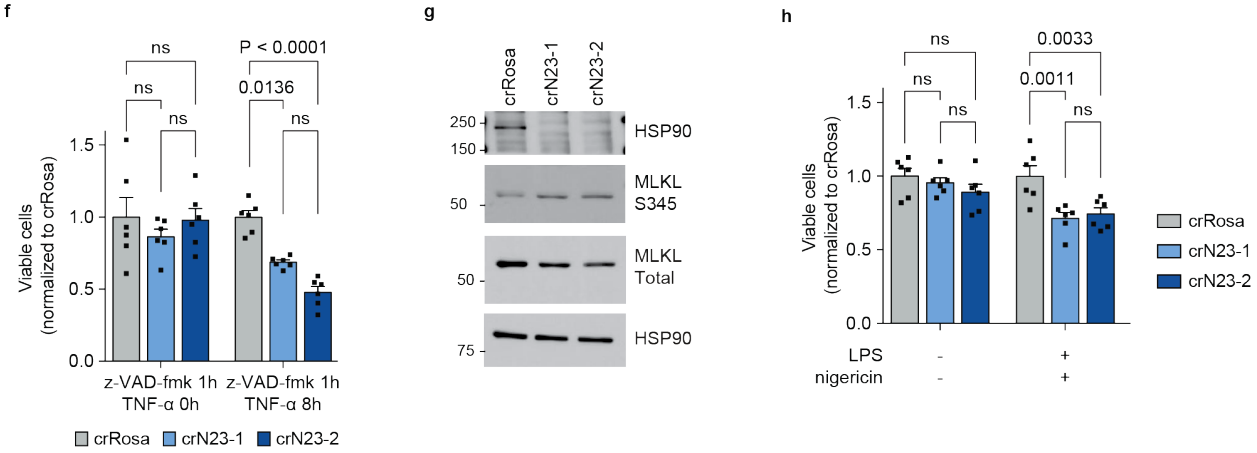
The apoptotic data in Fig. 8e look convincing, however the necroptotic and pyroptotic data in Fig. 8f and g are far less so. The phospho-MLKL levels in Fig. 8f are the same in the knock out as in the control, while in 8g it seems disingenious (and far less convincing) to not show cleaved GSDME (or GSDMD) or caspase-1 as the authors did in Fig. 2. I can't understand because there are certainly good antibodies for mouse proteins.

We think that is inappropriate for the reviewer to suggest that we have been disingenuous in our approach to this manuscript; any suggestion that we have tried deliberately to mislead the reader is without merit.

In follow up studies, we have taken steps to optimize the conditions for inducing necroptosis and pyroptosis. Initially, we used CHX plus z-VAD-fmk, based on the rationale that TNFR1 accumulates in PTPN23-deficient cells and activates NF-κB (Fig. 8a). CHX treatment suppresses NF-κB, and z-VAD-fmk inhibits caspases to trigger necroptosis. In the revised manuscript, we optimized necroptosis induction using z-VAD-fmk and TNF-α, resulting in a more robust necroptotic phenotype, as indicated by increased MLKL phosphorylation (Revised Fig. 8g). Furthermore, we used the CTG assay to demonstrate that Ptpn23-deficient cells are more sensitive to necroptotic stimuli (Revised Fig. 8f) and pyroptotic stimuli (Revised Fig. 8h).

Unfortunately, due to time and personnel constraints, we were unable to optimize conditions for detecting all pyroptosis-related markers, such as cleaved GSDME, GSDMD, and caspase-1. Nonetheless, we believe the data presented effectively supports our conclusions regarding the heightened sensitivity of PTPN23-deficient cells to necroptosis and pyroptosis stimuli.

Revised Fig. 8



Revised Fig. 8f, CTG luminescent cell viability assay in WT and Ptpn23-knockout BMDMs pretreated with 40 μM z-VAD-fmk for 1 hour, followed by treatment with 1 μg/ml TNF-α for the indicated time, 6 days post-nucleofection. **g,** Representative immunoblotting analysis of MLKL in BMDMs 7 days post-nucleofection with crRosa or crN23. Cells were treated with 40 μM z-VAD-fmk for 1 hour, followed by 1 μg/ml TNF-α for 4 hours (n = 2 independent experiments, each performed with cells from three mice). **h,** CTG luminescent cell viability assay in WT and Ptpn23-knockout BMDMs primed with 100 ng/ml LPS for 4 hours, followed by 2.5 μM nigericin for 4 hours, 6 days post-nucleofection. Data for **f** and **h** are mean ± SEM from six replicates derived from three mice, analyzed using two-way ANOVA followed by Tukey's multiple comparisons test.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

1) To illustrate the role PTPN23, the authors were asked to compare the turnover rate of TNRF1 with and without PTPN23 (in the presence of cycloheximide). However, I do not find the new data (Ex Data Fig 3C) very conclusive. The sgNEG and sgPTPN23 profiles are quite similar and the difference is mainly weighted by the 120min time-point. The bulk of TNRF1 degradation appears to occur rapidly, in a PTPN23-independent manner. This does not support the notion that PTPN23 plays an important role in sorting TNRF1 to degradation.

We conducted three independent experiments to assess TNFR1 degradation in the presence and absence of PTPN23, as illustrated in the figure below.

Our results consistently demonstrate that the TNFR1 degradation rate is notably prolonged in the PTPN23 knockout group across all experiments. This suggests that PTPN23 does indeed play a role in regulating TNFR1 degradation, supporting our hypothesis regarding its involvement in the sorting of TNFR1 for degradation.

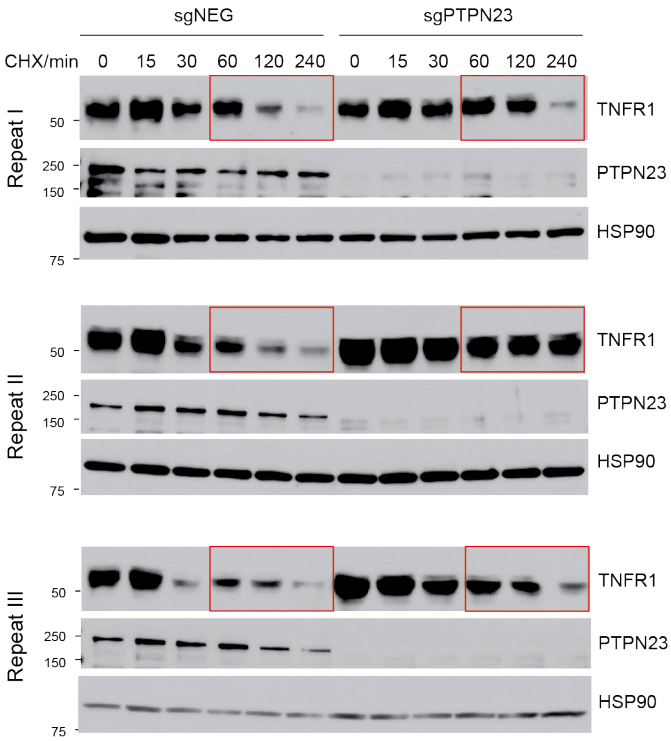


Figure. Immunoblotting analysis of endogenous TNFR1 in PTPN23-dTAG NOMO-1 cells following cycloheximide (CHX) treatment. Cells were pre-treated with 200 nM dTAG-13 for 24 hours before CHX treatment. Three independent replicates are shown.

2) The second comment was that the overall distribution of NAP1 is different from that of RAB5Q79L and TNFR1. Do the authors mean that many (most?) TNFR1mCherry-positive structures are early endosomes containing endogenous RAB5 but not the Q79L mutant? Is there evidence for the presence of “wt” endosomes (containing Rab5) in cells expressing the Q79L mutant? If so, wouldn’t it be difficult to explain the mutant’s phenotype in endosome dynamics and transport? In my opinion, it is not appropriate to compare the volume percentage of TNFR1 over RAB5-Q79L-eGFP in the presence and absence of NAP1, without introducing a bias into the analysis. Moreover, the overall distribution of NAP1 is different from that of RAB5Q79L and TNFR1, which is difficult to reconcile with the notion that NAP1 interacts with PTPN23 to promote TNFR1 sorting into RAB5-containing endosomes. I am afraid that these points are not consistent with the view that NAP1 interacts with PTPN23 in sorting TNRF1 to degradation, and I believe that authors should tone down their conclusions.

We have strong evidence demonstrating that NAP1 interacts with PTPN23 (Fig. 6c, d, e, and h) and with TNFR1 (Fig. 6i, and Fig. 7c). Additionally, we showed that in the presence of NAP1, PTPN23 can pull down more TNFR1 (Fig. 7d), which supports our claim that NAP1 functions as an adaptor protein. Additionally, we have shown that NAP1 regulates TNFR1 signaling (Fig. 7b and Extended Data Fig. 7a). We acknowledge the reviewer’s concern regarding the distinct distribution of NAP1 compared to RAB5Q79L and TNFR1, and we believe this difference arises because NAP1 has additional functions. However, we are open to adjusting the phrasing of our conclusions to better capture the complexity of this interaction.

In response, we revised the statement “NAP1 is a sorting adaptor protein to promote the spatial regulation of TNFR1” to “NAP1 functions as an adaptor protein that modulates TNFR1 signaling.” Additionally, we included the phrase, “although NAP1 displayed a different cellular distribution compared to Rab5-Q79L,” along with “suggesting that NAP1 ***may*** promote the endosomal sorting of TNFR1” to clarify the complexity of the interaction.

Rather than stating explicitly that NAP1 promotes TNFR1 sorting into early endosomes (as originally indicated in the title of Fig. 7), we revised this to “NAP1 acts as an adaptor protein in the regulation of TNFR1 signaling” to better align with the available data. We also removed the quantification in Fig. 7e, presenting it as a qualitative observation rather than a quantitative one.

Reviewer #4 (Remarks to the Author):

The authors have addressed most of my concerns. However I personally think that the existence of a human syndrome with a mutation in PTPN23 is highly relevant and should be acknowledged/cited. The explanation given in the rebuttal seems reasonable for why it may not be relevant in myeloid cells and would be a pertinent point in the discussion, in my opinion. Nevertheless I leave this point to the editors discretion.

Thank you for acknowledging our efforts to address your concerns.