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Paracaspase MALT1 regulates glioma cell survival by controlling endo-lysosome homeostasis

Kathryn A. Jacobs, Gwennan André-Grégoire, Clément Maghe, An Thys, Ying Li, Elizabeth Harford-Wright, Kilian Trillet, Tiphaine Douanne, Carolina Alves Nicolau, Jean-Sébastien Frénel, Nicolas Bidère, and Julie Gavard

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1st Editorial Decision 28th Apr 2019

Thank you for your interest and the submission of your manuscript (EMBOJ-2019-102030) to The EMBO Journal. Your manuscript has been sent to four referees for consideration, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential high interest and robustness of your work, although they also express a number of major issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. Referee #1 states that the dependence of GSC survival and Qki binding on MALT1 protease activity is currently unresolved and needs clarification (ref#1, pts. 3,5). Also, this referee is concerned that the mechanistic details of how MALT1 inhibition results in lysosomal leakage and the MALT1 link to Qki remain unclear. Both referees #2 and #3 state that a specific role of MALT1 activity in GSCs and lysosomal dysfunction is not well enough supported by the data (ref#2, pt. 1; ref#3, pts.4,9) and the functional link to GSC maintenance needs more characterized (ref#2, pt.3). These referees also request to explore roles of BCL10 and other CARD proteins in MALT1's activity further (ref#2, pt.2) and investigate the autophagy pathway (ref#3, pt.8) in more detail. In addition, the referees point to issues related to missing controls, additional literature background and textual clarity, that would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

REFEREE REPORTS:

Referee #1:

Jacobs et al present a really interesting story where MALT1 protease activity is suggested as a novel therapeutic target for glioma treatment, potentially even affecting the cancer stem cell compartment. Inspection of the The Cancer Genome Atlas (TCGA) for mRNA expression of several NF-kB pathway related genes in glioblastoma revealed that MALT1 expression inversely correlates with patient probability of survival. MALT1 was subsequently shown to be constitutively activated in patient derived glioblastoma stem-like cells (GSC) and knockdown of MALT1 largely impaired their expansion and stemness in vitro. Moreover, pharmacological inhibition of MALT1 using specific phenothiazines that were previously found to inhibit MALT1 also impaired the expansion of these stem cells in vitro and in vivo using ectopically implanted human GSC in nude mice.
The story does however not only provide an exciting prospect of novel therapeutic tools to treat glioma, it also points to an entirely novel role of MALT1 (protease activity) in regulation of lysosomes and autophagy. More specifically, when analyzing the type of cell death upon MALT1 inhibition with mepazine (MPZ), the authors found that cells die in a non-apoptotic manner but show increased vacuoles and lysosomes. Increased endo-lysosome abundance was followed by impaired autophagic flux, culminating in lysosomal mediated death. The authors then hypothesized that MALT1 inhibition increased lysosome abundance via an effect at the posttranscriptional level (as supported by RNAseq data) and related mTOR signaling. Indeed, they found that MPZ led to mTOR inactivation/displacement, which fits with the previously described link between mTOR and lysosomal biogenesis. Finally, they further show that MALT1 maintains low levels of lysosomes by sequestering the RNA binding protein QKI, which has recently been linked with the downregulation of endocytosis, receptor trafficking and endo-lysosome-mediated degradation.
Altogether, this is a very interesting paper that not only implicates a novel role for MALT1 proteolytic activity in glioblastoma, offering interesting perspectives for therapeutic targeting, but also reveals an intriguing novel role for MALT1 in endo-lysosome homeostasis. Although not addressed in the current paper, the latter could also point to a currently unexplored function of MALT1 in healthy cells and during normal development (for example, a fraction of Bcl10 and CARD10 mouse KO embryos die due to neuronal development defects, and MALT1 is highly expressed in neurons during embryo development). The authors suggest that already existing drugs with MALT1 protease-inhibiting activity could be used therapeutically, which is extremely interesting since the glioma patients are showing very poor survival chances and this could improve the chances for "compassionate use" attempts for drug repurposing.
The study is very well designed, with several independent and complementary experimental approaches that support the conclusions. Also the use of independent strategies to block MALT1 (small compound inhibitors as well as knockdown) increases confidence that the observed effects indeed reflect a role of MALT1. Although some questions remain (e.g. regarding the mechanism by which MALT1 inhibition drives lysosomal leakage and the exact role of QKI), the results reported in the present study significantly advance the field and form a strong basis for future research. Some issues still need to be addressed.
Major comments:
1) P5 L94-96 (Fig 1b): I do not see how this figure contributes to the rest of the paper. GCSs represent only a small number of cells within the tumor. Hence it is unlikely that the observed inverse correlation between MALT1 expression and patient survival is due to MALT1 dependent inhibition of cell death in GSCs. Increased MALT1 expression could also be due to increased immune cell infiltration, which is also correlated with poor survival. Of note MALT1 expression is not correlated with expression of the glioblastoma stem cell marker CD133 (see cBioportal).
2) P6 L116-127 (Fig 1f-j): The reported reduction in proliferation upon knockdown of MALT1 could be a secondary effect of the observed cell death upon inhibition of MALT1. Therefore, before stating that MALT1 plays a role in Glioblastoma cell proliferation, it is important to examine if reduction of proliferation is a direct or secondary effect of MALT1 inhibition.
3) P7 (Fig 2, Supplemental figure 2, 3): The authors heavily rely on the phenothiazine class of inhibitors, which could be problematic if this should be used as an argument for drug repurposing (for example, several phenothiazine compounds will influence Ca-dependent signaling, and all 3 active compounds selected in this study share this potential side-effect; PMID: 30513612). I think the authors should at least mention the non-specificity of the phenothiazines. A beautiful solution to this problem, which would strongly indicate that the effect from the inhibitor indeed comes from MALT1 protease inhibition would be to replicate what was done in ABC-DLBCL cells (PMID: 23946259; see fig 3): express wild-type or MPZ-resistant E397A mutant MALT1 (e.g. lentiviral) and test the effect from the different phenothazines on cells expressing either wt or mutant MALT1. To further prove that the biological effects are due to protease inhibition and not structural changes

of MALT1 due to the allosteric inhibitor, I would suggest to also include a E397A/C464A (protease-inactive) double mutant control, which should remain MPZ-sensitive (endogenous MALT1 inhibited).
On another note, it is very interesting that chloropromazine seems to be more active than MPZ, especially since this drug is still in use whereas MPZ has been discontinued. The higher activity was surprising, since promazine was about 10X less active than MPZ in the original discovery of MALT1 inhibition by this class of compounds (PMID: 23238017, ref #25).
4) P7 L140-143 (Suppl. Fig 1e-F): Knockdown of BCL10 does not seem to affect cell viability (whilst MALT1 knockdown was shown to reduce cell viability Fig). This raises the important question if MALT1 activation in GSC is independent from BCL10 (or maybe even CBM complex independent). The CYLD blot in supplemental figure 1f should be expanded to include the 70 kDa cleaved fragment. If MALT1 activation indeed is Bcl10-independent in GSCs, this is a very important message because only a few indications of Bcl10-independent roles of MALT1 have been suggested: Importantly, one such suggestion is related to mTOR activation in T cells (PMID: 24917592; ref #42) and the other suggestive link is that insects and nematodes have a MALT1 homolog but lack Bcl10 or CARD-CC family proteins, indicating alternative independent activation mechanisms of MALT1 (PMID: 29881386).
5) P12 L254-257 (Fig 5c): Is binding of QKI dependent on MALT1 protease activity or is the observed reduction in QKI binding upon MPZ treatment explained by competition between QKI and MPZ to bind MALT1 (MPZ is an allosteric inhibitor). Cells were exposed only 1h with MPZ hence it is less likely that binding of QKI depends on MALT1 protease activity. In general in many experiments cells are treated with MPZ for a relative short period (less than 24h). These experiments should be repeated with VRPR-fmk and/or with MPZ-resistant MALT1 mutant cells (see above).

Minor comments:
1) P3 L44: What is the relevance of using a cancer stem cell line when cancer stem cells are maintained in vivo in a quiescent slow-growing state.
2) P3 L56: QKI is abbreviated before mentioning the full name in the Result section P11 L238
3) P 6 L106: The authors mention the scaffolding function of MALT1 without introducing this to the reader. It would make more sense to devote a part of the introduction to MALT1 and MALT1 dependent signaling, which will be important for further discussion below (Bcl10 results).
4) P7 L133-136 (Fig 2e): Two different cell viability assays were used to show that MPZ treatment reduces cell viability of GSC (Uptiblue colorimetric assay) but does not affect viability of brain-originated human cells (Cell TiterGlo luminescent assay). Preferably this should have been done using the same assay.
5) Fig 4B: why are the most abundant genes that are downregulated not indicated in the vulcano plot? Also, the font of significant differentially expressed genes is so small that it's illegible on a printed copy.
6) Fig 4D: all p values and FDR values are indicated as 0. Please check.
7) The format of the suppl table is not readable and should be presented in a better way and include a legend.
8) P34 Legend Fig 2 Typo: "Fluophenazine" should be changed to "Fluphenazine"

Referee #2:

Jacobs et al present data correlating high MALT1 expression in glioblastomas with poor prognosis. They demonstrate that MALT1 protease is critical for stem-like cell viability from patient-derived glioblastomas. Knock-down and inhibitor studies suggest that MALT1 expression and protease activity are required for the expansion of patient-derived glioblastoma stem-like cells (GSC) in vitro and in a murine xenotransplantation model. Further studies suggest a non-canonical role of the MALT1 protease in maintaining endo-lysosomal homeostasis and mTOR activity in glioblastoma cells. Accordingly, MALT1 protease inhibition induces a non-apoptotic, lysosome-mediated cell death pathway in this tumor cells.

Extensive work has shed light on the critical role of MALT1 paracaspase in triggering adaptive and innate immune responses. In line, deregulations of MALT1 contribute to autoimmunity and hematologic malignancies and MALT1 inhibitors have been suggested as a therapeutic approach in these diseases. The manuscript unveils a new and quite unexpected role for the MALT1 protease in

brain cancers, which in the long run may guide new therapeutic approaches. However, a number of questions and concerns need to be addressed.

Major points:

1) A critical point is to unequivocally prove MALT1 protease activity in GSC. At present, all these data are somewhat hidden in Suppl. Fig. 1. These data should be moved to the main part and there needs to additional verification that MALT1 protease is indeed active in these cells. Is CYLD the only known MALT1 substrate that is cleaved in GSCs? The authors need to determine the cleavage of a larger panel of known MALT1 substrates such as RelB, BCL10, HOIL-1, Regnase-1 and Roquin1/2. Of course, other proteins besides the known MALT1 substrates may be cleaved under these conditions. Thus, the authors should also directly measure MALT1 activity in cell extracts, e.g. by using the in vitro fluorescence assay of peptide substrates (e.g. LRSR-AMC) as done previously (e.g. Rebeaud et al., Nat. Immunol, 2008).

2) How is MALT1 activated and/or activity maintained in GSC? It may go beyond the scope of this manuscript to unravel the exact signaling pathways involved, but at least it needs to be determined, if BCL10 knock-down affects MALT1 protease activity. Also, constitutive MALT1 cleavage activity should be analyzed after knock-down of putative CARMA/CARD proteins (e.g. CARD10 or CARD14). It would be interesting to see if auto-/paracrine mechanisms are involved, which could be analyzed by determining MALT1 protease activity after cells have been washed with fresh medium.

3) What is the function of MALT1 protease in maintaining GSC viability? Again, it may go beyond the scope to analyze which MALT1 substrates are involved. However, it should be determined, if knock-down of QKI not only alters alter MPZ-induced LAMP2 redistribution and LC3B lipidation (Figure 5), but also affects GSC viability after MALT1 knock-down and inhibition. Such data would significantly strengthen the assumption that regulation endo-lysosomal homeostasis is critical for the MALT1 function in these cells.

Specific points:

Fig. 1e: MALT1 protein expression is shown, but there is no evidence that MALT1 expression is increased in patient-derived GSC. Also, there seems to be no significant differences in expression when compared to Jurkat T cells. It may be difficult to obtain valid control cells/tissues, but a comparison to primary neurons, neuronal cell lines and glia cells should be performed. BCL10 expression could serve as an internal control to detect relative increases in MALT1 amounts.

Fig. 2a-c: The peptide inhibitor zVRPR-FMK should be used to confirm the findings.

Supp. Fig. 2: a) IkBa levels are quite different in GSC cells and it should be determined if there is constitutive NF-kB activity, e.g. by EMSA, p65 translocation and/or reporter assays. d) The effect of siMALT1 in CYLD cleavage is not convincing. e) Lack of cell death after BCL10 knock-down suggests that MALT1 is acting independent of BCL10. However, this needs to be confirmed in a similar setting as used for MALT1 in Fig. 1f (lentiviral shRNA knock-down). Also, MALT1 activity in response to BCL10 KD should be determined (see main point 2).

Fig.3: g) More xenografted samples should be analyzed to confirm increased LAMP2 expression upon MPZ treatment in the tumor cells. i-m) Does siMALT1 exert similar effects as MALT1 inhibition by MPZ?

Fig. 4l and 5i: Co-localization of LAMP2 and mTOR and re-positioning needs to be quantified. Also, it should be verified that knock-down of MALT1 exerts the same effect on mTOR positioning as MPZ treatment.

Fig. 5a: The negative correlation between MALT1 and QKI is not so persuasive. Can it be confirmed that the correlation is meaningful by showing that there is an inverse correlation of survival probability between MALT1 and QKI (see Fig. 1b)?

Minor points:

The lines in the graphs in Fig. 1g and 2a are hardly visible and thus the presentation is not convincing.

On page 11 the authors state that '... mTOR staining divorced LAMP2-positive structures'. What does this mean in this context?

Referee #3:

This is a great paper, because it
(i) provides a treatmemt option for Glioblastoma
(ii) this treatment is executed through a novel and unexpected pathway

Minor:
1) The presentation of data in some figures is rather cumbersome:
> especially the frequent use of odd scales (e.g. Fig1c,d, Fig2b) or unclear axes (Fig1i).
> Several immunoblot figures lack the proper loading controls for phosphorylated proteins (e.b. Fig4g (ULK1), Fig4i (AKT, S6)).
> In fig 4d p- and q-values seem to be incomplete
> Loading control in S-Fig1f does not seem to be derived from the same membrane / experiment and thus is not reliable.
Finally, please clearly label which figures are related to cells, or mice, or humans. Figures should be understandable at a glance.
2) While it is commendable that several different lines of GSCs are used in this work, many times data is only shown for a reduced (or even single) and often inconsistent set of cells.
3) In SFig 3a, caspase inhibition does seem to rescue cell death in at least one of the two tested GSCs upon MPZ treatment - to exclude involvement of this canonical way of apoptotic cell death, more replicates also in the other GSCs should be performed and other assays (Annexin V or TUNEL) should be considered, given that the specific cell death route is a major point of the manuscript (or the argument should be tamed down). This should also be incorporated into the main figures.
4) While the authors nicely show altered endo-lysosome homeostasis in GSCs upon MPZ treatment, it remains unclear if GSCs show altered lysosomes per se or if the MPZ effect is specific to GSCs.
5) In TEM images, arrows would help allocating subcellular structures
6) It would have been nice to have the lysosome-related phenotypes in the pharmacological setup confirmed genetically with a MALT1 knockdown
7) Co-localization and dispersion of mTOR and lysosomes in Fig4l does not look very convincing; alterations of mTOR activity due to (re-)localization upon MPZ treatment should be shown using additional methods or at least quantified in some way. Or the data should be eliminated, since they are not essential for the paper.
8) The role of autophagy in this anti-cancer scenario is still rather unclear - additional established autophagic flux assays using inhibitors like chloroquine or leupeptin could give more insight on that matter. Also it would be interesting to see in which way genetic/pharmaceutical activation or inhibition of autophagy affects GSC survival and lysosomal phenotypes in the setup of altered MALT1 activity or QKI expression.
9) Is the interaction of MALT1 and the Endo-lysosomal regulator QKI specific to the pathological condition in GSCs? Does MALT1 interaction with QKI alter QKI activity? Colocalization studies of MALT1 and QKI in GSCs with and without MPZ would strenghten the shown immunoprecipitation data.
10) While SFig4 suggests a pervasive expression of QKI, in Fig5 it seems to be exclusively expressed in the nucleus
11) In Fig5h, MPZ treatment seems to actually reduce QKI levels per se, which somewhat contradicts the finding that high levels of QKI are associated with increased endo-lysosomes.
12) Functionality of the increased lysosomes under MALT1 inhibition is still somewhat inconclusive, given the proposed early block in autophagic flux while lysosomes still seem functional. This can be adressed in more detail.

Referee #4:

The authors examined the effects of MALT1 inhibition on glioma cells and the mechanism of MALT1-induced cytotoxicity. First, the authors evaluated NF-kB signaling related molecules using TCGA data set and found that MALT1 expression level is associated with GBM patient survival. The authors evaluated the effects of an MALT1 inhibitor MPZ, and found that MPZ induced decrease of viability in both GSCs and DGCs, GSC death, suppression of tumor sphere formation and stemness marker expression in GSCs, and in vivo tumor growth inhibition. The authors also found that growth inhibition by MPZ might not be dependent on canonical MALT1 signaling pathways including NF-kB signaling or CYLD degradation. The authors further evaluated effects of other clinically available phenothiazines on GSC viability, and found that drugs that efficiently inhibit MALT1 activity in Jurkat cells induced in vitro growth suppression of GSCs.
Regarding the mechanism, the authors found that MALT1 inhibition upregulated endocytosis and increased lysosomes but affected autophagic degradation, associated with cathepsin D leakage. Phenothiazines and MALT1 knockdown inhibited AKT-mTOR signaling pathway that was associated with displacement of mTOR from lysosomes. The authors examined lysosome regulators, and found that QKI is bound with MALT1 and this binding was suppressed by MALT1 inhibitor. Overexpression of QKI increased LAMP2 and decreased cell survival, and knockdown of QKI suppressed MALT1 inhibition-induced mTOR displacement from lysosomes. Authors proposed a working model in which MALT1 binds and inhibits QKI and consequently induces downregulation of lysosomes that could contribute to lysosomal homeostasis and activated mTOR signaling in glioma cell survival.
Overall the experiments were performed well, using human GSCs and clinically available drugs is also a strength. The novel findings including growth suppression of glioma cells by MALT1 inhibition and binding of MALT1 with QKI are interesting. However, the manuscript suffered from many shortcomings that need to be addressed.

Major points
1) In Introduction, the authors described "Here, we repurpose several members of a family of drugs, phenothiazines, to disrupt GSC lysosomal homeostasis...". However, the rationale is not clear in Introduction why the authors had thought of using MALT1 inhibitors, phenothiazines. In addition, according to the authors' statement in Introduction, major aim of this study appears to be evaluation of repurposing of phenothiazines in glioma therapy. In Results, however, it appears that the authors' focus was primarily NF-kB signaling and then MALT1 signaling, and that discovery of lysosomal homeostasis impairment by phenothiazines is part of the consequences in MALT1 signaling study, rather than phenothiazine repurposing study. These sounds confusing. I think that the manuscript would be easier to follow if the authors clearly stated the authors' aim or hypothesis and described the results in a consistent manner regarding the aim/hypothesis.
2) I think that large part of experiments was performed with focus on MALT1. Therefore, providing background regarding MALT1 in Introduction would be helpful for readers.
3) In the first part of the results, the authors described that "Because the transcription factor NF-κB is instrumental in many cancers and because it centralizes the paracrine action of cytokines..." I think that more specific justification for studying NF-κB would be helpful for readers to understand the authors' initial aim/hypothesis. I wonder why the authors focused on NF-kB pathways among multiple signaling pathways that are thought to be important in neural stem cell niches.
4) Association between MALT1 expression and glioma grades has already been reported (Yang et al., 2017). This article also appears to suggest possible contribution of MALT1 to malignant behavior of glioblastoma. I think that the authors should appropriately cite articles regarding glioma and MALT1 and provide sufficient background information.
5) Apparently, the MALT1 inhibitor has a much stronger and complicated functions than MALT1 knockdown does. In the Figure1, knockdown of MALT1 reduces the GSC expansion but not survival, however, in the Figure2 the inhibitor affects the viability of the same cell lines. These results indicated that the inhibitor might have additional function beyond the MALT1 itself. Therefore, the RNA sequencing data in the Figure4 might not correctly represent the function of MALT1 in GSC.
6) When talking about of the blocking MALT1 protease activity, what does it exactly mean? Does it mean that MALT1 regulates the GSC endo-lysosome activity going through its proteolytic activity? Does MALT1 regulates mTOR activity dependent on this activity? Does a catalytically inactive MALT1 mutant recapitulate the phenotype found in this manuscript?

7) Regarding the interaction of MALT1 and QKI, as we know QKI has several isoforms, which isoform is the dominate one interacting with MALT1? Where do the authors think that the functional interaction will take place - nuclear or cytoplasm? In the Fig.5, QKI can bind MALT1, and also BCL10, which indicates that QKI may form the complex with MALT1 and BCL10, does this indicate that MALT1 works as a scaffold instead of the proteinase with QKI?
8) Growth inhibitory effects of some of drugs in Supplementary Fig 2 on glioma cells have been reported (reviewed by Tan et al., 2018, for example). I think that authors should mention those previous studies.
9) In Fig 1e, I wonder if non-tumor control cells are available.
10) Regarding the evaluation of NF-kB activation in GSCs, I think that examining not only IkB but also NF-kB subunits that activate transcription might be useful.
11) Providing more background information of MPZ, including target specificity, would be helpful for readers. Especially, I wonder if 20 uM MPZ would have off-target effect.
12) Given efficient transition of phenothiazines through blood brain barrier, justifying concentrations of these drugs used in this study would be convincing.
13) In Fig 2g, which band is SOX2? Is differentiation induction successful?
14) In Fig 3a, indicator of vacuoles would be helpful for readers. I also think that showing quantitative data would be convincing. In addition, the interpretation of this finding appears to be missing in the text.
15) Regarding Fig 3b, was increase of vacuoles induced by siMALT1 treatment?
16) I wonder what is indicated by "endo-lysosomal". In most part of text, "endo-lysosomal" is used while "lysosomes" is used in Supplementary Fig 4i. I wonder how they are differently used.
17) Regarding "endo-lysosomal" protein, I wonder if LAMP2 alone is sufficient to show "endo-lysosome". I also wonder if LAMP1 or late endosome marker such as RAB7 would show the same trend of alteration with LAMP2.
18) Regarding Supplementary Fig 2a, data of cell death and lysosome were obtained using GSCs but MALT1 activity data were from Jurkat cells. Showing MALT1 activity data using the corresponding GSCs would be informative.
19) Regarding Supplementary Fig 2a, MALT1 inhibition activity appears to correlate with cell death. However, lysosome increase might not correlate with cell death or MALT1 inhibition activity, suggesting possible lysosome-independent mechanism by which MALT1 inhibition induces cell death.
20) Regarding Fig 3m, I wonder how Baf rescues cell viability against MPZ induced cytotoxicity. Given that MPZ could increase lysosomal membrane permeability and consequently induce lysosomal enzyme leakage that could induce cytotoxicity, how could V-ATPase inhibitor rescue this cytotoxicity.
21) In Fig 4f, data of LAMP1 and LAMP2 are missing.
22) In Fig 4g, it appears that 50 nM rapamycin suppresses mTOR-S6K-S6 pathway as effectively as 20 uM MPZ dose. However, Supplementary Fig 4b shows remarkable difference of cytotoxicity between MPZ and RAPA. I wonder if there is a major contributor in MPZ-induced cytotoxicity other than mTOR signaling inhibition.
23) In Fig 5e and 5f, displacement of mTOR from lysosomes is not directly shown.
24) Regarding QKI overexpression and knockdown experiments, showing alterations of downstream molecules to mTOR, such as p-S6K or p-S6 would be convincing.
25) I wonder if QKI overexpression increases lysosomal membrane permeability and leakage of lysosomal enzymes.
26) I wonder if alteration in MALT1-QKI-lysosome axis is critical in MALT1 inhibition-induced cell death. In Fig 4, the authors showed inhibition of AKT activation by phenothiazines and siMALT1. Suppression of AKT could induce persistent activation of TSCs, resulting in inhibition of recruitment of mTORC1 to lysosomes (reviewed by Lawrence et al, 2019, for example). In addition, AKT is known to promote cell proliferation and survival via multiple signaling pathways independent to mTOR. Therefore, AKT signaling pathways could be important contributors to observed phenotype in this study. I think that examining or at least discussing those possible causative mechanisms that have already been reported would be useful to show the significance of the findings in this study.
27) Regarding the authors' description "These results suggest that MALT1 affects lysosomal homeostasis post-transcriptionally, and that this increase coincides with weak mTOR signaling, which may be due to displacement of mTOR from its lysosomal signaling hub", I wonder what "this increase" is.
28) According to proposed working model, MALT1 binds and inhibits QKI that downregulates

lysosomes, resulting in lysosomal homeostasis and mTOR activation, while MALT1 inhibition enables QKI to bind its targets and increase lysosomes but LMP and LCD are also increased. I wonder why downregulation of lysosomes could activate mTOR and why QKI activation affects LMP. More specific discussion would be useful.

Minor points
1) In Supplementary Fig 3a, QVD appears to partially rescue cell viability in GSC#9 treated with 10 uM MPZ. I wonder if this is statistically significant and if apoptosis is suggested.

1st Revision - authors' response 20th Sep 2019

Please see next page.

Referee #1

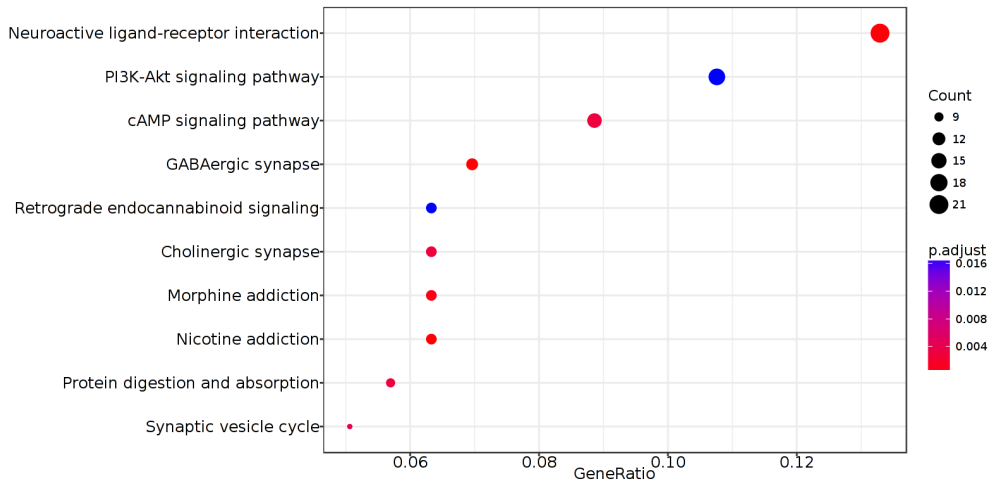
We thank the Reviewer who acknowledges that "*The story does however not only provide an exciting prospect of novel therapeutic tools to treat glioma, it also points to an entirely novel role of MALT1 (protease activity) in regulation of lysosomes and autophagy*." He/She also mentions that: "*The study is very well designed, with several independent and complementary experimental approaches that support the conclusions. Also the use of independent strategies to block MALT1 (small compound inhibitors as well as knockdown) increases confidence that the observed effects indeed reflect a role of MALT1*".

Major comments

1) P5 L94-96 (Fig 1b): I do not see how this figure contributes to the rest of the paper. GCSs represent only a small number of cells within the tumor. Hence it is unlikely that the observed inverse correlation between MALT1 expression and patient survival is due to MALT1 dependent inhibition of cell death in GSCs. Increased MALT1 expression could also be due to increased immune cell infiltration, which is also correlated with poor survival. Of note MALT1 expression is not correlated with expression of the glioblastoma stem cell marker CD133 (see cBioportal).

We agree with the Reviewer. This figure indeed only meant that there is an inverse correlation between MALT1 overall expression in GBM and survival prognosis. It does not formally prove the importance of the gene product in the progression of the disease and in the biology of GSCs. To challenge the potential involvement of MALT1 in GBM, we thus employed GSCs, as widely accepted cellular models for GBM. In fact, GSCs are patient-derived primary cells that can initiate, propagate and repopulate the tumor mass (Lathia et al., 2015; Bao et al., 2006; Chen et al., 2012; Singh et al., 2004). Nevertheless, we cannot discount that some of the MALT1 correlation with patient survival is indeed due to other cell types, such an increased immune infiltration. We thus have amended the text to reflect this opinion: «*this increased MALT1 expression may be due to tumor infiltrating immune cells"*

Further, our own TCGA analysis also returns no significant correlation between MALT1 and PROM1 (CD133) or any other relevant stemness genes (*eg* NES, SOX2, OCT4). Instead, KEGG analysis on high *versus* low MALT1 expression from TCGA RNAseq data directs towards neural associated functions and intracellular trafficking, but not immune cell related gene signature (*please see below*). It would be interesting in the future to dissect MALT1 expression in the different cellular components of GBM, through single cell analysis for instance.



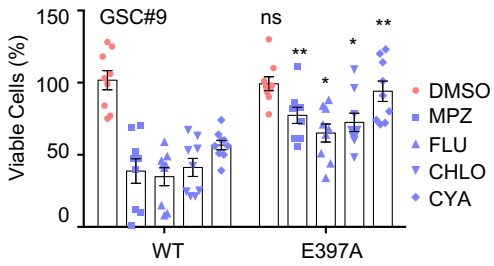
2) P6 L116-127 (Fig 1f-j): The reported reduction in proliferation upon knockdown of MALT1 could be a secondary effect of the observed cell death upon inhibition of MALT1. Therefore, before stating that MALT1 plays a role in Glioblastoma cell proliferation, it is important to examine if reduction of proliferation is a direct or secondary effect of MALT1 inhibition.

This is an insightful remark. We did observe a reduction in EdU incorporation as early as 16 hours post-MPZ treatment (Fig 2F), while the uptake of PI was significantly increased after 48 hours (Fig 2G). In MALT1 siRNA-mediated knocked down cells, EdU staining was decreased and PI incorporation was increased at the endpoint (3 days, Fig 1G-1H). However, whether reduction of proliferation precedes or results from cell death commitment cannot be ruled out. We have thus amended the text to comment on reduced cell viability rather than proliferation.

"*Likewise, cells transfected with siMALT1 had a lower percentage of EdU-positive cells as compared to non-silenced control cells (Fig 1G) and a higher incorporation of propidium iodide (PI) (Fig 1H). Additionally, GSCs either expressing shMALT1 or transfected with siMALT1 had less stem traits, as evaluated by limited dilution assay and tumorsphere formation (Fig 1I-J). Taken together, these results indicate that MALT1 expression may be important for glioblastoma cell expansion.*"

3) P7 (Fig 2, Supplemental figure 2, 3): The authors heavily rely on the phenothiazine class of inhibitors, which could be problematic if this should be used as an argument for drug repurposing (for example, several phenothiazine compounds will influence Ca-dependent signaling, and all 3 active compounds selected in this study share this potential side-effect; PMID: 30513612). I think the authors should at least mention the non-specificity of the phenothiazines. A beautiful solution to this problem, which would strongly indicate that the effect from the inhibitor indeed comes from MALT1 protease inhibition would be to replicate what was done in ABC-DLBCL cells (PMID: 23946259; see fig 3): express wild-type or MPZ-resistant E397A mutant MALT1 (e.g. lentiviral) and test the effect from the different phenothiazines on cells expressing either wt or mutant MALT1. To further prove that the biological effects are due to protease inhibition and not structural changes of MALT1 due to the allosteric inhibitor, I would suggest to also include a E397A/C464A (protease-inactive) double mutant control, which should remain MPZ-sensitive (endogenous MALT1 inhibited). On another note, it is very interesting that chlorpromazine seems to be more active than MPZ, especially since this drug is still in use whereas MPZ has been discontinued. The higher activity was surprising, since promazine was about 10X less active than MPZ in the original discovery of MALT1 inhibition by this class of compounds (PMID: 23238017, ref #25).

We agree with the Referee that phenothiazines may have additional targets beside MALT1, and this point is now mentioned in the revised manuscript (please see page 7). However, MALT1 was experimentally blocked by several means (chemically and genetically) in multiple assays, militating for a specific action of MPZ in the observed phenotypes. In addition, we now provided new viability data using MPZ-resistant MALT1 (E397A mutant). In fact, modified GSCs displayed significant resistance to phenothiazine-induced cell death (Fig EV1F).



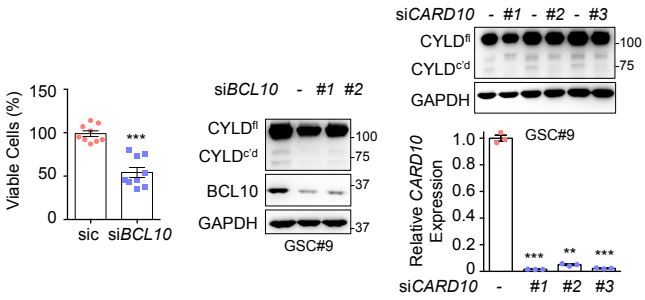
We too were surprised by the strong effects of chlorpromazine. However, as our experiments challenge functional impact of the drugs and do not directly test compound activity as was done in Nagel et al., we cannot therefore state with certainty whether chlorpromazine is more active than mepazine. Indeed, in terms of GSC viability, both compounds seem to

have a similar dose dependent effect on cell viability (Fig 2I). For sake of clarity, we have thus removed the relative (+/-) classification in the former Figure EV1A.

4) P7 L140-143 (Suppl. Fig 1e-F): Knockdown of BCL10 does not seem to affect cell viability (whilst MALT1 knockdown was shown to reduce cell viability). This raises the important question if MALT1 activation in GSC is independent from BCL10 (or maybe even CBM complex independent). The CYLD blot in supplemental figure 1f should be expanded to include the 70 kDa cleaved fragment. If MALT1 activation indeed is Bcl10-independent in GSCs, this is a very important message because only a few indications of Bcl10-independent roles of MALT1 have been suggested: Importantly, one such suggestion is related to mTOR activation in T cells (PMID: 24917592; ref #42) and the other suggestive link is that insects and nematodes have a MALT1 homolog but lack Bcl10 or CARD-CC family proteins, indicating alternative independent activation mechanisms of MALT1 (PMID: 29881386).

The Reviewer raises a crucial point, as to whether MALT1 activation requires BCL10 in GSCs. One caveat with our original experiment was that BCL10 knockdown was sub-optimal. We therefore optimized the procedure to improve siRNA-mediated BCL10 knockdown, and now conclusively show that BCL10 silencing mirrors the siMALT1-provoked phenotype (Fig 3K, and 4D). At the Reviewer's suggestion, CYLD cleavage upon BCL10 knockdown was also assessed. Prompted by the Referees #1 and #2 (please, see also our answer to his/her point #2), we investigated the expression of CARMAs in GSCs and found that they do indeed express CARMA3 (CARD10) at the RNA level. Its knockdown with three individual siRNA oligoribonucleotides prevented the constitutive cleavage of CYLD in GSCs (Fig 3J).

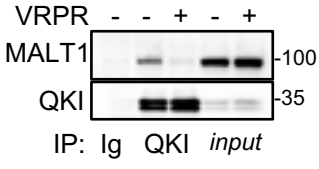
Altogether, our data suggest that CARMA3 and BCL10 participate in MALT1 activation in GSCs.



5) P12 L254-257 (Fig 5c): Is binding of QKI dependent on MALT1 protease activity or is the observed reduction in QKI binding upon MPZ treatment explained by competition between QKI and MPZ to bind MALT1 (MPZ is an allosteric inhibitor). Cells were exposed only 1h with MPZ hence it is less likely that binding of QKI depends on MALT1 protease activity. In general, in many experiments, cells are treated with MPZ for a relative short period (less than 24h). These experiments should be repeated with VRPR-fmk and/or with MPZ-resistant MALT1 mutant cells (see above).

Following this Reviewer's suggestion, co-IP experiments were carried out in cells treated with Z-VRPR-FMK for 4 hours. Similarly to what was found with MPZ, this led to a strong reduction in MALT1 binding to QKI (Fig 7C). Hence, MALT1-QKI binding most likely involves MALT1 protease activity. It is tempting to speculate that interfering with MALT1 activity alters its interactome, therefore reducing QKI binding.

Of note, many experiments were done in relative short period (few hours), in order to prevent from potential bystander effects of cell death. This relatively short kinetics was compatible with increase in lysosomal protein level (Fig 4B), signs of autophagy (Fig 5B), reduction in mTOR activation (Fig EV3F), and mTOR repositioning (Fig EV3G).



Minor comments

1) P3 L44: What is the relevance of using a cancer stem cell line when cancer stem cells are maintained in vivo in a quiescent slow-growing state.

We have selected GSCs as an *ex vivo* model of GBM, as they are derived from patient biopsies and therefore more representative of human tumors than established GBM cell lines such as U87. GSCs can indeed recapitulate properties of the tumor of origin including molecular subtype, mutational landscape, heterogeneity, resistance to treatment, and tumor-initiating capabilities (Galan-Moya et al., 2011; Harford-Wright et al., 2017; Lathia et al., 2015). Notably, GSCs used here represent the different molecular subtypes of GBM (mesenchymal, classical, neural), as well as diversity in patient gender (2 male, 2 female), and age. This is now clearly stated in the method section.

2) P3 L56: QKI is abbreviated before mentioning the full name in the Result section P11 L238

We thank the Referee for catching this error, and have now amended the text.

3) P 6 L106: The authors mention the scaffolding function of MALT1 without introducing this to the reader. It would make more sense to devote a part of the introduction to MALT1 and MALT1 dependent signaling, which will be important for further discussion below (Bcl10 results).

At the Reviewer's suggestion, a section about MALT1 was added to the introduction (please see page 4).

4) P7 L133-136 (Fig 2e): Two different cell viability assays were used to show that MPZ treatment reduces cell viability of GSC (Uptiblue colorimetric assay) but does not affect viability of brain-originated human cells (Cell TiterGlo luminescent assay). Preferably this should have been done using the same assay.

We have now performed all experiments using the same assay, namely Cell TiterGlo (Fig 2E, 2H).

5) Fig 4B: why are the most abundant genes that are downregulated not indicated in the volcano plot? Also, the font of significant differentially expressed genes is so small that it's illegible on a printed copy.

For sake of clarity, the gene names from the volcano plot were removed. All data from the RNAseq can be found in the Expanded Table 1.

6) Fig 4D: all p values and FDR values are indicated as 0. Please check.

We apologize for the lack of clarity. p-values/q-values are indeed not literally zero. They are less than the smallest representable positive double-precision floating point value in the software used. As such, they are the most significant ones. We have amended the table to $p<0.001$ and $q(FDR)<8.10^{-5}$, for sake of clarity.

7) The format of the supp table is not readable and should be presented in a better way and include a legend.

We have now amended the expanded table 1 and legend for clarity.

8) P34 Legend Fig 2 Typo: "Fluophenazine" should be changed to "Fluphenazine"

We have now amended this typo.

Referee #2:

We are grateful to this Reviewer who acknowledges that: "*the manuscript unveils a new and quite unexpected role for the MALT1 protease in brain cancers, which in the long run may guide new therapeutic approaches*."

Major points

1) A critical point is to unequivocally prove MALT1 protease activity in GSC. At present, all these data are somewhat hidden in Suppl. Fig. 1. These data should be moved to the main part and there needs to additional verification that MALT1 protease is indeed active in these cells. Is CYLD the only known MALT1 substrate that is cleaved in GSCs? The authors need to determine the cleavage of a larger panel of known MALT1 substrates such as RelB, BCL10, HOIL-1, Regnase-1 and Roquin1/2. Of course, other proteins besides the known MALT1 substrates may be cleaved under these conditions. Thus, the authors should also directly measure MALT1 activity in cell extracts, e.g. by using the in vitro fluorescence assay of peptide substrates (e.g. LRSR-AMC) as done previously (e.g. Rebeaud et al., Nat. Immunol, 2008).

We agree with the Reviewer that exploring further MALT1 activity in GSCs is of interest. As per this Reviewer's suggestion, we have now moved these data to the revised main Figure 3. In addition, new experiments were carried out to substantiate the idea that MALT1 is active in GSCs. We first tried to evaluate MALT1 activity with the LRSR-AMC peptide substrate. Unfortunately, our attempt to provide reproducible and robust measurements of MALT1 activity was not successful in our hands, regardless of cellular models. Nevertheless, we further assessed the status of additional MALT1 known substrates, and identified that CYLD and ROQUIN1/2 were cleaved in GSCs (Fig 3C-F). This was however not the case for HOIL1 (Fig 3C), or RELB (not shown). Of note, we failed to robustly detect Regnase-1 and BCL10 cleaved forms (not shown), and A20 was not expressed (not shown). Importantly, the processing of CYLD and ROQUIN1/2 was reduced upon MALT1 inhibition (via mepazine and Z-VRPR-FMK, as well as siRNA, please see Fig 3D-F). Again, arguing for a basal constitutive activity of MALT1 protease activity in GSCs, BCL10 and CARMA3 (CARD10) silencing reduced CYLD processing (Fig 3J-K, please see also our response to Reviewer#1, point #4). In addition, the expression of MALT1 protease-dead mutant C464A modifies basal CYLD cleavage (Fig 3G-H).

Overall, the new provided data, notably those now using Z-VRPR-FMK (Fig 2C, 3F, 4C, 4D, 4E, 4G, 5F, 6K, 7C, EV2C) and the protease-dead mutant form of MALT1 (Fig 3H, 4D, 6J) in most of the assays reinforce the idea that the MALT1 protease activity is indeed co-opted in GSCs.

2) How is MALT1 activated and/or activity maintained in GSC? It may go beyond the scope of this manuscript to unravel the exact signaling pathways involved, but at least it needs to be determined, if BCL10 knock-down affects MALT1 protease activity. Also, constitutive MALT1 cleavage activity should be analyzed after knock-down of putative CARMA/CARD proteins (e.g. CARD10 or CARD14). It would be interesting to see if auto-/paracrine mechanisms are involved, which could be analyzed by determining MALT1 protease activity after cells have been washed with fresh medium.

This is a very insightful point, also raised by the Referee#1. We therefore carried out several new experiments to challenge the importance of BCL10 and CARMA3 (CARD10) in MALT1 activity in GSCs (please, see our response to Reviewer#1, point#4). In brief, we now show that knocking down BCL10 or CARMA3 led to a significant reduction of CYLD processing (Fig 3J-K). Overall, this suggests that MALT1 activation in GSCs relies on CARMA/BCL10.

Further we have attempted to evaluate whether MALT1 is activated by autocrine or paracrine mechanisms. Our data indicate an increased MALT1 activity, based on the extend of CYLD cleavage, in cells cultured for 3 days as compared to cells that have been washed

with fresh medium (Fig 3I). Although further studies will dissect the exact upstream mechanisms involved in MALT1 activation in GSCs, outside signals and culture conditions might be contributing too.

3) What is the function of MALT1 protease in maintaining GSC viability? Again, it may go beyond the scope to analyze which MALT1 substrates are involved. However, it should be determined, if knock-down of QKI not only alters alter MPZ-induced LAMP2 redistribution and LC3B lipidation (Figure 5), but also affects GSC viability after MALT1 knock-down and inhibition. Such data would significantly strengthen the assumption that regulation endo-lysosomal homeostasis is critical for the MALT1 function in these cells.

We agree that determining whether any known or to be discovered substrate(s) of MALT1, which can bridge protease to viability is an exciting future field of intense research. At the Reviewer's suggestion, we evaluated cell viability (EdU and PI incorporation, Fig 7M, 7N, EV4F) in cells where both MALT1 and QKI were silenced. Interestingly, we found that the knockdown of QKI with MALT1 partially rescued GSCs from lethality driven by MALT1 depletion. This new set of data thus reinforces the mechanistic link between MALT1 and endo-lysosomal homeostasis via QKI.

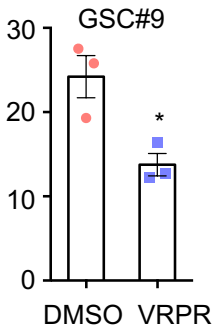
Specific points

a) Fig. 1e: MALT1 protein expression is shown, but there is no evidence that MALT1 expression is increased in patient-derived GSC. Also, there seems to be no significant differences in expression when compared to Jurkat T cells. It may be difficult to obtain valid control cells/tissues, but a comparison to primary neurons, neuronal cell lines and glia cells should be performed. BCL10 expression could serve as an internal control to detect relative increases in MALT1 amounts.

We agree with the Reviewer and have looked at MALT1 expression in GSCs compared to neuron-like cells (from neuroblastoma), fetal human astrocytes and human brain endothelial cells. However, there was no significant change in BCL10/MALT1 total protein expression. For sake of clarity, we have removed this panel from our revised manuscript.

b) Fig. 2a-c: The peptide inhibitor zVRPR-FMK should be used to confirm the findings.

This is an insightful suggestion. As presented in the new Fig 2C, we now show that MALT1 inhibition with Z-VRPR-FMK significantly reduced tumorsphere formation (Fig 2C).



c) Supp. Fig2: IκBα levels are quite different in GSC cells and it should be determined if there is constitutive NF-κB activity, e.g. by EMSA, p65 translocation and/or reporter assays.

We have now performed cellular fractionation experiments to explore p65, c-REL and RELB nuclear translocation (Fig 3B) at the basal level and upon stimulation with TNFα. Our data show no overt basal activation of NF-κB in GSCs.

d) The effect of siMALT1 in CYLD cleavage is not convincing.

We have now performed densitometric analysis to quantify the effect of MALT1 silencing on CYLD processing in repeated experiments (Fig 3E).

e) Lack of cell death after BCL10 knock-down suggests that MALT1 is acting independent of BCL10. However, this needs to be confirmed in a similar setting as used for MALT1 in Fig. 1f (lentiviral shRNA knock-down).

This is a good point, also raised by Reviewer#1 (Please see our response to his/her point#4). In brief, we optimized the procedure to improve siRNA-mediated BCL10 knockdown, and now conclusively show that it recapitulates MALT1 silencing (Fig 3JK and 4D).

f) Also, MALT1 activity in response to BCL10 KD should be determined (see main point 2).

We now report that BCL10 silencing reduces CYLD processing (Fig 3K), suggesting that MALT1 proteolytic activity in GSCs requires BCL10.

g) More xenografted samples should be analyzed to confirm increased LAMP2 expression upon MPZ treatment in the tumor cells.

At the Reviewer request, more xenograft samples showing LAMP2 increase were included (Fig 4F).

h) Fig 3i-m, Does siMALT1 exert similar effects as MALT1 inhibition by MPZ?

We have now included data in MALT1 siRNA-transfected cells that persistently aligned with mepazine treatment in terms of increased LC3 lipidation, P62 puncta, and CTSD release (Fig 5C, 5F, 5J).

i) Fig. 4l and 5i: Co-localization of LAMP2 and mTOR and re-positioning needs to be quantified. Also, it should be verified that knock-down of MALT1 exerts the same effect on mTOR positioning as MPZ treatment.

As per the Reviewer's suggestion, we have now quantified these data and included a panel related to MALT1-silenced cells (Fig 6K).

j) Fig. 5a: The negative correlation between MALT1 and QKI is not so persuasive. Can it be confirmed that the correlation is meaningful by showing that there is an inverse correlation of survival probability between MALT1 and QKI (see Fig. 1b)?

We agree that the negative correlation is not strong, yet significant. We now also show that higher QKI expression correlates with a higher probability of survival in GBM, thus mirroring observations with MALT1 (Fig 7A), and confirming previous findings (Shingu et al., 2016).

Minor points

The lines in the graphs in Fig. 1g and 2a are hardly visible and thus the presentation is not convincing.

We have now darkened the graph-lines for clarity.

On page 11 the authors state that '... mTOR staining divorced LAMP2-positive structures'. What does this mean in this context?

This phrasing has now been altered to read "*In fact, mTOR staining dissipated from LAMP2-positive structures*".

Referee #3

We thank the Reviewer who endorsed the quality of our manuscript.

Minor

1) The presentation of data in some figures is rather cumbersome especially the frequent use of odd scales (e.g. Fig 1c, d, Fig2b) or unclear axes (Fig 1i).

We have now amended scales for clarity (Fig 1D, 1E, 1I, 2A, 2B).

Several immunoblot figures lack the proper loading controls for phosphorylated proteins (e.b. Fig 4g (ULK1), Fig 4i (AKT, S6)).

We have now included requested loading controls (Fig 5G, 5I).

In fig 4d p- and q-values seem to be incomplete.

We apologize for this, and have now verified these values in new panel 6D. Please see also our answer to Reviewer#1, specific comment#6.

Loading control in S-Fig1f does not seem to be derived from the same membrane/ experiment and thus is not reliable.

Due to the number of antibodies used, samples were loaded twice onto two different gels, which had run independently (gel1 probed for MALT1 and BCL10, gel2 for CYLD, LAMP2 and LC3). This experiment has been removed from the current version of the manuscript. Data are now presented in Fig 3K.

Finally, please clearly label which figures are relatd to cells, or mice, or humans. Figures should be understandable at a glance.

We thank the Reviewer for this suggestion. We have now clearly labeled each panel for the source of the materials (patient, cells, xenografts).

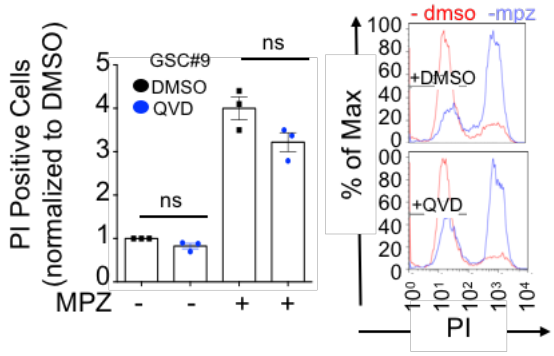
2) While it is commendable that several different lines of GSCs are used in this work, many times data is only shown for a reduced (or even single) and often inconsistent set of cells.

We apologize for this apparent inconstancy in the use of patient-derived cells. Most of the data were indeed obtained using GSC#9, including *in vivo* xenografts. We have now validated main findings with additional patient cells: this includes spheroid assays (Fig 1J, 2B-C), viability (Fig 1G-H, 2E, 2H, 4D, EV1E), NF-κB and MALT1 activity (Fig 3A-D), lysosome increase and autophagy (Fig 4D, 5C, EV2F), mTOR activity (EV3E), and QKI implication (7B, EV4A, EV4C, EV4E).

3) In SFig 3a, caspase inhibition does seem to rescue cell death in at least one of the two tested GSCs upon MPZ treatment - to exclude involvement of this canonical way of apoptotic cell death, more replicates also in the other GSCs should be performed and other assays (Annexin V or TUNEL) should be considered, given that the specific cell death route is a major point of the manuscript (or the argument should be tamed down). This should also be incorporated into the main figures.

This data is now incorporated in the main Fig 5G. Our careful examination of the statistics shows that none of the QVD treatments significantly rescued cell viability in both GSC#1 and GSC#9. We therefore have added 'ns' labels for clarity. To further investigate potential effects of caspase inhibition, we have performed PI incorporation experiments using QVD and MPZ (Fig 5H). While there is slightly less PI incorporation, it is not statistically significant. In comparison, lysosomal inhibitors significantly rescue cell viability, as measured by both Cell

TiterGlo and PI incorporation (Fig 5J). We therefore determine that canonical apoptosis is not the primary means by which GSCs die upon MPZ treatment.



4) While the authors nicely show altered endo-lysosome homeostasis in GSCs upon MPZ treatment, it remains unclear if GSCs show altered lysosomes *per se* or if the MPZ effect is specific to GSCs.

Whether the effect of MPZ is specific to GSCs is an interesting question we tried to address. To this end, MPZ was added to the ABC DLBCL-derived HBL1 cell line where MALT1 is constitutively active (Hailfinger et al, 2009). This led to a modest increase in lysosomes (Fig EV2E). Nevertheless, QKI was also found bound to MALT1 in these cells (Fig EV4E). Therefore, we cannot exclude that the effect of MALT1 on lysosomes may be present in other cells where MALT1 is constitutively activated.

In addition, we now discussed that GSCs exhibit an interesting vulnerability towards changes in lysosome content/abundance, reinforcing the concept that lysosome homeostasis could be an Achille's heel in GBM expansion and the basis for future treatments, as recently suggested by others (Shingu et al, 2016; Le Joncour et al, 2019).

5) In TEM images, arrows would help allocating subcellular structures

We have now added asterisks to denote Vacuoles (Blue) and Lysosomes (Red).

6) It would have been nice to have the lysosome-related phenotypes in the pharmacological setup confirmed genetically with a MALT1 knockdown

As per the Reviewer's suggestion, we now documented P62 accumulation and LC3 lipidation under siMALT1 settings (Fig 5C, 5F). We have also performed CTSD ELISA on siMALT1-transfected cell supernatants (Fig 5I).

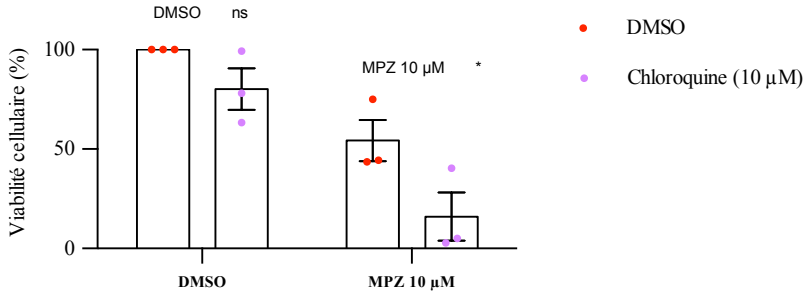
7) Co-localization and dispersion of mTOR and lysosomes in Fig4I does not look very convincing; alterations of mTOR activity due to (re-)localization upon MPZ treatment should be shown using additional methods or at least quantified in some way. Or the data should be eliminated, since they are not essential for the paper.

We feel that mTOR localization at the lysosomes and lysosomal positioning are well-admitted determinants for the canonical activity of this pathway (Korolchuk et al, 2011). Nevertheless, we have now added quantification for these data, which we hope makes the alterations in mTOR localization clearer (Fig 6K).

8) The role of autophagy in this anti-cancer scenario is still rather unclear - additional established autophagic flux assays using inhibitors like chloroquine or leupeptin could give more insight on that matter. Also it would be interesting to see in which way genetic/pharmaceutical activation or inhibition of autophagy affects GSC survival and lysosomal phenotypes in the setup of altered MALT1 activity or QKI expression.

As per the Reviewer's suggestion, we have now implemented additional assays to further investigate the intricacy of autophagy function in this context. We now dedicated a full figure to this matter (Fig 5), notably with P62 and LC3 immunostaining and immunoblots, in the setting of MALT1 inhibition (Fig 5A-5F, EV3B, EV3D) and QKI inhibition (Fig 7F, 7J). Blocking

autophagy did not rescue MALT1 inhibition (*please see below* and Fig EV3A-B), while lysosome drugs did so (Fig 5J). Overall, while MALT1 inhibition seems to trigger some autophagy flux defects, they may not be the primary cause of cell death engagement in this context. This is now discussed in the revised manuscript (please see page 13).



9) Is the interaction of MALT1 and the Endo-lysosomal regulator QKI specific to the pathological condition in GSCs? Does MALT1 interaction with QKI alter QKI activity? Colocalization studies of MALT1 and QKI in GSCs with and without MPZ would strengthen the shown immunoprecipitation data.

These are interesting points. We have performed co-immunoprecipitation experiments in HBL1 cells (an ABC DLBCL cell line with aberrant MALT1 activity) and were able to detect a QKI/MALT1 immunocomplex (Fig EV4E). This suggests that this interaction is not exclusive to GBM pathological condition, but might be rather associated with MALT1 basal activity and/or lysosome homeostasis. This will require further investigation.

We agree that it would have been interesting to evaluate whether the interaction between MALT1 and QKI modulate QKI activity. We feel these efforts would require a new investigation on its own, but we added this specific point in the discussion (please see page 14). We agree too that co-localization studies of MALT1 and QKI would strengthen immunoprecipitation data, however, in our hands four commercial anti-MALT1 antibodies cannot allow us to detect endogenous MALT1 by immunofluorescence in GSCs. We have, however, added immunoprecipitation data using Z-VRPR-FMK (Fig 7C) to further confirm the effect of MALT1 inhibition on MALT1/QKI interaction (please see our response to Reviewer #1, point #5). We also performed fractionation assays and found that a portion of QKI falls into the cytosolic fraction together with MALT1 (Fig EV4C).

10) While SFig4 suggests a pervasive expression of QKI, in Fig5 it seems to be exclusively expressed in the nucleus.

We agree with this statement, upon overexpression, QKI massively localizes into the nucleus, however, a closer look show some delicate staining into the cytosol, which matches the biochemical fraction data with endogenous proteins (Fig EV5C).

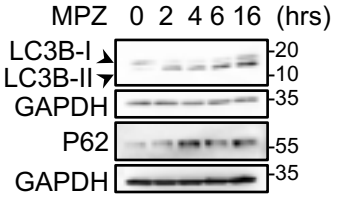
11) In Fig5h, MPZ treatment seems to actually reduce QKI levels per se, which somewhat contradicts the finding that high levels of QKI are associated with increased endo-lysosomes.

We agree too and we feel it might be due to the cell death commitment, we thus repeated this experiment using a lower, yet active, dose of MPZ (10 µM, Fig 7J).

12) Functionality of the increased lysosomes under MALT1 inhibition is still somewhat inconclusive, given the proposed early block in autophagic flux while lysosomes still seem functional. This can be addressed in more detail.

This Reviewer is correct. Functionality of lysosomes was indeed assessed via multiparametric analysis (DQ-OVA, Lysotracker, effect of Bafilomycin), which together suggests that despite the massive increase in LAMP2 expression and potential permeabilization (CSTD elisa), they do retain some of their hallmarks. Additionally, we have performed kinetics experiments (please see below), which do not allow to conclusively demonstrate whether autophagy flux defects precede lysosome increase, and *vice versa*. In

addition, we found that BECLIN1 siRNA does not alter MPZ-mediated death (Fig EV3B), while CQ did not change the effect of MALT1 inhibition (MPZ or siRNA) on LC3 lipidation (Fig 5C, 5D, and please see above our response point #8). We have thus clarified the text accordingly and now discuss on the interplay between lysosome phenotype with autophagy.



Referee #4

We would like to thank the Reviewer for his/her careful reading and examination of the manuscript, and who had acknowledged '*Overall the experiments were performed well, using human GSCs and clinically available drugs is also a strength. The novel findings including growth suppression of glioma cells by MALT1 inhibition and binding of MALT1 with QKI are interesting*.'

Major points

1) In Introduction, the authors described "Here, we repurpose several members of a family of drugs, phenothiazines, to disrupt GSC lysosomal homeostasis...". However, the rationale is not clear in Introduction why the authors had thought of using MALT1 inhibitors, phenothiazines. In addition, according to the authors' statement in Introduction, major aim of this study appears to be evaluation of repurposing of phenothiazines in glioma therapy. In Results, however, it appears that the authors' focus was primarily NF-κB signaling and then MALT1 signaling, and that discovery of lysosomal homeostasis impairment by phenothiazines is part of the consequences in MALT1 signaling study, rather than phenothiazine repurposing study. These sounds confusing. I think that the manuscript would be easier to follow if the authors clearly stated the authors' aim or hypothesis and described the results in a consistent manner regarding the aim/hypothesis.

We apologize for the inconsistency between our original introduction and results sections. We have now restructured the introduction to better reflect the aims and hypotheses consequentially.

2) I think that large part of experiments was performed with focus on MALT1.Therefore, providing background regarding MALT1 in Introduction would be helpful for readers.

We have now added a section about MALT1 to our introduction, as also suggested by Reviewer #1 (minor point #3).

3) In the first part of the results, the authors described that "Because the transcription factor NF-κB is instrumental in many cancers and because it centralizes the paracrine action of cytokines..." I think that more specific justification for studying NF-κB would be helpful for readers to understand the authors' initial aim/hypothesis. I wonder why the authors focused on NF-κB pathways among multiple signaling pathways that are thought to be important in neural stem cell niches.

We have now added more specific justification to the introduction and result sections for our selection of NF-κB signaling, in the context of non-oncogene addiction. We have also added one panel to better describe the reported interaction within this network (Fig 1A).

4) Association between MALT1 expression and glioma grades has already been reported (Yang et al., 2017). This article also appears to suggest possible contribution of MALT1 to malignant behavior of glioblastoma. I think that the authors should appropriately cite articles regarding glioma and MALT1 and provide sufficient background information.

We apologize for this oversight and have now cited this article. "*Moreover, levels of MALT1 RNA are elevated in GBM (Grade IV) when compared with lower grade brain tumors (Grades II and III) (Yang et al., 2017) or non-tumor samples.*" Nevertheless, it has to be noted that this paper is essentially based on established cell lines (U87), in which NF-κB status and function might differ from patient-derived primary cells.

5) Apparently, the MALT1 inhibitor has a much stronger and complicated functions than MALT1 knockdown does. In the Figure1, knockdown of MALT1 reduces the GSC expansion but not survival, however, in the Figure2 the inhibitor affects the viability of the same cell lines. These results indicated that the inhibitor might have additional function beyond the MALT1 itself. Therefore, the RNA sequencing data in the Figure4 might not correctly represent the function of MALT1 in GSC.

In fact, further testing of cell viability in cells knocked down for MALT1 has demonstrated increased PI incorporation in both of the tested cell lines (GSC#1 and #9) (Fig 1H), in addition to reduced proliferation, as reported by EdU incorporation in the original manuscript (Fig 1G), therefore the reduction in proliferation we see may be a consequence of cell death similar to what we show upon MPZ treatment. Please see also our response to Reviewer #1, main point #3.

Moreover, we have now validated the RNAseq data (Fig EV3C) by qPCR for 9 out of 10 tested up-regulated or down-regulated genes found in MPZ-treated conditions. In addition, we also showed that the overall phenotype (cell death, lysosomal increase, autophagy induction, reduced mTOR signaling, and lysosomal position) was validated in both pharmacological, molecular and knockdown settings.

6) When talking about of the blocking MALT1 protease activity, what does it exactly mean? Does it mean that MALT1 regulates the GSC endo-lysosome activity going through its proteolytic activity? Does MALT1 regulates mTOR activity dependent on this activity? Does a catalytically inactive MALT1 mutant recapitulate the phenotype found in this manuscript?

This is an interesting point also raised by the Reviewer#2 (please see also our answer to his/her point #1). First, the use of Z-VRPR-FMK catalytic inhibitor recapitulates our findings obtained with mepazine treatment and MALT1 siRNA, in terms of spheroid assays (Fig 2), CYLD processing (Fig 3), lysosome phenotype (Fig 4, 5), and mTOR positioning (Fig 6). Also, our new Figure 3 militates for a constitutive protease activity of MALT1 in GSCs. Second, catalytically inactive MALT1 protease dead mutant (C464A) recapitulates Z-VRPR-FMK and phenothiazine action over mTOR activity (Fig 6J), CYLD processing (Fig 3G) and lysosome abundance (Fig 4D).

7) Regarding the interaction of MALT1 and QKI, as we know QKI has several isoforms, which isoform is the dominate one interacting with MALT1? Where do the authors think that the functional interaction will take place - nuclear or cytoplasm? In the Fig.5, QKI can bind MALT1, and also BCL10, which indicates that QKI may form the complex with MALT1 and BCL10, does this indicate that MALT1 works as a scaffold instead of the proteinase with QKI?

QKI indeed has several isoforms; QKI-5 is exclusively nuclear, QKI-7 is exclusively cytoplasmic, whereas QKI-6 can shuttle between cytoplasm and nucleus (Darbelli et al, 2016). We have now performed cell fractionation experiments in GSC#1 and #9 to characterize localization of QKI and MALT1 (Fig EV4C). MALT1 remains exclusively in the cytoplasm with or without MPZ treatment, excluding theoretically the possibility of an interaction with QKI5. It is thus most likely then than MALT1 and QKI interacts within the cytoplasm. However, we cannot distinguish whether QKI6 and/or QKI7 interacts with MALT1 from our data. It might be interesting in the future to detail QKI isoforms and activity in this context.

With regards to the MALT1/BCL10/QKI complex, we performed immunoprecipitation

after treatment with Z-VRPR-FMK to block directly the protease activity (Fig 7C) (please see also our answer to Reviewer #1, point #5). In this setting, MALT1 binding to QKI was reduced, suggesting that the protease activity is important for this interaction to occur.

8) Growth inhibitory effects of some of drugs in Supplementary Fig 2 on glioma cells have been reported (reviewed by Tan et al., 2018, for example). I think that authors should mention those previous studies.

We apologize for this oversight and have now cited these studies.

9) In Fig 1e, I wonder if non-tumor control cells are available.

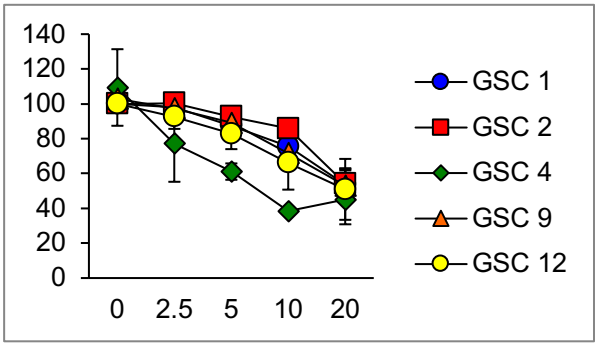
Please see our answer to Reviewer #2, specific point a. We have looked at MALT1 expression in GSCs compared to neuron-like cells (from neuroblastoma), fetal human astrocytes and human brain endothelial cells. However, there was no significant change in BCL10/MALT1 total protein expression. We have therefore removed this panel from our revised manuscript.

10) Regarding the evaluation of NF-κB activation in GSCs, I think that examining not only IκB but also NF-κB subunits that activate transcription might be useful.

As per the Reviewer's comments, we have now examined other NF-κB subunits (Fig 3B) and do not see much activation, in basal conditions, without TNFα stimulation, reinforcing the early observation with IκB.

11) Providing more background information of MPZ, including target specificity, would be helpful for readers. Especially, I wonder if 20 uM MPZ would have off-target effect.

We agree with the Reviewer and this is now added in the revised text (page 7). The 20μM dose of MPZ is consistent with the one used in previous studies (Nagel et al, 2012). Furthermore, a pilot dose response study in 5 patient-derived GSCs revealed that this was the lowest dose to induce more than 50% reduction in cell viability (please see below).



12) Given efficient transition of phenothiazines through blood brain barrier, justifying concentrations of these drugs used in this study would be convincing.

Doses for phenothiazines *in vitro* were determined using a dose response curve (Fig 2I), which showed that those which were killing did so in a similar range to MPZ. Furthermore, *in vivo* dosing of MPZ (8mg/kg) was half the dose used in Nagel et al (Cancer Cell 2012). We chose to use the dose determined by McGuire et al (J Neuroinflammation 2014). This information is now mentioned in the method section.

13) In Fig 2g, which band is SOX2? Is differentiation induction successful?

We apologize for this initial blot that we have removed for sake of clarity. Differentiation can be indeed better appreciated at the morphological level (Fig 2H) and from our previous characterization (Le Guelte et al JCS 2012, Treps et al Oncogene 2016, Harford-Wright et al Brain 2017).

14) In Fig 3a, indicator of vacuoles would be helpful for readers. I also think that showing

quantitative data would be convincing. In addition, the interpretation of this finding appears to be missing in the text.

We have now included asterisks for vacuoles (Blue) and lysosomes (Red), in the EM analysis from MPZ- and siMALT1-treated cells (Fig 4A, EV2A). The interpretation of EM remains qualitative, while deeper characterization and quantification have been achieved with immunofluorescence-based assays. This is now clarified in the revised manuscript.

15) Regarding Fig 3b, was increase of vacuoles induced by siMALT1 treatment?

We have now included an additional image where vacuole occurrence is more obvious in si*MALT1* conditions (Fig EV2A).

16) I wonder what is indicated by "endo-lysosomal". In most part of text, "endo-lysosomal" is used while "lysosomes" is used in Supplementary Fig 4i. I wonder how they are differently used.

We have now amended the text to stick to the term endo-lysosomes as it is based essentially on LAMP2 expression.

17) Regarding "endo-lysosomal" protein, I wonder if LAMP2 alone is sufficient to show "endo-lysosome". I also wonder if LAMP1 or late endosome marker such as RAB7 would show the same trend of alteration with LAMP2.

For sake of accuracy, we have substituted the term ‘endo-lysosome’ for LAMP2 when describing results obtained with LAMP2 immunofluorescence and western-blot. We have now included western-blots for RAB7 upon treatment with MPZ, Z-VRPR-FMK, and knockdown of MALT1 (Fig EV2C). RAB7 expression level follows similar trend than LAMP2. Interestingly, GSCs do not seem to express LAMP1 (western-blot and immunofluorescence), but we thought that this specific mention on LAMP1 was too preliminary to be included in the manuscript. In addition, we have used DQ-OVA (Fig EV2F) and Lysotracker (Fig 4G) to monitor endo-lysosomal compartments.

18) Regarding Supplementary Fig 2a, data of cell death and lysosome were obtained using GSCs but MALT1 activity data were from Jurkat cells. Showing MALT1 activity data using the corresponding GSCs would be informative.

This is a good point. We have added the MALT1 activity data concerning GSCs (Fig EV1C).

19) Regarding Supplementary Fig 2a, MALT1 inhibition activity appears to correlate with cell death. However, lysosome increase might not correlate with cell death or MALT1 inhibition activity, suggesting possible lysosome-independent mechanism by which MALT1 inhibition induces cell death.

Although all phenothiazines show increase in LAMP2 expression (Fig EV1D), the best inducers of cell death (Fig 2I) have more accumulation of lipidated LC3 which suggests an impaired autophagic flux in these treatments. Because it might be difficult to compare the extent of cell death as measured by Cell TiterGlo assays to lysosome increase evaluated by western-blot, we have thus removed the relative (+/-) classification in Table EV1A (please see also our answer to Reviewer#1, point #3). As cited in the discussion (please page 13-14), flux inhibition is often a downstream effect of LMP, which suggests lysosomal involvement in death.

20) Regarding Fig 3m, I wonder how Baf rescues cell viability against MPZ induced cytotoxicity. Given that MPZ could increase lysosomal membrane permeability and consequently induce lysosomal enzyme leakage that could induce cytotoxicity, how could V-ATPase inhibitor rescue this cytotoxicity.

We apologize for the lack of clarity in the initial version of the manuscript. In order for lysosomal enzymes to mature, acidification of the lysosome is required (Aits et al, 2015). In keeping with this, bafilomycin can reduce lysosomal cell death (Fig 5J) by preventing lysosomal enzyme acidification and therefore maturation. Bafilomycin appears to block

lysosomal cell death rather than membrane permeability specifically.

21) In Fig 4f, data of LAMP1 and LAMP2 are missing.

We actually left LAMP2 off, as we did not want to complexify the message of this figure more dedicated to mTOR activity. However, the effect of TFEB knockdown on LAMP2 can be appreciated with immunofluorescence (Fig EV3H).

22) In Fig 4g, it appears that 50 nM rapamycin suppresses mTOR-S6K-S6 pathway as effectively as 20 uM MPZ dose. However, Supplementary Fig 4b shows remarkable difference of cytotoxicity between MPZ and RAPA. I wonder if there is a major contributor in MPZ-induced cytotoxicity other than mTOR signaling inhibition.

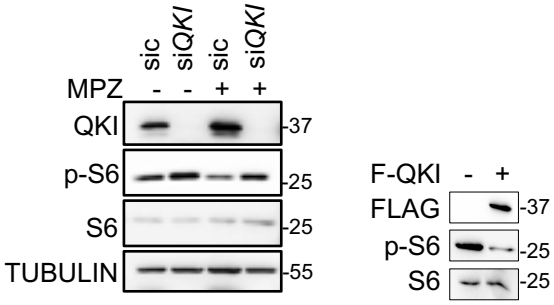
We agree with this Reviewer that mTOR signaling inhibition is most likely not the major cause of death. Instead, our data support the concept that the increased in lysosomes and LMP are instrumental to MALT1 inhibition-provoked cell demise (Fig 5J). For sake of clarity, we have removed this initial panel.

23) In Fig 5e and 5f, displacement of mTOR from lysosomes is not directly shown.

We agree with the Reviewer. However, we could not experimentally triple-stained for LAMP2 (IgG1), mTOR (rabbit) and Flag (IgG1); lysotracker cannot be used either because of the saponin treatment prior fixation. We have thus rephrased the text as follows "*mTOR dispersion from a focalized organization*".

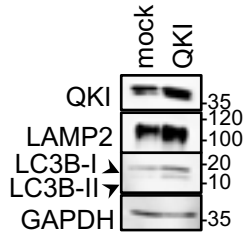
24) Regarding QKI overexpression and knockdown experiments, showing alterations of downstream molecules to mTOR, such as p-S6K or p-S6 would be convincing.

We thank the Reviewer for this suggestion. We have now performed western-blot experiments on p-S6 levels in cells expressing ectopic QKI (Fig 7H). Conversely, we have also added a western-blot of p-S6 in QKI silenced cells (Fig 7L).



25) I wonder if QKI overexpression increases lysosomal membrane permeability and leakage of lysosomal enzymes.

We agree with the Reviewer that this is an interesting point. However, this is quite technically challenging, as QKI overexpression is rapidly toxic for cells (Fig 7I) and that CSTD ELISA is done on collected supernatants from cells prior to signs of cell death. However, short-time QKI overexpression did trigger increase in LAMP2 staining and expression (Fig 7E-F), and a slight but reproducible LC3B lipidation, strongly suggesting lysosomal dysregulation.



26) I wonder if alteration in MALT1-QKI-lysosome axis is critical in MALT1 inhibition-induced cell death. In Fig 4, the authors showed inhibition of AKT activation by phenothiazines and siMALT1. Suppression of AKT could induce persistent activation of TSCs, resulting in inhibition of recruitment of mTORC1 to lysosomes (reviewed by Lawrence et al, 2019, for example). In addition, AKT is known to promote cell proliferation and survival via multiple signaling pathways independent to mTOR. Therefore, AKT signaling pathways could be important contributors to observed phenotype in this study. I think that examining or at least discussing those possible causative mechanisms that have already been reported would be useful to show the significance of the findings in this study.

The Reviewer is correct. Alteration in MALT1-QKI-Lysosomal signaling axis is critical for inhibition induced death. This was shown by the rescue of cell viability when lysosomal enzymes were inhibited (Fig 5J). Furthermore, knockdown of QKI significantly rescued MALT1 silencing-induced cell death (Fig 7M, 7N), highlighting the central role of MALT1/QKI. We now discussed that AKT can be instrumental to balance between proliferation and apoptosis by integrating multiple signaling network besides mTOR in GBM (please see discussion on page 13).

27) Regarding the authors' description "These results suggest that MALT1 affects lysosomal homeostasis post-transcriptionally, and that this increase coincides with weak mTOR signaling, which may be due to displacement of mTOR from its lysosomal signaling hub", I wonder what "this increase" is.

We apologize for our lack of clarity. We have now amended the text to read "*the increase in endo-lysosomes…*".

28) According to proposed working model, MALT1 binds and inhibits QKI that downregulates lysosomes, resulting in lysosomal homeostasis and mTOR activation, while MALT1 inhibition enables QKI to bind its targets and increase lysosomes but LMP and LCD are also increased. I wonder why downregulation of lysosomes could activate mTOR and why QKI activation affects LMP. More specific discussion would be useful.

This is an important point. The mTOR pathway has been shown to be highly activated in GBM tumors, which often have a PTEN deletion and/or EGFRvIII mutation (Verhaak et al, 2010). Moreover, in the recent study (Shingu et al, 2016), which identified QKI as a lysosomal regulator, authors suggest that down-regulation of lysosomes reduces receptor recycling of EGFR which allows signaling to continue even in unfavorable niche, where GSCs often reside and/or travel. Less turnover of EGFR may partially explain increased mTOR activation despite lysosomal downregulation. This is now discussed in the revised manuscript on page 14. We also decided to omit our initial working model, which might appear preliminary.

With regards to LMP, it appears to be a consequence of dysregulated lysosomal increase. QKI was shown to directly bind to lysosomal RNAs (Shingu et al, 2016). One can hypothesize that upon MALT1 inhibition QKI may tether and stabilize its known lysosomal targets and therefore biases the system toward lysosomal translation. In order to address how exactly LMP is induced, we would need to analyze the composition of newly formed lysosomes. Although of high interest, this specific point falls beyond the scope of the current study.

As per the Reviewer's suggestion, we have altered the discussion to better clarify this point *"It is thus tempting to speculate that QKI-dependent stabilization of lysosomal RNAs would preference the system towards more translation of these genes upon MALT1 inhibition, leading to dysregulated lysosome expression and LMP*."

Minor points

1) In Supplementary Fig 3a, QVD appears to partially rescue cell viability in GSC#9 treated with 10 uM MPZ. I wonder if this is statistically significant and if apoptosis is suggested.

Please see our answer to Reviewer #3, point #3.

2nd Editorial Decision 11th Oct 2019

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your amended study was sent back to the referees for re-evaluation, and we have received input from all of them, which I enclose below.

As you will see the referee finds that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some issues are conclusively addressed.

Please consider the additional minor issue expressed by referees #2 and #4 related to appropriate discussion of the findings, citations and see whether you would be able to add additional data to consolidate the findings, or alternatively introduce caveats where appropriate.

REFEREE REPORTS:

Referee #1:

The authors have done a very good job in addressing the comments of all referees and added several new experiments that further support the conclusions. The manuscript has thus significantly improved.

Referee #2:

The authors have carefully addressed my comments and provided new data that support a critical role of MALT1 protease activity in glioma cell survival. Before I can fully recommend publication, the authors should consider the following points:

1) The data on MALT1 cleavage inhibition by different phenothiazines presented in Fig. EV1B and EV1C are not consistent. I think the technical quality of the CYLD WB in EV1C is not sufficient, because CYLD FL and cd are decreasing from left to right in a very similar manner. It looks like an uneven loading/blotting and the blot needs to be repeated.

2) By using the MALT1 E397A mutant, the authors have elegantly shown that the effect of MPZ and other phenothiazines on GSC viability requires binding to the MALT1 allosteric side. Importantly, accumulation of LAMP2 and increased LC3B lipidation was detected after siRNA knock-down of MALT1 and BCL10. However, with regard to endo-lysosomal homeostasis, there are issues with the MALT1 inhibitors used in this study and this needs to be clearly mentioned in the Discussion:

a) All phenothiazine derivatives are affecting LAMP2 and LC3B-II increase to a very similar degree, which is very difficult to reconcile solely with their inhibitory effect on MALT1. A clear structure activity relationship (SAR) has been established between MALT1 and the phenothiazine mepazine (MPZ) and thioridazine (PMID: 23946259), which involves right positioning of the N-methylpiperidine nitrogen in MPZ and E397 of MALT1 to allow hydrogen bonding. Other phenothiazines like fluphenazine (FLU) or chlorpromazine (CHLO) nicely fit into this spacing of 4 methyl-groups outside the tricylic ring. Actually, the highly analogs compounds perphenazine and promazine (IC50 3.8 µM) have been shown to inhibit MALT1 (PMID: 23238017). However, promethazine (PRO) was shown to be a very poor MALT1 inhibitor with an in vitro IC50 of ~170 µM, which is more than 300 fold higher compared to MPZ (IC50 ~0.5 µM) (PMID: 23946259). The nitrogen outside the tricyclic ring system in PRO is improperly spaced (3 methyl-groups) and will not allow hydrogen bonding with E397 in MALT1. In line, PRO is not inhibiting CYLD cleavage in Jurkat T cells (Fig. EV1B) and I have some doubts that it does so in GSC#9 (see point 1). However, PRO is increasing LAMP2 expression and accumulation of lipidated LC3B-II to the same extent as

MPZ (Fig. EV1D), which clearly argues against a purely MALT1 protease dependent mechanism.

b) The irreversible tetra-peptide MALT1 inhibitor VRPR-FMK was used to confirm the results. However, VRPR-FMK is not a selective MALT1 inhibitor, but is nearly as efficient in inhibiting lysosomal Cathepsin B in vitro and in cells (PMID: 25556945). Especially with respect to endo-lysosomal homeostasis, this may be a relevant off-target effect. Also, several publications have shown that Cathepsin B participates in the processes of glioma tumor growth.

The authors need to consider and clearly mention such constraints in the Discussion. It does not argue against the use of these or other MALT1 inhibitors, but a critical discussion needs to be included.

3) Carefully check and revise the references. Schlauderer et al. 2013 is cited on page 7, but not in the list. What is the reference Ngo et al., 2019 on page 7 and in the reference list on page 28? I don't think it exists. Nat Rev Immunol is Ruland and Hartjes, but maybe not the right citation for MPZ models. There are a number of other references for the in vivo use of MPZ as MALT1 inhibitor, e.g. PMID: 29367251, PMID: 31138793, PMID: 31092922.

4) Figure 3F on page 9 should be 4F.

Referee #3:

No additional comments.

Referee #4:

I think that the manuscript was restructured and is now easier to follow than the previous form. My concerns include background information of MALT1 in glioma, LMP, dissipation/dispersion of mTOR from LAMP2, and roles of AKT.

1) Regarding description in Results "Moreover, levels of MALT1 mRNA are elevated in GBM (Grade IV) when compared with lower grade brain tumors (Grades II and III) or non-tumor samples (Yang et al, 2017) (Fig 1D-E)", I think that it is not clear what the citation means.
2) Regarding Fig 3C and "basal protease activity of MALT1", I wonder if non-tumor cells (control) are available.
3) I wonder if increase of extracellular cathepsin D (Fig 5I) is an appropriate indicator of LMP. Mechanism of secretion of lysosomal enzymes to extracellular space (Dragonetti et al., 2000, Liu et al., 2012, for example) might be different from that of leakage of lysosomal enzymes into cytosol in LMP.
4) Regarding mTOR dispersion/dissipation from LAMP2, I wonder if the IF findings really indicate dissipation or dispersion of mTOR from LAMP2. Increase of LAMP2 could cause relative decrease of colocalization of mTOR and LAMP2, but it does not necessarily mean dissipation of mTOR. Similarly, if increase of LAMP2 could affect tM1's coefficient, lower tM1's coefficient does not necessarily mean disperse of mTOR from LAMP2 by the treatments.
5) Regarding discussion of AKT signaling, I think that more specific discussion regarding possible roles of AKT signaling in mTOR activation and colocalization of mTOR and lysosome would be helpful for readers because authors showed that siMALT1 and MPZ decreased p-AKT.
6) In Fig 7M, I wonder how to interpret the data.
7) In Fig EV2C, GAPDH picture might not be correctly placed.

2nd Revision - authors' response 16th Oct 2019

The authors performed the requested editorial changes.

3rd Editorial Decision 25th Oct 2019

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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: Julie Gavard
Journal Submitted to: The EMBO JOURNAL
Manuscript Number: EMBOJ-2019-102030

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?	Sample size was chosen based on previous experiments. The number of independant experiments and type of statistical analyses are mentioned in the figure legends
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size was chosen based on previous experiments. Details about statistics can be found in the figure legends
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusions were made
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.	Yes animals were randomly assigned to each group and groups were mixed within cages.
For animal studies, include a statement about randomization even if no randomization was used.	Yes animals were randomly assigned to each group and were mixed within cages
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.	no
4.b. For animal studies, include a statement about blinding even if no blinding was done	no blinding was done
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.	Yes ANOVA and T test were used. Detailed information can be found in statistics section of materials and methods and in the figure legends.
Is there an estimate of variation within each group of data?	N/A
Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

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).	all antibodies were mentioned in the Materials and Methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were specified in the Materials and Methods section

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

D- Animal Models

USEFUL LINKS FOR COMPLETING THIS FORM

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http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

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.	Female Balb/C nude mice aged 6 to 7 weeks
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal procedures were conducted as outlined by the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (ETS 123) and approved by the French government (APAFIS#2016-2015092917067009)
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.	we confirm compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/A
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.	n/A
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.	N/A

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	RNA sequencing data is provided in EV Table 1 and raw files are available at GEO #GSE139018. Details about analysis of TCGA can be found in the Materials and methods section. All plasmids and primer sequences were provided in Materials and methods section.
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).	EV table 1 provides all data from RNA sequencing (GSE139018)
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).	N/A
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.	N/A

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.	N/A