EMBO Molecular Medicine

Synthetic lethality between VPS4A and VPS4B triggers an inflammatory response in colorectal cancer

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 15 May 2019

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript.

As you will see from the reports below, both referees mention the interest of the study. However, they also raise substantial concerns on your work, which should be convincingly addressed in a major revision of the present manuscript. In particular, both referees insist on including the effects of VPS4A inhibition in cancer cell lines, and on further increasing the clinical relevance of the manuscript by testing VPS4B loss/partial loss and improving the discussion.

Addressing the reviewers' concerns in full will be necessary for further considering the manuscript in our journal, and acceptance of the manuscript will entail a second round of review. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

The experiments are performed well and support the conclusions drawn in the manuscript. However, lethality induced by combined inhibition of two paralogs is not novel and has been observed before for other pairs of paralogs. Although the authors suggest that a large fraction of colorectal cancers have lost or reduced expression of VPS4B, they do not provide any examples of such CRC cell lines and their dependency on VPS4A. In addition, they do not test the inhibition of VPS4A and B in normal cells. As such it is difficult to conclude that VPS4B low or null CRC tumors are specifically

lethal to VPS4A inhibition. This is even more concerning when an VPS inhibitor is used that inhibits both VPS4A and B proteins.

Referee #1 (Remarks for Author):

The consequence of depletion of VPS4A and B on cell proliferation and survival is interesting and well documented. However, the claim that this supports the increased sensitivity of VPS4B low or null CRC is not substantiated due to the absence of experiments in CRC tumor cell lines with low or no VPS4B expression. If the authors would like to extrapolate the findings of combined knockdown, these experiments should also be performed in different normal cell lines, preferable in vivo to show the reduced of absence of effects. This is particular important when no specific inhibitors can be developed.
It is surprising that the data in the DRIVE database indicate that SK-CO-1 cells are the most sensitive cell line to VPS4A as well as VPS4B depletion. In addition, one can observe a correlation between the viability scores in the cell line panel for VPS4A and B.
One other point is the potential correlation between proliferation and VPS4B expression. Is the expression of VPS4B affected by proliferation rate of cell cycle progression?

Referee #2 (Comments on Novelty/Model System for Author):

The idea of targeting passenger mutations is still quite novel and interesting which is why I have rated technical and novelty highly. They seem to have done a good job identifying a synthetic lethal pair that could impact a subset of CRC patients but the medical impact is unknown at this point. It is probably a long way from clinical implementation but its a good idea.

Referee #2 (Remarks for Author):

In this manuscript the authors exploit coincidental loss of VPS4B in colorectal cancer as a biomarker to predict and test VPS4A depletion as a synthetic lethal strategy for this subset of cancers. Loss of the two VPS4 paralogues caused synthetic lethality in cell line and xenograft models. The manuscript characterizes the observed synergy with respect to gene expression changes, cell death mechanisms, and the potential to elicit an inflammatory response. Overall, the promise of targeting presumed 'passenger' mutations like VPS4B has been underexploited and this study represents an interesting and important addition to this field. The manuscript is quite thorough, the data quality look good, and the work tests important mechanisms of cell killing and immune stimulation. I have some suggestions to improve the manuscript below.

On Page 5, the authors propose to use SK-CO-1 cells as a model for a cell line with native loss of VPS4B but then find that the model is not appropriate for this purpose? Why do they include this section at all? It would be better to remove it, or screen other cell lines with natively low or lost VPS4B.

The rationale for looking at gene expression changes by RNA-seq is not clear because VPS4-depleted cells would be expected to have defects in secretion or the endomembrane system. The authors seem to observe a gene expression program consistent with dying cells, which is good, but does not add much to the mechanism of lethality. Did the authors look directly at secretory defects in the double-depleted VPS4A/4B cells?

The finding that both caspase-dependent and independent pathways lead to cell killing in the VPS4A/B depletion model is good but I thought could be framed in the text as eliminating potential resistance mechanisms (i.e. upregulation of anti-apoptotic factors seen in some cancers would NOT make them inherently resistant to this approach).

It was not clear in what genetic contexts the authors think this approach would be effective. The authors claim that 70% of CRCs have loss of VPS4B, but that only 2% have biallelic loss. Their model systems have a complete VPS4B loss, so would the synthetic lethality only be effective in the 2% with biallelic loss, or would there be some efficacy of VPS4A knockdown in the 70%? I felt that the manuscript should be explicit about this, and perhaps test partial VPS4B loss for the efficacy of

VPS4A-knockdown mediated killing. The future clinical impact and use of VPS4B as a biomarker depends on a clear statement here.

Minor points:
On page 10, the authors state that VPS4A and 4B are functionally redundant. I recommend saying 'partly' or 'partially' redundant since their own analysis (e.g. RNA-seq) shows they are not full redundant.
The grey inset images in Figure 2 are not very useful or easy to see.

Figure 2. What is the rationale for using a one-sample T-test?

Figure 3. I did not understand why the authors showed the right panel on Figure 3b. Are these just the endpoint values? Why is there no indication of statistical significance?

1st Revision - authors' response 17 October 2019

Referee #1
The experiments are performed well and support the conclusions drawn in the manuscript. However, lethality induced by combined inhibition of two paralogs is not novel and has been observed before for other pairs of paralogs. Although the authors suggest that a large fraction of colorectal cancers have lost or reduced expression of VPS4B, they do not provide any examples of such CRC cell lines and their dependency on VPS4A. In addition, they do not test the inhibition of VPS4A and B in normal cells. As such it is difficult to conclude that VPS4B low or null CRC tumors are specifically lethal to VPS4A inhibition. This is even more concerning when an VPS inhibitor is used that inhibits both VPS4A and B proteins.

Referee #1 Remarks for Author
1. The consequence of depletion of VPS4A and B on cell proliferation and survival is interesting and well documented. However, the claim that this supports the increased sensitivity of VPS4B low or null CRC is not substantiated due to the absence of experiments in CRC tumor cell lines with low or no VPS4B expression (*issue raised also by Referee #2).*

We do agree with the opinion of both Reviewers that our observation on increased vulnerability of *VPS4B* knockout cells to VPS4A depletion should be confirmed in non-engineered cancer cell line(s) with native loss (or partial loss) of *VPS4B* expression. To identify such cell lines we used datasets from the Dependency Map (DepMap) portal (https://depmap.org/portal/) that were updated after the initial submission of our manuscript (May 2019). This portal systematically catalogs genetic vulnerabilities in human cancer models (currently above 600) identified in genome-scale CRISPR/Cas9 and RNAi screens performed as a part of the following projects: Broad's Project Achilles (Broad Institute, USA), Novartis' Project DRIVE (Novartis Institutes for Biomedical Research, Switzerland) and Sanger's Project Score (Wellcome Sanger Institute, UK).

According to this portal, *VPS4A* (as well as *VPS4B*) are "strongly selective genes", meaning that certain cell lines demonstrate distinctive vulnerability to the perturbation of their expression across the panel of over 500 cancer cell lines tested. Importantly, this observation was cross-validated in a number of screens independently of the approach for gene perturbation (CRISPR/Cas9 vs. RNAi) applied. Specifically, in CRISPR/Cas9 screens 141 cell lines out of 625 tested were sensitive to the perturbation of *VPS4A* expression, while in RNAi screens these were 38 lines out of 550 tested. Moreover, genetic characterization of the investigated cell lines revealed that those most vulnerable to VPS4A depletion had a decreased copy number of the *VPS4B* gene (new Fig 2C). Yet another study, published in April 2019, listed *VPS4A* as one of priority cancer drug targets, based on the data from CRISPR/Cas9 screens combined with the genetic characterization within the Sanger's Project Score (Behan et al, Prioritization of cancer therapeutic targets using CRISPR-Cas9 screens. *Nature* 568:511). Altogether, these very recent data lend strong independent support to our conclusion that targeting the VPS4A activity is a promising target for precision oncotherapy.

To identify and validate a cancer cell model with low or null *VPS4B* expression, as requested by the Reviewers, we compared the top dependency scores of cell lines selected in various screens. We assumed that cells with complete or partial loss of *VPS4B* rely on VPS4A activity but probably are not or less sensitive to VPS4B perturbation. Thus, we aimed to fish out cell line(s) with an altered *VPS4B* gene copy number and differential dependency scores for *VPS4A* and *VPS4B*. For

further experiments we chose HOP62 and SNU410 cell lines (lung and pancreatic cancer, respectively). These cell lines had been tested in at least two independent screens, consistently reaching low *VPS4A* dependency score (CERES or DEMETER2 lower than -0.5, where score of -1 corresponds to the median of all common essential genes) and relatively high *VPS4B* dependency score (CERES or DEMETER2 higher than -0.5, the score of 0 is equivalent to a gene that is not essential) (new Fig EV2C). Importantly, in SNU410 cells *VPS4A* was scored among the top 10 preferentially essential genes (genes with the lowest dependency scores among all genes tested in a given cell line) in both CRISPR/Cas9 and RNAi screens according to DepMap. In HOP62 cells, *VPS4A* was identified among top essential genes only in CRISPR/Cas9 screens.

Having selected the two cell lines, we first confirmed the decreased number of *VPS4B* alleles in SNU410 and HOP62 cells (new Fig 2D). We also verified low VPS4B protein abundance in lysates of these cells in comparison to other cancer cell lines (new Fig 2E). Finally, we did confirm that RNAi depletion of VPS4A in SNU410 and HOP62 cells (new Fig EV2D) suppressed by over 40% their viability (new Fig 2F, G). Moreover, in case of HOP62 cells their clonal growth was also reduced (SNU410 cells did not exhibit clonal growth), as shown in new Fig 2F (right panels).

Cumulatively, we demonstrated that HOP62 and SNU410 are cell models with partial loss of *VPS4B* that exhibit increased dependency on *VPS4A*. These models might be used for further studies on the synthetic lethality between VPS4 paralogs in cancer.

2. If the authors would like to extrapolate the findings of combined knockdown, these experiments should also be performed in different normal cell lines, preferable in vivo to show the reduced of absence of effects. This is particular important when no specific inhibitors can be developed.

As requested, we analyzed the proliferation rate of two different non-tumor cell lines: CCD-841CoN and CCD-1070Sk (colon epithelium and skin fibroblasts, respectively) upon single and double knockdown of VPS4 paralogs (Figure 1 provided for the Reviewer below). Both cell lines are diploid and express *VPS4A* and *VPS4B* (new Fig 2E and Figure 1 provided for the Reviewer, A and B, left panels). In both cell lines we obtained very efficient siRNA-mediated silencing of *VPS4B* expression and quite efficient silencing of *VPS4A* (Figure 1 provided for the Reviewer, A and B, left panels). As a result, we confirmed that none of these cell lines were sensitive to single depletion of VPS4A or VPS4B (similarly to cancer cell lines with unperturbed *VPS4A* and *VPS4B* expression, e.g. HCT116, DLD1) (Fig 1 provided for the Reviewer, A and B, right panels). In turn, simultaneous depletion of both VPS4 paralogs severely decreased cell viability of normal cells as a consequence of irreplaceable functions of these proteins in maintaining cellular homeostasis. Based on our data, we believe that cells with unperturbed expression of *VPS4A* and *VPS4B* have some surplus of VPS4 activity that could be diminished (by siRNA or an inhibitor) without negative impact on cell growth. If this is true, then an inhibitor targeting both VPS4 paralogs when used in chemotherapy would first affect the growth of those cells that have low expression of one of VPS4 paralogs (in some patients these would be cancer cells with *VPS4B* loss).

By all means, we are aware that our conclusions are drawn on the basis of short-term *in vitro* studies on a limited number of cell lines and as such may not detect potential side effects that would be generated in a complex organism. However, in our opinion, to precisely evaluate the potential benefits and risks of using a VPS4 inhibitor in therapy, *in vivo* mouse studies should be performed once such an inhibitor is developed.

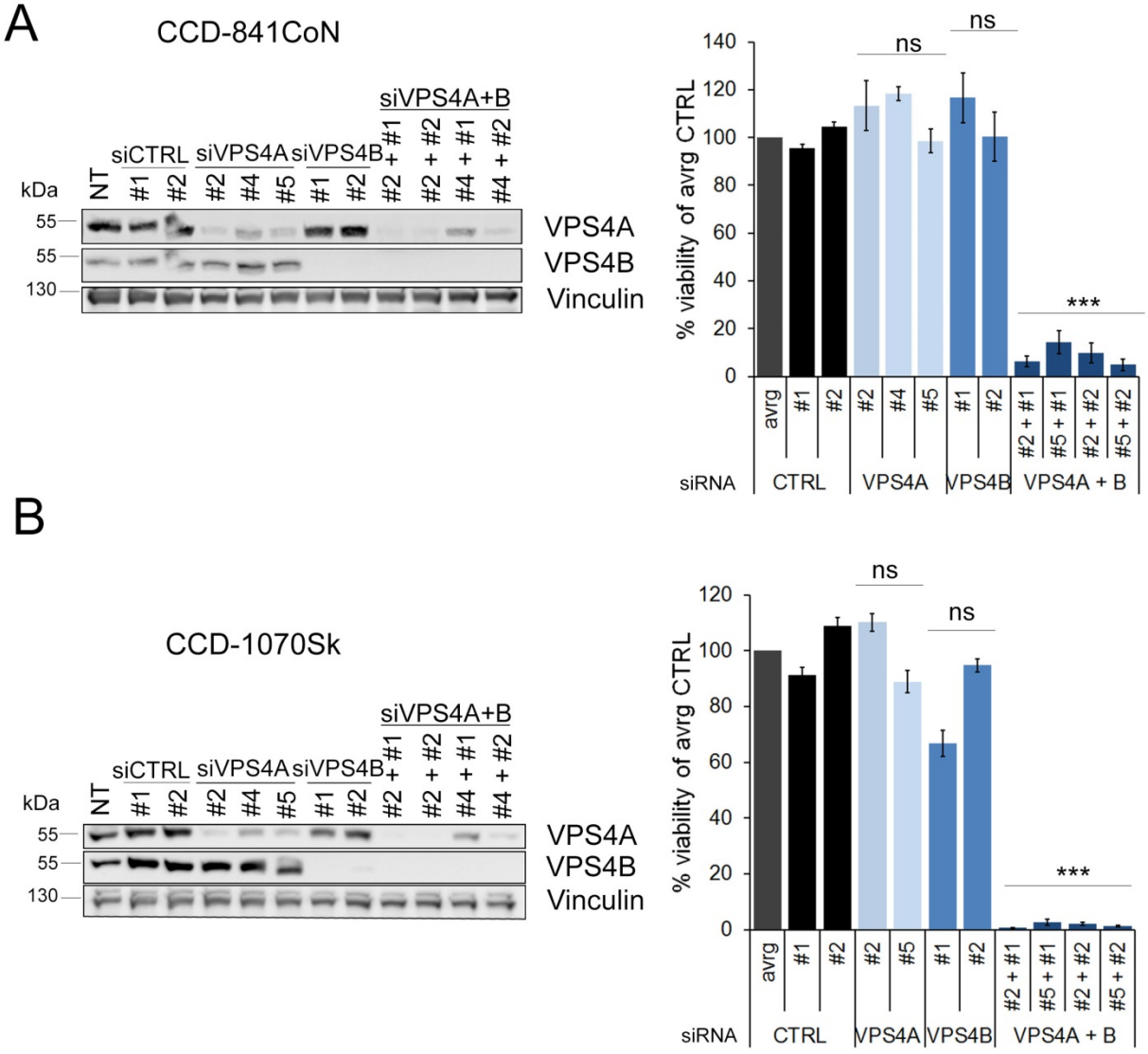

Figure 1 provided for the Reviewer. Silencing efficiency of VPS4A and VPS4B, and the viability of CCD-841CoN and CCD-1070Sk cells upon depletion of VPS4A, VPS4B and VPS4A+B.
A, B) Left panels, immunoblot analysis of the VPS4A and VPS4B protein abundance in lysates of CCD-841CoN (A) and CCD-170Sk (B) cells collected 72 h after transfection with control (siCTRL) or VPS4A- or VPS4B-targeting siRNA (siVPS4A or siVPS4B, various oligonucleotide sequences and their combinations were tested). Vinculin – loading control. Right panels, analysis of cell viability of CCD-841CoN (A) and CCD-170Sk cells (B) assessed 144 h after transfection with siRNA as indicated.
*Data were normalized (averaged value of siCTRL#1 and siCTRL#2 was set as 100) and are means ± SEM (n=4). Statistical significance was determined by Kruskal-Wallis followed by Dunn's multiple comparison post test. The following groups were compared: siCTRL#1,#2 group vs. siVPS4A#2,#4,#5 group; siCTRL#1,#2 group vs. siVPS4B#1,#2 group; siCTRL#1,#2 group vs. siVPS4A+B group). NS – non-significant ($p \geq 0.05$), ***$p < 0.001$.*

3. It is surprising that the data in the DRIVE database indicate that SK-CO-1 cells are the most sensitive cell line to VPS4A as well as VPS4B depletion. In addition, one can observe a correlation between the viability scores in the cell line panel for VPS4A and B.

Following the request of Referee #2, we removed the data concerning SK-CO-1 cells from the manuscript, instead including the new data on positively verified HOP62 and SNU410 cells (see point 1 above).

Nevertheless, to address the issue raised by the Reviewer, we re-analyzed the screening data available for SK-CO-1 cell line. According to DepMap, *VPS4A* and *VPS4B* are among the top

10 preferentially essential genes for this cell line among 8000 genes analyzed in shRNA screens within the Drive project (with DEMETER2 scores -2.08 and -1.76 for *VPS4A* and *VPS4B*, respectively; Figure 2A provided for the Reviewer). A similar strong dependency on both VPS4 paralogs was also observed for some other cell lines in this RNAi-based and other genome-wide CRISPR/Cas9-based projects (Figure 2B provided for the Reviewer).

We can only speculate that the dependency of SK-CO-1 and other cell lines on both VPS4 paralogs may result from unique proteomes of these cells arising from their individual mutagenic history. Possibly, survival and proliferation of these cell lines may require high rates of ESCRT-mediated processes, e.g. endocytosis (for nutrient or growth factor uptake) that in parallel with concomitant loss of compensatory pathway(s), make them highly sensitive to any VPS4 paralog perturbation. However, only dedicated experimental studies can clarify these issues in the future.

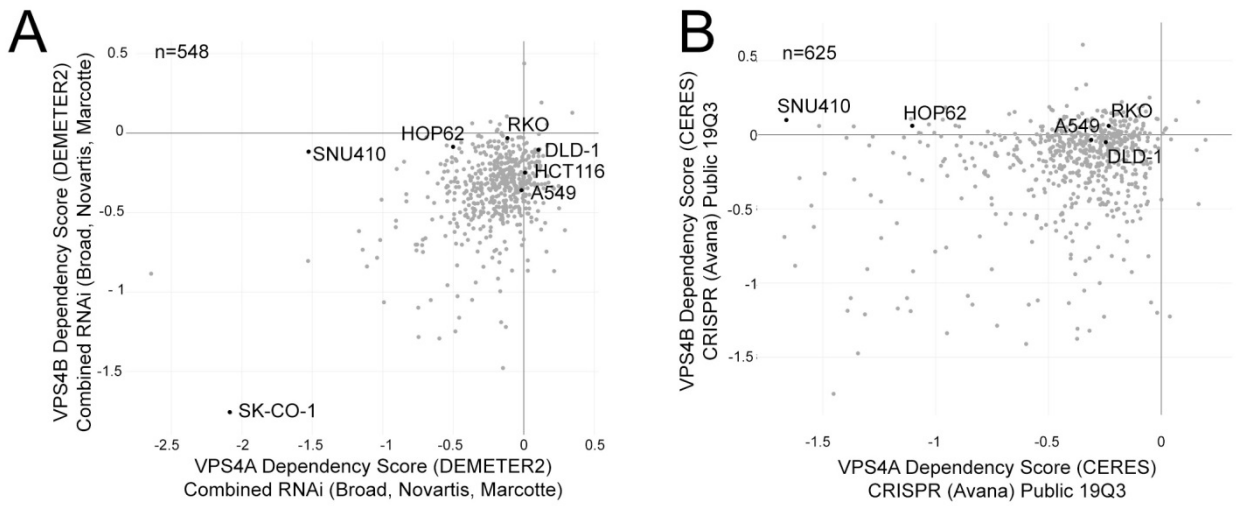

Figure 2 provided for the Reviewer. Various dependencies on VPS4A and VPS4B across cancer cell lines.
A, B) Distribution of dependency scores obtained for a panel of cell lines examined in RNAi-based (A) and CRISPR/Cas9-based (B) screens. Images were downloaded from the DepMap portal (https://depmap.org/portal/). Dependency scores of cell lines used in our study were marked in black.

4. One other point is the potential correlation between proliferation and VPS4B expression. Is the expression of VPS4B affected by proliferation rate of cell cycle progression?

To our knowledge, there have been no reports on *VPS4B* expression during cell cycle progression. Thus, to answer the Reviewer's question, we analyzed VPS4B and VPS4A protein abundance in HCT116 cells either serum-starved or treated with cell cycle inhibitors. We observed that both G1- and G2/M-arrested cells maintained unchanged VPS4A and B protein levels (Figure 3 provided for the Reviewer), thus we concluded that the expression of VPS4 paralogs is invariable during cell cycle progression.

In parallel, we addressed a related question of how simultaneous knockdown of VPS4 paralogs affected cell cycle progression. To this end, we depleted HCT116 *VPS4B*$^{-/-}$cells of VPS4A with siRNA for 72 h and analyzed the distribution of their cell cycle phases. We demonstrated that depletion of VPS4A caused G2/M arrest of HCT116 *VPS4B*$^{-/-}$ cells (new Fig EV4C), most probably due to the interrupted mitotic exit and cytokinesis, as it was previously shown by others (Vietri et al, 2015, Nature 522:231; Mierzwa et al, 2017 Nat Cell Biol 19:787).

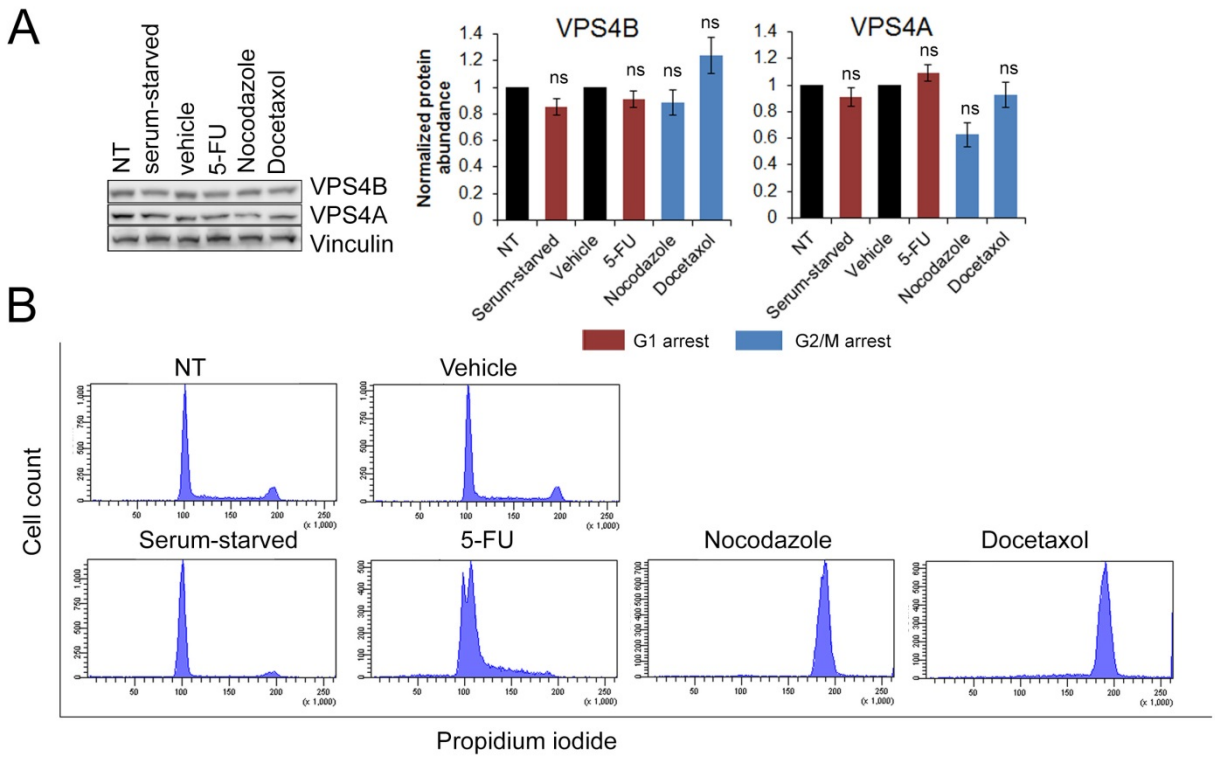

Figure 3 provided for the Reviewer. Abundance of VPS4A and VPS4B proteins in cell cycle-arrested cells.
A) Left panel, immunoblot analysis of VPS4B and VPS4A protein abundance in lysates of HCT116 cells treated for 24 h with 5–fluorouracil (5-FU, 10 µg/ml), nocodazole (100 µg/ml), docetaxol (20 nM), vehicle (0.1% DMSO) or serum-starved. Vinculin – loading control. NT – non-treated. Right panel, densitometry analysis of the abundance of the indicated proteins based on immunoblot images as shown on the left. Normalized VPS4B and VPS4A protein abundance in non-treated (NT) and vehicle-treated (Vehicle) samples was set as 1. Data are mean ± SEM (n=4). Statistical significance was assessed using one-sample t-test. ns – not significant, p≥0.05.
B) Example of flow cytometry analysis of the cell cycle phase distribution of HCT116 cells treated with cell cycle inhibitors as indicated in A.

Referee #2
In this manuscript the authors exploit coincidental loss of VPS4B in colorectal cancer as a biomarker to predict and test VPS4A depletion as a synthetic lethal strategy for this subset of cancers. Loss of the two VPS4 paralogues caused synthetic lethality in cell line and xenograft models. The manuscript characterizes the observed synergy with respect to gene expression changes, cell death mechanisms, and the potential to elicit an inflammatory response. Overall, the promise of targeting presumed 'passenger' mutations like VPS4B has been underexploited and this study represents an interesting and important addition to this field. The manuscript is quite thorough, the data quality look good, and the work tests important mechanisms of cell killing and immune stimulation. I have some suggestions to improve the manuscript below.

Referee #2 Remarks for Author
1. On Page 5, the authors propose to use SK-CO-1 cells as a model for a cell line with native loss of VPS4B but then find that the model is not appropriate for this purpose? Why do they include this section at all? It would be better to remove it, or screen other cell lines with natively low or lost VPS4B.

Following the Reviewer's recommendation, in the revised version of the manuscript we removed data for SK-CO-1 cell line. Instead we present the results obtained for other cancer cell lines with the confirmed decreased *VPS4B* gene copy number and low VPS4B protein abundance (new Fig 2C-G). We discuss this issue in detail in response to Referee #1, point 1.

2. The rationale for looking at gene expression changes by RNA-seq is not clear because VPS4-depleted cells would be expected to have defects in secretion or the endomembrane system. The authors seem to observe a gene expression program consistent with dying cells, which is good, but does not add much to the mechanism of lethality. Did the authors look directly at secretory defects in the double-depleted VPS4A/4B cells?

Indeed, we did not explain our rationale for RNA-seq experiments well enough. In new Fig EV4 we now present data showing that double depletion of VPS4A+B causes simultaneous perturbation of two well-established ESCRT-dependent processes: endocytosis and cell cycle progression. Thus, we are convinced that the lethal phenotype of double-depleted VPS4A+B cells arises from the perturbation of several ESCRT-dependent processes causing irreversible loss of cellular homeostasis. However, by performing RNA-seq analysis we aimed to identify further unknown cellular consequences of depleting either single or both VPS4 paralogs. The results of these experiments gave us hints to study e.g. inflammatory or apoptotic signaling, elaborated in the final part of the manuscript.

3. The finding that both caspase-dependent and independent pathways lead to cell killing in the VPS4A/B depletion model is good but I thought could be framed in the text as eliminating potential resistance mechanisms (i.e. upregulation of anti-apoptotic factors seen in some cancers would NOT make them inherently resistant to this approach).

We fully share the interpretation of the Reviewer but obviously we were not clear enough in the initial version of the manuscript. Now, in the revised Discussion we strengthened our conclusions on this issue, as suggested by the Reviewer.

4. It was not clear in what genetic contexts the authors think this approach would be effective. The authors claim that 70% of CRCs have loss of VPS4B, but that only 2% have biallelic loss. Their model systems have a complete VPS4B loss, so would the synthetic lethality only be effective in the 2% with biallelic loss, or would there be some efficacy of VPS4A knockdown in the 70%? I felt that the manuscript should be explicit about this, and perhaps test partial VPS4B loss for the efficacy of VPS4A-knockdown mediated killing. The future clinical impact and use of VPS4B as a biomarker depends on a clear statement here.

We thank the Reviewer for this important comment. We addressed this issue experimentally in cells with partial loss of *VPS4B*, as also requested by Referee #1 point 1, and these new results allowed us to strengthen the conclusions of our study. Since we confirmed the increased vulnerability of HOP62 and SNU410 cell lines with partial loss of *VPSB* to depletion of VPS4A (new Fig 2 F, G), we believe that a therapeutic approach based on VPS4 inhibitors could also potentially target tumors with incomplete loss of *VPS4B*. However, we are aware that based on our *in vitro* studies with cancer cells harboring incomplete loss of *VPS4B*, it is hard to predict whether and to what extent any inhibition of tumor growth upon VPS4A perturbation would be of therapeutic value. Nevertheless, even if targeting VPS4A were advantageous only for therapy of tumors with a complete loss of *VPS4B* (estimated at 2% of CRC), still a large number of patients could potentially benefit from it (each year there are 1.8 million new cases of CRC as the third most commonly diagnosed cancer according to WHO).

Minor points:

5. On page 10, the authors state that VPS4A and 4B are functionally redundant. I recommend saying 'partly' or 'partially' redundant since their own analysis (e.g. RNA-seq) shows they are not full redundant.

The Reviewer is right. We introduced the suggested phrasing in the text.

6. The grey inset images in Figure 2 are not very useful or easy to see.

The aim of presenting bright-field microscopy images was to strengthen the results of viability assays indicating cell death upon simultaneous VPS4A+B depletion. However, since they were not found useful, we removed them from the new Fig 2.

Figure 2. What is the rationale for using a one-sample T-test?

In our viability assays, we initially normalized growth readout for all transfection conditions to the value of non-targeting control siCTRL#1-transfected cells that was set as 100% in each biological repetition. Having no variation for the normalized control group, we used one-sample t-test to determine statistical significance. However, during the revision, we observed some variability in growth of SNU410 cells transfected with various control siRNA (#1, #2, #3; new Fig

2G) so we decided in each experiment to average values for all non-targeting siRNA and set this average to 100%. To test statistical significance between two groups (siCTRL group vs. siVPS4 group), we used two-tailed unpaired t-test (new Fig 2 F,G). To test statistical significance between more groups (siCTRL group vs. siVPS4 group, siCTRL group vs. siVPS4B, and siCTRL group vs. VPS4A+B group), we used Kruskal-Wallis or ANOVA test (with an appropriate post test) (new Fig 2 A, B). Thus, in the revised Figure 2 we used one sample t-test only to test significance in the colony formation assay for HCT116 cells, because we have no variation in the normalized control group (siCTRL#1, new Fig 2A, middle panel).

Figure 3. I did not understand why the authors showed the right panel on Figure 3b. Are these just the endpoint values? Why is there no indication of statistical significance?

The right panel in Figure 3B presents the endpoint values of tumor sizes and now we state it clearly in the figure legend. Statistical differences between doxycycline treated and untreated groups were presented in the left chart (showing averaged tumor volumes for each group).

2nd Editorial Decision 6 November 2019

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have received the referees' reports, and as you will see the reviewers are now supportive of publication of your study. I am therefore pleased to inform you that we will be able to accept your manuscript pending minor editorial amendments.

Please address referee #1's comments in writing. We would like you to discuss this referee's concerns regarding colorectal cancer versus other cancer types and rephrase as asked. If you do have data at hand, we would be happy for you to include it, however we will not ask you to provide any additional experiments at this stage.

I look forward to reading a new revised version of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

The synthetic lethal interaction between VPS4A and VPS4B has been described before and linked to copy number alterations due to passenger deletion in conjunction with SMAD4 (McDonald et al, Cell 2017). This work also showed that VPS4A dependency is associated with VPS4B copy number alterations (McDonald, fig 5C). It is unclear to me how the authors conclude that the paper by McDonald suggests that "another partner from 18q that remained unidentified". the experiments convincingly show the SL interaction in the cell line panel without the need for an unknown player..

The focus of this manuscript is on CRC for which they determine frequent down regulation of VPS4B expresion. However, this is not exclusively for CRC as it also occurs in lung, pancreas and other tissues. The validation of the dependency on VPS4A in VPS4B low cell lines is subsequently performed with a lung and pancreas cell line and not a CRC cell line. As consequence the conclusions drawn from this work with respect to CRC are more correlative than causative with no example of a CRC cell line that has lost or reduced VPS4B expression.

Referee #1 (Remarks for Author):

The authors have responded adequately to the reviewers comments with respect to the use of normal cell lines and the inclusion of cell lines characterized by the loss of VPS4B expression making use of existing databases. However, based on these additional data, it is no longer appropriate to conclude that CRC is particular dependent on this paralog SL interaction. The cell line examples are derived from lung and pancreatic cancer, two tumor types that also seem to most strongly show this SL interaction in Depmap. It is difficult to extrapolate the observations on the additional cell lines as they were selected "a priori" on their dependency on VPS4A and loss of VPS4B expression. One could ask if a set of CRC cell lines with low VPS4B would be chosen without the information on VPS4A dependency, the same result would be obtained.

In order to publish this work, either the authors include CRC cell lines in their analysis or they rephrase the title and other statements about the dependency of CRC on VPS4A to a more general statement about different cancers. They should also correct wording of the reference to the work of McDonald as this interaction was clearly identified and described.

Referee #2 (Comments on Novelty/Model System for Author):

As before I felt the article was technical sound and novel. The study is pre-clinical and so the medical impact is just hard to determine at this point. I do not view this as an impediment to publication but I do not know the EMBO MOL MED mandate.

Referee #2 (Remarks for Author):

I was happy to see a thorough and thoughtful revision of the manuscript and complete responses to reviewer comments. I have no further concerns.

2nd Revision - authors' response 4 December 2019

***** Reviewer's comments *****
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The focus of this manuscript is on CRC for which they determine frequent down regulation of VPS4B expresion. However, this is not exclusively for CRC as it also occurs in lung, pancreas and other tissues. The validation of the dependency on VPS4A in VPS4B low cell lines is subsequently performed with a lung and pancreas cell line and not a CRC cell line. As consequence the conclusions drawn from this work with respect to CRC are more correlative than causative with no example of a CRC cell line that has lost or reduced VPS4B expression.

Referee #1 (Remarks for Author):
The authors have responded adequately to the reviewers comments with respect to the use of normal cell lines and the inclusion of cell lines characterized by the loss of VPS4B expression making use of existing databases. However, based on these additional data, it is no longer appropriate to conclude that CRC is particular dependent on this paralog SL interaction. The cell line examples are derived from lung and pancreatic cancer, two tumor types that also seem to most strongly show this SL interaction in Depmap. It is difficult to extrapolate the observations on the additional cell lines as they were selected "a priori" on their dependency on VPS4A and loss of VPS4B expression. One could ask if a set of CRC cell lines with low VPS4B would be chosen without the information on VPS4A dependency, the same result would be obtained.

In order to publish this work, either the authors include CRC cell lines in their analysis or they rephrase the title and other statements about the dependency of CRC on VPS4A to a more general statement about different cancers. They should also correct wording of the reference to the work of McDonald as this interaction was clearly identified and described.

The Referee #1 wrote ***"This work also showed that VPS4A dependency is associated with VPS4B copy number alterations (McDonald, fig 5C). It is unclear to me how the authors conclude that the paper by McDonald suggests that "another partner from 18q that remained unidentified". the experiments convincingly show the SL interaction in the cell line panel without the need for an unknown player.*** We apologize for our unfortunate phrasing when citing the paper by McDonald and colleagues (doi: 10.1016/j.cell.2017.07.005). In their impressive large-scale shRNA screen the

authors found that some cancer cell lines were sensitive to VPS4A depletion and they linked this phenotype to an altered *VPS4B* copy number in these cells. However, the authors did not confirm this finding experimentally. Neither did they analyze the VPS4A/VPS4B protein abundance in sensitive and non-sensitive cells. For these reasons, we considered a synthetic lethal interaction between VPS4A and VPS4B proposed by McDonald and colleagues as a hypothesis that requires further dedicated experimental verification. By writing about a "partner from 18q that remained unidentified" we meant an unconfirmed and uncharacterized interaction of VPS4A with a gene from 18q. However, as rightly noticed by the Referee #1, this statement misrepresented the conclusions drawn by McDonald and colleagues. We corrected it in the final version of the manuscript. This paragraph now reads: "A large-scale screening for cancer vulnerabilities within the Sanger's Project Score (Behan et al, 2019) and the DRIVE project (McDonald et al, 2017) revealed that some cancer cell lines are very sensitive to perturbed VPS4A expression. The authors of the latter report suggested the existence of a synthetic lethality between VPS4A and VPS4B, however this hypothesis has not been experimentally verified."

Second, the Referee #1 wrote: "***However, based on these additional data, it is no longer appropriate to conclude that CRC is particular dependent on this paralog SL interaction (..)***" and ***"In order to publish this work, either the authors include CRC cell lines in their analysis or they rephrase the title and other statements about the dependency of CRC on VPS4A to a more general statement about different cancers".*** We respectfully disagree with the Referee. At no place in our paper did we claim that *VPS4B* loss is a unique feature of CRC. Neither did we narrow down the potential application of VPS4A+B synthetic lethality to CRC. Based on the findings from Fig 1B and 1C, we chose CRC as a cancer model with frequent deletions of *VPS4B*. Consistently, by using CRC cancer patient samples we confirmed the downregulation of VPS4B mRNA and protein levels. Subsequently, by using CRC cell lines we characterized the transcriptional and biochemical consequences of simultaneous depletion of VPS4A+B and analyzed its paracrine impact on macrophages. CRC was our only thoroughly characterized model and in our opinion, this entitles us to preserve the proposed title. Generalizing it to different types of cancer would be an overstatement unjustified by our data.

We wish to stress that although most of our work was performed in the CRC model, we were admitting in the text that other cancers may show a similar dependency. For example, in the abstract: "Here, we report that VPS4B gene, encoding an ATPase involved in ESCRT-dependent membrane remodeling, is such a passenger gene frequently deleted in many cancer types, notably in colorectal cancer (CRC)." In the discussion: "Importantly, our demonstration of synthetic lethality between druggable VPS4 paralogs provides a rationale to develop novel therapies targeting VPS4A activity in cancers with 18q deletion, such as CRC."

However, we followed the Reviewer's suggestion to emphasize this issue even further, so we have also rephrased other sentences in the discussion. They now read: "Here, by demonstrating the synthetic lethal interaction between two ubiquitously expressed human paralogs *VPS4A* and *VPS4B*, we uncovered a novel therapeutic target to treat patients bearing *VPS4B*-deficient cancers, for example CRC used as a model in our study. (…) Third, we demonstrated that various genetic backgrounds of cancer cell lines did not reverse the synthetic lethality between *VPS4* paralogs (Fig 2). Finally, we showed that the synthetic lethality between *VPS4A* and *VPS4B* is conserved across tumor types (CRC, lung, pancreas; Fig. 2) and species (Fig EV5). (…) In summary, our findings establish a foundation for future work aiming to develop a VPS4 inhibitor as a putative therapeutic for precision therapy of *VPS4B*-deficient cancers such as CRC".

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: Marta Miaczyńska
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2019-10812

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?	No statistical methods were used to predetermine the sample sizes. Sample sizes were sufficient to acquire statistical significance between samples in all experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical methods were used to predetermine the animal group sizes. Animal group sizes were sufficient to acquire statistical significance between groups in all experiments. The number of mice analyzed is indicated in the figure legend.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No inclusion/exclusion criteria were used.
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.	Animals/samples were randomly allocated into experimental groups prior to treatment.
For animal studies, include a statement about randomization even if no randomization was used.	See statement above.
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.	Yes. Immunohistochemistry samples were reviewed for abundance of VPS4 proteins in normal and neoplastic tissue by two pathologists, who were blinded to the outcome.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding methods were used to allocate animals into experimental gropus.
5. For every figure, are statistical tests justified as appropriate?	Yes, they are. If we had to compare one experimental group to the normalized control we used one-sample t-test or Wilcoxon signed rank test. If we had to compare two groups, we used two-tailed unpaired Welch t-test, t-test or the Mann-Whitney U test. If we had to compare more than two groups we used the Kruskal-Wallis test followed by Dunn's post test. The appriopriate test was selected based on sample distribution. A detailed statistical test description is included in the figure legends as well as Methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, data were analysed for Gaussian distribution using the Kolmogorov-Smirnov test with the Dallal-Wilkinson-Lillie for corrected p-value.

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Is there an estimate of variation within each group of data?	Yes, the variation of each group of data was estimated calculating its standard deviation or standard error of the mean as indicated in the figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes.

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).	The manufacturer and the catalog number for each antibody used in this study are provided in the Methods section in the manuscript.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The source of cell lines as well as authentication and mycoplasma tests are reported in the Methods section in the manuscript.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NU/J (nude) athymic mice were purchased from the Jackson Laboratory and maintained in a specific pathogen-free (SPF) facility. Mice were kept under 12 light/12 dark cycle and housed in individually ventilated cages (Tecniplast). In experiments we used randomly selected groups of males and females. All mice were over 6 weeks of age.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal work was performed in accordance with the protocol approved by the 2nd Local Ethics Committee for Animal Experimentation in Warsaw (decision no. WAW2/047/2018).
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.	The study protocol for analysis of protein levels of VPS4B and VPS4B in human normal colon and CRC samples was approved by the Bioethics Committee of the Maria Skłodowska-Curie Institute-Oncology Centre in Warsaw (decision no. 40/2017).
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.	The study was performed after obtaining the approval by Bioethics Comittee of the Maria Sklodowska-Curie Institute-Oncology Centre in Warsaw. Informed consent was obtained from all subjects. The experiments conformed to the principles set out in WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
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	"Data Availability" section was included. The RNA-seq datasets have been deposited to GEO under the accesion number GSE128070.
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).	N/A
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.	No