Author's response to reviews

Title: Low expression of the X-linked ribosomal protein S4 in human serous epithelial ovarian cancer is associated with a poor prognosis

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Author's response to reviews: see over
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Dr Stephan Polterauer
BMC Cancer
BioMed Central

Subject: Re-submission of manuscript MS: 8773655498427904

Dear Prof. Polterauer,

We are hereby re-submitting for your consideration the manuscript MS: 8773655498427904 entitled "Low expression of the X-linked ribosomal protein S4 in human serous epithelial ovarian cancer is associated with a poor prognosis." by Serges P. Tsolack, Liliane Meunier, Lilia Sanchez, Jason Madore, Diane Provencher, Anne-Marie Mes-Masson, and Michel Lebel. You will find files of the revised manuscript with the modifications, the tables and the new figures in the appropriate format, as well as a file with a point-by-point response to the reviewers’ comments. As you will see, we have responded to the referees’ comments by making the corresponding modifications in the manuscript.

We are very grateful for the suggestions made by the referees. We agree that the work has been appreciably improved by virtue of their thoughtful comments. We, therefore, are returning a revised version of the manuscript to you in the hopes that it will meet with your approval and will be judged suitable for publication as an article in BMC CANCER. Should you have any question about this manuscript, please do not hesitate to contact me. We look forward to hearing from you at your earliest convenience.

Yours sincerely,

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Response to comments from reviewer #1
Comments to the authors

1) Please add in to the text the relevance of using the OVCAR-3 cell line which, although being serous, is derived from a patient having malignant ascites resistant to combination chemotherapy treatment and then extrapolating that cell line to results seen in 193 HGS patients with FFPE samples taken at primary surgery (which are not chemotherapy resistant). Why did the authors choose this cell line?

Response: We added the following sentence in the appropriate results section

Although such cells were derived from patients with malignant ascites resistant to clinically relevant concentrations of cisplatin (www.atcc.org), we examined whether a depletion of RPS4X could increase cisplatin resistance further.

2) As FFPE samples were used, please expand on quality control done on these samples before immunohistochemistry was performed, specifically focusing on the variability of fixation and procedures as well as the effect of formalin on antigenicity.

Response: We added the following information in the materials and methods section. All samples were fixed with formalin and embedded in paraffin following a standard procedure. Formalin fixed paraffin embedded tumor blocks were then biopsied using a 0.6 mm diameter tissue arrayer and resultant cores were arrayed into a grid in a recipient paraffin block. It has previously been demonstrated using several different antibodies that the quality of the core samples on this TMA was suitable for immunohistochemistry and statistical analyses confirmed that the age of the paraffin blocks was not a confounder in these studies [18].

3) Please expand on the scoring done for the staining intensity of each marker-was this done by histopathologists-was a kappa coefficient determined? Is this what you mean by “inter-rating correlation” in methods?

Response: We added the following information in the materials and methods.

We use the inter-rating correlation (Cronbach's Alpha) to evaluate the overall correlation between the observers as described previously [18]. Inter-rating correlation was >75% for all three proteins.

4) The statistical analysis has major issues:
a) A Pearson correlation was used. This is problematic for several reasons: 1) It is unlikely that there was a linear dependence between the markers and clinicopathological variables. 2) It is unlikely that the clinicopathological variables were normally distributed (especially stage, grade and residual disease, which should have been treated as factors and not continuous). A Spearman correlation is more accurate to use for Age, YB1, PRS4X, and Ki67 (or a linear regression). However NO correlation should have been used for the other factors. Please rerun table 2.
Response: Since the survival data were not normally distributed, we performed a Spearman correlation test as requested by the reviewer. We thus reran Table 2. A new Table 2 is added in the revised version of the manuscript along with the appropriate modifications throughout the text. We added the following information in the materials and methods.

The Spearman correlation (two-tailed) and the Wilcoxon-Mann-Whitney test were used to estimate the correlation with clinicopathological variables and markers as continuous variables. Survival curves were calculated according to Kaplan Meier method coupled with a log-rank test for survival analysis. Since survival times are positively skewed, we took the median as the threshold value for each marker (YB-1 and RPS4X).

We also changed one sentence in the abstract. Immunohistochemistry studies indicated that high expression of RPS4X was associated with a high hazard risk for survival and with later disease progression (HR=0.716, p=0.001 and HR=0.760, p<0.001, respectively).

b) A Kruskal Wallis test (permutation) would be appropriate to use for association between residual disease, stage and grade and the markers. Alternatively logistic or multinominal regression models can be used. Please rerun table 2.

Response: Consistent with revised guidelines for ovarian cancer which described these cancers as either high or low grade, a pathologist reviewed all the intermediate grade 2 cases. They are now all considered high-grade samples in our analyses. Therefore, all grades were removed from Table 2. The text of the result sections was modified accordingly. Table 2 was rerun as requested by the reviewer. We also performed a Krustal Wallis test as advised by the reviewer and we found no significant difference between our biomarkers and stages or residual disease. We then performed a non-parametric Wilcoxon-Mann-Whitney test for these two variables: stage (Stage I vs Stage IV and low Stages (I,II) vs high Stages (III, IV)) and residual disease (no residual disease vs tumors more than 2cm and no residual disease vs any residual disease). We found that RPS4X expression was significantly different between low and high stages (sig=0.032). These results are now included in the new supplementary Table S1. The following information was added in the text.

RPS4X also correlated significantly with lower levels of residual disease (Table 2) and with a lower disease stage (Table 2 and supplementary Table S1).

c) It is completely inappropriate to use survival data in a correlation model. Survival data consists of an indicator and time-while it is ok to use them as descriptive factors in a demographic table-they should be used in Kaplan Meier and Cox models for any kind of associations. Please rerun table 2.

Response: We removed the survival data from Table 2 as requested by the reviewer.

d) It is unclear why ROC curves were even used and what is meant by “Since ROC curves were
not significant for each markers, we used the median as a threshold value for each marker—this is not a reason to dichotomize the markers. If you believe there is dose dependence or a threshold effect, then it is ok to dichotomize them—please rephrase or explain.

Response: To avoid confusion, we removed all references pertaining to the ROC curves in the text and added the following information in the materials and methods instead.

Since survival times were positively skewed, we took the median as the threshold value for each marker (YB-1 and RPS4X).

e) It appears in table 1 and in table 3, that the indicator was switched for survival. It is conventional that death or progression are coded as 1 and censoring as 0—please rewrite/rerun table 1, supplementary figure 1, and figure 2 to reflect this as well as change the interpretation of your results of table 3 to “lower risk progression”, “lower risk death”, “protective to progression” or “protective to poorer survival”. “Longer time to disease progression” is a completely wrong interpretation of survival results.

Response: Table 1, supplementary figure 1 and figure 2 have been modified accordingly to the reviewers suggestions. We also changed the wording in the text according to the reviewer suggestions.

f) Multivariate should be changed to multivariable throughout the text.

Response: We have made this correction throughout.

g) Why was grade not adjusted for in the multivariable Cox models?

Response: Our pathologist reviewed all the cases and classified them as low grade and high grade. We only kept the high-grade cases for this analysis.

h) Was any statistical test done for Figure 3C and D, Figure 4 and Figure 5—this could change the interpretation of the results—please do appropriate tests. Was figure 3 done in duplicates? If so where are the error bars?

Response: Experiments in figure 3 were performed twice. The error bars were added in the graphs of figure 3C and D as requested. The following information was added in the legend of figure 3.
All experiments were performed in duplicate.

Appropriate statistical analyses have been performed for figures 4 and now the new figure 6. The following information can be found in the legend of figure 4.
C, histogram representing the growth rate of OVCAR-3 transfected cells (from at least three
transfections for each siRNA sequences) calculated from the growth curves in A. Error bars represent the standard deviation. (Unpaired student t-test: $P = 7.6 \times 10^{-5}$ for siRPS4X-A vs siCTRL and $P = 2.4 \times 10^{-6}$ for siRPS4X-D vs siCTRL). D, histogram representing the growth rate of SK-OV-3 transfected cells (from at least three transfections for each siRNA sequences) calculated from the growth curves in B. Error bars represent the standard deviation (Unpaired student t-test: $P = 3.9 \times 10^{-7}$ for siRPS4X-A vs siCTRL and $P = 8.7 \times 10^{-6}$ for siRPS4X-D vs siCTRL). Growth rates were estimated as described in materials and methods.

The following information can be found in the legend of the new figure 6. E, histogram representing the IC50 of OVCAR-3 transfected cells (from at least three transfections for each siRNA sequences) calculated from the drug response curves in A. Error bars represent the standard deviation. (Unpaired student t-test: $P = 0.0084$ for siRPS4X-A vs siCTRL and $P = 0.025$ for siRPS4X-D vs siCTRL). F, histogram representing the IC50 of SK-OV-3 transfected cells (from at least three transfections for each siRNA sequences) calculated from the drug response curves in B. Error bars represent the standard deviation. (Unpaired student t-test: $P = 0.00066$ for siRPS4X-A vs siCTRL and $P = 0.0001$ for siRPS4X-D vs siCTRL). IC50 were estimated as described in materials and methods.

The following information can be found in the materials and methods.

The R software version 2.10.1 (http://www.r-project.org/) was used to estimate the growth rate, the IC50, and the associated standard deviation. Briefly, the growth curves were fitted to a mathematical model of the form $y = x_0 \times (1+r)^t$, where $x_0$ represents the 50,000 transfected cells plated on day 0, $r$ represents the growth rate, and $t$ represents the time unit (days). The dose response curves were fitted to a standard exponential decay mathematical model of the form $y = y_0 + A \times e^{kx}$ where $y_0$ represents the minimal normalized intensity, $A$ the intensity at time 0 and $k$ is the decay rate.

i) Since there was no statistical test done in Figure 5 (as far as I can see), how can the authors conclude that “RPS4X depletion causes cisplatin resistance in two different cell lines”. Insufficiently supported by the data. Please add.

Response: Figure 5 is now the new figure 6. Please see response to comment h) above.

j) I recommend that authors see a statistician for their analysis.

Response: This was done accordingly. The statistician guided us for the Pearson correlation, the Krustal Wallis test and the Wilcoxon-Mann-Whitney test as described above.

5) Why were two different siRNAs used for YB-1 and PRS4X respectively-please expand.

Response: Two different siRNAs were used to eliminate potential off target effects. The following information was added in the section entitled “Impact of RPS4X depletion on the growth of two serous epithelial ovarian cancer cell lines” with the appropriate reference.
Two different siRNAs against our target proteins were used in all experiments to avoid misinterpretation of the data due to potential off target effect of a single siRNA [15].

6) For the apoptosis data which is not shown (cleavage caspases and PARP1 by Western Blot in knockdown PRS4X cells) which cell line was used?

Response: We performed FACS analyses (for cell cycle) and FITC-Annexin V for apoptosis as suggested by a reviewer on both OVCAR-3 and SK-OV-3 cell lines. Because of space limitation we removed the PARP/caspase data. (Please see also response to reviewer #2).

7) The authors attempt to extrapolate cisplatin findings to carboplatin in the discussion due to them having “the same type of DNA damage”-please remove.

Response: This was done accordingly.

8) Please expand on what you mean by “intrinsic resistance” in the discussion

Response: The following information was added in the discussion section.
As the immunohistochemistry study was performed on serous high-grade ovarian tumors from patients who had not received chemotherapeutic treatment, the patients showing low expression of RPS4X in their tumor tissues at surgery could correlate with an intrinsic resistance to platinum-based drugs. More precisely, cancer cells with low expression of RPS4X present in high-grade tumors that have never been in contact with platinum would correspond to cells exhibiting a pre-existing mechanism for resistance to such drug. The exact mechanism by which a depletion of RPS4X confers cisplatin resistance is not known.

Minor Essential Revisions
1) In the abstract-please add “lines” to “cells” in the background.

Response: The correction was done.

2) Please rephrase “which one impact on cisplatin response upon depletion” in the background section.

Response: The phrase was changed by the following sentence
In a recent study of breast cancer cell lines, we identified the proteins that interact directly to YB-1 and impact on cisplatin response upon depletion [15].

3) When talking about associations with survival, please change “correlate” to “associate” and “low survival” to “poor survival” (Background)
Response: The corrections were made accordingly.

4) The authors should comment on the validity and quality of antibodies used in immunohistochemistry

Response: The following information was added in the first paragraph of the results section. The quality and validity of the antibodies against YB-1 and Ki67 used in this immunohistochemistry study have been described previously [18, 19]. To confirm the validity of the antibody against RPS4X, we performed Western blots and immunofluorescence tests on control and RPS4X-depleted SK-OV-3 ovarian tumor cell lines. As indicated in Figure 1D, the RPS4X antibody recognized a band of approximately 29 kDa that was depleted by two different siRNAs specific to RPS4X mRNAs. The immunofluorescence signal was also reduced in a population of SK-OV-3 cells transfected with a siRNA against RPS4X compared to siRNA control cells (Figure 1E). These results indicate that the antibody is specific to the RPS4X protein.

The following sentences were added in the legend of Figure 1.
D. Western blot analysis of total protein extracts from SK-OV-3 cell lines transfected with a siRNA sequence against RPS4X mRNA (siRPS4X-A and siRPS4X-D) or transfected with a control (scrambled) siRNA sequence. β-actin is used as a loading control. E, a representation of immunofluorescence signals in SK-OV-3 cells transfected with a siRNA sequence against RPS4X mRNA (siRPS4X-A) or transfected with a control scrambled siRNA sequence (siCtrl). Nuclei are revealed with DAPI staining (in blue).

Finally, the method for the immunofluorescence analysis was also added in the materials and methods section.

5) Please comment on the representativeness of staining and images seen in figure 1.

Response: For the figure 1, we selected images representative for each staining intensity of YB-1 and RPS4X from the TMA. The figure 1 legend now reads: **Figure 1** Representative staining for immunohistochemistry of YB-1, RPS4X, and Ki67 on a high-grade serous EOC TMA. A, representative staining of each intensity by immunohistochemistry for YB-1. From left to right: low to high intensity. B, representative staining of each intensity by immunohistochemistry for RPS4X. From left to right: low to high intensity. C, representative staining of each intensity by immunohistochemistry for Ki67. From left to right: 0% of total staining, 50% of total staining, and 100% of total staining.

Discretionary Revisions
1) If the authors had chemoresistance data on the FFPE patients—that would be best.

Response: Unfortunately, we do not have useful chemoresistance data from our cohort.
**Response to comments from reviewer #2**

1) On page 3, the authors refer to the involvement of YB-1 in patient survival and cisplatin resistance, but they did not discuss the contradictory results from previous studies: In 1999 and in 2007, Kamura at al. and Oda et al. showed the prognostic value of YB-1 expression in patients with stage III serous carcinoma who had receive cisplatin, epirubicin and cyclophosphamide. In contrast in 2004, Huang et al. in their study observed no correlation between YB-1 expression and survival in ovarian cancer patient. The authors should more broadly discuss the how YB-1 may not be the highest fidelity biomarker in ovarian cancer, which serves to only reinforce the potential importance of RPS4X as a prognostic biomarker in this disease.

**Response:** We have changed the first paragraph of the discussion section as requested by the reviewer.

The expression of YB-1 in ovarian carcinomas has been correlated with a poor prognosis in several studies including one study on serous ovarian cancer [7, 23]. However, there is one study that indicated no relationship between ovarian cancer patient survival and YB-1 expression [24]. Such differences in the results may be due to the small numbers of ovarian tumor samples specifically of the serous type (less than 40 samples of both low and high grades) that were used in past studies [7, 23, 24]. Another confounding parameter in the interpretation of the results is the anti-YB-1 antibodies used in the different the studies. Antibodies recognizing epitopes on the C-terminus [7, 24] or the N-terminus portion of the YB-1 protein (our study) as well as the immunohistochemistry protocol will have an impact on the results [25]. Finally, as our study focused exclusively on high-grade serous epithelial ovarian cancers, it is possible that within this subset of serous cancer YB-1 has little prognostic value. In contrast, the level of RPS4X may be a better prognostic biomarker than YB-1 in serous epithelial ovarian cancers.

2) In regard to Figure 3, the histograms were generated by densitometry of the western blot analysis in the panel above. However, the number of replicates of this experiment is not indicated, and error analysis is not provided. Please provide error analysis and error bars indicating the number replicates in the figure legend for these Western blots in panels B and D. In addition, please provide details of densitometry analysis in the Methods and Materials, including the software program used.

**Response:** We added the appropriate information in the figure 3 as indicated in the response to reviewer #1. We also added the following information in the materials and methods. Protein bands on the Western blots were quantified using LI-COR Image Studio software 2.0 (LI-COR Biosciences, Lincoln, NE). β-actin was used as a control for protein loading. The background signal for each band was determined using an identical area to the target band covering a region in the same lane where no protein signal was observed. Results were determined by calculating a ratio of target protein signal (minus background) over β-actin signal (minus background).
3) The caspase-3/9 and PARP cleavage data, referred to as “data not shown” on page 9 should be included in the manuscript. In addition, it might be useful to show flow data using propidium idodide and FITC-AnnexinV to characterize the affects of RPS4X depletion on the ovarian cancer cell lines, with respect to cell cycle, necrotic and apoptotic death. This data will enhance the interpretation of the proliferation inhibition shown in these cells upon RPS4X depletion. This analysis should also be repeated for cells treated with cisplatin, which may uncover a differential cellular response to the drug, rather than simply reporting the percentage inhibition.

Response: We performed FACS analyses (for cell cycle) and FITC-Annexin V as suggested by this reviewer. Because of space limitation we removed the PARP caspase data.

The following sentences were added in the Materials and Methods

**FACS and FITC-Annexin V analyses**

Cells were transfected with either control siRNA or siRNA against RPS4X. After 72 h, cells were fixed in 50% ethanol overnight. Cells were then washed in phosphate-buffered saline (PBS) and incubated for 30 min at 37°C in a buffer containing propidium iodide and RNAses. Cells were then analyzed on a Beckman-Coulter Epics Elite ESP (Cambridge, MA, USA) flow activated cell sorter. Data were analyzed with the MultiCycle software (Phoenix Flow System, San Diego, CA, USA). To estimate apoptosis and/or necrosis, we used the FITC Annexin V apoptosis detection kit I (BD Biosciences, Palo Alto, CA). Transfected cells were treated 48 h with the indicated concentration of cisplatin and then harvested to measure apoptosis/necrosis following the manufacturer’s instructions.

The following results were added in the text. We further analyzed the cell cycle of transfected cells by FACS analysis. As indicated in the summary histogram of Figure 4E, siRPS4X OVCAR-3 transfected with siRPS4X sequences showed an increase in S phase with a concomitant decrease in the G1 phase of the cell cycle compared to control siRNA transfected cells. Based on the growth rate (Figure 4C), these results suggest that the siRPS4X stalls OVCAR-3 cell proliferation in the S phase of the cell cycle. SK-OV-3 transfected with siRPS4X sequences exhibited an increase in the G2/M phase of the cell cycle with a concomitant decrease in the S phase. Based on the growth rate (Figure 4D), these results suggest that siRPS4X stalls SK-OV-3 cell proliferation in the G2/M phases of the cell cycle. Examples of FACS analyses are shown in supplementary Figure S2. The difference between RPS4X-depleted OVCAR-3 and SK-OV-3 cell cycle behavior is currently unknown. Nevertheless, siRPS4X decreased the proliferation rate in both cell lines.

To determine whether a depletion of RPS4X had an impact on apoptosis, we analyzed siRPS4X transfected cells with a FITC-Annexin V assay and compared them to control siRNA transfected cells. A depletion of RPS4X protein in OVCAR-3 cells did not increase the percentage of apoptotic or necrotic cells in culture (Figure 5). In contrast, RPS4X depletion in SK-OV-3 cells increased apoptosis by 17% (Figure 5). These results indicated that the SK-OV-3 cells are more sensitive to the depletion of RPS4X protein than the OVCAR-3 cells.

The following information was added in the legend of figure 4.

E, percentage of OVCAR-3 and SK-OV-3 transfected cells in each phase of the cell cycle. Cells were transfected with the indicated constructs in duplicates and subjected to FACS analysis 72 h later. (The siRPS4X represent data from cells transfected with siRPS4X-A and siRPS4X-D performed in duplicata). Data are the mean ± SE.
Also we added the new figure 5.

**Figure 5** Apoptotic and necrotic events in RPS4X-depleted cells were assessed in the presence of cisplatin by flow cytometry with Annexin V/PI staining. Cells were transfected with the indicated siRNA sequences (siControl and siRPS4X-A) and 48 hours later cells were treated for 48 hours with the indicated concentration of cisplatin.

Finally, we added the following information in the section of the results dealing with cisplatin resistance.

We next analyzed the impact of cisplatin on cell death in transfected cells with a FITC-Annexin V assay. OVCAR-3 cells transfected with a control siRNA showed a 14% increase in apoptosis when treated 48 hours with 2 μM cisplatin (Figure 5). There was no significant increase in necrosis. In contrast, RPS4X-depleted OVCAR-3 cells did not exhibit an increase in apoptosis or necrosis after 48 hours of cisplatin treatment. Similarly, SK-OV-3 cells transfected with a control siRNA showed a 30% and 2% increase in apoptosis and necrosis respectively when treated for 48 hours with 15 μM cisplatin (Figure 5). In contrast, RPS4X-depleted SK-OV-3 cells showed only a 7% increase in apoptosis after 48 hours of cisplatin treatment (Figure 5). There was no increase in necrosis. All together, these results indicate that RPS4X-depleted ovarian cancer cells are resistant to apoptosis induced by cisplatin.

4) On page 10, when presenting the drug response of the ovarian cancer cell lines treated with cisplatin with and without RPS4X depletion the error analysis should and P-value should be reported in the text not just in the legend of the Figure 5.

Response: We added the following sentences in the last paragraph of the results section for the new figure 6.

The calculated IC50 in OVCAR-3 cells for the control siRNA, siRPS4X-A, and siRPS4X-D were 0.9, 2.7, and 1.8 μM, respectively (Unpaired student t-test: $P = 0.0084$ for siRPS4X-A vs siCTRL and $P = 0.025$ for siRPS4X-D vs siCTRL) (Figure 6E). The calculated IC50 in SK-OV-3 cells for the control siRNA, siRPS4X-A, and siRPS4X-D were 9.1, 25.1, and 36.3 μM, respectively (Unpaired student t-test: $P = 0.0066$ for siRPS4X-A vs siCTRL and $P = 0.0001$ for siRPS4X-D vs siCTRL) (Figure 6F). These results indicate that cells that express low levels of RPS4X are more resistant to cisplatin and a depletion of RPS4X causes further cisplatin resistance in both serous epithelial ovarian cancer cell lines tested in this study.

5) What is the relative level of RPS4X between OVCAR-3 and SKOV-3 cells? This data should be included as it will aid in the interpretation of Figure 5 and the authors should consider the discretionary revision suggested below.

Response: We added the following information in the text as suggested by the reviewer. The original figure 5 is now changed for the new figure 6.

We first compared the expression of endogenous RPS4X in untransfected OVCAR-3 and SK-OV-3. As shown in Figure 6A and B, RPS4X protein level was 1.5-fold higher in OVCAR-3 cells than SK-OV-3 cells. Although such cells were derived from patients with malignant ascites
resistant to clinically relevant concentrations of cisplatin (www.atcc.org), we examined whether a depletion of RPS4X could increase cisplatin resistance further. As indicated in Figure 6C and D, RPS4X-depleted ovarian cancer cells were more resistant to cisplatin than control siRNA transfected cells. The calculated IC50 in OVCAR-3 cells for the control siRNA, siRPS4X-A, and siRPS4X-D were 0.9, 2.7, and 1.8 µM, respectively (Unpaired student t-test: $P = 0.0084$ for siRPS4X-A vs siCTRL and $P = 0.025$ for siRPS4X-D vs siCTRL) (Figure 6E). The calculated IC50 in SK-OV-3 cells for the control siRNA, siRPS4X-A, and siRPS4X-D were 9.1, 25.1, and 36.3 µM, respectively (Unpaired student t-test: $P = 0.00066$ for siRPS4X-A vs siCTRL and $P = 0.0001$ for siRPS4X-D vs siCTRL) (Figure 6F). These results indicate that cells that express low levels of RPS4X are more resistant to cisplatin and a depletion of RPS4X causes further cisplatin resistance in both serous epithelial ovarian cancer cell lines tested in this study.

The new figure 6 legend was added accordingly.

**Figure 6** RPS4X depletion increases cisplatin resistance in OVCAR-3 and SK-OV-3 cells. A, One example of a Western blot showing expression of RPS4X in untransfected OVCAR-3 and SK-OV-3 cells. β-actin is used as a loading control. B, histogram presenting the ratio of RPS4X signal over β-actin signal from Western blots. Experiments were performed in duplicate. C, cisplatin dose response curves for transfected OVCAR-3 cells. D, cisplatin dose response curves for transfected SK-OV-3 cells. Cells were transfected with the indicated siRNA molecules. Cisplatin dose response curves were determined by the sulforhodamine B colorimetric assay. E, histogram representing the IC50 of OVCAR-3 transfected cells (from at least three transfections for each siRNA sequences) calculated from the drug response curves in A. Error bars represent the standard deviation. (Unpaired student t-test: $P = 0.0084$ for siRPS4X-A vs siCTRL and $P = 0.025$ for siRPS4X-D vs siCTRL). F, histogram representing the IC50 of SK-OV-3 transfected cells (from at least three transfections for each siRNA sequences) calculated from the drug response curves in B. Error bars represent the standard deviation. (Unpaired student t-test: $P = 0.00066$ for siRPS4X-A vs siCTRL and $P = 0.0001$ for siRPS4X-D vs siCTRL). IC50 were estimated as described in materials and methods.

The following text was added in the discussion section (in the 3rd paragraph).
An important aspect of RPS4X protein expression is its potential association with cisplatin resistance in different cell lines. The SK-OV-3 cell line is more resistant to cisplatin than the OVCAR-3 cell line [26, 27]. Interestingly, the expression of endogenous RPS4X protein is lower in the more cisplatin resistant SK-OV-3 cell line than the OVCAR-3 cell line.

**Discretionary Revisions:**
1) The authors should comment on the dramatically different intrinsic resistance to cisplatin seen between the OVCAR-3 (sensitive) and SKOV-3 (resistant) cell lines, e.g. is this expected from previous literature? Is the mechanism of resistance known for SKOV-3 cell lines or sensitivity for OVCAR-3? This information will provide an important context for the fact that regardless of intrinsic sensitivity, cells depleted of RPS4X are more sensitive to cisplatin.

**Response:** We added the following information in the discussion section with the appropriate references.
The SK-OV-3 cell line is more resistant to cisplatin than the OVCAR-3 cell line [26, 27].
Interestingly, the expression of RPS4X protein is lower in the more cisplatin resistant SK-OV-3 cell line than the OVCAR-3 cell line.

As for the mechanism of resistance, it is not entirely known what is the difference between SK-OV-3 and OVCAR-3 and hypotheses are often based on microarray analyses with few proteomic studies. We thus decided not to speculate on this issue in the discussion because of lack of space.

2) Given the fact that in breast cancer cells YB-1 and RPS4X interact physically, do these proteins interact in ovarian cancer cells? This data could be included in the supplementary materials.

Response: We were unable to co-immunoprecipitate YB-1 and RPS4X with the antibodies available. However, we transfected GFP and GFP-YB-1 and found co-immunoprecipitation of RPS4X only with the GFP-RPS4X in SK-OV-3 cells.

We added the following information in the text (new subsection at the end of the results section).

**RPS4X interacts with YB-1 in ovarian cancer cells**

We previously showed that RPS4X interacts with a tagged YB-1 in a breast cancer cell line [15]. To confirm this interaction in an ovarian cancer cell line, GFP-YB-1 and a control GFP expression vectors were transfected into SK-OV-3 cells. The next day the GFP-YB-1 construct was precipitated with an antibody against the GFP tag and the presence of RPS4X in the immunoprecipitate was detected by immunoblotting (supplementary Figure S3). Endogenous RPS4X was only found in the GFP-YB-1 immunoprecipitate indicating an interaction between RPS4X and YB-1 in ovarian cancer cells as well.

We added the following in the Materials and Methods section.

Immunoprecipitation of GFP (Green Fluorescent Protein) and GFP-YB-1 constructs were performed as described previously [21].

We also added the following information as supplemental Figure S3.

**Supplementary Figure S3** Co-immunoprecipitation of endogenous RPS4X protein with GFP-YB-1 in transfected SK-OV-3 cells. Cells were transfected with GFP or GFP-YB-1 expression vectors and the next day GFP or GFP-YB-1 proteins were immunoprecipitated with an anti-GFP antibody. Endogenous RPS4X is co-immunoprecipitated only in cells transfected with the GFP-YB-1 construct. WCE = whole cell extract; anti-GFP = immunoprecipitation with an antibody against GFP. Bands corresponding to GFP-YB-1 and the endogenous YB-1 proteins are shown in the whole cell extract.

**Response to reviewer #3**

1. Page 4, line 16 – The eligibility criteria are described in the material and method section and state clearly “platinum-based post-operative chemotherapeutic treatment for ovarian cancer” as one of those criteria. In contrast in the discussion section the authors write: “As the immunohistochemistry study was performed on serous high-grade ovarian tumors from patients who had not received chemotherapeutic treatment, low expression of RPS4X could also correlate
with intrinsic resistance, although this remains to be determined”. Did the author not adhere to their own eligibility criteria, please explain?

Response: Because of the confusion with the criteria inclusion in the materials and methods section, we replace the sentence by the following phrase. Eligibility criteria for inclusion in the study were as follows: primary surgery, complete information on post-operative chemotherapeutic treatment, high grade serous histopathology subtype, and completed tumor banking informed consent.

We also added the following sentence in the TMA section of the materials and methods. The tissue array was composed of 260 ovarian cancer samples from patients that never received chemotherapy before their surgery and 11 samples of areas from normal fallopian tubes of cancer patients.

2. How can RPS4X levels be used in the clinic as prognostic marker? Have the authors tried more quantitative ways of measuring RPS4X expression e.g. RT-PCR? Please mention this in the discussion section.

Response: The following sentence was added at the end of the second paragraph of the discussion section.
In addition, a more quantitative way of measuring RPS4X expression, as for example real-time quantitative RT-PCR, could be envisioned.

3. The authors present evidence that RPS4X down regulation affects cellular proliferation and confers resistance to cisplatin. Exactly how RPS4X modulates cisplatin sensitivity is not known. In the discussion section (page 11) the authors speculate that the reduced growth rate caused by RPS4X depletion affects the cellular survival after cisplatin exposure. Is the RPS4X mediated resistance phenotype specific for cisplatin or is it a general effect seen with other (DNA damaging) chemotherapeutics as well? The authors should mention this in the discussion.

Response: As requested by the reviewer the following sentence was added in the last paragraph of the discussion section.
In addition, a thorough analysis of the impact of RPS4X levels on different types of reagents used in chemotherapy is also required.

Minor essential revisions
1. Page 7 – The word sulforhidamine is misspelled multiple times, correct is sulforhodamine.

Response: The corrections were made.

2. Page 8, line 2 – Line 2 mentions 193 clinical samples whereas Table 1 mentions 196. Please correct.
**Response:** We made the appropriate corrections. In the results section, we added the following information.

We determined the levels of expression of these proteins by immunohistochemistry in 192 clinical samples from women with ovarian cancer (Table 1). (Note that for each immunohistochemistry staining experiments, samples of poor quality were excluded from the statistical analyses).

3. **Page 9 –** Please include data not shown in a supplemental figure.

**Response:** We replaced the Western data by FACS and FITC-Annexin V analyses as requested by the previous reviewers. (Please see response to the previous reviewer).

4. **Page 9, line 26 –** Did not detect differences…….. What was the rationale behind analyzing p16, p21 and p53 expression? Please include in the text.

**Response:** We replaced such data by FACS analyses to determine the impact of RPS4X depletion on cell cycle. (Please see response to previous reviewer).

5. **Page 10, line 1 –** Figure 4 should be changed into Figure 5.

**Response:** We added new figures 4, 5, and 6 as we performed additional experiments as requested by all the reviewers.

6. **Table 1 –** Explain what censured (censored?) and non-censured (non-censored?) means in the legend.

**Response:** We removed those confusing terms from table 1.

7. **Figure 4 legend –** Figure 4 does not show cisplatin resistance data, correct figure title. OVCAR-1 should be OVCAR-3.

**Response:** The new title of figure 4 now reads:
**Figure 4** Cell growth in RPS4X-depleted OVCAR-3 and SK-OV-3 cells.

8. **Figure legend –** sulforhidamine should be sulphorhodamine.

**Response:** The correction was made accordingly.

9. **Is there any information whether the tumors that express relatively low levels of RPS4X grow more slowly than tumors that express high levels of RPS4X?**
Response: We have very little information on tumor growth rate in each patient of our cohort. However, the following information was added in the results section. As expected expression of RPS4X correlated significantly with expression of YB-1. It also correlated positively with the expression of the mitotic index marker Ki67.

Response to reviewer #4
1) Table 1 shows the summary of the characteristics of the tumours used. The tumours are divided into Grade 2 and Grade 3. The mean disease free interval and the mean overall survivals are substantially shorter for the Grade 2 group than for the Grade 3 group. Is this expected? It seems counterintuitive. Given that these clinical parameters are important factors in the subsequent univariate and multivariate analyses, this should be explained.

Response: Our pathologist reviewed all the cases and classified them as low grade and high grade. We kept the high-grade cases for this analysis.

2) In Table 2, the expression of RPS4X is positively associated with grade and mitotic index, and negatively associated with death and recurrence. This is confusing- does this mean that low levels of RPS4X expression are associated with higher rates of death, higher rates of recurrence, lower grade and lower mitotic index? It is not clear from this presentation of the data exactly what the correlations are.

Response: We removed the death and recurrence from Table 2 to avoid this confusing interpretation.

Minor essential revisions:
1) In M&M, the description of the tumours used should include the fact that these tumours were chemo naïve. This fact is mentioned in the discussion, and can be inferred from the description in the methods, but should be specified.

Response: The following sentence was added in the TMA section of the M&M. The tissue array was composed of 260 ovarian cancer samples from patients that never received chemotherapy before their surgery and 11 samples of areas from normal fallopian tubes of cancer patients.

2) The TMA data for YB-1 and RPS4X are reported as 1 through 5, representing no through high levels of staining. In the analyses, the tumours are grouped into low versus high expression. Low is defined as categories 1-3, and high is categories 4 and 5. How is this division of categories justified?

Response: We used the median of the scale staining to divide the scores in 2 groups for each biomarker. (Please also see response to comment 4b of reviewer #1 above.)
3) In the results, there is a statement that Kaplan-Meier plots for RPS4X showed that the overall survival of patients with low or no RPSX expression was significantly worse than those with high expression. There were, apparently, no cases with no expression, so this statement is not accurate and should be amended.

Response: The following new sentence replaces the old version.
In contrast, Kaplan-Meier plots for RPS4X showed that the overall survival of patients with low RPS4X expression was significantly worse than that of patients with high RPS4X expression ($P = 0.002$) (Figure 2A).

4) There is a statement in the results that Figure 3B shows that RPS4X and YB-1 do not regulate each other at either the transcriptional or translational level. Since Figure 3 is entirely based on protein-based assays, there is no data in this manuscript about possible effects at the transcriptional level.

Response: The text was revised as follows.
These results suggest that RPS4X and YB-1 do not regulate each other at the protein expression level in OVCAR-3 cells. Similarly, a depletion of RPS4X in SK-OV-3 cells did not have an impact on YB-1 protein levels (Figure 3C and D).

Discretionary revisions:
1) The experiments were aimed at examining both the expression patterns of YB-1 as well as RBS4X. YB-1 is discussed in the context of its direct interaction with RPS4X, and indeed previous studies showed that expression of this protein is correlated with prognosis. In this study, YB-1 does not correlate with outcome parameters, and the authors suggest that either the high-grade nature of this cohort, or, alternatively, the particular antibody used, may account for this difference. The fact that the use of a different antibody might radically change the interpretation of how protein levels might correlate with outcome would seem to be important for the discussions in this paper. This could be examined relatively easily in this same cohort and would further explore the true relationship of the expression of these proteins to clinical outcomes.

Response: While we agree this could shed light onto the differences we observed with the literature, it should be noted that the time to properly address this is significant, and cannot be accomplished in the time allotted for corrections. Finally, we prefer to remain focused on the RPS4X data that we are presenting as novel in the field.