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Reprogrammed mRNA translation drives resistance to therapeutic targeting of ribosome biogenesis

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 30th Mar 2020

Thank you for submitting your manuscript (EMBOJ-2020-105111) to The EMBO Journal. I have now read your study carefully and discussed your work with the other members of the editorial team. I regret to inform you that we have decided not to pursue publication of this manuscript in The EMBO Journal.

We appreciate that you assess the response to CX-5461 and everolimus (EV) treatment using polyribosome profiling and metabolomics and find that translation of mRNAs encoding factors involved in metabolic processes is suppressed in the Eu-Myc lymphoma model and AML cell lines. Furthermore, cells resistant to combination treatment exhibit increased translation of mRNAs encoding mitochondrial electron components and increased cAMP signaling, which depends on EPAC1/2, but not PKA. We recognize that this study extends your previous work on CX-5461, and that the proposed potential therapeutic applicability of a triple combination of CX-5461, EV and metformin, or possibly EPAC1/2 inhibition, will be of interest to the respective fields. However, we also find that molecular details of how metabolic reprogramming occurs and how it affects translation of specific mRNAs differentially in cells resistant to combination treatment, would need to be further defined. Thus, taking everything into consideration, we have concluded that the degree of mechanistic insight into synergy and resistance of the CX-5461/EV combination treatment and in turn the conceptual advance provided for a broader audience is not sufficient to warrant further consideration for publication at The EMBO Journal.

Authors' appeal of editorial decision **14th Apr 2020**

Thank you for handling the initial screening process of our manuscript "**Reprogrammed mRNA translation drives resistance to therapeutic targeting of ribosome biogenesis**."

We were disappointed with the decision not to consider the paper as high priority for further review because "the conceptual advance provided for a broader audience is not sufficient". Understanding how alterations in ribosome biogenesis and translation contribute to tumor progression is indeed a critical topic in cancer biology with the NCI convening an international workshop in April 2019 to facilitate development of ribosome targeting therapies ("Dysregulation of ribosome biogenesis and protein synthesis in cancer", co-led by Prof Davide Ruggero and Prof George Thomas). We do take your point that better definition of the molecular details of how metabolic reprogramming occurs and how it affects translation of specific mRNAs differentially would improve the manuscript. Unfortunately, we had not included some key data in order to simplify the story. In a revised manuscript, we will include data demonstrating that the increase in polysomes in resistant cells is associated with increased translation of metabolism-associated mRNAs with 5'UTRs that are shorter and contain a uridine-rich motif similar to the pyrimidine-rich translational element (PRTE). Short PRTE-containing mRNAs are a characteristic of mTOR targeted mRNAs (Hsieh et al., 2012) and our findings are consistent with upregulation of translation of mRNAs normally targeted by mTOR inhibition as a source of resistance to ribosome-directed therapies. Among the metabolism-associated mRNAs that are being translated significantly more efficiently in the *in vivo*-derived early-passage drug-resistant cells are mRNAs encoding components of the mitochondrial electron transport chain such as NDUFV1 and NDUFC2 (Complex I), CYC1 and UQCRC1 (Complex III), as well as multiple subunits of the ATP synthase (Complex V).

We hope that you will re-consider your decision and allow us to re-submit a manuscript that provides additional mechanistic insight while also commanding the attention of both cancer researchers and medical oncologists.

We thank you for considering this request.

2nd Editorial Decision 30th May 2020

Thank you again for submitting your manuscript analyzing the synergistic effects of targeting ribosome biogenesis and metabolism for consideration by The EMBO Journal. We have now received three referee reports on your study, which are included below for your information.

As you will see, the reviewers are overall positive and acknowledge the approaches taken to address molecular basis for the synergy of CX-5461 and everolimus (EV) treatment. Nonetheless they also raise some concerns that would need to be addressed in a revised manuscript. In particular, both referee #1 and referee #3 find that the methods are not discussed in sufficient detail and that this hinders the reader in easily following the conclusions drawn. This issue must be resolved throughout the revised manuscript. In addition, referee #1's point 2 regarding further discussion of the resistance phenotype should be addressed, as well as thoroughly taking into account the comments of referee #3 and revising the text and figures as necessary. Please also carefully respond to all other concerns raised by the referees and revise the manuscript and figures as applicable. Furthermore, please be advised that EMBO Journal's policy requires deposition of datasets in public repositories and these accession codes must be specified in a "Data Availability" section in the main manuscript (please also see point 8 below).

Please note that it is our policy to allow only a single round of major revision. We realize that lab work worldwide is currently affected by the COVID-19/SARS-CoV-2 pandemic and that experimental revisions may currently be delayed. We can extend the revision time when needed, and we have extended our 'scooping protection policy' to cover the period required for a full revision. However, it is nonetheless important to clarify any questions and concerns at this stage and we encourage you to discuss a revision plan and any potential issues you may foresee as soon as possible.

Please also feel free to contact me should you have any other further questions. Thank you for the opportunity to consider your work for publication, I look forward to receiving your revised manuscript.

Referee #1:

Kusnadi et al. aim to identify the biological mechanisms underlying the potent combinatorial effects of the RNA Pol I inhibitor, CX-5461 (CX), and the mTOR inhibitor, everolimus (EV), in blood cancers as well as how cancer cells acquire resistance to this treatment. Using genome-wide polysome profiling, the authors demonstrate that acute treatment with this combinational therapy decreases the translation of mRNAs that encode for many components of the translation apparatus itself, as well as mRNAs encoding for key metabolic enzymes. However, in acquired therapy resistance, the translation of mRNAs encoding for energy-producing pathway components increases, and the metabolomics analysis show that the metabolic products of these pathways are also increased, particularly in the cAMP-EPAC1/2-RAP1 pathway. Finally, in line with these proposed mechanisms of resistance, the authors demonstrate that the general energy decreasing drug metformin can re-sensitize resistant lymphoma cells to the combinatorial treatment in vivo.

This work provides interesting mechanistic insights into how the simultaneous targeting of the translation apparatus and metabolism may provide new therapeutic approaches. In addition, the experiments proposed demonstrate that the response to CX and EV therapy is due to translational rewiring and not just p53 induction. The authors elegantly pair a multi-omics approach with a mechanistic functional link to the cAMP-EPAC1/2-RAP1 pathway to identify a new clinically relevant treatment regimen for blood cancers. Additionally, the 5'UTR analysis that is used to detect an RNA element previously found in mTOR-regulated genes provides new insight into how the emerging concept of the cis regulon coordination of the translational response may mediate the resistance phenotype. However, the manuscript would benefit from some clarification on the methods used for the polysome profiling analyses. In addition, the authors should provide a clearer explanation on the linkage between the acute, decreased translational response and the increase in translation of metabolic pathways in the resistant cells. Strengthening what mediates this change in translational profiles, especially further validation of key translationally controlled target genes and how this contributes to, or results in, the dependence on the cAMP-EPAC-RAP1 survival pathway, would enhance the impact and clarity of the paper. Overall, this work uses multifaceted approaches to reveal a new metabolic mechanism of resistance and defines a new therapeutic vulnerability for more effective and tailored cancer treatments. With adequate addressing of the points below, it would be appropriate for publication in EMBO Journal.

Main Points:

1. The in vivo polysome profiling of acute treatment and the polysome profiling of in vivo-derived, treatment-resistant cells are useful data sets. However, the text lacks sufficient explanation on the analysis behind each panel presenting these data. A key example is Fig. 1E; has this analysis been normalized to total cytoplasmic/all fractions mRNA-seq? The methodology is opaque in both the text on p. 4 and in the methods, this is important to differentiate changes specifically at the translation level as well as the mRNA abundance level. Without this needed normalization, it is challenging to differentiate a change in translation from a change in mRNA abundance. This is made all the more pertinent given the fact that in human cancers, total mRNA levels of EPAC1/2 are increased, suggesting that transcriptional changes are equally likely as the translational rewiring in resistant cells.

2. The authors should provide a clearer discussion on how the repressed translation of specific cellular processes in acute treatments (Fig. 1E) shifts to a resistance phenotype, where key metabolic pathways are increased at the translational level. For example, are there specific

translationally regulated targets identified in Fig. 4F also present in the gene sets in Fig. 1E? Are these genes translationally derepressed during the transition from the acute to resistant phase to become more translated in a resistant state? Are the components of the cAMP downstream signaling, such as EPAC1 or EPAC2 themselves, translationally regulated? A better understanding of the shift in translation over the course of treatment will inform the optimal use of metformin, either concurrent or adjuvant, to CX-5461+EV treatment in order to prevent the development of resistance. Alternatively, Is pharmacologically/genetically reducing EPAC1/2 sufficient to prevent resistance to CX+EV? This would also shed light on how the cancer cells ramp up metabolic gene translation in the process of acquired resistance.

Minor Points:
1. Fig. 1B - It would be helpful to see the polysome traces for this experiment. Do the authors observe any changes in ribosome quantities?
2. Fig. 1D - What does the circle size indicate in this figure?
3. Fig. 2G - Do the authors observe a dose-dependent correlation between sensitivity to metformin and increased cellular metabolism levels as characterized in Fig. 2F? This would strengthen the hypothesis that metabolic rewiring is a potent vulnerability in CMB-resistant cells.
4. Fig. 4D - This clustered heatmap does not clearly convey the desired message of the panel. It would be helpful to the reader if the authors reconfigured this heatmap to better highlight the genes that are uniquely changed in the CMB cells as compared to CX and CTRL cells.
5. Fig. S1A - It would be beneficial to show Annexin V/PI staining of the GFP+ cells to confirm the lack of apoptosis at this early time point.
6. Fig. S1I-L - It is unclear why CX-5461 + EV has no impact on these cell lines, but single agent CX + metformin does. This result appears consistent with the hypothesis that the double agent CX + EV rewires translation to promote the metabolic dependency. Is this an artifact of the cell lines?
7. Fig. S3F - This figure is not referenced in the text of the manuscript.
8. p. 7 - The reference to Figure S3D appears to be for Figure S4A
9. Fig. S3G - It would be nice to confirm that Rap1-GTP is decreased in the treated cells to validate target activity.
10. p. 8 - Reference Figure S3A in the discussion of the mRNA 5'UTR motif analysis appears incorrect as that panel displas the quantification

Referee #2:

CX-5461 is an innovative drug that inhibits rRNA synthesis highly specifically and has shown promise in a phase I clinical trial. Its efficacy in preclinical models can be synergistically enhanced by combination with everolimus, an approved inhibitor of signaling through mTORC1. The current manuscript dissects the molecular basis of this combinatorial effect, revealing metabolic changes mediated through translational reprogramming. It goes on to show that drug resistance can arise through adaptive changes at the translational level that up-regulate cAMP-EPAC-RAP1 signaling to provide protection against drug-induced cell death. This discovery reveals a metabolic vulnerability that can be exploited therapeutically, as shown by the highly effective addition of the anti-diabetic drug metformin to the combination of CX-5461 and everolimus. The manuscript explains clearly the rationale for the experiments and the data are well-presented and convincing, building a strong case based on novel insights.

I have only minor suggestions for improvement.

P4. "...consistent with the reduced translation of translation initiation factors following CX-5461+EV treatment (Figure 1D), which are required for efficient translation of mRNAs with long 5'UTRs (19)." This sentence needs clarification.

P5. The authors do not comment on the fact that EV cells in Fig 2B have significantly reduced sensitivity to CX-5461, relative to CTRL.

P5. It would be worth including EV cells in the ATP data shown in Fig 2E.

Referee #3:

The manuscript by Kusnadi and colleagues seeks to gain a deeper understanding of the mechanisms underlying the previously reported positive synergistic effects of CX-5461 (a ribosome biogenesis inhibitor) and everolimus (a PI3K/AKT/mTORC1 inhibitor) observed in their pre-clinical studies of Myc-driven lymphoma and prostate cancer (REFs. 4 and 6). Evidently, combination therapy (CX-5461 + everolimus) outperformed treatments with either drug alone in vivo. They were also interested to understand how this combination therapy led to an apparent resistance to both CX-5461 alone or in combination with everolimus.

To gain these mechanistic insights, the authors set out to define the distinct impacts of both of these drugs on gene expression alone and in combination in order to reveal insights on how cells achieve resistance to drug treatment in order to optimize the clinical efficacy of Pol I-directed 'ribosometargeting' therapies. The goals of this study are laudable and important given the need for effective clinical therapies for the cancer treatment and the promising data in the literature - initiated and advanced by many of the authors on this body of work - indicating that CX-5461-mediated disruption of ribosome biogenesis is a viable treatment approach that has shown significant clinical promise, including low toxicities.

Over the course of several weeks, each time I tried to delve into this paper was thwarted by the presentation of the data, particularly the data presented in Figures 1 and 2. I certainly appreciate the complexities of the experiments presented and I can tell they were performed with care, but a better job needs to be done to present clearly the methods employed to carry out the massive amount of data presented. The procedures used to generate the data presented are, in my view, not adequately or completely described (see examples below).

In my review of this work, I came to realize only at a very late stage, that the compiled PDF that I was provided actually had concatenated files, where a second version of the manuscript was appended to the first. Given the letter to the editor, it would seem that the latter of the two was the revised version that included the data that is now present in Figure 4. The older version of Figure 4 is now Figure 5. The lengthy review provided below, appear to apply to both versions of the manuscript as they largely pertain to Figures 1 and 2. I was, however, unable to do a precise comparison of the specific language used in both versions of the text. I am reasonably confident that all comments apply. The purpose of these comments is not to harshly criticize the work. Rather, they are meant to help the authors revise the language in the main text, the figure legends and methods sections so that the reader can readily follow the presentation of data with confidence. I was unable to do so until in my review of Figures 1 and 2. The paper became much

more accessible thereafter, although specific issues, delineated below, should be addressed before the manuscript is accepted.

Figures 1 and 2 comments:

To begin this work, the authors state, "To interrogate the molecular basis of the response to CX-5461 and the CX-5461 plus everolimus combination, we first performed genome-wide translational profiling to characterize the acute changes in mRNA usage of MYC-driven B-cell lymphomas in mice treated with CX-5461 and the mTORC1 inhibitor, everolimus." They go on to present the data in Figure 1 and Figure S1, which I find extraordinarily confusing. This confusion, highlighted in the points to follow, was at least partly due to the manner in which the work was explained and this undermined my confidence in the data presented and my ability to follow the analyses presented subsequently.

Example issues: To begin, the authors state that, "E -Myc B-cell lymphoma (MSCV Gfp; clone #4242) cells were transplanted into C57BL/6 mice (3, 4)." Assuming that the authors are not referring the reader to the methods section of referenced papers 3 and 4, I turned to the current paper's methods section, which states under the "Animal Experiments" heading that they initiated their drug studies in the following way: "2 x 105 E -Myc lymphoma cells injected into the tail vein of 6-8 week old male C57BL/6 mice". They then state, "Lymphoma-bearing mice were treated on Day 10 post transplant for 2 hours with CX-5461 (35 mg/kg), everolimus (EV; 5 mg/kg), or both (CX-5461+EV; 35 mg/kg CX-5461 and 5 mg/kg EV)." The subsequent biochemical Figure 1 data are then discussed, without ever stating how the cells were harvested from these mice. I believe the information should be provided here as to how the cells that were subsequently harvested and analyzed. I deduced that the authors somehow physically harvested lymphomas by dissection and that they ground these cells up to isolate total RNA and polysomal RNA to then perform their comparative gene expression analysis. Fig. 1A starts with a Western Blot, which seemed perfectly reasonable. But then they present Fig. 1B, a polysome analysis profile, which from experience requires nearly 1 billion cells to obtain (this is a bit of a guess given than there are no units on the A260nm Y-axis in this figure). I then checked the Methods section as to how polysome profiles were performed. There, under the section heading, "Polysome profiling, RNA isolation and RNAseq analysis", the discussion begins with a description of cultured suspension cells. Initially, this made me think that the polysome analysis shown in Fig. 1B was actually from suspension cells, which would make sense to me given the data shown (I will add here that there is no mention made of how many cells were used for such experiments). I became further concerned that this was the case based on the legend of Figure 1B, which states that this figure is a schematic. However, isolation procedures for "in vivo polysome profiles" are described later in the same Methods section as well. There, the authors state that they extracted material by "grinding snap-frozen tissue samples". They make no mention of how much tissue was used and where it came from. Perhaps then this data is actually suspension cell lysate and is meant only as an illustration? To me it seems important to show all of the polysome profiles for the samples carried forward in the analyses to follow. How much do the polysomes vary? Is it possible that the data were biased in some way based on this isolation procedures? It seems important to present these data to the reader as the impacts reported could simply represent a non-specific metabolic impact on translation initiation or elongation rates that shift the polysomes differentially towards "lighter" polysome fractions (monosomes, disomes and trisomes) without actually changing the overall amounts of protein produced at the level of translation. Directly showing the polysome profiles would inform on this possibility.

Moving forward. The authors then present comparative gene expression analyses on cytoplasmic

and polysomal (tetrasomes and greater) for the "tissue samples" isolated from untreated, CX5461 treated (35 mg/kg), everolimus treated (5 mg/kg) and combination treated. These cells, wherever they came from, were evidently treated for just 2 hours each "to exclude any confounding effects of drug-induced apoptosis on our molecular analyses". The authors state that they verified that there was no apoptosis by counting the number of GFP-positive lymph cells they isolated from the lymph nodes of the same mice: "no change in the percentage of GFP-positive lymphoma cells was observed in lymph nodes isolated from treated animals (Figure S1A)". Again, although not explicitly stated, I deduced the information stated in the Figure 1C legend that this analysis was performed on the same source of the cells used to perform their gene expression analysis. How valid is the assumption that the tumors are uniformly comprised of the same types of cells and that the compositions of these samples don't vary in some way following the drug treatments? I suppose 2 hours is not very much time for significant restructuring to occur assuming the authors are isolating solid tumors (although not stated) but how do the authors verify that identical proportions of the tail injected, Eu-Myc lymphoma cells are present in the samples assessed? The cell compositions of the "tissue samples" seems like the relevant information to present for the reader to be confident in the data presented. If this is not the case, then the changes in gene expression shown in Figs. 1C-E and Fig. S1 may simply reflect changes in the population mix of cells present in these lymph tissues.

Figure 1 then goes on to present a gene set enrichment analysis using MetaCore® GeneGO of the "translationally" affected gene sets for CX-5461, everolimus and CX-5461 + everolimus "tissue samples" compared to control cells. Here the authors provide the striking finding that CX-5461 has "no significant" impact on translation, while everolimus significantly reduced the expression of "translation initiation" and "translation elongation" gene sets. Importantly, the authors state that this everolimus impact carries through to the combination treatment (CX-5461 + everolimus), where CX-5461 appears to profoundly amplify the magnitude and breadth of the number of genes impacted in these two categories. Here, additional questions arise. First, it seems that these two GeneGO "categories" are nearly identical in composition: there appear to be only 5 unique genes in translation initiation and 3 in translation elongation. The rest are ribosomal proteins (1 translation initiation factor eIF1a appears in both categories for some reason). Given CX-5461's published impact (supported by the data presented later in Figs S2D-I) of repressing ribosome biogenesis, this would make sense. As the gene sets principally represent ribosome biogenesis impacts not translation initiation or elongation. In my read of the data presented, the observed impacts on gene expression appear to be quite small. The LogFC color map indicated would imply maximally 1.3 fold or 1.5 fold changes on average depending on whether this is log based 2 or 10- (not stated). Are these "dramatic" effects as the authors later state? This raises the second question: why is it surprising that two drugs that both act to inhibit ribosome biogenesis (by distinct targets presumably) would act additively to do so? The inference of synergism seems unwarranted. The authors seem to argue that this additive impact is at the level of polysome fractionation specifically? To me, additive negative impacts on the production of ribosome components seems to be the expected outcome and may occur through subtle changes in translation initiation rates brought about by the actions of both drugs. This could arise, for example, via even a modest activation of eIF2-related aspects of the Integrated Stress Response. Was this checked? This possibility does not however appear to be considered or discussed.

Rather, the authors go on to do a distinct analysis of their polysomal RNAs, which appears to bypass important normalizations to total RNAs (single sample gene set enrichment analyses). This leads them to conclude that certain metabolic pathways are disproportionately downregulated. "Critically, despite this potent reduction in the efficiency of translation of components of the translational apparatus, cells treated with CX-5461+EV did not indiscriminately reduce the translation of all mRNAs globally". How do the authors know that this isn't simply a matter of cell

number? Are there other normalization procedures used? I won't dwell here on the term "potent", except to say that the impact, as explained above, seems quite modest given the expected impacts of the drugs on proliferation. Are the authors suggesting that 2 hours is too short to have transcriptional responses? The downregulation of metabolic gene expression at the level of transcription could very well be rapid and would be the expected response to a global down regulation mTORC signaling and ribosome biogenesis. Such a response should also disproportionately effect genes with longer 5'UTRs (ie. slower translation initiation rates by the definition of the scanning mechanism) given what the scientific community knows about translation initiation (no references provided by the authors - see for instance a recent review by Rachel Green's group on the subject). Slower initiation rates, which could arise via eIF2 modulation or other factors, would therefore tend to shift such mRNAs to "lighter" polysome fractions as a consequence, which does not necessarily mean that the protein levels are reduced, it just means that these mRNAs would drop out due to the authors isolation procedure.

Nonetheless, the authors follow these findings by stating, "We hypothesised that this CX-5461+EV-induced targeting of metabolism was a key driver of the improved response to the combination". What do the authors mean by "improved"? Synergistic? Here, although this is not stated, the authors appear to switch to studies of suspension cells (my inference), not the isolated tumor cells. This should be clarified. They then state, "To test this hypothesis, we used metformin, a well-tolerated anti-diabetic drug that lowers cellular energy levels (20). To my knowledge the mechanism of action of metformin is not known and thus seems like a poor choice for gaining mechanistic insights that inform on the problem statement. Metformin will simply add to the inhibition of proliferation through unknown mechanisms. The clearest indication I had that the experimental strategy had changed entirely was their statement, "Metformin treatment robustly increased cell death induced by CX-5461, consistent with a critical role for inhibition of metabolism in the improved efficacy of CX-5461+EV (Figure S1G)." As stated above, the authors clearly state at the onset of the results section that they gave CX-5461 and everolimus to mice for only 2 hrs specifically "to exclude confounding effects of drug-induced apoptosis on our molecular analyses". This seems like an important clarification to make for the reader. Also, where does the notion that CX-5461 causes cell death at the concentrations employed come from? Is this a new finding? There is no reference provided.

Figure S1G and figure legend indicate the application of 5 millimolar metformin for 48 hrs onto a presumed culture of Eu-Myc lymphoma cells that were grown either in the absence or presence of CX-5461. Here it is not at all clear how CX-5461 was administered, how the cells were grown or for how long the cells were subjected to 7.5 nM CX-5461. I infer that the drug was added for 48 hrs given the induction of cell death. It is not stated why a concentration of 7.5 nM CX-5461 was chosen. Does this reflect the authors anticipated equivalent to 35 mg/kg in vivo? At this juncture, the authors also refer to Figure S1H, which shows a similar test of propidium iodide staining of cells treated in an analogous way but including everolimus co-treatment. Again, the durations of the drug administrations and the rationales for the drug concentrations used are not given (here, for some reason CX-5461 is administered at a concentration of 5 nM - presumably for 48 hrs.).

The authors further conclude from this study that "Moreover, metformin also markedly improved the therapeutic potency of CX-5461+EV (Figure S1H), emphasizing the importance of the targetable metabolic vulnerability in response to Pol I-directed therapy." Here, I am confused by what the authors mean by "therapeutic potency" in regard to combined CX-5461 + everolimus treatment. I presume we are referring to cultured cell lines and propidium iodide staining. This statement implies that cell death is the desired outcome, rather than a simple inhibition of growth. Is this the case? The authors further state in the conclusion of this paragraph, "Thus, we propose that the reduced

translational activity in CX-5461+EV combination therapy treated cells (Figure 1C), which selectively impaired translation of mRNAs encoding metabolic regulators, is a key mechanism in the synergistic effect of the two drugs and highlights the intimate coupling of mRNA translation and energy metabolism (13, 21)." Here, I do not believe the data presented support the claim, that the data in Figure 1C show selectively reduced translational activity. It may be the case that genes for metabolic enzymes were down regulated, this again makes sense given the MOAs of the administered drugs, but it is not at all clear why these genes only came to light in the single sample GSEA analysis, which does not appear to control of total RNA levels. Why were these not found to be enriched in the earlier GeneGo analyses. Measurements of total protein levels or metabolites that would be needed to substantiate this conclusion are not given - at least in the data presented up to this point. This conclusion therefore requires additional clarification.

The authors then go on to state, "Despite the dramatic initial impact of the combination treatment on tumor growth, animals eventually succumb to lymphomagenesis (3, 4). The initial clause of this statement appears to be a conclusion that is drawn from the present set of experiments but I do not see such data presented? Did I miss something or are the authors trying to indicate that tumor growth measurements at the 2 hour time point were made in referenced works 3 and 4? This should be clarified. Either way, this would seem to provide supporting evidence that short drug treatments do indeed affect tumor morphology and hence composition - at least potentially - drawing question to the interpretations presented in Figure 1.

The authors then immediately go on to examine the stated hypothesis: "we hypothesized that metabolic rewiring driven by specific changes in mRNA translation would confer this resistance to therapy." What resistance to therapy? Cell death? Are the authors referring to an inference that lymphogenesis results from drug-resistant cells? How do they know this is the case? Does this model assume that all cells subjected to drug treatment must die or become drug resistant? I am not sure I follow the certainty of this logic. This requires clarification.

The authors go on to test this hypothesis by examine the metabolic activities of the cells extracted from the tumors of mice that had been subjected to one or both drugs as well as control cells, "These early-passage cell lines were derived from lymph node extracts isolated from 12 different mice that were transplanted with Eμ-Myc B-lymphoma cells (clone #107) as indicated in Figure 2A." Figure 2A does not provide any information on how these cells were extracted, sorted or for how long they were passaged before the experiments were conducted. I was also unable to find any information in the Methods section of the paper. The authors then state," To confirm that CX and CMB [early passage cells isolated from lymph node extracts] maintained their drug resistance phenotypes, they were treated with EV, CX-5461 or CX-5461+EV in vitro. The drug-naive CTRL cell lines retained sensitivity to all treatments, while CX cells were resistant to CX-5461 and sensitive to CX-5461+EV and the CMB cells were unresponsive to all the treatments (Figure 2B). This is not my interpretation of the data shown in Fig. 2B. I see that cells isolated from CX-treated and everolimus-treated mice remain sensitive to both drugs. Is the argument that the differences observed relative to the control studies are statistically significant? I did not see support for this in their analyses. The clearest result appears to be that cells isolated from combination-treated drugs exhibit significant resistance to both drugs. But how is the reader to know that these the same cell types in the absence of a detailed methods section on how they were extracted and cultured? Thus, the importance of clarifying the isolation method and verification of what cells are being examined precisely.

Regardless, the authors go on to show in control cells that CX-5461 and everolimus both exhibit expected changes in cellular markers 47S rRNA transcription and ribosomal protein S6

phosphorylation, respectively, while in early passage cells isolated from CX-5461 treated mice and combination-treated mice are less sensitive to short exposures to CX-5461 treatment. For some unexplained reason, the CX-treatment was 50 nM and the duration of time was 3 hrs - roughly 10x the concentration used for the propidium iodine experiments shown in Fig.S1. This should be clarified. Nonetheless, this finding appears to be quite remarkable, even if the cell cultures are heterogeneous in nature.

Beyond Figure 2:

The later sections of the paper seem to be easier to follow, and much stronger in terms of their presentation, although specific clarifications are needed.

Specific points of clarification needed:

The authors state on page 6, "This analysis identified the cyclic-adenosine 3',5'-monophosphate (cAMP) signaling pathway as the top hit when comparing CMB versus CTRL (Figure 3A), with increased translation efficiency of mRNAs encoding essential components of this pathway, including adenylate cyclase and cAMP-guanine nucleotide exchange factors (cAMP-GEFs) 1 and 2, which is also known as exchange protein directly activated by cAMP (EPAC) 1 and 2." Do these mRNAs exhibit any 5'-UTR characteristics that may help explain the upregulation - i.e. short 5-UTRs?

The axes on Fig.S3C and D need to be made legible.

The text surrounding discussion of Figure 4 seems to need editing.

For instance, the authors state," To determine whether the energy- and cAMP-dependent changes observed in drug-resistant cells are driven by specific reprogramming of mRNA translation, we evaluated the expression patterns of mRNAs encoding proteins that are known to be involved in the regulation of mRNA". Are these the early passage cells? Again, how were these cells isolated and verified.

They go on to state, "Importantly, the heatmap indicated that the CMB cells displayed distinct polysome-association patterns and therefore translation efficiency of of mRNAs encoding the majority of ribosomal proteins, translation initiation (such as eIF4G1 and eIF2A) and elongation factors (such as eEF1A1 and eEF2) as compared to the CTRL and CX cells (Figure 4A)." First, this sentence has typos and is incompletely constructed. It also states that they are making conclusions about translation efficiency, but it is not clear how this was done. Are the samples isolated as schematized in Figure 1B? Are these analyses normalized for total RNA? Or is this a single-sample GSEA analysis? There is no mention of the methods for cell growth, harvest, polysome fractionation and the data analyses methods employed so it's entirely unclear how conclusions about translation efficiency can be drawn.

Figure 4A presents data on the mRNA abundance levels

Figure 4A needs to have the cell types indicated more clearly so that the reader knows which column is which.

In referring to Figure 4F, the authors state, "In addition to changes in the cAMP pathway,

comparison of the RNAseq data from polysomal mRNAs from CTRL, CX and CMB cell lines revealed that the CMB cells strikingly upregulated the translation of mRNAs encoding multiple components of the mitochondrial electron transport chain, such as such as NDUFV1 and NDUFC2 (Complex I), CYC1 and UQCRC1 (Complex III), as well as multiple subunits of the ATP synthase (Complex V) (Figure 4F)." First, I believe the authors mean to reference Figure 3 to support the first clause of this sentence. Second, I am also not sure where these data come from. Were they from the same analyses that led to the generation of Figure 4A? Details are needed here. Do the authors plan to simply provide their gene expression data? It seems like the subsets presented are a bit of cherry picking in a manner that support the model. What were the criteria for selecting these specific genes and what is the statistical significance? There is no FDR information provided on the X-axis of the figure as is presented in the other figures.

I do believe that there are potentially very important data presented that may ultimately be of clinical significance. However, I am left with the impression that the authors need to revise this manuscript to make it more scientifically grounded, readable and interpretable particularly in the early sections (surrounding discussion of Figs. 1 and 2). My recommendation is that the authors carefully revise the manuscript to address the points raised above, particularly as it relates to informing the reader on the details of how the experiments were performed to ensure reproducibility. This may be addressed through careful revision of the manuscript in its present form without major restructuring. I would be more than happy to re-review the work should that be undertaken and should the editor request further review.

We thank you and the reviewers for their constructive feedback and insightful comments to improve the clarity and readability of our manuscript. As you and the reviewers have commented, we agree that a more detailed explanation of some experimental and analysis methods will significantly facilitate the interpretation of the data presented in our study. We have resolved this issue throughout the manuscript. We have included further discussion of the resistance phenotype as suggested by Reviewer #1 in the discussion section (page 12) and have updated Fig 1D, 2E and 4A, as well as included additional data (Fig EV5A) as suggested by the Referees. Importantly, we have responded to all the other concerns raised by the referees as detailed in the point-by-point response below. We note that Reviewer #3 was confused by the two versions of the manuscript they were sent and have attempted to distill the key issues that pertain to the revised manuscript from a very complex response. We have now split the first supplementary figures into Fig EV1 and Fig EV2 to resolve clarity issue highlighted by Reviewer #3

We thank you for reviewing our manuscript and hope that the revised version is now acceptable for publication in The EMBO Journal.

Referee #1:

Kusnadi et al. aim to identify the biological mechanisms underlying the potent combinatorial effects of the RNA Pol I inhibitor, CX-5461 (CX), and the mTOR inhibitor, everolimus (EV), in blood cancers as well as how cancer cells acquire resistance to this treatment. Using genome-wide polysome profiling, the authors demonstrate that acute treatment with this combinational therapy decreases the translation of mRNAs that encode for many components of the translation apparatus itself, as well as mRNAs encoding for key metabolic enzymes. However, in acquired therapy resistance, the translation of mRNAs encoding for energy-producing pathway components increases, and the metabolomics analysis show that the metabolic products of these pathways are also increased, particularly in the cAMP-EPAC1/2-RAP1 pathway. Finally, in line with these proposed mechanisms of resistance, the authors demonstrate that the general energy decreasing drug metformin can re-sensitize resistant lymphoma cells to the combinatorial treatment in vivo.

This work provides interesting mechanistic insights into how the simultaneous targeting of the translation apparatus and metabolism may provide new therapeutic approaches. In addition, the experiments proposed demonstrate that the response to CX and EV therapy is due to translational rewiring and not just p53 induction. The authors elegantly pair a multi-omics approach with a mechanistic functional link to the cAMP-EPAC1/2-RAP1 pathway to identify a new clinically relevant treatment regimen for blood cancers. Additionally, the 5'UTR analysis that is used to detect an RNA element previously found in mTOR-regulated genes provides new insight into how the emerging concept of the cis regulon coordination of the translational response may mediate the resistance phenotype.
However, the manuscript would benefit from some clarification on the methods used for the polysome profiling analyses. In addition, the authors should provide a clearer explanation on the linkage between the acute, decreased translational response and the increase in translation of metabolic pathways in the resistant cells. Strengthening what mediates this change in translational profiles, especially further validation of key translationally controlled target genes and how this contributes to, or results in, the dependence on the cAMP-EPAC-RAP1 survival pathway, would enhance the impact and clarity of the paper.
Overall, this work uses multifaceted approaches to reveal a new metabolic mechanism of resistance and defines a new therapeutic vulnerability for more effective and tailored cancer treatments. With adequate addressing of the points below, it would be appropriate for publication in EMBO Journal.

<u>Main Points:</u>
1. The in vivo polysome profiling of acute treatment and the polysome profiling of in vivo-derived, treatment-resistant cells are useful data sets. However, the text lacks sufficient explanation on the analysis behind each panel presenting these data. A key example is Fig. 1E; has this analysis been normalized to total cytoplasmic/all fractions mRNA-seq? The methodology is opaque in both the text on p. 4 and in the methods, this is important to differentiate changes specifically at the translation level as well as the mRNA abundance level. Without this needed normalization, it is challenging to differentiate a change in translation

from a change in mRNA abundance. This is made all the more pertinent given the fact that in human cancers, total mRNA levels of EPAC1/2 are increased, suggesting that transcriptional changes are equally likely as the translational rewiring in resistant cells.

The manuscript has been modified with additional detail of the polysome profiling analysis. As stated in the manuscript text, Fig 1C and 1D were generated based on the anota2seq analysis, in which polysome-associated RNA-seq data was indeed normalized to total cytoplasmic mRNA to demonstrate that acute CX-5461+EV treatment inhibited mRNA translation (Table EV1). In contrast, the single sample gene set enrichment analysis (ssGSEA) shown in Fig 1E is a complementary analysis to anota2seq, which is focused on identifying the resulting functional alterations in the levels of translation of individual mRNAs, independent of total mRNA levels. We have clarified this in the Results section on page 5, Methods section on page 15-16 and in the Appendix on page 6. The ssGSEA revealed a selective reduction in the translation of mRNAs encoding key regulators of oxidative phosphorylation and importantly, this was validated by anota2seq analysis that has been added as Table EV2 and described on page 5 of the modified manuscript. These metabolic genes were not identified in the Anota2seq analysis shown in Fig 1C because while the genes from within the relevant ontology groups include key translationally controlled mRNAs (listed Table EV2), others are not and this meant that the group as a whole did not meet the adjusted P value cutoffs for the enrichment analysis performed with MetaCore® GeneGO (Clarivate Analytics).

In the ssGSEA, mRNAs associated with actively translating polysomes are ranked based on their levels, followed by calculation of Gene Set Enrichment Score as now defined in the Methods section. It measures whether genes from multiple defined gene sets, which represent key biological processes involved in cellular growth, proliferation and metabolism obtained from Gene Ontology, KEGG and Reactome databases, are over-represented at the top or bottom of each ranked list. Subsequently, for each gene set, we calculated the difference between the ssGSEA scores in the CX-5461, EV and CX-5461 + EV samples and the vehicle sample. This was then normalised by the ssGSEA scores of the gene set in the vehicle sample. The results represent the percentage of change of ssGSEA scores between the different conditions and the vehicle. These results were presented as “Direction” that visualises the percentage of genes associated with indicated pathways that are up- (red) or down- (blue) regulated upon each treatment. Data were obtained from n=6 mice per treatment group. As noted above, the manuscript text on page 5 has been updated and detailed description of the ssGSEA methodology is now included in the Methods section on page 16-17

2. The authors should provide a clearer discussion on how the repressed translation of specific cellular processes in acute treatments (Fig. 1E) shifts to a resistance phenotype, where key metabolic pathways are increased at the translational level. For example, are there specific translationally regulated targets identified in Fig. 4F also present in the gene sets in Fig. 1E? Are these genes translationally derepressed during the transition from the acute to resistant phase to become more translated in a resistant state?

We have provided more explanation on how the development of acquired resistance caused the biological processes repressed by the acute treatments to be upregulated in the drug-resistant cells in the discussion section (pages 11-12) according to ref #1 and ref #3's suggestions. Similar to the rationale for ssGSEA presented above, our analysis of the mechanisms driving resistance to ribosome targeting therapy specifically examines functional changes in metabolism and in the levels of translation of individual mRNAs independent of total mRNA levels. Our focus is to characterise the key long-term functional changes induced by chronic *in vivo* exposure of tumour cells to ribosome targeting therapy. Indeed, it is likely that altered transcription of these mRNAs may contribute to these functional changes in translation given the long-term in vivo adaptation required to generate resistance. This explains why genes translationally repressed by acute treatment were not identified by the Anota2seq analysis as described for ssGSEA. We have included this detail and rationale on page 8 of the revised manuscript and discussed this further on pages 11-12.

The data analysis revealed specific alterations in the actively translating polysome-associated mRNAs encoding proteins that are involved in the processes of mRNA translation, mitochondrial oxidative phosphorylation and cAMP pathway, consistent with metabolic re-wiring being required for resistance to ribosome targeting therapy.

In particular, anota2seq analysis identified mRNA translation to be significantly downregulated in CX-5461+EV-treated mice (Fig 1C), but after resistance developed *in vivo*, the CX-5461+EV-resistant CMB cells exhibit higher polysome:monosome ratio (Fig 4B and 4C) and altered expression profile of mRNAs encoding proteins implicated in protein synthesis (Fig 4A). Furthermore, we have demonstrated very clearly that acute targeting of translation results in compromised metabolism (Fig 1E, Table EV2). The drug-resistant cells then re-establish elevated metabolism (Fig 2) as shown by Seahorse and metabolomics data (Figs 2C-F) and polysome-association of mRNAs encoding proteins involved in oxidative phosphorylation (Fig 4F).

Are the components of the cAMP downstream signaling, such as EPAC1 or EPAC2 themselves, translationally regulated? A better understanding of the shift in translation over the course of treatment will inform the optimal use of metformin, either concurrent or adjuvant, to CX-5461+EV treatment in order to prevent the development of resistance. Alternatively, Is pharmacologically/genetically reducing EPAC1/2 sufficient to prevent resistance to CX+EV? This would also shed light on how the cancer cells ramp up metabolic gene translation in the process of acquired resistance. Alternatively, Is pharmacologically/genetically reducing EPAC1/2 sufficient to prevent resistance to CX+EV?

The translation of EPAC1 and EPAC2 mRNA was not altered by acute *in vivo* treatment with CX-5461+EV. We cannot monitor changes occurring during the transition to resistance in vivo as this transition begins from very minimal/undetectable disease and no tumour samples were collected until the mice reached the ethical endpoint of relapsed disease over months. However, we demonstrate that drug resistant cells rewire translation by increasing the polysome association of specific mRNAs encoding

proteins involved in cAMP pathway in Fig 3A. We also show that this can be targeted by specific inhibitors of EPAC1/2 and by metformin (Fig 3F and Fig 5A). Nevertheless, we agree that future studies that define this transition may enable improved treatment scheduling.

Minor Points:

1. Fig. 1B - It would be helpful to see the polysome traces for this experiment. Do the authors observe any changes in ribosome quantities?

Unfortunately, the sensitivity of detection of A260 required to observe the integrity and quality of the polysomal peaks, meant that the 80S signal was off scale and thus not quantifiable. There are no consistent changes in the levels of polysomal ribosomes by A260nm between treatments although the traces from individual tumours vary between mice within the same treatment group and hence the analysis is performed on tumours from 6 mice per group (24 traces). In fact, we used one axillary lymph node and one brachial lymph node for each sample (40-50 mg per sample; methods section page 15) and the numbers of ribosomes as indicated by western blot analysis for ribosomal protein S6 were consistent across treatment groups (Fig 1A). We have included the scans of the 24 traces in *EMBOJ-2020-105111_SourceDataForFig1C.pdf*.

2. Fig. 1D - What does the circle size indicate in this figure?

Figure 1D has now been updated with a graphic to show that circle sizes indicate adjusted P value (false discovery rate).

3. Fig. 2G - Do the authors observe a dose-dependent correlation between sensitivity to metformin and increased cellular metabolism levels as characterized in Fig. 2F? This would strengthen the hypothesis that metabolic rewiring is a potent vulnerability in CMB-resistant cells.

CMB cells have elevated metabolic activity compared with CX-cells and indeed LC/MS data indicated that ATP levels are only significantly up in CMB (Fig 2E). Consistent with metabolic rewiring being a potent vulnerability in CMB-resistant cells, CMB cells are sensitive to metformin-induced apoptosis (Fig 2I) while CX cells are not (Fig 2J).

4. Fig. 4D - This clustered heatmap does not clearly convey the desired message of the panel. It would be helpful to the reader if the authors reconfigured this heatmap to better highlight the genes that are uniquely changed in the CMB cells as compared to CX and CTRL cells.

We have now improved the presentation of this heatmap.

5. Fig. S1A - It would be beneficial to show Annexin V/PI staining of the GFP+ cells to confirm the lack of apoptosis at this early time point.

We have included PI-staining of GFP+ cells in Fig EV1A and Appendix Fig S1A, confirming lack of cell death at this time point. The manuscript text in the first paragraph of the Results section has been updated accordingly.

6. Fig. S1I-L - It is unclear why CX-5461 + EV has no impact on these cell lines, but single agent CX + metformin does. This result appears consistent with the hypothesis that the double agent CX + EV rewires translation to promote the metabolic dependency. Is this an artifact of the cell lines?

We believe that this difference reflects the distinct genetic backgrounds and metabolic profiles of human AML cells compared with mouse lymphoma cells rather than being an artefact.

7. Fig. S3F - This figure is not referenced in the text of the manuscript.
The text of the manuscript has been updated to indicate that high expression of EPAC1 was associated with poorer survival on page 8. The updated figure nomenclature is Fig EV4E.

8. p. 7 - The reference to Figure S3D appears to be for Figure S4A
This sentence has now been updated to correctly refer to Fig EV5C.

9. Fig. S3G - It would be nice to confirm that Rap1-GTP is decreased in the treated cells to validate target activity.
The Rap1-GTP pull down data has now been included in Fig EV5A and referred to on p8 of the modified manuscript.

10. p. 8 - Reference Figure S3A in the discussion of the mRNA 5'UTR motif analysis appears incorrect as that panel displays the quantification
The text has now been corrected to only refer to Fig 4E.

Referee #2:

CX-5461 is an innovative drug that inhibits rRNA synthesis highly specifically and has shown promise in a phase I clinical trial. Its efficacy in preclinical models can be synergistically enhanced by combination with everolimus, an approved inhibitor of signaling through mTORC1. The current manuscript dissects the molecular basis of this combinatorial effect, revealing metabolic changes mediated through translational reprogramming. It goes on to show that drug resistance can arise through adaptive changes at the translational level that up-regulate cAMP-EPAC-RAP1 signaling to provide protection against drug-induced cell death. This discovery reveals a metabolic vulnerability that can be exploited therapeutically, as shown by the highly effective addition of the anti-diabetic drug metformin to the combination of CX-5461 and everolimus. The manuscript explains clearly the rationale for the experiments and the data are well-presented and convincing, building a strong case based on novel insights.

I have only minor suggestions for improvement.
P4. "...consistent with the reduced translation of translation initiation factors following CX-5461+EV treatment (Figure 1D), which are required for efficient translation of mRNAs with long 5'UTRs (19)." This sentence needs clarification.

On re-working our original manuscript, we decided that this concept was confusing and removed it from the revised version prior to the submission process.

P5. The authors do not comment on the fact that EV cells in Fig 2B have significantly reduced sensitivity to CX-5461, relative to CTRL.

It is unclear why these cells show some resistance to CX-5461 and we have not studied the EV cells in detail, focusing our analysis on CX and CMB cell lines given the efficacy of these treatments.

P5. It would be worth including EV cells in the ATP data shown in Fig 2E.
We have further analysed ATP levels in all cell lines by LC-MS and updated Figure 2E and its associated manuscript text on page 7 of the modified manuscript. ATP levels are unchanged in EV cells relative to the drug naïve CTRL cells.

Referee #3:

The manuscript by Kusnadi and colleagues seeks to gain a deeper understanding of the mechanisms underlying the previously reported positive synergistic effects of CX-5461 (a ribosome biogenesis inhibitor) and everolimus (a PI3K/AKT/mTORC1 inhibitor) observed in their pre-clinical studies of Myc-driven lymphoma and prostate cancer (REFs. 4 and 6). Evidently, combination therapy (CX-5461 + everolimus) outperformed treatments with either drug alone in vivo. They were also interested to understand how this combination therapy led to an apparent resistance to both CX-5461 alone or in combination with everolimus.
To gain these mechanistic insights, the authors set out to define the distinct impacts of both of these drugs on gene expression alone and in combination in order to reveal insights on how cells achieve resistance to drug treatment in order to optimize the clinical efficacy of Pol I-directed 'ribosometargeting' therapies. The goals of this study are laudable and important given the need for effective clinical therapies for the cancer treatment and the promising data in the literature - initiated and advanced by many of the authors on this body of work - indicating that CX-5461-mediated disruption of ribosome biogenesis is a viable treatment approach that has shown significant clinical promise, including low toxicities.
Over the course of several weeks, each time I tried to delve into this paper was thwarted by the presentation of the data, particularly the data presented in Figures 1 and 2. I certainly appreciate the complexities of the experiments presented and I can tell they were performed with care, but a better job needs to be done to present clearly the methods employed to carry out the massive amount of data presented. The procedures used to generate the data presented are, in my view, not adequately or completely described (see examples below).

In my review of this work, I came to realize only at a very late stage, that the compiled PDF that I was provided actually had concatenated files, where a second version of the manuscript was appended to the first. Given the letter to the editor, it would seem that the latter of the two was the revised version that included the data that is now present in Figure 4. The older version of Figure 4 is now Figure 5. The lengthy review provided below, appear to apply to both versions of the manuscript as they largely pertain to Figures 1 and 2. I was, however, unable to do a precise comparison of the specific language used in both versions of the text. I am reasonably confident that all comments apply. The purpose of these comments is not to harshly criticize the work. Rather, they are meant to help the authors revise the language in the main text, the figure legends and methods sections so that the reader can readily follow the presentation of data with confidence. I was unable to do so until in my review of Figures 1 and 2. The paper became much more accessible thereafter, although specific issues, delineated below, should be addressed before the manuscript is accepted.

Figures 1 and 2 comments:
To begin this work, the authors state, "To interrogate the molecular basis of the response to CX-5461 and the CX-5461 plus everolimus combination, we first performed genome-wide translational profiling to characterize the acute changes in mRNA usage of MYC-driven B-cell lymphomas in mice treated with CX-5461 and the mTORC1 inhibitor, everolimus." They go on to present the data in Figure 1 and Figure S1, which I find extraordinarily confusing. This confusion, highlighted in the points to follow, was at least partly due to the manner in which the work was explained and this undermined my confidence in the data presented and my ability to follow the analyses presented subsequently.

We believe that these issues have been largely addressed in our response to Reviewer 1 and will add additional comments as required below.

Example issues: To begin, the authors state that, "E -Myc B-cell lymphoma (MSCV Gfp; clone #4242) cells were transplanted into C57BL/6 mice (3, 4)." Assuming that the authors are not referring the reader to the methods section of referenced papers 3 and 4, I turned to the current paper's methods section, which states under the "Animal Experiments" heading that they initiated their drug studies in the following way: "2 x 105 E -Myc lymphoma cells injected into the tail vein of 6-8 week old male C57BL/6 mice". They then state, "Lymphoma-bearing mice were treated on Day 10 post transplant for 2 hours with CX-5461 (35 mg/kg), everolimus (EV; 5 mg/kg), or both (CX-5461+EV; 35 mg/kg CX-5461 and 5 mg/kg EV)." The subsequent biochemical Figure 1 data are then discussed, without ever stating how the cells were harvested from these mice. I believe the information should be provided here as to how the cells that were subsequently harvested and analyzed.

In the methods section we stated “Snap-frozen lymph nodes or spleens were homogenized using a Precellys 24/Cryolys cryomill (Bertin Technologies) (6,800 rpm; 2 x 30-second pulse, 45 seconds interval between pulses; 0 °C).” We have now made it clear in the text (first paragraph of the results section) that this western blot is from extracts of lymph nodes from individual mice.

I deduced that the authors somehow physically harvested lymphomas by dissection and that they ground these cells up to isolate total RNA and polysomal RNA to then perform their comparative gene expression analysis. Fig. 1A starts with a Western Blot, which seemed perfectly reasonable. But then they present Fig. 1B, a polysome analysis profile, which from experience requires nearly 1 billion cells to obtain (this is a bit of a guess given than there are no units on the A260nm Y-axis in this figure). I then checked the Methods section as to how polysome profiles were performed. There, under the section heading, "Polysome profiling, RNA isolation and RNAseq analysis", the discussion begins with a description of cultured suspension cells. Initially, this made me think that the polysome analysis shown in Fig. 1B was actually from suspension cells, which would make sense to me given the data shown (I will add here that there is no mention made of how many cells were used for such experiments). I became further concerned that this was the case based on the legend of Figure 1B, which states that this figure is a schematic. However, isolation procedures for "in vivo polysome profiles" are described later in the same Methods section as well. There, the authors state that they extracted material by "grinding snap-frozen tissue samples". They make no mention of how much tissue was used and where it came from. Perhaps then this data is actually suspension cell lysate and is meant only as an illustration? To me it seems important to show all of the polysome profiles for the samples carried forward in the analyses to follow. How much do the polysomes vary? Is it possible that the data were biased in some way based on this isolation procedures? It seems important to present these data to the reader as the impacts reported could simply represent a non-specific metabolic impact on translation initiation or elongation rates that shift the polysomes differentially towards "lighter" polysome fractions (monosomes, disomes and trisomes) without actually changing the overall amounts of protein produced at the level of translation. Directly showing the polysome profiles would inform on this possibility.

We have now provided a more detailed description of the methodology including the procedure for harvesting multiple lymph nodes from 6 mice per treatment and the amounts of material required for polysomal RNAseq analysis as detailed in the response to reviewer 1 (Minor point 1) and in the methods section.
It is important to note that engraftment of GFP+ Eμ-*Myc* cells is monitored (tail-vein blood collection at Day 9 post-injection) before randomising mice to the treatment groups and we saw no change in the GFP positive cells in the lymph nodes of treated animals (Fig EV1A-1B) confirming the effects we measured are independent of cell death and reductions in tumour burden.

Moving forward. The authors then present comparative gene expression analyses on cytoplasmic and polysomal (tetrasomes and greater) for the "tissue samples" isolated from untreated, CX5461 treated (35 mg/kg), everolimus treated (5 mg/kg) and combination treated. These cells, wherever they came from, were evidently treated for just 2 hours each "to exclude any confounding effects of drug-induced apoptosis on our molecular analyses". The authors state that they verified that there was no apoptosis by counting the number of GFP-positive lymph cells they isolated from the lymph nodes of the same mice: "no change in the percentage of GFP-positive lymphoma cells was observed in lymph nodes isolated from treated animals

(Figure S1A)". Again, although not explicitly stated, I deduced the information stated in the Figure 1C legend that this analysis was performed on the same source of the cells used to perform their gene expression analysis. How valid is the assumption that the tumors are uniformly comprised of the same types of cells and that the compositions of these samples don't vary in some way following the drug treatments? I suppose 2 hours is not very much time for significant restructuring to occur assuming the authors are isolating solid tumors (although not stated) but how do the authors verify that identical proportions of the tail injected, Eu-Myc lymphoma cells are present in the samples assessed? The cell compositions of the "tissue samples" seems like the relevant information to present for the reader to be confident in the data presented. If this is not the case, then the changes in gene expression shown in Figs. 1C-E and Fig. S1 may simply reflect changes in the population mix of cells present in these lymph tissues.

We have clarified this above. Tumour bearing mice are randomised before treatment. These samples are from lymph nodes harvested after 2 hours treatment of the mice, before any changes in tumour burden are observed. We have provided extensive additional detail as to how these experiments were performed in the methods section.

Figure 1 then goes on to present a gene set enrichment analysis using MetaCore® GeneGO of the "translationally" affected gene sets for CX-5461, everolimus and CX-5461 + everolimus "tissue samples" compared to control cells. Here the authors provide the striking finding that CX-5461 has "no significant" impact on translation, while everolimus significantly reduced the expression of "translation initiation" and "translation elongation" gene sets. Importantly, the authors state that this everolimus impact carries through to the combination treatment (CX-5461 + everolimus), where CX-5461 appears to profoundly amplify the magnitude and breadth of the number of genes impacted in these two categories. Here, additional questions arise. First, it seems that these two GeneGO "categories" are nearly identical in composition: there appear to be only 5 unique genes in translation initiation and 3 in translation elongation. The rest are ribosomal proteins (1 translation initiation factor eIF1a appears in both categories for some reason). Given CX-5461's published impact (supported by the data presented later in Figs S2D-I) of repressing ribosome biogenesis, this would make sense. As the gene sets principally represent ribosome biogenesis impacts not translation initiation or elongation. In my read of the data presented, the observed impacts on gene expression appear to be quite small. The LogFC color map indicated would imply maximally 1.3 fold or 1.5 fold changes on average depending on whether this is log based 2 or 10- (not stated). Are these "dramatic" effects as the authors later state?

These changes were indeed determined by anota2seq to be significantly altered (FDR <0.05) in terms of their polysomal association with no change in their cytosolic abundance, i.e. they are translationally regulated. The number of affected mRNAs is also dramatically greater in the combination treated lymph nodes in comparison to everolimus alone (Fig 1D). We have now corrected the $\log_2$FC labelling on the figure and updated the figure legend.

This raises the second question: why is it surprising that two drugs that both act to inhibit ribosome biogenesis (by distinct targets presumably) would act additively to do so?

The most surprising aspect of these acute studies is that the combination effect is NOT through alterations in ribosome numbers as indicated by consistent expression of ribosomal protein S6 (Fig 1A) (ribosomes are extremely stable, with half lives of 5-10 days under normal physiological conditions (Nikolov EN, Dabeva MD, Nikolov TK. Turnover of ribosomes in regenerating rat liver. Int J Biochem. 1983 15(10):1255-60. p.1255)).

The inference of synergism seems unwarranted. The authors seem to argue that this additive impact is at the level of polysome fractionation specifically? To me, additive negative impacts on the production of ribosome components seems to be the expected outcome and may occur through subtle changes in translation initiation rates brought about by the actions of both drugs. This could arise, for example, via even a modest activation of eIF2-related aspects of the Integrated Stress Response. Was this checked? This possibility does not however appear to be considered or discussed.

We agree that it would have been of interest to interrogate the possible role of eIF2 activity and have added this concept to the first paragraph of the discussion (Page 11).

Rather, the authors go on to do a distinct analysis of their polysomal RNAs, which appears to bypass important normalizations to total RNAs (single sample gene set enrichment analyses). This leads them to conclude that certain metabolic pathways are disproportionately downregulated. "Critically, despite this potent reduction in the efficiency of translation of components of the translational apparatus, cells treated with CX-5461+EV did not indiscriminately reduce the translation of all mRNAs globally". How do the authors know that this isn't simply a matter of cell number? Are there other normalization procedures used?

Addressed in the response to reviewer 1 above

I won't dwell here on the term "potent", except to say that the impact, as explained above, seems quite modest given the expected impacts of the drugs on proliferation. Are the authors suggesting that 2 hours is too short to have transcriptional responses? The downregulation of metabolic gene expression at the level of transcription could very well be rapid and would be the expected response to a global down regulation mTORC signaling and ribosome biogenesis. Such a response should also disproportionately effect genes with longer 5'UTRs (ie. slower translation initiation rates by the definition of the scanning mechanism) given what the scientific community knows about translation initiation (no references provided by the authors - see for instance a recent review by Rachel Green's group on the subject).

This is in fact the case but we removed this data from the initial manuscript version to simplify the story and focus on the characteristics of mRNAs upregulated in resistant cells.

Slower initiation rates, which could arise via eIF2 modulation or other factors, would therefore tend to shift such mRNAs to "lighter" polysome fractions as a consequence, which does not necessarily mean that the protein levels are reduced, it just means that these mRNAs would drop out due to the authors isolation procedure.

Figure EV1A and EV1B indicated that the downstream analyses were independent of cell death and reductions in tumor burden. A more detailed explanation of the ssGSEA methodology has been included in response to reviewer 1, in the manuscript text (page 5) and in Appendix Supplementary Methods.

Nonetheless, the authors follow these findings by stating, "We hypothesised that this CX-5461+EV-induced targeting of metabolism was a key driver of the improved response to the combination". What do the authors mean by "improved"? Synergistic?

We deliberately used this terminology, as there is no way to definitively measure in vivo synergy as opposed to cooperation.

Here, although this is not stated, the authors appear to switch to studies of suspension cells (my inference), not the isolated tumor cells.

We have clarified the transition to in vitro studies on Eμ-Myc B-lymphoma cell lines by including a new sub-section titled “Co-targeting of energy metabolism improved the efficacy of CX-5461-mediated inhibition of ribosome biogenesis *in vitro*” on page 5 of the revised manuscript.

This should be clarified. They then state, "To test this hypothesis, we used metformin, a well-tolerated anti-diabetic drug that lowers cellular energy levels (20). To my knowledge the mechanism of action of metformin is not known and thus seems like a poor choice for gaining mechanistic insights that inform on the problem statement. Metformin will simply add to the inhibition of proliferation through unknown mechanisms. The clearest indication I had that the experimental strategy had changed entirely was their statement, "Metformin treatment robustly increased cell death induced by CX-5461, consistent with a critical role for inhibition of metabolism in the improved efficacy of CX-5461+EV (Figure S1G)." As stated above, the authors clearly state at the onset of the results section that they gave CX-5461 and everolimus to mice for only 2 hrs specifically "to exclude confounding effects of drug-induced apoptosis on our molecular analyses". This seems like an important clarification to make for the reader.

The major purpose for using metformin was to inhibit ATP synthesis with a drug that could be used *in vivo* and test the hypothesis that resistant cells require elevated levels of energy metabolism. Our mechanistic studies focused on defining the role of EPAC signalling in Fig 3.

We have modified the text to make it clear that for *in vitro* cell line experiments using metformin, the treatment time was 48 hr rather than the 2 hr treatment of the mice for the acute studies (Page 5).

Also, where does the notion that CX-5461 causes cell death at the concentrations employed come from? Is this a new finding? There is no reference provided.

We apologise for not specifically referring to our previous studies showing CX-5461 induced apoptosis in the Eu-Myc mouse model and have added the references (Bywater et al., 2012, Cancer Cell) and (Devlin et al., 2016 Cancer Discovery) at the start of the results subsection titled "Co-targeting of energy metabolism improved the efficacy of CX-5461-mediated inhibition of ribosome biogenesis *in vitro*" (page 5)

Figure S1G and figure legend indicate the application of 5 millimolar metformin for 48 hrs onto a presumed culture of Eu-Myc lymphoma cells that were grown either in the absence or presence of CX-5461. Here it is not at all clear how CX-5461 was administered, how the cells were grown or for how long the cells were subjected to 7.5 nM CX-5461. I infer that the drug was added for 48 hrs given the induction of cell death. It is not stated why a concentration of 7.5 nM CX-5461 was chosen. Does this reflect the authors anticipated equivalent to 35 mg/kg in vivo? At this juncture, the authors also refer to Figure S1H, which shows a similar test of propidium iodide staining of cells treated in an analogous way but including everolimus co-treatment. Again, the durations of the drug administrations and the rationales for the drug concentrations used are not given (here, for some reason CX-5461 is administered at a concentration of 5 nM - presumably for 48 hrs.).

To test the combination of metformin with CX-5461, we used CX-5461 at 7.5nM, which is the concentration of GI20 for CX-5461 single agent based on previous studies from our group referenced in the manuscript text. We tested the combination of metformin with CX-5461 with everolimus, we used CX-5461 at 5nM and everolimus at 10nM, which is also the concentration of GI20 for CX-5461 plus everolimus. The treatment was for 48 hours as indicated in the figure legend.

The authors further conclude from this study that "Moreover, metformin also markedly improved the therapeutic potency of CX-5461+EV (Figure S1H), emphasizing the importance of the targetable metabolic vulnerability in response to Pol I-directed therapy." Here, I am confused by what the authors mean by "therapeutic potency" in regard to combined CX-5461 + everolimus treatment. I presume we are referring to cultured cell lines and propidium iodide staining. This statement implies that cell death is the desired outcome, rather than a simple inhibition of growth. Is this the case?

Cytoxocity is a major predictor of in vivo response, especially at levels >60%. However we have shown that CX-5461 can also have efficacy in other models based on activation of G2/M cell cycle arrest (Quin et al., 2016, Oncotarget, Inhibition of RNA polymerase I transcription initiation by CX-5461 activates non-canonical ATM/ATR signaling)

The authors further state in the conclusion of this paragraph, "Thus, we propose that the reduced translational activity in CX-5461+EV combination therapy treated cells (Figure 1C),

which selectively impaired translation of mRNAs encoding metabolic regulators, is a key mechanism in the synergistic effect of the two drugs and highlights the intimate coupling of mRNA translation and energy metabolism (13, 21)." Here, I do not believe the data presented support the claim, that the data in Figure 1C show selectively reduced translational activity.

The data presented in Fig 1C is generated by Anota analysis and does control for total mRNA levels.

It may be the case that genes for metabolic enzymes were down regulated, this again makes sense given the MOAs of the administered drugs, but it is not at all clear why these genes only came to light in the single sample GSEA analysis, which does not appear to control of total RNA levels. Why were these not found to be enriched in the earlier GeneGo analyses.

These metabolic genes were not identified in the Anota analysis shown in Fig 1C because while the genes from within the relevant ontology groups include key translationally controlled mRNAs (listed Table EV2), others are not and this meant that the group as a whole did not meet the adjusted P value cutoffs for the enrichment analysis performed with MetaCore® GeneGO (Clarivate Analytics). This has been addressed in referee #1 major point 1.

Measurements of total protein levels or metabolites that would be needed to substantiate this conclusion are not given - at least in the data presented up to this point. This conclusion therefore requires additional clarification.

The rationale for the ssGSEA analysis in Fig 1E is described in our response to Reviewer 1 and we have now included data indicating that several mRNAs encoding oxidative phosphorylation proteins are translationally upregulated as determined by anota2seq (Table EV2).

The authors then go on to state, "Despite the dramatic initial impact of the combination treatment on tumor growth, animals eventually succumb to lymphomagenesis (3, 4). The initial clause of this statement appears to be a conclusion that is drawn from the present set of experiments but I do not see such data presented? Did I miss something or are the authors trying to indicate that tumor growth measurements at the 2 hour time point were made in referenced works 3 and 4? This should be clarified. Either way, this would seem to provide supporting evidence that short drug treatments do indeed affect tumor morphology and hence composition - at least potentially - drawing question to the interpretations presented in Figure 1.

This statement was not referring to the response of the tumours to the 2hr treatments presented here but simply a succinct summary of the data in the cited references generated following long term treatments.

The authors then immediately go on to examine the stated hypothesis: "we hypothesized that metabolic rewiring driven by specific changes in mRNA translation would confer this resistance to therapy." What resistance to therapy? Cell death? Are the authors referring to an inference

that lymphogenesis results from drug-resistant cells? How do they know this is the case? Does this model assume that all cells subjected to drug treatment must die or become drug resistant? I am not sure I follow the certainty of this logic. This requires clarification.

The resistance to therapy is simply the re-lapse of the mice to their disease during continued treatment.

The authors go on to test this hypothesis by examine the metabolic activities of the cells extracted from the tumors of mice that had been subjected to one or both drugs as well as control cells, "These early-passage cell lines were derived from lymph node extracts isolated from 12 different mice that were transplanted with Eμ-Myc B-lymphoma cells (clone #107) as indicated in Figure 2A." Figure 2A does not provide any information on how these cells were extracted, sorted or for how long they were passaged before the experiments were conducted. I was also unable to find any information in the Methods section of the paper.

We apologise for this lack of experimental detail and have included a comprehensive description of the methods section on page 13 of the manuscript.

The authors then state," To confirm that CX and CMB [early passage cells isolated from lymph node extracts] maintained their drug resistance phenotypes, they were treated with EV, CX-5461 or CX-5461+EV in vitro. The drug-naive CTRL cell lines retained sensitivity to all treatments, while CX cells were resistant to CX-5461 and sensitive to CX-5461+EV and the CMB cells were unresponsive to all the treatments (Figure 2B). This is not my interpretation of the data shown in Fig. 2B. I see that cells isolated from CX-treated and everolimus-treated mice remain sensitive to both drugs. Is the argument that the differences observed relative to the control studies are statistically significant? I did not see support for this in their analyses.

We apologise for not being more specific about the rationale for our statements about sensitivity to the in vitro treatments. More than 75% of the CX cells remained viable under the conditions compared with less than 20% of drug naïve cells. Most importantly, we observe in the next sentence that "*Moreover, the CX and CMB cells maintained drug resistance in vivo when re-transplanted into mice and re-challenged with CX-5461 (Fig EV3A) and CX-5461+EV (Fig EV3B) respectively. EV cells showed little change in sensitivity to EV treatment, consistent with our previous finding that EV treatment did not provide a significant survival benefit in the Eμ-MycB-lymphoma cells (clone #107)*"

The clearest result appears to be that cells isolated from combination-treated drugs exhibit significant resistance to both drugs. But how is the reader to know that these the same cell types in the absence of a detailed methods section on how they were extracted and cultured? Thus, the importance of clarifying the isolation method and verification of what cells are being examined precisely.

This has been addressed above and we have now updated Figure 2B with the statistical analysis using two-way ANOVA.

Regardless, the authors go on to show in control cells that CX-5461 and everolimus both exhibit expected changes in cellular markers 47S rRNA transcription and ribosomal protein S6 phosphorylation, respectively, while in early passage cells isolated from CX-5461 treated mice and combination-treated mice are less sensitive to short exposures to CX-5461 treatment. For some unexplained reason, the CX-treatment was 50 nM and the duration of time was 3 hrs - roughly 10x the concentration used for the propidium iodine experiments shown in Fig.S1. This should be clarified. Nonetheless, this finding appears to be quite remarkable, even if the cell cultures are heterogeneous in nature.

We now make note specifically (pages 6-7) and referenced that 50 nM CX-5461 is routinely used for short term experiments in studies from our group as those shown in Fig S2 (now Expanded View Figure 3) while for longer term (48-hour c.f. 3-hour treatment) we use sub-10 nM concentrations.

Beyond Figure 2:
The later sections of the paper seem to be easier to follow, and much stronger in terms of their presentation, although specific clarifications are needed.

Specific points of clarification needed:
The authors state on page 6, "This analysis identified the cyclic-adenosine 3',5'-monophosphate (cAMP) signaling pathway as the top hit when comparing CMB versus CTRL (Figure 3A), with increased translation efficiency of mRNAs encoding essential components of this pathway, including adenylate cyclase and cAMP-guanine nucleotide exchange factors (cAMP-GEFs) 1 and 2, which is also known as exchange protein directly activated by cAMP (EPAC) 1 and 2." Do these mRNAs exhibit any 5'-UTR characteristics that may help explain the upregulation - i.e. short 5-UTRs?

Some confusion was generated by the two manuscript versions – the UTR analysis is presented in Fig 4 of the revised manuscript and discussed in the text on page 12.

The axes on Fig.S3C and D need to be made legible.

We have improved the presentation of these figures. The new figure nomenclature is Fig EV4B and EV4C

The text surrounding discussion of Figure 4 seems to need editing.
For instance, the authors state," To determine whether the energy- and cAMP-dependent changes observed in drug-resistant cells are driven by specific reprogramming of mRNA translation, we evaluated the expression patterns of mRNAs encoding proteins that are known to be involved in the regulation of mRNA". Are these the early passage cells? Again, how were these cells isolated and verified.

The origin of these cells has now been clarified in the text as tracked on p5 of the manuscript file under the subheading “Elevated energy metabolism is associated with resistance to ribosome targeting therapy” and detailed in the Methods section on page

13. We have also clarified on page 9 that Fig 4A was generated using the same polysome profiling dataset (Table EV3) as Fig 3A.

They go on to state, "Importantly, the heatmap indicated that the CMB cells displayed distinct polysome-association patterns and therefore translation efficiency of of mRNAs encoding the majority of ribosomal proteins, translation initiation (such as eIF4G1 and eIF2A) and elongation factors (such as eEF1A1 and eEF2) as compared to the CTRL and CX cells (Figure 4A)." First, this sentence has typos and is incompletely constructed. It also states that they are making conclusions about translation efficiency, but it is not clear how this was done. Are the samples isolated as schematized in Figure 1B? Are these analyses normalized for total RNA? Or is this a single-sample GSEA analysis? There is no mention of the methods for cell growth, harvest, polysome fractionation and the data analyses methods employed so it's entirely unclear how conclusions about translation efficiency can be drawn.

The typo has now been corrected.
In Fig 4A we focused particularly on the distinct polysome-association patterns of mRNAs encoding proteins that are known to be involved in the regulation of mRNA translation (genes in the "Translation" category in the Gene Ontology Consortium Pathway database) in CTRL, CX and CMB cells. The rationale for the focus on mRNAs associated with actively translating polysomes in early passage resistant cells isolated from chronically treated lymphoma bearing mice is presented in the response to Reviewer #1.

The expression values were normalized using voom based on the same polysome profiling dataset (Table EV3) used to generate Fig 3A. Individual P values and log2 fold changes for specific mRNAs when comparing the cell lines with each other can be examined in Table EV3

Figure 4A presents data on the mRNA abundance levels
Figure 4A needs to have the cell types indicated more clearly so that the reader knows which column is which.

We have now improved the presentation of Figure 4A by indicating the CTRL, CX and CMB cell lines more clearly and updating the clustering of the analysis output using a newer version of the script.

In referring to Figure 4F, the authors state, "In addition to changes in the cAMP pathway, comparison of the RNAseq data from polysomal mRNAs from CTRL, CX and CMB cell lines revealed that the CMB cells strikingly upregulated the translation of mRNAs encoding multiple components of the mitochondrial electron transport chain, such as such as NDUFV1 and NDUFC2 (Complex I), CYC1 and UQCRC1 (Complex III), as well as multiple subunits of the ATP synthase (Complex V) (Figure 4F)."
First, I believe the authors mean to reference Figure 3 to support the first clause of this sentence. Second, I am also not sure where these data come from. Were they from the same analyses that led to the generation of Figure 4A? Details are needed here. Do the authors plan

to simply provide their gene expression data? It seems like the subsets presented are a bit of cherry picking in a manner that support the model. What were the criteria for selecting these specific genes and what is the statistical significance? There is no FDR information provided on the X-axis of the figure as is presented in the other figures.

The mRNAs shown in Figure 4F represent the OXPHOS-associated mRNAs that were selected based on an initial filtering based on a nominal p-value < 0.01 cut-off in either the CMB vs. CTRL or CX vs. CTRL as analysed by limma according to the same polysome profiling dataset used to generate Fig 3A (Table EV3). In the version that was sent to reviewers, FDR (adjusted P value) information had already been provided on the side as indicated by the size of the circles. Nevertheless, the legend for this figure has been updated to indicate that mRNA species with an adjusted P value ≤ 0.05 were considered to be significant, and are denoted with a black border.

I do believe that there are potentially very important data presented that may ultimately be of clinical significance. However, I am left with the impression that the authors need to revise this manuscript to make it more scientifically grounded, readable and interpretable particularly in the early sections (surrounding discussion of Figs. 1 and 2). My recommendation is that the authors carefully revise the manuscript to address the points raised above, particularly as it relates to informing the reader on the details of how the experiments were performed to ensure reproducibility. This may be addressed through careful revision of the manuscript in its present form without major restructuring. I would be more than happy to re-review the work should that be undertaken and should the editor request further review.

3rd Editorial Decision 4th Aug 2020

Thank you for submitting your revised manuscript, we have now received the reports from the three initial referees (see comments below). I am pleased to say that they overall find that their comments have been satisfactorily addressed and now support publication. Referee #2 has some minor issues left that can however be addressed in a final revised version. In addition, I would like to also ask you to address a number of editorial issues that are listed in detail below. Please make any changes to the manuscript text in the attached document only using the "track changes" option. Once these remaining issues are resolved, we will be happy to formally accept the manuscript for publication.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving your final revision. Please feel free to contact me if you have further questions regarding the revision or any of the specific points listed below.

REFEREE REPORTS

Referee #1: The authors have addressed all of my comments. This is a very interesting manuscript that, in my opinion, is ready for publication in EMBO Journal.

Referee #2:

This manuscript describes high-quality experiments that effectively address an issue of high priority and general significance.

I would, however, recommend rewording of the description of Fig 2B on page 6, which I consider inadequate at present. I pointed out previously that the authors do not comment on the fact that EV cells in Fig 2B have significantly reduced sensitivity to CX-5461, relative to CTRL. The authors have replied that they don't know the reason and have focused elsewhere; that is fine, but they should explain that in the manuscript and not just in a reviewer's response. I also have reservations about the statement that "the CMB cells were unresponsive to all the treatments", when a highly significant (***) response is indicated to everolimus. I accept that the response is blunted, but not that it has disappeared, as implied by the statement. This is an easy change to make and will remove my only remaining concern regarding an otherwise excellent piece of work.

Referee #3:

The authors have made a concerted effort to revise the manuscript in order to clarify the majority of the key points raised during review to an acceptable level.

3rd Authors' Response to Reviewers 7th Aug 2020

The Authors' have addressed all remaining editorial concerns.

Accepted 8th Aug 2020

Thank you again for submitting the final revised version of your manuscript. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Richard B. Pearson
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-105111R2

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.

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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?	For in vitro experments, the sample size was chosen based on common practice and previous experience. The sample size was not determined based on power calculations. However, we consistently achieved at least three biological replicates for all in vitro experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For in vivo experiments, an estimate was made for the number of mice required. The sample size was not determined based on power calculations because there was no previous data available on the expected size effects.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For in vitro experiments, no data exclusions were undertaken in this manuscript. For in vivo experiments, exclusion criteria were defined by the experimental protocol approved by the AECC.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	When lymphoma establishment is confirmed (GFP+ blood cells by FACS), mice were randomly assigned to four groups: (1) vehicle; (2)metformin; (3)CX-5461+everolimus; (4) Cx-5461+everolimus+metformin.
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done for animal studies.
5. For every figure, are statistical tests justified as appropriate?	Yes. We described the stastical tests in the figure legends whereever it is applicable and in the methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution was not tested. Since there are limited numbers of data points, it is difficult to assess the normal distribution.
Is there an estimate of variation within each group of data?	There was no estmiate of variation within each group of data. In this manuscript, the standard deviation (or standard error of the mean) is shown whereever it is applicable.
Is the variance similar between the groups that are being statistically compared?	When the equality of variances was not assumed, Welch t-test was applied.

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 information is included in Appendix Table S1.
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 information is included in the subsection "cell culture and reagents" of the Methods section.

* 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.	The information is included in the subsection "animal experiments" of the Methods section. Mice were maintained in the animal facility of Peter Maccallum Cancer Centre with a relative humidity of approximately 50%, a temperature at 21°C and a 14 hours light-10 hours dark cycle. Eu-Myc lymphoma cells were injected into the tail vein of 6-8 week old male C57BL/6 mice (Water and Eliza Hall Institute, Australia).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The information is included in the subsection "animal experiments" of the Methods section. All anaimal experiments were performed in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals. All protocols were approved by the Animal Experimentation Ethics Committee at the Peter MacCallum Cancer Centre (Ethics number E462 and E557).
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.	Animal studies were reported according to the ARRIVE guidelines.

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The datasets and computer code produced in this study are available in the following databases: • RNA-Seq data: Gene Expression Omnibus GSE154614 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154614) • Modeling computer scripts: https://github.com/cancer-evolution/CX5461_translation_reprogramming
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).	The polysome profiling data for both the acute and resistant studies, and the code required to reproduce the bioinformatics figures is publicly available: https://github.com/cancer-evolution/CX5461_translation_reprogramming. The analysis results including lists of significantly regulated genes are provided as Expanded View Tables.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO