EMBO reports

Che-1 is targeted by c-Myc to sustain proliferation in pre-B-cell acute lymphoblastic leukemia

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

1st Editorial Decision 11 August 2017

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all three referees acknowledge the potential interest of the findings. However, they also raise several concerns to be addressed and point out that your manuscript requires significant revision before the study can be considered for publication here. In particular, they point to missing controls, statistics, validations and quantifications, and also suggest additional experiments to further support the conclusions.

From the analysis of the referee comments it is clear that a significant revision is required before publication can be considered and I would also understand your decision if you chose to rather seek rapid publication elsewhere at this stage. However, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the all referee concerns must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. Please address all referee concerns in a complete point-by-point response.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further.

Please refer to our guidelines for preparing your revised manuscript:
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Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph describing this to the methods section.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

Please format the references according to EMBO reports style. See:
http://embor.embopress.org/authorguide#referencesformat

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The manuscript by Folgiero, V et al. reports data on the role of Che-1, an RNA pol II-binding protein known to contribute to proliferation and cell cycle progression, in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Overall the manuscript shows some interesting data, but there are major issues with the manuscript, including multiple conclusions not sufficiently supported, some data not properly controlled, poor quality data in multiple places, and poor writing of the manuscript.

The data are not thorough. For example, one time point is shown for all the data. Multiple times points showing kinetics would help support conclusion. Also, it is not stated how viable the cells tested are at the time point chosen for multiple figures and subfigures. Knockdown of Che-1 has previously been shown to impact apoptosis, but no data are shown indicating the time point chosen for analysis in multiple figures the cells were viable, partially viable, or mostly dead. This same question also refers to knockdown of Myc, which is known to kill B cell leukemia and lymphoma cells. This is critical information that needs to be included in the manuscript as at least supplemental data so the other data can be evaluated in the proper context.

Data show that Che-1 is overexpressed in patient samples of BCP-ALL at diagnosis and upon relapse, but not during remission. The authors do not describe what remission samples are, so it is unclear if they are comparing similar cell types.

There are multiple poor quality western blots with questionable data: Figure 2A che-1 for LAL-B#2; Figure 2F both Puma blots for NALM-6, Che-1 for LAL-B for both blots, caspase-7 for LAL-B for shCHe-1; Figure 5A c-Myc LAL-B#2 cells, Che-1 LAL-B cells, Figure 6E Myc; Figure 7D c-Myc P493-6.

There are multiple places in the manuscript that are missing controls (both positive and negative). Figure 1B and figure 2F, there are no positive controls or samples that were included on both bots; comparisons between different membranes need to have controls to do this. This is particularly true for figure 2F, when you are comparing low/neg protein levels to each other they need to be on the same membrane. Also, cannot compare results from figure 5E and 5F, since 5E has no positive controls. For Figure 1E, there are no positive controls for the Che-1 blot. Figure 4D shows ChIP at the Che-1 promoter, but it is missing ChIP outside the promoter for negative controls.

Not clear whether the Che-1 siRNA is the same sequence as the shRNA. Not clear whether two different sequences were used. If the same sequence, the authors need to show the effects on cell number and cell cyle can be rescued with Che-1 overexpression showing specificity for the knockdown.

For figure 2C, the authors indicate a reduction in G2/M cells with siChe-1, but there is also a decrease in S phase that is not mentioned. Is there a G1 arrest? Moreover, Che-1 was shown by the senior investigator to be involved in the G2/M checkpoint, so knockdown should have showed reduced G2/M checkpoint, but this was not tested.

No experimental follow up to the ChIP results are done to verify the PTEN, PLK1, and CDK13 shown in fig 3D.

The fact that Myc levels are higher in leukemia cells in figure 5 and that Myc is necessary for the growth of BCP-ALL cells in figure 6 has been known for years, but this is not how it is presented in the text.

The data in figure 2E and 2F are not convincing. There is at most a 10% difference in vitality (should be viability) with siChe-1. It is also difficult to understand how such small differences in fig 2E could actually be due to the large changes in caspase 7 and puma shown in 2F. Also, it is unclear why puma protein would change, but no explanation is given. Additional experiments would need to be done to make the claims that authors make for cooperation between siChe-1 and chemo.

The authors also make the bold claim that Che-1 is necessary for Myc to bind to promoter regions only by showing one piece of data in figure 6G. This is grossly insufficient to make this claim. Only one cell line is evaluated, but more importantly, is that if siChe-1 caused cells to growth arrest as figure 2C shows, there would be less Myc on the promoters because they are not growing. Levels of Myc are cell cycle regulated with low to undetectable amounts in G1 with increasing amounts as cells near and enter S-phase. Claiming cause and effect requires more experiments, as there are other interpretations to the data shown.

The authors make an overstatement in the discussion, "Che-1 overexpression in c-Myc-silenced cells was able to rescue the effects of c-Myc inhibition, indicating that, at least in part, Che-1 could act as a transcriptional effector of c-Myc activity." The data show a possible partial rescue, but provide no explanation for this. How does an an RNA pol II associated protein replace Myc transcription factor function? Does another transcription factor replace Myc?

Figure 7 claims that Che-1 and Myc physically interact only by showing co-IP of endogenous proteins. This is grossly insufficient to make this claim. Additional experiments are required to make this conclusion.

The exact same Che-1 and beta-actin western blots are shown in figure 1B and figure 5C, but no where in the manuscript do the authors indicate this.

The abstract is poorly written along with multiple other parts of the manuscript. There are many places of poor grammar. Abstract needs some re-writes. Abstract claims RNA-seq suggests Che-1 is required for c-Myc recruitment onto DNA. RNA-seq does not test recruitment of transcription

factors. Abstract states "these results..., acting as c-Myc transcriptional cofactor..." No data described in the abstract indicates Che-1 is a Myc cofactor. Check grammar throughout. The manuscript is missing a lot of "the". The phrases, "and the relapsed status" in the abstract, "where c-Myc expression resulted negative ", "a direct correlation between these two proteins resulted statistically significant" ,"whereas it resulted expressed in relapsed BM samples ", "that resulted down-regulated from the RNA-seq experiment" in the results does not make sense grammatically.

Minor points:
The authors write, "Consistent with these findings, a published RNAseq experiment in the E$\boldsymbol{u}$-Myc mouse model of lymphoma (Sabo et al. 2014), demonstrated that Che-1 expression increased along with c-Myc activation and with lymphoma aggressiveness (Fig. EV2E)." This is not entirely accurate. It is a figure of control, pre-tumor, and tumor. It is not a figure showing aggressiveness, but instead a figure showing stages of lymphoma development.

Figures 4F and 4G are missing a key, so it is difficult to decipher the data.

For figure 4G, the authors say "two different mutations in the E-box sequence" but the mutations are not described in the text, material and methods or the figure legend.

Graph with non-readable text figure 5G.

Figure 6A not cited in the text.

Abbreviations not defined (e.g., HBM, BM)

Greek symbol used for Eμ-myc was not correct on page 9.

Che-1 has been previously linked to p53 regulation and the DNA damage response, but this was not mentioned.

It is not clear whether the authors filtered out lowly expressed genes from of their analysis after normalization and before DEseq2.

Referee #2:

In this manuscript, Valentina et al., show that Che-1 is a potential marker, which plays a pivotal role in regulating BCP-ALL cell proliferation. They further show that c-Myc is able to regulate Che-1 expression by direct binding to its promoter and Che-1 and c-Myc cooperate to regulate gene expression.
Overall, this manuscript has established a new connection between Che-1 and c-Myc in BCP-ALL. More importantly, the results are supported directly by human BCP-ALL samples.
Major comments:
1. The data in figure 1 are scattered. As the authors have shown the Che-1 level in 9 BCP-ALL human samples in figure 1B, it's necessary to show the progression of these 9 patients: how many has relapsed, and how many are in remission. It is better to show Che-1 levels in all 9 patients instead of some of the selected ones in figure 1D, 1E and 1G.
2. The same western blotting panels are used in both figure 1B and 5C (panels of Che-1 and β-actin). Is it acceptable by the standard of the journal ?
3. In figure 5C and 5D, authors claim a positive correlation between Che-1 and c-Myc. But in figure 5F, in relapsed BCP-ALL samples, authors only show the c-Myc levels, but not Che-1 levels. Could the authors provide the results of Che-1 level in these samples too ?
It's better to do a FACS analysis to show whether the cell populations with high c-Myc level also have high Che-1 level.
4. In figure 6A, it shows that the inhibition efficiency of cell proliferation after siChe-1 or si-c-Myc silencing is very similar. Especially in LAL-B cells, siChe-1 results in a stronger inhibition effect than that of si-c-Myc. However, in figure 6F, Che-1 overexpression only partially rescues the impaired proliferation ability in c-Myc silenced cells. Regarding the regulation of cell proliferation, what is the relationship between c-Myc and Che-1?

Minor Comments:
1. In figure 2A, the authors claim that difference in NALM-6 group is "***", whereas "**" in LAL-B group. However the error bars indicate that the difference may be similar or less significant.
2. In figure 2A, the proliferation assay did not show the start time point after siRNA transfection and the experiment period. There is same concerns in figure 6A and 6G too.
3. In figure 2B, the authors claim that difference in NALM-6 group is "*", whereas "**" in LAL-B group. However the error bars indicate that the difference may be more significant in LAL-B group.
4. In figure 2E, authors claim that Che-1 silencing affected blast cell viability increasing the efficiency of Adriamycin (Adr) treatment in three different B-ALL cell lines. However, in this figure, the differences in NT groups are "***", whereas "* or **" in Adr groups. By looking at the columns in this figure, it looks like the differences in Adr treatment group should be more significant than that in NT groups, especially in NALM-6 cells. Could the authors provide clarifications on the statistical analysis they used?
5. In figure 4F and 4G, the figure legend showed that the luciferase assay was executed in -tet and +tet conditions. However, there is no such data in the figures.

Referee #3:

The authors have identified a protein called Che-1 as being elevated in BCP-ALL at the onset and recurrence of disease, but is reduced during remission. Che-1 is involved in RNA polymerase function, and the authors contend it also has a role in apoptotic effects. More importantly, the authors have identified a link between Che-1 expression and Myc expression in these cancers. Loss of Che-1 reduces proliferation of such tumor cells, as does loss of Myc expression. More interestingly, the authors present evidence that Che-1 expression is regulated by Myc, and Che-1 and Myc regulate a similar set of genes based on RNA-seq approaches. Finally, the authors present some evidemnce that Che-1 and Myc may reside at some promoters together and interact in complxes together in cells. The authors conclude that Che-1 functions with Myc and presents an alternative target, besides Myc itself, for tumor suppression in these tumors.

The work is overall thorough and interesting. There are some technical concerns with certain experiments, indicated below, and some conclusions the authors state must be modified to be fair and in-line with their actual data. At time the authors over-interpret their findings to yield a broadewr model for Che-1/Myc than what their data can truly support at this time. With modifications as such, this paper will be suitable for publication.

Concerns:

1. Fig2 has several issues. The authors need to identify what is being targeted (sequences if possible) with their si-Che1 reagents. The authors also need to explain what is being targeted by the sh-Che1 viruses. How are these different in such a way as to be able to say (as they do) that the sh-Che1 tests are done for specificity purposes? At the moment, the authors may be using similar targeting and therefore there is a chance that both approaches have off-target issues that are not addressed experimentally. If the si-Che1 assays are not reloiable, then the manuscript will lose much of its strength.

2. Fig2: what is meant by nucleoporated? I am assuming they mean transfected. There is likely an issue here with wording that must be fixed.

3. Fig2: the manner in which the viruses are used to do the sh-Che1 assays must be clarified. Timing? inductions? The experimental procedures are non-existant.

4. Fig2: The authors fail to describe what their assays truly are for Proliferation and Viability/Vitality. In Methods or legends, this must be clarified.

5. Fig2: There is a major concern with the data presented for proliferation and viability in Figure2. When the authors use siChe1 to show loss of proliferation (Fig2A), the data seem reasonable and in support of the hypothesis that loss of Che1 reduces cell growth of the tumor cells. However, in the same Figure2 they show almost no reduction in viability (Fig2E). They attempt to statistically validate the latter results with p-values, but taken at face value, there is virtually no loss of viability

in the absence of Adriamycin. Then Adriamycin causes a loss of viability that is likewise only slightly affected by co-reduction of Che1. It is very difficult to reconcile a loss of prioliferation with virtually no loss of viability, especially if the authors contend that Che1 is an effective target for suppression (in the future) in these tumors. The authors need to re-think these data and not rely on p-values. Perhaps they are simply not knocking-down enough Che1 or using enough Adriamycin? Too little of either would give such data in Fig2E.

6. Fig2F: In order to draw fair and related comparisons between the western blots shown, the authors need to run the samples from each horizontal pair of westerns in the same gel and on the same filter. As it stands, the arguments that protein levels are changing could be due to exposure differences in the individual westerns, which this reviewer does see.

7. Fig1E and Fig5E: In both of these panels the authors need to run remission samples next to at least one non-remission/relapsed sample, to verify that the antibody actually worked on the filter. Trying to say nothing is there without control comparisons is not proper, as a low exposure would easily show no signal.

8. Fig6E: change esogenous to exogenous.

9. Fig6: There are no controls for 6E,F,G panels. teh authors need to compare empty vector transfections to Che1 transfections, and even better, the authors should compare as a control the transfection of a different protein to Che1, to verify that the effects they see are not simply due to transfecting a protein in general. This can have an effect on any experiment, and their rescuing by Che1 expression is not very strong.

10. Fig7: Description of results in Fig7 must be changed. The authors state that a physical interaction between Myc and Che1 exists, but only using co-IP assays. All that I can agree with is that in some cases Che1 and Myc co-reside in larger protein complexes in cells. A physical interaction usually requires bacterial expression of both factors followed by in vitro co-IP testing, and then often involves domain loss that leads to loss of such co-IP interactions. Unless the authors want to extend their work with such experiments, they need to be more fair in how they state their findings.

11. I do not feel their data are strong enough to conclude that Che1 regulates Myc binding to promoters (discussed for Fig7). the authors did identify some overlapping binding between the two, and they can state they hypothesize this may be the situation for Some promoters, and not others. their data do support this interpretation. However, they show only two promoters in Fig7E that Che1 regulates Myc binding toward. In the paper just prior, the authors state that Che1 regulates many promoters, but fail to look broadly at them and focus on just the two aforementioned promoters. I suspect that the Che1 regulating Myc binding is not universal in any way, and the authors must be fair in stating so. If they want to make such claims as currently exist in the paper, then they would need to present more compeling data. They might also need to add data showing that Che1 is a cofactor for Myc similar to how Max is a cofactor. No such data exist in this paper.

1st Revision - authors' response 8 November 2017

Referee #1:

The manuscript by Folgiero, V et al. reports data on the role of Che-1, an RNA pol II-binding protein known to contribute to proliferation and cell cycle progression, in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Overall the manuscript shows some interesting data, but there are major issues with the manuscript, including multiple conclusions not sufficiently supported, some data not properly controlled, poor quality data in multiple places, and poor writing of the manuscript.

- The data are not thorough. For example, one time point is shown for all the data. Multiple times points showing kinetics would help support conclusion. Also, it is not stated how viable the cells tested are at the time point chosen for multiple figures and subfigures. Knockdown of Che-1 has previously been shown to impact apoptosis, but no data are shown indicating the time point chosen for analysis in multiple figures the cells were viable, partially viable, or mostly dead. This same

question also refers to knockdown of Myc, which is known to kill B cell leukemia and lymphoma cells. This is critical information that needs to be included in the manuscript as at least supplemental data so the other data can be evaluated in the proper context.
Following the reviewer's comment, we have included in the revised manuscript the requested time points upon Che-1 and c-Myc interference, to support our choice to show protein down-regulation at 36 hours. As shown in the new Figures 2A and EV3B we observed the strongest inhibition of cell proliferation at 36 hours post Che-1 or c-Myc down-regulation, whereas after 48 hours from the nucleoporation the cells re-started to proliferate probably because they lost the effect of the transient protein downregulation. Moreover, as shown in the new Figures EV1B and EV3C after 48 hours from the nucleoporation the cell viability started to be affected by Che-1 or c-Myc silencing. For these reasons, we performed the experiments shown in this paper at 36 hours post nucleoporation, with the exception of Adriamycin treatment shown in Figure 2E, where cells were analyzed 48 hours after transfection.

- Data show that Che-1 is overexpressed in patient samples of BCP-ALL at diagnosis and upon relapse, but not during remission. The authors do not describe what remission samples are, so it is unclear if they are comparing similar cell types.
We apologize for having not been clear about this point. We rewrote this section of the manuscript to explain that the remission BM samples are from BCP-ALL patients and were collected after induction/consolidation therapy when remission had been achieved. To this aim, we reloaded the representative 9 samples at the onset of BCP-ALL next to sample from the same patient collected at the time of remission.

- There are multiple poor quality western blots with questionable data: Figure 2A che-1 for LAL-B#2; Figure 2F both Puma blots for NALM-6, Che-1 for LAL-B for both blots, caspase-7 for LAL-B for shCHe-1; Figure 5A c-Myc LAL-B#2 cells, Che-1 LAL-B cells, Figure 6E Myc; Figure 7D c-Myc P493-6.
As suggested by this reviewer, we reloaded the indicated samples or performed new experiments to improve the western blots quality.

- There are multiple places in the manuscript that are missing controls (both positive and negative). Figure 1B and figure 2F, there are no positive controls or samples that were included on both bots; comparisons between different membranes need to have controls to do this. This is particularly true for figure 2F, when you are comparing low/neg protein levels to each other they need to be on the same membrane. Also, cannot compare results from figure 5E and 5F, since 5E has no positive controls. For Figure 1E, there are no positive controls for the Che-1 blot. Figure 4D shows ChIP at the Che-1 promoter, but it is missing ChIP outside the promoter for negative controls.
In agreement with the reviewer's concern regarding the figures 1B, 1E, 5C, 5E, we reloaded the samples at time of diagnosis next to samples collected at time of remission status achievement as positive or negative control. In Figure 1B, we loaded the HBM samples together with a Che-1 positive control (BCP-ALL#1). A HBM sample was loaded together with relapsed samples as negative control. With the aim to address the reviewer's criticism, concerning figure 2F, we performed new experiments with Adr and loaded samples from both cell lines on the same membrane. Moreover, as requested, we added the negative control for ChIP experiment in figure 4D.

- Not clear whether the Che-1 siRNA is the same sequence as the shRNA. Not clear whether two different sequences were used. If the same sequence, the authors need to show the effects on cell number and cell cyle can be rescued with Che-1 overexpression showing specificity for the knockdown.
We apologize for not mentioning in the text that Che-1 siRNA has not the same sequence as shRNA:
Che-1 siRNA (AATFHSS120157) (nucleotides 893-917):
5'-CCAGUACCCAGACACUAGAUAUCUA-3'
5'- UAGAUAUCUAGUGUCUGGGUACUGG-3'

Che-1 siRNA (AATFHSS120158) (nucleotides 994-1018)
5'-AGCAACGAAGAAGGGUCCCUGCAAA-3'
5'-UUUGCAGGGACCCUUCUUCGUUGCU-3'

Che-1 siRNA (AATFHSS120159) (nucleotides 1093-1117)
5'-GCACACUUCAGAAAUGGCACGAUAA-3'
5'-UUAUCGUGCCAUUUCUGAAGUGUGC-3'

shChe-1 sequence (nucleotides 824–842):
5′-gatccccAAAGTTTCTGAGGAAGTGGttcaagagaCCACTTCCTCAGAAACTTTtttttggaaa-3′ 5′-agcttttccaaaaaAAAGTTTCTGAGGAAGTGGtctcttgaaCCACTTCCTCAGAAACTTTggg-3′shControl sequence:
5′-cgcgtCTATAACGGCGCTCGATATttcaagagaATATCGAGCGCCGTTATAGtttttggaaat-3′, 5′-cgatttccaaaaaCTATAACGGCGCTCGATATtctcttgaaATATCGAGCGCCGTTATAGa-3′.

- For figure 2C, the authors indicate a reduction in G2/M cells with siChe-1, but there is also a decrease in S phase that is not mentioned. Is there a G1 arrest? Moreover, Che-1 was shown by the senior investigator to be involved in the G2/M checkpoint, so knockdown should have showed reduced G2/M checkpoint, but this was not tested.
It has been shown that Che-1 in response to genotoxic stress is phosphorylated by several specific kinases and, because of these modifications, it contributes to the activation of the G2/M checkpoint (Bruno et al., Cancer Cell 2006). Conversely, in the absence of DNA damage, Che-1 plays an important role during the G1/S transition by promoting the activity of the transcription factor E2F1 (Bruno et al., Cancer Cell 2002). Therefore, we think that the cell cycle arrest in G1 observed in the siChe-1 cells results entirely in accordance with our previous results.

- No experimental follow up to the ChIP results are done to verify the PTEN, PLK1, and CDK13 shown in fig 3D.
To validate ChIP results of Che-1 enrichment on CDK13, PLK1 and PTEN promoters, we performed new qRT-PCR experiments in NALM-6 and LAL-B cell lines. Graph was added in Figure 3 as Figure 3E.

- The fact that Myc levels are higher in leukemia cells in figure 5 and that Myc is necessary for the growth of BCP-ALL cells in figure 6 has been known for years, but this is not how it is presented in the text.
c-Myc has been demonstrated to strongly regulate cell proliferation in BCP-ALL (Kohrer et al. 2016, Hiratsuka et al. 2016, Saba et al. 2015, Ma et al. 2010). In accordance with the literature, we show time course experiments upon c-Myc silencing, confirming that in this pathological context c-Myc downregulation induces a strong inhibition of cell proliferation (Figure EV3B).

- The data in figure 2E and 2F are not convincing. There is at most a 10% difference in vitality (should be viability) with siChe-1. It is also difficult to understand how such small differences in fig 2E could actually be due to the large changes in caspase 7 and puma shown in 2F. Also, it is unclear why puma protein would change, but no explanation is given. Additional experiments would need to be done to make the claims that authors make for cooperation between siChe-1 and chemo.
In agreement with this reviewer's comment, we performed new experiments to reinforce the cooperation between Che-1 downregulation and the administration of Adriamycin. As shown in the new Figure 2E, a new experimental procedure adding a higher amount of Adriamycin (2 mM for 48 hours) at the same time of the nucleoporation showed a marked increase in cell death in cooperation with Che-1 silencing. The weak activation of pro-apoptotic genes observed in the untreated cells was probably due to the effect of nucleoporation (Figure 2F of the revised version). Puma protein was chosen because we have previously demonstrated that Che-1 depletion strongly induces p53 recruitment onto promoters of cell death genes (Desantis et al., 2015).

- The authors also make the bold claim that Che-1 is necessary for Myc to bind to promoter regions only by showing one piece of data in figure 6G. This is grossly insufficient to make this claim. Only one cell line is evaluated, but more importantly, is that if siChe-1 caused cells to growth arrest as figure 2C shows, there would be less Myc on the promoters because they are not growing. Levels of Myc are cell cycle regulated with low to undetectable amounts in G1 with increasing amounts as cells near and enter S-phase. Claiming cause and effect requires more experiments, as there are other interpretations to the data shown.
To try to assess whether the reduction of c-Myc levels on DNA is a direct effect of Che-1 or a consequence of the reduced protein c-Myc levels in response to growth arrest, we performed ChIP experiments showing reduced amounts of c-Myc only on promoters shared with Che-1 (Fig.7F).

This result reinforces the hypothesis that Che-1 may regulate c-Myc activity at least on a particular cluster of genes.

- The authors make an overstatement in the discussion, "Che-1 overexpression in c-Myc-silenced cells was able to rescue the effects of c-Myc inhibition, indicating that, at least in part, Che-1 could act as a transcriptional effector of c-Myc activity." The data show a possible partial rescue, but provide no explanation for this. How does an an RNA pol II associated protein replace Myc transcription factor function? Does another transcription factor replace Myc?
We apologize for having not fully explained this concept. Bruno Amati has affirmed in his review: "Two different models have been proposed to describe the function of the MYC oncoprotein in shaping cellular transcriptomes: one posits that MYC amplifies transcription at all active loci, while the other that MYC differentiates discrete sets of genes, products of which affect global transcript levels "(Kress et al., Nat Rev Cancer 2015). Thus, in accordance with the second model, we considered the hypothesis that Che-1, as target of c-Myc, may contribute to activating the transcription by its binding to RNA polymerase II.

- Figure 7 claims that Che-1 and Myc physically interact only by showing co-IP of endogenous proteins. This is grossly insufficient to make this claim. Additional experiments are required to make this conclusion.
To support the Co-IP experiments aimed at demonstrating the physical interaction between Che-1 and c-Myc, we performed a GST pull-down assay, observing that GST-Che1 fusion protein is able to bind directly in vitro translated c-Myc protein. In addition, the use of GST-Che-1 deleted fusion proteins demonstrated the necessity of the C-terminal domain for the physical interaction between the two molecules. Results were added to Figure 7 as Figure 7E.

- The exact same Che-1 and beta-actin western blots are shown in figure 1B and figure 5C, but no where in the manuscript do the authors indicate this.
We apologize for having not explicitly stated that beta-actin and Che-1 blots in Figure 1B and 5C are the same. We now mentioned it in the text. We chose to use the same representative samples to reinforce the message of a complete co-expression of c-Myc and Che-1 in BCP-ALL at time of diagnosis.

- The abstract is poorly written along with multiple other parts of the manuscript. There are many places of poor grammar. Abstract needs some re-writes. Abstract claims RNA-seq suggests Che-1 is required for c-Myc recruitment onto DNA. RNA-seq does not test recruitment of transcription factors. Abstract states "these results..., acting as c-Myc transcriptional cofactor..." No data described in the abstract indicates Che-1 is a Myc cofactor. Check grammar throughout. The manuscript is missing a lot of "the". The phrases, "and the relapsed status" in the abstract, "where c-Myc expression resulted negative ", "a direct correlation between these two proteins resulted statistically significant" ,"whereas it resulted expressed in relapsed BM samples ", "that resulted down-regulated from the RNA-seq experiment" in the results does not make sense grammatically.
We apologize for the quality of the abstract and we rewrote the text following the reviewer's indications. We hope that the quality of writing be improved in this revised version of the text.

Minor points:

*-The authors write, "Consistent with these findings, a published RNAseq experiment in the E**u**-Myc mouse model of lymphoma (Sabo et al. 2014), demonstrated that Che-1 expression increased along with c-Myc activation and with lymphoma aggressiveness (Fig. EV2E)." This is not entirely accurate. It is a figure of control, pre-tumor, and tumor. It is not a figure showing aggressiveness, but instead a figure showing stages of lymphoma development.*
Following the reviewer's suggestion, we performed the indicated changes to the Figure EV2E comment.

- Figures 4F and 4G are missing a key, so it is difficult to decipher the data.
We uniformed the bar's color in the graphs for a simpler interpretation of the data in Figure 4F and 4G.

- For figure 4G, the authors say "two different mutations in the E-box sequence" but the mutations are not described in the text, material and methods or the figure legend.

Mutagenesis of mChe-1 promoter was performed using the QuikChange Mutagenesis kit (Stratagene, CA), altering the canonical E-box sequence CACGTG in TTCGAC. Sequencing was realized by Genechron (Rome, IT). As suggested by the reviewer, we added this paragraph in the Material and methods section.

- Graph with non-readable text figure 5G.
We improved the readability of the text

- Figure 6A not cited in the text.
We checked Figure 6A citation in the text

- Abbreviations not defined (e.g., HBM, BM)
We are sorry for the abbreviations not defined. We defined them in the text as requested

- Greek symbol used for Eu-myc was not correct on page 9.
We corrected the symbol on page 9. Many apologies for the mistake.

- Che-1 has been previously linked to p53 regulation and the DNA damage response, but this was not mentioned.
Following the reviewer's suggestion, we mentioned that Che-1 is involved in the DNA damage response by regulating p53 in the introduction of the manuscript.

- It is not clear whether the authors filtered out lowly expressed genes from of their analysis after normalization and before DEseq2.
As for DESeq2, we didn't filter out for lowly expressed genes.

Referee #2:

In this manuscript, Valentina et al., show that Che-1 is a potential marker, which plays a pivotal role in regulating BCP-ALL cell proliferation. They further show that c-Myc is able to regulate Che-1 expression by direct binding to its promoter and Che-1 and c-Myc cooperate to regulate gene expression.

Overall, this manuscript has established a new connection between Che-1 and c-Myc in BCP-ALL. More importantly, the results are supported directly by human BCP-ALL samples.

Major comments:

1. The data in figure 1 are scattered. As the authors have shown the Che-1 level in 9 BCP-ALL human samples in figure 1B, it's necessary to show the progression of these 9 patients: how many has relapsed, and how many are in remission. It is better to show Che-1 levels in all 9 patients instead of some of the selected ones in figure 1D, 1E and 1G.
Following the reviewer's suggestions, we reloaded the 9 samples at the onset of BCP-ALL each one close to the sample of the same patient collected at the time of achievement of remission. The nine BMs analyzed resulted all in the remission status, with any observed relapse. In addition, we reloaded all the 14 relapse samples (#R1-#R14) in Figures 1C and 5C.

2. The same western blotting panels are used in both figure 1B and 5C (panels of Che-1 and β-actin). Is it acceptable by the standard of the journal ?
We preferred to use the same panel of representative samples to reinforce the message that Che-1 and c-Myc are co-expressed at the onset of the disease and at time of relapse.

3. In figure 5C and 5D, authors claim a positive correlation between Che-1 and c-Myc. But in figure 5F, in relapsed BCP-ALL samples, authors only show the c-Myc levels, but not Che-1 levels. Could the authors provide the results of Che-1 level in these samples too ?
Following the reviewer's request, we added western blot of Che-1 levels in the same samples. Che-1 expression in these samples was already shown in Figure EV1A (now Figure 1E) as percentage of Che-1 expression by FACS analysis.

It's better to do a FACS analysis to show whether the cell populations with high c-Myc level also have high Che-1 level.
As requested by the reviewer, we added in Figure 5E a FACS analysis showing that BCP-ALL cells with high levels of c-Myc also have high Che-1 levels

4. In figure 6A, it shows that the inhibition efficiency of cell proliferation after siChe-1 or si-c-Myc silencing is very similar. Especially in LAL-B cells, siChe-1 results in a stronger inhibition effect than that of si-c-Myc. However, in figure 6F, Che-1 overexpression only partially rescues the impaired proliferation ability in c-Myc silenced cells. Regarding the regulation of cell proliferation, what is the relationship between c-Myc and Che-1?
From the data produced in this study, we propose a model in which Che-1 is a target of c-Myc, necessary for cell proliferation, and in accordance with this model Che-1 overexpression is able to rescue, at least in part, the effects of c-Myc inhibition. To strengthen the rescue experiment in NALM-6 cells, we also performed a new proliferation assay in LAL-B #2 cells confirming that Che-1 was able to rescue the impaired proliferation in c-Myc silenced cells (new Figures 6F, EV4G). However, it is not possible to exclude that other transcription factors involved in cell proliferation can perform this function by activating Che-1. In fact, as described by Kaul et al. (2006), the Che-1 promoter contains Sp1 and E2F putative binding sites, thus suggesting that Che-1 can also contribute to the activity of these two important factors.

Minor Comments:

*1. In figure 2A, the authors claim that difference in NALM-6 group is "***", whereas "**" in LAL-B group. However the error bars indicate that the difference may be similar or less significant.*
In the old version of the paper the number of * indicated the absolute P value in the chronological order inside each experiment. As correctly pointed out by the Reviewer, this representation is confusing and we now adopted a more conventional approach following this legend: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. We also added a paragraph describing the statistical analysis in the material and methods section.

2. In figure 2A, the proliferation assay did not show the start time point after siRNA transfection and the experiment period. There is same concerns in figure 6A and 6G too.
Following reviewer's comment, we prepared a new Figure 2A showing a detailed time course after siRNA transfection. In the Figure 6A legend, we specified the timing of the experiment. The experimental conditions of Figure 6G are the same of Figure 6E, and are now specified in the legend of Figure 6E.

3. In figure 2B, the authors claim that difference in NALM-6 group is "", whereas "**" in LAL-B group. However the error bars indicate that the difference may be more significant in LAL-B group.*
We modified the statistical analysis data as indicated in the answer to the minor point 1.

*4. In figure 2E, authors claim that Che-1 silencing affected blast cell viability increasing the efficiency of Adriamycin (Adr) treatment in three different B-ALL cell lines. However, in this figure, the differences in NT groups are "***", whereas "* or **" in Adr groups. By looking at the columns in this figure, it looks like the differences in Adr treatment group should be more significant than that in NT groups, especially in NALM-6 cells. Could the authors provide clarifications on the statistical analysis they used?*
For the statistical analysis, we calculated each P value using the student T test. In the revised text, we modified each figure as indicated in the answer to the minor point 1

5. In figure 4F and 4G, the figure legend showed that the luciferase assay was executed in -tet and +tet conditions. However, there is no such data in the figures.
We greatly apologize for the missing indication about Tet administration in Figure 4F. We added this information in the graph as suggested. The experiments in Figure 4G were performed only at basal conditions.

Referee #3:

The authors have identified a protein called Che-1 as being elevated in BCP-ALL at the onset and recurrence of disease, but is reduced during remission. Che-1 is involved in RNA polymerase function, and the authors contend it also has a role in apoptotic effects. More importantly, the authors have identified a link between Che-1 expression and Myc expression in these cancers. Loss of Che-1 reduces proliferation of such tumor cells, as does loss of Myc expression. More interestingly, the authors present evidence that Che-1 expression is regulated by Myc, and Che-1 and Myc regulate a similar set of genes based on RNA-seq approaches. Finally, the authors present some evidemnce that Che-1 and Myc may reside at some promoters together and interact in complxes together in cells. The authors conclude that Che-1 functions with Myc and presents an alternative target, besides Myc itself, for tumor suppression in these tumors.

The work is overall thorough and interesting. There are some technical concerns with certain experiments, indicated below, and some conclusions the authors state must be modified to be fair and in-line with their actual data. At time the authors over-interpret their findings to yield a broadewr model for Che-1/Myc than what their data can truly support at this time. With modifications as such, this paper will be suitable for publication.

Concerns:

1. Fig2 has several issues. The authors need to identify what is being targeted (sequences if possible) with their si-Che1 reagents. The authors also need to explain what is being targeted by the sh-Che1 viruses. How are these different in such a way as to be able to say (as they do) that the sh-Che1 tests are done? At the moment, the authors may be using similar targeting and therefore there is a chance that both approaches have off-target issues that are not addressed experimentally. If the si-Che1 assays are not reloiable, then the manuscript will lose much of its strength.
As suggested by the reviewer, we used two different approaches to downregulate Che-1 expression for specificity purposes. We added the sequences in the materials and methods section.

2. Fig2: what is meant by nucleoporated? I am assuming they mean transfected. There is likely an issue here with wording that must be fixed.
With the word "nucleoporated", we mean a nuclear electroporation performed by Amaxa 4D-Nucleofector. We have now described better this method in the materials and methods section of the revised version.

3. Fig2: the manner in which the viruses are used to do the sh-Che1 assays must be clarified. Timing? inductions? The experimental procedures are non-existant.
In agreement with reviewer's concern, in the revised version of the manuscript, we added the requested experimental procedure in the materials and methods section. Thank you for this useful suggestion.

4. Fig2: The authors fail to describe what their assays truly are for Proliferation and Viability/Vitality. In Methods or legends, this must be clarified.
As requested by the reviewer, we added the description of the proliferation and viability assay in the materials and methods section of the revised version.

5. Fig2: There is a major concern with the data presented for proliferation and viability in Figure2. When the authors use siChe1 to show loss of proliferation (Fig2A), the data seem reasonable and in support of the hypothesis that loss of Che1 reduces cell growth of the tumor cells. However, in the same Figure2 they show almost no reduction in viability (Fig2E). They attempt to statistically validate the latter results with p-values, but taken at face value, there is virtually no loss of viability in the absence of Adriamycin. Then Adriamycin causes a loss of viability that is likewise only slightly affected by co-reduction of Che1. It is very difficult to reconcile a loss of prioliferation with virtually no loss of viability, especially if the authors contend that Che1 is an effective target for suppression (in the future) in these tumors. The authors need to re-think these data and not rely on p-values. Perhaps they are simply not knocking-down enough Che1 or using enough Adriamycin? Too little of either would give such data in Fig2E.
As suggested by the reviewer, we re-thought these experiments modifying the amount of Adriamycin and testing different time points. In Figure 2E of the revised version we combined an higher amount of Adriamycyn (2 mM) with Che-1 interference for 48 hours, since as shown in Figure EV1B, Che-1 silencing affects viability of the cells at this time point.

6. Fig2F: In order to draw fair and related comparisons between the western blots shown, the authors need to run the samples from each horizontal pair of westerns in the same gel and on the same filter. As it stands, the arguments that protein levels are changing could be due to exposure differences in the individual westerns, which this reviewer does see.
As requested by the reviewer, we performed a new western blot analysis running the samples on the same gel as shown in the new Figure 2F of the revised manuscript.

7. Fig1E and Fig5E: In both of these panels the authors need to run remission samples next to at least one non-remission/relapsed sample, to verify that the antibody actually worked on the filter. Trying to say nothing is there without control comparisons is not proper, as a low exposure would easily show no signal.
As suggested by this reviewer, we run remission samples close to samples from the same patients at the onset of leukemia for a better comparison.

8. Fig6E: change esogenous to exogenous.
We modified the indicated word in the text and in the figure.

9. Fig6: There are no controls for 6E,F,G panels. teh authors need to compare empty vector transfections to Che1 transfections, and even better, the authors should compare as a control the transfection of a different protein to Che1, to verify that the effects they see are not simply due to transfecting a protein in general. This can have an effect on any experiment, and their rescuing by Che1 expression is not very strong.
We apologize for a not clear description of the above indicated figures. We only modified these figures and the respective legends because we already performed these experiments using the empty control vector necessary for comparing the effect of Che-1 overexpression in the old version of the paper. However, to strengthen the rescue experiment in NALM-6 cells, we also performed a new proliferation assay in LAL-B #2 cells confirming that Che-1 was able to rescue the impaired proliferation in c-Myc silenced cells (new Figures 6F, EV4G).

10. Fig7: Description of results in Fig7 must be changed. The authors state that a physical interaction between Myc and Che1 exists, but only using co-IP assays. All that I can agree with is that in some cases Che1 and Myc co-reside in larger protein complexes in cells. A physical interaction usually requires bacterial expression of both factors followed by in vitro co-IP testing, and then often involves domain loss that leads to loss of such co-IP interactions. Unless the authors want to extend their work with such experiments, they need to be more fair in how they state their findings.
To reinforce the Co-IP data, we performed a GST-pull down assay by using GST-Che1 fusion protein and *in vitro* translated c-Myc protein. In addition, the use of GST-Che-1 deleted fusion proteins demonstrated the necessity of the C-terminal domain for the physical interaction between the two molecules. Results were added to Figure 7 as Figure 7E.

11. I do not feel their data are strong enough to conclude that Che1 regulates Myc binding to promoters (discussed for Fig7). the authors did identify some overlapping binding between the two, and they can state they hypothesize this may be the situation for Some promoters, and not others. their data do support this interpretation. However, they show only two promoters in Fig7E that Che1 regulates Myc binding toward. In the paper just prior, the authors state that Che1 regulates many promoters, but fail to look broadly at them and focus on just the two aforementioned promoters. I suspect that the Che1 regulating Myc binding is not universal in any way, and the authors must be fair in stating so. If they want to make such claims as currently exist in the paper, then they would need to present more compeling data. They might also need to add data showing that Che1 is a cofactor for Myc similar to how Max is a cofactor. No such data exist in this paper.
In agreement with the concerns of this reviewer, we have corrected our conclusion by stating that Che-1 is able to regulate c-Myc recruitment on only a particular cluster of genes.

2nd Editorial Decision 30 November 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study (you will find enclosed below). As you will see, the referees now support the publication of your manuscript in

EMBO reports. Referee #1 has some further suggestions to improve the paper that we ask you to address in a final revised version of the manuscript. Please have the final manuscript proofread by a native speaker.

Further, I have the following editorial requests that also need to be addressed:

Please provide a title without commas (and without using more than 100 characters including spaces).

Please format the references according to EMBO reports style. See: http://embor.embopress.org/authorguide#referencesformat

Part of the labelling in figures 3C, 4B, 5G, 6C/D and EV3D are too small, and will not be readable in the final online or pdf-version of the paper. Please provide these panels with bigger fonts.

In Figure EV5 the items shown in the second and third column are also too small, and can be hardly seen/read. Pleas provide this significantly larger. As there seem not to be other panels here, please remove the A.

Some of the Western blot panels look over-contrasted (e.g. in Fig. 2F), or show very different background levels within one sub-figure. Please provide the Western blot images with similar background intensities, with as little modification and contrast-adjustment compared to the original source files. For some panels, the contrast levels differ quite clearly comparing the source data image with the final version (e.g. in 1C).

I could not find the source data for the Western blots shown in Figures EV3 and EV4. Could you provide also these?

Further, please provide ONE pdf-file with all the image source data for one figure (even if some panels will then show up more than once - e.g. 1B and 5B). Finally, please zip these together with the related excel sheets.

Finally, you provided one file (Supplemental bioinformatic materials) with supplemental data S3-S7. Is there also S1 and S2? We would require that you upload these as separated EV dataset excel files (named Dataset EV1, Dataset EV2 .. etc.), with a title and a legend on the first tab. Please also update the callouts for these in the manuscript accordingly.

Please contact our editorial assistants, if you have questions regarding this: contact@emboreports.org

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The revised manuscript by Folgiero et al on Che-1 in B-ALL is significantly improved. The authors have addressed most of my concerns and it is now a much better manuscript. However, there are still multiple places where English was a problem for the authors resulting in wrong word choices, missing words, and exaggerated conclusions. The authors should carefully go through their manuscript to make sure they do not overstate or misrepresent their results in the text due to their choice of an English word. Additionally, there are multiple places in the manuscript where the conclusion could be correct or it could have another explanation that was not tested or considered. For example, it is still a stretch for the authors to conclude Myc and Che-1 function together at the same promotor at the same time when the proper experiments to demonstrate this were not done. Nevertheless, the data overall are more convincing, understandable, and better controlled than the first submission. The results are interesting and provide insight into B-ALL.

Referee #2:

The authors have successfully addressed previous concerns and also provided additional evidence in response to previous comments. It is now suitable for publication in EMBO Reports.

Referee #3:

The authors have made numerous changes to the revised manuscript, and it appears most of these satisfy my previous issues. Reading of the the other two reviewers' comments and rebuttal comes to a similar conclusion, that the authors also appear to have adequately addressed the reviews/critiques of all three reviewers. The paper is now suitable for publication.

2nd Revision - authors' response 13 December 2017

As required, we have modified the manuscript and the figures following the editorial requests. The final version of the manuscript has been proofread by a native speaker.

Please provide a title without commas (and without using more than 100 characters including spaces).
We modified the title of the manuscript excluding the commas as requested.

Please format the references according to EMBO reports style.
We formatted the references following the EMBO reports style as requested.

Part of the labelling in figures 3C, 4B, 5G, 6C/D and EV3D are too small, and will not be readable in the final online or pdf-version of the paper. Please provide these panels with bigger fonts.
We provided each indicated panel with larger fonts as requested. The only exception is Figure 4B, where LASAGNA software output didn't provide a format in which font size was in any way editable, nor with high quality raster images (e.g. 300 DPI PNG). For this reason, we kept the LASAGNA reference for the result itself but we generated a more readable, vector-based image via the UCSC Genome Browser with predicted Transcription Factor Binding Sites for AATF/Che-1 gene.

In Figure EV5 the items shown in the second and third column are also too small, and can be hardly seen/read. Pleas provide this significantly larger. As there seem not to be other panels here, please remove the A.
The indicated items in Figure EV5 are now larger, we downloaded the largest version of the CENTDIST output and embedded it in the final panel.

Some of the Western blot panels look over-contrasted (e.g. in Fig. 2F), or show very different background levels within one sub-figure. Please provide the Western blot images with similar background intensities, with as little modification and contrast-adjustment compared to the original source files. For some panels, the contrast levels differ quite clearly comparing the source data image with the final version (e.g. in 1C).
As suggested we modified the background intensities in Figure 2F.

I could not find the source data for the Western blots shown in Figures EV3 and EV4. Could you provide also these?
We provided the original source data for the indicated figures. Furthermore, following the editorial request to show the source data, fortunately we realized that in Fig. EV4A (NALM-6 and LAL-B) the western blots for Che-1 and β -actin were the same showed in Fig.2A. The same mistake was done for c-Myc western blot in Fig. EV4A (NALM-6) and Fig.5A. In this current revised version we have now inserted the right images in Fig. EV4A. We strongly apologize for not being careful enough in controlling images.

Further, please provide ONE pdf-file with all the image source data for one figure (even if some panels will then show up more than once - e.g. 1B and 5B). Finally, please zip these together with the related excel sheets.
We generated a unique PDF file containing the image source data together with the related excel sheets.

Finally, you provided one file (Supplemental bioinformatic materials) with supplemental data S3-S7. Is there also S1 and S2?
In the Supplemental bioinformatic materials, the number of the table was related to the number of the figure but, as suggested, this nomenclature is confusing so we renamed the supplemental dataset following the editorial indications.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Valentina Folgiero and Maurizio Fanciulli
Journal Submitted to: Embo Reports
Manuscript Number: EMBOR-2017-17

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We used 80 samples from patients at the onset of BCP-ALL, 28 at the time of remission and 14 from relapsed patients (pag 18)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We analyzed samples of patients with either newly diagnosed or relapsed BCP-ALL, who were diagnosed and/or treated at IRCCS Bambino Gesù Children's Hospital (Rome), a center affiliated to the 'Italian Association of Pediatric Hematology/Oncology' (AIEOP) network. The only exclusion criteria were absence of available blasts or absence of parent informed consent (pag.18).
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.	NA
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	NA
5. For every figure, are statistical tests justified as appropriate?	Each experiment was performed three times and analyzed as appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The student T-test was applicated to data with normal distribution. Mann– Whitney U test was used to compare patients data because of distribution (pag. 26).
Is there an estimate of variation within each group of data?	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	pagg.19,20
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines used in the paper were produced from bone marrow samples derived from BCP-ALL patients by Epstein Barr virus infection. NALM-6 cell line was bought from AATC. All cell lines were tested for Mycoplasma contamination by PCR (pagg. 18, 19).

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Ethical committee of the Bambino Gesù Children's Hospital in Rome (pag. 18).
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	The statement has been included on pag. 18
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.	Thre was no restriction to use the cryopreserved blast samples of the patients enrolled in this study
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	All high-throughput data (ChIP-seq and RNA-seq) have been submitted to the GEO archive (GSE93628) (pag.26).
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).	NA
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.	NA