EMBO reports

LRH-1/NR5A2 interacts with the glucocorticoid receptor to regulate glucocorticoid resistance

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1st Editorial Decision **16th Nov 2021**

Dear Prof. Brunner,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your 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 of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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 file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

See also the guidelines for figure legend preparation:
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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We 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. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments were performed, if these were biological or technical replicates, 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 detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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10) Please order the manuscript sections like this (using the same expressions):
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods -DAS - Acknowledgements - Author contributions - Conflict of interest statement - References - Figure legends - Expanded View Figure legends.

I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision:
https://embor.msubmit.net/cgi-bin/main.plex

Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

Based on previous research observations (their own and many other independent groups), the authors hypothesized that glucocorticoid receptor (GR) and NR5A2 (also known as LRH-1) interact physically and modulate each other's function. They found that GR physically interact with LRH-1. The interaction of these two transcription factors reciprocally inhibits each other's function. They moved on to corelate this observation with glucocorticoid resistance of T-ALL. They show that LRH-1 is a key regulator of T-ALL cell proliferation and survival. Genetic or pharmacologic inhibition of LRH-1 results in growth defects and apoptosis. They went on to show evidence that the mutual antagonism of the GR and LRH-1 plays a key role in GC resistance in T-ALL. Pharmacologic or genetic inhibition of LRH-1 sensitizes T-ALL cells for GC-induced apoptosis. Furthermore, they show that such sensitization is possibly mediated by up-regulation of the GR and Bim induction. Finally, the authors extended their research to test whether the induction of synergistic cell death by combined treatment with dexamethasone and LRH-1 inhibitor in primary tumour cells from T-ALL patients in vitro and in vivo in a xenograft transplantation model. Overall, it is an interesting and important study, which opens new perspectives in developing novel therapeutic strategies for glucocorticoid resistant T-ALL.

The study addressed coherent and important questions of nuclear receptor function, which is physiologically relevant and clinically important. The study provides detailed mechanistic insight into the mechanism of action of GR and LRH-1. Functional relevance is demonstrated in cell culture models and also using human patients' samples.

The experimental evidences are strong enough to support the conclusions drawn except the experiment described in Fig 7B. I don't find any overclaim. The conclusions constitute a single key message, which is reflected in the title of the article.

The study addressed a new question which was never been tested/reported up to my knowledge. I assure that I had a substantial knowledge in this area, and I performed a few hours literature search to up to date my previous knowledge in the area glucocorticoids receptor biology when I am reviewing this. However, the sentence with this claim "Taken together, this study reports for the first time a physical interaction and a mutual antagonism between the GR and LRH-1 that plays a role in GC sensitivity", in the end of introduction section, can be revised by removing the phrase "for the first time". It would not affect the merit of the study.

The study has broad biological significance. Particularly, it is very important observation in the field of nuclear receptor functioning. GR itself have the capacity to regulate approximately 22% of the protein coding genes. GR resistance of T-ALL is a clinical challenge. At least a subset of patients is expected to fall in this category. Therefore, for those patients, this study opens up a new possibility to overcome GR resistance in T-ALL.

It is worthy of publication in EMBO reports with minor corrections.

I have no major concern. However, addressing following minor concerns may improve the quality of the manuscript.

1. mh-hGR is myc and his tagged human GR. It is explained in the Expanded View Figure. However, it seems not spelled out in the main manuscript.
2. The length of the manuscript can be shortened by tightening the writing of the discussion. Overall, the "discussion" section is reiterating introductions in many places. Authors undertook an approach to discuss too much detailed to put the findings into right context. It goes beyond the interpretation of the data. This approach may distract the general readers. I admit that the introduction and results are very appropriate.
3. Fig 1A and C: Representative of how many experiments?
4. Fig 1D: Please provide gating strategy and a sample FACS profile in the Extended View Fig
5. Fig 2: I would suggest to specify which statistical method was used for which experiment.
6. Fig 3C: If the authors really want to keep this Fig, then I would suggest to include at least three independent repeats and show the statistical significance.
7. Fig 3D: I am curious to know why non-specific bands follow similar pattern as of Bim. I would appreciate if the authors clarify this. Maybe I am misunderstanding. If those are non-specific band then they supposed to follow tubulin. Are they modified form of Bim or a protein that is also similarly regulated? It would be good to mention how many repeats have been done for what the fig represents.
8. Fig 3: Differences are obvious. However, statistical significance can enhance the strength.
9. Fig 4D: Which band is PARP? And which one is Bim? There are two bands that follow same pattern. Please label the bands clearly.
10. Fig 5F: Flow cytometry gating and a sample representative FACS plot in the Extended View Fig may add extra value and clarity.
11. Fig 6: Please specify/label which band is PARP, Bim and cleaved Caspase 3
12. Fig 6 F/G: I would suggest to perform one more repeat and show the statistical significance (if you really want to keep this fig in the main figs)
13. Fig 7B: This experiment is weak. It is difficult to draw a conclusion from this. I assume this is because of heterogeneity associated with T-ALL and drug resistance. In addition, a very low number of patients' sample is involved/analysed. If patients were stratified based on LRH-1 expression and number of samples increased, a conclusion could be made. I would suggest either remove this panel (at least from the main Figures) or include more patients sample in the experiment.

14. In many places authors used "resp." possibly to denote "respectively" or respective. I would suggest to avoid this short form and write full word.
15. The present title is appropriate. However, "Reciprocal inhibition of the glucocorticoid receptor and LRH-1 regulates glucocorticoid resistance" might be a better title. No?
16. In some places authors wrote YFP1-GR and GR-YFP in another places. Is this to denote fusion protein was clones N-ter or C-ter? Otherwise, a consistent nomenclature would be good to follow throughout the manuscript.
17. What was the basis of choosing the control substance Cpd2?
18. Page 30, line 872: "Leukemia progression was followed by flow cytometry..." . I think "Leukemia progression was monitored by flow cytometry..." might be better. Similar corrections are suggested throughout.

Referee #2:

Michalek et al studied the role of LRH-1 in regulating glucocorticoid response in T-ALL and stated that GR and LRH-1 inhibitors synergistically induced apoptosis of T-ALL cells. The paper revealed a novel strategy of treating glucocorticoid-resistant T-ALL patient who are usually with poor prognosis, thus holding a certain promise of clinical translation. However, mechanisms of the interaction between GR and LRH-1 were not illustrated well or some parts not convincing. Even though quite a lot of data were presented, there are still some key experiments missing in related to either the model or the mechanism. Therefore, in this reviewer's opinion, the paper should be substantially improved before getting published in EMBOR. My major comments are:

1. Figure 1 was performed using 293 cells. As we know GR resistant cells are often resistant in their own ways, which could be but not limited to cell-type-specific mechanisms. So, in my opinion, these experiments should be performed in T-ALL cell lines instead.
2. Figure 1 show only a few cells with positive GFP in the photos. Are these cells representative? Also, it is hard to identify the location where GR and LRH-1 interact with each other. Considering GRs translocate from cytoplasm to nucleus upon dex treatments, the location could be critical.
3. It is still lack of evidence leading to a conclusion of a direct interaction between GR and LRH-1, despite of evidence in Figure 1 and EV1. Before the dex treatment, why is there no coIP (GFP signal) in cytoplasm, shouldn't the tag-GR locating in cytoplasm before the treatment? Did the authors see a translocation of GR?
4. In Figure 3, in my opinion, the authors should check GR function first in Jurkat, Molt-4, CEM cells, as several publications has demonstrated dysfunctional/low-active GR in ALL cell lines and the CEM cell may have one allele of GR gene mutated. The experiments may include translocation of GR from cytoplasm to nuclei and/or ELISA assays.
5. In Figure 3, the flowcytometry data showed apoptosis increased in CEM-C7. Could the authors please explain the difference between this cell line with other CEM cell lines?
6. Figure 3 C and D should show the translocation of GR besides its expression.
7. Figure 3 E showed in most cases no luciferase signal after treatment. So, besides the points above, is it possible that LRH1 has up-specific binding?
8. Related to my comment #1, experiments performed in figure 1 should actually be presented here using ALL cell lines.
9. In Figure 4, inhibiting LRH-1 seems killing T-ALL cells without dex, so I am just wondering if the killing happened via GR pathway or any other pathways. Actually these data made the story more complicated. Furthermore, cMyc and Cycline data seem to represent another mechanism, so how was it associated with GR pathway? It is interesting to see no BIM induction here, so it further indicated an alternative mechanism.
10. In Figure 5, the authors should calculate the cytotoxicity values to find out if an additive or synergistic effect happened.
11. In Figure 6, shLRH-1 upregulated GR mRNA expression and induced BIM by itself, and no enhanced BIM induction was observed in 6H. These data do not seem to support a synergy between GR and LRH-1, certainly not a direct interaction between those two.
12. Does the GR binding at ALL genomes get influence by LRH-1 or its inhibition?

Referee #3:

This study finds that the glucocorticoid recepter (GR) binds the transcription factor LRH-1, which results in mutual antagonism. Based primarily on four T cell lines, which are likened to T-ALL cells, the authors find that pharmacologic inhibition of LRH-1 results in enhanced sensitivity to glucocorticoid-mediated growth arrest and apoptosis, which was true of a subset of human T-ALL organoids as well. They argue that GR-LRH-1 inhibitory interaction is a new mechanism for glucocorticoid resistance and presents an attractive target for therapeutic intervention. Although this is an interesting possibilty, some of the data on mechanism are overinterpreted and, more importantly, the correlation between LRH-1 levels and its inhbition are often not shown or, when they are, don't correlate well with the responses to LRH-1 inhibition.

Specific comments:

1. Of the 4 T cell lines tested, 3 were GC-resistant and expressed low levels of GR and moderate to high levels of LRH-1 as

measured by qRT-PCR. A fourth, CEM-C7, expressed high levesl of GR and very low levels of LRH-1. CEM-C7 cells, therefore, should provide a control for "off-target" effects of LRH-1 inhibitors (that is, effects that aren't due to inhibition of GR/LRH-1 interactions). There were a number of experiments whose intepretations would benefit from analysis of CEM-C7, which will be mentioned in other comments. Although it was noted that LRH-1 knockout cells could not be obtained, shRNA knockdown was successful, and at the least one could compare the effect of the two inhbitors on mock vs. LRH-1 knockdown cells.

2. In Fig. 3F it is noted that the predicted effect of 3d2 on Jurkat cell LRH-1 reporter activity was not observed. Although reporter assays are notoriously prone to arefact, this result was mentioned as "of interest" but wasn't explored,. Instead the expected result with MOLT-4 was taken as proof that 3d2 was acting as expected. Fig. 3E and 3F are examples where analysis of CEM-C7 cells would be helpful.

3. In Fig. 4A, B, and E, the growth-inhibiting effects of 3d2 and shLRH-1 need to be tested with CEM-C7 cells as well (where they would be predicted to have little effect). The assumption that the RNAi studies "rule out unspecific off-target effects" of 3d2 (line 280) is incorrect: 3d2 may well have off-target effects on growth and apoptosis in addition to (or instead of) its effects on LRH-1 conformation.

4. Line 297: "In contrast, LRH-1 inhibition caused rather dynamic changes in protein abundance of both cell cycle regulators, with a marked reduction in MOLT-4 after only 2 h and in CEM-C1 cells after 6 h (Figure 4D)." I'm not sure what "rather dynamic changes" is in reference to, but even after close inspection I see only small changes in protein levels that don't even trend (e.g. c-Myc levels in MOLT-4 at 0, 4, and 6 hr are comparable, with perhaps a very small dip at 2 hr. ECL is only linear over a very narrow range and small differences in intensity can't be interpreted with certainty. To my eye Fig. 4D shows no obvous effect of 3d2 on cell cycle proteins. This is in contrast with the shRNA data in Fig. EV2C, which makes one wonder about the mechanism by which 3d2 inhibits cell proliferation.

5. In keeping with unexpected behavior, although 3d2 inhibits the growth of CEM-C1 as well as it does for Jurkat and MOLT-4, it only affected the cell cycle phase distribution in the latter two (.e.g, no G1 arrest). It is difficult to draw conclusions about mechanism when different cells behave differently

6. In Fig. 4G CEM-C7 cells were included, but instead of showing little to no effect of 3d2 on cell death, they died with the same kinetics and almost to the same extent as Jurkat and MOLT-4, despite the statement that they were "comparably insensitive". In fact, at 48 hr they were similar except at the highest 3d2 dose, where the % annexin V was ~30% to 50%, despite the fact that MOLT-4 is the highest expressor of LRH-1 (Fig. 3C). In keeping with these inconsistensies, the patterns of sensitivity for 3d2 and SR1848 were quite different. The hierarchy for 3d2 was CEM1-C1 > Jurkat = MOLT-4, but for SR2848 Jurkat was completely resistant. If these inhibitors are converging on the same target (LRH-1) why this variability?

7. In Fig. 4I it is clear that cleavage of PARP and Caspase-3 increased over time. But the changes in p-p38 and Bim are small, fluctuate about a mean, and do not trend (e.g. p-p38 levels are similar at 0, 4, and 8 hr, and slightly higher at 2 and 6 hr). I would suggest that the experiment's point that the death is apoptotic is made without having to overinterpret these small and inconsisent changes.

8. Fig. 5A. In the text (line360-361) it is stated that "In all GC-resistant T-ALL cell lines tested, dexamethasone significantly enhanced 3d2-mediated cell death induction". While there are small enhancements at the highest 3d2 dose for Jurkat and CEM-C1, the dose-response curves are remarkably similar, and given that n=4 the claim is unconvincing. The results are only clear for MOLT-4, where the curve is clearly shifted. The liklihood of off-target affects is once again raised by the "surprising" large effect on CEM-C7 cells(Fig. 5C), which express little LRH-1.

9. Fig. EV4E, panel E, supposedly shows that GR deficiency decreased Bim protein levels (line 454). But it's not clear what the blot shows. There are 3 "Bim" bands. The topmost is very similar in the GR KO to the intensity in the WT cells, the most striking difference being an increase in mobility (a postranslational modification?), and the lower 2 bands are identical. The text doesn't match the data.

10. Lines 502-503, it is said that the patient-derived cells were insensitive to dexamethasone alone, but there no dexamethasone-only data in Fig. 7A.

11. The data with human T-ALL are welcome, but to tie the putative mechanism by which 3d2 affects cell growth and viability to its effects on cell lines, one must at least have some information about such critical parameters as GR and LRH-1 levels. As well, there are marked differences in the response of the 4 samples, with 4 being unresponsive, 2 and 3 responding at very low doses of 3d2 (10 nM or less, compared to the {greater than or equal to} 40000 nM levels used for the cell lines), and 1 responding only when the concentration reached 1000 nM or greater. An analysis of pertinant variables may help understand these differences.

EMBOR-2021-54195-T: Novel interaction of the glucocorticoid receptor and LRH-1 regulates glucocorticoid resistance

Point-by-point reply

Reply in green and *italic*

We would like to thank all reviewers for the critical reading of our study. Their suggestions helped to significantly improve the presentation and discussion of the results obtained, and to clarify remaining questions and concerns.

Comments Referee 1:

1. mh-hGR is myc and his tagged human GR. It is explained in the Expanded View Figure. However, it seems not spelled out in the main manuscript.

The abbreviation was introduced in line 141/142

2. The length of the manuscript can be shortened by tightening the writing of the discussion. Overall, the "discussion" section is reiterating introductions in many places. Authors undertook an approach to discuss too much detailed to put the findings into right context. It goes beyond the interpretation of the data. This approach may distract the general readers. I admit that the introduction and results are very appropriate.

As suggested by this reviewer we have now shortened the discussion section

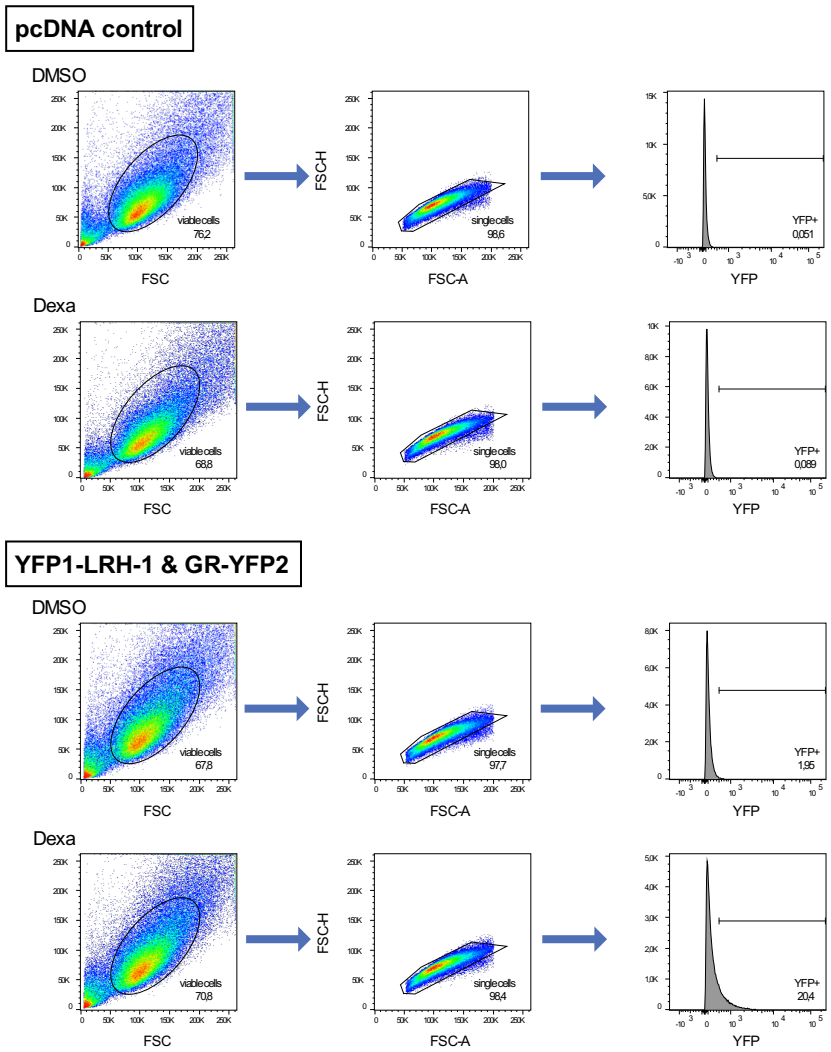
3. Fig 1A and C: Representative of how many experiments?

Fig 1A: Representative of four independent experiments.
Fig 1C: Representative of three independent experiments.
(also added to the respective Figure Legend)

For all performed BiFC experiments, presence/localization of the Dexamethasone-induced BiFC was further routinely evaluated via fluorescence microscopy prior to quantification of YFP+ cells via flow cytometry (additional n = 4 experiments).

4. Fig 1D: Please provide gating strategy and a sample FACS profile in the Extended View Fig

Due to limited space in the corresponding EV Figure, the gating strategies are provided for reviewers only below.



Gating Strategy for quantification of BiFC via flow cytometry.

HEK 293H (and similarly Jurkat T-ALL cells) were co-transfected with YFP1- and YFP2-tagged GR and LRH-1 expression vectors at a ratio of 1:1 as described in the "Materials and Methods" section. After treatment with dexamethasone or DMSO as solvent control for indicated time periods, cells were harvested and flow cytometry was performed using the LSR Fortessa (BD Biosciences) and the FACS Diva Software (BD Biosciences). Data were analyzed using FlowJo software (FlowJo LLC). Sideward (SSC) and forward (FSC) scatter blots were used to determine viable cells and subsequently perform doublet discrimination. YFP negative controls cells transfected with an equal amount of the corresponding empty vector pcDNA3.1 were used to set the gate for the determination of YFP positive cells (YFP+) as percentage of the viable, single cell population.

5. Fig 2: I would suggest to specify which statistical method was used for which experiment.

The statistical methods for Figure 2 were already specified in the figure legend of the first manuscript version – see line 1409-1411.

6. Fig 3C: If the authors really want to keep this Fig, then I would suggest to include at least three independent repeats and show the statistical significance.

Figure 3C aims for the characterization/comparison of different untreated T-ALL cell lines and does not display an experiment. Thus, assessment of LRH-1 and GR mRNA expression levels in T-ALL cells could only be repeated with samples of the respective cell lines taken from different frozen stocks and time-points. GC sensitive CEM-C7 cells displayed statistically significant higher GR mRNA expression and GR:LRH-1 expression

ratio in comparison to all GC resistant T-ALL cell lines as calculated by classic one-way ANOVA and depicted in the revised Figure 3C.

7. Fig 3D: I am curious to know why non-specific bands follow similar pattern as of Bim. I would appreciate if the authors clarify this. Maybe I am misunderstanding. If those are non-specific band then they supposed to follow tubulin. Are they modified form of Bim or a protein that is also similarly regulated? It would be good to mention how many repeats have been done for what the fig represents.

We thank this reviewer for pointing out this potential confusion. While in the apoptosis and Bcl-2 field it is commonly known that Bim has 3 splice variants, we keep forgetting that this is not common knowledge outside of the apoptosis field Bim has three isoforms of different size that are generated by alternative splicing: Bim ExtraLong (Bim_{EL}; 22.1 kDa), Bim Long (Bim_L; 15.9 kDa) and Bim Short (Bim_S; 12.7 kDa). All three isoforms induce apoptotic cell death, with Bim_S being the most potent (O'Connor et al, 1998; Sionov et al, 2015). We indicated these isoforms in all Western Blots and clarified the issue in the main text (line 247/248).

8. Fig 3: Differences are obvious. However, statistical significance can enhance the strength.

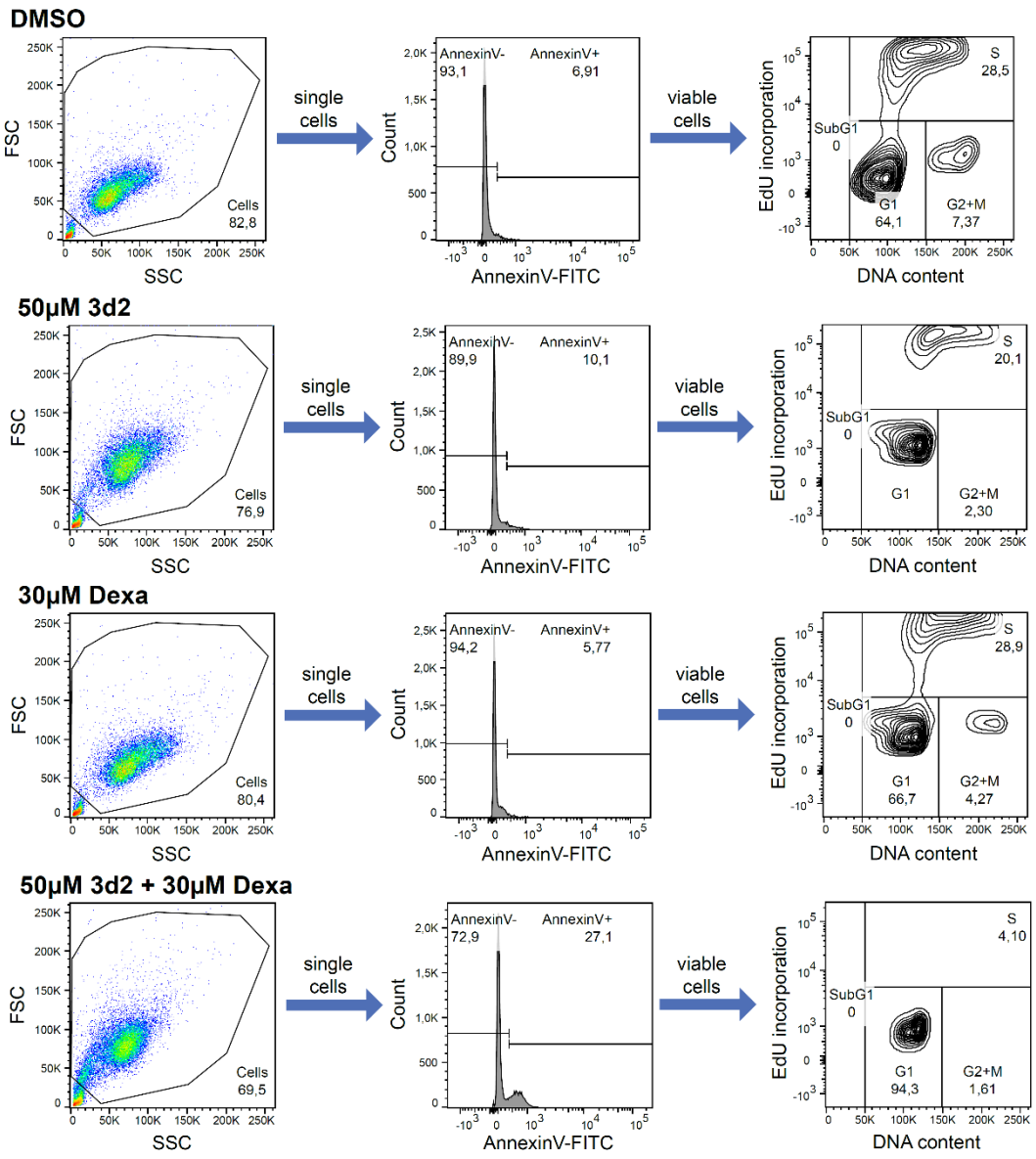
Since graphs in Figure 3E&F needed to show technical replicates of one representative out of ≥3 biological repeats in some cases, due to variable transfection efficiencies, we initially decided to exclude statistical analysis. Statistically significant results were included in the revised figure and number of technical/biological replicates detailed in the corresponding figure legend.
Furthermore, GR and LRH-1 activities of the GC-sensitive CEM-C7 cell line were included following suggestions of Reviewer 3 (line 256-265).

9. Fig 4D: Which band is PARP? And which one is Bim? There are two bands that follow same pattern. Please label the bands clearly.

We again apologize for our "apoptosis slang"! Western Blots labels were now revised. PARP and its cleaved form, indicating apoptosis induction (Cl. PARP), Cl. Caspase 3 as well as the different Bim isoforms are now labeled in the blots.

10. Fig 5F: Flow cytometry gating and a sample representative FACS plot in the Extended View Fig may add extra value and clarity.

Similar to Comment 4, representative FACS plots are provided for reviewers only, due to limited space in the corresponding EV Figure. A description of the gating strategy was included in the "Structured Methods" section of the manuscript (line 1020-1030)t.



Gating strategy for flow cytometric analysis of bidimensional cell cycle distribution and apoptosis

After 48h treatment with 3d2 and/or dexamethasone, MOLT-4 cells were stained using the 647 EdU Click Proliferation Kit (BD Bioscience, San Jose, USA) according to the manufacturers protocol and flow cytometry was performed using the LSR Fortessa (BD Biosciences) and the FACS Diva Software (BD Biosciences). Data were analyzed using FlowJo software (FlowJo LLC). Sideward (SSC) and forward (FSC) scatter blots were used to gate cellular events and subsequently perform doublet discrimination (not shown). AnnexinV histograms were used to determine the number apoptotic cells as percentage of total cells. Contour blots were used to assess the cell cycle distribution of AnnexinV negative (AnnexinV-) viable cells based on EdU incorporation and DNA content (DAPI staining).

11. Fig 6: Please specify/label which band is PARP, Bim and cleaved Caspase 3

Western Blot labels have been revised.

12. Fig 6 F/G: I would suggest to perform one more repeat and show the statistical significance (if you really want to keep this fig in the main figs)

Generation of true biological replicates would require further elaborate generation, selection, characterization and experimentation of/with another set of knockdown cell lines. Even though similar trends have been observed in experiments using different

control/knockdown pairs, absolute results can often not be compared directly, respectively hamper statistical analysis because of variable knockdown efficiencies. Due to the multitude of challenging non-established revision experiments, limited time and human resources, we decided to generate additional technical replicates to confirm the robustness of the method. Statistically significant differences calculated by two-way ANOVA were included in the revised figure.

13. Fig 7B: This experiment is weak. It is difficult to draw a conclusion from this. I assume this is because of heterogeneity associated with T-ALL and drug resistance. In addition, a very low number of patients' sample is involved/analysed. If patients were stratified based on LRH-1 expression and number of samples increased, a conclusion could be made. I would suggest either remove this panel (at least from the main Figures) or include more patients sample in the experiment.

In response to the reviewers comment we have now characterized in more detail the 8 patient samples regarding LRH-1, GR and Bim expression, and the GR:LRH-1 ratio. Furthermore, we have correlated the synergy score (Z score) with the LRH-1:GR ratio, LRH-1 and Bim expression (new Figure 7B-D). These analyses strongly support our hypothesis that synergistic induction of cell death by the combination of the LRH-1 inhibitors 3d2 and dexamethasone is largely defined by the expression levels of LRH-1 and GR. While it would be desirable to include more patient samples in the in vivo transplantation experiments, we would like to remind the reviewer that the duration of these experiments are completely unpredictable, as individual patient-derived tumor cells engraft and grow with variable efficiency. Thus, experiments can take up to 4 months, as seen in former Figure 7B (now figure 7E). Due to time restrictions for this revision, we have thus abstained from repeating these in vivo treatment experiments with additional patient samples. Nonetheless, we are convinced that, even though the sample size is limited, these in vivo experiments are informative as they demonstrate the potential of LRH-1 inhibition in sensitizing resistant T-ALL tumors to dexamethasone.

14. In many places authors used "resp." possibly to denote "respectively" or respective. I would suggest to avoid this short form and write full word.

Has been corrected throughout the manuscript!

15. The present title is appropriate. However, "Reciprocal inhibition of the glucocorticoid receptor and LRH-1 regulates glucocorticoid resistance" might be a better title. No?

Since we aim to focus on the novel GR/LRH-1 interaction resulting in GR inhibition we prefer to keep the original title but appreciate your suggestion.

16. In some places authors wrote YFP1-GR and GR-YFP in another places. Is this to denote fusion protein was clones N-ter or C-ter? Otherwise, a consistent nomenclature would be good to follow throughout the manuscript.

Correct: YFP1-GR indicates N-terminal tagging of the GR with the YFP1 fragment, while GR-YFP2 denotes C-terminal tagging with the YFP2 fragment. We introduced the used abbreviation in the results part and hence aimed to sufficiently clarify this issue (line 151-152 and 158).

17. What was the basis of choosing the control substance Cpd2?

Compound 2 (Cpd2) was first identified as a potential LRH-1 inhibitor during the molecular docking screening conducted by Benod et al (2013) that resulted in the discovery of 3d2. Even though structurally related, Cpd2, unlike 3d2 is unable to inhibit the transcriptional activity of LRH-1. It was thus used as a control substance to rule out potential 3d2-related off-target effects as well as in any way possible.

18. Page 30, line 872: "Leukemia progression was followed by flow cytometry...". I think "Leukemia progression was monitored by flow cytometry..." might be better. Similar corrections are suggested throughout.

Thanks for the helpful tip – we rephrased the manuscript.

Comments Referee 2:

1. Figure 1 was performed using 293 cells. As we know GR resistant cells are often resistant in their own ways, which could be but not limited to cell-type-specific mechanisms. So, in my opinion, these experiments should be performed in T-ALL cell lines instead.

The first two figures of our manuscript solely aimed to demonstrate, resp. visualize the novel GR-LRH-1 interaction and characterize its impact on the transcriptional activity of both transcription factors. We deliberately chose to present this biochemical discovery in a rather generic manner, as it may also have yet to be described implications in other cell types than T-ALL, for example intestinal epithelial cells, where we previously described a role for LRH-1 in regulating glucocorticoid synthesis (Mueller, J. Exp. Med 2006). The presented experiments are based and highly dependent on the effective transfection of several relatively large expression vectors. Known for a high transfection efficiency and capability to express recombinant proteins, we hence chose HEK 293 cells as a heterologous expression, respectively reporter system to show the interaction and mutual antagonism of the GR and LRH.

We agree that it is important to show that the interaction of LRH-1 and the GR also in T-ALL cells. However, the efficient transfection of different T-ALL cell lines and subsequent sufficing expression of YFP-tagged recombinant proteins – at least in our hands – has been extremely difficult. During the review process, a lot of effort was been put into optimization of transfection protocols of T-ALL cells. This finally allowed us to successfully demonstrate bimolecular fluorescence complementation (i.e. LRH-1-GR interaction) also in Jurkat cells transfected with split YFP GR and LRH-1 fusion constructs. Hopefully meeting the reviewer's request to prove that the GR-LRH-1 interaction also occurs in leukemic T cells, flow cytometric analysis of BiFC in Jurkat cells was included in revised Figure 3 (line 270-272). The in comparison to HEK 293 cells lower percentage of Dexa-induced BiFC positive cells is most likely due to the in general far lower transfection efficiency in Jurkat cells.

2. Figure 1 show only a few cells with positive GFP in the photos. Are these cells representative? Also, it is hard to identify the location where GR and LRH-1 interact with each other. Considering GRs translocate from cytoplasm to nucleus upon dex treatments, the location could be critical.

We assume that the reviewer is referring to figure 1A when discussing GFP-positive cells. Yes, these pictures are representative of many different experiments. The reason why there are relatively few LRH-1, resp. GR expressing cells is likely not the transfection efficiency, which is generally very high in HEK 293T cells, but intrinsic regulatory processes, which control the expression of these nuclear receptors. Especially for LRH-1 we have seen always lower frequencies of LRH-1-expressing cells, likely because the expression of a transcription factor, which regulates so many different biochemical processes in a cell has to be tightly regulated.
It is important to mention that under steady-state conditions LRH-1 is always nuclear and the GR is cytoplasmic. As we believe this can be nicely seen in Figure 1A, where the nuclear DAPI staining allows to properly identify the cell nuclei. Here it can be clearly seen that in the absence of dexamethasone GR and LRH-1 do not co-localize, whereas after dexamethasone stimulation they do.
Certainly, more informative than the colocalization experiment is, however, the bimolecular fluorescence complementation experiment (BiFC) employing split-YFP-tagged LRH-1 and GR. Also these experiments have been repeated many times, and

representative pictures are shown. Depending on the split YFP label position (C- or N-terminus), we further only observed Dexa-induced nuclear fluorescence when using certain GR and LRH-1 fusion protein combinations. Since no YFP signal was observed in the absence of Dexa and/or transfection of other combinations, it can be assumed that the BiFC signal is specific and not resulting from a random reassembling of the YFP fragments. We are thus more than confident that Dexa-induced BiFC resembles the specific interaction of the GR with LRH-1. Furthermore, as can be seen in Figure 1C merge, the YFP fluorescence nicely colocalizes with the DAPI stain, clearly supporting the idea that GR and LRH-1 interact in the nucleus.

3. It is still lack of evidence leading to a conclusion of a direct interaction between GR and LRH-1, despite of evidence in Figure 1 and EV1. Before the dex treatment, why is there no coIP (GFP signal) in cytoplasm, shouldn't the tag-GR locating in cytoplasm before the treatment? Did the authors see a translocation of GR?

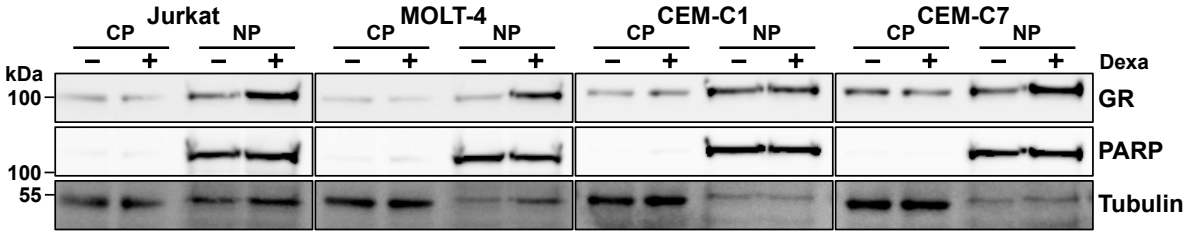
The lack of a Co-IP from cytosolic fractions in the absence of Dexamethasone can simply be explained by the different subcellular localization of the GR and LRH-1. It is correct that the GR is located in the cytoplasm before the GC treatment. In contrast, LRH-1 exhibits a constant nuclear localization (Fayard et al, 2004). As verified in Figure 1A, LRH-1 and the GR are thus naturally/physically separated in the absence of GCs. Thus, the direct interaction of the GR and LRH-1 is critically dependent on the presence of the GR ligand Dexamethasone (Figure 1 and EV1). Together with the points discussed in the reply to question No. 2., we think the presented results provide convincing evidence for a direct GC-dependent GR-LRH-1 interaction in the nucleus and the specificity of this interaction. GC-induced translocation of the GR could be shown in all used T-ALL cell lines (see answer to comment No. 4.)

4. In Figure 3, in my opinion, the authors should check GR function first in Jurkat, Molt-4, CEM cells, as several publications has demonstrated dysfunctional/low-active GR in ALL cell lines and the CEM cell may have one allele of GR gene mutated. The experiments may include translocation of GR from cytoplasm to nuclei and/or ELISA assays.

In line with the reviewer's comment that low-active GR due to haploinsufficiency has been reported for T-ALL cell lines tested, Figure 3F confirms that Jurkat as well as MOLT-4 cells do not display dexamethasone-induced GR activity. Importantly, however, the LRH-1 inhibitor 3d2 reverses this functional resistance. As suggested by Referee No. 3, assessment of GR activity in the GC-sensitive CEM-C7 cell line was now also included in the revised Figure 3. In contrast to GC-resistant cell lines, the GR activity of CEM-C7 cells was induced by dexamethasone alone, which could be slightly enhanced by additional 3d2 treatment (line 256-265). This confirms our hypothesis that interaction of the GR with LRH-1 likely contributes to GR inhibition and associated GC resistance. Despite considerable effort, CEM-C1 cells could unfortunately not be included in these analyses since transfection of this cell line seems to be impossible with the methods, resp. equipment available at our institution.

We may miss the point here, but we do not know how or what kind of ELISA could be performed to assess GR function, resp. GR translocation. We must assume that the reviewer means subcellular fractionation and detection of GR by ELISA. We agree with this reviewer that nuclear localization of the GR is a prerequisite for the induction of GR-LRH-1 interaction. Thus, following the reviewer's helpful suggestion, we now analyzed the subcellular localization of the GR in presence and absence of GC to confirm that the GR translocates in response to dexamethasone and that GR-LRH-1 interaction is thus

possible in the T-ALL cell lines analyzed. These data are now part of the revised Figure 3E (line 244-247).



Immunoblots of GR, PARP (nuclear loading control) and Tubulin (cytoplasmatic loading control) from cytoplasmic (CP) and nucleoplasmic (NP) extracts of T-ALL cells treated for 2 h either with 10 µM (Jurkat, MOLT-4; CEM-C1) or 1 µM (CEM-C7) dexamethasone (Dexa). Note: Nuclear extracts were more concentrated (approx. 6-fold) than cytosolic fractions, explaining differences in overall GR protein levels present in the cytosolic versus nuclear fraction.

Even though the nuclear fractions were slightly contaminated with cytosolic proteins (presence of Tubulin in nucleoplasm fractions), Dexa-induced translocation of the GR from the cytoplasm (CP) to the nucleoplasm (NP) could be observed in the GC-sensitive CEM-C7 cell line, as well as in Jurkat and MOLT-4 cells. Only minimal Dexa-induced GR translocation could be observed in CEM-C1 cells, which, however, exhibited rather high nuclear GR protein levels already in the absence of dexamethasone, indicating that also in this cell line the GR can translocate to the nucleus. This might be explained by the presence of GC in the serum/cell culture medium that in case of CEM-C1 may suffice to induce nuclear translocation of the GR.
In summary, we think that the presented experiment convincingly confirms the GC-induced nuclear localization of the GR in all T-ALL cell lines used and supports the hypothesis that the newly identified GR-LRH-1 interaction can occur also in the GC-resistant cell lines investigated.

5. In Figure 3, the flowcytometry data showed apoptosis increased in CEM-C7. Could the authors please explain the difference between this cell line with other CEM cell lines?

Both, CEM-C1 and CEM-C7 cells are subclones of the CCRF-CEM cell line, which was derived from primary cells of a pediatric patient suffering from T-ALL (Foley et al, 1965). They were both subcloned from the initial mixed wild type population without selective pressure and are widely used as experimental models for a comparably GC-resistant, resp. -sensitive T-ALL phenotype. An initial characterization of both subclones (Zawydiwski et al, 1983) revealed that GC-resistant CEM-C1 cells contain a normal, functional and active GR when compared to the GC-sensitive CEM-C7 cells. In line with this, later studies suggest that resistance to GCs results from the altered expression of distinct GR-target genes, including for instance the pro-apoptotic BH3-only protein Bim_{EL} (Medh et al, 2003).

6. Figure 3 C and D should show the translocation of GR besides its expression.

According to the reviewer's suggestion, a Western Blot showing GR translocation in response to Dexamethasone treatment (depicted in reply to Comment 4) was included in Figure 3E.

7. Figure 3 E showed in most cases no luciferase signal after treatment. So, besides the points above, is it possible that LRH1 has up-specific binding?

Since translocation of the GR seems to be unimpaired in the analyzed T-ALL cell lines, we assume that the translocated GR is most likely sequestered by LRH-1 and therefore kept inactive in these T-ALL cells. In favor of this interpretation, Dexamethasone plus 3d2 treatment potently induces/restored GRE luciferase activity in leukemia cells. Furthermore, 3d2, as well as LRH-1 knockdown, also restored GC-induced apoptosis in these cell lines. Similarly, 3d2 also enhanced Dexa-induced GR promotor activities also in HEK 293T cells (Figure 2) and seemed to reduce this direct inhibitory interaction of the GR and LRH-1 as assessed by BiFC (Figure1).

8. Related to my comment #1, experiments performed in figure 1 should actually be presented here using ALL cell lines.

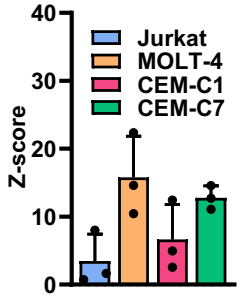
Please, refer to our reply to comment #1.

9. In Figure 4, inhibiting LRH-1 seems killing T-ALL cells without dex, so I am just wondering if the killing happened via GR pathway or any other pathways. Actually these data made the story more complicated. Furthermore, cMyc and Cycline data seem to represent another mechanism, so how was it associated with GR pathway? It is interesting to see no BIM induction here, so it further indicated an alternative mechanism

We believe that this observation relates to various aspects. The role of LRH-1 as a positive cell cycle regulator has been reported previously for various cell types, including cancer cells and primary T cells. We for instance discovered a critical pro-proliferative function of LRH-1 in primary T cells where it regulates expression of c-Myc and Cyclins (Seitz et al, 2019). Furthermore, it is clear from various publications that LRH-1 also regulates cell survival, which we also see here in T-ALL cell lines. As summarized in a review article by us, enhanced LRH-1 levels in general seem to protect from cell death induction, while a reduction of LRH-1 activity was shown to trigger spontaneous cell death in different cell types (Michalek & Brunner, 2020). The survival-promoting functions of LRH-1 can be attributed to its direct role in controlling expression of apoptotic proteins and to its function in cellular energy metabolism and protection from ER stress, oxidative as well as DNA damage.
Furthermore, this observation might be also mediated by restoration of basal GR signaling. In line with this idea, we have seen that 3d2-induced apoptosis in the absence of GCs is reduced in GR knockout cells (Figure 6E). Since most of the experiments have been performed in medium supplemented with fetal calf serum, which also contains glucocorticoids, it may be feasible that removing the "LRH-1 break" allows the "spontaneous" reactivation of the GR by serum GCs to trigger cell death.

10. In Figure 5, the authors should calculate the cytotoxicity values to find out if an additive or synergistic effect happened.

As suggested, similar to analysis performed for human patient-derived T-ALL, the expected drug combination responses were calculated based on ZIP reference model using SynergyFinder (Ianevski et al, 2020). Referring to the user manual provided, positive ZIP values (referred to as Z-score) denote drug synergy (especially above 1). According to this definition, synergistic drug effects are observable in all T-ALL lines tested, as depicted in the figure below (for review only). In line with the results presented in Figure 5, MOLT-4 and CEM-C7 exhibit the highest, CEM-C1 intermediate and Jurkat the lowest Z-scores and hence synergistic drug responsiveness.



Synergy scores (Z-scores) between 3d2 and dexamethasone of human T-ALL cell lines were calculated from the dose-response matrix of Jurkat, MOLT-4, CEM-C1 and CEM-C7 cells treated for 48h with 0-60µM 3d2 and 0-30µM dexamethasone (results exemplified in Figure 5B) and analyzed by standard flow cytometric quantification of cell death after AnnexinV staining. Individual Z-scores of 3 independent experiments calculated using SynergyFinder tool and mean values ± SD are shown.

11. In Figure 6, shLRH-1 upregulated GR mRNA expression and induced BIM by itself, and no enhanced BIM induction was observed in 6H. These data do not seem to support a synergy between GR and LRH-1, certainly not a direct interaction between those two.

We are not really sure what the reviewer is referring to. As shown in Figure 6D and H, LRH-1 knockdown leads to the upregulation of GR and Bim protein levels. This is likely caused by reduced LRH-1-mediated GR inhibition resulting in an improved GR auto-induction and consequent GR-induced Bim induction. Given that this is observed already in control treated shLRH-1 knockdown cells, we must assume that this is the result of low-level GC in the serum-containing medium, as discussed above. It can be assumed that inhibition of the remaining LRH-1 protein pool by 3d2 further enhances dexamethasone-induced GR signaling, as seen by increased Caspase 3 and PARP processing. We agree that this experiment does not demonstrate a direct physical interaction, but clearly confirms a functional/regulatory interaction of GR and LRH-1.

12. Does the GR binding at ALL genomes get influence by LRH-1 or its inhibition?

We must certainly assume so as LRH-1 knockdown or inhibition results in GR auto-upregulation and Bim induction, which was shown to be regulated by GR-mediated transactivation (as reviewed by Clarisse et al, 2020) and which is a pre-requisite for GC-induced cell death.

Comments Referee 3:

Specific comments:

1. Of the 4 T cell lines tested, 3 were GC-resistant and expressed low levels of GR and moderate to high levels of LRH-1 as measured by qRT-PCR. A fourth, CEM-C7, expressed high levesl of GR and very low levels of LRH-1. CEM-C7 cells, therefore, should provide a control for "off-target" effects of LRH-1 inhibitors (that is, effects that aren't due to inhibition of GR/LRH-1 interactions). There were a number of experiments whose intepretations would benefit from analysis of CEM-C7, which will be mentioned in other comments. Although it was noted that LRH-1 knockout cells could not be obtained, shRNA knockdown was successful, and at the least one could compare the effect of the two inhbitors on mock vs. LRH-1 knockdown cells.

As suggested by this reviewer, a number of experiments were repeated in GC-sensitive CEM-C7 cells, as also discussed in the upcoming replies to individual comments. While CEM-C7 were already sensitive to dexamethasone, 3d2 alone and especially in combination with dexamethasone had potent cell death-inducing effects in this cell line. We thus assume that even though expressed at low levels, LRH-1 still negatively regulates GC sensitivity and/or has further critical functions in the general regulation survival of CEM-C7 cells. Inhibition of LRH-1 might therefore especially prime already somewhat GC-sensitive T-ALL cells to dexamethasone-induced cell death by mechanisms that are directly, but possibly also indirectly related to GR-LRH-1 interaction. In line with this hypothesis, enhanced LRH-1 levels in general protect from cell death induction, while a reduction of LRH-1 activity triggers spontaneous cell death in certain cell types (as recently reviewed in: Michalek & Brunner, 2020). The survival-promoting functions of LRH-1 can be attributed to its direct role in controlling expression of anti-apoptotic proteins, to its function in cellular energy metabolism and to protection from ER stress, oxidative as well as DNA damage.

A comparison between LRH-1 knockdown and inhibition by 3d2 was analyzed in Figure 6H. As can be seen, LRH-1 knockdown has similar effects as 3d2-mediated LRH-1 inhibition. However, we also observed that 3d2 further enhances the effect of shRNA-mediated LRH-1 downregulation. While at a first glance, this might be counterintuitive and be interpreted as a confirmation of non-specific/off-target effects of 3d2, it represents likely the effect of 3d2 on the remaining LRH-1 protein levels, given that shRNA-mediated knockdown is not complete. However, the fact that both, 3d2 and LRH-1 knockdown result in similar gene expression patterns and sensitization to dexamethasone strongly supports the idea that the effect of 3d2 is via inhibition of LRH-1.

2. In Fig. 3F it is noted that the predicted effect of 3d2 on Jurkat cell LRH-1 reporter activity was not observed. Although reporter assays are notoriously prone to artefact, this result was mentioned as "of interest" but wasn't explored. Instead the expected result with MOLT-4 was taken as proof that 3d2 was acting as expected. Fig. 3E and 3F are examples where analysis of CEM-C7 cells would be helpful.

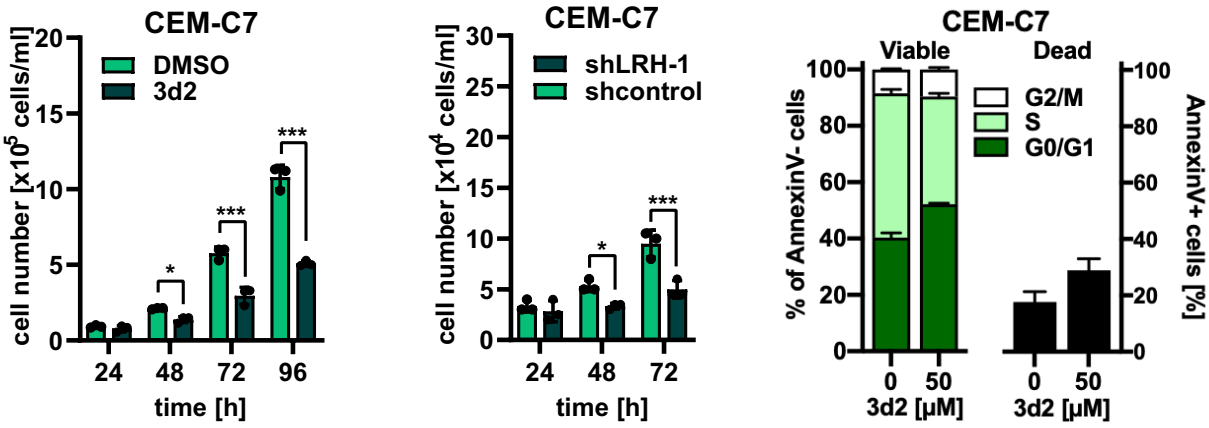
We thank this reviewer for this valuable comment and suggestion. We thus invested a lot of time and effort to optimize transfection protocols and luciferase reporter assays for individual T-ALL cell lines to further investigate this issue. Moreover, as suggested we analyze LRH-1, resp. GR activity also in CEM-C7 cells. Adjustment of transfection protocols, buffers, incubation times and concentrations allowed for generation of highly reproducible results, demonstrating that LRH-1 activity can also be potently and significantly inhibited in Jurkat, as well as CEM-C7 cells. In line with their observed GC

sensitivity, dexamethasone applied as a single treatment was sufficient to potently induce GR activity in CEM-C7 cells, which could be slightly enhanced by additional treatment with 3d2. This is in line with our observation that also in CEM-C7 cells 3d2 further enhances and accelerates dexamethasone-induced apoptosis (Figure 5C). Revised figures have now been integrated in Figure 3F and G (line 256-265).

3. In Fig. 4A, B, and E, the growth-inhibiting effects of 3d2 and shLRH-1 need to be tested with CEM-C7 cells as well (where they would be predicted to have little effect). The assumption that the RNAi studies "rule out unspecific off-target effects" of 3d2 (line 280) is incorrect: 3d2 may well have off-target effects on growth and apoptosis in addition to (or instead of) its effects on LRH-1 conformation.

As suggested by this reviewer, the effect of LRH-1 inhibition or knockdown on the growth of CEM-C7 cells were investigated. Like the other T-ALL cell lines tested, 3d2 (graph included in Figure 4A) and shRNA-mediated LRH-1 knockdown (for review only) reduced the proliferation of CEM-C7 cells. Combined quantification of dead cells using Annexin V staining and bidimensional cell cycle analysis of Annexin V-negative, viable cells revealed that CEM-C7 cells that survive 3d2 treatment accumulate in the G1 phase of the cell cycle (similar to results observed in MOLT-4 cells, Figure 4 D).

As discussed above, while this result appears at a first glance a confirmation that 3d2 has unspecific effects, we observe again comparable effects of LRH-1 knockdown and pharmacological inhibition in CEM-C7 cells, supporting the idea that 3d2 indeed targets LRH-1. In line with this notion is the observation that although CEM-C7 are GC-sensitive LRH-1 further sensitizes them to dexamethasone, resulting in increased and in particular accelerated cell death induction. While one would predict that pharmacological LRH-1 inhibition would have a stronger effect in cells expressing higher levels of LRH-1, this may not necessarily be the case. Of note, while LRH-1 deletion in hepatocytes and intestinal epithelial cells (Lee et al, 2008; Mataki et al, 2007), expressing excessive levels of LRH-1 still results in the development of a liver, resp. intestinal epithelium, the deletion of LRH-1 in primary T cells, expressing 100-1000-fold less LRH-1 than hepatocytes, results in a massive reduction of peripheral T cells (Seitz et al., 2019). Thus, cells expressing already low levels LRH-1 appear to be even more sensitive to further downregulation, resp. inhibition of LRH-1, whereas in cells with high LRH-1 expression levels residual LRH-1 activity may be still sufficient to compensate.



Daily cell counting-based comparison of cellular proliferation of CEM-C7 cells after (**left**) treatment with 20µM 3d2 and (**middle**) small hairpin RNA (shRNA)-mediated LRH-1 knockdown. Individual and mean values of technical triplicates of one representative experiment ± SD are shown. Two-way ANOVA; * $p < 0.05$, *** $p < 0.001$. (**right**) Simultaneous flow cytometric analysis of cell cycle distribution by EdU/DAPI staining and cell death by AnnexinV staining in CEM-C7 cells upon treatment with 50µM 3d2. Dead cells are represented by the number of AnnexinV positive (AnnexinV+), apoptotic cells as a percentage (%) of total cells and cell cycle phase distribution as % of viable, AnnexinV negative (AnnexinV-)

cells. Representative results from three independent experiments are shown. Bars represent the mean of technical triplicates ± SD (n=2 independent experiments).

According to your helpful suggestion, the sentence “Nonetheless, to rule out unspecific off-target effects of pharmacological LRH-1 antagonists, we employed RNA interference-mediated silencing to down-regulate LRH-1 expression in T-ALL cells.” *was rephrased to:* “Nonetheless, to confirm LRH-1 inhibitor-mediated effects, we employed RNA interference-mediated downregulation of LRH-1 in T-ALL cells.” *in order to be more specific (line 285/286).*

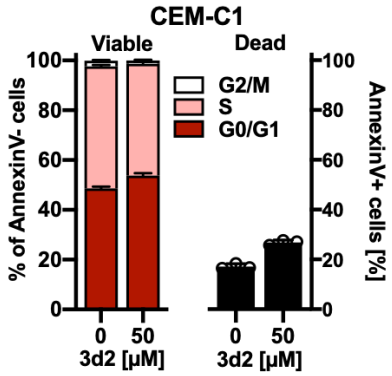
4. Line 297: "In contrast, LRH-1 inhibition caused rather dynamic changes in protein abundance of both cell cycle regulators, with a marked reduction in MOLT-4 after only 2 h and in CEM-C1 cells after 6 h (Figure 4D)." I'm not sure what "rather dynamic changes" is in reference to, but even after close inspection I see only small changes in protein levels that don't even trend (e.g. c-Myc levels in MOLT-4 at 0, 4, and 6 hr are comparable, with perhaps a very small dip at 2 hr. ECL is only linear over a very narrow range and small differences in intensity can't be interpreted with certainty. To my eye Fig. 4D shows no obvious effect of 3d2 on cell cycle proteins. This is in contrast with the shRNA data in Fig. EV2C, which makes one wonder about the mechanism by which 3d2 inhibits cell proliferation.

A major difference between results shown in revised Figure EV2C and D is that in Figure EV2C mRNA levels of c-Myc and Cyclin E1 were analyzed, whereas in Figure EV2D protein levels were monitored. As it takes time until changes in mRNA levels translate in altered protein levels, it makes in our view sense that changes observed in Figure EV2C are more pronounced than in FigureEV2D. However, the same pattern as seen for 3d2-triggered changes in mRNA expression was also seen in LRH-1 knockdown cells (Figure EV2E), indicating that changes in c-Myc and cyclin E1 are specific.

Nonetheless, in order not to overinterpret the changes in protein levels we rephased the text and transferred the graphs showing cell cycle regulators after 3d2 treatment from the main to the expanded view figures (line 299-304).

5. In keeping with unexpected behavior, although 3d2 inhibits the growth of CEM-C1 as well as it does for Jurkat and MOLT-4, it only affected the cell cycle phase distribution in the latter two (.e.g, no G1 arrest). It is difficult to draw conclusions about mechanism when different cells behave differently.

Similar to CEM-C1 cells, we also didn’t observe changes in the proportion of CEM-C7 cells in the G1, S and G2/M phase of the cell cycle even though cell growth was potently inhibited by 20µM 3d2 (reference to comment No. 3). We think that absence of effects on cell cycle distribution in these cell lines are related to cell death induction as seen in the mild increase in subG1 upon treatment with concentrations <40µM 3d2. Additional analysis of dead cells using Annexin V staining combined with bidimensional cell cycle analysis of Annexin V-negative, viable cells revealed that CEM-C1 cells that survive 3d2 treatment accumulate in the G1 phase of the cell cycle (similar to results observed in CEM-C7 and MOLT-4 cells).



Simultaneous flow cytometric analysis of cell cycle distribution by EdU/DAPI staining and cell death by AnnexinV staining in CEM-C7 cells upon treatment with 50µM 3d2. Dead cells are represented by the number of AnnexinV positive (AnnexinV+), apoptotic cells as a percentage (%) of total cells and cell cycle phase distribution as % of viable, AnnexinV negative (AnnexinV-) cells. Representative results from three independent experiments are shown. Bars represent the mean of technical triplicates ± SD (n=2 independent experiments).

Since CEM cells divide comparably fast and were not synchronized before 3d2 was applied for only 24h, effects on cell cycle phase distribution are extremely slight and are hence missed in standard DNA staining assays shown in Figure 4. We thus believe that more detailed analysis of 3d2 effects on cell cycle progression in CEM-C7 cells confirms our observations made in the other cell lines.

6. In Fig. 4G CEM-C7 cells were included, but instead of showing little to no effect of 3d2 on cell death, they died with the same kinetics and almost to the same extent as Jurkat and MOLT-4, despite the statement that they were "comparably insensitive". In fact, at 48 hr they were similar except at the highest 3d2 dose, where the % annexin V was ~30% to 50%, despite the fact that MOLT-4 is the highest expressor of LRH-1 (Fig. 3C). In keeping with these inconsistensies, the patterns of sensitivity for 3d2 and SR1848 were quite different. The hierarchy for 3d2 was CEM1-C1 > Jurkat = MOLT-4, but for SR1848 Jurkat was completely resistant. If these inhibitors are converging on the same target (LRH-1) why this variability?

We are not entirely sure whether we understand this comment correctly. Former Figure 4G and revised Figure 4E shows clearly that after 48h treatment CEM-C7 cells are the least sensitive ones to 3d2. Concerning the at first glance unexpected 3d2 sensitivity of CEM-C7 cells please refer also to our reply to comment #3. We certainly agree that expression of LRH-1 must be critical for LRH-1-dependent, 3d2-induced cell death. However, mRNA levels of LRH-1 might not necessarily reflect protein levels, functionality and transcriptional activity of LRH-1, and therefore might not directly correlate with 3d2 sensitivity. However, based on the reviewer's criticism, the description of the results shown in former Figure 4G (revised Figure 4E) was rephrased to describe differences in 3d2-induced cell death rates in a more neutral manner.
Variability between sensitivity to the two tested LRH-1 inhibitors can be explained by their entirely different mode of action. While 3d2 only interferes with co-activator binding to LRH-1 (Benod et al., 2013), SR1848 induces the translocation of LRH-1 from the nucleus to the cytosol (Corzo et al, 2015). Like knockdown, SR1848 prevents any scaffold functions of LRH-1 in the nucleus of cells, which could account for its toxicity already at 10 times lower concentrations in CEM-C1 and MOLT-4 cells.
As pointed out by this reviewer, Jurkat cells were found to be rather insensitive to SR1848-mediated LRH-1 inhibition. Interestingly, also LRH-1 knockdown had the least effects in Jurkat cells. As the case for other nuclear receptors, effector functions of LRH-1 depend on interaction with co-regulatory proteins that likely differ between distinct cell types and individual leukemia cell lines/patients. Depending on the levels and

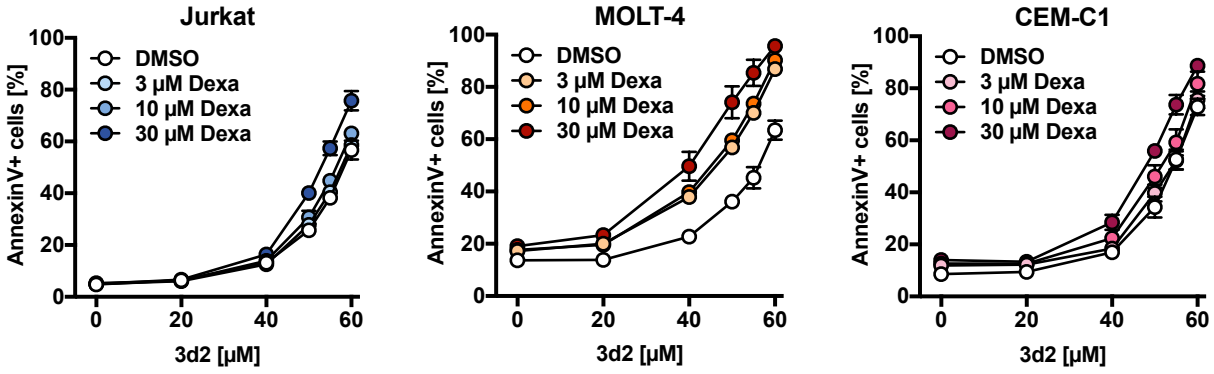
composition of LRH-1 interaction partners, knockdown or SR1848-mediated reduction of nuclear LRH-1 could have different outcome in distinct T-ALL cells. While nuclear presence of LRH-1 seems to be critical for the growth and survival of MOLT-4 and CEM cells, it only seems be essential for proliferation of Jurkat cells. Thus, it is possibly that GR sensitivity in Jurkat cells is also regulated by other mechanisms than interactions with LRH-1. Furthermore, SR1848 resistance in Jurkat cells might also be explained by differential drug uptake and/or export rates of certain chemical compounds. Along these lines, in our lab Jurkat cells also display the lowest drug sensitivity to some but not all other cell death inducing agents.

7. In Fig. 4I it is clear that cleavage of PARP and Caspase-3 increased over time. But the changes in p-p38 and Bim are small, fluctuate about a mean, and do not trend (e.g. p-p38 levels are similar at 0, 4, and 8 hr, and slightly higher at 2 and 6 hr). I would suggest that the experiment's point that the death is apoptotic is made without having to overinterpret these small and inconsisent changes.

We thank the reviewer for this suggestion and have now rephased the corresponding result section accordingly (line 348-353).

8. Fig. 5A. In the text (line 360-361) it is stated that "In all GC-resistant T-ALL cell lines tested, dexamethasone significantly enhanced 3d2-mediated cell death induction". While there are small enhancements at the highest 3d2 dose for Jurkat and CEM-C1, the dose-response curves are remarkably similar, and given that n=4 the claim is unconvincing. The results are only clear for MOLT-4, where the curve is clearly shifted. The liklihood of off-target affects is once again raised by the "surprising" large effect on CEM-C7 cells (Fig. 5C), which express little LRH-1.

While the statistical analysis of a number of independently repeated experiments is mandatory to prove significant differences, it has the disadvantages that due to combining different experiments in one graph clear differences are not so obvious anymore. As shown in the figure below, individual experiments out of the n=4 independent experiments showed a clear, dose-dependent difference in the 3d2-mediated sensitization to dexamethasone.



Flow cytometric analysis of cell death by AnnexinV staining in T-ALL cell lines upon 48h of treatment with Dexamethasone (Dexa) and 3d2. Mean values of technical triplicates of one representative experiment (n=4) ± SD are shown.

Since the basal and compound-induced cell death rates generally differed between single experiments, we think that the presented results showing combined data from n=4 individual experiments show statistically significant differences and are indeed convincing. Again, referring to previous comments, we would like to point out that low

LRH-1 mRNA expression levels do not necessarily indicate that LRH-1 does not have important effector functions in these cells. It rather seems that cells expressing low levels of LRH-1 are especially sensitive to interfering with LRH-1 activity or expression.

9. Fig. EV4E, panel E, supposedly shows that GR deficiency decreased Bim protein levels (line 454). But it's not clear what the blot shows. There are 3 "Bim" bands. The topmost is very similar in the GR KO to the intensity in the WT cells, the most striking difference being an increase in mobility (a postranslational modification?), and the lower 2 bands are identical. The text doesn't match the data.

We would like to apologize for not having better explained the protein expression data of Bim. As discussed in our answer to comment 7 of Referee No. 1, there are 3 isoforms (splice variants) of Bim that can be detected by Western Blot. Especially Bim_{EL} is known to be subject of post-translational modifications, such as phosphorylation by ERK, PKA and JNK. Depending on the gel electrophorectical resolution up to 5-6 distinct bands of Bim_{EL} can be identified. Usually, a reduced molecular weight is associated with reduced phosphorylation. Likely, the downregulation of GR indirectly also changes the activity of Bim-phosphorylating kinases. Given that changes in protein expression levels are not so obvious as mRNA expression levels, we have now changed the text accordingly (line 456-459).

10. Lines 502-503, it is said that the patient-derived cells were insensitive to dexamethasone alone, but there no dexamethasone-only data in Fig. 7A.

We thank the reviewer for pointing out this mistake. Viability of human PDX after dexamethasone single treatment at concentrations that was used for the combined treatment was now included in Figure 7A.

11. The data with human T-ALL are welcome, but to tie the putative mechanism by which 3d2 affects cell growth and viability to its effects on cell lines, one must at least have some information about such critical parameters as GR and LRH-1 levels. As well, there are marked differences in the response of the 4 samples, with 4 being unresponsive, 2 and 3 responding at very low doses of 3d2 (10 nM or less, compared to the 40000 nM levels used for the cell lines), and 1 responding only when the concentration reached 1000 nM or greater. An analysis of pertinant variables may help understand these differences.

According to the reviewer's suggestion, the used human T-ALL PDX have been analyzed regarding GR, LRH-1 as well as Bim mRNA expression. The corresponding results plus a correlation of the expression levels with drug responsiveness were included and discussed in the main figures and manuscript (revised Figure 7; line 519-528). In short, our analyses demonstrate that synergistic induction of cell death by 3d2 and dexamethasone, as documented by the Z-scores, nicely correlated with the expression levels of LRH-1 and Bim. Confirming in vitro results, the presented data suggests that the combined toxicity of 3d2 and dexamethasone is LRH-1-mediated and dependent on Bim. Furthermore, we also observed a negative correlation between the GR:LRH-1 expression ratio and Z-score (line 520-529). One possible interpretation of this finding is that the lower LRH-1 levels are (i.e. the higher GR:LRH-1 ratio), the less effective 3d2-mediated sensitization of cells to dexamethasone might be. But clearly, an increase in sample size in the analysis of follow-up studies might help to clarify this aspect.
A most important aspect in terms of a potential translational application of our study is the observation that patient-derived T-ALL cells were much more sensitive to this drug combination than cell lines. Even nanomolar dexamethasone concentrations in

combination with low nanomolar doses of 3d2 were sufficient to potently induce cell death at least in some of the samples tested.
In line with the fact that leukemia is a highly heterogenous disease, we thus propose the here described LRH-1/GR interaction as a novel mechanism among various others contributing to GC resistance in individual patients. Other established mechanisms, such as variations in Bcl-2 homolog expression causing a general drug/apoptosis resistance or GR mutations are likely critical determinants of the treatment outcome in different patients. Nonetheless, our data on the synergistic induction of cell death by the LRH-1 inhibitor 3d2 and dexamethasone provide a first proof of principle. According to our results, this combination treatment could be successful in those patients which show LRH-1 expression and display minimal GC sensitivity.

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Dear Prof. Brunner,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you can see, all referees now fully support the publication of your study. Referee #3 has a minor comment/question I ask you to address in a final revised version of the manuscript.

Moreover, I have these editorial requests:

- Please remove the word 'novel' from the title. It is the aim of EMBO reports always to publish novel findings. How about: LRH-1 interacts with the glucocorticoid receptor to regulate glucocorticoid resistance

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

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Achim Breiling
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Referee #1:

This reviewer is satisfied with the amended version and thanks to the authors for resolving the queries.

Referee #2:

The authors have addressed all my questions properly. I would recommend publishing this paper in EMBOR.

Referee #3:

The authors have addressed my major concerns. A minor point -- on the new line 518, do they mean < 10 nM rather than < 2 μM?

University of Konstanz · Box 660 · 78457 Konstanz

To the
Editor
EMBO Reports
Dr. Achim Breiling

EMBOR-2021-54195V2, submission of revised manuscript

Dear Editor, dear Dr. Breiling

Many thanks for your kind mail and the formal acceptance of our manuscript. According to your suggestions, we have now changed the title to „LRH-1/NR5A2 interacts with the glucocorticoid receptor to regulate glucocorticoid resistance". Furthermore, we have done all changes in the text as suggested. Please, find the detailed reply below.

We hope that the manuscript can now be published in EMBO Reports.

Kind regards

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Reply

- Please remove the word 'novel' from the title. It is the aim of EMBO reports always to publish novel findings. How about:
LRH-1 interacts with the glucocorticoid receptor to regulate glucocorticoid resistance
Title was changed.

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.
Was updated in the revised manuscript. According to your bullet point number 4 and Author Guidelines we placed the 'Disclosure and Competing Interests Statement' after the "Author contributions".

- Please remove the section 'The paper explained' from the manuscript text. You can use part of this for the synopsis blurb and the bullet points (see below). Please also remove the section 'LEAD CONTACT AND MATERIALS AVAILABILITY' and name this part 'Materials and Methods' (combining the sections 'EXPERIMENTAL MODEL AND SUBJECT DETAILS', 'METHODS AND PROTOCOLS' and 'QUANTIFICATION AND STATISTICAL ANALYSIS').
Was updated in the revised manuscript.

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The RTT was updated and removed from the main manuscript. However, our three EV tables contain forward and reverse oligonucleotide sequences used for cloning, RT-PCR and CRISPR/Cas9-mediated gene knockout. It does not seem possible to include all relevant information in the RTT due to the given format restriction of the RTT. Similar to the example you linked and recent publications in EMBO reports we therefore retained the primer sequence-containing EV Tables in the main manuscript.

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A 2 sentence summary is attached
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A summary figure is attached

Referee #3:

The authors have addressed my major concerns. A minor point -- on the new line 518, do they mean < 10 nM rather than < 2 µM?
Thank you very much for pointing out this mistake. We changed the sentence to: "Nevertheless, in one out of the four tested T-ALL PDX (Sample 2) more than 2 µM 3d2 was sufficient to notably reduce cellular viability."

Prof. Thomas Brunner
Universität Konstanz
Biochemical Pharmacology, Dept. Biology
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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.

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The data shown in figures should satisfy the following conditions:

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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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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 have been in the lab for the past 20 years. They have been frequently tested for mycoplasm contamination

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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.	mice, NOD.Cg-Prkdc(scid)Il2rg(tm1Wjl/SzJ), females, 6 wekks old. Housed under SPF conditions.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animals experiments were performed in compliance with ethical regulations and approved by the veterinary office of the canton of zurich.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance with ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Ethics committee of the Canton of Zurich, Switzerland
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.	A statement is included in the Materials and Methods section
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	na
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