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BCL11B and the NuRD complex cooperatively guard T-cell fate and inhibit OPA1-mediated mitochondrial fusion in T cells

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Dear Prof. Li,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

Given the referees' recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. It is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. It would be good to discuss your plan to address the referee concerns and I will be available to do so by email or by zoom in the coming weeks. I have also attached a guide for revisions for your convenience.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Kelly M Anderson, PhD
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The EMBO Journal
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Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (10th Jun 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

In this study, Liao and colleagues investigated the role of Bcl11b and NuRD complex in human T cells. The authors knocked-out various NuRD complex members as well as performed ATAC-seq and CUT&Tag approaches. They show that Bcl11b/NuRD negatively regulate the expression of genes associated with NK cell-mediated cytotoxicity and thus repress a cytolytic program in human T cells. Furthermore, the authors noticed fused mitochondria and enhanced metabolic function in the ITNK cells. Mechanistically, the authors demonstrated that Bcl11c/NuRD is recruited to the OPA1 gene locus and that the loss of Bcl11b/NuRD leads to an upregulation of OPA1. They suggest that part of the mechanism is due to the upregulation of OPA1, since OPA1 knockout reverted the effect. Finally, the authors measured acetyl-CoA levels in INKT cells and observed increased levels in CD4+ and CD8+ Bcl11b-deficicent INKT cells, which was reverted in the absence of OPA1. Based on these data the author conclude that Bcl11b/NuRD complex maintains T cell fate via directly repressing NK cell-associated transcriptional programs as well as via repressing an metabolomic/epigenetic axis that would result in the upregulation of NK-like transcriptional programs. Overall, this is a nicely performed study.

Specific comments:

1) The OPA1 knockout shows that the process is OPA1-dependent, but does not indicate that OPA1 upregulation is the cause of the elongated mitochondria and increased OXPHOS. It would be interesting to overexpress OPA1 (long and short isoforms) in human T cells to test whether this induced similar mitochondrial alterations as seen in the NuRD member knockdowns? The authors mention some of these aspect in the discussion section (unfortunately, page numbers are missing) and write that "Further studies are warranted to investigate whether BCL11B suppresses mitochondrial fusion via OPA1 during T-cell

development and differentiation.", however adding this data is important for providing insight into the a potential mechanism.

2) The authors observed an upregulation of NK-like cytolytic programs in CD4+ and in CD8+ T cells. With respect to CD4+ T cells: it is know that certain subsets of CD4+ T cells have cytotoxic activity (CD4+ cytotxic T lymphocytes, CD4+ CTLs). Have the authors tested whether Bcl11b/NuRD knockout results in the induction of genes associated with CD4+ CTL subset? This could be easily tested by performing GSEA with CD4 CTL-specific gene lists (e.g. taken from the following publication: PMID: 29352091).

Minor issue:
3) The authors indicate in several figure legends that the results are representative of 3 independent experiments. I assume the cells for the independent experiments were also isolated from independent donors. Perhaps one could mention this.

Referee #2:

Summary

Liao et al. investigate mechanisms by which T cells are reprogrammed into induced T-to-NK (ITNK) cells in culture upon deletion of BCL11B. ITNK cells express a T-cell receptor as well as NK-cell surface markers, and can lyse NK-cell targets.

The authors demonstrate that BCL11B binds components of the NuRD repressor in human T cells including MTA2, MBD2 and CHD4. Deletion of each NuRD component resulted in reprogramming of some cells into ITNKs with similar properties to cells produced by BCL11B deletion, although reprogramming by NuRD deletion was much less efficient. RNA-seq revealed some genes similarly regulated in BCL11B- and NuRD-deletion induced ITNKs, including NK-associated genes. H3K27Ac is increased at a set of transcriptional start sites, including NK-cell associated genes, and the loci of several of these genes are bound by BCL11B.

Genes upregulated in ITNKs were enriched in signatures associated with metabolic processes. The authors show evidence that the OPA1 locus is bound by BCL11B. OPA1, which regulates mitochondrial fusion, is upregulated in BCL11B-ablated ITNKs, and this is associated with elongation of mitochondria in BCL11B- and NuRD-ablated ITNKs. BCL11B-deficient ITNKs showed more OXPHOS metabolism and less reactive oxygen species. Deletion or inhibition of OPA1 inhibited ITNK reprogramming, and reduced their cytotoxicity. Acetyl-CoA, an OXPHOS metabolite that is the substrate for H3K27Ac, was increased after BCL11B deletion, but this requires OPA1 expression. Decreased Acetyl-CoA levels, INTK reprogramming, and INTK cytotoxicity in BCL11B/OPA1 double deletion T cells can be rescued by exogenous acetate, which is associated with increased H3K27Ac across the chromatin, and at specific NK-associated loci.

Significance

Several findings are consistent with previous work in mouse models where BCL11B recruits repressor complexes (including NuRD) in T cell development to repress the expression of genes associated with other lineages (including NK-cell genes).

The authors' conclusion that T-cell fate is maintained by BCL11B/NuRD repressing OPA1, resulting in less mitochondrial fusion, less OXPHOS metabolism, and therefore less H3K27Ac at NK-cell genes is more novel, but more evidence is required to support this metabolic-epigenetic axis, especially in physiologically relevant cells. The evidence provided in support of key parts of this conclusion must be strengthened (e.g. BCL11B binding to the OPA1 locus, and upregulation of OPA1 during the loss of T-cell identity during reprogramming). Furthermore, much of the data is from an unusual cell population selected during 10 days culture after deletion of a gene, and it is therefore not possible to conclude that the molecular changes observed in them are the same as those that controlled the fate of physiological T cells 10 days earlier.

Major concerns

1. Evidence of BCL11B binding to OPA1 locus
This finding, central to the metabolic-epigenetic axis hypothesis proposed, is only supported by a single, very small, increase in Cut&Tag signal in Fig 2E. This evidence is not sufficient to conclude BCL11B binds the OPA1 locus TSS. The authors must provide statistical analysis that the peak is significant or, better, independent evidence that BCL11B binds the TSS of the OPA1 locus, but not at nearby control regions.

2. Evidence of OPA1 upregulation
OPA1 RNA and protein are upregulated in CD3+NKp46+ ITNKs compared to T cells and NK cells. The authors also conclude that "OPA1 expression increases over time upon BCL11B ablation" in Fig S3A and "MTA2-, MBD2-, and CHD4-deficient ITNKs also showed upregulation of OPA1 protein" in Fig 3C. These conclusions are also central to the metabolic-epigenetic regulation

of T-cell fate hypothesis. However, the referred figures don't seem to support this. Better quantitation of OPA1 protein levels after BCL11B or NuRD ablation are required, ideally with multiple replicates, and Western blots images without signal saturation.

3. Evidence that metabolic and epigenetic changes contribute to maintaining T-cell fate
The authors conclude that NuRD and BCL11B maintain T-cell fate at least partly through a metabolic-epigenetic axis. Evidence supporting this is obtained from subpopulations of cells cultured for 10 days after gene deletion - T-cell identity may have been lost much earlier than this time.
For example: possible BCL11B binding OPA1 locus; OPA1 upregulation; increased mitochondrial length, increased OXPHOS. For the authors to conclude that these processes contribute to maintaining T-cell fate they must show that they occur prior to or at least concurrently with loss of T-cell identity. Otherwise they cannot rule out they are unrelated events that are associated with the late stages of ITNK differentiation in culture that happen after the loss of T-cell fate. Experiments showing evidence of metabolic and epigenetic changes at multiple timepoints, alongside the loss of T-cell specific mRNA and protein expression, are required.

4. Incomplete reporting of RNA-seq, Cut&Tag and ATAC-seq data
Excellent experiments to determine the changes to the transcriptome and chromatin in ITNK cells have been performed, but only select parts of the data have been presented.
For RNA-seq, the methods used to generate the gene set enrichment analysis in Figs S2C and S2D must be described in more detail. The authors conclude that "the transcriptional profiles of ITNKs derived from shMTA2-, sgMBD2-, and sgCHD4-transducted T cells were similar to those for BCL11B-deficient ITNKs but not to those of sgCtrl-transduced T cells", but the principal component analysis in Fig 2A does not support this at all, showing that BCL11B-deficient ITNKs and controls T cells are both very different from the NuRD-ablated ITNKs. The authors must correct this and analyse the differences in gene expression between the BCL11B-deficient and NuRD-deficient ITNKs - not just the similarities.
For H3K27me3, H327Ac, BCL11B, and CHD4 Cut&Tag, as well as ATAC-seq a summary of the data should be provided: how many peaks in each data set, where are they in the genome (promoters, enhancers, gene bodies, etc.), and which gene sets are they near. Without this it is impossible to interpret the significance of the handful of loci shown.

Minor concerns

5. Experiments use data from slightly different cell types thoughout, depending on whether a sorted population was used, and which surface markers. It would make the manuscript much easier to read and interpret if the source of cells used in an experiment was indicated clearly in each figure panel.

Referee #3:

In this study, Liao et al. performed CRISPR knockouts of BCL11B and NuRD subunits in T cells to investigate the importance of the interaction between these components for the acquisition of NK cell-like attributes in these cells. Data from these experiments confirm interactions between BCL11B and NuRD subunits and show that knockout of NuRD subunits generally phenocopy results seen with BCL11B knockout with respect to NK cell signature gene expression and cytotoxic function against K562 cells. During their investigation, the authors noted elevated expression of genes associated with metabolism in BCL11B- and NuRD subunit-deficient T cells. They also observed binding of BCL11b within the OPA1 locus in T cells. The authors then showed that ITNK cells had higher levels of OPA1 (particularly the long isoform) relative to T cells and NK cells. This correlated with mitochondrial length and measures of OXPHOS. Further experiments showed that knockout of OPA1 along with BCL11B blunted the conversion of T cells into ITNK cells, and this could be rescued through acetate supplementation. Overall, the findings connecting BCL11B, OPA1, and OXPHOS in this manuscript are of interest and provide potentially important new insights into the nature of ITNK cells, which may have some therapeutic relevance. There are a additional points that should be addressed to strengthen this manuscript for publication.

Major comments:

1. In Figure 3A, B the authors provide data showing the relative amounts of OPA1 mRNA and protein in T cells, NK cells, and ITNK cells. It would be of interest to compare the levels of BCL11B mRNA and protein in these cells alongside this data to determine to what extent there is a correlation between BCL11B levels and OPA1 levels. This is particularly important for the comparison between NK cells and T cells. It appears that T cells and NK cells have similar levels of OPA1 mRNA, but T cells have significantly more short OPA1 protein. It's not clear what accounts for this disconnect. Also, if NK cells have significantly lower amounts of Bcl11b protein, the model put forth by the authors would suggest that NK cells should have higher levels of OPA1, which isn't evident in the qRT-PCR or Western blot data.

2. In Figure 5A, the authors show that BCL11B knockout in T cells results in significant elevations in acetyl-CoA, which is lost when OPA1 is also knocked out. It is not clear why OPA1 knockout would have such a major effect on acetyl-CoA levels. The authors should at least address this in the discussion. I may have missed it, but it is not clear either in the figure legend or methods how acetyl-CoA levels were being measured. Was it total protein, histones, or concentrations of this molecule in cells?

This needs to be clarified.

3. In the model proposed in Figure 6, it seems as though the authors suggest that ITNK cells have elevated TCA cycle activity that can generate more acetyl-CoA for histone modification at NK cell-related genes. However, the authors don't provide data to show this. The authors should acknowledge the limitations of their current study and gaps in knowledge that still exist.

Response Letter to The EMBO Journal Submission

Paper ID: EMBOJ-2023-113448

Paper Title: BCL11B and the NuRD complex cooperatively guard T-cell fate and inhibit OPA1-mediated mitochondrial fusion in T cells

REVIEWERS' COMMENTS

Reviewer 1#

In this study, Liao and colleagues investigated the role of Bcl11b and NuRD complex in human T cells. The authors knocked-out various NuRD complex members as well as performed ATAC-seq and CUT&Tag approaches. They show that Bcl11b/NuRD negatively regulate the expression of genes associated with NK cell-mediated cytotoxicity and thus repress a cytolytic program in human T cells. Furthermore, the authors noticed fused mitochondria and enhanced metabolic function in the ITNK cells. Mechanistically, the authors demonstrated that Bcl11c/NuRD is recruited to the OPA1 gene locus and that the loss of Bcl11b/NuRD leads to an upregulation of OPA1. They suggest that part of the mechanism is due to the upregulation of OPA1, since OPA1 knockout reverted the effect. Finally, the authors measured acetyl-CoA levels in INKT cells and observed increased levels in CD4+ and CD8+ Bcl11b-deficicent INKT cells, which was reverted in the absence of OPA1. Based on these data the author conclude that Bcl11b/NuRD complex maintains T cell fate via directly repressing NK cell-associated transcriptional programs as well as via repressing an metabolomic/epigenetic axis that would result in the upregulation of NK-like transcriptional programs. Overall, this is a nicely performed study.

Specific comments:

1) The OPA1 knockout shows that the process is OPA1-dependent, but does not indicate that OPA1 upregulation is the cause of the elongated mitochondria and

increased OXPHOS. It would be interesting to overexpress OPA1 (long and short isoforms) in human T cells to test whether this induced similar mitochondrial alterations as seen in the NuRD member knockdowns? The authors mention some of these aspect in the discussion section (unfortunately, page numbers are missing) and write that "Further studies are warranted to investigate whether BCL11B suppresses mitochondrial fusion via OPA1 during T-cell development and differentiation.", however adding this data is important for providing insight into the a potential mechanism.

Our response: We appreciate the comments. According to the first suggestion, we overexpressed the long- and short-form of OPA1 in human T cells and assessed mitochondrial alterations. Previous studies show that the isoform 1 functions as the fusion-active species, giving rise to long forms of OPA1 that can be cleaved into short forms. The isoform 1 ΔS1 produces an uncleavable isoform 1. Conversely, the isoform 5 produces a short-form of OPA1 with little fusion activity (PMID: 28636943, 16778770, 17709429). We found that the overexpression of the long-form (Isoform 1 and its mutant S1) resulted in tubulation of mitochondria in T cells, while overexpression of the short-form (Isoform 5) produced little fusion activity. The results are shown in Figs. EV3G and EV3H (page 11, lines 8-18) of the revised manuscript. In addition, we induced upregulation of OPA1 in human T cells with M1 (mitochondrial fusion promoter) treatment (PMID: 22907892, 36349989, 31840416) and found that mitochondria elongated in T cells. We have shown the results in Figs. EV3I and J (page 11, lines 18-21) of the revised manuscript.

Regarding the second comment, I am afraid we do not have an ideal model to study human T-cell development in the lab, though it is a very important research question. Furthermore, we mainly focus on maintaining T cell identity, rather than T cell development and differentiation in this study. Therefore, we think the study on "whether BCL11B suppresses mitochondrial fusion via OPA1 during T-cell development and differentiation" should be addressed in a separate study. We have acknowledged the limitations of our current study in the discussion of the revised

manuscript (page 21, lines 10-13).

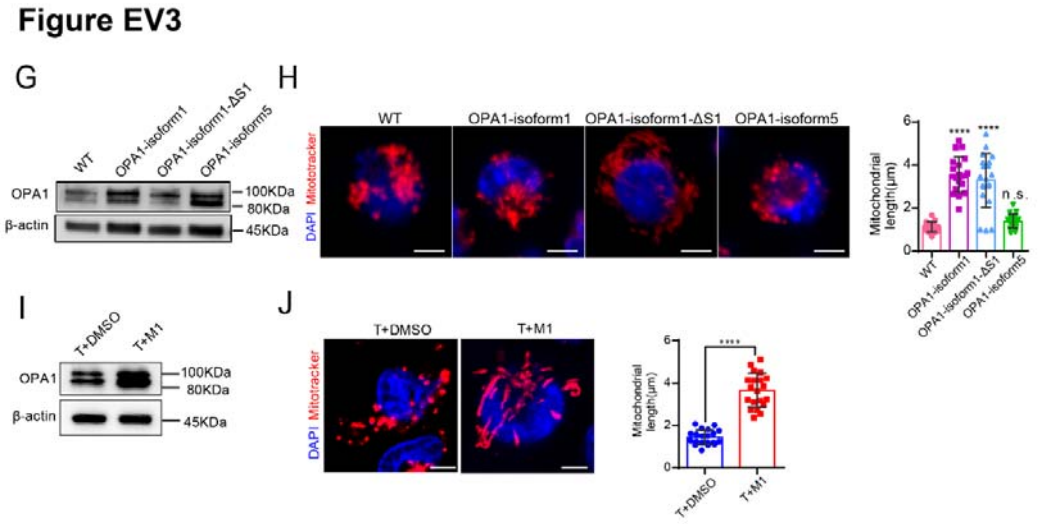

Figure EV3. (G) Representative western blot of OPA1 in T cells with different OPA1 isoforms overexpression. β-actin was used as a control. Isoform 1 is the long form and its mutant S1 is only a single long form of OPA1. Isoform 5 is the short form of OPA1.

(H) Mitochondrial morphology of T cells with different OPA1 isoform overexpression in which the mitochondria (MitoTracker; red) and nuclei (Hoechst; blue) are stained. Scale bars: 5 μm. Each dot represents the mean relative length of the mitochondria in a sample. ****P≤0.0001, one-way ANOVA with Tukey's multiple comparisons test.

(I) Representative western blot of OPA1 in T cells with M1 treatment. β-actin was used as a control.

(J) Mitochondrial morphology of T cells with M1 treatment in which the mitochondria (MitoTracker; red) and nuclei (Hoechst; blue) are stained. Scale bars: 5 μm. Each dot represents the mean relative length of the mitochondria in a sample. ****P≤0.0001, two-tailed paired Student's t-test.

2) The authors observed an upregulation of NK-like cytolytic programs in CD4+ and in CD8+ T cells. With respect to CD4+ T cells: it is know that certain subsets of CD4+ T cells have cytotoxic activity (CD4+ cytotxic T lymphocytes, CD4+ CTLs). Have the authors tested whether Bcl11b/NuRD knockout results in the induction of genes associated with CD4+ CTL subset? This could be easily tested by performing GSEA with CD4 CTL-specific gene lists (e.g. taken from the following publication: PMID: 29352091).

Our response: Thanks for your suggestion. We performed GSEA with the CD4 CTL-specific gene lists from the publication (PMID: 29352091) on the RNA-seq analysis of BCL11B-/-, MBD2-/-, CHD4-/-, or MTA2-/- human CD4+ T cells and

found that the "natural killer cell-mediated cytotoxicity" gene set was enriched in CD4+ BCL11B-/-, MBD2-/-, CHD4-/-, and MTA2-/- human T cells. However, "the CD8 T cells effector" gene set was only enriched in CD4+ MTA2-/- human T cells. We have cited the publication and shown these results in Fig. EV1E (page 7, lines 8-14) of the revised manuscript.

Figure EV1

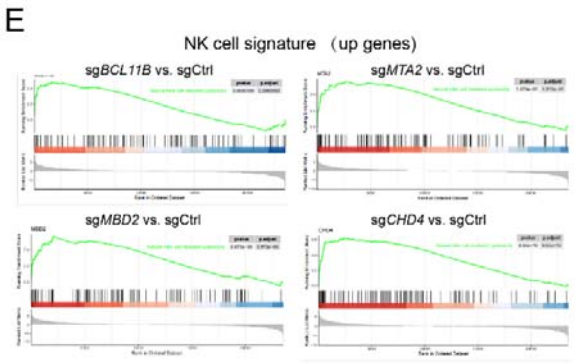

Figure EV1. (E) GSEA enrichment plots for the indicated gene sets in the transcriptome of CD4+ CTL (enrichment plot: NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY; Table EV1). The top portion of the plot shows the running enrichment score (RES) for the gene set as the analysis walks down the ranked list of genes and reflects the degree to which the gene set is overrepresented at the top or bottom of the ranked list of genes. The middle portion of the plot shows where the members of the gene set (indicated as black lines) appear in the ranked list of genes. The bottom portion of the plot shows the value of the ranking metric.

Minor issue:

3) The authors indicate in several figure legends that the results are representative of 3 independent experiments. I assume the cells for the independent experiments were also isolated from independent donors. Perhaps one could mention this.

Our response: We appreciate the suggestion. Yes, the independent experiments were isolated from independent donors. We specified this point in the "Data information" of each figure legend in the revised manuscript, including Figs. 1, 4-5; Figs EV3-5; Appendix Figs. S1-4.

Referee #2:

Summary

Liao et al. investigate mechanisms by which T cells are reprogrammed into induced T-to-NK (ITNK) cells in culture upon deletion of BCL11B. ITNK cells express a T-cell receptor as well as NK-cell surface markers and can lyse NK-cell targets.

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Significance

Several findings are consistent with previous work in mouse models where BCL11B recruits repressor complexes (including NuRD) in T cell development to repress the expression of genes associated with other lineages (including NK-cell genes).

The authors' conclusion that T-cell fate is maintained by BCL11B/NuRD repressing OPA1, resulting in less mitochondrial fusion, less OXPHOS metabolism, and therefore less H3K27Ac at NK-cell genes is more novel, but more evidence is required to support this metabolic-epigenetic axis, especially in physiologically relevant cells. The evidence provided in support of key parts of this conclusion must be strengthened (e.g. BCL11B binding to the OPA1 locus, and upregulation of OPA1 during the loss of T-cell identity during reprogramming). Furthermore, much of the data is from an unusual cell population selected during 10 days culture after deletion of a gene, and it is therefore not possible to conclude that the molecular changes observed in them are the same as those that controlled the fate of physiological T cells 10 days earlier.

Major concerns

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This finding, central to the metabolic-epigenetic axis hypothesis proposed, is only supported by a single, very small, increase in Cut&Tag signal in Fig 2E. This evidence is not sufficient to conclude BCL11B binds the OPA1 locus TSS. The authors must provide statistical analysis that the peak is significant or, better, independent evidence that BCL11B binds the TSS of the OPA1 locus, but not at nearby control regions.

Our response: We thank the reviewer for the critical comments. We have added the peak calling analysis of BCL11B at OPA1 loci in T cells in Figure EV2A and EV2B of the revised manuscript and shown the details of peak information in Dataset EV1.

We have also analyzed the peak from 3kb upstream to downstream of the TSS region and found the BCL11B peaks were significantly enriched at the TSS of OPA1 (Fig. 2F) (page 9, lines 18-21). In addition, to further confirm that BCL11B binds to the TSS of OPA1 loci, rather than the nearby control regions, we performed BCL11B CUT&Tag-qPCR assay using six primer sets for OPA1, targeting the TSS region as well as nearby control regions. The results show that BCL11B bind to the TSS of the OPA1 locus, but not to its nearby control region. We have shown the results in Figs. 3A and EV3A (page 9, lines 21-22; page 10, lines 1-2).

Figure 2F

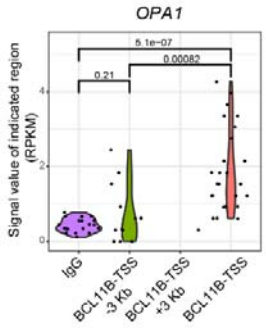


Figure 3A

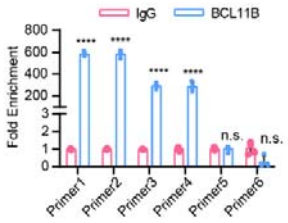


Figure EV3A

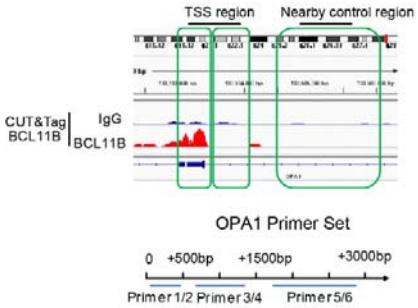


Figure 2. (F) Violin plot of the distribution of BCL11B binding peaks spanning 3kb upstream to downstream of the TSS region of the OPA1 gene.

Figure 3. (A) Quantifying the fold enrichment of OPA1 at different binding sites of BCL11B using CUT&Tag-qPCR. The results represent mean ± SD (N=4 individual healthy donors); **** $P \leq 0.0001$, two-way ANOVA with Sidak's multiple comparisons test.

Figure EV3. (A) Schematic diagram of BCL11B binding sits in the TSS region of OPA1 and OPA1 qPCR primer design strategies. OPA1 qPCR Primers 1 and 2 were designed in the range of 500bp downstream of the OPA1 TSS; primers 3 and 4 were designed in the range of 500bp to 1500bp; and primers 5 and 6 were designed in the nearby control region from 1500bp to 3000bp.

2. Evidence of OPA1 upregulation

OPA1 RNA and protein are upregulated in CD3+NKp46+ ITNKs compared to T cells and NK cells. The authors also conclude that "OPA1 expression increases over time upon BCL11B ablation" in Fig S3A and "MTA2-, MBD2-, and CHD4-deficient ITNKs also showed upregulation of OPA1 protein" in Fig 3C. These conclusions are also central to the metabolic-epigenetic regulation of T-cell fate hypothesis. However, the referred figures don't seem to support this. Better quantitation of OPA1 protein levels after BCL11B or NuRD ablation are required, ideally with multiple replicates, and Western blots images without signal saturation.

Our response: We appreciate the critics. We have repeated the Western blot experiments with three biological replicates and shown improved blot images of Fig. 3D and Fig. 6A (page 10, lines 20-22; page 16, lines 14-20) with statistical analysis in the revised manuscript.

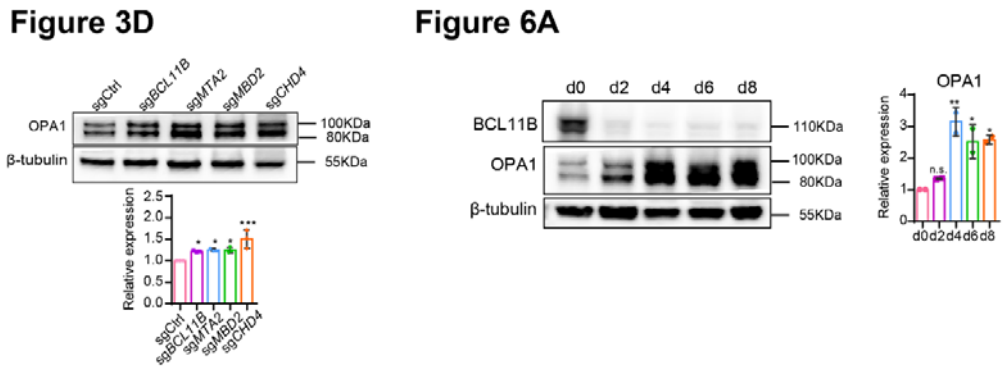


Figure 3. (D) Western blot analysis of OPA1 protein levels in ITNKs that were derived from T cells transduced with sgBCL11B, sgMTA2, sgMBD2 or sgCHD4 and sgCtrl-transduced T cells. The results represent mean ± SD (N=3 individual healthy donors). *P≤0.05 and ***P≤0.001, two-tailed paired Student's t-test.

Figure 6. (A) Representative western blot analysis of BCL11B and OPA1 protein levels in ITNKs from Day 0 to Day 8. The results represent mean ± SD (N=2 individual healthy donors). *P≤0.05 and **P≤0.01, one-way ANOVA with Dunnett's multiple comparisons test.

3. Evidence that metabolic and epigenetic changes contribute to maintaining T-cell fate

The authors conclude that NuRD and BCL11B maintain T-cell fate at least partly

through a metabolic-epigenetic axis. Evidence supporting this is obtained from subpopulations of cells cultured for 10 days after gene deletion - T-cell identity may have been lost much earlier than this time.

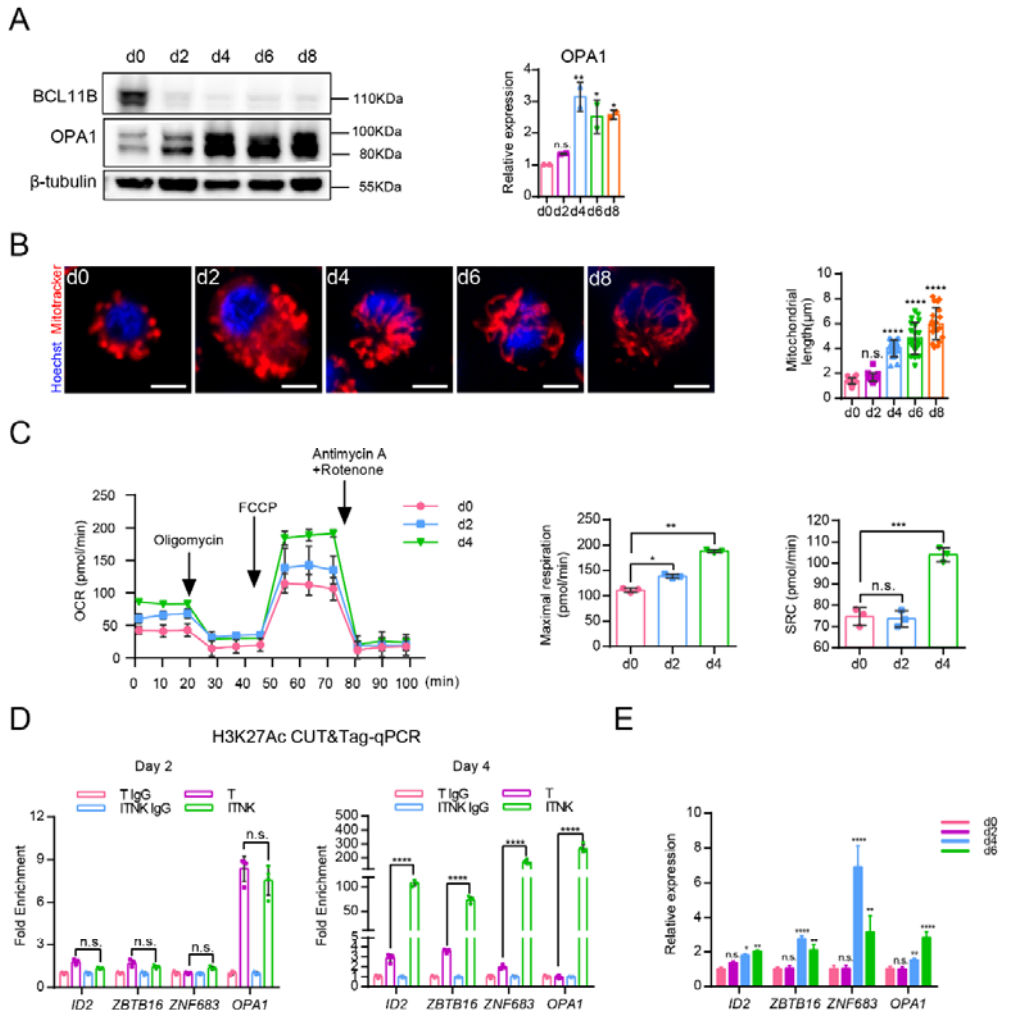
For example: possible BCL11B binding OPA1 locus; OPA1 upregulation; increased mitochondrial length, increased OXPHOS.

For the authors to conclude that these processes contribute to maintaining T-cell fate they must show that they occur prior to or at least concurrently with loss of T-cell identity. Otherwise they cannot rule out they are unrelated events that are associated with the late stages of ITNK differentiation in culture that happen after the loss of T-cell fate. Experiments showing evidence of metabolic and epigenetic changes at multiple timepoints, alongside the loss of T-cell specific mRNA and protein expression, are required.

Our response: We agree with the critics and appreciate the suggestions. Accordingly, we measured the levels of OPA1 protein and observed the morphology of mitochondria in T cells at different time-points post BCL11B ablation to determine when OPA1-mediated mitochondrial fusion occurred. We found a gradual increase of OPA1 expression following BCL11B ablation, with a significant elevation of OPA1 expression on Day 4, along with the occurrence of mitochondrial fusion. The results of the revised manuscript are shown in Fig. 6A and 6B (page 16, lines 14-20). Subsequently, we performed a mitochondrial-stress test by Seahorse on Day 2 and Day 4 post-BCL11B ablation at the early stage of the reprogramming and found that OCR of T cells with BCL11B ablation was significantly improved on Day 4 (Fig. 6C) (page 16, lines 20-22). Therefore, the metabolic changes happened in T cells on Day 4 upon BCL11B ablation. Then, we confirmed whether epigenetic changes and gain of NK-cell lineage fate in these T cells occurred on Day 4 upon BCL11B ablation by measuring the levels of H3K27Ac modification at the loci of OPA1 and NK cell-associated transcription factors (ID2, ZBTB16, and ZNF683) and their expression. Indeed, H3K27Ac CUT&Tag-qPCR showed that H3K27Ac modification at the loci of OPA1 and NK cell-associated transcription factors (ID2, ZBTB16, and ZNF683) was also significantly increased on Day 4 upon BCL11B ablation but not earlier (Fig.

6D) (page 17, lines 5-8). In addition, RT-qPCR analysis shows that the expression of ID2, ZBTB16, and ZNF683 was upregulated on Day 4 but not earlier (Fig. 6E and Appendix Figs. S4A and B) (page 17, lines 8-10). Taken together, these results show that metabolic and epigenetic changes alongside the upregulation of mRNA and protein levels of NK cell markers all started to occur on Day 4 upon BCL11B ablation. Thus, in the revised manuscript, we have modified our original conclusion that BCL11B and NuRD suppress NK cell-potential at least partially through a metabolic-epigenetic axis (page 17, lines 10-14).

Figure 6



Appendix Figure S4

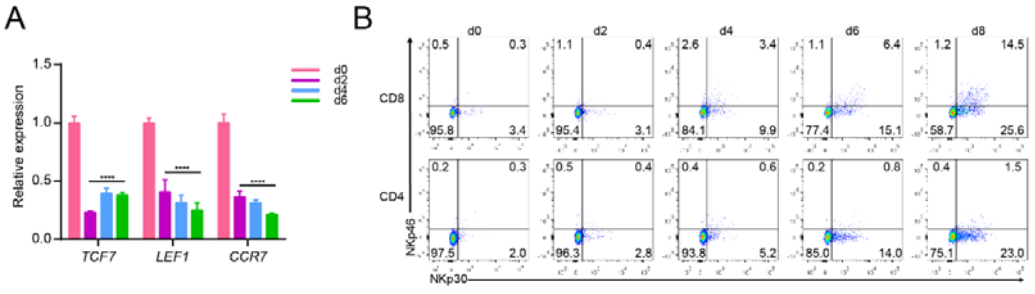


Figure 6. (A) Representative western blot analysis of BCL11B and OPA1 protein levels in ITNKs from Day 0 to Day 8. The results represent mean ± SD (N=2 individual healthy donors). *P≤0.05 and **P≤0.01, one-way ANOVA with Dunnett's multiple comparisons test. (B) Confocal microscopy images showing ITNKs from Day 0 to Day 8 in the reprogramming process in which the mitochondria (MitoTracker; Red) and nuclei (Hoechst; blue) are stained. Scale bars: 5 μm. The images were digitized using ImageJ software. Each dot represents the mean relative length of the

mitochondria in a sample. ****P≤0.0001, one-way ANOVA with Dunnett's multiple comparisons test. (C) Mitochondrial-stress test on Day 2 and Day 4 of ITNKs. Curves represent mean ± SD (N=3 individual healthy donors). *P≤0.05, **P≤0.01 and***p≤0.001, one-way ANOVA with Dunnett's multiple comparisons test. (D) CUT&Tag-qPCR analysis of H3K27Ac enrichment in the promoter regions of indicated genes on Day 2 and Day 4 of ITNKs. The results represent mean ± SD (N=4 individual healthy donors); ****p≤0.0001, two-way ANOVA with Bonferroni's multiple comparisons test. (E) Time-course of NK-related transcription factors and OPA1 expression by RT-qPCR. The results represent mean ± SD (N=4 individual healthy donors); *p≤0.05, **p≤0.01, ***p≤0.001, and ****p≤0.0001, two-way ANOVA with Dunnett's multiple comparisons test.

Appendix Figure S4. (A) Time-course of T cell-related genes expression by RT-qPCR. The results represent mean ± SD (N=4 individual healthy donors); ****p≤0.0001, two-way ANOVA with Dunnett's multiple comparisons test. (B) T cells transduced sgBCL11B (from (Fig. 6A)) were cultured in vitro. The markers (NKp30 and NKp46) of CD4+ and CD8+ ITNKs were detected using flow cytometry at multiple time-points.

4. Incomplete reporting of RNA-seq, Cut&Tag and ATAC-seq data

Excellent experiments to determine the changes to the transcriptome and chromatin in ITNK cells have been performed, but only select parts of the data have been presented.

For RNA-seq, the methods used to generate the gene set enrichment analysis in Figs S2C and S2D must be described in more detail. The authors conclude that "the transcriptional profiles of ITNKs derived from shMTA2-, sgMBD2-, and sgCHD4-transducted T cells were similar to those for BCL11B-deficient ITNKs but not to those of sgCtrl-transduced T cells", but the principal component analysis in Fig 2A does not support this at all, showing that BCL11B-deficient ITNKs and controls T cells are both very different from the NuRD-ablated ITNKs. The authors must correct this and analyse the differences in gene expression between the BCL11B-deficient and NuRD-deficient ITNKs - not just the similarities.

For H3K27me3, H327Ac, BCL11B, and CHD4 Cut&Tag, as well as ATAC-seq a summary of the data should be provided: how many peaks in each data set, where are they in the genome (promoters, enhancers, gene bodies, etc.), and which gene sets are they near. Without this it is impossible to interpret the significance of the handful of loci shown.

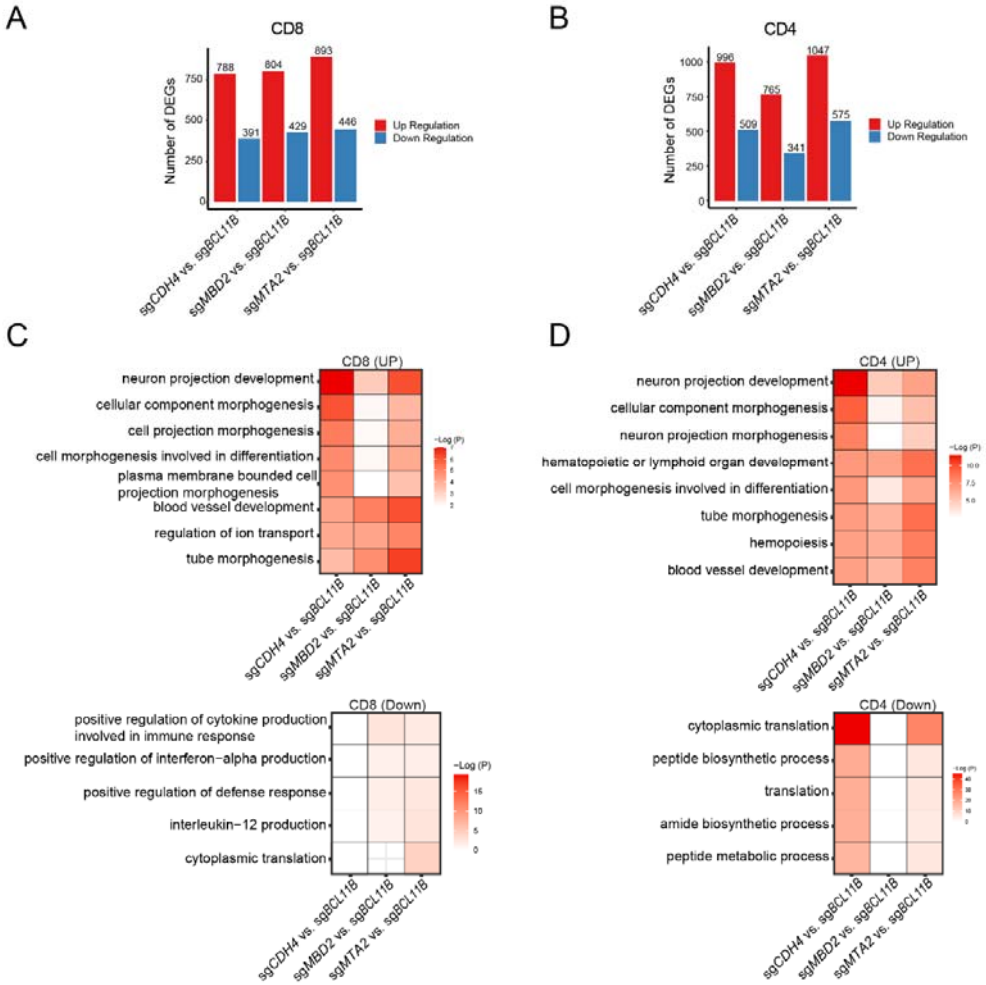
Our response: We apologize for the inadequate presentation of the data analysis. We

have added the detailed methods of set enrichment analysis in methods (page 31, lines 4-20) and figure legends of Figs. EV1C and D (page 54, lines 11-18).

We agree that BCL11B-deficient ITNKs and control T cells are very different from the NuRD-ablated ITNKs. The PCA analysis shows that the expression profiles of sg*MTA2*-, sg*MBD2*-, and sg*CHD4*-transduced ITNKs were similar but different from that of sg*BCL11B*-transduced ITNKs (Fig. 2A and Appendix Figs. S2A and S2B) (page 7, lines 15-17). We then performed GO analysis and found that upregulated genes in MTA2-, MBD2-, and CHD4-deficient ITNKs were mainly involved in cellular morphogenesis pathways, while downregulated genes were enriched in immune responses and biosynthetic processes, compared with BCL11B-deficient ITNKs. We have added these analyses on the differences between NuRD-ablated ITNKs and BCL11B-deficient ITNKs (Appendix Figs. S2C and D) (page 7, lines 17-21) and deleted the previous statement in the revised manuscript.

Of note, we reanalyzed the H3K27Ac CUT&Tag data and observed a significant upregulation of H3K27Ac modification in gene sets associated with T cell activation, NK cell-mediated immunity, and oxidative phosphorylation in the sg*BCL11B* and sgNuRD groups compared to the sgCtrl group, which is consistent with the transcriptome data. These results were added in Figs. EV2D-F (page 8, lines 10-11 and page 9, lines15-17). In addition, we have provided a summary of each CUT&Tag- and ATAC-seq data in Dataset EV1, including the number of peaks, enriched regions, and associated genes in Figs. EV2A and B of the revised manuscript.

Appendix Figure S2



Appendix Figure S2. (A) Bar chart indicating the numbers of up- and down-regulated genes from differentially expressed genes (DEGs) between NuRD-subunit-deficient CD8+ ITNKs and CD8+ BCL11B-deficient ITNKs. Gene differential expression analysis was performed by R package DESeq2 (version 1.38.1). The number of differentially expressed genes was counted as visualized by customed R scripts using ggplot2 (version 3.4.0). (B) Bar chart indicating the numbers of up- and down-regulated genes from DEGs between NuRD-subunit-deficient CD4+NKp30+ ITNKs and CD4+NKp30+ BCL11B-deficient ITNKs. (C) Gene ontology analysis of up- and down-regulated genes from DEGs between NuRD-subunit-deficient CD8+NKp30+ ITNKs and BCL11B-deficient CD8+NKp30+ ITNKs. GO enrichment analysis was performed by R package clusterprofile (version 4.6.2). The result was visualized as heatmaps generated by customed R scripts using ggplot2 (version 3.4.0). (D) Gene ontology analysis of up- and down-regulated genes from DEGs between NuRD-subunit-deficient CD4+NKp30+ ITNKs and BCL11B-deficient CD4+NKp30+ ITNKs.

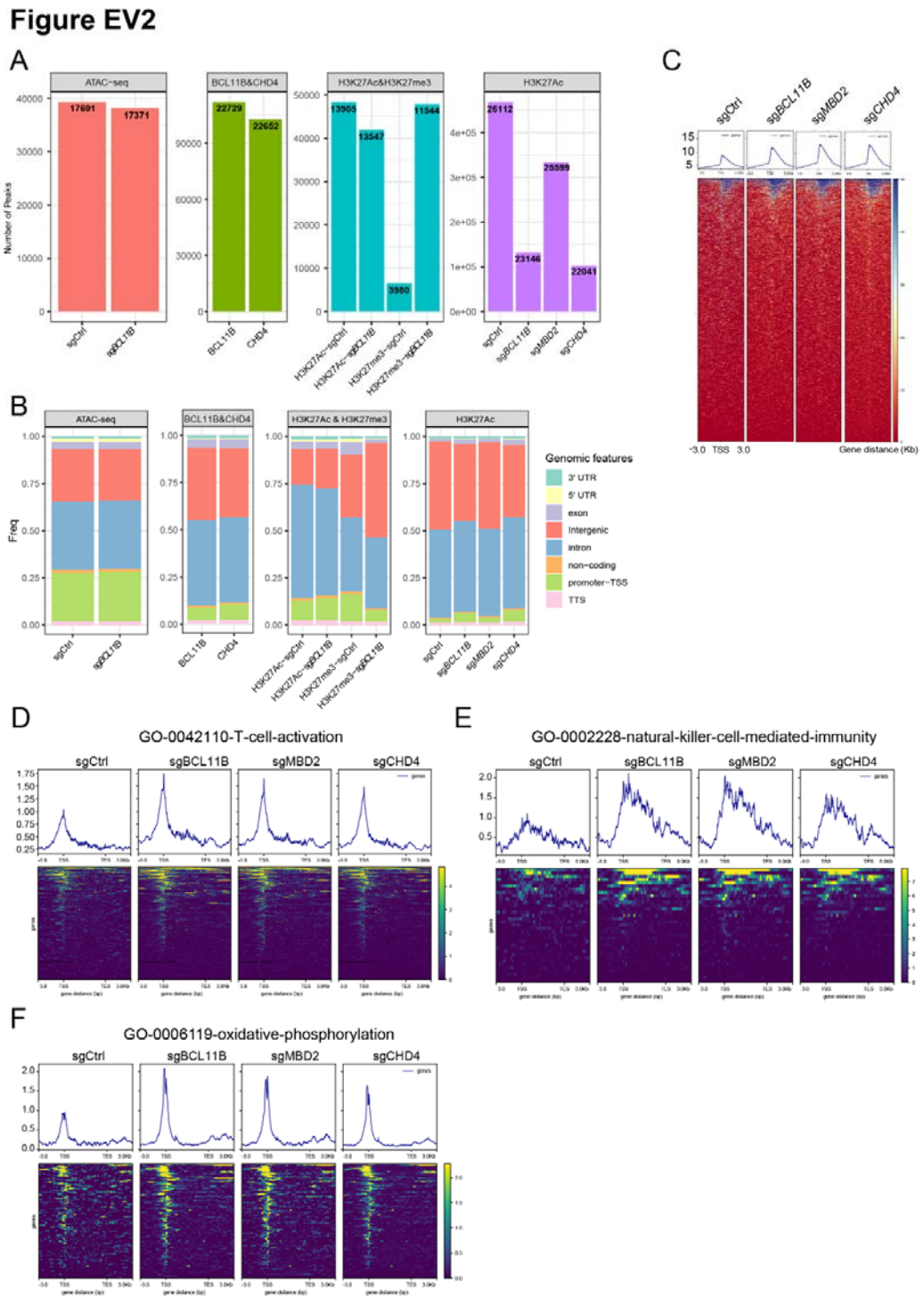


Figure EV2. (A) Bar chart indicating the number of peaks and the number of genes at the peak position in these omics-seq. (B) Bar chart demonstrating the percentage of peaking with a TSS, TSS-promoter, transcription end site (TES), and other sites in these omics-seq. (C) Tag density pileups of H3K27Ac peaks in CD8+ T cells transduced with sgBCL11B, sgMBD2, or sgCHD4 and CD8+ sgCtrl-transduced T cells. Two individual healthy donors were used for the CUT&Tag assay. (D) Tag density pileups of H3K27Ac peaks at the T-cell-activation gene set (see Table EV2)

in CD8+ T cells transduced with sgBCL11B, sgMBD2, or sgCHD4 and CD8+ sgCtrl-transduced T cells. (E) Tag density pileups of H3K27Ac peaks at the natural-killer-cell-mediated-immunity gene set (see Table EV3) in CD8+ T cells transduced with sgBCL11B, sgMBD2, or sgCHD4 and CD8+ sgCtrl-transduced T cells. (F) Tag density pileups of H3K27Ac peaks at the oxidative-phosphorylation gene set (see Table EV4) in CD8+ T cells transduced with sgBCL11B, sgMBD2, or sgCHD4 and CD8+ sgCtrl-transduced T cells.

Minor concerns

5. Experiments use data from slightly different cell types thoughout, depending on whether a sorted population was used, and which surface markers. It would make the manuscript much easier to read and interpret if the source of cells used in an experiment was indicated clearly in each figure panel.

Our response: We appreciate the advice. We have stated the source of cells clearly in each figure panel of Figs. 2D-E and Figs. EV4D-F in the revised manuscript.

Referee #3:

In this study, Liao et al. performed CRISPR knockouts of BCL11B and NuRD subunits in T cells to investigate the importance of the interaction between these components for the acquisition of NK cell-like attributes in these cells. Data from these experiments confirm interactions between BCL11B and NuRD subunits and show that knockout of NuRD subunits generally phenocopy results seen with BCL11B knockout with respect to NK cell signature gene expression and cytotoxic function against K562 cells. During their investigation, the authors noted elevated expression of genes associated with metabolism in BCL11B- and NuRD subunit-deficient T cells. They also observed binding of BCL11b within the OPA1 locus in T cells. The authors then showed that ITNK cells had higher levels of OPA1 (particularly the long isoform) relative to T cells and NK cells. This correlated with mitochondrial length and measures of OXPHOS. Further experiments showed that knockout of OPA1 along with BCL11B blunted the conversion of T cells into ITNK cells, and this could be rescued through acetate supplementation. Overall, the findings

connecting BCL11B, OPA1, and OXPHOS in this manuscript are of interest and provide potentially important new insights into the nature of ITNK cells, which may have some therapeutic relevance. There are a additional points that should be addressed to strengthen this manuscript for publication.

Major comments:

1. In Figure 3A, B the authors provide data showing the relative amounts of OPA1 mRNA and protein in T cells, NK cells, and ITNK cells. It would be of interest to compare the levels of BCL11B mRNA and protein in these cells alongside this data to determine to what extent there is a correlation between BCL11B levels and OPA1 levels. This is particularly important for the comparison between NK cells and T cells. It appears that T cells and NK cells have similar levels of OPA1 mRNA, but T cells have significantly more short OPA1 protein. It's not clear what accounts for this disconnect. Also, if NK cells have significantly lower amounts of Bcl11b protein, the model put forth by the authors would suggest that NK cells should have higher levels of OPA1, which isn't evident in the qRT-PCR or Western blot data.

Our response: We appreciate these critics. Accordingly, we now show the mRNA and protein levels of BCL11B and OPA1 in T cells, ITNKs, and NK cells in Figs. EV3C and D (page 10, lines 6-15) of the revised manuscript. Although BCL11B protein level in NK cells was lower than that in T cells, the levels of OPA1 mRNA and protein in NK cells were similar to that in T cells, suggesting that the transcription of OPA1 may be regulated by other factors, but not BCL11B in NK cells. Further investigation is warranted to reveal how OPA1 transcription is regulated in NK cells in a separate study. We have acknowledged the limitations of our current study in the discussion of the revised manuscript (page 21, lines 14-18).

It is interesting to notice that NK cells expressed a lower amount of short OPA1 protein than T cells, though they had similar levels of OPA1 mRNA. The disconnect of short OPA1 protein is possibly due to lower translation efficiency of the short form OPA1 in NK cells, or accelerated protein degradation following translation (PMID:

20038678). As short OPA1 promotes mitochondrial fragmentation (PMID: 16778770), lower amount of short OPA1 may result in elongated mitochondria in NK cells. In addition, we found that levels of Drp1^{pS616}, which promotes Drp1-mediated mitochondrial fission (PMID: 324335447, 31636463), were lower in NK cells than in T cells (Fig. EV3E of the revised manuscript) (page 10, lines 19-20). This could be another reason why the mitochondria in NK cells were longer than that in T cells. We have explained the disconnect in the discussion of the revised manuscript (page 19, lines 3-12).

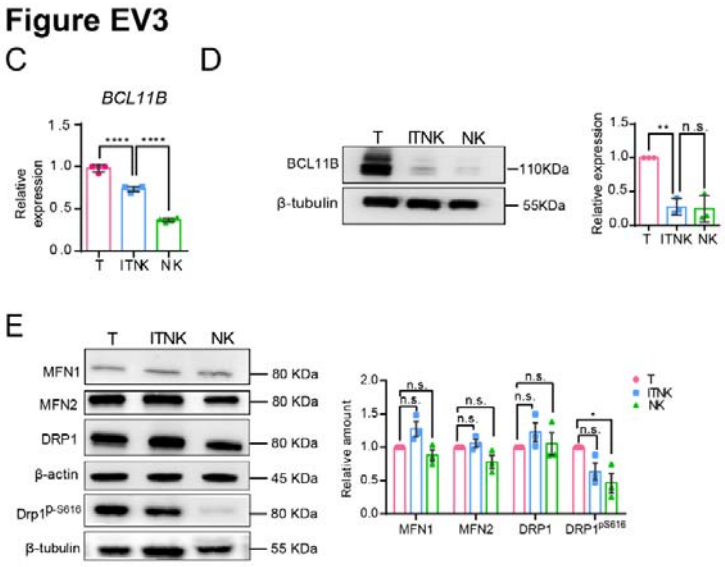


Figure EV3. (C) Relative mRNA levels of BCL11B in samples of human T cells, ITNKs (CD3+NKp46+), and NK cells (CD3-CD56+) based on quantitative RT-PCR. The results represent mean ± SD (N=3 individual healthy donors). ****P≤0.0001, Data were analyzed by two-tailed paired Student's t-test. (D) Western blot analysis of BCL11B levels in samples of human T cells, ITNKs (CD3+NKp46+), and NK cells (CD3-CD56+). Graph summarizing the relative protein levels of BCL11B, the results represent mean ± SD (N=3 individual healthy donors). **P≤0.01, two-tailed paired Student's t-test. (E) Western blot analysis of mitochondrial fusion (MFN1, MFN2) and fission (DRP1, DRP1^{pS616}) protein levels in samples of PBMC-derived T cells, ITNKs (CD3+NKp46+), and NK cells (CD3-CD56+). A graph summarizing the relative protein levels of MFN1, MFN2, DRP1, and DRP1^{pS616} in ITNKs compared to T cells is shown. The results represent three individual healthy donors.

2. In Figure 5A, the authors show that BCL11B knockout in T cells results in significant elevations in acetyl-CoA, which is lost when OPA1 is also knocked out. It is not clear why OPA1 knockout would have such a major effect on acetyl-CoA levels.

The authors should at least address this in the discussion. I may have missed it, but it is not clear either in the figure legend or methods how acetyl-CoA levels were being measured. Was it total protein, histones, or concentrations of this molecule in cells? This needs to be clarified.

Our response: We appreciate the suggestions. To reveal how OPA1 knockout has a major effect on acetyl-CoA levels, we re-analyzed the bulk RNA-seq data of ITNKs and T cells from our previous study (PMID: 35331335) and found that the tricarboxylic acid (TCA) and fatty acid oxidation (FAO) processes-related genes were upregulated in ITNKs, compared to T cells. We now show this analysis in Fig. EV3B (page 10, lines 4-6) of the revised manuscript. Previous studies demonstrated that mitochondrial fusion favors TCA and FAO (PMID: 36171294; 27293185). In addition, Acetyl-CoA and α-KG are key metabolites in the TCA cycle and participate in the regulation of histone modification (PMID: 26440431, 31534224). We have found that the levels of Acetyl-CoA but not α-KG were increased in ITNKs, compared to T cells (Fig. EV5B of the revised manuscript) (page 14, lines 19-22 and page 15, line 1). Consistently, ITNKs expressed OPA1 at higher levels than T cells (Fig. 3C). Taken together, it is likely that OPA1 increases Acetyl-CoA levels through promoting mitochondrial fusion and TCA during ITNK reprogramming. Therefore, loss of OPA1 decreased Acetyl-CoA levels in ITNKs (Fig. 5A). Consistently, a recent publication demonstrates that Th17 cells have fused mitochondria and require OPA1 for its control of the TCA cycle to regulate effector programs (PMID: 36171294). We have shown the results in the revised manuscript and provide possible mechanisms how OPA1 knockout has a major effect on acetyl-CoA levels in the discussion part of the revised manuscript (page 19, lines 15-22 and page 20, lines 1-9).

We measured the Acetyl-CoA and α-KG levels in ITNKs and T cells by ELISA. We have added this information in the methods section of the revised manuscript (page 27, lines 3-21). We understand that a specific source of acetyl-CoA needs to be determined by isotope-based metabolic flux analysis. We are sorry that we do not have the facility to perform this experiment and have admitted the limitations in the discussion of the revised manuscript (page 20, lines 8-9).

Figure EV3B

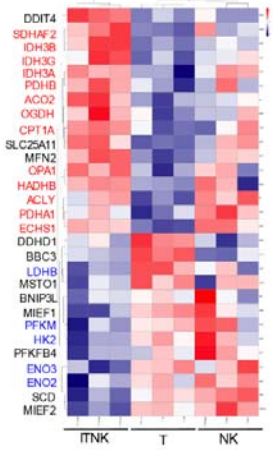


Figure EV5B

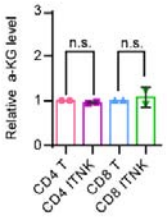


Figure EV3. (B) The heatmap shows OPA1 and those transcripts related to TCA and FAO processes upregulated in ITNKs (N = 3 individual donors). Cutoff: absolute log2 (fold change) ≥ 1; adjusted P value ≤0.05.

Figure EV5. (B) Relative α-KG levels in CD4+ (left) and CD8+ (right) ITNKs on day 10 that were reprogrammed from T cells transduced with sgBCL11B and sgCtrl-transduced T cells. Two-tailed paired Student's t-test. The data are presented as the mean ± SD (N=2 individual healthy donors).

3. In the model proposed in Figure 6, it seems as though the authors suggest that ITNK cells have elevated TCA cycle activity that can generate more acetyl-CoA for histone modification at NK cell-related genes. However, the authors don't provide data to show this. The authors should acknowledge the limitations of their current study and gaps in knowledge that still exist.

Our response: We appreciate the critics. We have replaced the "TCA, FAO" with "OXPHOS" in Figure 7 and acknowledged the limitations of our current study in the discussion of the revised manuscript (page 19, lines 15-22 and page 20, lines 1-9).

Figure 7

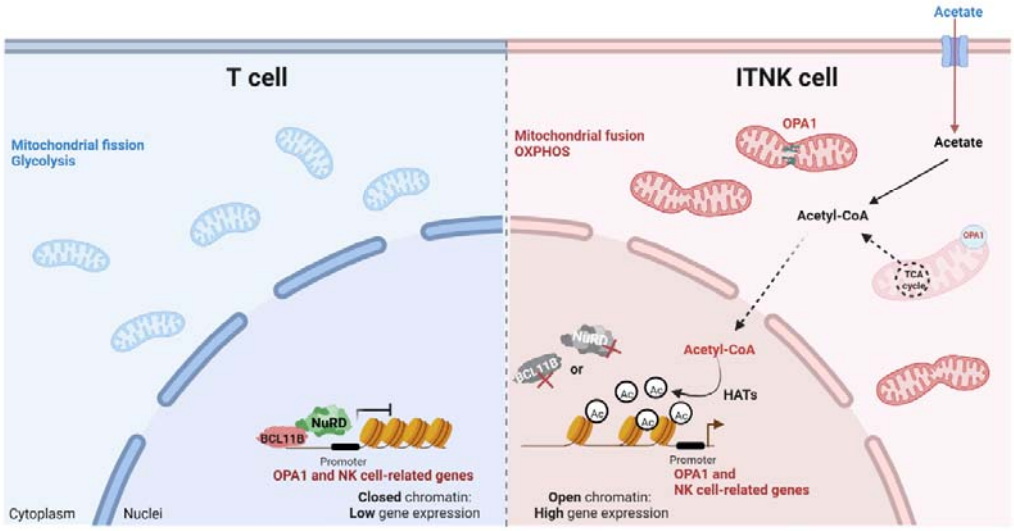


Fig. 7. Schematic representation of the mechanism by which BCL11B and the NuRD complex inhibit the expression of OPA1 and NK-cell-associated genes in human T cells (left). OPA1-mediated mitochondrial fusion regulated by the BCL11B-NuRD complex promotes the reprogramming from T cells into ITNKs by regulating H3K27Ac modification (right). The graphics were created with BioRender.com.

1st Revision - Editorial Decision **1st Aug 2023**

Dear Prof. Li,

Congratulations on a great revision! Overall, the referees have been positive. However, referee 3 has a few minor concerns which should be addressed in a new revision.

When you submit your revised version, please also take care of the following editorial items and add this also to your point-by-point response:

1. Please upload the following grant information into our online system: 2017YFE0131600 (Y.L.), 2019YFA0111500 (X.L.); 82273377(L.S), 82202031(Q.L.); Guangdong Natural Science Foundation, No. 2022A1515012569 (J.Z.), 2022A1515012484 (L.S.), 2021A1515220077 (L.S.), 2021A1515110005 (Q.L.), 2022A1515012360 (Q.L.), 2019A1515010062 (Y.Y.), 2022A1515010604 (Y.Y), 2020B1212060052; Science and Technology Program of Guangzhou, No. 202002020083 (X.L.), 202102080470 (Y.Y.); Open project of State Key Laboratory of Respiratory Disease, SKLRD-OP-202002 (Z.Z.). Partially supported by a grant from the University Grants Committee / Research Grants Council of the Hong Kong Special Administrative Region, China (Project No. AoE/M-401/20), Innovation and Technology Fund (ITF).

2. Please review our new policy on conflict of interests on the EMBO author guide website and update the title of this section to: Disclosure and competing interests statement

3. Please remove the author contribution section from the main manuscript file.

4. We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper. 12. We also need a summary figure for the synopsis. The size should be 550 wide by 200-440 high (pixels). You can also use something from the figures if that is easier.

5. For appendix figure S3A - BCL11B. The western blot is cropped too closely, please re-crop this figure.

6. Our publisher has also done their pre-publication check on your manuscript. Please take a look at the attached word file and the comments regarding the figure legends and respond to the issues. For example in Figure 6A and EV5B, we do not allow statistics to be performed on N=2 data.

7. Please upload Tables 1-3 and EV tables individually instead of zipped together.

8. Please visit our website and correct the section order of the main manuscript accordingly.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Kind regards,

Kelly

Kelly M Anderson, PhD
Editor
The EMBO Journal
k.anderson@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Use the link below to submit your revision:
https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors responded properly to my comments.

Referee #2:

The authors have provided a substantial amount of new data in this revised manuscript, which has significantly improved it. Experiments and analyses effectively addressing all of my comments on the first submission have been included.

I am satisfied that the inclusion of these new data support the authors' conclusion that the induction of ITNK cells from T cells by BCL11B deletion is associated with OPA1-dependent changes in mitochondria and metabolism that promote ITNK development and function.

In my view, given the novelty and significance of this finding for understanding the biology of these cells, which have already entered clinical trials, this manuscript should be published in EMBO J.

A couple of minor errors I noticed in the revised manuscript:

The new figure 2F appears to be missing data in the third column of the plot.

Page 7, lines 6-10: Fig 2B does not show NK-cell-associated genes. These venn diagrams show differentially expressed gene.

Referee #3:

The critiques have been thoughtfully addressed by the authors and important new data has been added to increase definiteness and clarity. The manuscript is improved. Suggest accept.

Response Letter to The EMBO Journal Submission

Paper ID: EMBOJ-2023-113448R

Paper Title: BCL11B and the NuRD complex cooperatively guard T-cell fate and inhibit OPA1-mediated mitochondrial fusion in T cells

Editorial items

1. Please upload the following grant information into our online system: 2017YFE0131600 (Y.L.), 2019YFA0111500 (X.L.); 82273377(L.S), 82202031(Q.L.); Guangdong Natural Science Foundation, No. 2022A1515012569 (J.Z.), 2022A1515012484 (L.S.), 2021A1515220077 (L.S.), 2021A1515110005 (Q.L.), 2022A1515012360 (Q.L.), 2019A1515010062 (Y.Y.), 2022A1515010604 (Y.Y), 2020B1212060052; Science and Technology Program of Guangzhou, No. 202002020083 (X.L.), 202102080470 (Y.Y.); Open project of State Key Laboratory of Respiratory Disease, SKLRD-OP-202002 (Z.Z.). Partially supported by a grant from the University Grants Committee / Research Grants Council of the Hong Kong Special Administrative Region, China (Project No. AoE/M-401/20), Innovation and Technology Fund (ITF).
Our response: We have uploaded the grant information in the online system.

2. Please review our new policy on conflict of interests on the EMBO author guide website and update the title of this section to: Disclosure and competing interests statement
Our response: We have revised the title of this section. Please find it on page 33, line 14 (Manuscript Text.docx).

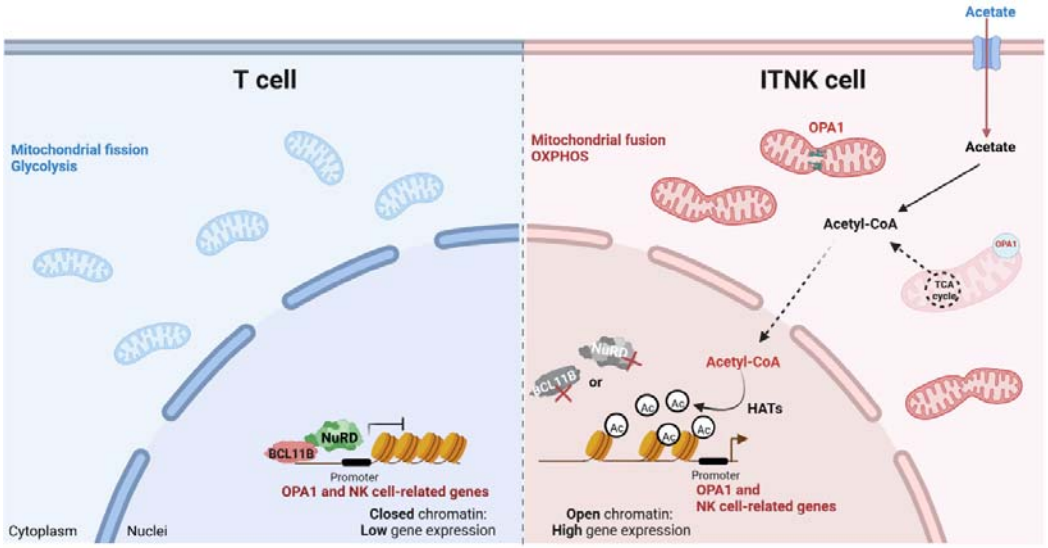
3. Please remove the author contribution section from the main manuscript file.
Our response: We have removed the author contribution section.

4. We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper. 12. We also need a summary figure for the synopsis. The size should be 550 wide by 200-440 high (pixels). You can also use something from the figures if that is easier.
Our response: Please find the bullet points and graphical abstract below. The graphical abstract (550 w * 300 h pixels) also have been uploaded into the online system.

Highlights:

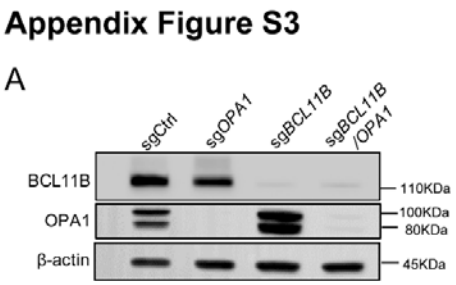
1. T cells are reprogrammed into ITNKs upon deletion of MTA2, MBD2, or CHD4;
2. BCL11B associates with the NuRD complex to repress OPA1 expression in T cells;
3. OPA1-mediated mitochondrial fusion facilitates ITNK reprogramming and cytotoxicity via increasing OXPHOS;
4. Acetyl-CoA elevated by OXPHOS promotes NK-cell-associated genes expression and antitumor effects in ITNKs by regulating the acetylation of H3K27.

Graphical Abstract:



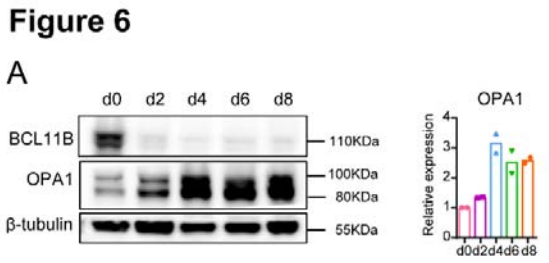
5. For appendix figure S3A - BCL11B. The western blot is cropped too closely, please re-crop this figure.

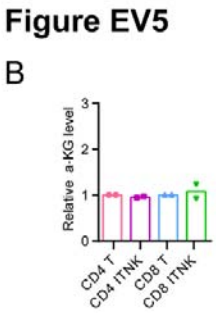
Our response: We have replaced the western blot photo of appendix figure S3A - BCL11B.



6. Our publisher has also done their pre-publication check on your manuscript. Please take a look at the attached word file and the comments regarding the figure legends and respond to the issues. For example in Figure 6A and EV5B, we do not allow statistics to be performed on N=2 data.

Our response: We have removed the statistics of the revised Figure 6A and EV5B. Besides, we have added the information of " sample size " in the figure legends and replied to the comments (page 41, lines 11-15; page 42, lines 16 and 25-26; page 44, lines 22-23; page 49, lines 3, 9, 17 and 21) (Manuscript Text.docx).





7. Please upload Tables 1-3 and EV tables individually instead of zipped together.
Our response: We have uploaded each of the Tables and EV Tables respectively.

8. Please visit our website and correct the section order of the main manuscript accordingly.
Our response: We have re-ordered the main manuscript according to the journal policy.

could you in this case also update the Dataset EV1 file and add a short legend with a short description of the dataset? Please also add a callout to Dataset EV1 in the manuscript text.
Our response: We have added a short description of Dataset EV1 in a readme document and the callouts to the revised manuscript (page 8, line 4; page 9, line 4; page 9; line 22) (Manuscript Text.docx).

Reviewers' comments

Referee #1:

The authors responded properly to my comments.
Our response: Thank you for your positive comment on our work and help us strengthen this research.

Referee #2:

The authors have provided a substantial amount of new data in this revised manuscript, which has significantly improved it. Experiments and analyses effectively addressing all of my comments on the first submission have been included.

I am satisfied that the inclusion of these new data support the authors' conclusion that the induction of ITNK cells from T cells by BCL11B deletion is associated with OPA1-dependent changes in mitochondria and metabolism that promote ITNK development and function.

In my view, given the novelty and significance of this finding for understanding the biology of these cells, which have already entered clinical trials, this manuscript should be published in EMBO J.
Our response: Thank you for your valuable comments and suggestion that has helped us

strengthen our research and motivated us to strive for higher quality.

A couple of minor errors I noticed in the revised manuscript:

The new figure 2F appears to be missing data in the third column of the plot.
Our response: Thank you for the comment. We checked the figure 2F, and the third column is indeed empty because no reads was enriched at 3kb downstream of the TSS region of the OPA1. Please find the details of reads that are enriched at different regions of OPA1, as depicted in Figure 2F of the source data.

Page 7, lines 6-10: Fig 2B does not show NK-cell-associated genes. These venn diagrams show differentially expressed gene.
Our response: We apologize for the mistake and appreciate your comment. Yes, the veen diagrams show the differentially expressed genes, not the NK-cell-related genes. We have corrected it in the revised manuscript (page 7, lines 1-2) (Manuscript Text.docx).

Referee #3:

The critiques have been thoughtfully addressed by the authors and important new data has been added to increase definiteness and clarity. The manuscript is improved. Suggest accept.
Our response: Thank you for your valuable comments that has helped us identify the limitations of our research and further strengthen its inherent value.

2nd Revision - Editorial Decision **17th Aug 2023**

Dear Peng,

Congratulations on an excellent manuscript, I'm pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. It has been a pleasure to work with you to get this to the acceptance stage.

I will begin the final checks on your manuscript before submitting to the publisher next week. Once at the publisher, it will take about 3 weeks for your manuscript to be published online. As a reminder, the entire review process, including referee concerns and your point-by-point response, will be available to readers.

I will be in touch throughout the final editorial process until publication. In the meantime, I hope you find time to celebrate!

Kind regards,
Kelly

Kelly M Anderson, PhD
Editor, The EMBO Journal
k.anderson@embojournal.org

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Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 χ^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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Table 1

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Table1, 2, 3

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

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Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends, Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends, Materials and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends, Materials and Methods
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends, Materials and Methods

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Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
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Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
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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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Figure legends, References