Reviewer #2 did ask about RNAseq for KO (dLckCre+) vs control. I believe that data is in P435-2, and I wonder if we can make some quick and focused queries. The reply is in this thread because it’s the last I have in the conversation about P435-2. This is my understanding to date:

* Find The WT vs KO figures from P435-2

 \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

- DYRK1A expression by RNAseq does not appear different by looking at exons individually or in aggregate, although counts are low so this is hard to assess by this method. I will look to see if we have or can generate qPCR data to complement (eg maybe more signal if we can exclude a poorly deleted sample)

THE: Alex Hu and I reviewed the P435-2 data with IGV and confirmed Alex’s original findings that the DYRK1A expression does not appear different when looking at the exons.

 \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

- broad testing of harmine signatures suggest that harmine-upregulated genes are up in DYRK1A KO (I presume this is 5h Th17 stim harmine vs no-harmine, compared to 5h Th17 stim, KO vs WT)

THE: In P435-2, there is stim at 5h and no stim at 0h.

* Venn diagram + .csv of (5h Th17 stim harmine vs noHar) & (5h Th17 stim KO vs WT)
  + All DGE
  + Up in Harmine / Up in KO
  + Up in Harmine / Down in KO
  + Up in noHar / Up in WT
  + Up in noHar / Down in WT

There is no overlap. There are only 3 DGEs in 5h Th17 stim KO vs WT

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

- In the manuscript, there are 3 Th17 signatures we focused on.

The Th17 signature (4185 genes, Th17 5h vs 0h)

(NoHar5hVsNaive\_HarVsNoHar5h\_intersections, NoHar5hVsNaive\_unique)

The harmine-dysregulated Th17 signature (482 genes)

(NoHar5hVsNaive\_HarVsNoHar5h\_intersections, HarVsNoHar5h\_NoHar5hVsNaive\_intersection)

The harmine-counter-regulated Th17 signature (272 genes).

(NoHar5hVsNaive\_HarVsNoHar5h\_intersection\_counterregulated\_bySign)

I propose we ask how these signatures are dysregulated in KO vs WT @ 5h stim.

Load these gene signatures into P435-2 analysis script

Label these genes on volcano plots

Do GSEA with Roast on the 5h KO vs WT, show volcano plots

P435-1\_HarDysregTh17Sig\_in\_Dyrk1aKO\_stim.csv

Dyrk1aKOminWT\_gsea\_highlight\_p435\_1HarDysregTh17Sig.svg

P435-1\_Th17Signature\_in\_Dyrk1aKO\_stim.csv

Dyrk1aKOminWT\_gsea\_highlight\_p435\_1Th17Sig.svg

P435-1\_CounterRegGenes\_in\_Dyrk1aKO\_stim.csv

Dyrk1aKOminWT\_gsea\_highlight\_p435\_1\_counterRegGenes.svg

$`counterRegGenes Dyrk1aKO\_stim`

NGenes PropDown PropUp Direction PValue FDR PValue.Mixed FDR.Mixed

Set1 242 0.1487603 0.1694215 Up 0.3562644 0.7543817 0.00069993 0.00539946

$`Th17Signature Dyrk1aKO\_stim`

NGenes PropDown PropUp Direction PValue FDR PValue.Mixed FDR.Mixed

Set1 875 0.1097143 0.104 Up 0.840216 0.9482802 0.02659734 0.04517548

$`HarDysregTh17Sig Dyrk1aKO\_stim`

NGenes PropDown PropUp Direction PValue FDR PValue.Mixed FDR.Mixed

Set1 3439 0.1049724 0.1285257 Up 0.5155484 0.7543817 0.01509849 0.03577142

counterRegGenes

NoHar5hVsNaive\_HarVsNoHar5h\_intersection\_counterregulated\_bySign.csv

Th17Signature

Har5hVsNaive\_NoHar5hVsNaive\_intersections.csv

DGE: Har5hVsNaive

HarDysregTh17Sig

Har5hVsNaive\_NoHar5hVsNaive\_intersections.csv

DGE: Har5hVsNaive + NoHar5hVsNaive

 \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

- I’d also like to address Th17-related cytokines in P435-1 and P435-2. Specifically, I’d like to compare Harmine vs control at 4d, and KO vs WT at 4d, to look at IL-17 (will RNAseq distinguish between the isoforms?), IL-21 and IL-22 at least.

Note: we only have 0h and 5h timepoints in the P435-2 KO/WT data

HarVsNoHar96h = (stim\_10uM\_Harmine\_96h) - (stim\_none\_96h)

Har96hVsNaive = stim\_10uM\_Harmine\_96h - no\_stim\_none\_0

NoHar96hVsNaive = stim\_none\_96h - no\_stim\_none\_0

P435\_2\_Dyrk1aKOminWT\_stim.csv

P435\_2\_Dyrk1aKOminWT\_nostim.csv

* IL-17 (A, B, C, D, E, F), IL-17 Receptor, IL-21, IL-22, IL-23, IL-1beta, IL-6, TGF-beta, TNF-beta
* IFN-gamma, IL-10, IL-4, IL-12, IL-33, IL-2, IL-9
* RORalpha, RORgammat ?
* Label all of these genes on both DGEs
* Run ROAST with the top bullet point as a module
* Ask Bernard for an explicit gene list.

Reviewer #1 (Comments for the Author (Required)):   
  
This is an interesting manuscript that documents the role of DYRK1A in regulating Th17 differentiation and function. Previous work from this group demonstrated that blocking DYRK1A with the plant alkaloid harmine inhibited Th17 differentiation, however harmine is not specific to DYRK1A hence this study was performed using a small molecule inhibitor that exhibits greater specificity for DYRK1A, as well as T cell specific DYRK1A deficient mice.   
  
The authors do not clearly articulate how Th17 cells are identified. Is this based on IL-17A staining or RORgt staining? This is necessary information for the conclusion of the manuscript. If the authors utilize RORgt to identify Th17 cells, a measurement of IL-17A production should also be provided.   
  
There is only one representative flow cytometry plot for gp130 in Figure 4. The authors need to show representative staining of other staining parameters that are used to make major conclusions, even if this is in supplemental data.   
  
The authors aim to connect DYRK1A activity to gp130 levels and STAT3 phosphorylation after IL-6 signaling. The majority of the data in figure 4 utilizes DYRK1A knockout cells to conclude that in the absence of DYRK1A activity there is lower gp130 expression and reduced STAT3 phosphorylation after IL-6 stimulation, and this is fine. However there is one panel that demonstrates increased gp130 expression on cells that overexpress DYRK1A, as well as one panel to show increased pSTAT3 after IL-6 stimulation of cells from individuals with Down Syndrome. It would be nice if the reciprocal experiments could be performed: pSTAT3 levels in DYRK1A BAC CD4 T cells and gp130 levels on CD4 T cells from Down Syndrome individuals. This is not a major critique.   
  
  
Reviewer #2 (Comments for the Author (Required)):   
  
The manuscript "Defining a novel DYRK1A-gp130/IL-6R-pSTAT axis that regulates Th17 differentiation" added novel mechanistic insight in cytokine signaling-mediated Th17 cell differentiation, which is intriguing. However, there are several concerns on this manuscript:   
  
Major:   
1. Due to the side effects and off-target effects, the inhibitors are not necessarily target only the kinase DYRK1A. For instance, the authors showed that LCTB better inhibited Th17 differentiation than the conditional KO, although this may be caused by insufficient deletion of the gene. Therefore, the transcriptome analysis in Figure 2 using an inhibitor cannot be attribute to merely DYRK1A function. Similar studies should also use KO vs WT cells.   
2. The efficiency of conditional knockout Dyrk1a was not shown. It can be detected by western blot or RT-PCR in T cells. LCK-Cre mediates the deletion in thymus with a high efficiency in many published studies. Data shown in Fig. 1F suggest WT but not Dyrk1a-deificient cells may overgrowth during differentiation. If this is true, introduction of a floxed-stop Rosa reporter allele may help indicate the deletion events.   
3. The authors did a lot work on in vitro differentiation but not any in vivo experiments, even not using the bone marrow chimeric mice.   
4. Does the inhibition or KO affect the expression of Th17-related cytokines?   
5. Unlike STAT3, STAT1 and STAT5 play negative roles in Th17 differentiation. Authors should discuss it.   
6. STAT3 not only promotes Th17 differentiation but is also important for the function of these cells by mediating IL-23 and IL-21 signals. Will DYRK1A affect these cytokine signals? How about those in DS? Do DS T cells produce more cytokines?   
  
Minor:   
1. Curiously, does DYRK1A affect other TH cells (TH1, TH2, Treg)?   
2. Fig. 1A-B, no inhibitor concentration unit was shown.   
3. Fig. 2D, DMSO samples were not shown.   
4. Any sex differences?   
5. Fig. 4I, which residue phosphorylation of STAT3 was tested?   
6. In Line 296, "As expected, neither CKO nor naïve CD4+ T cells expressed significant levels of phospho-STAT3Y705 at baseline." CKO should be compared with WT.

20240823: Alex Hu

Can’t detect KO with the RNA methods here – Alex Hu.

Exon 6. Looked up the coordinates of exon 6 in genome and used a tool called ??? counts reads that overlap a particular region. Counts gene where any part that overlaps DYRK1A

Might be worth doing normalized expression of exon 6.

Loaded bam files into IGV (genome viewer). Found DYRK1A. Worth getting a better visualization of exactly where Exon 6 is. KO would show difference in the pileups. Overall looks like not a visual difference between WT and DYRK1A. Maybe look particularly at Exon 6.

Proportion of exonic reads in particular exon y-axis is good.

Not optimistic but, instead of looking at raw counts or proportion of exonic counts, include all the exons in a matrix and do regular TMM normalization and see if that makes a difference. Or maybe ask Matt D.

Alex remembers another exon KO and it was subtle, so maybe our analysis isn’t getting the whole story.

20240903 Bernard:  
The key goal here is to respond to Reviewer 2 comment 1. I think finding no shared DEGs between harmine & KO is expected because the KO showed minimal DEGs. I think the best we can expect is to try to show if harmine can act as a lens to identify genes that are dysregulated to a lesser extent in the KO. An alternative might be to ask if some subset of those genes shows correlation with DYRK1A expression levels (to the point that DYRK1A expression overlaps between WT & KO). It’ll be helpful to meet because I’m a little confused that your code doesn’t reflect the expected # genes (eg for counter-regulated I expect 272 genes, not 242. Also I expect the Th17 signature to be bigger than the harmine-dysregulated, which seems opposite in your data below, but is probably just inadequate understanding on my part.

20240916 Bernard:

I think the core question is the extent to which the harmine-dysregulated signature is somewhat recapitulated by the DYRK1A-KO. What I had in mind was a simple search query:

If we start with the “counter-regulated” 272 genes, these should be broken up into 2 groups:

Source\_counter\_up\_in\_Har: These would be genes that are down in NoHar5hVsNaive, and up in HarVsNoHar5h

Source\_counter\_down\_in\_Har: These would be genes that are up in NoHar5hVsNaive, and down in HarVsNoHar5h

If I’ve thought about this right, each would reflect one half of the Th17 genes dysregulated (by sign) by Harmine.

The next query would be:

Of the genes in Source\_counter\_up\_in\_Har, how many are up in KO v WT

Of the genes in Source\_counter\_down\_in\_Har, how many are down in KO v WT

If KO reflects a weak version of harmine, you might expect the sign of dysregulation in KO to recapitulate the sign of dysregulation in harmine. The reason to use this convoluted method of deriving the harmine signature (you could envision just using the genes up/down in NoHar5hVsNaive and that could be worth testing) is that harmine hits targets other than DYRK1A, so the hope is narrowing down to a filtered Th17 geneset gets us some specificity. If this comparison shows that genes moved up in Har also tend to move up in KO and vice versa, I would stop and report that.

20240917 Bernard:

1. Genes in sourceCounterXXInHar but not in KO vs WT - should U understand these to be genes expressed at low levels and probably filtered out in the KO vs WT DGE? (Otherwise they should be present somewhere in the DGE?)

Correct, these genes are not present in the gene counts matrix after the gene filtering step.

2. The plots in your email - the titles are kind of the opposite of those in the text (text describes genes that HAR turned up AND that KO turned up and vice versa. Figs seem to describe genes that HAR turned up AND the KO turned down)

Good catch! I had an indexing error in a string-assembly loop.

3. Is there some documentation where I can learn how roast calculates p values? Just trying to get a sense of that vs chi-sq test

4. Once we clarify the comparisons I’ll probably want the following 3 plots in PDF:

Background: KO vs WT

A. Highlight CounterUpInHar genes in red

B. Highlight CounterDownInHar genes in blue

C. Both A and B in the same plot