Supplementary Information

Bcl-6 is the nexus transcription factor of T follicular helper cells (T$_{FH}$) via repressor-of-repressor circuits

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SUPPLEMENTARY NOTE

T$_{FH}$ differentiation is not a default pathway

In addition to KLH-gp$_{61}$ immunization model, we also examined the T$_{FH}$ differentiation capacity of *Bcl6*$^{f/f}$*Prdm1*$^{f/f}$*Cre*^{CD4} CD4$^+$ T cells in the context of an acute viral infection to assess whether *Bcl6*$^{f/f}$*Prdm1*$^{f/f}$*Cre*^{CD4} CD4$^+$ T cells failing to differentiate into proper GC-T$_{FH}$ cells was a general principle of T$_{FH}$ biology. *Bcl6*$^{f/f}$*Prdm1*$^{f/f}$*Cre*^{CD4}, *Bcl6*$^{f/f}$*Cre*^{CD4}, *Prdm1*$^{f/f}$*Cre*^{CD4}, and wild-type CD45.1$^+$ SMARTA cells were transferred into C57BL/6 host mice and then host mice were infected with LCMV Armstrong (LCMV$_{Arm}$) (Extended Data Fig.1e). *Bcl6*$^{f/f}$*Cre*^{CD4} CD4$^+$ T cells did not differentiate into T$_{FH}$ cells, as expected. *Prdm1*$^{f/f}$*Cre*^{CD4} CD4$^+$ T cells primarily differentiated into T$_{FH}$ cells (CXCR5^{hi}SLAM^{lo} and CXCR5^{hi}PSGL1$^{int/lo}$) and GC-T$_{FH}$ cells (CXCR5^{hi}PSGL1^{lo}) (Extended Data Fig.1f-g). Removal of Blimp-1 expression did allow for CXCR5 expression by Bcl-6–deficient *Bcl6*$^{f/f}$*Prdm1*$^{f/f}$*Cre*^{CD4} CD4$^+$ T cells; however, CXCR5 expression was lower than that of wild-type T$_{FH}$ cells (Extended Data Fig.1f-g), and most other T$_{FH}$ cell features were defective (see main text).

Bcl-6 is an autoregulatory repressor in CD4$^+$ T cells

While an increase of reporter activity was seen in T$_H$1 cells for the SIN RV vector ΔBPS1 construct containing the *Bcl6* promoter, when ΔBPS1 mice were constructed the enhancement of Bcl-6 protein expression was selectively observed in T$_{FH}$ and GC-T$_{FH}$ cells, not T$_H$1 cells (Fig.2c). This indicated that the chromatin context of the *Bcl6* locus is relevant for the *Bcl6* gene expression and it is not fully recapitulated by the RV vector containing only the *Bcl6* promoter. Limitations of the SIN RV vector were expected, and therefore our interpretations focus on the data from the ΔBPS1 mice, as the more physiological model.

Simple circuitry repressor-of-repressors model of Bcl-6
Regarding syntenic ChIP-Seq analysis

Assuming Bcl-6 binding to genes in mouse GC-T$_{FH}$ cells based on synteny between human and mouse alone is imperfect. Active enhancers can diverge substantially between species[1] (though promoters are much more conserved[1], and the majority of Bcl-6 binding sites are in promoter regions[2]). Of note, evolutionarily conserved TF-binding sites have been observed to be sites that are more frequently important for gene regulation than TF-binding sites found only in one species[3]. Most GC-T$_{FH}$ gene expression is conserved between humans and mice. Thus, while observation of sequence conservation does not suffice to demonstrate Bcl-6 regulation of a given gene in mouse T$_{FH}$ cells, global analysis of all mouse T$_{FH}$ gene expression and syntenic Bcl-6 binding sites from human GC-T$_{FH}$ is likely to be a reasonably robust process for assessing global patterns of Bcl-6 gene regulation in T$_{FH}$ cells.

Additionally, the human BCL-6 ChIP-seq was from authentic GC-T$_{FH}$ cells directly *ex vivo*, and thus definitively represents physiological BCL-6 binding sites in humans (unlike most published TF ChIP-seq data for helper T cells, which are not from cells directly *ex vivo*). We used these BCL-6 GC-T$_{FH}$ ChIP-seq data in combination with mouse T$_{FH}$ RNA-seq data to ask: Does the gene expression pattern when Bcl-6 is absent (DKO cells [*Bcl6*$^{f/f}$*Prdm1*$^{f/f}$*Cre*^{CD4}] vs *Prdm1*$^{f/f}$*Cre*^{CD4} in Fig.3d) match the prediction from a simple circuitry model with Bcl-6 acting as a repressor at the apex of a repressor-of-repressors program (Fig.3e)?

If the model was correct, Bcl-6–bound genes should predominantly be represented in Cluster 1 (downregulated in T$_{FH}$), and not Cluster 4 (upregulated in T$_{FH}$), even though both have signatures of gene expression patterns controlled by the availability of Bcl-6. When cross-referencing to BCL-6–bound genes in human GC-T$_{FH}$ by GSEA, the gene expression data passed that test, with clear enrichment for Bcl-6–bound genes among Cluster 1 (FDR < 0.0098) and not Cluster 4 (FDR = 0.43). The value of using the BCL-6 GC-T$_{FH}$ ChIP-seq in this manner is that it allows us to make a valuable conclusion without depending on perfection of the synteny analysis, because the data set is relatively large, and the T$_{FH}$ gene expression profiles are conserved, and GSEA enriches for conserved events; thus, mismatches between species do not drive the outcome. The fact that no Bcl-6 association was seen for Cluster 4 (or Cluster 2 or Cluster 3) reinforced that the analytical approach was trustworthy.

This analysis was meant to be one step of a process for testing the Bcl-6 repressor-of-repressors model. Subsequent wet lab experiments were required to validate the conclusions made from this bioinformatics experiment, particularly utilizing ATAC-seq and Bcl-6 ChIP. *Tox2* and *Tox* are grouped in Cluster 4 genes in our RNA-seq analyses (Fig.3d and Extended Data Fig.9h), suggesting that *Tox2* and *Tox* are Bcl6-rr genes. Recent data proposed that *Tox2* and *Tox* may be direct positive targets of Bcl-6[4]. The available data are consistent with both models. Future mechanistic experiments are necessary to determine how *Tox2* and *Tox* gene expression is regulated.

Regarding Bcl-6 functioning as a repressor and an activator

The repressor-of-repressors Bcl-6 model does not exclude the possibility of Bcl-6 acting as an activator at some

gene loci. The logic of the experimental approach taken here has been to develop the simplest possible models for Bcl-6 gene regulation of T_{FH} cells and test those models. It is well defined in B cells, and now in CD4$^+$ T cells (Fig. 2) that Bcl-6 acts as a transcriptional repressor. Occam's Razor posits that the hypothesis with the fewest number of assumptions is more likely to be correct. Therefore, the simplest model that can be proposed is that Bcl-6 acts via its one known mechanism, repression.

Bcl-6 is known to be able to accomplish gene repression via pairing with a number of co-repressors and a diverse array of chromatin regulators[5,6].

Here we started with a logical conceptual approach to T_{FH} differentiation, with as few assumptions as possible, and developed the simplest possible hypothetical model of Bcl-6 activity and T_{FH} differentiation, followed by rigorous testing of that model to determine if the experimental results would be consistent with the model. The logic of a repressor-of-repressors gene circuit is the most direct gene circuit that can explain the main gene expression changes observes in T_{FH} cells (e.g., gene upregulation and gene downregulation) and the central role of Bcl-6. The goal of testing such a model is to determine whether the overall structure of T_{FH} differentiation and gene regulation can be explained by a simple model of Bcl-6 acting at the apex of a repressor-of-repressors gene circuit.

A failure of such a test would demonstrate that invoking more complex gene regulation is required to explain the role of Bcl-6 in T_{FH} biology. A successful test of this model would indicate that the Bcl-6 repressor-of-repressors gene circuitry model can explain the overall structure of T_{FH} differentiation and gene regulation in a parsimonious manner. We reiterate that the Bcl-6 repressor-of-repressors model does not exclude that (1) Bcl-6 may act as an activator at certain genes, or (2) additional TFs or additional mechanisms of action may occur; but, a successful test of the model would demonstrate that the overall structure and majority of the features of the gene regulatory network of T_{FH} differentiation and function can be accounted for by a repressor-of-repressors Bcl-6 gene regulatory network, without broadly invoking a need for additional mechanisms.

Bcl-6 repressor-of-repressors circuits

The gene disruption studies of *Runx3*, *Runx2*, and *Klf2* focused on using the *Bcl6*$^{f/f}$*Prdm1*$^{f/f}$*Cre*^{CD4} DKO mice as background strain, rather than WT CD4$^+$ T cells. The model posits these genes act downstream of Bcl-6. If that is the case, disruption of these TFs should increase ('rescue') expression of T_{FH}-associated genes in the absence of Bcl-6. The complementary experiment of gene disruption studies of *Runx3*, *Runx2*, and *Klf2* in a WT background is not as straightforward to interpret as experiments in DKO cells since those TFs are already being actively repressed by the presence of Bcl-6. Therefore the downstream gene expression regulation may be relatively insensitive to Bcl6-r TF gene disruptions. Results of gene disruption studies of *Runx3*, *Runx2*, and *Klf2* in a WT background were consistent with the results from DKO background for some genes, with moderate changes (data not shown). It was not unexpected that additional impairment of these TFs (beyond what Bcl-6 is already doing in a WT background) generated a relatively minimal effect. Separately, we reasoned that enforced expression of the Bcl-6–target TFs (e.g., pMIG-Runx2) in a WT background were better experiments to investigate the capacity of these putative Bcl6-r TFs to repress T_{FH} genes. Expression of these TFs is relatively low in T_{FH} cells because they are repressed by Bcl-6, and thus exogenous expression from an RV vector bypasses Bcl-6 as a relatively direct approach to assess the functionality of the putative Bcl6-r TFs.

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