Molecular Mechanisms of LKB1 Function During B Cell Activation

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1. Abstract

Germinal center B cells are crucial for generating effective antibody responses to immunization or infection. It is important to understand the mechanisms by which resting B cells differentiate to become activated, rapidly proliferating germinal center B cells. The liver kinase B1 (LKB1) and its substrates, AMPK and the AMPK-related kinases (ARKs) are key regulators of proliferation and differentiation in various cell types. Still, their role in activated and germinal center B cells remains largely unexplored. In this study, we investigate the underlying mechanism of LKB1 and the ARKs by using an *ex vivo* model of germinal center B cells in which LKB1 deficiency causes proliferation and activation defects. We demonstrate that the function of LKB1 is specifically dependent on the salt-inducible kinase (SIK) subfamily of the ARKs and not the other ARK subfamilies. We show that the SIKs largely function by repressing the CRTC transcriptional coactivators. Finally, we characterize the kinetics of the SIK-CRTC axis in response to BCR and CD40 stimulation. We also analyze other elements of B cell signaling, which show that LKB1 limits NF-kB signaling in B cells through the canonical but not the non-canonical pathway. We conclude that LKB1 is an important B cell regulatory node downstream of BCR and CD40 signaling pathways partly through its independent regulation of the SIK-CRTC axis and canonical NF-kB pathway.

2. Résumé

Les cellules B du centre germinatif sont essentielles pour générer des anticorps efficaces à la suite de l'immunisation ou une infection. Il est important de comprendre les mécanismes par lesquels les cellules B au repos se différencient pour devenir des cellules B du centre germinatif actives et à prolifération rapide. La kinase hépatique B1 (LKB1) et ses substrats, AMPK et les kinases liées à l'AMPK (ARK), sont des régulateurs importants de la prolifération et de la différenciation dans divers types de cellules. Pourtant, leur rôle dans les cellules B activées et du centre germinatif reste largement inexploré. Dans cette étude, le mécanisme qui sous-tend LKB1 et les ARK sont explorés en utilisant un modèle *ex vivo* de cellules B du centre germinatif dans lesquelles la carence de LKB1 provoque des défauts de prolifération et d'activation. Cette étude démontre que la fonction de la LKB1 dépend spécifiquement de la sous-famille kinase inductible par le sel (SIK) des ARK et non des autres sous-familles ARK. En plus, les SIK fonctionnent en grande partie en désactivant les coactivateurs transcriptionnels CRTC. Dernièrement, la cinétique de l'axe SIK-CRTC en réponse à la stimulation du BCR et du CD40 a été caractériser. D'autres éléments de la signalisation des cellules B montrent que LKB1 limite la signalisation du NF-kB dans les cellules B par la voie canonique plutôt que par la voie non canonique. Pour conclure, LKB1 est un important nœud de régulation des cellules B en aval des voies de signalisation BCR et CD40, en partie grâce à sa régulation indépendante de l'axe SIK-CRTC et de la voie canonique NF-kB.

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Contributions:

The thesis was written by Paul Donnelly (P.D., McGill University), with assistance from Dr. Javier M. Di Noia (J.M.D., Institut de Recherches Cliniques de Montreal). The experiments or analyses performed in this thesis were all performed by P.D. with the following exceptions. Dr. Astrid Zahn (A.Z., Institut de Recherches Cliniques de Montreal) contributed to this work by helping to establish the retroviral transduction protocol used to for genetic complementation assays in iGBs. A.Z. also performed the experiments and analyses shown in Appendix Figure S1 (except for FACs histograms that are from experiments performed by P.D., based on previous results found by A.Z.). A.Z. and J.M.D. performed the experiments and analyses shown in Appendix Figure S4. A.Z. also performed one of the two biological replicates shown in Appendix Figure S2B. Additionally, A.Z. performed the two experiments using the p65 inhibitor (SC75741) that are shown in Figure 6I.

5. List of Abbreviations

ARKs	-	AMPK-Related Kinases
BCR	-	B Cell Receptor
bZIP	-	Basic Leucine Zipper
CA	-	Constitutively Active
CI	-	Catalytically Inactive
EV	-	Empty Vector
GC	-	Germinal Center
LZ	-	Light Zone
GZ	-	Gray Zone
DZ	-	Dark Zone
HDAC-IIAs	-	Class II-A HDACs
iGBs	-	Induced Germinal Center B Cells
MFI	-	Median Fluorescence Intensity
NP-CGG	-	Nitrophenyl-Chicken Gamma Globulin
SIKs	-	Salt Inducible Kinases
SRBC	-	Sheep Red Blood Cell
TCR	-	T Cell Receptor

6. Chapter 1: Introduction

To compete with the persistent evolution of pathogens, B cells can evolve their antibody genes in a complicated and fascinating process. This fundamental concept underlies the basis of most immunization strategies, which all seek to provide the same result: antibody-mediated immunity against infection and disease. This can only be achieved by following a precisely regulated sequence of cellular and molecular processes. This is illustrated by the series of B cell stage transitions that follow activation. If these events are not precisely regulated, then the entire antibody response that follows is at risk. This is critical to human health, as dysregulated B cell activation is implicated in the pathogenicity of many human diseases involving B cells, such as autoimmune diseases, immunodeficiencies, and cancers.

6.1. Cell Signaling Controls Important Processes in B cells

6.1.1. B cell Development

During B cell development, B cells undergo somatic recombination of the DNA sequences that will eventually encode their B cell receptor (BCR), which is the membrane bound form of antibody[8]. This process involves the random rearrangement of variable (V), diversity (D), and joining (J) gene segments together in a process known as VDJ recombination[8]. This generates unique DNA sequences for the genes that encode the portion of the BCR that binds to antigen in each B cell. Successful progression through this stage requires tonic signals to be transmitted through the developing BCR[9]. The result of this process is a pool of mature B cells that contain different BCRs, each with the ability to bind and respond to different antigens that an organism may be exposed to. The resulting diversity provides a foundation for adaptive immunity because it allows for the recognition of antigen from essentially any pathogen[8].

6.1.2. B cell Activation

Before exposure to antigen, mature B cells receive tonic survival signals[10] but are mostly metabolically quiescent. They are poised to respond to cognate antigen that is able to bind to and crosslink the BCR[11]. This stimulates a cascade of cell signaling pathways in the cell, activating transcriptional programs that govern the fate of the cells during the first stage of B cell activation[11]. This signaling activates MYC and MTORC1 growth pathways and changes the profile of molecules expressed on the surface of the B cell, which affects their interaction with and sensing of their environment[12]. Chemokine receptors, such as chemokine receptor 7 (CCR7), are expressed early after B cell activation, allowing B cells to migrate from the B cell follicle to the border of the T cell zone, where they can interact with T cells[11]. B cells internalize the antigens that they bind through the BCR and present them to T cells as antigenic peptide MHC-II complexes on the cell surface[11]. Linked recognition occurs when a B cell encounters a T cell that can recognize these peptide MHC-II complexes through the T cell receptor (TCR)[11].

This interaction with T cells, which express CD40L, stimulates the B cells through the CD40 receptor, providing a secondary signal that controls the next stages of B cell activation[11]. One effect of this is the downregulation of CCR7 and upregulation of the oxysterol receptor EBI-2, which allows B cell migration to the edges of the B cell follicle, where they can either differentiate into short-lived plasma cells, or migrate back into the follicle to proliferate and initiate the process of germinal center formation[11,13,14]. Another key effect of B:T interactions is the upregulation of costimulatory molecules expressed on the surface of B cells, such as B7-2 ligands (CD80 and CD86) and OX40L, which provide secondary signals for T cell activation[15-17]. This activation of T cells during the early stages of an immune response is critical for stimulating the production of T follicular helper (T_{FH}) and T follicular regulatory (T_{FR}) cells that

are key players in the germinal center[16,18]. Ultimately, proper transduction of cell signals during each stage of B cell activation is crucial for the eventual formation, structural organization, and maintenance of germinal centers[13,19].

6.1.3. Germinal Center B cells

Mature germinal centers are highly organized structures that are functionally separated into two main compartments, the dark zone, and the light zone[19,20]. This segregates the processes of somatic hypermutation (SHM) from BCR affinity-based B cell selection. In the dark zone, cells proliferate and undergo SHM, which involves the introduction of random mutations into the variable region of the antibody genes[13]. Recent studies suggest that B cells face a checkpoint at this stage that limits progression to the light zone only for cells that mutate the BCR in such a way that it can still be expressed on the cell surface[21]. This indicates that a BCR signaling mechanism controls progression from the dark zone to the light zone[21].

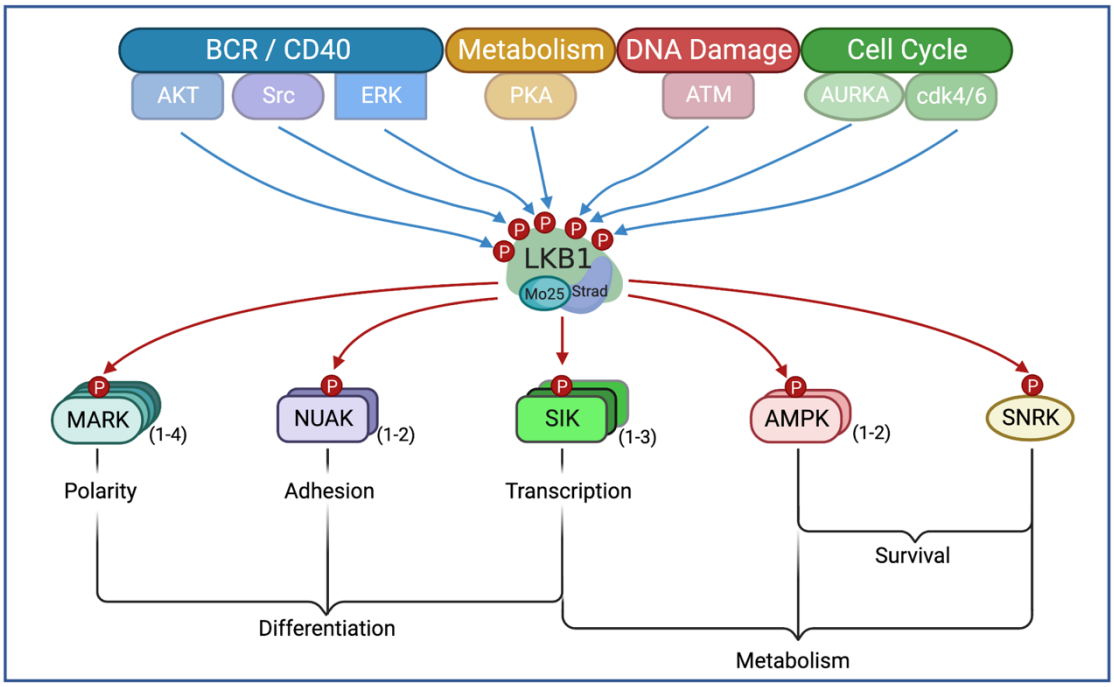
In the light zone, B cells extract antigen from follicular dendritic cells, which they can then process and present to a limited pool of T_{FH} cells, which stimulate the germinal center response[19]. This activity is regulated by T_{FR} cells that limit the magnitude of the germinal center reaction[18]. B cells containing more productive mutations in their antibody genes bind more efficiently to antigen, which increases the chance of stable interactions with T_{FH} cells[20]. This leads to stimulation of B cells through the CD40 receptor by CD40L expressed on T_{FH} cells[22]. This interaction can either renew the proliferative capacity of B cells so that they can recycle into the dark zone or signal them to differentiate and eventually exit the germinal center as memory B cells and plasma cells[5]. This cell fate decision is regulated by the strength of the B-T interaction[5] but the underlying mechanism is not fully understood. Fate decisions culminate in the activation of master transcription factors that regulate either the germinal center B cell program, or

differentiation programs[23,24]. For example, the germinal center B cell master transcription factor Bcl6 represses the master transcription factors of the plasma cell program, Irf4 and BLIMP-1[23]. The expression and function of these transcription factors depends on the signals received through the BCR and interactions with T cells (through CD40 and other receptors), which conveys information about the affinity of the BCR[5].

Signaling is also rewired in germinal center B cells in a process that is still incompletely understood. This leads to the separation of BCR signals primarily to Foxo1, and CD40 signals to NF-kB[12,25]. BCR and CD40 signals must also synergize in germinal center B cells to induce the activation of MYC and MTORC1 growth pathways[12]. Interestingly, either pathway alone is sufficient to activate these pathways in naive B cells[12]. This provides additional stringency to the activation of the growth program, which is critical for the generation of high affinity antibodies but can result in germinal center B cell derived lymphomas or germinal center collapse if the process is dysregulated[19]. Therefore, the identification of novel cell signaling networks that are active downstream of the BCR and CD40 is critically important to achieving a better understanding of B cell biology and the humoral response.

6.2. The LKB1 Signaling Hub

Liver kinase B 1 (LKB1) is a ubiquitously expressed kinase that is encoded by the Serine threonine kinase 11 gene (*Stk11*)[26]. Across the various cell types that LKB1 has been studied in, it has been shown to be directly phosphorylated downstream of signal transduction pathways that activate AKT, ERK, CDK4, CDK6, AURK, Src-family kinases and others[27]. These kinases are active downstream of important signaling cascades that occur during B cell activation[24,28,29] (**Graphical Figure 1**). Although many signals may converge on LKB1 in B cells, it has not yet been studied in this context.



Graphical Figure 1: B cell signaling pathways converge on the LKB1 signaling hub
LKB1 is shown with its upstream and downstream kinases. The upstream kinases shown have been demonstrated to phosphorylate LKB1 in various systems. The downstream kinases are involved in the indicated processes. *Created with BioRender.com*

Despite the identification of many post-translational modifications of LKB1, upstream regulation of LKB1 remains incompletely understood[27]. Unlike many other kinases[30], LKB1 is not phosphorylated at the activation loop to stabilize its catalytically active conformation[31-33]. Instead, LKB1 regulation relies on nuclear to cytoplasmic shuttling, endosomal/lysosomal membrane association, and allosteric interaction with two other proteins which allow for the activation loop to mimic the conformation of a phosphorylation-induced active state [27,34-38]. The lack of a nuclear export signal confines LKB1 to the nucleus until it interacts with the pseudokinase STRADα, which acts as an adaptor between LKB1 and exportins that shuttle the complex to the cytosol[31]. This also inhibits LKB1 import to the nucleus by blocking importin-α from binding LKB1[34]. Furthermore, STRADα stimulates LKB1 catalytic activity, and the molecular scaffold protein MO25α stabilizes the STRADα/LKB1 interaction[39]. The high affinity

ternary complex formed by MO25α/STRADα/LKB1 induces a 10-fold increase in the catalytic activity of LKB1 to its substrates[33]. Catalytic activity may thereby be modulated by phosphorylation (or other modifications) of the interaction interfaces between LKB1 and STRADα/MO25α[38,40]. However, the effect of many phosphorylation events on mediating the LKB1/STRADα/MO25α complex formation remains unknown due to the lack of structural data for the N and C terminus of LKB1[33].

6.3. The AMPK Related Kinase Family (ARKs)

LKB1 is the upstream kinase for 14 kinases in the ARK family[26], which includes AMPKα1/α2, SNRK and the subfamilies SIK, MARK and NUAK (**Graphical Figure 1**). Direct phosphorylation by LKB1 at a conserved threonine in the activation loop of these kinases stimulates a 50-fold increase in their catalytic activity[26]. Through these kinases, LKB1 controls critical cellular processes such as metabolism, transcription, cell polarity, and adhesion,[41] which are critical processes in activated B cells[11]. Despite this, the role of the LKB1 signaling hub during B cell activation remains unclear.

The most well characterized of these substrates is AMPKα1/α2, which regulates cellular energy homeostasis by repressing MTOR, thereby limiting anabolic processes and cell growth[42,43]. Although the other ARKs are not as well characterized as AMPK, various studies have identified certain key roles for each subfamily. The most recently identified of these is SNRK, which is implicated in metabolic pathways such as glucose uptake and fatty acid oxidation[44]. The MARK subfamily includes four members, MARK1/2/3/4, that phosphorylate microtubule associated proteins (MAPs), leading to their dissociation from microtubules, which allows for restructuring of the cytoskeleton and regulation of cell polarity[45]. The NUAK subfamily, which includes NUAK1/2, phosphorylates the MYPT1- PP1β myosin phosphatase

complex, leading to its interaction with 14-3-3 proteins and the suppression of PP1β phosphatase activity[46]. This results in an increase of myosin light chain 2 phosphorylation, allowing for cell detachment to occur[46]. Thus, the LKB1-NUAK pathway regulates cell adhesion.

6.4. The LKB1-SIK Pathway

In addition to being activated by LKB1, the three members of the SIK subfamily, SIK1/2/3, are negatively regulated by PKA, which phosphorylates regulatory regions that mediate the binding of 14-3-3 proteins to inhibit their catalytic activity[47,48]. The major substrates identified downstream of the SIKs are two families of transcriptional regulators: the CREB-regulated transcriptional coactivators (CRTCs, previously known as TORCs) and the class IIA histone deacetylases (HDAC-IIAs)[49]. Their phosphorylation by SIK stimulates their binding to 14-3-3 proteins that sequester these proteins in the cytosol[6,49]. In this way, the LKB1-SIK pathway regulates the activity of various transcription factors, such as MEF2 family members that are regulated through interactions with HDAC-IIAs, and basic leucine zipper (bZip) transcription factors such as CREB/ATF and AP-1 (Fos/Jun), that are regulated by CRTCs[50-52]. Although the MARKs and AMPKs also phosphorylate the CRTCs, they only phosphorylate a fraction of the sites phosphorylated by the SIKs, and with less potency[6].

The main target of CRTC-mediated transcriptional coactivation is CREB[51,53]. The three members of the CRTC bind to the bZIP domain of CREB through their evolutionarily conserved CREB-binding domain (CBD)[54]. CREB function has been extensively studied in neurons, where it is stimulated downstream of cAMP and calcium signaling to induce the transcription of immediate early genes[55,56]. The majority of immediate early genes are transcription factors that act collectively to express genes that control diverse cellular phenotypes of neurons[56-59]. This is a critical mechanism for synaptic plasticity, which is the foundation of learning and memory[56-59].

Interestingly, it was found that the role of CREB in synaptic plasticity and memory formation is dependent on its transcriptional coactivation by CRTC1, the predominant CRTC expressed in neurons[53,54]. Additionally, it was found that transcriptional activation by CREB-CRTC1 has a negative feedback mechanism involving SIK1 in neurons[60,61]. Specifically, CREB-CRTC1 stimulated the transcription of *Sik1*, leading to LKB1 activation of SIK1 and termination of the CREB-CRTC1 transcriptional response[60,61]. Thus, the LKB1-SIK-CRTC axis can regulate the levels of a broad range of transcription factors, which affect key processes in ways that are beginning to be explored in neurons[49,53,60-62] and other cell types[63-65].

Interestingly, a similar process may occur in immune cells, as CREB-CRTC2/3 induces the expression of multiple transcription factors that regulate broad transcriptional programs, such as KLF4 and CEBPβ, that have been shown to regulate macrophage polarization[63-65]. This was shown to be controlled by the LKB1-SIK pathway, which limits the production of macrophages with an anti-inflammatory phenotype by restricting the function of CRTC3[66,67]. Furthermore, this demonstrates that the LKB1 signaling hub can respond to external stimuli by regulating the transcriptional output of diverse cell types, from neurons to macrophages.

6.5. Functions of LKB1 in the Immune System

6.5.1. Dendritic Cells

Similar to the effect described in macrophages, LKB1 has been found to regulate immunosuppressive responses of dendritic cells[22,68-70]. This occurs through LKB1-mediated regulation of cell surface molecules that promote migration towards T cells (CCR7), costimulation of T-cells (OX40L and CD86) and cytokines (IL2, others) that favor the expansion of T regulatory cells (T_{Regs})[22,68-70]. This highlights a cell-extrinsic role for LKB1 in the

maintenance of the immune microenvironment. Furthermore, these phenotypes were regulated independently of AMPK[22,68-70]. Although the other ARKs downstream of LKB1 were not characterized in these studies, a small molecule inhibitor screen identifies the SIKs as regulators of anti-inflammatory phenotypes in bone marrow derived-dendritic cells[71]. This suggests a possible role for the LKB1-SIK pathway in dendritic cells as well.

6.5.2. T cells

LKB1 has been established to have key roles in T cell processes[72-77]. In thymocytes, LKB1 was shown to be phosphorylated by Lck downstream of TCR signaling[77]. This recruited LKB1 to the LAT signalosome, where it regulated cell signaling downstream of the TCR, primarily through effects on PLCγ (calcium signaling)[77]. This is consistent with the function of LKB1 as a signaling hub[26]. LKB1 has also been demonstrated to play important roles in T cell proliferation, cytokine secretion, metabolism, survival, and lineage identity[72-76]. Importantly, studies have shown that many T cell phenotypes controlled by LKB1 cannot be explained by AMPK activity[72-76]. This suggests roles for one or more of the ARKs, but these roles remain largely unexplored. In T_{Regs}, LKB1-mediated control of energy homeostasis was shown to occur through the SIKs and the MARKs, independently of AMPK[72]. Additionally, a recent report showed that the SIKs are required for thymic T cell development[78]. Although other potential roles for the SIKs remain unexplored in adaptive immunity, the SIK substrate CRTC2 has been studied. It is reported that CRTC2 is required for Th17 cell polarization, but unnecessary for thymic T cell development[79]. The role of CRTC1 and CRTC3 in T cells has not been characterized.

6.5.3. Mature B cells

LKB1 has previously been studied in B lymphocytes using *Stk11*$^{F/F}$, *Rosa26*^{YFP} CD19-Cre mice, which delete the *Stk11* gene early during B cell development and are marked for deletion by expression of YFP[80]. However, analysis of the mature, resting B cell compartment in these mice demonstrated that 70% of these cells still expressed LKB1 (YFP-)[80]. This indicates that there is a strong selective pressure to maintain the expression of LKB1 in mature B cells, as the cells that escaped deletion had an advantage over the cells that had deleted LKB1. To further assess the importance of LKB1 in mature B cells, our lab assessed protein levels of LKB1 by western blot in resting B cells isolated from *Stk11*$^{F/F}$ CD21-Cre mice, which delete the *Stk11* gene at the mature B cell stage. This showed maintained expression of LKB1 in resting B cells that was comparable to control mice (data not shown), which confirmed the importance of LKB1 in the maintenance of mature B cell homeostasis[80,81].

In the *Stk11*$^{F/F}$, *Rosa26*^{YFP} CD19-Cre mice, the ~30% of mature B cells that had deleted LKB1 were further characterized. These cells showed activation of NF-kB signaling and induction of apoptosis in non-immunized mice, which indicates that resting B cells may rely on LKB1 to repress NF-kB and apoptotic signaling pathways[80], which is consistent with another study of *Stk11*$^{F/F}$ CD19-Cre mice showing LKB1-mediated regulation of apoptosis in mature B cells[81]. These cells also secreted IL-6 after LKB1-loss, which resulted in an expansion of the T_{FH} cell pool, and the production of spontaneous germinal centers, which largely consisted of cells that still expressed LKB1[80]. Ultimately, the cell extrinsic effects of LKB1 loss in resting B cells complicates the identification of B cell intrinsic roles for LKB1 after B cell activation in these mouse models.

6.5.4. Germinal Center B cells

Our lab used *Stk11*$^{F/F}$ Cγ1-cre mice to study the role of LKB1 in B cells post-activation. For clarity, these mice will hereafter be referred to as *Lkb1*$^{F/F}$ Cγ1-cre or simply KO. These mice show a defective antibody response, diminished GC B cell numbers, defective germinal center formation and structure, and expansion of CD4+ T cells (**Appendix Figure S1A-C**) with preferential expansion of T_{Regs} and T_{FR} cells (data not shown). This indicates that LKB1 plays a critical role after B cell activation for germinal center formation. Interestingly, no increase in apoptosis was found (data not shown) which contrasts with a previously described role of LKB1 in mature B cells. This demonstrates that LKB1 can play context specific roles during different B cell stages, where it likely restrains apoptotic processes before activation, but not after.

6.5.5. Induced Germinal Center B cells (iGBs)

In this thesis, we define the mechanism by which LKB1 regulates B cell activation by using an *ex vivo* model of activated mouse B cells that was previously characterized[82]. Activation of naive B cells in this system was demonstrated to produce B cells that are consistent with germinal center B cells *in vivo,* thus the cells in this system are referred to as induced germinal center B cells (iGBs)[82]. iGBs recapitulate key germinal center traits, such as the expression of the transcription factor Bcl6 and cell surface molecules GL7, FAS, and cell surface ligands for PNA[82]. They also downregulate non-germinal center B cell markers such as CD38 and IgD[82]. Finally adoptive transfer of these cells back into mice after expansion *ex vivo* demonstrated that these cells have the capacity to differentiate into the two known fates of germinal center B cells, memory B cells and plasma cells[82].

This system functions by culturing naive B cells on a monolayer of fibroblasts that co-express CD40L and BAFF in the presence of IL-4. This stimulates B cell signaling pathways that

are present during activation and within germinal centers[19]. BAFF is a survival factor that is secreted by stromal cells in lymphoid organs and follicular dendritic cells within germinal centers[19]. Additionally, CD40 stimulation by CD40L mimics the secondary activation signals that would normally be provided by T cells[19]. Finally, IL-4 is added because it is a key cytokine produced by T-cells in the germinal center to promote B cell cycle entry[83]. Thus, the iGB cell culture system can be used to study key cell signaling processes in activated B cells.

Analysis of B cells from *Lkb1*$^{F/F}$ Cγ1-cre mice in the iGBs system demonstrated that LKB1 plays a role in proliferation, expression of the T cell costimulatory molecules OX40L and CD86, and expression of CCR7, a key receptor involved in migration (**Appendix Figure S1D-G**). Additionally, LKB1 was shown to play a strong role in transcriptional regulation, as major changes in the transcriptional program were found in *Lkb1*$^{F/F}$ Cγ1-cre iGBs. This includes major decreases in genes related to proliferation (data not shown), and major increases in genes related to receptor signaling pathways, such as downstream of CD40 signaling (**Appendix, Figure S4**). This suggests multiple cell intrinsic roles for LKB1 in B cells after activation. Finally, the *in vivo* and *ex vivo* phenotypes were found to occur independently of AMPK, as *Prkaa1*$^{F/F}$ Cγ1-cre mice did not recapitulate the phenotypes observed in *Lkb1*$^{F/F}$ Cγ1-cre mice (data not shown).

6.6. Central Hypothesis and Research Questions

The central hypothesis of this thesis is that **LKB1 regulates B cell activation through one or more of the ARK downstream pathways.** To test this hypothesis, we sought to answer the following research questions:

1. What are the downstream ARKs that mediate the regulation of proliferation and expression of OX40L, CD86, and CCR7 by LKB1 in activated B cells?
2. What is the downstream mechanism of the ARKs function?
3. How does LKB1 activate the ARKs during B cell activation?
4. How is cell signaling altered in the absence of LKB1?

7. Chapter 2: Materials and Methods

7.1. Mice

Stk11$^{F/F}$ Cγ1-cre mice (referred to as *Lkb1*$^{F/F}$ Cγ1-cre mice for simplicity) and Cγ1-cre mice in C57BL/6J background were bred at the specific pathogen-free+ facility of the Institut de Recherches Cliniques de Montreal (IRCM). Male and female mice of 6 weeks to 8 months old were used as a source of B cells. Animals were euthanized in CO_2 chamber. All animal work was approved by the IRCM animal protection committee (AUP 2019-07 JDN) in accordance with the guidelines of the Canadian Council for Animal Care.

7.2. B cell Isolation

After euthanasia and spleen necropsy, mouse spleen tissue was homogenized by mashing through a 70 μm filter with addition of PBS 1% BSA. Homogenate was then passed through a 40 μm filter and resuspended by gentle aspiration. Naive B cells were isolated by negative selection with the EasySep™ Mouse B Cell Isolation Kit (Stemcell, Cat. # 19854) with EasyEights magnet (Stemcell, Cat. # 18103), following the manufacturer's instructions.

7.3. DNA Constructs

Additional information about DNA constructs is listed in **Appendix Table 1**. The open reading frames (ORFs) of mouse LKB1, ARKs, CBD of CRTC1 (AA1-AA55)[84] and kinase domain of CAMK-I (AA1-AA293)[85], were amplified from cDNA by PCR with High-fidelity KOD polymerase (Millipore, Cat. # 71085) or High-fidelity Q5 DNA polymerase (NEB, Cat. # E0555S). cDNA was sourced from primary mouse B cells activated *ex vivo* with 5 μg/ml LPS and 1 ng/ml IL-4, or from mouse skeletal muscle cDNA for CAMK-I. Constitutively active ARK

(CA-ARK) constructs were generated by PCR mutagenesis of the T-loop threonine encoding region (T>D in LNTNCGS) using non-overlapping primers for exponential PCR amplification (using KOD or Q5 polymerase). LKB1 mutants were also generated using the same PCR mutagenesis strategy. An N-terminal flag tag was added to each CA-ARK construct by PCR, except for SIK2-CA. LKB1 and CA-ARKs constructs were cloned into pMXs-IRES-GFP-gateway[86] by gateway cloning or into pMXs-IRES-GFP via pGEM-T easy cloning. pMXs-dnCRTC-nls-GFP was cloned by removing the IRES sequence from pMXs-IRES-GFP and replacing with the evolutionarily conserved CBD of CRTC1 with SV40 NLS, following a procedure that was previously described elsewhere[84]. The kinase domain of CAMK-I lacks the regulatory domains and has been previously characterized as constitutively active[85]. For attempts to lower LKB1 expression levels, weak Kozak (GCC-TGG-ATG) and very weak Kozak (TAT-TGT-ATG) translation initiation sequences were added upstream of the LKB1-ORF by PCR mutagenesis. Following an additional strategy reported[87] to lower gene expression, one or two strong upstream Kozaks (ACA-ATG) were added upstream of the LKB1-ORF in a different reading frame with the aim of triggering nonsense-mediated decay. A separate strategy for lowering retroviral-mediated gene expression employed a self-inactivating retroviral vector, pMSCV-SIN-PGK-GFP (Addgene, cat.#35615). In brief, the vector was modified to add gateway-IRES GFP via NEBuilder HIFI (NEB, cat.#E2621S), with subsequent removal of the PGK promoter via PCR mutagenesis. LKB1 was then cloned into the vector by gateway cloning.

7.4. Cell Culture and Transduction

Primary B cells were cultured at 37 °C with 5% (vol/vol) CO_2 in RPMI 1640 media (Wisent), supplemented with 10% fetal bovine serum (Wisent), 1% penicillin/streptomycin (Wisent), 50 μM 2-mercaptoethanol (Bioshop),10 mM HEPES and 1 mM sodium pyruvate. Anti-BCR

stimulations were performed with α-IgM and α-IgG, ($F(ab')_2$) (H+L), at 10 µg/mL each (Jackson); anti-CD40 stimulations were performed with α-CD40 at 5 µg/mL (mAb #FGK45, homemade) + IL-4 (1 ng/mL, PeproTech). Induced germinal center B cells (iGBs) were generated using 40LB feeder cells[82]. One day before B cell plating, 40LB cells were arrested by treatment with mitomycin-c (10 µg/mL, AG scientific and Bioshop) and plated at 0.09 x 10^6 cells per well (24-well plate) in 0.5 mL DMEM media supplemented with 10% fetal bovine serum (Wisent) and 1% penicillin/streptomycin (Wisent). Purified naïve B cells were plated on 40LB feeders at 2 x 10^4 cells per well in 1 mL of B cell media (24-well plate), supplemented with 1 ng/mL IL-4. For retroviral mediated genetic complementation of iGBs, pMXs vectors were transfected into the PLAT-E packaging cell line[88] using Trans-IT LT-1 (Mirus Bio, cat.# MIR 2305) with 2.5 µg DNA added per well (6-well plate). PLAT-E was cultured in RPMI 160 media, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 50 µM 2-mercaptoethanol. The culture media was exchanged 24 hours after transfecting PLAT-E to include 10mM HEPES and 1 mM sodium pyruvate. At day 3 post-plating, iGBs were infected by removing 0.5 mL of media and replacing with 1 mL of PLAT-E supernatant (at 48 h post-transfection), in the presence of 8 µg/mL polybrene (Sigma, Hexadimethrine bromide Cat.# H9268) with centrifugation at 600 x g for 90 min at 32 °C. Medium was replaced 4 hours later.

7.5. Competitive Growth Assay

The effect on growth was analyzed by comparing *Lkb1*-deficient iGBs that were retrovirally transduced with pMXs vectors encoding eGFP and proteins of interest such as LKB1, LKB1 mutants (ie. catalytically inactive D194A), downstream proteins (ARKs, dnCRTC), or empty vector (ires-GFP). The uninfected GFP- cells and retrovirally infected GFP+ cells were

monitored 18-24hrs after infection to measure transduction efficiency. The GFP+ to GFP− cell ratio over time was monitored by flow cytometry.

7.6. Flow cytometry

iGBs were detached from 40LB cells 5-6 days post-plating by removing media and adding 1 mL of PBS with 0.5% BSA and 2 mM EDTA and incubating at 37°C for 3 min. After counting, 5 x 10^6 cells were washed with PBS and pre-incubated with 20 µl FcR receptor block (Miltenyi) diluted 1/10 in PBS (+1% BSA, 0.1% sodium azide) for 10 mins at RT. Primary antibody was appropriately diluted in PBS (+1% BSA, 0.1% sodium azide) and 80 µl was added to cells without washing. Cells were stained with CD86-biotin (1/2000, eBioscience, cat.#13-0862) for 30 min at 4°C, washed, then stained with OX40L-PE (1/200, eBioscience) for 30 min at 37°C, washed, then incubated with streptavidin-APC (Miltenyi, 1/100) for 10 min at 4°C. Cells were also stained with CCR7-PE (1/200, eBioscience, cat.#12-1971-80) for 45 min at 37°C. Data were acquired using BD Facscalibur (BD biosciences, cat.#12-5905-81) or Sony SA-3800 and analyzed using FlowJo.

7.7. Western Blots

For stimulation experiments, resting B cells (unstimulated) were kept on ice in PBS (+0.25% BSA, 1 mM EDTA) before the preparation of cell lysates. Stimulated and unstimulated cells were washed once with PBS at 4°C before adding cell lysis buffer. Protein extracts were performed by lysing cells in NP-40 lysis buffer (1% NP-40, 20mM Tris pH 8, 137mM NaCl, 10% glycerol, 2 mM EDTA), containing protease and phosphatase inhibitor (Thermo Scientific, cat. #78440). For analysis of histone H3 modifications, samples were lysed in RIPA Buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8, 1mM EDTA,

containing proteinase Inhibitor (Roche) and benzonase endonuclease (Sigma, cat. #71205-3) diluted 1:250. Protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes (BIO-RAD). Membranes were blocked in TBS 5% milk and probed with primary antibodies overnight, washed 3x ~5 min in TBS + 0.1% Tween (TBS-T) before incubating with secondary antibodies conjugated to AlexaFluor680 or IRDye800 for 1 h, washed 3x ~5 min in TBS-T and read on the Odyssey CLx imaging system (LI-COR). Proteins were quantified using ImageStudiolite software. The antibodies used are listed in **Appendix Table 2**.

7.8. Reagents

More information regarding these reagents is listed in **Appendix Table 3**. The following reagents were kept in the dark and resuspended in DMSO for long-term storage at −80 °C. Mitomycin-c was kept in single-use aliquots (diluted fresh before each experiment). SC75741 (p65 inhibitor), IT-603 (c-REL inhibitor) and BI605906 (IKK- β inhibitor) were aliquoted and resuspended for long-term storage at −80 °C, and intermediate dilutions in DMSO (from 25 to 0.4 mM) were kept at −20 °C and frozen/thawed up to two times for individual experiments. DMSO was always diluted 1/1000 in final volume with cells.

8. Chapter 3: Results

8.1. Genetic complementation of *Lkb1*-deficient activated B cells

Lkb1$^{F/F}$ Cγ1-cre iGBs, hereafter referred to as KO iGBs, were previously demonstrated to have defective proliferation and to overexpress the cell surface molecules CD86 and OX40L (T-cell costimulation) and CCR7 (activated B cell chemokinesis) (**Appendix Figure S1E-G**). These phenotypes may partly explain the dysregulated immune response that was characterized *in vivo,* such as defective germinal center formation/organization, deficient antibody response, and elevated CD4+ T cell activation (**Appendix Figure S1A-C**). To investigate the mechanisms by which LKB1-loss produces these phenotypes, we established an assay by which we could genetically complement KO iGBs *in vitro* with retroviral constructs (**Figure 1A**). We demonstrate that retroviral transduction of LKB1, but not an empty vector (EV) control, can complement the proliferation defect and normalize cell surface molecule expression of OX40L, CD86, and CCR7 (**Figure 1B-E**). We also show that a catalytically inactive mutant of LKB1 (D194A), is unable to rescue proliferation, which confirms that the kinase activity of LKB1 is necessary (**Figure 1C**). The GFP+/- ratio (competitive growth) corresponds to the GFP+ cell count of complemented iGBs, which shows proliferation of KO iGBs complemented with LKB1, but not the EV control (**Appendix Figure S6)**.

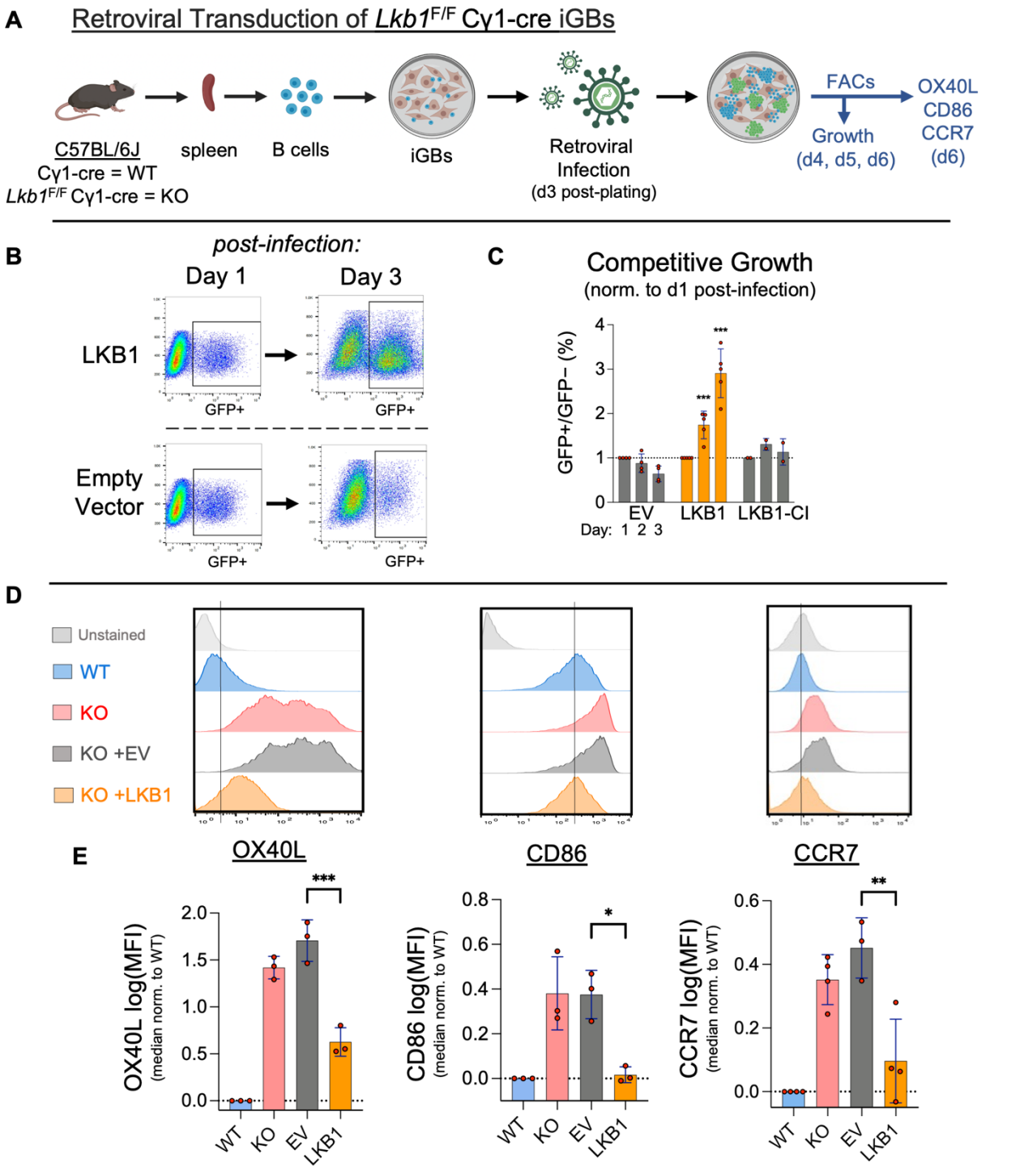


Figure 1: Establishing a genetic complementation assay in iGBs to study LKB1 function
A. *Ex vivo Lkb1*$^{F/F}$ Cγ1-cre (KO) iGBs genetic complementation protocol. **B.** Representative FACs plots for measurement of competitive growth between infected iGBs (GFP+) and uninfected iGBs (GFP-). **C.** Quantification of competitive growth between iGBs transduced with empty vector (IRES-GFP), LKB1, and catalytically inactive (CI) LKB1 (D194A), (n=2-5). **D.** Representative histograms of OX40L, CD86, and CCR7, with line drawn at MFI of WT iGBs. (n=3-4) **E.** OX40L, CD86, and CCR7 expression in transduced KO iGBs normalized to WT control: log(Sample MFI / WT MFI). **C.** Two-way ANOVA **E.** One-way ANOVA. **C.&E.** Mean ±SD are plotted. Dunnett's multiple comparison test, compared to EV control. (*≤0.05, **≤0.01, ***≤0.001, ****≤0.0001).

In an attempt to identify upstream kinases acting upon LKB1 in B cells (**Appendix Figure S2A**), we measured the competitive growth of KO iGBs transduced with the canonical isoform of LKB1, a short isoform of LKB1 (LKB1-S) and single alanine substitution mutants of LKB1 made at evolutionarily conserved post translational modification sites (**Appendix Figure S3**). We reasoned that if the function of LKB1 in B cells requires phosphorylation by an upstream kinase at a specific site, then mutation of that site to a residue that cannot be phosphorylated will result in a loss of LKB1 function that may prevent or diminish the ability to rescue phenotypes that are mediated by LKB1, such as proliferation. Other post-translational modifications that may affect the function of LKB1 such as cysteine farnesylation can be identified using this structure-function approach as well. However, all of the mutants were able to rescue iGB cell proliferation (**Appendix Figure S2B**). We suspect that overexpression of LKB1 bypasses its normal regulatory mechanisms. Therefore, we attempted to lower the expression of LKB1 in our retroviral transduction system. We manipulated the 5' untranslated region of *Stk11* (LKB1) in our construct to limit the initiation of protein translation through two previously described mechanisms (weak Kozak sequences and strong upstream open reading frames)[87]. We also employed a retroviral vector with a limited viral promoter (truncated LTRs). However, these strategies either produced alternative N-terminal isoforms of LKB1 (**Appendix Figure S2C-D**) that are reported to affect LKB1 activity[89], or did not sufficiently lower LKB1 expression levels (~60% expression relative to original vector) (**Appendix Figure S2E**), so they were no longer pursued. Future strategies could employ a PEST domain to target LKB1 to the proteasome, or an N-terminal GFP fusion that will distance the alternative internal start codons of LKB1 from manipulations of the Kozak sequence.

8.2. SIKs act downstream of LKB1 in activated B cells

We then focused on determining the pathway downstream of LKB1 that controls iGB cell proliferation and expression of OX40L, CD86 and CCR7. To do this, we genetically complemented KO iGBs with the downstream substrates of LKB1, the ARKs, by retroviral transduction. LKB1 activates 14 of the ARKs by phosphorylation of a conserved threonine in the activation loop of the kinase domain[26,90], and we found that 10 of these kinases are expressed in activated B cells (**Figure 2A**). Constitutively active (CA) mutants of the ARKs[26] that were the most highly expressed across the various ARK subfamilies (AMPK, SNRK, and SIK/MARK/NUAK subfamilies) were retrovirally transduced into KO iGBs (**Figure 2B**). CA-SIK1 rescued the defective proliferation and expression of OX40L, CD86 and CCR7, while CA-AMPK, MARK, NUAK and SNRK kinases did not (**Figure 2C-E**), suggesting that the LKB1-SIK pathway specifically mediates these phenotypes in activated B cells.

We further examined the expression of the SIKs by analysis of previously published RNAseq data in activated B cells. Comparing SIK1/2/3 showed that *Sik1* mRNA is highest in *in vitro* activated B cells (**Figure 3A**). Despite some discrepancies, *Sik3* mRNA appears to be highest in *in vivo* datasets (germinal center B cells) (**Figure 3B-C**) whereas *Sik1* mRNA expression may only be enriched in subsets of activated B cells (**Figure 3C-D**).

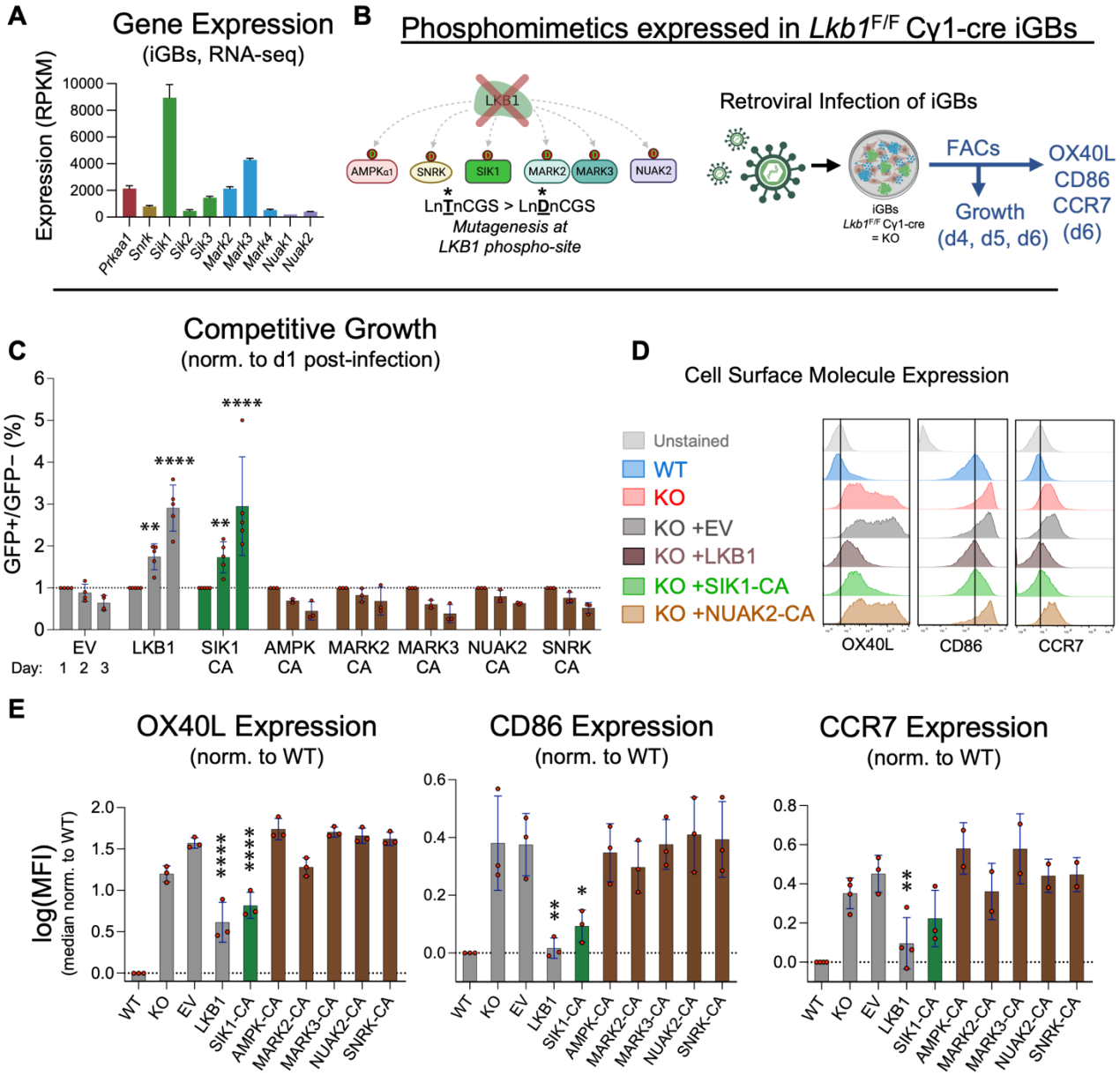


Figure 2: SIK1 regulates the proliferation and expression OX40L, CD86, and CCR7 downstream of LKB1

A. RNA-seq: expression of ARK transcripts in iGBs. **B.** Strategy to study the function of downstream kinases activated by LKB1 in iGBs. **C.** Quantification of competitive growth between transduced KO iGBs, (n=3-5). **D.** Representative histograms of OX40L, CD86, and CCR7, with line drawn at MFI of WT iGBs. **E.** OX40L, CD86, and CCR7 expression in transduced KO iGBs normalized to WT control: log(Sample MFI / WT MFI). (OX40L and CD86, n=3), (CCR7, n=2-4) **C.** Two-way ANOVA **E.** One-way ANOVA. **C.&E.** Mean ±SD are plotted. Dunnett's multiple comparison test, compared to EV control. (*≤0.05, **≤0.01, ***≤0.001, ****≤0.0001). For SIK1-CA effect on CCR7, p = 0.0727

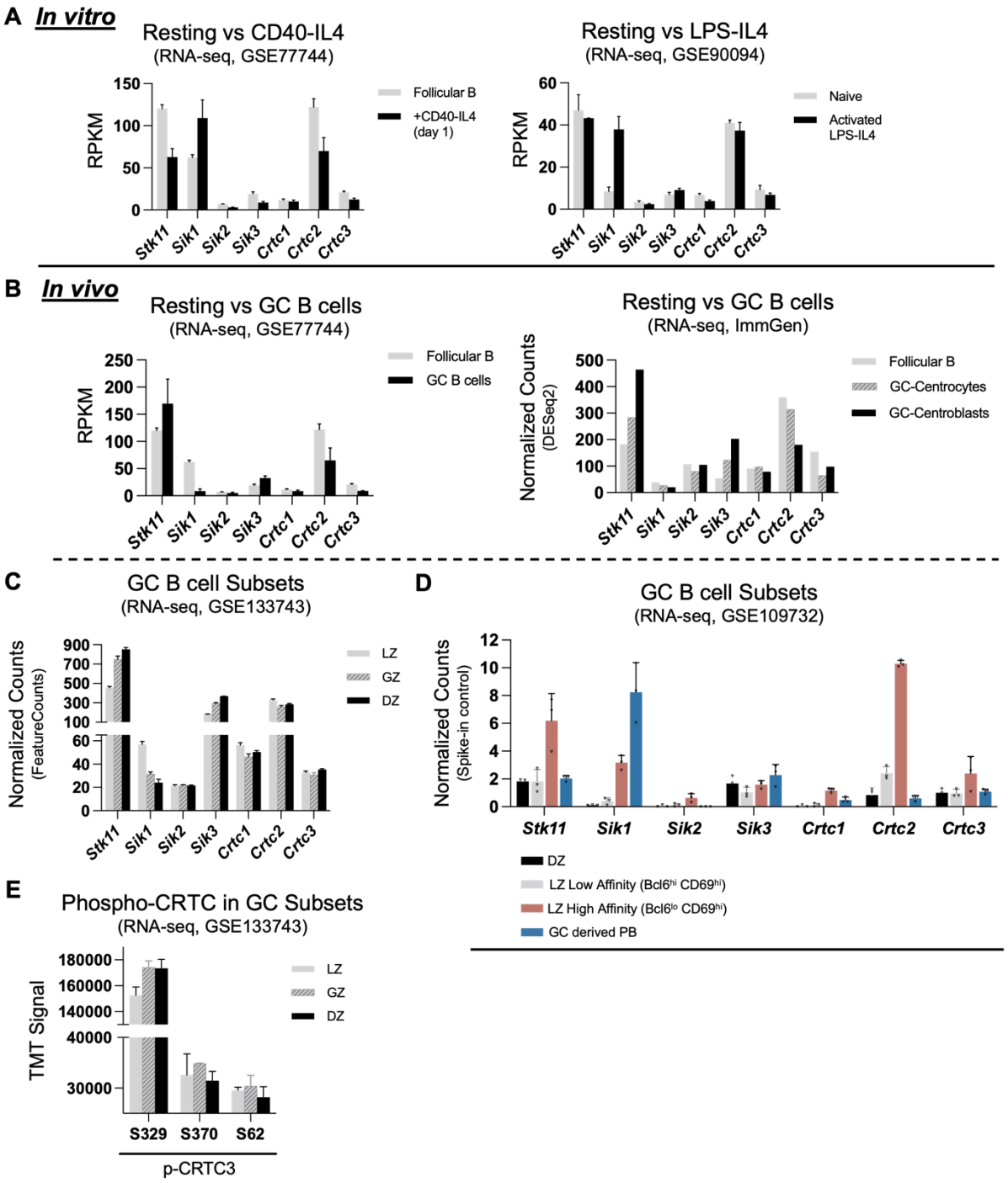


Figure 3: Expression of LKB1-SIK pathway signaling components in B cell system datasets
A. RNA-seq of B cells activated *in vitro* from the following, previously reported, public datasets: GSE77744-[1] and GSE90094-[2]. **B.** RNA-seq of resting vs germinal center B cells from GSE77744-[1] and the Immunological Genome Project (ImmGen)[3]. **C.** RNA-seq of GC B cell subsets, light zone (CXCR4–CD83+), gray zone (CXCR4+CD83+), and dark zone (CXCR4+CD83–). Cells progress from LZ > GZ > DZ. From GSE133743-[4]. **D.** RNA-seq of GC B cell subsets, with separation of the light zone into high- and low-affinity BCR subsets. From GSE109732-[5]. **E.** Phosphoproteomics data from GSE133743-[4]. Germinal center B cell subsets show phosphorylation of CRTC3 at sites previously found to be phosphorylated by SIKs in other systems[6]. Note, lack of detection of other phospho-proteins cannot be determined as biological absence in this assay[7].

Because all three *Sik* family mRNAs are expressed in iGBs, we tested the ability of each one to complement proliferation and expression of OX40L, CD86 and CCR7 in our retroviral transduction assay. While CA-SIK1 and CA-SIK2 rescued defective proliferation, CA-SIK3 did not (**Figure 4E**). Additionally, CA-SIK1 and CA-SIK2 rescued the expression of OX40L, CD86 and CCR7, while CA-SIK3 only showed a partial rescue of OX40L, and to a lesser extent, CD86 (NS in Dunnett's test compared to EV, however, comparison to KO shows a significant reduction, $p = 0.0245$). This suggests that SIK1 and SIK2 are the downstream mediators of these LKB1-dependent phenotypes in iGBs.

8.3. CRTCs are repressed by the LKB1-SIK pathway in activated B cells

To determine the downstream targets of the LKB1-SIK pathway that control iGB cell proliferation and expression of OX40L, CD86 and CCR7, we genetically complemented KO iGBs with constructs targeting the most frequently reported[49] downstream substrates of the SIKs: the class IIa histone deacetylases (HDAC-IIAs) and the CREB-regulated transcriptional coactivators (CRTCs) by retroviral transduction. SIK phosphorylation of HDAC-IIAs and CRTCs inhibits the nuclear functions of these enzymes by tagging them for nuclear export[49] so we sought to reproduce the nuclear export of these enzymes in our strategy (**Figure 4A**).

The SIKs are evolutionarily classified as part of the calmodulin kinase family[91] and share limited substrate specificity with calmodulin kinases (CAMKs) I and V. HDAC-IIAs are phosphorylated by both the SIKs and CAMK I/V at shared sites[92]. Similarly to the effect of the SIKs, the CA-calmodulin kinase I (CAMK-CA) was demonstrated by others[85] to stimulate cytoplasmic retention of HDAC-IIAs in other systems[93]. Thus, we used CAMK-CA to study the HDAC-IIA pathway downstream of SIK1/2. To study the CRTCs, we used a dominant negative CRTC that blocks the binding of endogenous CRTCs to transcription factors such as CREB in

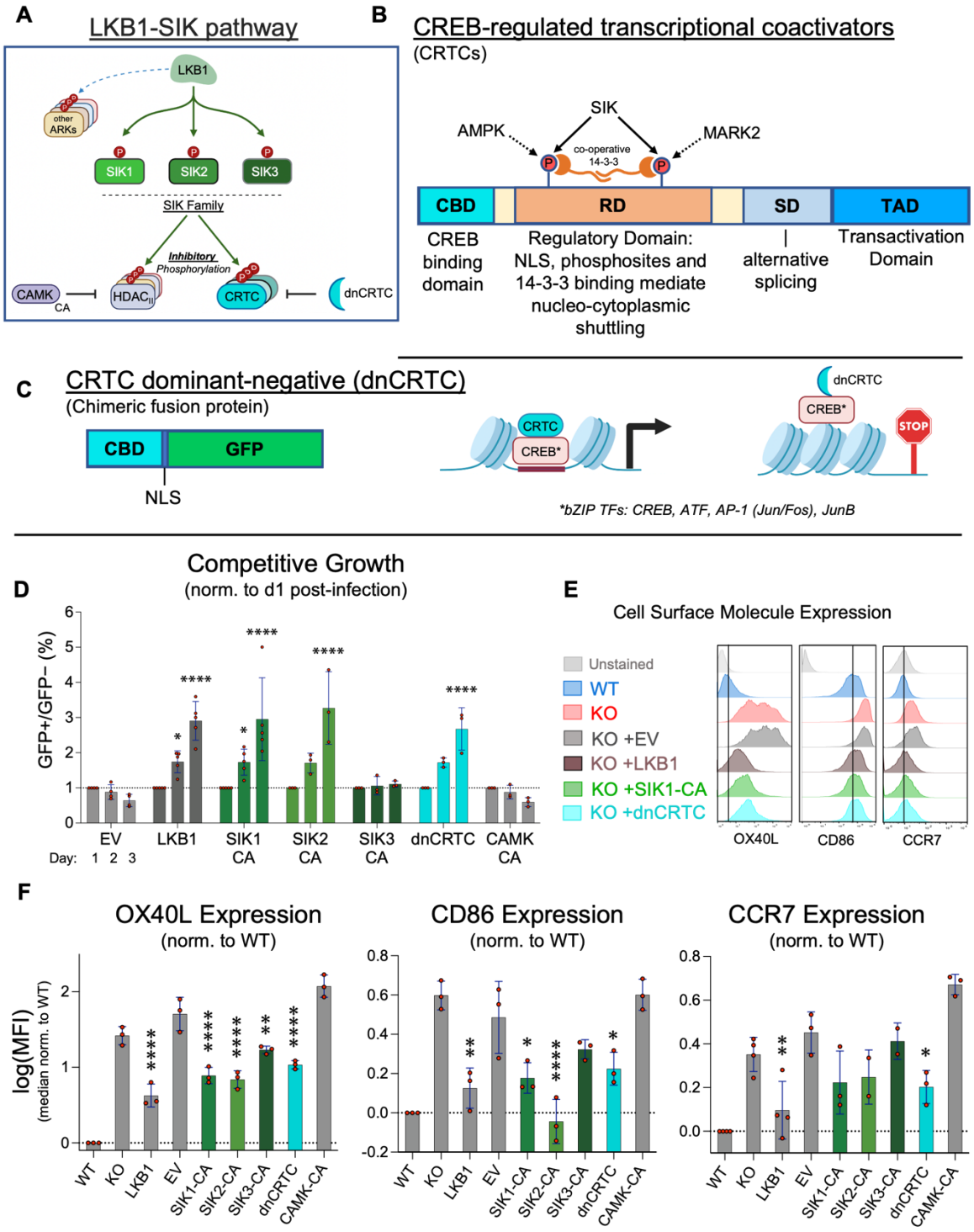


Figure 4: CRTCs are repressed downstream of SIK1 and SIK2 in activated B cells
A. Scheme of major LKB1-SIK downstream pathways **B.** General structure of CRTC(1-3) family. **C.** Structure and expected function of dnCRTC chimeric fusion protein. **D.** Quantification of competitive growth between transduced KO iGBs, (n=3-5). **E.** Representative histograms of OX40L, CD86, and CCR7, with line drawn at MFI of WT iGBs. **F.** OX40L, CD86, and CCR7 expression in transduced KO iGBs normalized to WT control: log(Sample MFI / WT MFI). (OX40L and CD86, n=3), (CCR7, n=2-4) **D.** Two-way ANOVA. **F.** One-way ANOVA. **D.&F.** Mean ±SD are plotted. Dunnett's multiple comparison test, compared to EV control. (*≤0.05, **≤0.01, ***≤0.001, ****≤0.0001). Note that effect of SIK3 on CD86 expression is non-significant compared to EV, but when compared to LKB1-KO, the Dunnett's multiple comparison is significant (*, p=0.0245).

the nucleus[84] (**Figure 4B-C**). We found that dnCRTC rescued the defective proliferation and expression of OX40L, CD86 and CCR7, while CAMK-CA did not (**Figure 4E-F**), suggesting that the LKB1-SIK pathway regulates iGB cell proliferation and expression of OX40L, CD86 and CCR7, by repressing the CRTCs.

8.4. Analysis of publicly available B-cell datasets

In our analysis of different datasets, we found variable transcript expression levels of the CRTCs, but *Crtc2* mRNA was consistently highest (**Figure 3**). This was consistent with our RNAseq data in iGBs.

This analysis also included a previous study of germinal center B cells that characterized a compartment of cells within the light zone ($Bcl6^{lo}$, $CD69^{hi}$) that is marked by a higher affinity BCR and stronger interactions with T cells[5]. Our analysis of RNAseq data from this study showed enrichment of *Stk11* (LKB1), *Sik1* and *Crtc2* in this compartment (**Figure 3D**), suggesting a possible signaling axis in cells receiving strong BCR and CD40 signaling. This may also reflect CREB-CRTC mediated transcription of the *Sik1* gene, which has previously been implicated as a negative feedback mechanism for CREB-CRTC activity[60,61]. However, it must be noted that there are inconsistencies between the different *in vivo* RNA seq datasets, therefore they must be interpreted with caution.

Our analysis also showed evidence for CRTC3 phosphorylation at SIK2 mediated[6] sites (**Figure 3E**). If this reflects SIK activity in B cells, the observed trend shows SIK activity peaking in the gray zone, which was characterized as a proliferative B cell subset that is progressing from the light zone to the dark zone[4]. This could suggest that SIKs respond to BCR and CD40 signaling events in the light zone by phosphorylating CRTCs. It also suggests a potential role for multiple CRTCs in germinal center B cells, not only CRTC2. Notably, the

inability to detect phosphorylated peptides in this dataset (such as LKB1-mediated phospho-SIK peptides) does not indicate that they are not present, it rather reflects the failure to enrich some phosphopeptides during sample processing depending on the TiO_2 enrichment protocol used[7].

8.5. BCR and CD40 signaling activates the LKB1-SIK pathway

We investigated the upstream signals that activate the LKB1-SIK-CRTC axis in B cells by stimulating B cells through the BCR and/or CD40 receptor and assessing various pathway components by western blot. We investigated SIK phosphorylation status at the site phosphorylated by LKB1 (the activation loop), which serves both as a marker of LKB1 activity and SIK activation. We also chose to investigate CRTC2 because it is expressed the highest in iGBs (**Figure 5A**) and other B cell system datasets (**Figure 3A-D**).

In the iGB system, we activated and expanded B cells for 4 days (CD40L, IL-4 and BAFF) and then stimulated the iGBs through the BCR, to model signals that germinal center B cells would receive in the light zone[20] (**Figure 5B**). p-SIK was present in the CD40-stimulated iGBs prior to BCR stimulation. These levels increased after BCR stimulation (**Figure 5C**), suggesting that LKB1 activity is induced in activated B cells receiving BCR signals. In the CRTC2 western blot, we observed modest levels of a lower mobility band corresponding to the phosphorylated form of CRTC2[84,94]. This lower mobility band in the CRTC2 western blot was demonstrated to be phosphorylation-dependent in previously published studies, by treatment of protein lysates with phosphatase, resulting in collapse of CRTC2 into a single band[84,94]. This band was less visible in KO iGBs (**Figure 5C**), possibly reflecting the loss of SIK phosphorylation(s) on the protein after LKB1-loss. Notably, this band can also indicate phosphorylation by kinases that may affect CRTC2 activity in other ways[95,96], which could potentially explain the minor presence of this band in KO iGBs.

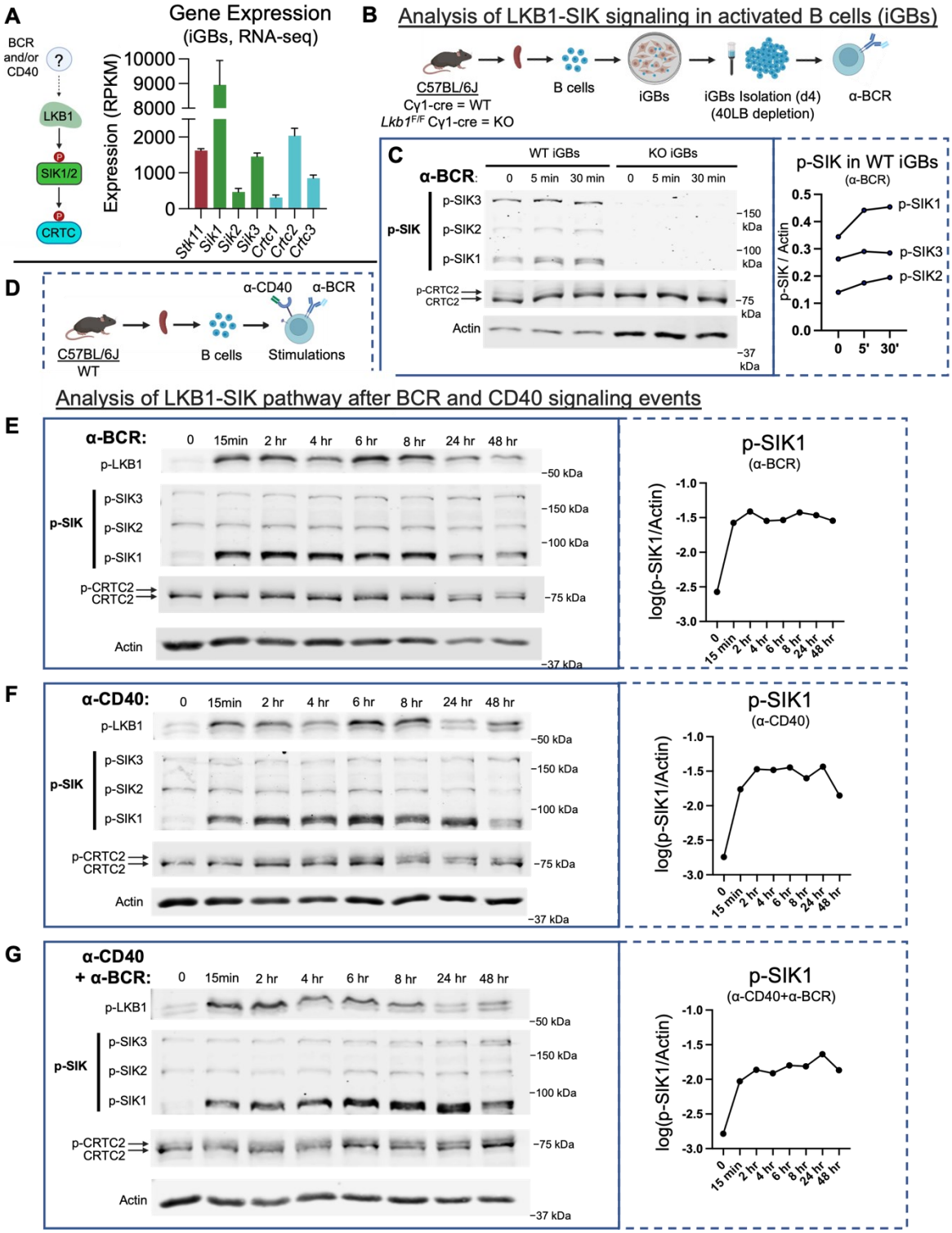
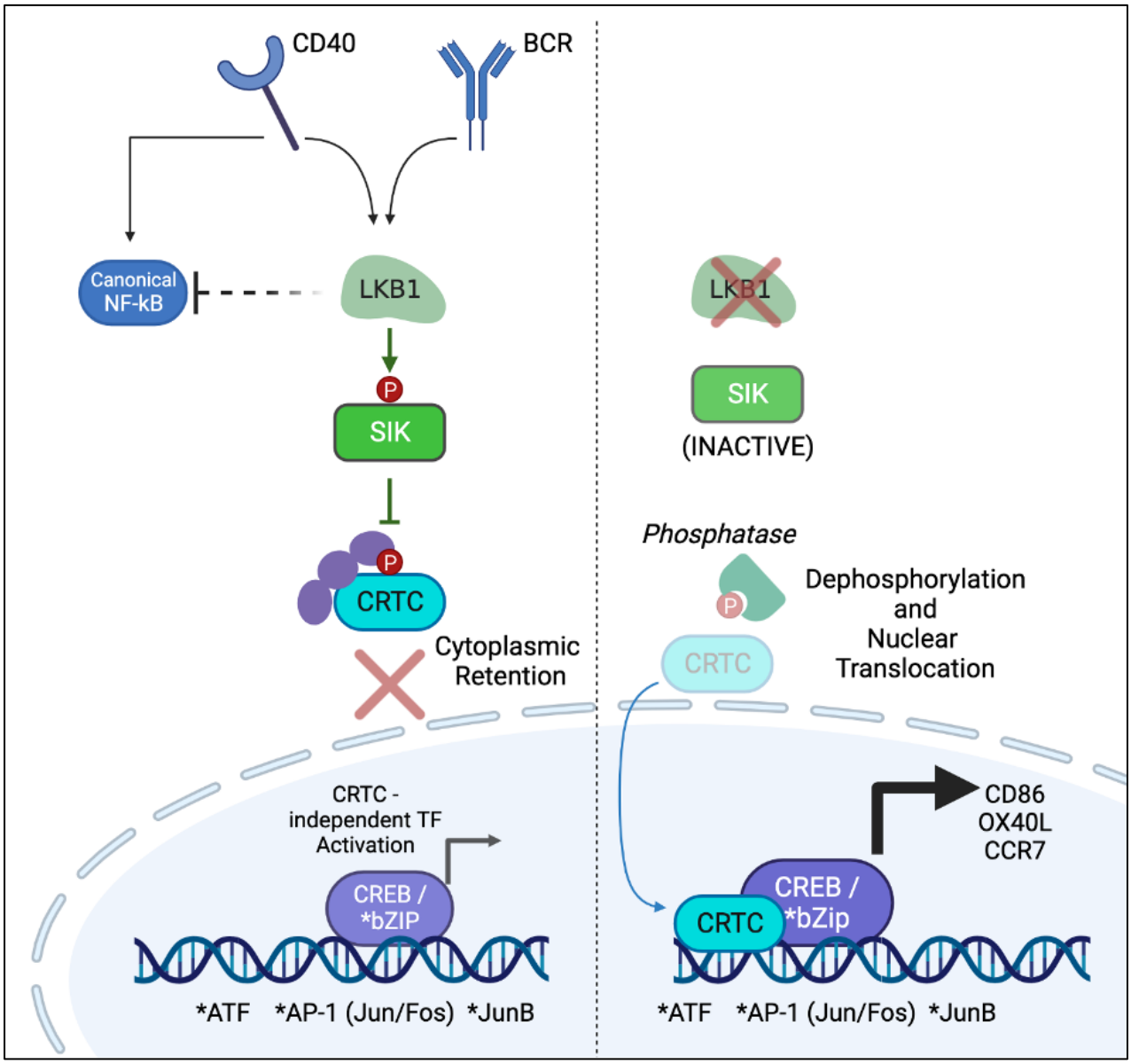


Figure 5: LKB1 responds to BCR and CD40 signaling by phosphorylating SIKs
A. RNA-seq: expression of CRTCs and SIKs in iGBs show SIK1 and CRTC2 are most highly expressed. Scheme of the tested signaling axis is shown on the right. **B.** Protocol for BCR stimulation of iGBs. **C.** WT or KO iGBs were stimulated with αIgM+αIgG (BCR) for the indicated times. The cell lysates were analyzed by western blotting with antibodies to the indicated proteins and phosphorylated (p-) proteins. Quantification of p-SIK1-3/actin is shown on the right (representative of 2 experiments). **D.** Protocol for BCR and/or CD40 stimulation of WT mouse B cells is shown. **E-G.** Primary B cells from WT mice were stimulated with αIgM+αIgG (BCR) and/or αCD40 for the indicated times. The cell lysates were analyzed by western blotting with antibodies to the indicated proteins and phosphoproteins (representative of 3 experiments). Quantification of p-SIK1/actin for the representative blots are on the right.

The next question we sought to address was whether BCR or CD40 signaling activated the LKB1-SIK-CRTC axis during initial B cell activation events. Therefore, we stimulated naive, resting B cells through either the BCR alone, CD40 (+IL4) alone or both BCR and CD40 (+IL4). We also included p-S431-LKB1 in our analysis because it is associated with LKB1 activation[27,62,97-99] downstream of potential BCR and CD40 signaling kinases. We found that within the first 15 minutes of stimulation, p-LKB1 (Ser-431) and p-SIK1 (T182D) levels increased over the baseline (unstimulated cells), while p-SIK2 and p-SIK3 levels did not (**Figure 5D-G**). We also show that this fast activation of the LKB1-SIK1 pathway is sustained for at least 24 hours downstream of either BCR or CD40 signaling. Analysis of CRTC2 levels showed the p-CRTC2 band appearing late after BCR activation (at 24 hrs and 48 hrs), but earlier in the context of stimulations that included CD40 (at ≤8 hrs). While this suggests different kinetics of CRTC2 inactivation in BCR vs CD40 signaling pathways, it could also indicate differential phosphorylation at other sites downstream of BCR or CD40 signaling kinases that may affect CRTC2 activity in other ways [95,96]. Altogether, these results suggest that the LKB1-SIK-CRTC axis is activated downstream of BCR and CD40 signaling events in B cells (**Graphical Figure 2**).

8.6. LKB1 limits canonical NF-kB signaling downstream of CD40

Previously, our laboratory used RNAseq to characterize the transcriptional defects in KO iGBs, which showed an upregulation of genes in the CD40 signaling pathway (**Appendix Figure S4A**). Furthermore, the promoters of upregulated genes were enriched for NF-kB response elements (**Appendix Figure S4B**), suggesting that LKB1 limits NF-kB signaling.



Graphical Figure 2: The LKB1-SIK pathway regulates CRTC activity in B cells
In B cells, the LKB1-SIK pathway is activated downstream of BCR and CD40 signaling pathways. This leads to the phosphorylation and cytoplasmic retention of CRTCs, allowing for regulation of the transcriptional response in activated B cells. A separate effect of the LKB1 pathway is the limiting of canonical NF-kB signaling pathways downstream of the CD40 receptor. When the LKB1-SIK pathway is not active (such as after *Lkb1*-deletion or other contexts), dephosphorylation of CRTCs allows them to translocate to the nucleus where they act as transcriptional coactivators for CREB (and potentially other transcription factors that are indicated*) through interactions with the bZip DNA binding domain.

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To characterize the aspects of NF-kB signaling that are regulated by LKB1 we analyzed NF-kB signaling components in KO iGBs by western blot (**Figure 6A**). We observed an increase in canonical NF-kB signaling components, including increased p65 Ser534 phosphorylation (1.47 fold over control, n=3) (**Figure 6B-E**), but no increase in non-canonical NF-kB signaling components (**Figure 6F**). Additionally, we observed a modest increase in NF-kB induced transcription of a retroviral reporter construct that was introduced to WT and KO iGBs (**Figure 6K**). Together, these results suggest that LKB1 limits the canonical pathway of NF-kB signaling in activated B cells, specifically through increased IKK-β mediated[100] phosphorylation of p65 at Ser534.

Our next question was whether the regulation of OX40L, CD86 and CCR7 expression in iGBs by the LKB1-SIK-CRTC axis depended on NF-kB induced transcription, as the genes encoding OX40L, CD86 and CCR7 contain potential NF-kB recognition motifs in their promoter regions (**Appendix Table 4 - Eukaryotic Promoter Database**[101]). Additionally, it has previously been shown that NF-kB and CRTCs can coexist at some promoters[51]. To answer this question, we treated KO iGBs with inhibitors of canonical NF-kB signaling components (**Figure 6G**). We observed that inhibition of upstream or terminal components of the canonical NF-kB signaling cascade (IKK-β and p65, respectively), had a modest effect on OX40L and CCR7 expression levels, but no effect on CD86 levels (**Figure 6H-I**). Inhibition of the transcription factor c-REL also had no effect (**Figure 6J**). Comparing the effects of the inhibitors to our genetic complementation assay shows a stronger rescue by SIK1-CA (**Figure 6I**). Additionally, canonical NF-kB inhibitors did not rescue defective proliferation (**Appendix Figure S7**). This suggests that the LKB1-SIK-CRTC axis regulates proliferation and the expression of OX40L, CD86 and CCR7 independently of the effect that LKB1 has on canonical NF-kB signaling.

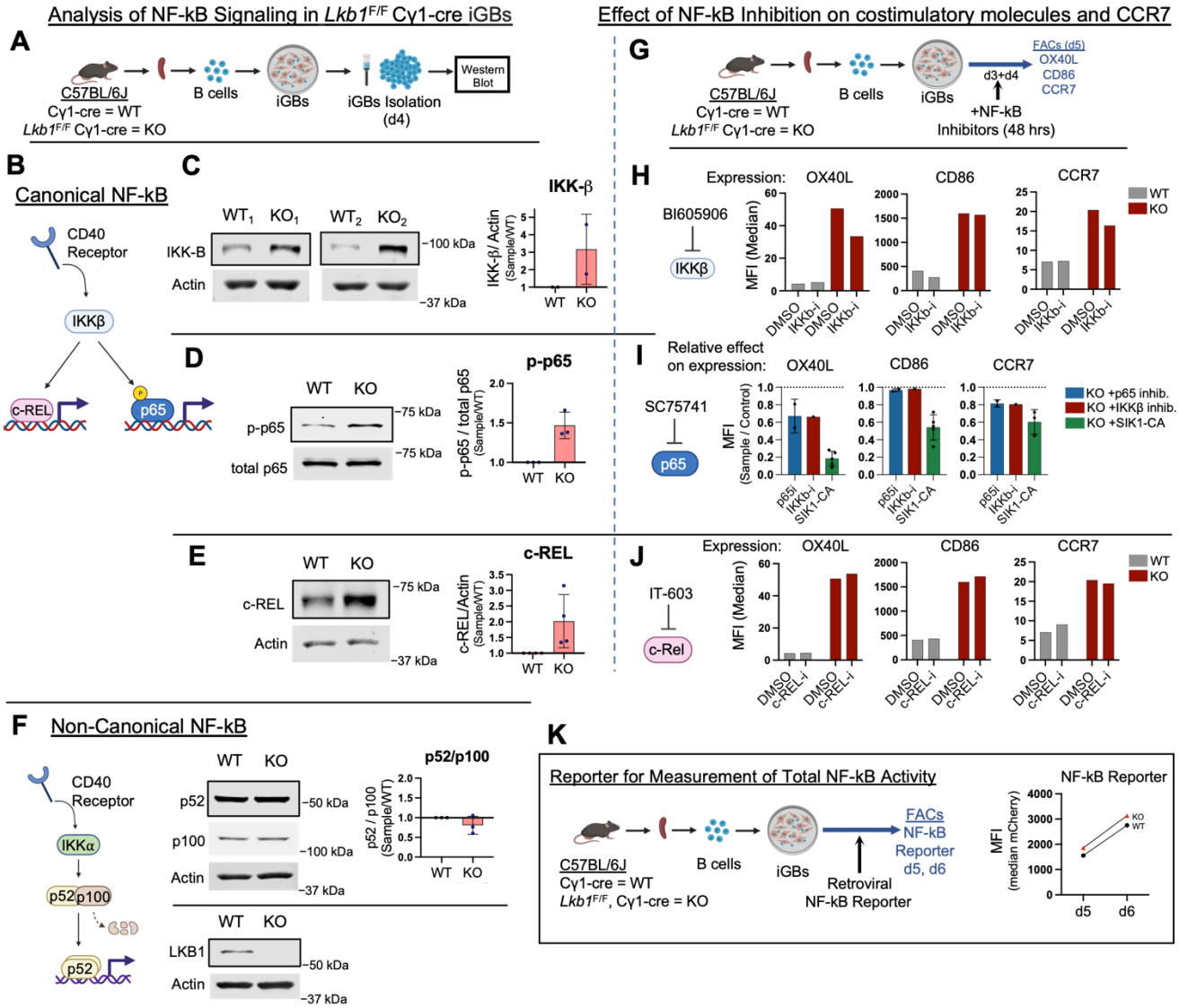

Figure 6: LKB1 limits canonical NF-kB signaling

A. Protocol for analysis of NF-kB signaling in iGBs, used for the experiments shown in C-F. **B.** Scheme of canonical NF-kB pathways assessed. **C-F.** The iGB cell lysates were analyzed by western blotting with antibodies to the indicated proteins and phosphoproteins for canonical (C-E.) and non-canonical pathways (F.). Quantification of fold change from controls is shown on the right. Mean ±SD are plotted. **G.** Protocol for analysis of NF-kB effect on costimulatory molecule and CCR7 expression in iGBs, used for the experiments shown in H-J. **H, J.** OX40L, CD86, and CCR7 expression in WT or KO iGBs treated with indicated NF-kB inhibitor or vehicle (DMSO) for 48hrs before flow-cytometry measurement on day 5. (n=1) **I.** Relative effect (1= no effect) of either treatment with either BI605906 (IKKβ inhibitor, 5 μM) or SC75741 (p65 inhibitor, 1 μM) on OX40L, CD86, and CCR7 expression (MFI) in KO iGBs. Effect of genetic complementation of SIK1-CA (retroviral transduction) is shown for comparison. MFI was measured on day 5 for inhibitor experiments (n=1-2) and on day 6 for SIK1-CA retroviral transduction (n=3-5). **K.** Protocol for analysis of NF-kB activity in iGBs by retroviral reporter construct (pSIRV-NF-kB-mCherry-PEST). Activity was measured in WT or KO iGBs (n=1).

9. Chapter 4: Discussion

B cell activation involves coordinated signaling events that trigger stage-specific changes in B cell surface molecules and proliferative capacity. Expression of B cell surface molecules is finely tuned during activation to control interactions with T cells and the migratory pattern of B cells along chemokine gradients in lymphoid organs. This controls the formation and organization of B and T cells in germinal centers. Thus, signaling nodes that can regulate the precise expression of these molecules during B cell activation are essential for achieving and maintaining the germinal center response. In this report, I focus on a signaling hub kinase that we find to regulate important aspects of B cell activation but remains largely uninvestigated in B cells.

The major transcriptional changes in *Lkb1* KO iGBs indicate that the transcriptional program of activated B cells is regulated by LKB1. In this study, we measured the cell surface molecule expression of OX40L, CD86, and CCR7 as a marker of the genes that are transcriptionally upregulated, and iGB cell proliferation as a marker of the genes that are transcriptionally downregulated. Our genetic complementation experiments suggest that both the transcriptional upregulation and downregulation occurs through a signaling axis involving SIK1/2 and the CRTCs. As we also find that the upregulated genes are dominated by NF-kB targets, we explored the potential role of NF-kB signaling in affecting the expression of the selected cell surface molecules. Our results suggest that the LKB1-SIK1/2-CRTC axis regulates NF-kB target gene transcription independently of the effect that LKB1 has on canonical NF-kB signaling. A major caveat is that we never measure transcription itself in these assays. Therefore, further research will be required to determine the full extent to which the SIK1/2-CRTC pathway contributes to LKB1-regulated transcription in activated B cells.

Interestingly, the proteins that we show are regulated by the LKB1-SIK-CRTC axis are induced during B cell activation with very different kinetics. CCR7 expression occurs in the initial activation stage downstream of BCR signaling, and remains until after the initial B:T interactions, when its expression must be downregulated for before returning to the follicle[11]. CD86 expression is rapidly induced after B cell activation until terminal B cell differentiation, where it is maintained in memory B cells but lost in plasma cells[102]. Finally, the expression of OX40L occurs early in B cell activation during initial B:T interactions, but decreases in germinal center B cells before appearing again later in a limited subset of germinal center B cells[103]. It is possible that the activity of the CRTCs is fine-tuned by LKB1/SIK to regulate the expression pattern of OX40L, CD86, CCR7 and other genes in activated B cells. In this way, LKB1 could coordinate the transcriptional response in B cells according to the signaling cues in the immune microenvironment. Interestingly, we predominately observed increased activation of T_{FR} cells after LKB1-loss in germinal center B cells (data not shown), even though increased costimulatory molecule expression would be expected to increase the production of T_{FH} cells. Thus, LKB1 may regulate the ability of B cells to shape the surrounding immune microenvironment as well. Therefore, further studies should seek to define the precise role of LKB1/SIK and the CRTCs in activated B cells.

We monitored the levels of CRTC2 in this study after BCR and CD40 stimuli, which show the relative levels of phosphorylated CRTC2 (associated with its repression), and unphosphorylated CRTC2 (associated with its activation). Our results suggests that CRTC2 is only repressed late after activation, which unexpectedly does not correlate with the early activation of SIK1. This is still consistent with our findings in *Lkb1* KO and genetically complemented iGBs because we define the LKB1-SIK-CRTC axis late after B cell activation. To

further characterize this process, studies can be performed in activated B cells or iGBs to monitor the kinetics of CRTC activity at longer timepoints after B cell activation. This could be done by directly measuring CRTC localization (by immunofluorescence or subcellular fractionation). This will build on our previous studies because we never measured if the intracellular distribution of CRTC2 is affected, only the phosphorylation, which does not always correlate with its distribution[84]. Additionally, characterization of CRTC2 activity by localization is important because reports indicate that CRTCs can be phosphorylated at residues that promote their function instead of repressing them[95,96].

Compared to our results, a previous study involving the Ramos Human B cell line showed robust phosphorylation of CRTC2 1-hour after BCR stimulation (by western blot) and cytoplasmic retention of CRTC2 (by immunofluorescence) 3-hours after BCR stimulation[104]. This contrasts with our observation of CRTC2 phosphorylation occurring ~24hrs after BCR stimulation, which likely reflects the different signaling mechanisms occurring in immortalized cells compared to primary B cells. However, this suggests that the LKB1-SIK axis is also activated downstream of BCR signaling in this system, albeit with different kinetics.

The regulation of CRTCs is not fully understood, but a recently published study shows that calcium dependent phosphatases (calcineurin) and other phosphatases (PP1 and PP2A) dephosphorylate the CRTCs to activate or maintain their activity[105]. The SIKs were shown to counteract the activation of CRTCs by limiting the quantity of signals transduced by CRTCs[6,47,105]. Therefore, the activity of CRTCs may be precisely regulated by the activation of the LKB1-SIK axis and phosphatases that are activated downstream of BCR and CD40 signaling[106,107]. This may serve to manipulate the expression pattern of genes targeted by the transcription factors that the CRTCs interact with, such as CREB and potentially other bZip

transcription factors[54]. In B cells, CREB is phosphorylated and activated downstream of PKA or calcium signaling pathways[108]. Interestingly, an additional mechanism for CREB activation has been described in other cell types. This allows activation of CREB to bypass the requirement of phosphorylation by interacting with the CRTCs[94,109]. Therefore, a consequence of LKB1-loss may be the aberrant activation of the transcription factor CREB. Moreover, the observation that the CRTCs and CREB are involved in the transcription of a diverse transcription factor repertoire that can regulate broad transcriptional programs in neurons, macrophages, and adipocytes[49,53,60-65], warrants the further investigation of the LKB1-SIK-CRTC axis in regulating transcriptional programs in B cells.

Another remaining question is whether the increase in canonical NF-kB signaling seen by western blot analyses can fully explain the upregulation of genes enriched with the NF-kB transcription factor motif that we find in our RNAseq analysis. The promoters of the genes encoding the cell surface molecules that we analyzed in this study, *Tnfsf4* (OX40L), *Cd86,* and *Ccr7*, all contain NF-kB motifs. However, we find that they are regulated primarily by the LKB1-SIK1/2-CRTC axis, not by increased NF-kB signaling. This suggests that the effect of LKB1 on canonical NF-kB signaling occurs independently of the CRTC-mediated effect on transcription.

A previous report on CRTC2 in B cells contrasts with our findings[110]. Whereas we find that inhibiting CRTCs with a dominant negative rescues activated B cell proliferation after LKB1-loss, another study suggests that CRTC2 activation (downstream of LKB1 inactivation) plays a role in activated B cell proliferation[110]. This was assayed after lentiviral transduction of naive human B cells *ex vivo* with hyperactive CRTC2 or with shRNA targeting the LKB1 transcript, followed by activation with αCD40 and cytokines[110]. An increased proportion of

BrdU+ cells was shown at a single time point, but no other evidence of increased proliferation (cell number, divisions, etc.) was reported[110]. Notably, this analysis of LKB1-downstream pathways differs from our analysis, as our system involves LKB1 deletion and retroviral transduction after B cell activation, not during the resting B cell stage. This is important for LKB1, which has strong context-dependent roles in B cells that are illustrated by the drastically different phenotypes observed in *Lkb1*$^{F/F}$ CD19-cre & CD21-cre mice, which delete LKB1 before or at the resting B cell stage (resulting in spontaneous activation and apoptosis) and *Lkb1*$^{F/F}$ Cγ1-cre mice, which delete LKB1 after activation (resulting in defective proliferation, without any indication of apoptosis). Thus, dysregulation of LKB1-regulated pathways before B cell activation would be expected to produce different phenotypes. For instance, given that LKB1 regulates apoptosis in naive B cells, BrdU could become incorporated in apoptotic cells that are in the S-phase DNA-damage checkpoint[111], potentially explaining the BrdU+ increase even if proliferation was reduced. Additionally, the indication that B cells in *Lkb1*$^{F/F}$ CD19-cre mice showed a strong competitive disadvantage in the resting and germinal center B cell compartments[80] is inconsistent with the proposed interpretation that LKB1 loss led to increased proliferation.

Our results also contrast with the interpretation in this study that LKB1 inhibits the germinal center B cell transcriptional program in favor of the plasma cell fate[110]. This is inconsistent with our findings and those of a previously published study[104] which indicate that BCR signaling stimulates LKB1 to repress CRTC2. BCR signaling occurs during the earliest stages of B cell activation and onwards[13], thus, the LKB1-SIK pathway is likely activated continually in B cells. Taken together with the analysis of the impaired germinal center B cell response in *Lkb1*$^{F/F}$ Cγ1-cre mice, there is a strong indication that LKB1 activity is important in

the germinal center. Therefore, it is unlikely that activation of LKB1 inhibits the germinal center B cell transcriptional program.

Studies have extensively characterized the negative regulation of MTOR by the LKB1-AMPK axis[43]. Interestingly, MTORC1 is a critical component of the germinal center response, where it is activated in B cells by interactions with T_{FH} cells in the light zone[112]. Along with MYC induction, this mediates the positive selection of B cells and their clonal expansion in the dark zone[112]. Consistent with the importance of LKB1 in germinal center B cells, we find that SIK1/2, and not AMPK, are the major downstream substrates of LKB1 in B cells. The mechanism by which LKB1 mediates its substrate specificity in B cells is still unknown, but it likely occurs through mechanisms that regulate the subcellular localization of LKB1 and its substrates. This has previously been described for the activation of LKB1 by AMPK, which requires endosomal/lysosomal membrane association[37]. Similar mechanisms may exist for the SIKs, which could explain the specific activation of SIK1 phosphorylation downstream of BCR and CD40 signaling that we observed. Previous reports in other cell types indicate that SIK1 is predominately nuclear (in the absence of PKA signaling)[47,49,113,114], while SIK2 and SIK3 are predominately cytoplasmic[47]. This suggests a possible role for nucleocytoplasmic shuttling in mediating the LKB1-SIK1 interaction.

Subcellular localization[35,36,115] and protein-protein interactions[33] of LKB1 can be regulated by a multitude of upstream signaling inputs[27,33,36,97,115] that remain uncharacterized in B cells. We show that downstream of BCR and CD40 stimulation, LKB1 is phosphorylated at Ser-431, which can occur either through PKA, RSK, or PKC-ζ[97,98] (**Appendix Figure S2A**). p-S431-LKB1 appears in parallel to the increased phosphorylation of its substrate, SIK1, suggesting possible LKB1 activation through S431 phosphorylation (**Figure 5E-G**). While this

phosphorylation is indeed associated with LKB1 activation[97,98,116]; a mouse knock-in of the S431A phospho-null mutant of LKB1 lacks any apparent phenotype[36], indicating that the function of this phosphorylation is unclear, but is perhaps context-specific, as the residue is highly conserved[36] (**Appendix Figure S3**). Future experiments, such as the structure-function approach that the lab is pursuing (**Appendix Figure S2**), will help answer this question by allowing us to screen the multitude of post-translation modifications of LKB1[27]. Additionally, the use of mouse knock-ins, such as the S431A phospho-null mutant (or other phospho-null mutants) would be an even better approach as they would bypass the limitation of overexpression in our structure function assay.

Although our analysis showed that SIK3 was generally the highest expressed SIK in publicly available RNAseq datasets of germinal center B cells, CA-SIK3 failed to rescue LKB1-dependent phenotypes in our assay. There are limitations to this assay, such as the possibility that the kinase activity of our phosphomimetic mutant is not as effective as it is in SIK1 and SIK2, which rescue the defective phenotypes we see in iGBs after LKB1-loss. Although previous studies in other systems show that the T to D mutant of SIK3 displays a similar level of rescue as SIK2 in their assay[117], we cannot rule out the possibility that the CA-SIK3 mutation is not as effective, as the biochemical kinase activity assay performed in CA-mutants of SIK3 involved the T to E mutation[26], instead of the T to D that we used. This limitation of our phosphomimetic approach may apply to other ARKs that did not rescue as well. However, most of the phosphomimetic ARKs show high activity in peptide assays[26,118-120] except for the T to E phosphomimetic of MARK2/3, which shows low activity against certain peptides[26] but high activity (4-fold increase) against others[119]. Another possible limitation of our assay is that CA-SIK3 may not have been expressed as highly as CA-SIK1/2. The ORF lengths in these constructs

are significantly different (Sik1= 2.3kb, Sik2= 2.8kb, Sik3 = 4.1kb), which affected retroviral transduction efficiency, but we specifically measure transduced B cells in our assay. Unfortunately, attempts to compare the levels of expression through the N-terminal flags of SIK1 and SIK3 failed because we could not recuperate enough transduced B cells for western blot. However, a previous study involving the same three constitutively active SIK mutants showed equal protein expression levels in their system, but only CA-SIK1/2 could rescue defects after LKB1-loss while CA-SIK3 (T221D) could not[62]. This is consistent with our findings, suggesting that there may indeed be a separation of function between SIK1/2 and SIK3.

To characterize the effect of LKB1 on the epigenetic profile of activated B cells, we measured bulk histone H3 modifications by western blot (**Appendix Figure S5**). A previous analysis of RNAseq data comparing WT and KO iGBs indicated that LKB1 is required to maintain the H3K27me3 repressive mark in B cells, as these genes were overexpressed after LKB1-loss (data not shown). LKB1 was also shown to maintain this mark in lung cancer, where loss of LKB1 led to concomitant loss of global H3K27me3 levels and cancer lineage switching[121]. Despite this, we found no difference in the total levels of this mark between WT and KO iGBs. This either indicates an alternative mechanism, or that LKB1 only regulates a fraction of genes containing the H3K27me3 mark in B cells, as bulk western blot would not be able to detect a change in global H3K27me3 levels.

By the same token, we were able to detect a large decrease in H3K27 acetylation levels after LKB1 loss (0.54 fold over control, n=3) (**Appendix Figure S5B-C**), indicating a sizeable genome-wide effect. The H3K27ac reduction implies that LKB1 regulates either the deposition *or* the maintenance of H3K27ac in activated B cells. Although the mechanism remains undefined, this could occur either through an independent LKB1 downstream pathway, or

through LKB1-SIK downstream substrates, the CRTCs, or HDAC-IIAs. As previously discussed, CRTC and CREB can broadly affect transcription in other cell types by inducing the expression of various transcription factors, which can lead to changes in the epigenetic profile[63,122,123]. LKB1, SIK, and the CRTCs are also implicated in metabolic pathways that affect the metabolite pool[124], which includes metabolites such as acetyl-CoA, the primary substrate for acetyl histone modifications[125]. However, we did not find a difference in bulk histone acetylation (H3K(9,14,18,23,27)acetyl), which suggests that this is not the mechanism. In any case, H3K27ac is associated with enhancer formation and transcriptional activation, so this change could potentially relate to the downregulated genes we found in *Lkb1* KO iGBs, including those related to proliferation. It is also possible that the loss of H3K27ac results in the reduced expression of a protein that negatively regulates transcription in activated B cells. This could potentially explain the upregulation of genes, including *Tnfsf4* (OX40L), *Cd86*, and *Ccr7.* Thus, the regulation of H3K27ac by LKB1 warrants further study.

The other major substrate of the SIKs, the HDAC-IIAs, cannot be ruled out because we did not measure H3K27ac in our genetic complementation assay. Furthermore, we cannot be sure that the CA-CAMK used to study the HDAC-IIAs has a specific effect on only the HDAC-IIAs in B cells, which is a current limitation of our assay. This could be tested in the future by using novel class IIa HDAC specific inhibitors[126] and measuring H3K27ac levels. As the HDAC-IIAs have reportedly very low deacetylase function[49], the mechanism would likely involve interactions of the HDAC-IIAs in protein complexes containing other HDACs that do have deacetylase function[127].

10. Chapter 5: Conclusion

In this thesis, we outline a mechanism by which LKB1 regulates the proliferation of activated B cells and the expression of key cell surface molecules related to their migratory pattern and interactions with T cells. We identify that this occurs through the downstream substrates SIK1/2, but not AMPK or the other ARKs. We then identify the CRTCs as the major downstream substrate of the SIKs. Furthermore, we show that the LKB1-SIK pathway is activated downstream of BCR and CD40 signaling pathways. We also provide evidence suggesting that the LKB1-SIK-CRTC axis regulates the expression of the potential NF-kB targets OX40L, CD86, and CCR7 independently of the effect that LKB1 has on canonical NF-kB signaling.

We conclude that the LKB1-SIK pathway is critical during B cell activation because it regulates the precise expression of key genes downstream of the main signaling events occurring in activated B cells. These signaling events trigger stage-specific changes in B cell surface molecules and proliferative capacity, potentially affecting the normal formation and organization of B and T cells in germinal centers.

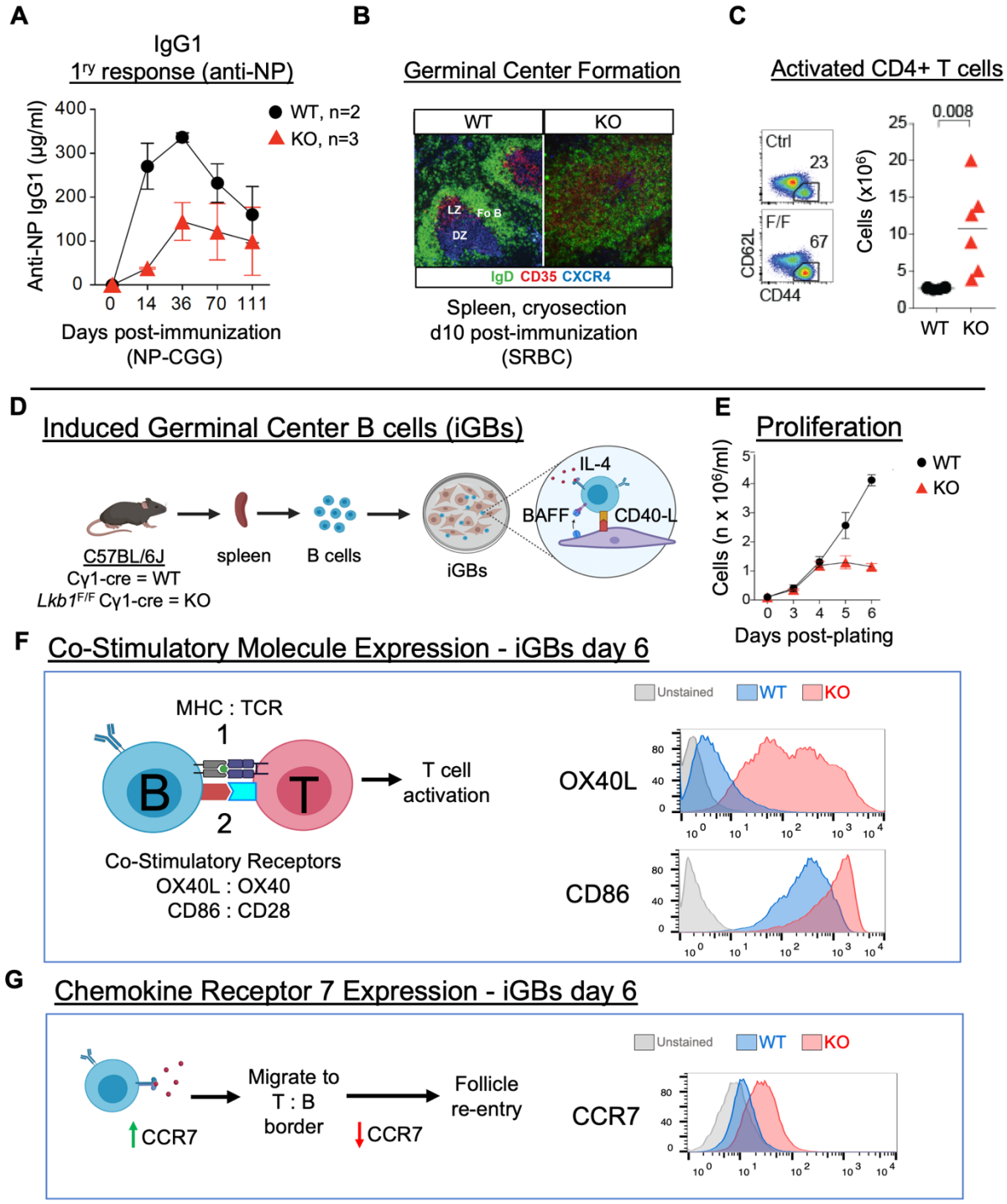

Figure S1: Dysregulation of GCBC traits when LKB1 is deleted from activated B cells

A-C. *In vivo* phenotypes of *Lkb1*$^{F/F}$ Cγ1-cre (KO) mice compared to controls. **A.** Mice immunized i.p. with NP-CGG in alum. ELISA measurement of antigen-specific IgG1 at day 14. **B.** Cryosections of mouse spleens stained for CD35 (Follicular Dendritic cells), IgD (Follicular B cells) and CXCR4 (DZ B cells); day 8 post-immunization (SRBC). **C.** Splenic CD4+ T cells were stained with CD44 (activation marker) and CD62L (naive T cell marker) for flow cytometry, counts of the CD44+, CD62L- population shown; day 8 post-immunization (SRBC). **D-G.** *Ex vivo* phenotypes of activated B cells in KO mice compared to controls **D.** Protocol for generation of iGBs. **E.** Cell count of iGBs grown 6 days in culture. **F.** Expression of OX40L and CD86 on d6 of iGB culture. **G.** Expression of CCR7 on d6 of iGB culture.

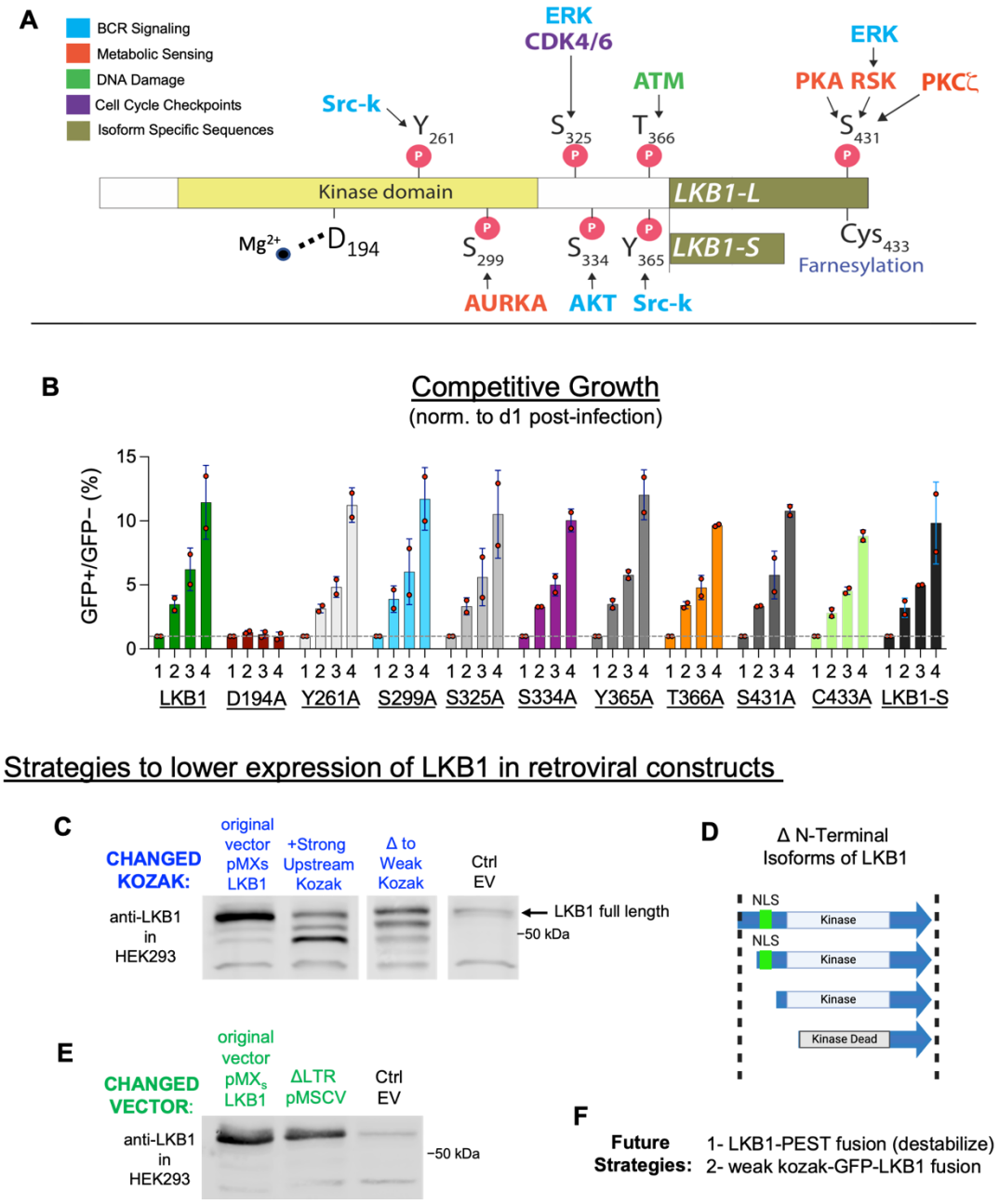

Figure S2: Technical limitations for structure-function analysis of LKB1 by genetic complementation of iGBs

A. A schematic representation of LKB1 is shown, with the phosphorylation sites that were mutated in this report indicated along with their corresponding upstream kinases, which are colored based on the type of signaling input. The cysteine residue (C433) that is farnesylated and the aspartate residue (D194) that binds to Mg2+ (essential for ATP binding and kinase catalytic activity) are also shown. A second isoform (LKB1-Short) only differs in the last 63 amino acids and is indicated below the canonical isoform (LKB1-Long). **B.** Quantification of competitive growth between KO iGBs transduced with indicated constructs (n=2). Catalytically inactive LKB1 (D194A) was used as a negative control. **C.** Various manipulations of the Kozak sequence in pMXs-LKB1-ires-GFP were tested for expression level by transfection into HEK293. The cell lysates were analyzed by western blotting for LKB1. **D.** Depiction of predicted LKB1 isoforms that were translated when Kozak was manipulated. **E.** An alternative vector to pMXs was tested for expression of LKB1 by transfection into HEK293. The cell lysates were analyzed by western blotting for LKB1.

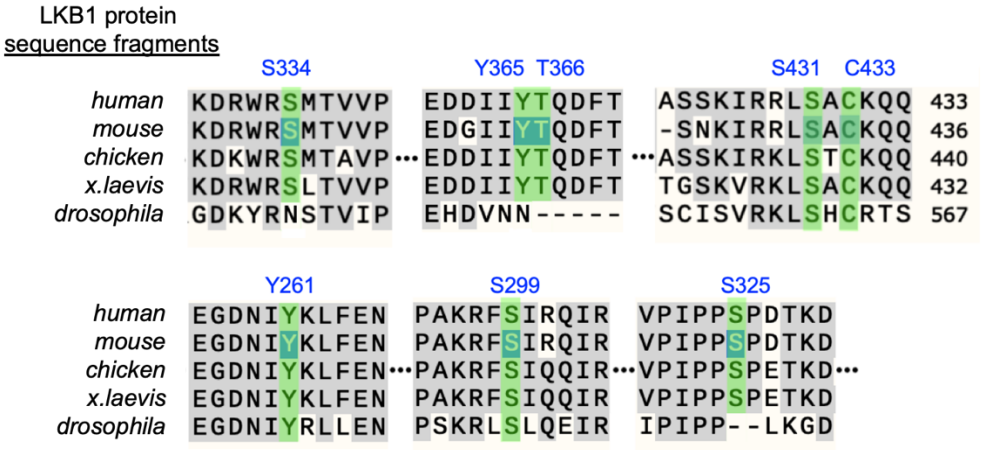

Figure S3: Conservation of LKB1 post-translational modification sites across species

Alignment of LKB1 protein sequence fragments across species showing conservation of phosphorylated and farnesylated residues. Mouse-LKB1 amino acid positions that were mutated in this study are indicated above in blue.

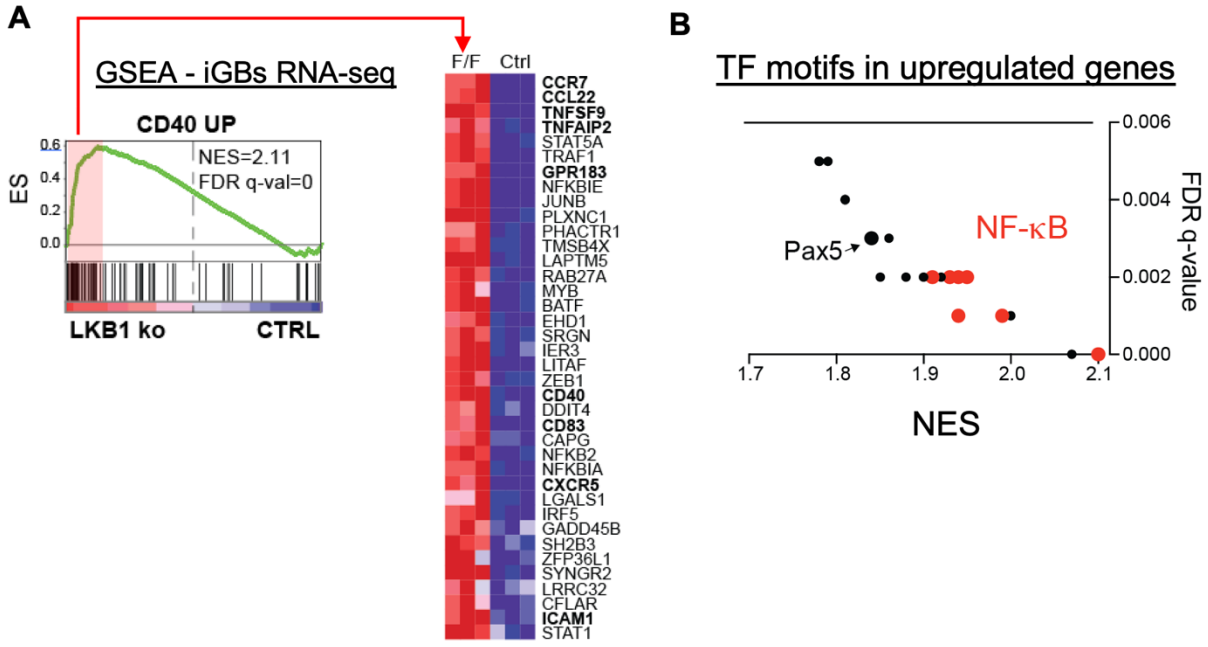

Figure S4: *Lkb1*$^{F/F}$ Cγ1-cre iGBs show upregulated transcription NF-kB targets downstream of CD40 signaling

A. GSEA of CD40 upregulated genes and RNA-seq triplicates of KO vs control. **B.** Transcription factor binding motif analysis in RNA-seq of KO vs control shows enrichment of genes with NF-kB motif.

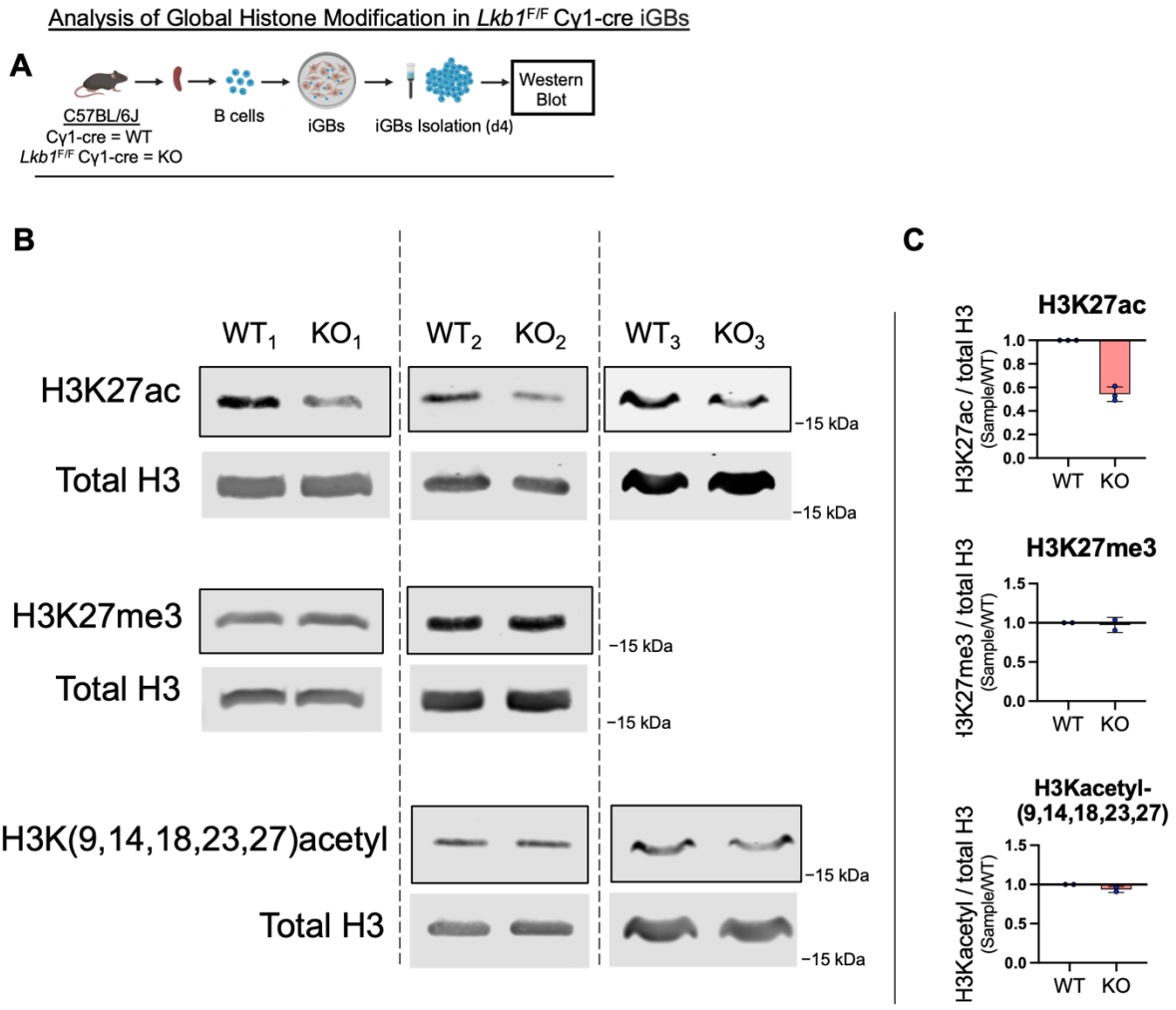

Figure S5: LKB1 is necessary for the maintenance of H3K27ac in activated B cells
A. iGBs protocol for western blots. **B.** The cell lysates from WT or KO iGBs were analyzed by western blotting with antibodies to the indicated proteins (n=2-3). **C.** Quantification of fold change from controls is shown on the right. Mean ±SD are plotted.

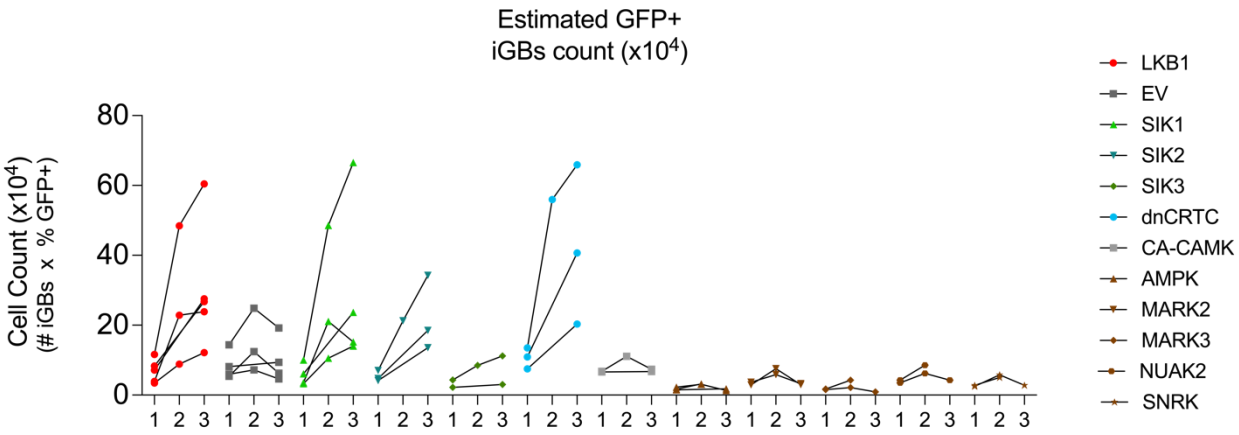

Figure S6: Genetic complementation of LKB1-KO iGBs rescues proliferation
Retroviral infection of KO iGBs with LKB1-SIK axis signaling components leads to increased proliferation compared to the EV control. The number of total iGBs day 1 post-infection was estimated based on the previously determined growth curve of iGBs in a 24-well plate. Total iGBs were counted on day 2 and day 3 post-infection, and the percentage of GFP+ cells determined by flow cytometry was multiplied by the number of total iGBs to estimate the number of GFP+ cells.

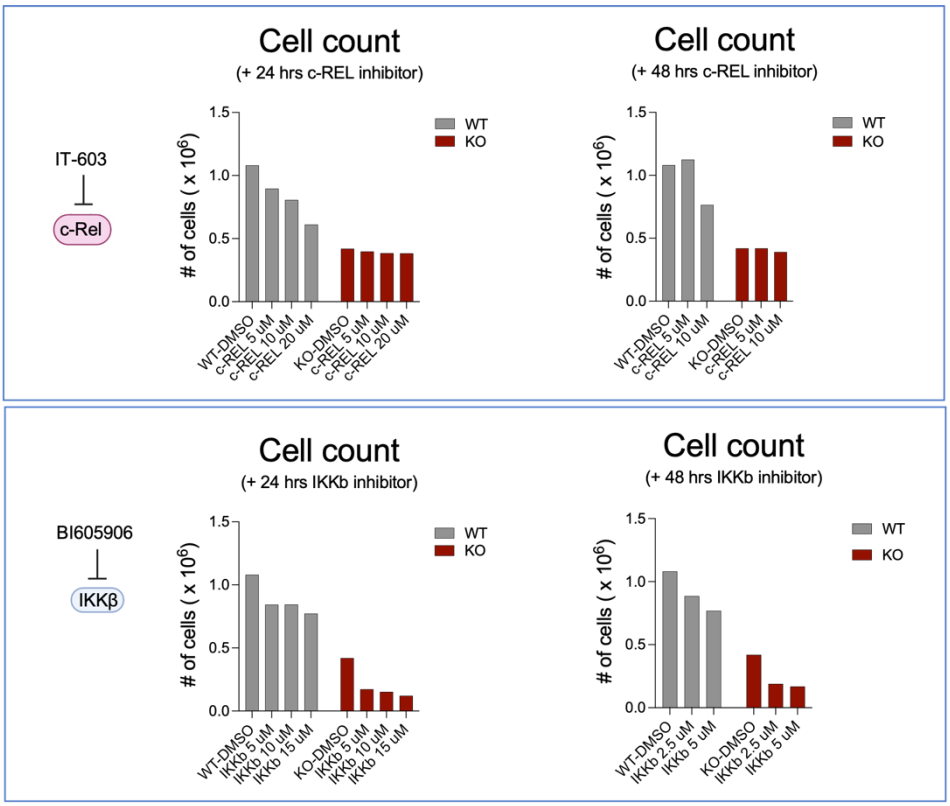

Figure S7: LKB1 regulates B cell proliferation independently of canonical NF-kB regulation
WT or KO iGBs treated with indicated NF-kB inhibitor or vehicle (DMSO) for 24hrs or 48hrs before flow cytometry measurement of cell counts on day 5. (n=1)

Table 1: Recombinant DNA Constructs

Recombinant DNA Identifier	Source
Downstream LKB1 pathway	
pMXs-ires-GFP	Cell Biolabs cat. #RTV-013
pMXs-FLAG-SIK1-T182D-ires-GFP-gateway	This manuscript
pMXs-SIK2-T175D-ires-GFP	This manuscript
pMXs-FLAG-SIK3-T221D-ires-GFP-gateway	This manuscript
pMXs-dnCRTC-NLS-GFP	This manuscript. dnCRTC is AA1-AA55 of mouse CRTC1
pMXs-CAMK-CA-ires-GFP-gateway	This manuscript. CA-CAMK is AA1-AA293 of mouse CAMK-I
pMXs-FLAG-SNRK-T173D-ires-GFP-gateway	This manuscript
pMXs-FLAG-NUAK2-T212D-ires-GFP-gateway	This manuscript
pMXs-FLAG-AMPKα1-T183D-ires-GFP-gateway	This manuscript
pMXs-FLAG-MARK2-T208D-ires-GFP-gateway	This manuscript
pMXs-FLAG-MARK3-T211D-ires-GFP-gateway	This manuscript
Upstream LKB1 pathway	
pMXs-LKB1-ires-GFP-gateway	This manuscript
pMXs-LKB1-S-ires-GFP-gateway	This manuscript
pMXs-LKB1-D194A-ires-GFP-gateway	This manuscript
pMXs-LKB1-Y261A-ires-GFP-gateway	This manuscript
pMXs-LKB1-S299A-ires-GFP-gateway	This manuscript
pMXs-LKB1-S325A-ires-GFP-gateway	This manuscript
pMXs-LKB1-S334A-ires-GFP-gateway	This manuscript
pMXs-LKB1-C433A-ires-GFP-gateway	This manuscript
pMXs-LKB1-T366A-ires-GFP-gateway	This manuscript
NF-kB Reporter	
pSIRV-NFkB-mCHERRY-PEST	Subcloned from Addgene #118093 and Addgene #176626 for this manuscript

Table 2: Antibodies used for Western Blot

Antibody Target	Manufacturer	Catalog #	Dilution
LKB1-SIK-CRTC pathway:			
phospho-LKB1	Cell Signaling	3482T	1:1000 in 5% Milk TBS-T
LKB1	Santa Cruz	sc-32245	1:1000 in 5% Milk TBS-T
phospho-SIK: SIK1 (p-T182) + SIK2 (p-T175) + SIK3 (p-T163)	Abcam	ab199474	1:1000 in 5% Milk TBS-T
CRTC2 (TORC2)	Invitrogen (Thermo)	PA5-34547	1:1000 in 5% Milk TBS-T
NF-kB pathway:			
IKK-β	Biotechne	MAB7155	0.5 ug/mL in 5% BSA TBS-T
p52/p100	Cell signaling	4882	1:1000 in 5% BSA TBS-T
phospho-p65 (Ser536)	Cell signaling	3033	1:1000 in 5% BSA TBS-T
total p65	Cell signaling	6956	1:1000 in 5% BSA TBS-T
c-REL	Biotechne	AF2699	1:2000 in 5% BSA TBS-T
Histone H3 Modifications:			
H3K27me3 (tri-methylation)	Cell Signaling	9733	1:5000 in 5% BSA TBS-T
H3K27ac	Active Motif	39135	1:5000 in 5% BSA TBS-T
Histone H3	Cell Signaling	3638	1:5000 in 5% BSA TBS-T
acetyl H3 K9 + K14 + K18 + K23 + K27	Abcam	ab47915	1:5000 in 5% BSA TBS-T
Loading Control:			
ACTIN	Sigma	A2066	1:20,000 in 5% BSA TBS-T

Table 3: List of Reagents

Reagent	Manufacturer	Use	Stock Concentration	Treatment Concentration
Mitomycin C	AG Scientific and BioShop	Arrest of 40LB growth	2 mg/ml	10 ug/ml
SC75741	MedChem Express	p65 Inhibitor	10 µM	1 µM
IT-603	Sigma	c-Rel Inhibitor	20 µM	5 µM
BI605906	MedChem Express	IKK-beta inhibitor	20 µM	5 µM

Table 4: Transcription Factor Binding Motifs in Selected Genes

Gene Symbol	Protein Name	p65 (REL-A) Motifs Relative to TSS	CREB1 Motifs Relative to TSS	p-value cut-off
Tnfsf4	OX40L	-79*, -143, -584*, -1203, -1297	-1141, -1142, -1528, -1529	0.001 (*≤0.0001)
Cd86	CD86	-185, -225, -549, -1239, -1740, -1901*, -1968	-399, -400, -1717, -1718	
Ccr7	CCR7	-152, -1803	-391, -392, -820, -821, -1646, -1647	

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