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The mTORC1/4E-BP/eIF4E Axis Promotes Antibody Class Switching in B Lymphocytes

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Abstract

During an adaptive immune response, activated mature B cells give rise to antibody secreting plasma cells to fight infection. B cells undergo antibody class switching to produce different classes of antibodies with varying effector functions. The mammalian/mechanistic target of rapamycin (mTOR) signaling pathway is activated during this process and disrupting mTOR complex-1 (mTORC1) in B cells impairs class switching by a poorly understood mechanism. In particular, it is unclear which mTORC1 downstream substrates control this process. Here we used an *in vitro* murine model in which the mTORC1 inhibitor rapamycin, when added after a B cell has committed to divide, suppresses class switching while preserving proliferation. Investigation of mTORC1 substrates revealed a role for eukaryotic translation initiation factor 4E (eIF4E) and eIF4E-binding proteins (4E-BPs) in class switching. Mechanistically, we show that genetic or pharmacological disruption of eIF4E binding to eIF4G reduced cap-dependent translation, which specifically affected the expression of activation-induced cytidine deaminase (AID) protein but not *Aicda* mRNA. This translational impairment decreased antibody class switching independently of proliferation. These results uncover a previously undescribed role for mTORC1 and the 4E-BP/eIF4E axis in AID protein expression and antibody class switching in mouse B cells, suggesting that cap-dependent translation regulates key steps in B cell differentiation.

Introduction:

During a response to infection, B cells become activated to produce different isotypes of antibodies with varying effector functions (1). Early in the immune response, some activated B cells differentiate into plasmablast cells that mainly secrete low affinity immunoglobulin

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M (IgM) antibodies. Others become germinal center B cells and through T-cell-dependent interactions undergo class switch recombination (CSR) to produce other classes of antibodies including IgG, IgA and IgE. During the germinal center reaction, they can also undergo somatic hypermutation (SHM) to diversify and produce higher affinity antibodies. The resulting B cells that survive selection will then either become plasma cells that secrete these antibodies to fight off the infection or become long-lived memory B cells that initiate a faster response during a second infection.

Class switching is initiated when the B cell receptor (BCR) recognizes antigen and B cells are further stimulated through CD40 and cytokine receptors. All of these signals activate the mammalian (also known as mechanistic) target of rapamycin (mTOR). This ubiquitously expressed serine/threonine kinase integrates receptor and nutrient signals to promote many cellular processes including mRNA translation, lipid biogenesis, and nucleotide synthesis. The mTOR kinase forms two complexes, mTOR complex 1 (mTORC1) defined by the Raptor subunit and mTORC2 defined by the Rictor subunit. A major function of mTORC2 is to phosphorylate AKT, a kinase that promotes survival and metabolic reprogramming. mTORC1 is activated downstream of PI3K and AKT and phosphorylates many substrates to promote biosynthetic pathways that support cell growth. Key mTORC1 substrates include S6 kinases and a family of mRNA translation inhibitors known as eIF4E-binding proteins (4E-BPs). Phosphorylation of 4E-BPs by mTORC1 leads to the formation of the eIF4F translational initiation protein complex composed of the cap-binding protein eIF4E, eIF4A helicase and eIF4G scaffold that promote cap-dependent mRNA translation.

The immunosuppressive drug rapamycin (Rap), which allosterically binds and inhibits mTORC1 formation, has long been known to be a potent inhibitor of B cell antibody production (2). Due to the profound anti-proliferative effect of Rap on both B and T cells, it has been difficult to uncouple the roles of mTORC1 in B cell proliferation and differentiation. However, several studies have now presented conclusive evidence that mTORC1 activity is dynamically regulated in germinal center (GC) B cells (3, 4), and that mTORC1 has a B cell-intrinsic role to promote antibody class switching from IgM to IgG and other isotypes (5–8). While these findings highlight the important role of mTORC1 in B cell differentiation, the mechanism by which mTORC1 promotes class switching has not been addressed. Specifically, a key question is which mTORC1 downstream effectors control B cell commitment to isotype switching. Mechanistic investigation of eIF4E activity using both genetic and pharmacological tools show that inhibiting eIF4E decreases antibody class switching and AID protein. Our findings suggest that cap-dependent translation plays a role in class switched antibody production and is a novel mechanism of regulating AID and B cell differentiation.

Materials and Methods:

Mice and reagents

C57Bl/6J (B6) mice were bred at the University of California, Irvine, and used at between 6 and 12 weeks of age. All animals were studied in compliance with protocols approved by the Institutional Animal Care and Use Committees of the University of California, Irvine. Mice carrying an AID-GFP reporter on a B6 background were obtained from the Jackson

Laboratory (stock number 018421). Mice harboring a transgenic allele encoding a constitutively active form of 4E-BP (4E-BP1^{M}) under a tetracycline-responsive element were described previously (18, 44). These mice were crossed to a strain harboring an optimized form of rtTA (rtTA-M2) inserted downstream of the Rosa26 promoter, which was purchased from the Jackson Laboratory (stock number 006965). MLN0128 and MK-2206 were purchased from Active Biochem. Rapamycin was purchased from Cell Signaling Technology. S6K1 inhibitor LY294002 was purchased from Tocris Bio. SBI-756 was synthesized as described (19). Inhibitors were included throughout the indicated cell treatment periods.

Mouse B cell culture

Splenic B cells were purified by negative selection (eBioscience Magnisort Mouse B cell enrichment kit). B cell purity measured by FACS analysis (FACSCalibur and CellQuest software; BD Biosciences) using anti-B220 antibody (Biolegend) yielded >95% B220+. Purified B cells were seeded at a final concentration of 1.5 × 10^6 cells per milliliter. For class switching experiments, B cells were stimulated 5 μg/mL anti-CD40 (HM40–3) agonistic antibody (eBioscience), or 5 μg/mL LPS (Sigma), together with 5 ng/mL mIL-4 (R&D Systems) for 96 h to induce switching to IgG1. 5 μg/mL LPS (Sigma) induced switching to IgG3. 5 μg/mL LPS (Sigma) with 5 ng/mL mIL-4 (R&D), 5 ng/mL mIL-5 (Tonbo Bioscience), 5 nM retinoic acid (Sigma Aldrich) and 5 ng/mL TGF-β (Thermo Fisher Scientific) induced switching to IgA. All cells were cultured with RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated FCS, 5 mM Hepes, 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol.

Western Blotting Analysis

Phospho-AKT S473 (Cell Signaling), phospho-S6 S244/240 (Cell Signaling), phospho-4E-BP-1 T37/46 (Cell Signaling), and AID (eBioscience) protein on western blots were analyzed using ImageJ to quantitate signal of each band. Signal was normalized with actin measurements and fold change was calculated using the stimulated/no drug control.

Immunizations

Mice were immunized i.p. with 100 μL of a final count of 5 × 10^{8} washed SRBCs (Innovative Research) and sacrificed 8 days after immunization. Mice were immunized with 100 μL of NP(18)-LPS (Biosearch Technologies) mixed at a 1:1 ratio with Imject alum (Pierce) at a final concentration of 0.05 mg/mL and sacrificed on day 10. Mice were injected i.p. with 100 μL PBS vehicle, 100 μL at 1 mg/kg, 0.3 mg/kg or 0.075 mg/kg Rapamycin. Blood was collected for serum and spleen was harvested for measuring germinal center and T follicular helper cells.

SRBC-antibody flow cytometry assay

Serum SRBC-antibody production was measured as described in (45).

Immunoglobulin ELISA

For ELISA to measure NP-IgM, Nunc Maxisorp plates (Thermo Fisher) were coated with anti-NP at 10 μg/mL in 50 μL of total sample in PBS and allowed to incubate overnight at 4 °C. Serum was harvested from mice and diluted 1:100 in 2% (wt/vol) BSA in PBS and incubated on coated plates for 1 h at 37 °C. HRP-conjugated rabbit anti-mouse IgM secondary antibody (Zymed) was used.

Flow cytometry, CFSE labeling, and antibodies

Cells were incubated with TruStain fcX in FACS buffer (0.5% BSA + 0.02% NaN3 in 1× HBSS) for 10 min on ice. Staining with antibodies was performed with FACS buffer and on ice for 20 min. Flow cytometry antibodies and other reagents used: B220 (eBioscience, RA3–6B2), IgG1 (BD, A85–1), IgG3 (BD, R40–82), IgA (BD, Cl0–3), IgD (Biolegend, 11–26c.2a), and 7-Aminoactinomycin D (Invitrogen). CFSE or eFluor 670 labeling of B cells was performed by resuspending cells to a concentration of 10×10^6 cells per mL with a concentration of 2.5 μM. Flow cytometric data were analyzed using FlowJo software (TreeStar).

Proximity Ligation assay

eIF4E-eIF4G interactions were detected in situ using the DuoLink detection kit (Sigma Aldrich) according to manufacturer instructions. Mouse anti-eIF4E (BD Biosciences) and Rabbit anti-eIF4G (Cell Signaling) primary monoclonal antibodies were used. Confocal images were acquired on a Leica SP8 fluorescence microscope using the UV laser for DAPI and an excitation laser of 561. PLA and DAPI signal areas were measured by ImageJ. Fold change relative to vehicle was quantified with PLA signal divided by DAPI signal.

Luciferase assay

Cap-dependent translation was measured using the luciferase reporter construct pRSTF-CVB3 containing the 5’ non-coding region of the Coxsackie B3 virus cloned between the firefly and renilla luciferase (46). The construct was electroporated in serum-free media and cells recovered in complete RPMI media for 2 hours followed by inhibitor treatment for 8 hours. Following treatment, cells were lysed and renilla and firefly luciferase expression were measured using the Dual luciferase assay kit (Promega) using a luminometer. Renilla luciferase expression was normalized to Firefly luciferase expression and results were expressed relative to vehicle treated.

RNA Extraction, RT, and Quantitative RT-PCR

RNA was extracted using TRIzol reagent (Invitrogen), followed by purification with Quick-RNA MiniPrep according to the manufacturer’s protocol (Zymo Research). For *Aicda,* purified B cells were stimulated with αCD40+IL4, LPS+IL4, or LPS for 72 hours. For germline transcripts Iγ-Cγ, Iγ3-Cγ3, and Iα-Cα, B cells were stimulated for either 48 hours before harvesting, or with 48hr Rap treatment, an additional 24 hours for a total of 72 hours. cDNA synthesis was carried out on 300 ng to 1 μg of total RNA using iScript Reverse Transcription Supermix (BioRad) for RT-quantitative PCR (qPCR). Gene expression of *Aicda* or germline transcripts was performed using iTaq Universal SYBR Green Supermix

(BioRad), appropriate primers, and StepOnePlus Real-Time PCR System by Applied Biosystems. Mouse L32 was used as the housekeeping gene. Ratio of gene expression change was determined by the delta-(delta-Ct) method.

The following primers were used for qPCR:

Aicda forward primer 5′-TGCTACGTGGTGAAGAGGAG-3′ and reverse primer 5′-TCCCAGTCTGAGATGTAGCG-3′, mL32 forward primer 5′-AAGCGAAACTGGCGGAAAC-3′ and reverse primer 5′-TAACCGATGTTGGGCATCAG-3′, Iγ-Cγ forward primer 5'-TCGAGAAGCCTGAGGAATGTG 3'and reverse primer 5'-GGATCCAGAGTTCCAGGTCACT-3', Iγ3-Cγ3 forward primer 5'-GAGGTGGCCAGAGGAGCAAGAT-3' and reverse primer 5'-AGCCAGGGACCAAGGGATAGAC-3', and Iα-Cα forward primer 5'-CAAGAAGGAGAAGGTGATTCAG-3' and reverse primer 5'-GAGCTGGTGGGAGTGTCAGTG-3'

FOXO nuclear localization assay

Mouse splenic B cells were stimulated with LPS+IL-4 for 48 hours and treated with inhibitors for 4 hours before harvesting. Cells were then fixed in 4% PFA and stained with anti-B220 PE and rabbit anti-FOXO (Cell Signaling) primary and anti-rabbit secondary. Cells were then stained with Hoescht before collection. Samples were analyzed using Amnis ImageStream Mark II Imaging Flow Cytometer (Millipore). FOXO nuclear localization was analyzed using the IDEAS software onboard Nuclear Localization Wizard algorithm. Ratio of Nuclear to Cytoplasmic was determined and expressed as fold change to vehicle.

Results

mTORC1 regulates antibody class switching independently from proliferation

Antibody class switching occurs after activated B cells commit to proliferation (9–12). Since rapamycin inhibits B cell proliferation, further studies were required to establish a separable role for mTORC1 in switching. Experiments in which mice were treated with low doses of Rap after viral infection provided evidence that partial mTORC1 inhibition selectively affects class switching while preserving or enhancing IgM responses that also require B cell clonal expansion (6, 8). In accord, we found that low dose Rap suppressed IgG1 production and GC B cell percentage without reducing IgM responses in mice immunized with the T-dependent antigen sheep red blood cells (SRBC) (Figure 1A-C) or the T-independent antigen NP-LPS (Figure 1D-F). Another study found that deletion of raptor in B cells post-activation (using Cγ1-Cre) suppresses switching to IgG1 while preserving IgM responses *in vivo* (7). Together these findings support the conclusion that mTORC1 promotes class switching in a manner independent from proliferation. This is an important distinction because it may be possible to develop inhibitors of mTORC1 downstream effectors to selectively modulate B cell differentiation without the broad immunosuppressive effects of rapamycin.

We sought to apply *in vitro* methods to study the B cell-intrinsic effects of different genetic and pharmacological perturbations on antibody class switching. Stimulation of purified B

cells *in vitro* with αCD40+IL-4 or LPS+IL-4 mimics T cell-dependent or T cell-independent signals (13, 14), respectively, to induce switching to IgG1. By labeling B cells with the cell division tracker CFSE prior to activation, it is possible to control for differences in proliferation by gating on divided cells (CFSE-lo). In B cells stimulated with αCD40+IL-4, Rap caused a dose-dependent decrease in switching to IgG1 among divided cells (Figure 2A). We used the cyclin-dependent kinase inhibitor palbociclib to suppress proliferation (Figure 2A) without affecting mTORC1 signaling (Figure 2B). Notably, palbociclib did not reduce the % IgG1+ among the residual dividing cells (Figure 2A). These data confirm that the ability of rapamycin to suppress switching is not due simply to slower proliferation.

To identify conditions where complete mTORC1 inhibition does not block proliferation, we tested different time points at which we added 20 nM Rap or 50 nM MLN0128 (a dual mTORC1/mTORC2 inhibitor). We stimulated splenic B cells with αCD40+IL-4 or LPS +IL-4 and added the inhibitors at the same time as activation (0hr addition), or 24 hours after activation (24hr addition), or 48 hours after activation (48hr addition). 0hr addition completely blocked division as expected, while 24hr addition partly reduced division and 48hr addition had no effect (Figure S1A). Western blot confirmed that 48hr addition Rap reduced p-S6 and partially inhibited 4E-BP2 phosphorylation while the mTOR kinase inhibitor MLN0128 completely reduced S6, 4E-BP1, 4E-BP2, and AKT phosphorylation (Figure S1B). Nevertheless, the B cells were still able to divide and seemed to have committed to cell cycle progression as seen by cyclin D2 expression; 0hr rapamycin addition decreased cyclin D2 measured 4 hours after activation, whereas Rap added at 48 hours did not affect ongoing cyclin D2 expression (Figure S1C). 48hr addition of Rap significantly decreased %IgG1+ in divided cells compared to 0.1% DMSO control (Figure 2C, Figure S1D); similar effects were observed over a range of LPS and IL-4 concentrations (data not shown). 48hr addition of MLN0128 did not affect %IgG1+ (Figure S1D), probably due to opposing effects of mTORC1 and mTORC2 inhibition on CSR (5). Over multiple experiments, measurement of proliferation index showed that 48hr addition of Rap had a significant but minor effect on the proliferation of B cells stimulated with αCD40+IL-4 or LPS+IL-4 (Figure S2A). Moreover, 48hr addition of Rap reduced %IgG1+ in each division in cells stimulated with αCD40+IL-4 (Figure S2B). Focusing on cells in the third division showed significantly reduced %IgG1+ in the Rap-treated condition (Figure S2B).

We also tested other stimuli that promote switching to IgG3 (LPS alone) or IgA (LPS +IL-4+IL-5+TGF-β+Retinoic Acid; henceforth referred to as IgA stimuli). 48hr Rap addition reduced %IgG3+ in B cells activated with LPS but did not significantly decrease switching to IgA (Figure 2C). To control for changes in proliferation efficiency, we also measured IgG1+ among cells that have divided three times, as determined by CFSE dilution (Figure S2B). This analysis revealed similar trends with 48hr addition of Rap reducing switching under IgG1 and IgG3 conditions. Using this analysis approach, switching efficiency to IgA was slightly reduced (Figure S2C). Overall, this suggests that the role of mTORC1 in switching is confined to a subset of isotypes but not selective to only IgG1.

To exclude the possibility that the lower percentage of IgG+ cells was due to reduced translation of Ig mRNAs, we measured the surface expression of IgG1 (determined by mean fluorescence intensity, MFI) in cells that did switch following Rap treatment. Notably, the

MFI was not lower than the vehicle treated cells (121.1 ± 40.6 vs. 109 ± 34.1) (Figure 2C). We also assessed the percentage of non-switched B cells (IgD+) and found that 0hr and 48hr addition of Rap increased %IgD+ cells following αCD40+IL-4, LPS+IL-4, or LPS but not IgA stimulation (Figure 2D). This suggests that Rap reduces switching overall rather than rerouting the switch to other antibody isotypes.

Since mTORC1 is a sensor of environmental inputs including nutrients and growth factors, we wanted to test the effect of physiological mTORC1 inhibition by nutrient restriction. Glutamine is used as an energy source by activated lymphocytes (15) and is also required for import of essential amino acids that activate mTORC1 (16). B cells require glutamine to proliferate and undergo Ig synthesis (17); however, the effect of glutamine restriction on antibody class switching has not been tested. Typical lymphocyte cell culture media contains 4 mM of glutamine. We found that 1 and 2 mM glutamine still preserved proliferation and class switching (%IgG1+) (Figure 3A, 3B). Complete glutamine withdrawal strongly reduced proliferation and %IgG1. As glutamine concentration increased to 0.4 mM, proliferation was mostly restored while switching to IgG1 was still reduced, similarly to low concentrations of rapamycin. B cells stimulated in 0.25mM glutamine had reduced mTORC1 activity as measured by pS6 and cell size (Fig. 3C, 3D). Gating on cells in the third division confirmed that decreasing glutamine impairs switching to %IgG1 among cells that have divided similarly (Figure 3E). Overall, these results demonstrated that chemical (Rap) or physiological (glutamine deprivation) inhibition of mTORC1 suppresses class switching independently from proliferation.

Targeting eIF4E function reduces antibody class switching

Two major mTORC1 downstream effectors are known to regulate gene expression: S6Ks and the 4E-BP/eIF4E axis. We assessed the role of these pathways in Ig class switching. To test the effects of S6K inhibition, we treated B cells with an S6K1-selective inhibitor (S6K1i) and confirmed reduced phosphorylation of the direct S6K substrate, ribosomal protein S6 (Figure S3A). S6K1 inhibition also caused increased p-AKT that frequently occurs due to loss of feedback (Figure 3C). S6K1 inhibition significantly reduced switching to IgG1 following αCD40+IL4 or LPS+IL4 stimulation but had no effect in LPS (IgG3) or IgA stimulating conditions when measured in total divided cells or in division 3 (Figure S3B, S3C). These results suggest that S6K1 activity contributes to mTORC1-driven switching under some conditions. Interestingly, S6K1 inhibition partially blocked proliferation with α-CD40+IL4 but not in cells stimulated with LPS+IL-4 (Figure S3D).

Next, we investigated the role of the 4E-BPs, a family of mTORC1 substrates that in non-phosphorylated forms compete with eIF4G for the same binding domain of eIF4E and inhibit eIF4F complex formation. We previously showed that Rap inhibits eIF4E function in lymphocytes more than other cell types, in part due to expression of the 4E-BP2 isoform that is more rapamycin sensitive than 4E-BP1 ((18) and (Figure S1B)). Thus, to test the role of eIF4E in class switching, we used transgenic mice in which a mutant form of 4E-BP1 (4E-BP1M) can be inducibly expressed by doxycycline (DOX) treatment. The 4E-BP1M protein has five S/T sites changed to alanine, such that the protein can no longer be phosphorylated by mTORC1. 4E-BP1M binds more avidly to eIF4E and inhibits initiation of cap-dependent

translation regardless of mTORC1 activity (Figure 4A). When 4E-BP1M is induced prior to B cell activation, proliferation is strongly suppressed (18). Therefore we used a late addition of DOX (48hr) and confirmed 4E-BP1M protein expression by western blotting (Figure 4B). In B cells stimulated with αCD40+IL4 or LPS+IL4 and treated at 48hr addition of increasing DOX concentrations, 4E-BP1M reduced %IgG1+ without reducing proliferation (Figure 4C-F). 4E-BP1M expression also reduced %IgG3+ but not %IgA+ (Figure 4D, 4E, Table I).

To complement this genetic model, we tested the compound SBI-756 which binds the scaffolding protein eIF4G and prevents its interaction with cap-binding protein eIF4E in melanoma cells (19). In B cells stimulated with LPS+IL4, 150 nM SBI-756 partially reduced switching to IgG1 while proliferation was mainly preserved (Figure 5A). As SBI-756 concentration was increased, switching and proliferation were reduced further (Figure 5A). We also tested 48hr addition of SBI-756 (Figure 5B). Again, switching to IgG1 or IgG3 but not IgA was significantly reduced (Figure 5C, 5D, Table I).SBI-756 did slightly reduce cell accumulation under LPS+IL4 and IgA stimulating conditions, however the number of cell divisions was similar under all conditions (Figure 5E). Hence, direct inhibition of eIF4F function does not affect ongoing B-cell proliferation, yet specifically inhibits Ig class switching. This selective effect is consistent with previous studies showing that 4E-BP/eIF4E axis affects the translation of subsets of specific mRNAs in a cell context-specific manner (20–22).

Rapamycin and SBI-756 disrupt cap-complex formation

To confirm that SBI-756 disrupts the cap-binding complex and to assess the effect of Rap, we used an *in situ* proximity ligation assay (PLA) (23). Analysis by confocal microscopy allows visualization and quantitation of the interactions between eIF4E and eIF4G (Figure 6A) in activated B cells. We stimulated B cells with LPS+IL-4 and used the mTOR kinase inhibitor, MLN0128, as a control since this compound strongly disrupts eIF4E interaction with eIF4G (18). MLN0128 (100 nM), Rap (2 nM or 20 nM) and SBI-756 (300 nM) all significantly reduced eIF4E:eIF4G association compared to DMSO vehicle control (Figure 6A). In activated B cells treated with Rap or SBI-756 at 48hr and tested 4hr later, we observed trends of reduced PLA signal that were not statistically significant (data not shown). To increase sensitivity, we performed a dual luciferase reporter assay using a bicistronic dual Renilla-Firefly luciferase reporter construct. This construct measures cap-dependent translation by synthesis of the Renilla luciferase as well as cap-independent, polio IRES mediated translation by synthesis of firefly luciferase as an internal control. In B cells activated with LPS+IL-4, 48hr addition of Rap or SBI-756 significantly reduced cap-dependent translation by 30–40% compared to vehicle (Figure 6B).

Rapamycin and SBI-756 reduce AID protein but not *Aicda* mRNA

Overall, these results suggest that cap-dependent translation is necessary for CSR. To identify at which step of the recombination event the mTORC1/eIF4E axis is involved, we first investigated the production of germline transcripts. When a B cell is activated, secondary signals such as IL-4, TGF-β and IFN-γ can direct the class of antibody to switch by promoting production of non-coding sterile RNAs (germline transcripts) encoded by the

DNA of the accepter switch region. During transcription elongation of the region, the chromatin becomes more accessible for the CSR machinery to facilitate recombination to the targeted switch region (24). Using RT-PCR we measured germline transcripts Iγ-Cγ, Iγ3-Cγ3, and Iα-Cα, which are produced from IgG1, IgG3, and IgA switch regions respectively. As expected, Iγ-Cγ was detected under αCD40+IL-4 or LPS+IL-4 stimulating conditions but not under IgG3 (LPS only) or IgA conditions (Figure S4A). Similarly, Iγ3-Cγ3 and Iα-Cα were detected only under IgG3 or IgA switching conditions, respectively. In all conditions, 0hr addition of Rap strongly inhibited germline transcript production (Figure S4A), consistent with a study of Raptor-deficient B cells (7). S6K1 inhibition reduced Iγ-Cγ transcripts in B cells stimulated with LPS+IL-4 (Figure S3E), suggesting a novel role for S6K1 in mTORC1-driven germline transcription under these conditions. However, the S6K1 inhibitor did not reduce germline transcripts under αCD40+IL-4, IgG3 or IgA switching conditions (Figure S3E). We also tested 48hr addition of Rap or SBI-756 which only reduced germline transcript production in LPS+IL-4 and IgA stimulating conditions but not with αCD40+IL-4 or LPS (Figure S4B). These results did not follow the same trends in our flow-based class switching experiments (Table I). This lack of correlation suggest that germline transcript production is not the mechanism by which mTORC1 activity and cap-dependent translation regulates class switching at the 48hr timepoint.

Class switching requires activation-induced cytidine deaminase (AID), a DNA mutator protein that initiates CSR by introducing mutations into the targeted switch regions of the Ig heavy chain locus (25, 26). Western blotting (Figure 7A) showed that Rap added at 0hr or 48hr timepoints after LPS+IL4 stimulation significantly reduced AID protein amounts. We used a GFP knock-in reporter of AID (AID-GFP mice) (27) to measure AID protein amounts using flow cytometry. Under all stimulating conditions, we found that 0 hr addition of a low concentration of Rap (0.2 nM) reduced AID expression per cell and that 48hr addition of Rap or SBI-756 also significantly reduced expression (Figure 7B, C, Table I). The AKT inhibitor MK-2206 was used as a control to increase AID expression (5).

Reduced AID expression under IgA conditions was surprising given that 48hr addition of Rap or SBI-756 did not affect switching to IgA. We tested the effect of adding Rap at earlier timepoints (24, 40, and 44 hr) and found that switching to IgA was minimally affected even when Rap reduced proliferation (data not shown). However, Rap did reduce %IgG3+ in the same experiment, suggesting that the reduction in AID mainly affects switching to other isotypes under the IgA stimulating conditions (data not shown).

To determine if AID expression is regulated post-transcriptionally in activated B cells, we compared changes to *Aicda* mRNA and AID protein. Under LPS+IL4 stimulating conditions, 0hr addition of Rap strongly reduced *Aicda* mRNA (Figure 7D), consistent with studies of B cells lacking Raptor (7). However, 48hr Rap addition had a lesser effect, and did not reduce *Aicda* mRNA under αCD40+IL4 or LPS stimulating conditions. Notably, 48hr addition of SBI-756 did not reduce *Aicda* mRNA under any switching conditions. Considering that Foxo proteins promote *Aicda* transcription (28) and Rap treatment can lead to increased AKT activity, we used ImageStream analysis to determine whether Rap reduced nuclear localization of Foxo1 in activated B cells (Figure S4C). Instead we found that 48hr addition of either Rap or SBI-756 had the opposite effect, increasing the ratio of

nuclear:cytoplasmic Foxo1 (Figure S4D). Together these data suggest that the reduction in AID protein is not due to a decrease in *Aicda* transcription.

Discussion

Following the initial discovery of rapamycin as a potent immunosuppressant over 40 years ago (29), this natural product was found to inhibit proliferation and impair differentiation of both T and B lymphocytes (2, 30, 31). High doses of rapamycin profoundly impair antibody production (32), yet low doses have a selective effect on class switched antibody responses (6–8). mTORC1 is dynamically regulated in GCs (3) and responsive to environmental conditions such as hypoxia (4); furthermore, genetic deletion of Raptor has established that the mTORC1 complex has a B cell-intrinsic role in antibody class switching (7). However, the mechanisms by which mTORC1 regulates class switching remain unknown. Here, we show that Rap reduces eIF4E and eIF4G association to inhibit cap-dependent translation in activated B cells, and that directly inhibiting eIF4E activity can also reduce antibody class switching. We also show that the mechanism involves post-transcriptional inhibition of AID protein expression, which is a novel mechanism of regulating AID and B cell differentiation.

We reported previously that the 4E-BP/eIF4E axis is rapamycin-sensitive in lymphocytes and required for both growth and proliferation following antigen receptor stimulation (18). Supporting the role of eIF4E in B cell proliferation, we found that 0hr addition of SBI-756 suppresses B cell proliferation and eIF4E:eIF4G association. However, mTORC1/eIF4E activity is not necessary for proliferation 48 hours after activation. The finding that ongoing proliferation is mTORC1-independent is consistent with early studies showing lesser effects of Rap when added at late time points after B or T cell activation (2, 33). More recent work has confirmed this *in vivo* by showing that mTORC1 activity is not necessary to drive B cell proliferation during the dark zone stages of the germinal center (3). Since mTORC1 is active in light zone B cells (3) and required for IgG1 switching *in vivo* (7), it will be interesting to see how eIF4E signaling regulates GC differentiation. We tested SBI-756 in the SRBC immunization model and did not observe any differences in IgG1 secretion or GC B cell percentages (data not shown). This might be due to the pharmacokinetics of SBI-756 *in vivo*, which might not facilitate sustained inhibition of the eIF4E:eIF4G interaction. Further studies with genetic models in which mutations disrupt eIF4G and eIF4E binding will be useful to address this question and to study B-cell intrinsic effects.

We found that 48 hour addition of rapamycin or SBI-756 reduced switching to IgG3 and IgG1, but did not reduce IgA switching even though AID protein was reduced. One possible explanation is that the cells switching to IgA are enriched in marginal zone and B-1 cells (34) and that mTORC1 might not regulate AID and switching in these subsets. Marginal zone cells make up about 10% of B cells purified from the spleen and perhaps the overall AID being produced is outweighed by the reduction of AID in the follicular B cells.

B cell activation is accompanied by rapid and sustained activation of S6 kinases (35); however, little is known about the role of S6Ks in B cell function. Consistent with our previous work (18), we show here that S6K1 inhibition has minimal effect on proliferation of B cells stimulated with αIgM+IL4. However, S6K1 inhibition did partially reduce

proliferation induced by αCD40+IL4 and diminished switching to IgG1. While S6K1 inhibition may be partially responsible for the effects of early Rap addition on antibody class switching, additional studies are required to better understand how S6K1 responds under the different stimuli to promote switching or proliferation.

Our findings demonstrate that the mTORC1/eIF4E axis plays a novel role in B cell differentiation. We and others have previously published that PI3K/AKT signals are involved in B cell differentiation and transcription of AID (5, 36, 37). However, the predominant role of PI3K/AKT in activated B cells is to oppose CSR as determined by treating cells with PI3K/AKT inhibitors or by deleting PTEN (5, 36, 37). The uncoupling of PI3K/AKT and mTORC1 function in B cell differentiation may be due in part to mTORC1 receiving signals from other inputs (35), along with the independent and opposing role of mTORC2/AKT/FOXO in CSR (5, 28). The finding that AID expression can be regulated post-transcriptionally by mTORC1 illustrates the need for better understanding and investigation of the translatome during B cell differentiation and how different signals control translation efficiency of different mRNAs. The 4E-BP/eIF4E axis downstream of mTORC1 has been studied extensively in cancer and shown to preferentially promote translation of mRNAs involved in metastasis and invasion, and growing evidence supports a key role for regulated translation in CD4 and CD8 T cell differentiation (38, 39). However, which mRNAs in activated B cells are regulated by translation efficiency remains an important question in the field. Additional experiments are required to establish whether AID, and other proteins involved in class switch recombination, are regulated at the level of translation. Cap-dependent translation is an attractive target for cancer therapy and efforts are underway to develop small molecule inhibitors of eIF4F components (eIF4E, eIF4G, eIF4A) for oncology (19, 23, 40–42). Further study of this pathway may provide mechanistic insight into how the immune system will be affected by eIF4F-targeted therapies. Indeed, the finding that the eIF4G antagonist SBI-756 reduces AID protein suggests that it may be useful as a combination therapy with idelalisib, a PI3Kδ inhibitor, which increases AID expression and mutational activity in activated B cells and in human patients with chronic lymphocytic leukemia (43). Further studies are needed to determine whether eIF4F inhibitors suppress AID expression in human B leukemia and lymphoma cells.

Our findings emphasize that 4E-BP/eIF4E can regulate expression of important proteins necessary for B cell differentiation. Study of this pathway may provide mechanistic insight into antibody-mediated autoimmune diseases such as lupus and arthritis as well as the formation of protective antibody responses following vaccination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Bibliography

1. Limon JJ, and Fruman DA 2012 Akt and mTOR in B Cell Activation and Differentiation. Front Immunol 3: 228. [PubMed: 22888331]
2. Aagaard-Tillery KM, and Jelinek DF 1994 Inhibition of human B lymphocyte cell cycle progression and differentiation by rapamycin. Cell Immunol 156: 493–507. [PubMed: 7517796]
3. Ersching J, Efeyan A, Mesin L, Jacobsen JT, Pasqual G, Grabiner BC, Dominguez-Sola D, Sabatini DM, and Victora GD 2017 Germinal Center Selection and Affinity Maturation Require Dynamic Regulation of mTORC1 Kinase. Immunity 46: 1045–1058.e6. [PubMed: 28636954]
4. Cho SH, Raybuck AL, Stengel K, Wei M, Beck TC, Volanakis E, Thomas JW, Hiebert S, Haase VH, and Boothby MR 2016 Germinal centre hypoxia and regulation of antibody qualities by a hypoxia response system. Nature 537: 234–238. [PubMed: 27501247]
5. Limon JJ, So L, Jellbauer S, Chiu H, Corado J, Sykes SM, Raffatellu M, and Fruman DA 2014 mTOR kinase inhibitors promote antibody class switching via mTORC2 inhibition. Proc Natl Acad Sci U S A 111: E5076–85. [PubMed: 25385646]
6. Ye L, Lee J, Xu L, Mohammed A-U-R, Li W, Hale JS, Tan WG, Wu T, Davis CW, Ahmed R, and Araki K 2017 mTOR Promotes Antiviral Humoral Immunity by Differentially Regulating CD4 Helper T Cell and B Cell Responses. J Virol 91.
7. Raybuck AL, Cho SH, Li J, Rogers MC, Lee K, Williams CL, Shlomchik M, Thomas JW, Chen J, Williams JV, and Boothby MR 2018 B Cell–Intrinsic mTORC1 Promotes Germinal Center–Defining Transcription Factor Gene Expression, Somatic Hypermutation, and Memory B Cell Generation in Humoral Immunity. The Journal of Immunology.
8. Keating R, Hertz T, Wehenkel M, Harris TL, Edwards BA, McClaren JL, Brown SA, Surman S, Wilson ZS, Bradley P, Hurwitz J, Chi H, Doherty PC, Thomas PG, and McGargill MA 2013 The kinase mTOR modulates the antibody response to provide cross-protective immunity to lethal infection with influenza virus. Nat Immunol 14: 1266–1276. [PubMed: 24141387]
9. Tangye SG, Ferguson A, Avery DT, Ma CS, and Hodgkin PD 2002 Isotype switching by human B cells is division-associated and regulated by cytokines. J Immunol 169: 4298–4306. [PubMed: 12370361]
10. Hodgkin PD, Lee JH, and Lyons AB 1996 B cell differentiation and isotype switching is related to division cycle number. J Exp Med 184: 277–281. [PubMed: 8691143]
11. Hasbold J, Lyons AB, Kehry MR, and Hodgkin PD 1998 Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4. Eur J Immunol 28: 1040–1051. [PubMed: 9541600]
12. Rush JS, Liu M, Odegard VH, Unniraman S, and Schatz DG 2005 Expression of activation-induced cytidine deaminase is regulated by cell division, providing a mechanistic basis for division-linked class switch recombination. Proc Natl Acad Sci U S A 102: 13242–13247. [PubMed: 16141332]
13. Bai L, Deng S, Reboulet R, Mathew R, Teyton L, Savage PB, and Bendelac A 2013 Natural killer T (NKT)-B-cell interactions promote prolonged antibody responses and long-term memory to pneumococcal capsular polysaccharides. Proc Natl Acad Sci U S A 110: 16097–16102. [PubMed: 24043771]
14. Gaya M, Barral P, Burbage M, Aggarwal S, Montaner B, Warren Navia A, Aid M, Tsui C, Maldonado P, Nair U, Ghneim K, Fallon PG, Sekaly R-P, Barouch DH, Shalek AK, Bruckbauer A, Strid J, and Batista FD 2018 Initiation of Antiviral B Cell Immunity Relies on Innate Signals from Spatially Positioned NKT Cells. Cell 172: 517–533.e20. [PubMed: 29249358]
15. Brand K, Fekl W, von Hintzenstern J, Langer K, Luppa P, and Schoerner C 1989 Metabolism of glutamine in lymphocytes. Metab Clin Exp 38: 29–33. [PubMed: 2569663]
16. Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B, Yang H, Hild M, Kung C, Wilson C, Myer VE, MacKeigan JP, Porter JA, Wang YK, Cantley LC, Finan PM, and Murphy LO 2009 Bidirectional transport of amino acids regulates mTOR and autophagy. Cell 136: 521–534. [PubMed: 19203585]
17. Crawford J, and Cohen HJ 1985 The essential role of L-glutamine in lymphocyte differentiation in vitro. J Cell Physiol 124: 275–282. [PubMed: 4044655]

18. So L, Lee J, Palafox M, Mallya S, Woxland CG, Arguello M, Truitt ML, Sonenberg N, Ruggero D, and Fruman DA 2016 The 4E-BP-eIF4E axis promotes rapamycin-sensitive growth and proliferation in lymphocytes. Sci Signal 9: ra57. [PubMed: 27245614]
19. Feng Y, Pinkerton AB, Hulea L, Zhang T, Davies MA, Grotegut S, Cheli Y, Yin H, Lau E, Kim H, De SK, Barile E, Pellecchia M, Bosenberg M, Li J-L, James B, Hassig CA, Brown KM, Topisirovic I, and Ronai ZA 2015 SBI-0640756 Attenuates the Growth of Clinically Unresponsive Melanomas by Disrupting the eIF4F Translation Initiation Complex. Cancer Res 75: 5211–5218. [PubMed: 26603897]
20. Thoreen CC, Chantranupong L, Keys HR, Wang T, Gray NS, and Sabatini DM 2012 A unifying model for mTORC1-mediated regulation of mRNA translation. Nature 485: 109–113. [PubMed: 22552098]
21. Gandin V, Masvidal L, Hulea L, Gravel S-P, Cargnello M, McLaughlan S, Cai Y, Balanathan P, Morita M, Rajakumar A, Furic L, Pollak M, Porco JA, St-Pierre J, Pelletier J, Larsson O, and Topisirovic I 2016 nanoCAGE reveals 5' UTR features that define specific modes of translation of functionally related MTOR-sensitive mRNAs. Genome Res 26: 636–648. [PubMed: 26984228]
22. Hsieh AC, Liu Y, Edlind MP, Ingolia NT, Janes MR, Sher A, Shi EY, Stumpf CR, Christensen C, Bonham MJ, Wang S, Ren P, Martin M, Jessen K, Feldman ME, Weissman JS, Shokat KM, Rommel C, and Ruggero D 2012 The translational landscape of mTOR signalling steers cancer initiation and metastasis. Nature 485: 55–61. [PubMed: 22367541]
23. Boussemart L, Malka-Mahieu H, Girault I, Allard D, Hemmingsson O, Tomasic G, Thomas M, Basmadjian C, Ribeiro N, Thuaud F, Mateus C, Routier E, Kamsu-Kom N, Agoussi S, Eggermont AM, Désaubry L, Robert C, and Vagner S 2014 eIF4F is a nexus of resistance to anti-BRAF and anti-MEK cancer therapies. Nature 513: 105–109. [PubMed: 25079330]
24. Xu Z, Zan H, Pone EJ, Mai T, and Casali P 2012 Immunoglobulin class-switch DNA recombination: induction, targeting and beyond. Nat Rev Immunol 12: 517–531. [PubMed: 22728528]
25. Muramatsu M, Nagaoka H, Shinkura R, Begum NA, and Honjo T 2007 Discovery of activation-induced cytidine deaminase, the engraver of antibody memory. Adv Immunol 94: 1–36. [PubMed: 17560270]
26. Neuberger MS 2008 Antibody diversification by somatic mutation: from Burnet onwards. Immunol Cell Biol 86: 124–132. [PubMed: 18180793]
27. Crouch EE, Li Z, Takizawa M, Fichtner-Feigl S, Gourzi P, Montaño C, Feigenbaum L, Wilson P, Janz S, Papavasiliou FN, and Casellas R 2007 Regulation of AID expression in the immune response. J Exp Med 204: 1145–1156. [PubMed: 17452520]
28. Dengler HS, Baracho GV, Omori SA, Bruckner S, Arden KC, Castrillon DH, DePinho RA, and Rickert RC 2008 Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation. Nat Immunol 9: 1388–1398. [PubMed: 18978794]
29. Martel RR, Klicius J, and Galet S 1977 Inhibition of the immune response by rapamycin, a new antifungal antibiotic. Can J Physiol Pharmacol 55: 48–51. [PubMed: 843990]
30. Kay JE, Kromwel L, Doe SE, and Denyer M 1991 Inhibition of T and B lymphocyte proliferation by rapamycin. Immunology 72: 544–549. [PubMed: 1709916]
31. Wicker LS, Boltz RC, Matt V, Nichols EA, Peterson LB, and Sigal NH 1990 Suppression of B cell activation by cyclosporin A, FK506 and rapamycin. Eur J Immunol 20: 2277–2283. [PubMed: 1700753]
32. Jones DD, Gaudette BT, Wilmore JR, Chernova I, Bortnick A, Weiss BM, and Allman D 2016 mTOR has distinct functions in generating versus sustaining humoral immunity. J Clin Invest.
33. Terada N, Franklin RA, Lucas JJ, Blenis J, and Gelfand EW 1993 Failure of rapamycin to block proliferation once resting cells have entered the cell cycle despite inactivation of p70 S6 kinase. J Biol Chem 268: 12062–12068. [PubMed: 8389370]
34. Kaminski DA, and Stavnezer J 2006 Enhanced IgA class switching in marginal zone and B1 B cells relative to follicular/B2 B cells. J Immunol 177: 6025–6029. [PubMed: 17056527]
35. Donahue AC, and Fruman DA 2003 Proliferation and survival of activated B cells requires sustained antigen receptor engagement and phosphoinositide 3-kinase activation. J Immunol 170: 5851–5860. [PubMed: 12794110]

36. Omori SA, Cato MH, Anzelon-Mills A, Puri KD, Shapiro-Shelef M, Calame K, and Rickert RC 2006 Regulation of class-switch recombination and plasma cell differentiation by phosphatidylinositol 3-kinase signaling. Immunity 25: 545–557. [PubMed: 17000121]

37. Wang J, Liu S, Hou B, Yang M, Dong Z, Qi H, and Liu W 2018 PTEN-Regulated AID Transcription in Germinal Center B Cells Is Essential for the Class-Switch Recombination and IgG Antibody Responses. Front Immunol 9: 371. [PubMed: 29541074]

38. Bjur E, Larsson O, Yurchenko E, Zheng L, Gandin V, Topisirovic I, Li S, Wagner CR, Sonenberg N, and Piccirillo CA 2013 Distinct translational control in CD4+ T cell subsets. PLoS Genet 9: e1003494. [PubMed: 23658533]

39. Araki K, Morita M, Bederman AG, Konieczny BT, Kissick HT, Sonenberg N, and Ahmed R 2017 Translation is actively regulated during the differentiation of CD8+effector T cells. Nat Immunol 18: 1046–1057. [PubMed: 28714979]

40. Moerke NJ, Aktas H, Chen H, Cantel S, Reibarkh MY, Fahmy A, Gross JD, Degterev A, Yuan J, Chorev M, Halperin JA, and Wagner G 2007 Small-molecule inhibition of the interaction between the translation initiation factors eIF4E and eIF4G. Cell 128: 257–267. [PubMed: 17254965]

41. Pelletier J, Graff J, Ruggero D, and Sonenberg N 2015 Targeting the eIF4F translation initiation complex: a critical nexus for cancer development. Cancer Res 75: 250–263. [PubMed: 25593033]

42. Malka-Mahieu H, Newman M, Désaubry L, Robert C, and Vagner S 2017 Molecular Pathways: The eIF4F Translation Initiation Complex-New Opportunities for Cancer Treatment. Clin Cancer Res 23: 21–25. [PubMed: 27789529]

43. Compagno M, Wang Q, Pighi C, Cheong TC, Meng FL, Poggio T, Yeap LS, Karaca E, Blasco RB, Langellotto F, Ambrogio C, Voena C, Wiestner A, Kasar SN, Brown JR, Sun J, Wu CJ, Gostissa M, Alt FW, and Chiarle R 2017 Phosphatidylinositol 3-kinase δ blockade increases genomic instability in B cells. Nature 542: 489–493. [PubMed: 28199309]

44. Hsieh AC, Costa M, Zollo O, Davis C, Feldman ME, Testa JR, Meyuhas O, Shokat KM, and Ruggero D 2010 Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E. Cancer Cell 17: 249–261. [PubMed: 20227039]

45. McAllister EJ, Apgar JR, Leung CR, Rickert RC, and Jellusova J 2017 New Methods To Analyze B Cell Immune Responses to Thymus-Dependent Antigen Sheep Red Blood Cells. J Immunol 199: 2998–3003. [PubMed: 28916524]

46. Jang GM, Leong LE-C, Hoang LT, Wang PH, Gutman GA, and Semler BL 2004 Structurally distinct elements mediate internal ribosome entry within the 5’-noncoding region of a voltage-gated potassium channel mRNA. J Biol Chem 279: 47419–47430. [PubMed: 15339906]

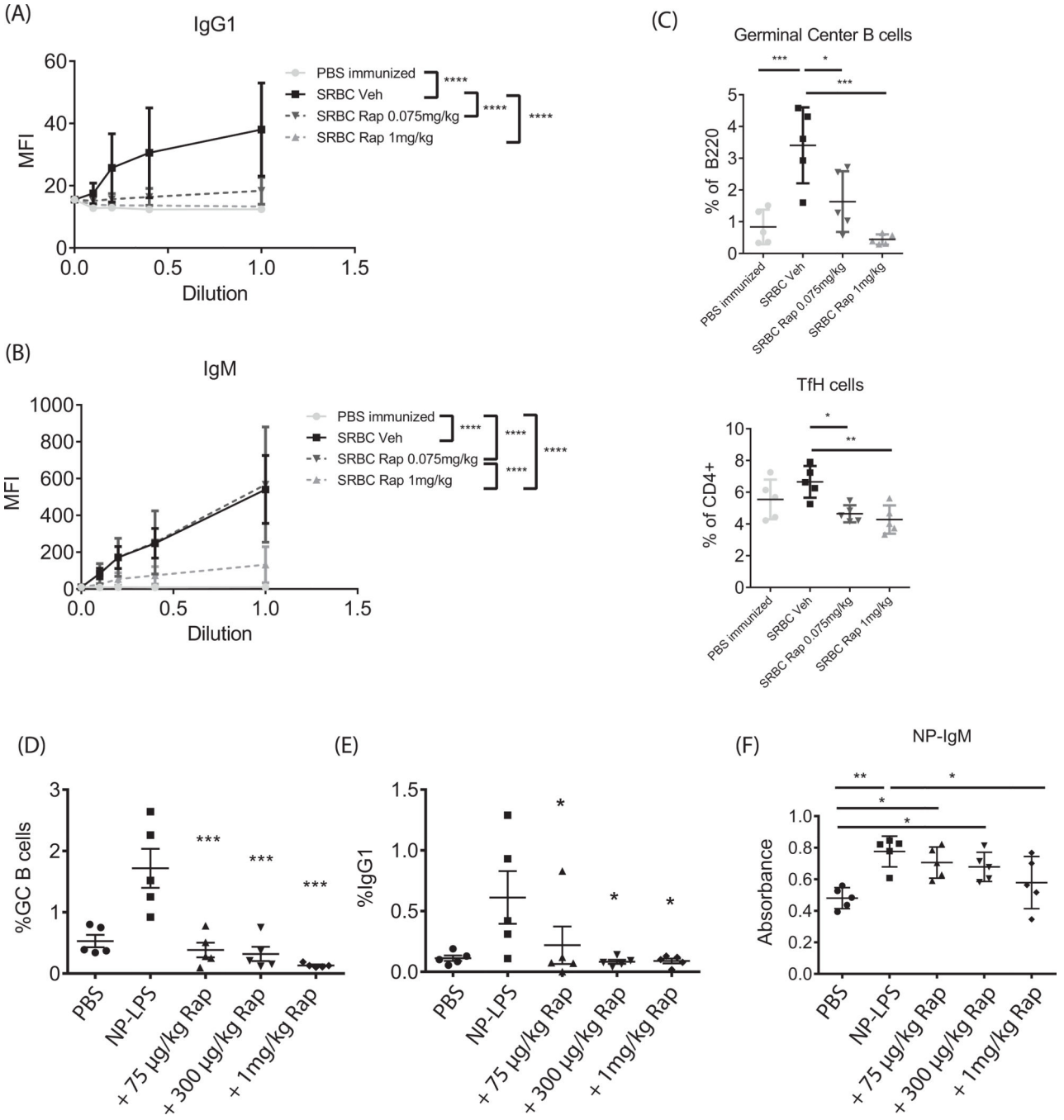

Figure 1. Low concentrations of rapamycin reduce IgG1 production during SRBC immunization

Mice (5 per group) were immunized with a T-dependent antigen, sheep red blood cells (SRBC) and treated for 7 days i.p. with vehicle control PBS, a low Rap dose of 75 µg/kg that has been shown to preserve lymphocyte activation, or an immunosuppressive dose of 1mg/kg. Serum was obtained from mice on day 8 and SRBC-specific IgG1 (A) and IgM (B) production were measured by detection of serum antibodies bound to SRBC using flow cytometry as described in (45). Significance was calculated using two-way ANOVA with Newman Keuls multiple comparison test. (C) Percentage of spleen cells with germinal

center (B220+GL7+Fas+) and T follicular helper cell (CXCR5+PD-1+CD4+) phenotype were analyzed by flow cytometry. (D-F) Mice (5 per group) were immunized with 5 μg NP-LPS and treated for 10 days with PBS, 75 μg/kg, 300 μg/kg or 1mg/kg Rapamycin by IP injection daily. (D) Percentage of spleen cells with germinal center (B220+GL7+Fas+) phenotype and (E) %IgG1+B220+ cells were analyzed by flow cytometry. (F) NP-specific serum IgM was measured by ELISA. Significance was calculated using one-way ANOVA with Newman Keuls multiple comparison test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)

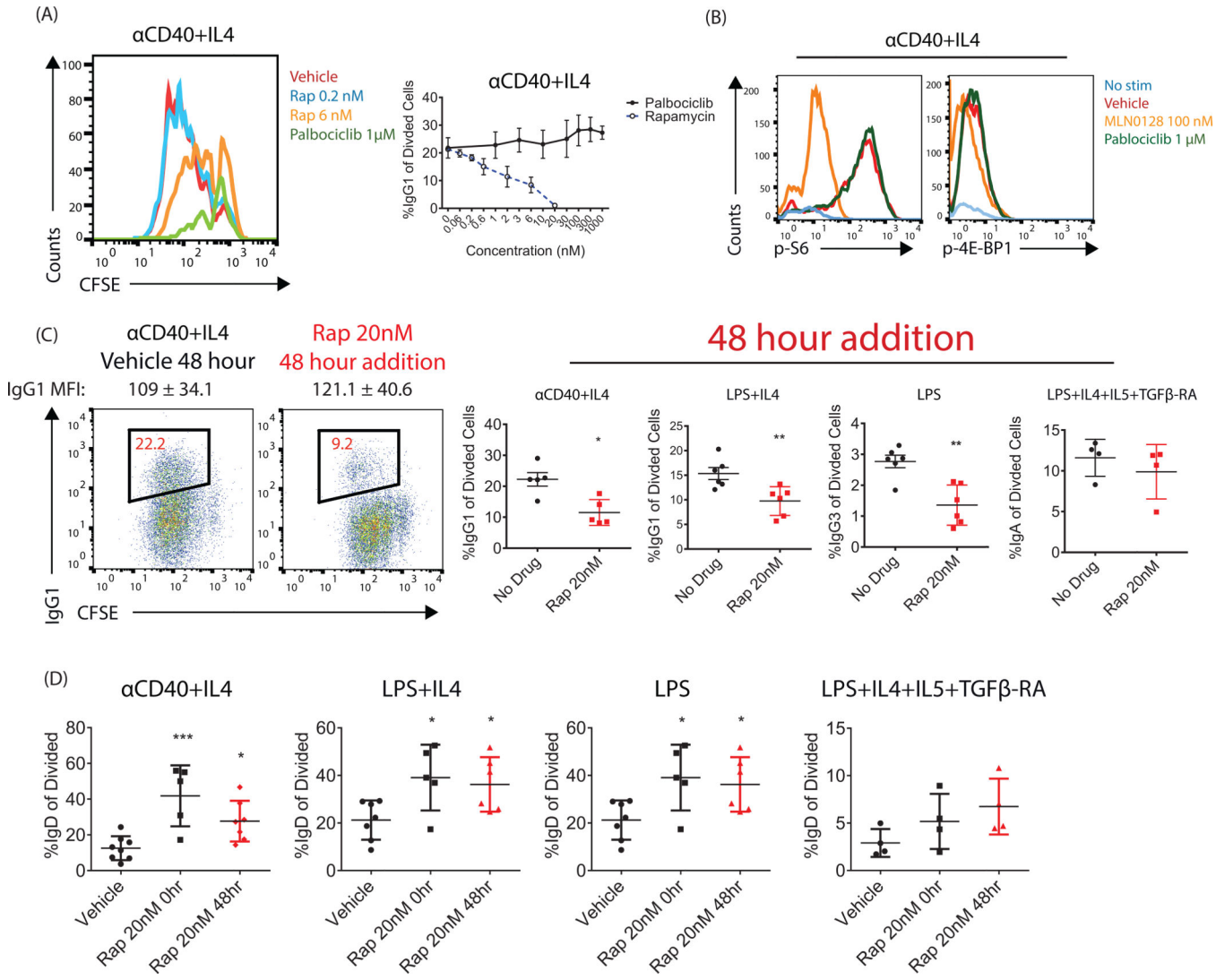

Figure 2. mTORC1 control of IgG1 switching is not due to inhibition of division

(A) Purified mouse splenic B cells were stimulated with αCD40+IL-4 and treated with rapamycin or the CDK4/6 inhibitor Palbociclib at the indicated concentrations. The percent of live B cells that have divided at least once (based on CFSE dilution; CFSE-low) expressing IgG1 was determined by flow cytometry at 96h. (B) Intracellular staining of p-S6 and p-4E-BP-1 was measured by flow cytometry. (C-D) Cells were stimulated with αCD40+IL-4, LPS+IL-4, LPS alone, or LPS+IL-4+IL-5+TGF-β+RA. (C) Percent of live B cells that have divided at least once expressing IgG1, IgG3, IgA and (D) IgD was determined by flow cytometry at 96h. Data are representative of three or more independent experiments (3–8 mice) and shown as mean ±SD. Significance was calculated using a paired two-tailed student's *t* test. (*P<0.05; **P<0.01; ***P<0.001)

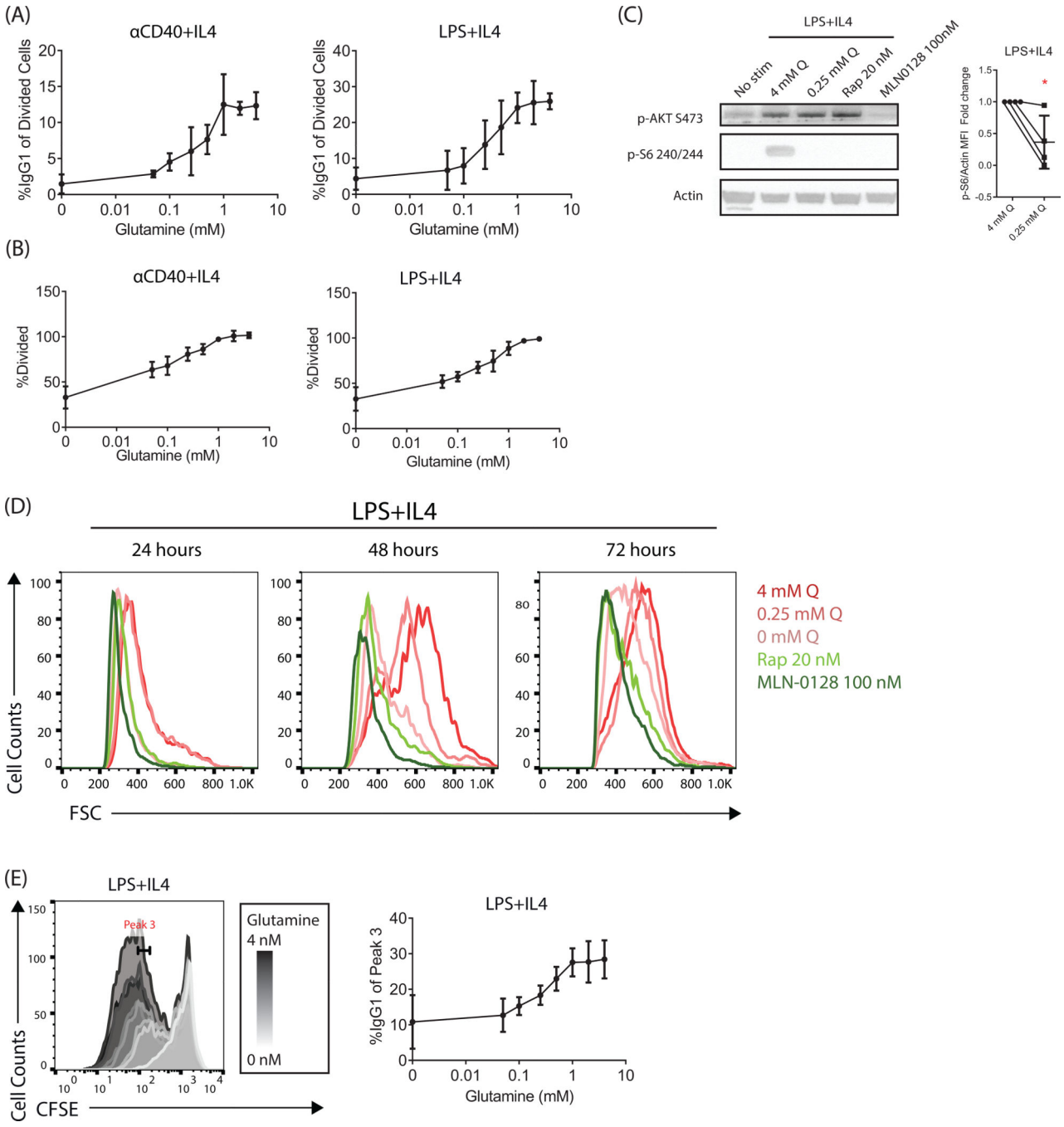

Figure 3. Glutamine deprivation reduces switching to IgG1

Cells were stimulated with αCD40+IL-4 or LPS+IL-4 in media with different glutamine concentrations as indicated. Percent IgG1 of the divided cells (A) and percent divided (B) is graphed. (C) Cells were stimulated with LPS+IL-4 in media with indicated glutamine concentrations and inhibitors for 24 hours before harvesting for western. p-S6 signal was quantified over multiple experiments. Data are representative of three or more independent experiments (3–4 mice). Significance was calculated using a paired one-tailed student's *t*

test. (*P<0.05; **P<0.01; ***P<0.001). (D) Cell size was determined by flow cytometry at 24, 48 and 72 hours. (E) %IgG1 gating on Peak 3 was determined by flow cytometry at 96h.

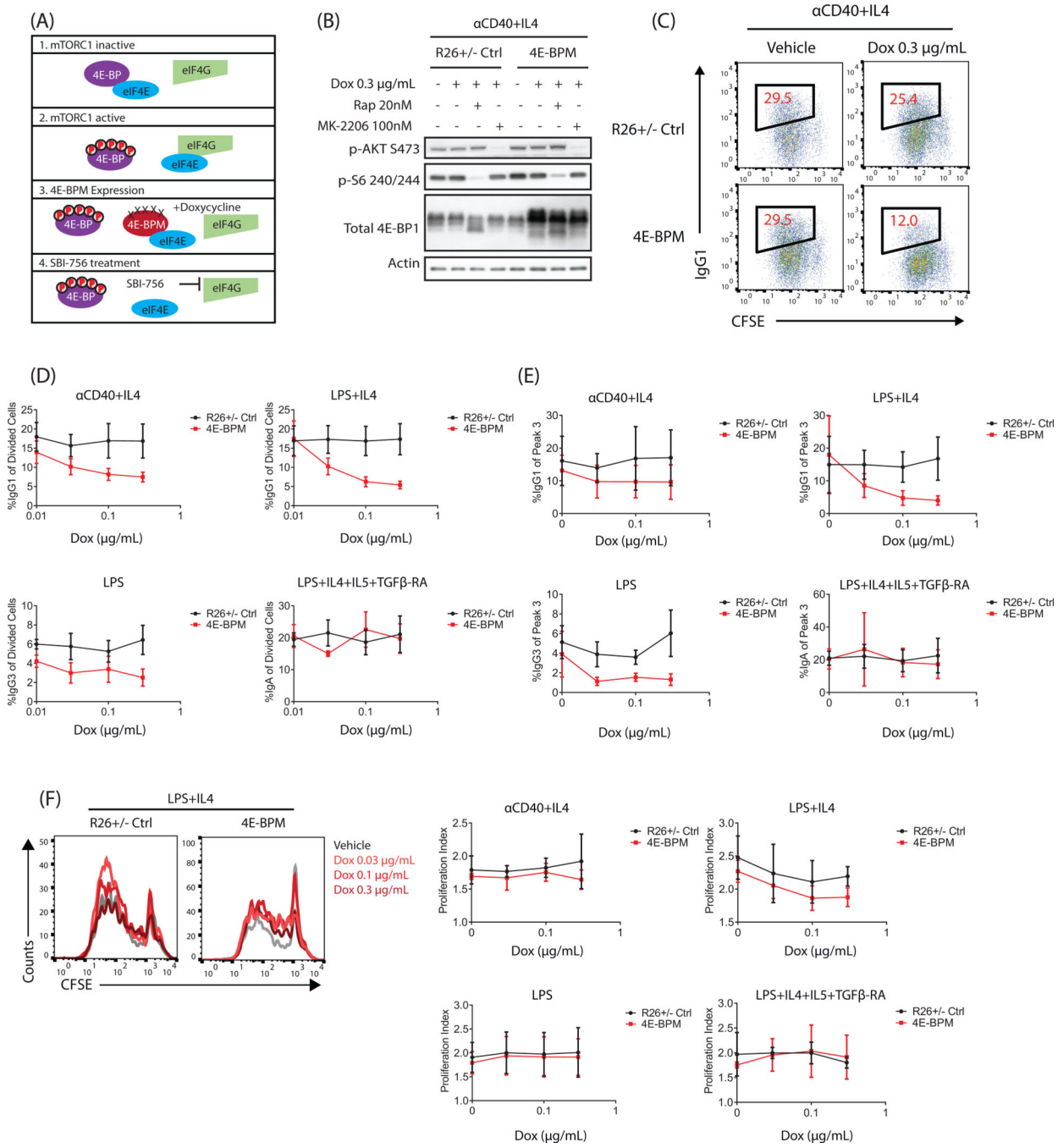

Figure 4. Genetic targeting of eIF4E activity reduces antibody class switching

(A) (1–2) Model of 4E-BP and eIF4E signaling. (3) Genetic and (4) pharmacological approaches to inhibit eIF4E activity. (B, C) Purified mouse splenic B cells from control mice or DOX-inducible 4E-BP mutant mice were stimulated with αCD40+IL-4 for 48 hours then treated with DOX and harvested at 54 hours to measure (B) transgene expression by western and (C) 96 hours for IgG1 switching among divided B cells. (D) B cells stimulated under the conditions indicated for treated at 48hr with titrations of DOX. The percent of live B cells that have divided at least once (measured by CFSE or eFluor670) or (E) in Peak 3 expressing

IgG1, IgG3, or IgA was determined at 96 hours. (F) The histograms on the right show cell division tracking by CFSE in cells treated with different concentrations of DOX and proliferation index, which is the average number of divisions among all divided cells, was also measured. Data are representative of three or more independent experiments (3–4 mice) and shown as mean ±SD. Significance was calculated using a paired one-tailed student's *t* test. (*P<0.05; **P<0.01; ***P<0.001)

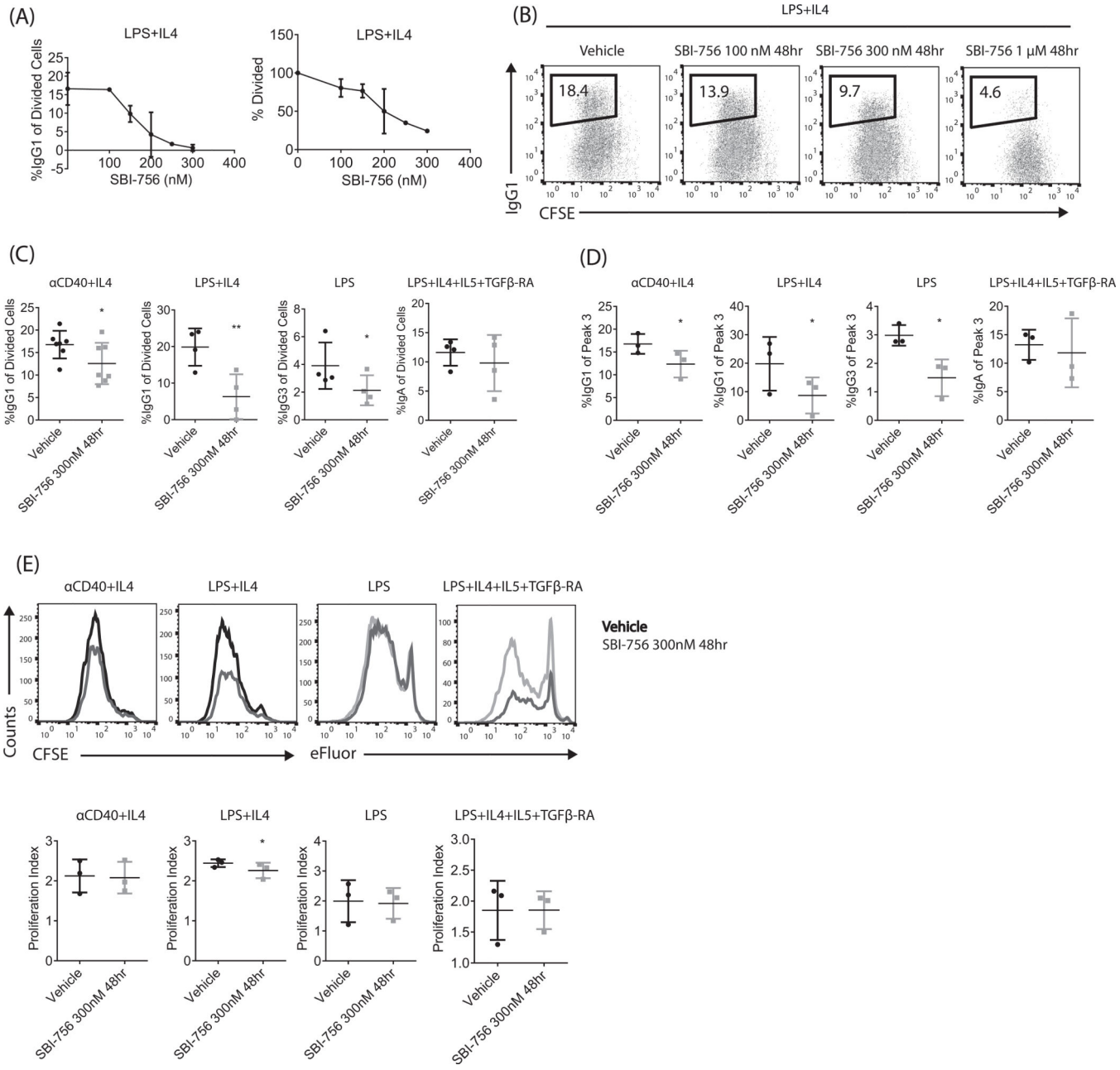

Figure 5. Pharmacological targeting of eIF4E activity with SBI-756 reduces antibody class switching

Cells were stimulated then treated with various concentrations of SBI-756 at 0 hrs (A), or at 48 hrs after activation (B). Percent of live B cells that have (C) divided at least once or (D) in Peak 3 expressing IgG1, IgG3, IgA and (E) proliferation index by CFSE or eFluor670 were measured at 96 hours by flow cytometry. Data are representative of three or more independent experiments (4–7 mice) and shown as mean ±SD. Significance was calculated using a paired one tailed student's t test. (*P<0.05; **P<0.01; ***P<0.001)

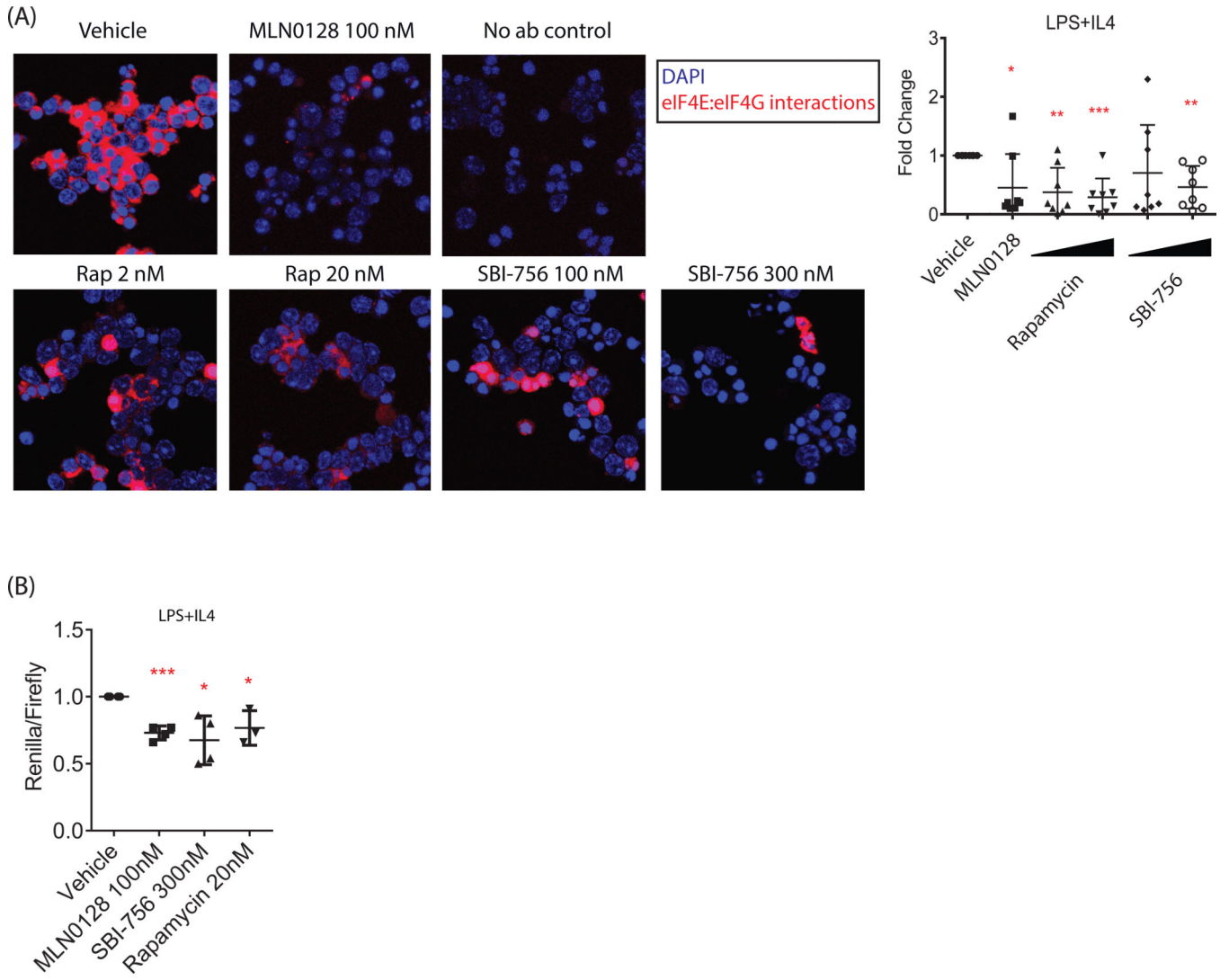

Figure 6. Inhibitors that reduce switching disrupt cap-complex formation

(A) eIF4E-eIF4G interactions detected by proximity ligation assay (PLA) in purified mouse splenic B cells that were treated with inhibitors as indicated and stimulated with LPS+IL-4 for 48 hours before fixing and permeabilizing. Interactions are visualized as red signal. Nuclei are stained with DAPI (blue). 5 images in separate fields were taken for each condition. All data are shown as binary area analysis of PLA/DAPI ±SD. Fold change was calculated by normalizing to vehicle. Each point represents one independent experiment. Significance was calculated using a paired one tailed student's t test (*P<0.05; **P<0.01; ***P<0.001). (B) We stimulated B cells for 48 hours with LPS+IL-4 and used a bicistronic dual Renilla-Firefly luciferase reporter construct to measure cap-dependent translation (Renilla luciferase) relative to cap-independent, polio IRES mediated translation (firefly luciferase) as an internal control. Each point represents one independent experiment (8 mice). Fold change was calculated using vehicle condition. Significance was calculated using a paired one tailed student's t test. (*P<0.05; **P<0.01; ***P<0.001)

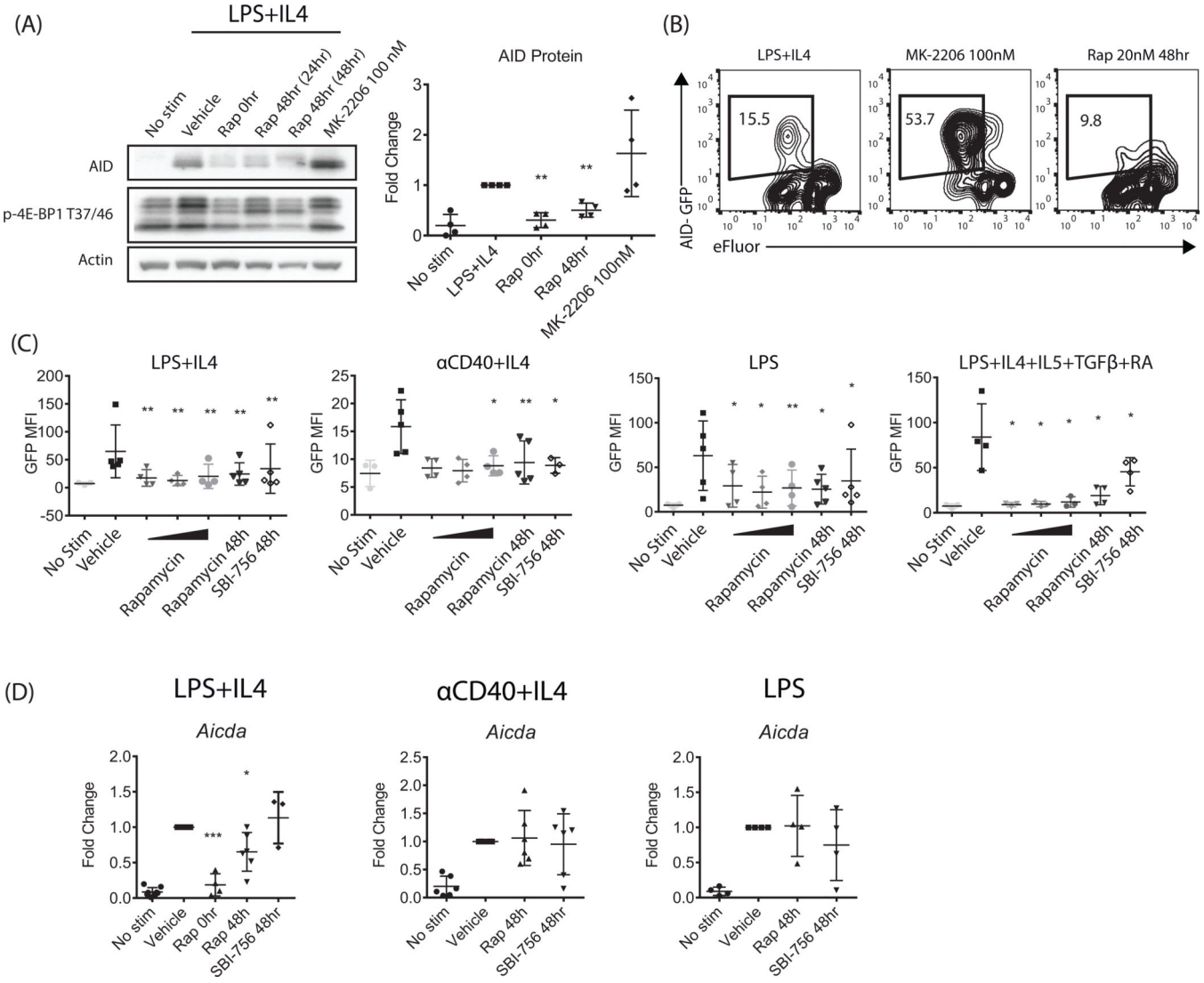

Figure 7. 48 hour addition of Rap or SBI-756 reduces AID protein but not *Aicda* mRNA

(A) B cells were purified and stimulated with LPS+IL-4 for 48 hours then treated with inhibitors as indicated. MK-2206 is an AKT inhibitor. In lanes 2–4 and 6, cells were harvested at 72 hours. Lane 5 cells were activated for 48 hours then treated with Rapamycin for an additional 48 hours before harvesting. AID amounts and 4E-BP phosphorylation were quantitated by western. (B,C) B cells were purified from AID-GFP mice and stimulated with LPS+IL-4, αCD40+IL-4, LPS, or LPS+IL-4+IL-5+TGF-β+RA and treated with inhibitors as indicated. Divided cells were gated on eFluor670-lo cells and AID-GFP MFI among divided cells was analyzed at 96 hrs using flow cytometry. Each point represents one independent experiment (4–5 mice). (D) Purified B cells were stimulated with left to right: LPS+IL-4, αCD40+IL-4, or LPS and treated with inhibitors as indicated. Cells were harvested at 72 hours to measure *Aicda* mRNA by RT-qPCR. Each point represents one individual experiment (3–6 mice). Fold change was calculated in comparison to vehicle

treated. Significance was calculated using a paired one tailed student's t test. (*P<0.05; **P<0.01; ***P<0.001)

Table I.

Summary of results by antibody isotype

Isotype	Rap 48hr	SBI-756 48hr	4E-BP^{M} 48hr	AID-GFP Rap	AID-GFP MFI SBI-756	Germline
IgG1 (αCD40+IL-4	↓*	↓*	↓**	↓*	↓*	No change
IgG1 (LPS+IL-4)	↓↓**	↓↓**	↓↓*	↓↓**	↓↓**	↓*
IgG3 (LPS)	↓*	↓*	↓*	↓*	↓*	No change
IgA (LPS+IL-4+IL-5+TGF-β+RA)	No change	No change	No change	↓*	↓*	↓*

* P<0.05

** P<0.01

Paired one-tailed t-test