SH2 domain-containing inositol 5-phosphatases support the survival of Burkitt lymphoma cells by promoting energy metabolism

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Abstract

Burkitt lymphoma cells (BL) exploit antigen-independent tonic signals transduced by the B-cell antigen receptor (BCR) for their survival, but the molecular details of the rewired BL-specific BCR signal network remain unclear. A loss of function screen revealed the SH2 domain-containing 5`-inositol phosphatase 2 (SHIP2) as a potential modulator of BL fitness. We characterized the role of SHIP2 in BL survival in several BL cell models and show that perturbing SHIP2 function renders cells more susceptible to apoptosis, while attenuating proliferation in a BCR-dependent manner. Unexpectedly, SHIP2 deficiency did neither affect PI3K survival signals nor MAPK activity, but attenuated ATP production. We found that an efficient energy metabolism in BL cells requires phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$), which is the enzymatic product of SHIP proteins. Consistently, interference with the function of SHIP1 and SHIP2 augments BL cell susceptibility to PI3K inhibition. Notably, we provide here a molecular basis of how tonic BCR signals are connected to energy supply, which is particularly important for such an aggressively growing neoplasia. These findings may help to improve therapies for the treatment of BL by limiting energy metabolism through the inhibition of SHIP proteins, which renders BL cells more susceptible to the targeting of survival signals.

Introduction

Burkitt lymphoma (BL) derives from germinal center B cells and represents a clinically aggressive non-Hodgkin lymphoma. Currently, BL is treated with intensive chemotherapy that result in high cure rates in younger patients. Treatment options for elderly patients are more challenging with lower cure rates and particularly problematic in areas with limited medical infrastructure, emphasizing the need for more targeted therapeutic options.

The hallmark of BL is a translocation of the *MYC* oncogene into an immunoglobulin locus leading to augmented *MYC* expression.[1] In order to counterbalance the apoptosis-sensitizing effect of c-Myc, the majority of BL subtypes employ the tonic signaling network of the B-cell antigen receptor (BCR), which is transduced in the absence of antigen binding and usually ensures the survival of mature B cells.[2,3] Accordingly, BL cell fitness depends on the expression of the BCR and its co-receptor CD19.[4]

Genetic analyses revealed that a substantial part of BL cells exhibits mutations in the genes encoding the transcription factor TCF3 or its inhibitor ID3, leading to a complex rewiring of tonic BCR signals.[5,6] In germinal center B cells, TCF3 is the key regulator of the centroblast program, which apparently is utilized by BL cells.[7,8] TCF3 represses the expression of the SHP1 coding gene, which is an important negative regulator and dephosphorylates BCR effectors such as the tyrosine kinase Syk.[2] Syk has been shown to be required

for BL-specific tonic BCR signaling and the destabilization of Syk by inhibition of the chaperone HSP90 limits BL cell survival.[9]

Since the TCF3-induced changes in the tonic BCR signal network result in augmented phosphatidylinositol 3-kinase (PI3K) activity, which facilitates activation of the AKT-mTOR survival pathway, a pivotal role of PI3K signals in BL-specific signaling was suggested and experimentally validated.[2,10] While cell line and murine models confirm the importance of PI3K, a recent study indicates that AKT activity is not the main driver of BL survival, suggesting that an exact regulation of tonic PI3K signals is required in BL cells.[11] Downstream of the BCR, PI3K activity can be counterbalanced by the SH2-containing inositol 5-phosphatases SHIP1 and 2, which dephosphorylate phosphatidylinositol trisphosphate (PIP_3) and disrupt plasma membrane docking sites for BCR effectors such as Bruton's tyrosine kinase (BTK).[12] While SHIP1 is required to adjust the efficiency of BCR signals in B-cell activation,[13] little is known about the role of SHIP proteins in tonic BCR signaling, except that both SHIP1 and SHIP2 are constitutively active in BL cells.[14] A comprehensive mass spectrometry analysis of the phospho-proteome in BL cell lines revealed a complex tonic BCR signal network, which goes far beyond PI3K-dependent pathways.[14] It also includes proteins that are involved in metabolism, which is consistent with findings that BL cells undergo a metabolic shift towards aerobic glycolysis to maintain their energy status.[15] One-carbon metabolism may also play an important role in BL cells, since it is upregulated in comparison to diffuse large B-cell lymphoma cells.[16] SHMT2, a protein of the mitochondrial glycine biosynthetic pathway, indirectly influences tonic BCR signaling in BL cells by controlling the TCF3-driven survival program.[17] However, little is known about how tonic BCR signaling initiates the required metabolic changes in BL cells.

Here, we report that SHIP1 and SHIP2 contribute to the fitness of BL cells by a previously unknown mechanism. While having no significant impact on PI3K signaling, SHIP proteins sustain the energy metabolism in BL cells. Inhibition or interference with SHIP expression renders BL cells more sensitive to inhibition of tonic BCR signals, which might give rise to new options for combinatorial therapies of BL.

Methods

Cell culture, proliferation, and apoptosis assays

Ramos (DSMZ ACC603), DG75 (DSMZ ACC83), Daudi (DSMZ ACC78) and Raji (DSMZ ACC319) cells were cultivated in RPMI-1640 GlutaMax (Gibco) containing penicillin/streptomycin, 50 μM β-mercaptoethanol and 1-20% fetal calf serum depending on the application.

The XTT proliferation assay (Biozol) was performed according to the manufacturer's protocol and analyzed with an enzyme-linked immunosorbant assay plate reader (Powerwave340, BioTek). For cell counting, cells were seeded at constant densities in 48-well plates and the number of living cells or GFP-positive cells was determined by flow cytometry.

Annexin V-APC/7-AAD assays (Biolegend) were carried out according to the manufacturers protocol. BH3-profiling was conducted as published[18,19] and details can be obtained from the *Online Supplementary Appendix*.

Stable isotope labeling with amino acids in cell culture (SILAC) and subsequent mass spectrometric analysis was performed as published.[17] Details can be obtained from the *Online Supplementary Appendix*.

Antibodies and flow cytometry

All primary and secondary antibodies are listed in the *Online Supplementary Appendix*. For flow cytometry analysis either FACS Celesta or LSRII, and FlowJo Version 10.6.2 (BD) was used.

Cell staining

A total of 10^6 cells were stained on ice for 20-30 minutes (min) and washed with phosphate-buffered saline (PBS). For intracellular labeling cells were fixed in Cytofix buffer (BD) diluted 1:2 with serum-free RPMI1640 (Gibco) for 15 min at 37°C and centrifuged at 450 rpm. Cells were resuspended in 200 μL 1x Perm/Wash buffer I (BD) containing 2% bovine serum albumin and incubated for 10 min at room temperature (RT), followed by 1:1 dilution with Perm/Wash buffer I and further 10 min incubation. Cells were stained in 100 μL 1x Perm/Wash I + primary antibody for 45 min at RT, centrifuged at 450 rpm and washed.

Glucose uptake

Cells were starved for 60 min at 37°C in RPMI1640 without glucose (Gibco™), followed by addition of 2-NBDG (100 μM; Thermo Scientific). Glucose uptake was stopped by the addition of ice-cold 1x PBS at indicated time points, washed and incubated for 20 min with 5 μg/mL 7-AAD.

Seahorse assay

On poly-D-lysine (PDL)-coated Seahorse 96-well cell culture plates $3x10^4$ cells were seeded in XF RPMI medium containing 10 mM glucose and 2 mM glutamine (Agilent). Plates were centrifuged at 300 rpm for 1 min and cells were incubated for 30 min in a non-CO_2 incubator at 37°C. Metabolic parameters were measured on a Seahorse XFe 96 extracellular flux analyzer (Agilent). In order to analyze the ATP production rate, 2.5 μM oligomycin and 500 nM rotenone together with antimycin A were sequentially added to the cells (Cayman Chemicals). Data were processed using Wave Desktop (Version 2.6.0.31) and ATP rates were calculated using the Seahorse Analytics online tool (Version March 2022).

Immunoblotting and western blot analysis

Cleared cellular lysates (CCL) were obtained by incubating cells on ice in lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM Na_3VO_4, 5 mM NaF, 0.1% sodium deoxycholate, 5 mM β-glycerophosphate, 10 mM N-ethylmaleimide, 1:50 protease inhibitor cocktail). CCL were subjected to SDS-PAGE and blotted on nitrocellulose membranes. Membranes were developed in enhanced chemiluminescence solution using ChemoStarECL (Intas).

Imaging flow cytometry

For imaging flow cytometry, we resuspended cells in Krebs-Ringer buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 4 mM KCl; 1 mM $MgCl_2$; 10 mM glucose; 1 mM $CaCl_2$) and collected data with an Amnis ImageStream X MKII. IDEAS (Version 6.2) software (Amnis) was used for analysis and cells with plasma membrane-localized signal were identified by using Homogeneity and Entropy parameters.

Results

SHIP2 supports the proliferation of Burkitt lymphoma cells

We aimed at identifying those tonic BCR components that support the survival of BL cells with a focus on PI3K-related proteins and reassessed a recently performed shRNA-based "drop-out" screen of different BL cell lines.[20] As highlighted in Figure 1A, targeting expression of the SHIP2-encoding gene *INPPL1* (P=0.019) was identified as one of the first BCR-proximal effectors in rank 151 of genes with relevance for BL fitness and has a similar significance as PAX5 (P=0.004), which was reported as one of the most critical among the 5,045 included genes.

We sought to corroborate these findings and treated different BL cell lines with the small molecule inhibitor AS1949490, which has been shown to be SHIP2-specific.[21] In order to consider the diversity among BL, we chose tonic BCR signal-dependent BL cell lines of different entities, such as Daudi,[22] which represent endemic BL, and Ramos[23] as well as DG75,[24] which derive from patients with sporadic BL. The surface BCR (sBCR)-negative BL cell line Raji (*Online Supplementary Figure S1F*) served as control. We evaluated the cell proliferation with an XTT assay after treatment with increasing inhibitor concentrations and the values of three independent experiments were normalized to solvent controls. As depicted in Figure 1B, all BCR-dependent BL cell lines exhibit an attenuated growth under SHIP2 inhibition in a dose-dependent manner starting at 5 μM, while Raji control cells appear to be unaffected up to 20 μM, implying that SHIP2 supports BL fitness in a BCR-dependent manner.

In order to further examine the SHIP2 function in BL-specific survival signaling, we targeted the SHIP2-encoding gene *INPPL1* in Daudi, Ramos, DG75, and Raji control cells by using the CRISPR/Cas9 method (*Online Supplementary Figure S1A*). We confirmed successful targeting and flow cytometry revealed unaffected sBCR expression (*Online Supplementary Figure S1B-E*). In order to exclude off-target and further clonal effects, we reconstituted the SHIP2-deficient cell lines with constructs encoding for a citrine-tagged version of SHIP2 (*Online Supplementary Figure S1D*). The results of XTT assays as well as cell counting (Figures 1C and D, respectively) confirmed compromised growth in the absence of SHIP2 in all sBCR-positive cell lines. Reconstitution of SHIP2-deficiency significantly improved the proliferation of Ramos and DG75 cells, thereby excluding off-target effects of the employed sgRNA, while in Daudi cells the expression of CitSHIP2 partly rescued cell proliferation. Inducible downregulation of SHIP2 expression in Ramos cells by tetracycline-dependent expression of three different shRNA confirmed these results (*Online Supplementary Figure S1G*). Notably, the proliferation of two independent *INPPL1*$^{-/-}$ clones of sBCR-deficient Raji cell lines was unaffected (Figure 1 E), further indicating that the supportive role of SHIP2 for the BL cell fitness is part of the tonic BCR signal network.

Burkitt lymphoma cells are more sensitive to apoptosis in the absence of SHIP2

In order to assess if SHIP2-deficiency renders BL cells more sensitive to programmed cell death, we stained the generated cell lines with Annexin V/7-AAD, which revealed a significant increase of total apoptosis in the absence of SHIP2 expression in Daudi and Ramos cells (Figure 2A; *Online Supplementary Figures S2A, B*). Since DG75 cells lack expression of the pro-apoptotic proteins BAX and BAK, an Annexin V-based assay is not possible.[26,27] Therefore, we complemented the apoptosis analyses by intracellular staining for active caspase-3, which confirmed the higher sensitivity to apoptosis in all three SHIP2-deficient cell lines (Figure 2B; *Online Supplementary Figures S2A, C*). We employed BH3 profiling in our Daudi cell lines to identify the molecular details of the augmented apoptosis in the absence of SHIP2. Here, BH3-peptides are used to induce apoptosis and the sensitivity to certain peptides correlates with the involved apoptotic pathways.[27,28] Apoptosis was determined according to the cytochrome c release, in which DMSO and alamethicin served as negative and positive controls, respectively. As shown in Figure 2C, SHIP2-deficient Daudi cells are markedly more sensitive to the apoptosis-inducing BIM peptide, thereby confirming their increased susceptibility to apoptosis. Moreover, in the absence of SHIP2 all BH3 peptides that inhibit specific anti-apoptotic proteins induce more cytochrome c release compared to parental or reconstituted cells. These data imply that SHIP2 deficiency does not affect a specific pathway, such as the FS-1-sensitive BFL-1 pathway, which is important for the survival of B cells,[29] but renders BL cells in general more susceptible to apoptosis.

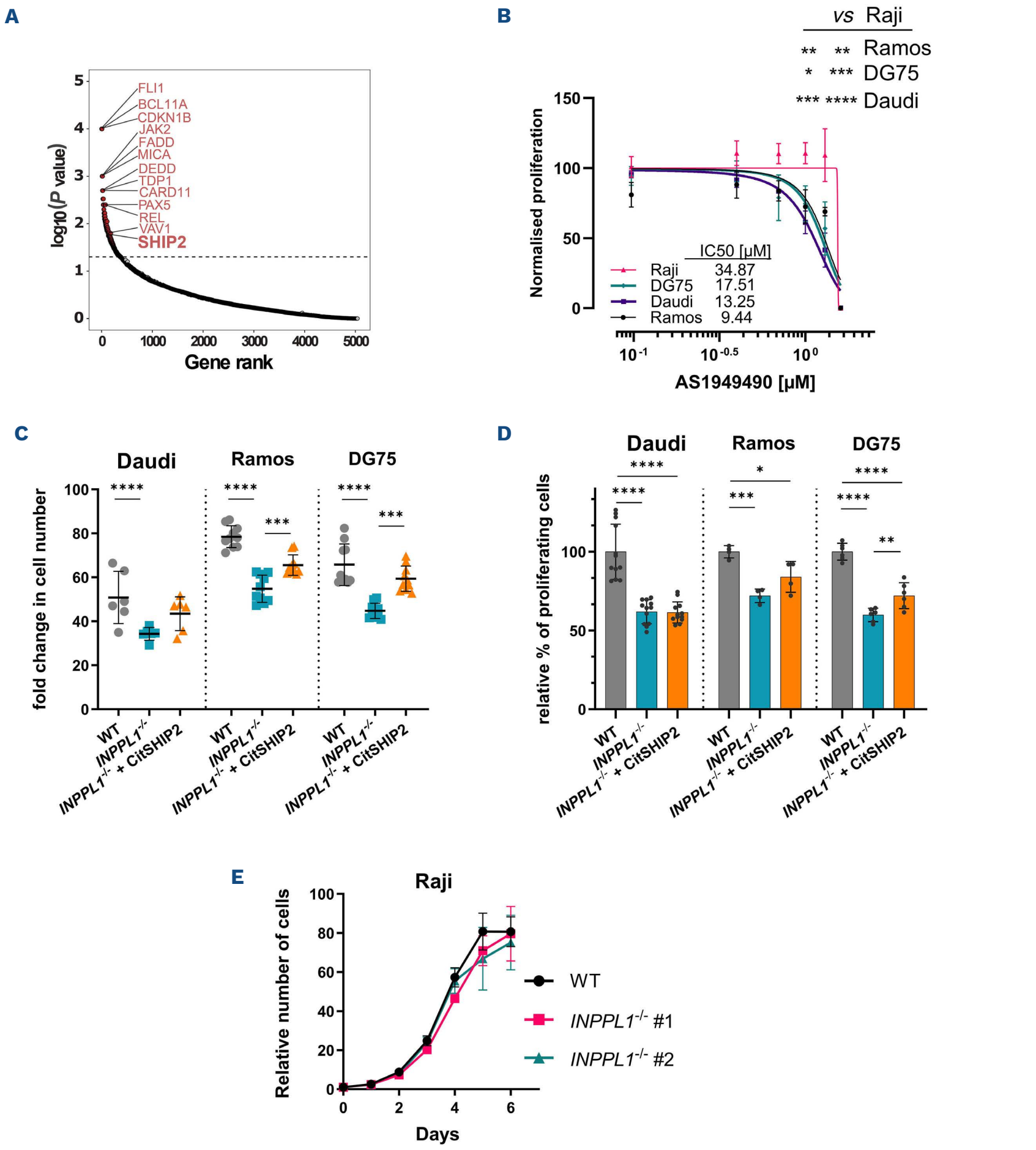

Figure 1. SHIP2 supports the proliferation of Burkitt lymphoma cells. (A) Data revealed from a previous short hairpin RNA (shRNA) screen in N=8 different Burkitt lymphoma (BL) cell lines are reassessed and plotted according to their significance for BL fitness (y-axis) and their rank among the 5,045 genes (x-axis).[20] Highlighted are the top relevant genes together with SHIP2. (B) Dose-response curve of BL cell lines treated with increasing concentrations of AS1949490. The proliferation was assessed by XTT after 24-hour treatment and data were normalized to respective vehicle controls. Half-maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism 10. (C) Cell counting of SHIP2-negative and CitSHIP2-reconstituted BL cell lines. Living cells were counted on day 7 (Daudi) or day 4 (Ramos/DG75) and normalized to day 0. (D) XTT proliferation assay of SHIP2-negative and CitSHIP2-reconstituted cells confirmed the cell counting assay. Proliferation was obtained by XTT assay after day 4 (Daudi) or day 1 (Ramos/DG75). Data was normalized to respective wild-type (WT) controls and the significance was calculated by one-way ANOVA. (E) Cell counting of Raji *INPPL1*$^{-/-}$ cells revealed no difference in proliferation in surface B-cell receptor (BCR)-negative cells. Cell count on each day was normalized to day 0. Significance was calculated using one-way ANOVA. If not indicated otherwise experiments were performed N≥3. Error bars indicate the standard deviation. Significance is indicated by *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

SHIP2 is not involved in the regulation of major tonic B-cell receptor signaling pathways

Based on the evidence that SHIP2 contributes to BL cell fitness, we analyzed the involved tonic BCR signal processes. Since PI3K-dependent signals are believed to be pivotal for BL survival and SHIP2 opposes PI3K activity, we first analyzed the activity of the PI3K effector AKT by determining the phosphorylation at the regulatory residue S473 in resting cells (*Online Supplementary Figure S3A*). However, western blot analyses and flow cytometry of intracellularly stained cells revealed only marginal changes in SHIP2-deficient cells (Figure 3A, B) or after SHIP2 inhibition (*Online Supplementary Figure S3B, C*). In order to test for further downstream signaling that may support BL cells, we determined phosphorylation levels of the MAPK JNK, ERK, and p38 in Daudi and Ramos cells (Figure 3C-E). These nalyses revealed a mild decrease of phospho-JNK signals in SHIP2-deficient Daudi cells, while in Ramos cells MAPK

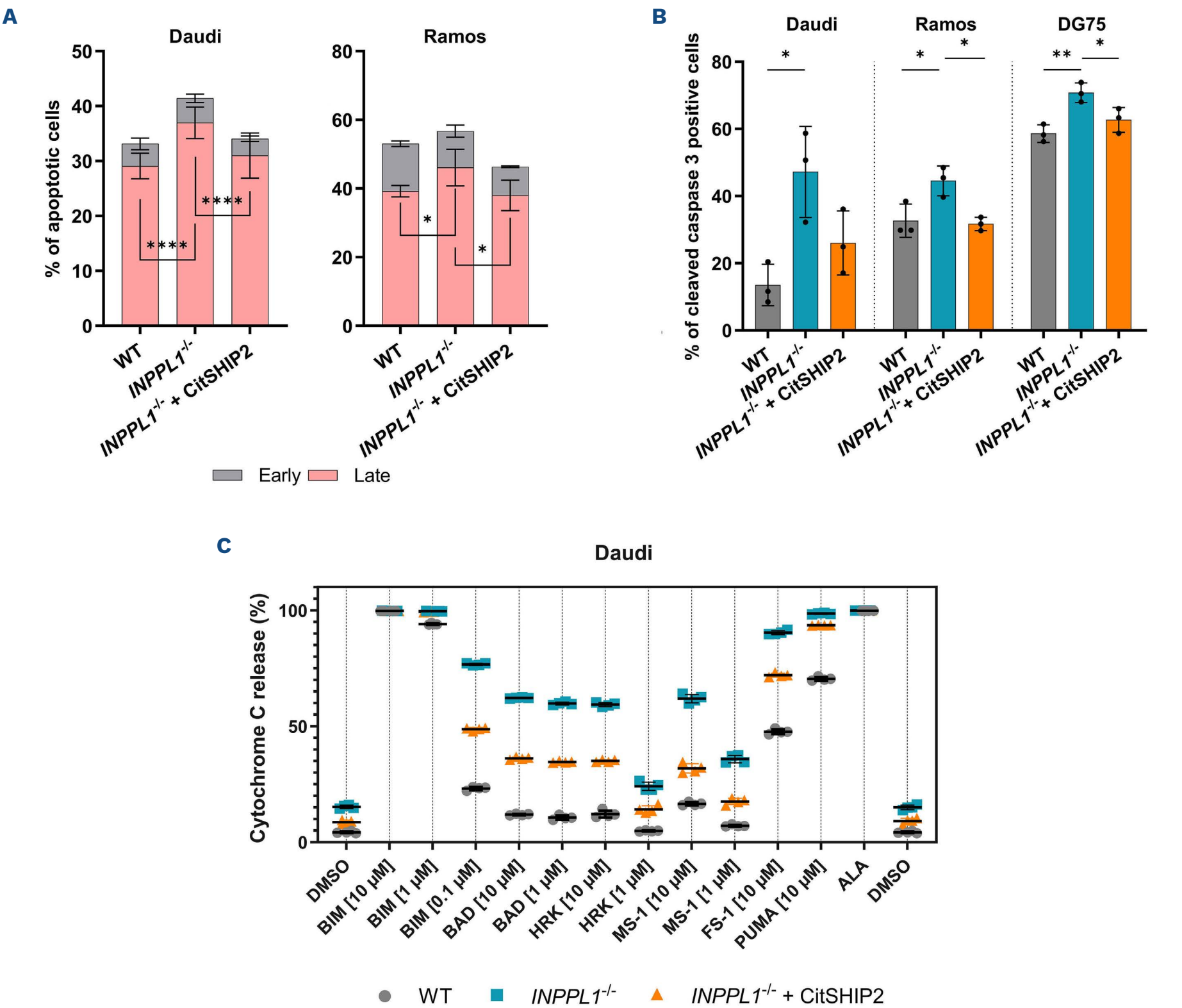

Figure 2. Burkitt lymphoma cells are more sensitive to apoptosis in the absence of SHIP2. (A) Apoptosis assay of Daudi and Ramos parental, SHIP2-negative and CitSHIP2-expressing cells as indicated by Annexin V/7-AAD staining. Apoptosis was induced by cultivation in 1% fetal calf serum medium prior to measurement for 2 or 3 days for Ramos and Daudi, respectively. Significance is calculated by two-way-ANOVA. (B) Cells, including the DG75 cell lines, were treated the same way as (A) followed by intracellular staining for cleaved caspase 3. Significance is calculated by one-way ANOVA. (C) BH3 profiling of Daudi wild-type (WT), SHIP2-deficient and CitSHIP2-expressing cells to assess the sensitivity to apoptosis. Cells were cultivated in medium containing 10% fetal calf serum prior to profiling. The displayed graph is 1 representative replicate and statistical analyses was performed by two-way ANOVA. If not indicated otherwise experiments were performed N≥3. Error bars indicate the standard deviation. Significance is indicated by *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

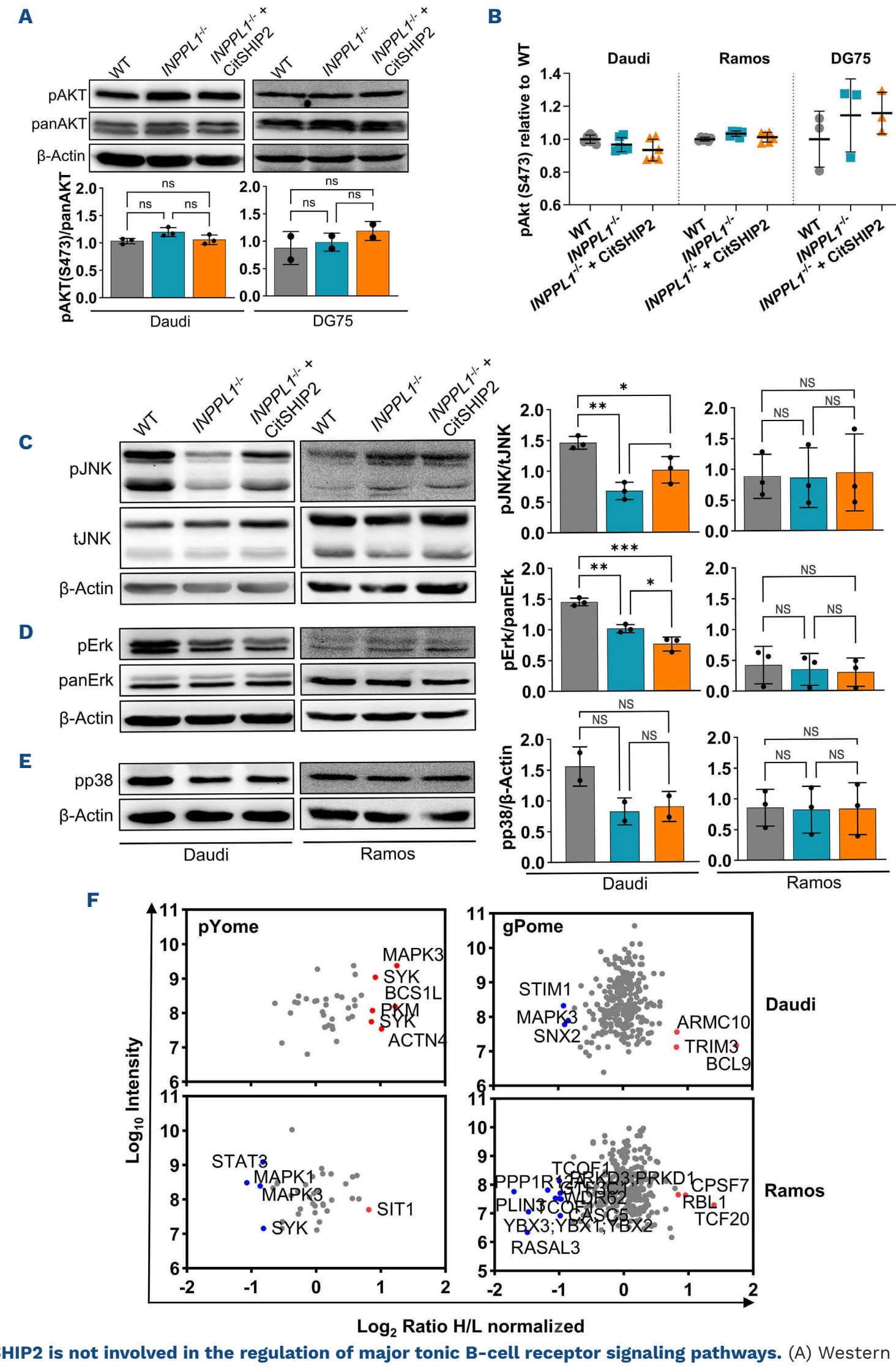

Figure 3. SHIP2 is not involved in the regulation of major tonic B-cell receptor signaling pathways. (A) Western blot analysis of phospho-Akt S473 in Daudi and DG75 cell lines. (B) Intracellular staining for phospho-Akt S473 in the Daudi, Ramos and DG75 cell lines. (C-E) Western blot analysis revealing the phosphorylation levels of phospho-JNK T183/Y185, phospho-Erk T202/Y204 and phospho-p38 T180/Y182 upon loss of SHIP2. Quantification of blots was performed using ImageJ. Normalization was carried

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out either to β-actin or the non-phosphorylated protein levels. (F) Phosphoproteomic analysis of the phosphorylation of tonic B-cell receptor (BCR) signaling contributors upon loss of SHIP2. The global phosphatome (gPome) and tyrosine-phosphatome (pYome) of SHIP2-deficient Ramos and Daudi cells were compared to reconstituted cells in a stable isotope labeling with amino acids in cell culture (SILAC) approach and the resulting data was queried for known phosphosites involved in tonic BCR signaling. Blue and red dots resemble proteins with significantly augmented and attenuated phosphorylation sites, respectively, in the absence of SHIP2. Data shown consists of single SILAC measurements. If not indicated otherwise experiments were performed N≥3. Error bars indicate the standard deviation. Significance was calculated by one-way ANOVA and is indicated by **P*<0.05, ** *P*<0.01, ****P*<0.001, *****P*<0.0001.

phosphorylation remained unaltered or appeared to be a clonal feature, because reconstitution did not re-establish the phosphorylation levels to those of parental cells.

In order to confirm that pivotal tonic BCR-dependent processes were not affected by SHIP2, we utilized a comprehensive mass spectrometry-based phosphoproteome analysis. For this purpose, we performed stable isotope labeling with amino acids in cell culture (SILAC) allowing for quantitative mass spectrometry. The obtained data set (*Online Supplementary Tables S1-4*) was aligned with the published BL-related global and tyrosine-based tonic BCR phosphoproteomes.[14] The changes in the identified tonic BCR effectors according to SHIP2 expression are depicted in Figure 3F. While we identified some SHIP2-dependent tonic BCR effector phosphorylation, this regulation is not consistent among Daudi (upper panel) and Ramos cells (lower panel). Notably, this approach did not reveal regulators or effectors of PI3K signaling.

Lack of SHIP2 function augments Burkitt lymphoma cell sensitivity to inhibition of survival signals

Since we could not identify any impact of SHIP2 on PI3-kinase-dependent survival signaling, we examined how SHIP2-deficient BL cells respond to inhibition of PI3K-dependent processes. Daudi and especially Ramos cells showed sensitivity to the selective PI3K inhibitor copanlisib in a dose-dependent manner, while DG75 cells were only mildly affected (Figure 4A). XTT-based proliferation measurements (Figure 4B) as well as cell counting (Figure 4C) revealed that SHIP2-deficient Daudi and Ramos cells were markedly more sensitive to copanlisib than their WT and reconstituted counterparts, while in the more resistant DG75 cells these differences were less pronounced. Correspondingly, in Daudi and Ramos cells the absence of SHIP2 causes augmented proportions of apoptotic cells after copanlisib treatment (Figure 4D; *Online Supplementary Figure S4A, B*). Consistently, the combined inhibition of PI3K and SHIP2 caused a more efficient reduction of BL cell proliferation compared to copanlisib only treatment (Figure 4E). In DG75 cells, supposedly due to their lower sensitivity, this effect, however, was moderate. In all analyzed cell lines, we did not observe an additional effect of SHIP2 inhibition on pAKT levels (*Online Supplementary Figure S4D*). Notably, we did not observe the strong sensitizing effect of SHIP2 deficiency after AKT inhibition (*Online Supplementary Figure S4C*). These data imply that the SHIP2 function in BL survival is beyond the regulation of PI3K signaling, and that SHIP2 deficiency renders BL cells more sensitive to the inhibition of tonic BCR survival signals.

SHIP2 is required for an efficient energy supply in Burkitt lymphoma cells

In order to assess the PI3K-independent role of SHIP2 in BL fitness, we analyzed the proteome of SHIP2-deficient and reconstituted BL cell lines. The generated data (*Online Supplementary Table S5*) were aligned with the STRING database to assign the identified proteins to their respective KEGG pathways (Figure 5A). This approach revealed a mildly reduced abundance of several cell cycle regulators (green dots/spots) in SHIP2-deficient cells, which, however, does not correlate with changes in the cell cycle of the different Daudi and Ramos cell lines (*Online Supplementary Figure S5A, B*). We also found a slightly altered abundance of proteins involved in energy metabolism (red dots) in the absence of SHIP2 (Figure 5A). This prompted us to analyze the plasma membrane expression of the glucose transporters GLUT1 and GLUT4, which were unaltered in SHIP2-deficient Daudi and Ramos cells when compared to reconstituted cells (Figure 5B). Consistently, the glucose uptake was almost identical in *INPPL1*$^{-/-}$ and parental cells, indicating a role of SHIP2 independent of glucose supply (Figures 5C; *Online Supplementary Figure S5C*). Therefore, we tested apoptosis rates in cells that were treated with the competitive glycolysis inhibitor 2-DG, which abrogates energy metabolism early in glycolysis.[30] Under these conditions, SHIP2 deficiency did not increase apoptosis rates of Ramos cells, which indicates a role of SHIP2 in the energy metabolism of BL cells downstream of glucose-6-phosphate production (Figure 5D). In order to assess this effect in more detail, we utilized a Seahorse assay to determine the rates of ATP production in the different BL cell lines. This approach revealed a significant decrease in glycolytic and mitochondrial ATP production in all SHIP2-deficient BL cell lines while the mitochondrial mass remained unaltered (Figure 5E; *Online Supplementary Figure S5D*). Expression of CitSHIP2 largely re-established ATP production, albeit in Daudi cells the reconstitution was marginal. These data imply that SHIP2 is required for efficient ATP production of BL cells.

The enzymatic SHIP2 product PI(3,4)P_2 contributes to Burkitt lymphoma cell fitness

In order to assess, if the SHIP2 enzymatic product phosphatidylinositol-3,4-bisphosphate (PI(3,4)P_2) is responsible for its energy supporting role, we employed a GFP-tagged

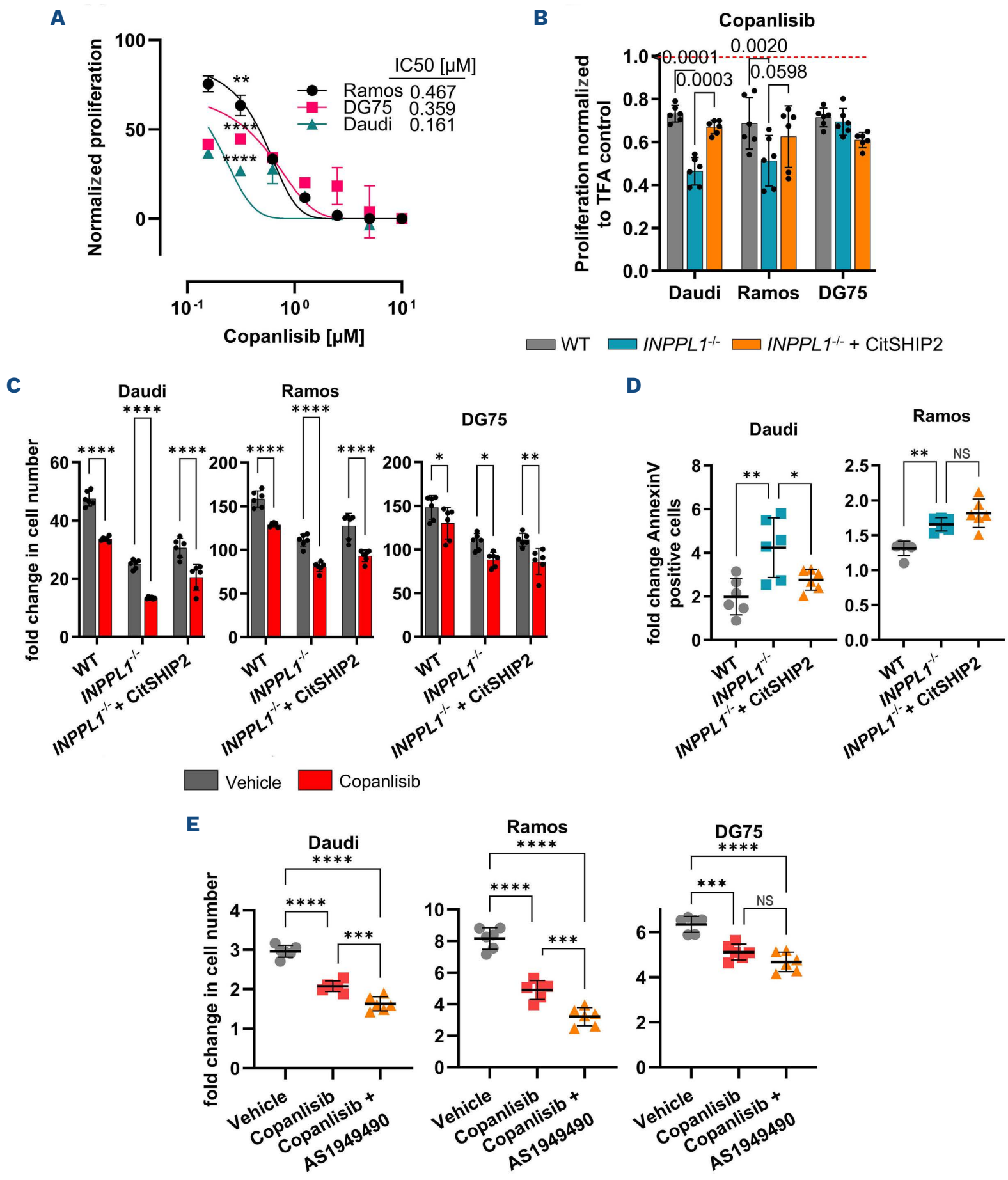


Figure 4. Lack of SHIP2 function augments Burkitt lymphoma cell sensitivity to inhibition of survival signals. (A) Dose-response curve of Burkitt lymphoma (BL) cell lines treated with increasing concentrations of copanlisib for 24 hours (h) followed by determination of proliferation by XTT assay. The proliferation is given by normalization to the vehicle control. The statistics indicate the significant difference between treated and control cells. Half maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Prism 10. (B) Proliferation of Daudi, Ramos and DG75 cell lines after treatment with copanlisib. The proliferation was assessed by XTT assay after 24-h treatment with 200 nM copanlisib. Treated cells were normalized to the respective solvent control of each cell line. (C) Cell counting experiment with the same set up as in (B). Living cells were counted on day 7 (Daudi) or day 4 (Ramos/DG75) and normalized to day 0. Treatment with 200 nM copanlisib was started 1 day prior to read-out. (D) Apoptosis assay of Daudi and Ramos cell lines after treatment with copanlisib. The cells were stained with Annexin V after treatment with 200 nM copanlisib for 24 h; 5% trifluoroacetate (TFA) was used as vehicle control (A-D). (E) Cell counting experiment in Daudi, Ramos and DG75 cell lines to assess the effects of copanlisib/AS1949490 combination. Cells were treated for 48 h either with 200 nM copanlisib or a combination of copanlisib and 5 µM AS1949490 followed by normalization to day 0. A respective mixture of 5% TFA and dimethyl sulfoxide (DMSO) served as vehicle control. If not indicated otherwise experiments were performed N≥3. Error bars indicate the standard deviation. Significance was calculated by two-way ANOVA and is indicated by *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

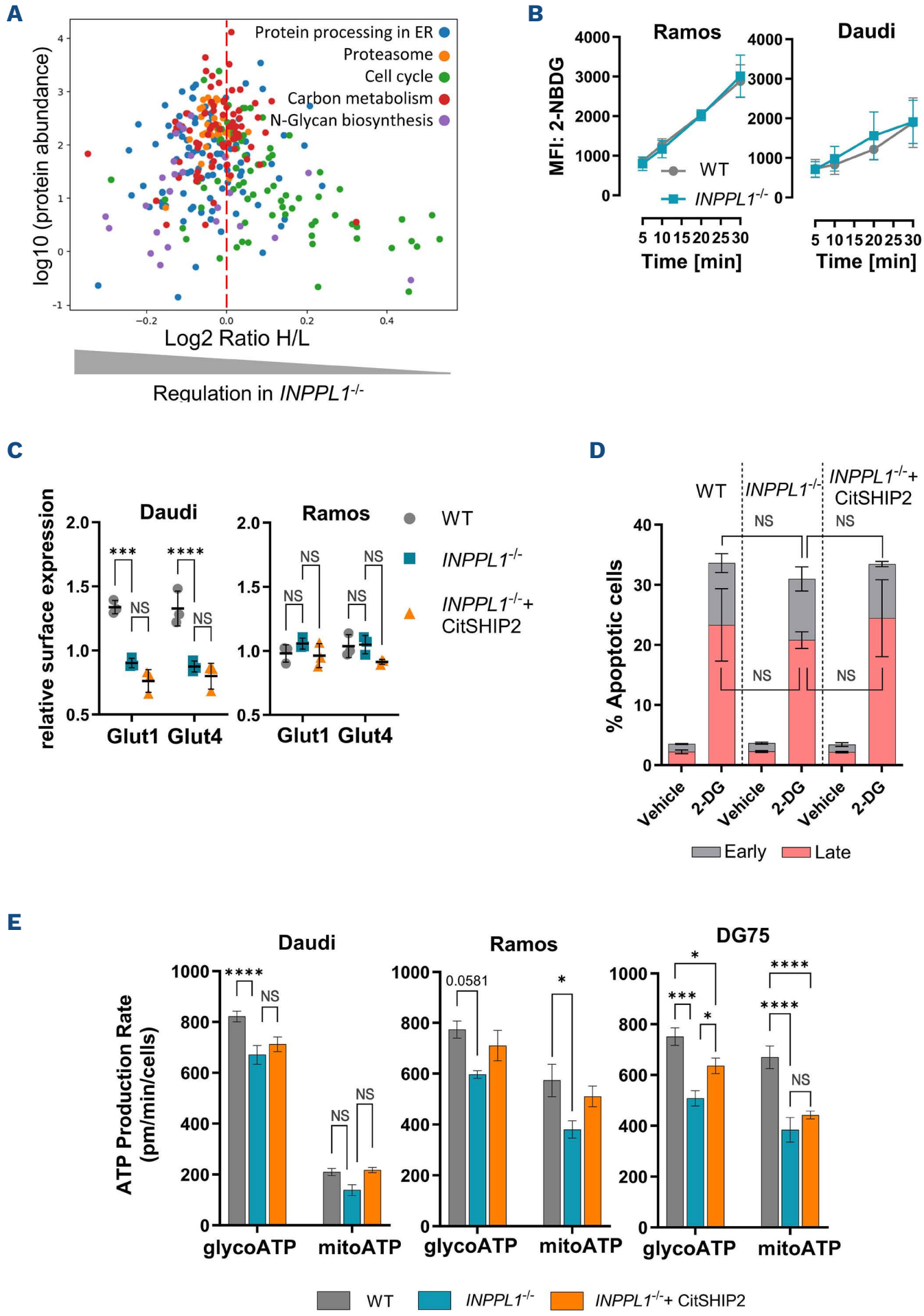


Figure 5. SHIP2 is required for an efficient energy supply in Burkitt lymphoma cells. (A) Stable isotope labeling with amino acids in cell culture (SILAC) mass spectrometry of Ramos *INPPL1*$^{-/-}$ and reconstituted cells followed by cross referencing with the STRING database. The significantly altered KEGG pathways were extracted. Each point represents a changed abundance of a protein involved in the indicated pathway. The log2 ratio H/L on the x-axis indicates the protein abundance in SHIP2-deficient compared to reconstituted cells with the dotted line indicating a ratio of 1. The y-axis indicates the typical protein abundance in the human body as given by the STRING database. (B) The glucose uptake of Daudi and Ramos wild-type (WT) and SHIP2-deficient cells was measured by incubation with 2-NBDG for indicated time points followed by counterstain with 7-AAD to exclude dead cells. (C) Surface staining for GLUT1 and GLUT4 on Daudi and Ramos cell lines. (D) Apoptosis levels of Ramos cell lines after treatment with the glycolysis inhibitor 2-DG. Cells were subjected to Annexin V/7-AAD staining to distinguish between apoptotic phases after 24-hour treatment with 10 mM 2-DG. ddH_2O was used as vehicle control. (E) Seahorse assay was performed to determine the ATP production rate in Daudi, Ramos and DG75 cell lines. If not indicated otherwise experiments were performed N≥3. Error bars indicate the standard deviation. Significance was calculated by two-way-ANOVA and is indicated by **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. MFI: median fluorescence intensity.

tandem TAPP1-PH domain (GFP-2x-TAPP1-PH)[31] and imaging flow cytometry to show that plasma membrane PI(3,4)P_2 is reduced in the absence of SHIP2 (Figure 6A, B). Because of the strong and specific binding of the TAPP1-PH protein to PI(3,4)P2 it may obstruct binding motifs for proteins that support energy metabolism. Consistently, expression of the tandem TAPP1-PH domain in Ramos, DG75, and also the sBCR-deficient Raji WT cells led to a significant growth

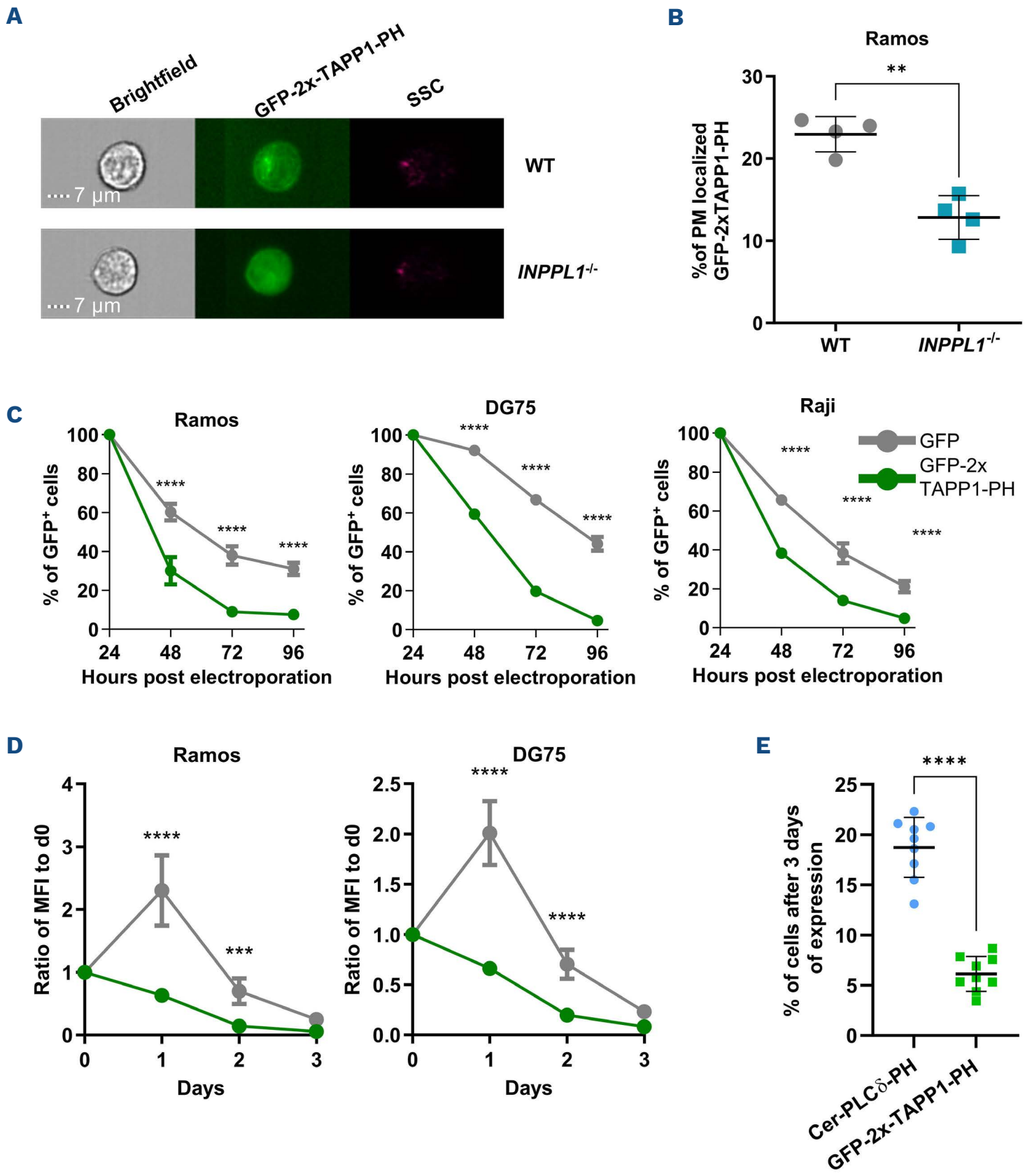


Figure 6. The enzymatic SHIP2 product PI(3,4)P2 contributes to Burkitt lymphoma cell fitness. (A, B) Percentage of plasma membrane-localized GFP-2xTAPP1-PH31 in Ramos wild-type (WT) and SHIP2-deficient cells. Constitutively expressing Ramos cells were analyzed by imaging flow cytometry. Statistical analysis was conducted using an unpaired student *t* test. (C) Constitutive expression of GFP-2xTAPP1-PH in Ramos, DG75 and Raji WT cells. Cells were electroporated with a plasmid encoding either GFP-2xTAPP1-PH or GFP as control followed by analysis of their GFP signal at indicated time points and normalization to the baseline at 24 h after electroporation. (D) Repeat of the experimental setup of (C) but with an induced expression of GFP-2xTAPP1-PH or GFP in Ramos and DG75 WT cells to exclude effects based on electroporation. The cells were induced on day 0 (d0) followed by washing and tracking over the next 3 days. (E) Competitive growth assay of Ramos WT cells expressing either GFP-2xTAPP1-PH or Cer-PLCδ-PH. Cells were mixed in a 1:1 ratio and expression of both constructs was induced on day 0 followed by washing. The percentage of GFP-positive or cerulean-positive cells was determined by fluoresecence-activated cell sorting at day 3. Statistical analysis was performed by two-way-ANOVA. If not indicated otherwise experiments were performed N≥3. Error bars indicate the standard deviation. Significance was calculated by two-way ANOVA and is indicated by *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. MFI: median fluorescence intensity.

disadvantage in comparison to GFP controls (Figure 6C; *Online Supplementary, Figures S6A-C*). This toxic effect was also observed after inducible expression of GFP-2x-TAPP1-PH when compared to either GFP (Figure 6D) or the PH domain of PLCδ, which is specific for $PI(4,5)P_2$ (Figure 6 E), thereby excluding an impact of the transfection procedure and general stability of PH domains, respectively. Hence, blocking $PI(3,4)P_2$ motifs interferes with BL cell fitness, strongly implying a role of this phosphoinositide in energy metabolism.

SHIP1 and 2 contribute to Burkitt lymphoma cell fitness in an additive manner

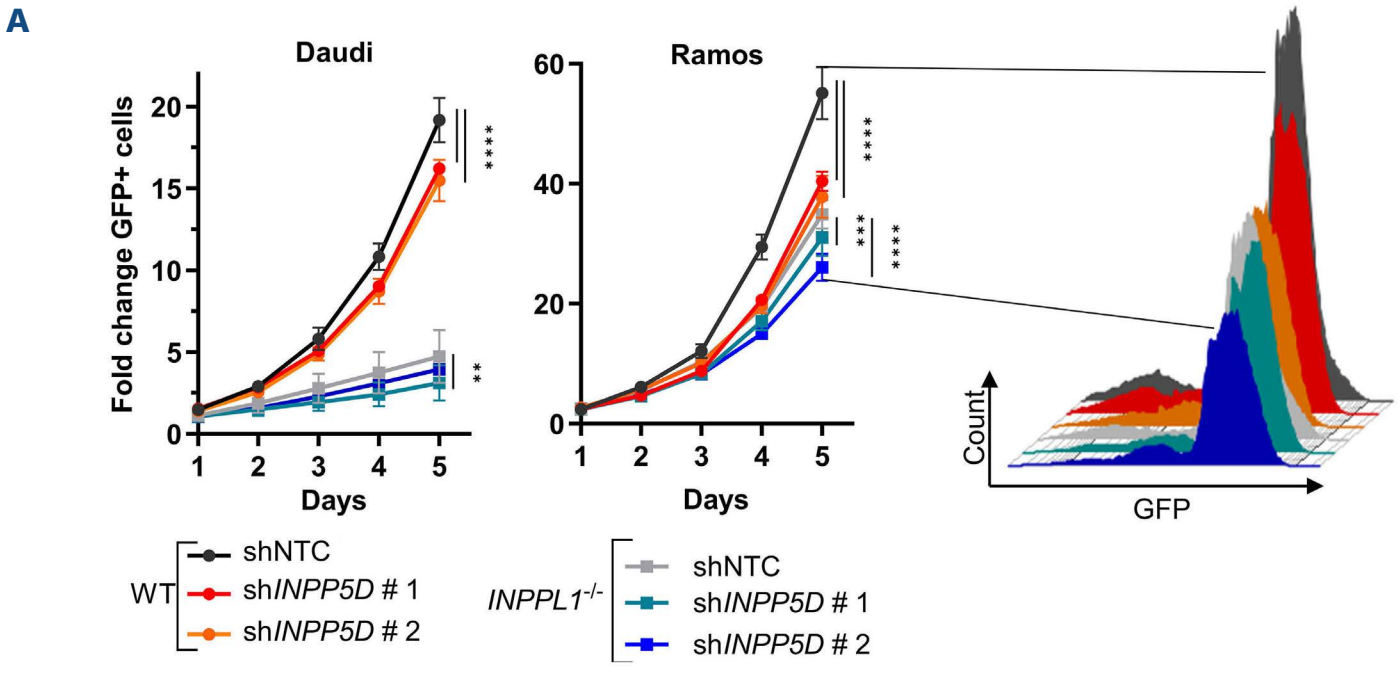
The importance of $PI(3,4)P_2$ is further corroborated by the fact that inhibition of SHIP1, which is the second producer of this PI in B cells, markedly attenuated Ramos cell proliferation, also in the absence of SHIP2 (*Online Supplementary Figure S7A*). In order to confirm this finding, we inducibly expressed two different short hairpin (sh) RNA that, while not significantly affecting AKT phosphorylation (*Online Supplementary Figure S7B*), efficiently reduce SHIP1 levels (gene *INPP5D*) in WT and SHIP2-deficient Daudi and Ramos cells compared to non-targeted (NTC) control shRNA-expressing cells (*Online Supplementary Figure S7C*). Since GFP is induced together with the shRNA, we monitored GFP-positive cells for up to day 6. As shown in Figure 7A, interference with SHIP1 expression significantly reduced the proliferation of WT and $SHIP_2$-deficient BL cells, which substantiates the contribution of PI(3,4)P2 to the fitness of BL cells. Consistently, the compromised SHIP1 expression renders BL cells more sensitive to PI3K inhibition, leading to severe proliferation defects and elevated apoptosis rates in *INPPL1*$^{-/-}$/SHIP1-low BL cells (Figure 7B-E). Compared to their Ramos counterparts (Figure 7C, E) this effect is mild in SHIP2-deficient Daudi cells, since their survival is already severely abrogated (Figures 7B, D). We observed similar sensitivity increases to inhibition of PI3K-dependent effectors and *INPPL1*$^{-/-}$/SHIP1-low cells almost completely stopped proliferation under inhibition of mTORC1 and PDK1, while the effect of two AKT inhibitors was moderate (*Online Supplementary Figure S7D-G*). Consistent with these findings, SHIP1-downregulation attenuated ATP production in a similar manner as SHIP2 deficiency. We did not observe additive effects in *INPPL1*$^{-/-}$/SHIP1-low BL cells, indicating a minimal rate of ATP production that can be measured with the SeaHorse assay in BL cells. Collectively, these data provide evidence that the production of $PI(3,4)P_2$ by SHIP proteins contributes to BL fitness by refining their energy metabolism. Hence, interference with SHIP function renders cells more sensitive to the inhibition of survival signals.

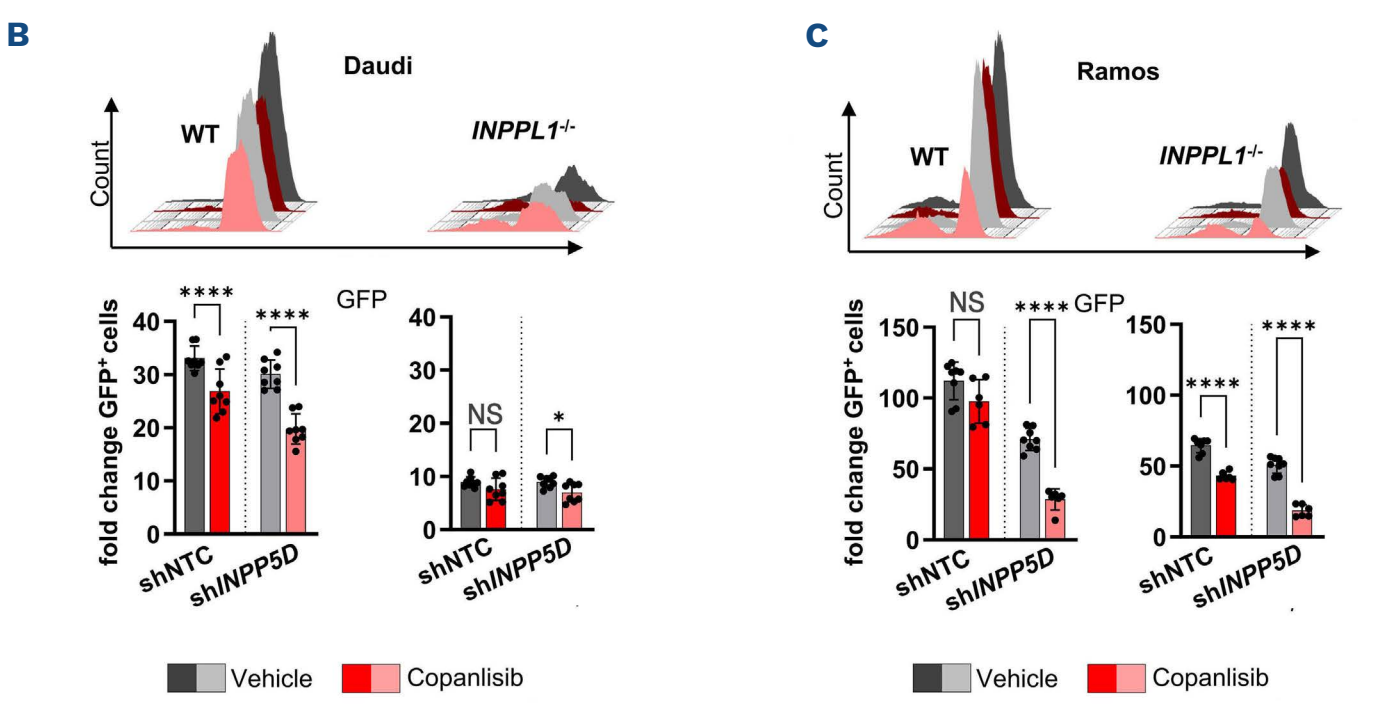
Discussion

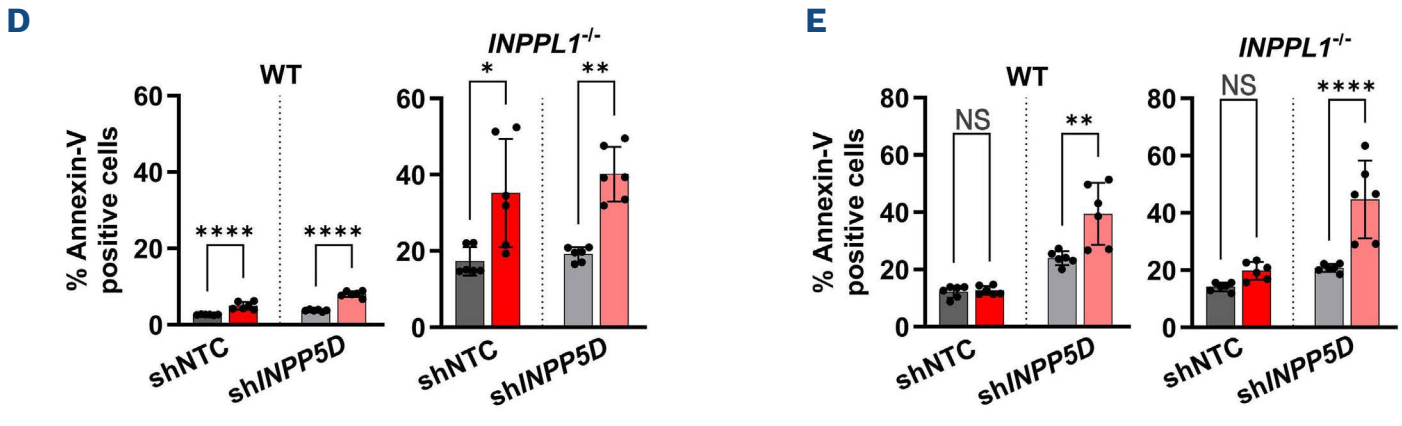
Since a substantial proportion of BL employ a reprogramming of the tonic BCR signaling network for their survival, the molecular details of the dysregulated processes may provide opportunities for a targeted treatment of this aggressive B-cell lymphoma. Here, we show that the inositol 5-phosphatases SHIP1 and 2 contribute to BL fitness by coupling tonic BCR signals to efficient energy metabolism. In the absence of SHIP2 activity or after silencing of SHIP1 expression, BL cell lines representing different entities exhibit attenuated proliferation and augmented sensitivity to apoptosis. Notably, the increased susceptibility to apoptosis is not based on interference with specific anti-apoptotic proteins, such as BFL1, which has been reported to be pivotal for B-cell survival.[29] This is supported by our BH3 profiling assay, which revealed an in general augmented sensitivity to all BH3 peptides. Silencing SHIP1 expression in the absence of SHIP2 expression further compromises proliferation and markedly enhances apoptosis rates. While we cannot exclude that also other signaling pathways integrate SHIP proteins, the fact that the sBCR-negative BL cell line Raji[32] is not affected by SHIP2 deficiency suggests the supportive function of SHIP proteins for BL cell fitness to be part of the tonic BCR signaling network. Both SHIP proteins have been shown to be part of the tonic BCR signaling network in BL cells[14] and in particular SHIP1 is a well described BCR effector protein,[33] which argues in favor of this conclusion. Despite this being the first study showing an oncogenic role of SHIP proteins for B-cell lymphoma, SHIP proteins have been reported as promotors of several types of tumors. High SHIP2 levels correlate with poor prognosis in breast cancer,[34] non-small cell lung cancer,[35] and laryngeal squamous cell carcinoma.[36] Inhibition of SHIP1 and 2 compromise the survival of multiple myeloma cells.[37] SHIP proteins are opposing the enzymatic function of PI3K, which was shown to be a main driver of BL cell survival[10] and initiates a number of pro-survival and anti-apoptotic processes, including an augmented energy metabolism through its pivotal effector AKT.[38] Accordingly, AKT is frequently dysregulated in a number of human cancers.[39] Since the pleckstrin-homology (PH) domain of AKT and its activating kinase PDK1 both bind to PIP3, SHIP may be considered as an inhibitor of AKT-dependent signals. On the other hand, many PIP3-binding PH domains including those of PDK1 and AKT also bind the SHIP product $PI(3,4)P_2$.[40-42] Henceforth, the full activation of AKT can correlate with $PI(3,4)P_2$ levels, indicating that SHIP protein function is even required for an efficient activation of AKT.[43,44] Moreover, the AKT family members 1 and 2, which both have been shown to be important in B-cell survival and activation, have distinct affinities to $PI(3,4)P_2$, which may result in a complex and SHIP-dependent activation pattern of AKT proteins.[45] However, we did not find any SHIP-dependent alterations in AKT-activating effectors, AKT activity, or AKT-dependent downstream processes within the tonic BCR signal network of the cell models used in this study. Although this finding might be counterintuitive, there is evidence in the literature that BL cell survival does not rely

on enhanced AKT activity. In CRISPR/Cas9-based "drop-out" screening approaches, BL cell lines turned out to be insensitive to AKT deficiency, which is in contrast to BCR-dependent diffuse large B-cell lymphoma (DLBCL) cells.[17,46] Moreover, a recent study showed that primary BL cells are sensitive to augmented AKT signals and BL survival requires an exactly controlled AKT activity.[11] Hence, the fact that the supportive function of SHIP proteins for BL cells does not correlate with altered AKT activity is consistent. Other PIP3-regulated BCR processes, such as the recruitment of PLCγ2 and the Ras GEF SOS, which could support cell proliferation through the activation of MAPK, are according to our data unlikely to be involved in SHIP-dependent survival signals in BL cells.

Instead of augmenting AKT or MAPK-dependent BCR signals, SHIP proteins appear to be required for efficient energy metabolism in BL cells. The provisioning of energy is an important aspect of BL cell fitness, because of their aggres-







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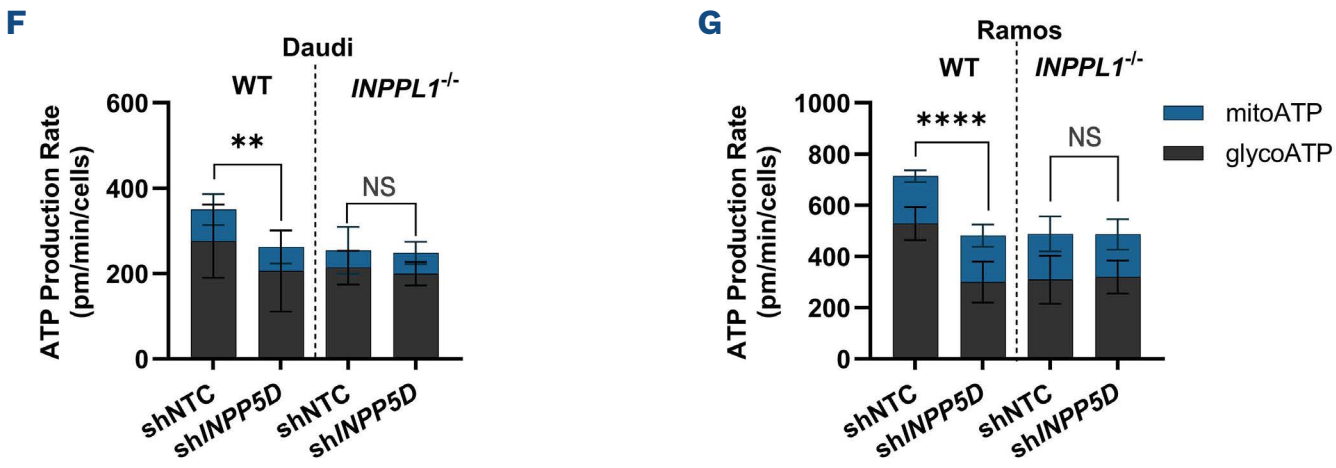

Figure 7. SHIP1 and 2 contribute to Burkitt lymphoma cell fitness in an additive manner. (A) Induction of short hairpin RNA (shRNA) targeting *INPP5D* (sh*INPP5D*) or the non-targeting control (shNTC) in Ramos and Daudi wild-type (WT) and *INPPL1*$^{-/-}$ cells. Since GFP is expressed together with shRNA, the proliferation was assessed by monitoring the increase of GFP-positive cells over 5 days followed by normalization to day 0. The histogram represents the final read out after day 5 in Ramos WT cells. Effect of copanlisib on the proliferation of Daudi (B) and Ramos (C) WT and SHIP2-deficient cells expressing either the control or sh*INPP5D*. The histograms represent the respective levels of GFP-positive cells after 6 days of sh*INPP5D* induction and 2 days treatment with 200 nM copanlisib. The number of GFP-positive cells was normalized to day 0. The same cells were subjected to Annexin V staining to assess the levels of apoptosis (D, E). Seahorse assay of Daudi (F) and Ramos (G) WT and SHIP2-deficient cells after downregulation of SHIP1. Shown are the glycolytic and mitochondrial ATP production rates after 5 days of sh*INPP5D* induction. The depicted significances refer to the glycolytic ATP production rate. If not indicated otherwise experiments were performed N≥3. Error bars indicate the standard deviation. Significance was calculated by two-way ANOVA and is indicated by **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

sively proliferating nature. In all studied BL cell models, we found a significantly attenuated production of ATP mainly in the cytosol in the absence of SHIP2 and after down-regulation of SHIP1. While the similar impact of both PI(3,4)P_2 producers in the tonic BCR signaling network on the ATP production is consistent, we did not observe additive effects in *INPPL1*$^{-/-}$/SHIP1-low BL cells. This finding might suggest a minimal ATP production rate in BL cells that is required to be measured by a Seahorse assay, thereby indicating that the observed SHIP-dependent reduction of cellular ATP is substantial for these cells. Currently, it is unclear how tonic BCR signals are connected to the efficiency of energy metabolism, particularly in an AKT-independent manner. According to our findings, the supportive effect of SHIP proteins relies on their enzymatic product PI(3,4)P_2, which is evident by the fact that the expression of the PI(3,4)P_2-blocking tandem TAPP1 PH domains causes a marked survival disadvantage to BL cells. Consistently, SHIP1 contributes to BL cell fitness in a manner, which is additive to SHIP2. As also Raji cells, which are insensitive to SHIP2 inhibition or deficiency, did not tolerate GFP-2x-TAPP1-PH, PI(3,4)P_2 may have a more general function in energy metabolism, while SHIP proteins produce this phosphoinositide as a part of tonic BCR signaling. It has been shown in other cell models that PI(3,4)P_2 is involved in the trafficking of vesicles thereby altering the plasma membrane abundance of glucose transporters,[47] but we could neither correlate SHIP2 activity to the plasma membrane abundance of GLUT1 and GLUT4 nor to the influx of glucose. Notably, under inhibition of glycolysis downstream of glucose-6-phosphate production by 2-DG,[30] SHIP2 does not have a supportive function in BL cells, which argues against an indirect impact and implies that PI(3,4)P_2 is directly supporting the efficiency of downstream energy-providing processes. In general, BL cells have metabolic features that are related to their overexpression of *MYC*, such as an increased glucose consumption and increased supply of glucose-derived carbon to the TCA cycle.[48] Moreover BL cells with rewired tonic BCR signaling are susceptible to the inhibition of the one carbon metabolism[17,49] and the direct comparison of the metabolome of B-cell lymphoma cells revealed that in BL cells components of one carbon metabolism are more abundant compared to DLBCL cells.[16] Our mass spectrometry analysis does not reveal any significant links between SHIP protein function and these BL-typical energy metabolism processes and hence, the molecular details of how PI(3,4)P_2 couples tonic BCR signals to a more efficient ATP production remain to be elucidated.

Due to their role in the energy metabolism, interference with SHIP function not only impairs the fitness of BL cells, but also renders them more sensitive to the inhibition of PI3K-related survival signals. Inhibition of PI3K in SHIP2-deficient and SHIP1-silenced BL cells almost completely blocks their survival. The inhibition of PI3K effectors such as PDK1 and mTORC2, which both have been reported to be important for BL survival,[17] is more efficient in the absence of SHIP activity. Hence, targeting SHIP function may be suitable for combinatorial therapies, which appear to be required for the targeted treatment of cancers, since adaptation processes are less probable compared to single-drug therapies. For example, the BTK inhibitor ibrutinib, which is approved for the treatment of mantle cell lymphoma, chronic lymphatic leukemia, and marginal zone lymphoma, has been shown to be associated with the development of resistance.[50] A study about the treatment of DLBCL with ibrutinib revealed that a combination with other drugs such as lenalidomide leads

to markedly better prognosis.[51] For the treatment of BL, it might be beneficial to combine the inhibition of tonic BCR survival signals, such as PI3K, with SHIP protein inhibition. Because of its ubiquitous expression, inhibition of SHIP2 might cause more side effects than the inhibition of SHIP1, which is mainly found in cells of hematopoietic origin. In our BL cell models, interference with either SHIP protein was effective, which opens the opportunity of testing different SHIP inhibitors for the treatment of BL.

Disclosures

No conflicts of interest to disclose.

Contributions

ME conceived, designed and supervised the study. FM performed most of the experiments and contributed to the study design. VK performed experiments and contributed to the study design. DF and SW performed the Seahorse assay. JL conducted BH3 profiling. SA and NP contributed to data acquisition and provided technical support. BH performed mass spectrometry analyses. FM, VK, DF, JL, KL, BC, BB, TZ, BH, TO, and ME analyzed and interpreted data. ME and FM wrote the manuscript and all other co-authors reviewed it.

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Data-sharing statement

For original data or protocols, please contact the corresponding author. Phosphoproteomic and proteomic data are included in the Online Supplementary Appendix.

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