ZAP-70 constitutively regulates gene expression and protein synthesis in chronic lymphocytic leukemia

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Key points:

1. ZAP-70 is essential to maintain a constitutive survival signal for CLL cells and is directly involved in the expression of CCL3 and CCL4.
2. Upon BCR-activation ZAP70 increasingly forms complexes with ribosomes and promotes MYC-expression and protein synthesis.

ABSTRACT

The expression of ZAP-70 in a subset of CLL patients strongly correlates with a more aggressive clinical course, though the exact underlying mechanisms remain elusive. The ability of ZAP-70 to enhance B cell receptor (BCR) signaling, independently of its kinase function, is considered to contribute. Here we employed RNA-sequencing and proteomic analyses of primary cells differing only in their expression of ZAP-70 to further define how ZAP-70 increases aggressiveness of CLL. We identified that ZAP-70 is directly required for cell survival in the absence of an overt BCR signal, which can compensate for ZAP-70 deficiency as an anti-apoptotic signal. In addition, the expression of ZAP-70 regulates the transcription of factors regulating recruitment and activation of T cells, such as CCL3, CCL4 and IL4I1. Quantitative mass spectrometry of double-cross linked ZAP-70 complexes further demonstrated constitutive and direct protein-protein interactions between ZAP-70 and BCR-signaling components. Unexpectedly, ZAP-70 also binds to ribosomal proteins, which is not dependent on, but further increased by BCR-stimulation. Importantly, decreased expression of ZAP-70 significantly reduced MYC-expression and global protein synthesis, providing evidence that ZAP-70 contributes to translational dysregulation in CLL. In conclusion, ZAP-70 constitutively promotes cell survival, microenvironment-interactions and protein synthesis in CLL cells, likely to improve cellular fitness and to further drive disease progression.

INTRODUCTION

The tyrosine kinase ZAP-70 is an essential molecule for upstream T cell receptor signaling, required for cell-activation and development[1,2]. Its expression in malignant B cells of a subset of patients with CLL (thereafter named ZAP-70^{pos}) has been shown to correlate with unmutated *IGHV* genes[3,4]. It was therefore not surprising that its presence is predictive of a more aggressive clinical course. However, analyses of discordant cases demonstrated that it is an independent biomarker and possesses stronger predictive power than the *IGHV* status[5]. Ultimately, this also suggested that ZAP-70 has a biological function relevant to disease progression. In several studies, it was demonstrated that the presence of ZAP-70 enhances BCR-signaling induced by anti-IgM stimulation, though tyrosine kinase activity appears to be dispensable for this[6–8]. Since the presence of a BCR-activating (auto)-antigen seems to be a feature predominantly of M-CLL (and ZAP-70^{neg}) based on the expression of sIgM[9], it remains a conundrum when precisely this function of ZAP-70 becomes relevant *in vivo*.

Several studies have described properties of ZAP-70^{pos} cells which are likely relevant to the pathogenesis of the disease, such as increased secretion of CCL3 and CCL4[10]. In addition, ZAP-70 expression is associated with an increased migration potential[11–13]. These studies provided valuable information to better understand the biology of ZAP-70 positive CLL. However, due to the correlative nature of these studies, it remains largely unknown whether ZAP-70 is a biomarker indicating the presence of these properties, mediated by other factors, or whether ZAP-70 actively contributes. The difficulties inherent to manipulating primary CLL cells were the hitherto major problem to address this question. Therefore, functional studies were often performed only in cell lines, which in several aspects are poor models of CLL. We here provide evidence that ZAP-70 is directly implicated in the provision of survival signals, protein synthesis and recruitment of T cells.

MATERIAL AND METHODS

Cell culture

After patients' informed consent and in accordance with the Helsinki Declaration, peripheral blood was obtained from CLL patients. Studies were approved by the Cambridgeshire Research Ethics Committee (07/MRE05/44). Mononuclear cells were isolated from heparinized blood by centrifuging over a Ficoll-Hypaque layer (PAN-Biotech). Cells were further isolated with anti-CD2 and anti-CD14 beads, resulting in 95%-99% pure CD5^{+}CD19^{+} cells. After thawing of cryopreserved samples, cells were cultured in RPMI 1640 (Gibco), supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin 50 U/ml, sodium pyruvate 1mM, L-glutamine 2mM, L-asparagine 20 mg/ml, 50µM 2-mercaptoethanol, 10mM HEPES, and MEM non-essential amino acids (Gibco). ZAP-70 status was assessed by flow cytometry using intracellular staining with an anti-ZAP-70 antibody (Becton Dickinson

Biosciences). Samples with >20% positive cells compared to isotype control were considered ZAP-70^{pos}.

Nucleofection of primary CLL cells

Nucleofector technology (Lonza) was used to deliver siRNA to primary cells. 5x10^6 CLL cells were re-suspended in 100µl Solution-V with 7.5µL of non-silencing (NSC) control siRNA (Thermofisher Scientific 12935300) or 2.5µL of each ZAP-70 siRNA (Life Technologies HSS187732, HSS187733, HSS187734). The cell-siRNA suspension was nucleofected using the Nucleofector 2b device (Lonza) (program Cell type-4, X-001). Cell pellets were collected and re-suspended in 3ml fresh media. siRNA-transfected CLL cells were co-cultured with 5x10^4 stromal cells (plated 24 hours prior to co-culturing CLL cells) per well in a 6-well plate for 7 days.

Pull down of endogenous ZAP-70 from primary CLL cells

For each condition 20x10^6 purified CLL cells were used. Post BCR-activation using a rabbit-anti-human anti-IgM antibody, cells were fixed with DSG (disuccinimidyl glutarate) for 20 minutes at room temperature. Formaldehyde (1%) in serum-free media was then added for 10 minutes at room temperature, followed by quenching with 125µM glycine for 5 minutes[14]. Cells were then washed by cold 1xPBS twice. Pellets were re-suspended in IP lysis buffer (Thermo Scientific) (supplemented with protease inhibitors) and went through three 'freeze and thaw' cycles on dry ice. Samples were then span at 13000rpm for 6 minutes and supernatant containing the whole cell lysates were collected. An aliquot of supernatant was saved as input for further analysis, the remaining supernatant was incubated overnight at 4^{o}C with an anti-ZAP-70 antibody or IgG. Prewashed protein-A beads were added and further incubated for 4 hours at 4^{o}C. After incubation, the beads were bound to a magnetic rack, then wash buffer removed and processed for mass spectrometry.

Measurement of protein synthesis

5x10^6 primary CLL cells were activated by anti-human IgM coated streptavidin beads (M280) (Thermo Scientific) in 100µl media for 20 minutes. After an additional 30 minutes, OPP intensities were assessed by flow cytometry (Click-iT™ Plus OPP Alexa Fluor™ 488 Protein Synthesis Assay Kit, Thermo Scientific). Mean fluorescence intensity (MFI) on FITC channel was calculated as representation of OPP labeling intensity.

Statistical analyses

All experiments were repeated at least three times, and the means ± SEM were calculated. The exact sample size for each experiment, biological or technical repeats are provided in the figure legends. Statistical analyses of results were performed using two-tail Student paired t-tests. For experiments where more than two groups are compared, statistical analyses were performed using one-way ANOVA followed by two-tail Student paired t-tests. Statistical annotations as previously noted were denoted with asterisks according to the following, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, and ns $p > 0.05$.

Data sharing

RNA-seq data are available at GEO under accession number GSE149022. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository[15] with the dataset identifier PXD019465. Data not included in the main manuscript can be requested from the corresponding author.

Additional information can be found in Supplementary Data.

RESULTS

Slow turnover of ZAP-70 in primary CLL cells requires extended *in vitro* culture for siRNA studies

The BCR-associated kinases Syk and ZAP-70 are presumed to share functional properties in transmitting signals in CLL cells[6]. Due to the high degree of homology between ZAP-70 and Syk, experiments employing small molecule inhibitors do not allow distinguishing between effects on ZAP-70 from those on Syk. To overcome these limitations, we knocked-down ZAP-70 in primary CLL cells using RNA-interference (RNAi). (Patient characteristics are listed in Supplementary Table 8). Our initial experiments were unsuccessful and suggested that ZAP-70 has a half-life of >72 hours. After extended *ex vivo* culture, however, we observed a significant down-regulation of ZAP-70 (Figures 1A,B). To maintain cell survival for further functional studies, cells were temporarily cultured on stroma cells, which substantially limited transfection-induced toxicity and spontaneous apoptosis of cells in suspension culture (Figure 1C). Importantly, RNAi with ZAP-70 expression did not affect Syk-expression, which allowed us to investigate ZAP-70 functions in primary CLL cells without the simultaneous inhibition of Syk (Figures 1D,E).

ZAP-70 promotes cell survival but is not essential for proximal BCR-signaling

Further down-stream analyses were performed on mono-culture by physically removing CLL cells from adherent stroma cells (Figure 2A), achieving pure lymphocyte populations of >98% (data not shown). Since we were particularly interested in understanding how ZAP-70 modulates BCR-signaling, we first assessed whether culturing CLL cells on stroma cells affected the expression of surface IgM, which could possibly impinge on our experiments. Notably, surface IgM levels remained stable over time and were not affected by temporarily culturing cells on stroma cells (Figure 2B). Similarly, removal of CLL cells from stroma monolayers did not alter the expression of surface IgM (Figure 2C), indicating that our experimental system did not skew BCR signaling by modulating surface receptor expression. BCR-activation with anti-IgM treatment rapidly induced Ca^{2+}-flux, which unexpectedly was not altered by the protein levels of ZAP-70 (Figures 2D,E). We subsequently analyzed whether the presence of ZAP-70 affected the activation of proximal BCR-kinases following receptor activation by cross-linking IgM for 20 minutes. This stimulation caused strong phosphorylation of BLNK, Syk and PLCγ2, which was not significantly different between ZAP-70 proficient and depleted cells, although we consistently detected a weaker phosphorylation of PLCγ2 in the ZAP-70 knock-down cells (Figure 2F,G). Similarly, anti-IgM mediated AKT-activation was unaffected by the levels of ZAP-70 (Figure 2H). However, we observed a small, but consistent and significant difference in the baseline phosphorylation of AKT threonine 308, which is a

known target for PDK1. The baseline phosphorylation of AKT serine 473, mediated by mTORC2, remained mostly unchanged by the expression of ZAP-70 (Figures 2H,I).

While proximal BCR signaling was largely unaffected by the expression of ZAP-70 in these experiments, extended *in vitro* culture demonstrated that the survival of CLL cells was significantly dependent on the presence of ZAP-70 in the absence of an induced BCR-signal (Figure 2J). Importantly, the reduced survival of ZAP-70 depleted CLL cells was rescued by IgM crosslinking (Figure 2K).

In conclusion, ZAP-70 expression in CLL cells is required for cell survival in the absence of an induced BCR-signal.

ZAP-70 is required for the constitutive expression of *CCL3, CCL4 and Il4I1*

To further explore how ZAP-70 contributes to disease progression, we performed bulk RNA-sequencing on primary CLL cells from two patients transfected with either ZAP-70 or control siRNA. The principal component analysis (PCA) of the raw gene abundances showed a clear separation between patients and ZAP-70 expression (Figure 3A). We reasoned that ZAP-70 dependent changes in gene expression could easily be masked by the enormous genetic heterogeneity of CLL samples[16] (Figure 3B), and therefore initially used technical replicates on 2 patients, followed by validation-qPCR on additional samples. In total, we identified 40 differentially expressed (DE)-genes, with 27 commonly down-regulated and 13 up-regulated genes in ZAP-70-deficient samples (adjusted p-value <0.05, Log_2(FC)>0.5) (Figure 3C and Supplementary Table 1). Importantly, *MYC*, *CCL4 and CCL3* showed a significant down-regulation by knocking-down ZAP-70 (Figure 3D). In addition, *Cd1c* and *IL4I1*, the latter contributing to immune-suppression in CLL[17], were also downregulated by depleting ZAP-70 expression. ZAP-70 dependent expression of these genes was validated by qRT-PCR in additional ZAP-70^{pos} patient samples (Figures 3E,F and Supplement Figure 1A). CCL3 and CCL4 are chemokines able to attract T cells and have previously been demonstrated to be regulated by BCR-signaling[10]. In line with this observation, inhibition of BTK with ibrutinib completely blunted the constitutive expression of *CCL3* and *CCL4* mRNA (Figure 3G).

Gene ontology (GO) enrichment analysis of the DE genes against the expression gene set also showed ZAP-70 involvement in ‘cell migration’ and ‘antigen binding’ (Figure 3H). Importantly, BCR-receptor activation with anti-IgM treatment (24h) did not significantly alter the ZAP-70 dependent gene expression profile (Supplement Figure S1B). Conclusively, these data provide evidence that ZAP-70 expression not only correlates with the expression of CCL3 and CCL4, but that it is *actively* involved in their constitutive gene expression. This regulation does not require additional triggering of a BCR-signal.

BCR-signaling enhances binding of ZAP-70 to ribosomes

To gain further insights into the biology of ZAP-70 in CLL, we next isolated ZAP-70 immune-complexes from primary CLL cells to identify binding-partners by mass spectrometry. For this, we employed the RIME (Rapid Immunoprecipitation Mass spectrometry of Endogenous proteins) protocol, which provides a sensitive method to identify protein complexes formed by endogenous proteins[18]. Proteins were double cross-linked with formaldehyde and the homobifunctional lysine cross-linker DSG prior to pull down. For the simultaneous analysis of cells either treated with anti-IgM or not, we combined RIME with isobaric labelling using Tandem Mass Tags (TMT-10plex)[14], which allows for the quantitative comparison between replicates and conditions. These combined methods enabled us to detect and to quantify endogenous, transient protein complexes isolated from intact cells. We decided to first perform technical repeats from one patient given the enormous heterogeneity of CLL cells, followed by co-immunoprecipitation studies to validate binding partners on additional patient samples. The TMT-10plex-RIME raw data quantified 1476 unique proteins at a peptide false discovery rate (FDR) of <0.01. PCA showed a clear separation of ZAP-70 protein-complexes from IgG controls and between non-IgM and anti-IgM treated cells (Figure 4A). Expectedly, 20 minutes after anti-IgM treatment, binding of ZAP-70 to known bona-fide BCR signaling components was significantly enhanced (Figure 4B), indicating that this method reliably reports protein-binding partners of ZAP-70. Comparison of IgG controls to proteins isolated from ZAP-70 complexes identified 1387 proteins with enhanced binding to ZAP-70 (Supplementary Table 2). Notably, we identified numerous BCR-related kinases constitutively bound to ZAP-70 in the absence of a triggered BCR-signal. Unexpectedly, a large proportion of bound proteins were ribosomal proteins, followed by proteins involved in metabolic processes and cell adhesion (Figures 4C,D). Further comparison of IgG controls to anti-IgM treated CLL cells showed an overall similar binding profile with a further enrichment of ribosomal proteins and BCR signaling proteins (Figures 4E,F and Supplementary Table 2). We then analyzed which proteins were differentially bound to ZAP-70 in the absence or presence of anti-IgM treatment and found enhanced binding of 214 proteins, while 162 proteins were less abundant after BCR stimulation (Figures 4G,H and Supplementary Table 2). Following BCR-activation, significantly more BCR-related kinases and ribosomal proteins were bound to ZAP-70.

ZAP-70 binding to ribosomal proteins is associated with increased protein synthesis in CLL cells

Cross-reactivity of the ZAP-70 antibody remained a major concern for us to discriminate ‘noise’ from true interacting proteins. To overcome this problem, we engineered the human Burkitt lymphoma B cell line BJAB to express FLAG-tagged full-length ZAP-70 fused to a BirA recognition peptide. BJAB cells, rather than MEC-1 cells, were chosen because they lack

endogenous ZAP-70 expression (data not shown), which we considered a confounding factor in further experiments. Although BJAB cells are fundamentally different from primary CLL cells, we reasoned that the preserved dependency on BCR-signals[19] for cell survival would allow to further investigate the function of ZAP-70 in B cells. For this BJAB-ZAP-70 cells were co-transfected with the *E.coli* derived biotin ligase BirA (thereafter named BJAB^{ZAP-70}) to allow for *in vivo* biotinylation of ZAP-70[20]. Control cells were infected with the same constructs lacking the ZAP-70 coding region (Supplement Figure 2A). Surface IgM expression was not changed by expressing ZAP-70 (Supplement Figure 2B). Anti-IgM treatment of BJAB^{ZAP-70} or BJAB^{control} cells induced calcium^{2+}-flux without significant differences evoked by ZAP-70 expression (Supplement Figure 2C). Notably, sIgM levels were generally higher than on CLL cells (data not shown), which may contribute to the absence of differences in calcium^{2+}-flux between ZAP-70 expresser and non-expresser. To identify proteins interacting with ZAP-70 in response to BCR-activation, cells were treated with anti-IgM for 20 minutes, prior to cross-linking with formaldehyde and labelling with TMT-10plex. In total, we identified 3015 unique proteins in the combined analysis (FDR<0.01). The PCA showed a clear separation of BJAB-cells expressing ZAP-70 or not and between anti-IgM treated and untreated cells (Supplement Figure 2D). Comparison of BJAB^{control} to BJAB^{ZAP-70} cells in the absence of an induced BCR-signal identified 545 unique proteins constitutively bound in ZAP-70-complexes (p-value<0.05). Proteins known to mediate BCR-signals were identified with significantly enhanced binding after anti-IgM treatment (Supplement Figure 2E). Other detected proteins clustered in cell adhesion/ migration and metabolic processes (Supplement Figures 2F,G and Supplement Table 3). Importantly, nearly twice as many proteins were bound to ZAP-70 after BCR-stimulation compared to unstimulated cells (Supplement Figure 2H and Supplement Table 3). The majority of these newly recruited proteins belong to ribosomes (Supplement Figure 2I). Importantly, we performed mass-spectrometry on whole cell lysates from BJAB^{ZAP-70} +/- anti-IgM treatment and did not find an enrichment of ribosomal proteins, indicating that increased binding to ZAP-70 is not related to a shift in the overall expression of those proteins (Supplement Figure 3A and Supplement Table 4).

Since primary CLL cells and BJAB cells shared substantial similarities in ZAP-70 protein-complexes, we rationalized that combining mass spectrometry data obtained in CLL- and BJAB cells would be an opportunity to refine our analysis by further reducing the number of non-specifically bound proteins. For this, we subtracted unique proteins only found to be ZAP-70 bound in either 'BJAB^{ZAP-70} or primary cells after BCR-stimulation. We thereby identified more than twice as many proteins (n=147) interacting with ZAP-70 compared to unstimulated cells (Figure 5A and Supplement Table 5). Besides a strong enrichment of proteins essential for BCR-signaling (Figures 5B,C), a large proportion of these proteins were ribosomal proteins

(Figures 5B-E). RPS-17 was one of the most enriched proteins in ZAP-70 complexes. Co-immunoprecipitation assays on additional patient samples confirmed the constitutive binding of ribosomal proteins to ZAP-70, further increased after BCR-activation (Figures 5F,G). Importantly, we did not observe binding of Syk to ribosomal proteins, suggesting that this property of ZAP-70 is not shared with Syk (Supplement Figure 3B). Interestingly, a recent study using light-affinity purification of ZAP-70 complexes from Jurkat cells also incidentally reported binding to ribosomes in T cells (Supplement Figure 4A)[21].

Based on these results, we hypothesized that ZAP-70 is recruited to ribosomes in response to BCR-activation. To generate further evidence for this, we sought to demonstrate co-localization of ZAP-70 to ribosomal proteins by applying a method not relying on cross-linkage of proteins. For this, we performed polysome profile analysis, which employs ultracentrifugation of cell lysates over a sucrose gradient to separate different ribosome units from total cell lysates. Quantification of RNA by spectrophotometry in each of the sucrose-fraction indicated that the separation of ribosomes was successful (Figure 5H). We detected few polyribosomes, possibly related to the quiescent state of cells and altered ribosome maturation in CLL compared to normal B cells, as reported previously[22]. Following BCR-receptor activation with anti-IgM we observed a significant increase of ZAP-70, co-migrating with the 60-80 ribosome fraction and to a lesser extent in the polyribosome fraction (Figure 5I, J).

We hypothesized that BCR-induced binding of ZAP-70 to ribosomal proteins could have effects on translation. To test this, we applied O-propargyl-puromycin (OP-Puro) to assess the total level of protein synthesis in unstimulated and in anti-IgM activated, primary cells, differing only in their expression of ZAP-70. Metabolic labelling demonstrated that protein synthesis was significantly reduced in CLL cells depleted of ZAP-70 (Figure 5K). Since many of its functions depend on kinase activity, we hypothesize that ZAP-70 mediates phosphorylation of ribosomal proteins. The hypothesis that ZAP-70 is a RBP-modifying kinase is further supported by previously published experiments in T cells: Employing a modified kinase domain of ZAP-70, tyrosine modifications of 15 ribosomal proteins were observed within minutes after inhibition of ZAP-70 in activated Jurkat T cells (Supplement Figure 4B)[23]. The kinetics of these phosphorylation-changes suggest that ZAP-70 may function, either directly or indirectly, as a ribosomal protein kinase, with the net effect of promoting protein biosynthesis.

A previous study has identified MYC being a target for translational regulation in CLL and increased by BCR-stimulation[24]. We therefore tested whether ZAP-70 levels could impact on MYC expression in CLL cells. Interestingly, constitutive MYC-levels were significantly reduced in ZAP-70 depleted cells (Figures 5L,M). In contrast, BCR-mediated upregulation of MYC was unaffected by ZAP-70 levels, indicating again that ZAP-70 levels are critical for the regulation

of biological processes in the absence an induced BCR-signal (Figure 5N and Supplement Figure 4C).

DISCUSSION

Nearly two decades ago ZAP-70 expression was identified in malignant B cells in a subset of CLL patients[4], possessing strong prognostic value for an aggressive disease course[5]. Since then, several studies have demonstrated that ZAP-70 can enhance BCR-signaling[8], though kinase-activity was dispensable for this[6,7]. This suggested that ZAP-70 acts predominantly as an adaptor protein, promoting phosphorylation of other BCR signaling components, such as Syk[6]. Our data further advance our understanding of the biology of ZAP-70 in CLL by demonstrating biological functions which do not depend on a strong BCR signal. In contrast to previously published work[7], we have not observed significant, ZAP-70 dependent changes in calcium-flux or the activation of proximal BCR-kinases following receptor ligation with anti-IgM. A possible explanation for this apparent conundrum is that in our experiments the residual ZAP-70 activity after (an incomplete) knockdown of the protein may fully compensate. The observed moderate, but statistically not significant decrease in PLCγ2-phopsphorylation would support this idea. However, our experimental approach also differs significantly from others, which have overexpressed ZAP-70 in negative, *IGHV* mutated CLL cells[7]. Therefore, it remains possible that the described BCR signaling-enhancing effects of ZAP-70 are more prominent in intrinsically ZAP-70 negative cells, predominantly harboring mutated *IGHV* genes[5] and differing in the cell-of-origin[25].

Regardless of these differences, which require further experimental work to clarify, we identified that ZAP-70 constitutively contributes to cell survival, protein synthesis and gene expression in the absence of an overt, strong BCR-signal. The dependency of these effects on ZAP-70 varied between patients in our experiments, which is likely related to substantial variations in the baseline expression of ZAP-70 among positive-patients, siRNA-mediated degree of ZAP-70 depletion, presence of chromosomal abnormalities and *IGHV* mutational status. Larger studies are needed to systematically address whether ZAP-70 has more or less pronounced affects in any of these subgroups.

The constitutive binding of ZAP-70 to BCR-signaling kinases as well as the moderate, but statistically significant decrease in AKT activation associated with decreased expression of ZAP-70 suggest that ZAP-70 could enhance a tonic BCR-signal. The constitutive activation of PI3K in CLL cells supports this idea and may contribute to the baseline activation of AKT[26]. However, activation of alternative, pro-survival signaling pathways must also be considered.

Our data demonstrate that the depletion of endogenous ZAP-70 not only negatively affected cell survival, but also the expression of genes known to be important for the recruitment and

interaction with T cells. CCL3 and CCL4 are both chemokines able to attract $CD4^+$ T cells, which can further induce CLL proliferation through the activation of CD40. *In vivo* evidences supporting this hypothesis derive from high CCL3 plasma levels found in ZAP-70 positive patients, associated with a shorter time to first treatment[27]. Furthermore, increased CCL3 expression in CLL lymph nodes correlate with $KI67^+$ expression and number of $CD3^+$ T cells[28]. Our data further advance these correlative findings and demonstrate that ZAP-70 *actively* contributes to the expression of these chemokines and suggest that ZAP-70 positive cells can create a more favorable, disease-promoting microenvironment in the absence of a strong BCR-signal.

Another, unexpected finding from our experiments was the identification of protein complexes between ZAP-70 and ribosomal proteins, particularly enhanced upon BCR activation. This observation begs the question of its functional implications. Previous proteomic analyses have described distinct profiles between ZAP-70^{pos} UM- and ZAP-70^{neg} M-CLL; these differences were constitutively present while BCR-activation only increased further changes in ZAP-70^{pos} UM-CLL but not in M-CLL[29]. In context of these results, our data indicate that protein-binding to ZAP-70 is modified by BCR-signals and suggest that it may contribute to these differences in global protein expression through transcriptional and post-transcriptional mechanisms.

Our data are in keeping with a recent report, showing that BCR-signaling drives mRNA translation, partly through *MYC*[24]. We here identified ZAP-70 as an enhancing factor for this. De-regulation of protein biosynthesis in CLL has also been reported by the Willis group, who identified significantly reduced ribosomal maturation and activity related to reduced translation of ribosomal RNA[22]. However, no subtype analyses were performed dependent on ZAP-70 status. A more recent, extensive proteomic characterization of CLL cells identified 'RNA-processing' and 'RNA-binding' as major discriminators between malignant and non-malignant B cells, though these findings were not restricted to a particular subset of patients[30]. Lastly, mutations in RPS15, found in a fraction of patients, cause alterations in translation initiation and fidelity, associated with global protein changes[31]. In conclusion, alterations in protein-biosynthesis are a common finding in CLL and a result of distinct underlying mechanisms. Our data identify ZAP-70 as another factor contributing to de-regulated protein synthesis. It remains unknown from our work how exactly ZAP-70 promotes mRNA translation. Since we used double-cross linking to identify binding-partners, we cannot specify which protein(s) are essential for its association with ribosomes. The possibility that ZAP-70 directly interacts with ribosomal proteins is supported by ZAP-70 associated changes in tyrosine-phosphorylation of ribosomal proteins observed in activated T cells[23]. Post-translational modifications of ribosomal proteins have been shown to modulate protein synthesis[32,33], though the precise mechanisms are poorly defined. We also consider other possibilities of ZAP-70 binding to ribosomes, such as binding to RNA or through functioning as a scaffold protein bound to newly

synthesized proteins. An alternative, though not mutually exclusive, possibility is that ZAP-70 regulates protein synthesis throughs its effects on MYC expression, since MYC itself can promote translation through direct and indirect mechanisms[34,35].

In conclusion, our data show that ZAP-70 is directly involved in the regulation of gene expression and protein synthesis, with the net-effect of improved cell survival. Therefore, ZAP-70 may increase disease aggressiveness through its function as a coordinating signaling node in malignant B cells, for which a strong BCR-signal is not essential.

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AUTHORSHIP CONTRIBUTION

J.C., V.S., A.M., C.J., S.T. performed wet-lab experiments, analyzed data and created figures. RNA-seq data were analyzed by A.S. and I.M.. Proteomic experiments were planned in close collaboration with C.D'S. and analyzed by V.N.R.F. and C.S.R.C., who also provided raw data and figures. The study was planned by I.R. with guidance of S.B.H and A.W.. I.R. wrote the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

All authors declare no conflict of interest. I.R. has previously received a research grant from AstraZeneca and advised the company as a consultant, both activities are irrelevant to this work.

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FIGURE LEGENDS

Figure 1. SiRNA effectively depletes ZAP-70 without affecting Syk expression in primary CLL cells.

A. Representative ZAP-70 and β-actin immunoblots of primary CLL cells at indicated time points post siRNA nucleo-transfection with a non-specific siRNA and ZAP-70 siRNA.
B. Quantification of ZAP-70 expression using ImageJ© and calculated as ratio (siZAP-70/NSC) after normalization to β-actin signal. Time points indicate time after nucleofection; (n=3).
C. Percentage of live (Annexin V-, DAPI-) CLL cells on day 7 of transfection. Primary CLL samples (n=7) were transfected with NSC or ZAP70 siRNA and cultured for 7 days on stroma cells.
D. Representative immunoblots of ZAP-70, Syk and β-actin of primary, purified CLL cells 7 days post siRNA transfection.
E. Quantification of ZAP-70 and Syk expression, normalized to β-actin, 7 days post siRNA transfection; (n=7).

Statistical analyses of results were performed using one-way analysis of variance (ANOVA), following paired two-tailed Student t-tests. ns, not significant. * $p< 5\times10^{-2}$; ** $p<10^{-2}$.

Figure 2. ZAP-70 contributes to cell survival, but is not essential for IgM-stimulated BCR-signaling

A. Scheme shows the strategy for siRNA knock-down of ZAP-70 in primary ZAP-70 positive CLL cells and co-culture on feeder cells. Down-stream analyses were performed on purified CLL cells in mono-culture. Created with BioRender.com
B. Mean Fluorescence Intensity (MFI) of cell surface IgM on $CD19^+$ CLL cells. Primary ZAP-70 positive CLL cells (n=5) were mono-cultured or co-cultured on stromal cells for 1 or 2 days before assessment of surface IgM by FACS analysis.
C. Graph shows the Mean Fluorescence Intensity (MFI) of cell surface IgM on $CD19^+$ CLL cells transfected with non-specific siRNA or ZAP-70 siRNA. Primary ZAP-70 positive CLL cells (n=6) were transfected with siRNA and co-cultured with stromal cells for 7 days and then separated from stromal cells for additional 24 hours in mono-culture.
D. Representative kinetic plots show the calcium flux of one primary CLL sample after transfection with NSC or ZAP-70 siRNA. CLL cells were harvested and labeled with Fluo-4 (FITC) after 24 hours in mono-culture. Anti-IgM activation was triggered 30 seconds after flow cytometric measurement started.
E. Quantification of calcium-flux response of CLL samples (n=7) transfected with NSC or ZAP-70 siRNA. Ratios were calculated by using kinetic plots, dividing peak Fluo-4 (FITC-A) intensity induced by anti-IgM activation by baseline Fluo-4 intensity.
F. Representative phospho-BLNK (Tyr96), phospho-Syk (Tyr525/526), phospho-PLCγ2 (Tyr759), total BLNK, total Syk, total PLCγ2, ZAP-70 and β-actin immunoblots of primary CLL cells mono-cultured for 24 hours after non-specific siRNA or ZAP-70 siRNA transfection. To induce BCR-signaling, mono-cultured CLL cells were treated with beads-bound anti-IgM for 20 minutes.
G. Quantification of phospho-kinases relative to total (unphosphorylated) proteins after 24 hours in mono-culture after siRNA transfection, using ImageJ© software (n=6).
H. Representative phospho-AKT (Ser473), phospho-AKT (Thr308), total AKT and β-actin immunoblots of primary CLL cells mono-cultured for 24 hours after non-specific siRNA or ZAP-70 siRNA transfection. For anti-IgM stimulated samples, mono-cultured CLL cells were treated with beads-bound anti-IgM for 20 minutes. [SE=short exposure].
I. Quantification of phospho-AKT relative to total (unphosphorylated) AKT after 24 hours in mono-culture after siRNA transfection, using ImageJ© software (n=4 or 5, as indicated).
J. Percentage of live (Annexin V^-, $DAPI^-$) CLL cells, cultured for 48 or 72 hours in mono-culture post transfection (n=10 and n=8, respectively).
K. Percentage of live (Annexin V^-, $DAPI^-$) CLL cells, cultured in mono-culture post transfection (n=7) and treated with anti-IgM for 48 hours (n=6).

Figure 3: ZAP-70 constitutively regulates gene expression relevant for T cell interactions

A. Principal component analysis (PCA) created on the top 2000 most abundant genes, illustrates the separation across patients (mainly captured in PC1) and treatments (PC2).
B. Venn Heatmap representing the Jaccard Similarity Index (JSI), on the top 2000 most abundant genes, across all pairwise comparisons on patients and treatments. The JSI spans the 0 to 1 range, which corresponds to no overlap (0) to identical sets of genes (1). The clear separation between patients is contrasted by closer similarity on replicates and treatments.
C. Volcano blot illustrating the DE genes (x-axis presents the log2(FC) between the Control and si70 samples; y-axis presents the -log10(p-value)); grey points correspond to non-DE genes, red points correspond to genes for which the p-value < 0.05 (but |log2(FC)| < 0.5), blue points are the DE genes called this analysis, color-intensity is proportional to the log2 of the average normalized expression levels across the compared samples.
D. Heat map of genes from two individual primary patients CLL cells treated with NSC or ZAP-70 siRNA. Red and blue indicate relatively high and low expression, respectively. Each condition analyzed depicts technical replicates, derived from 2 individual siRNA transfections. Genes were ranked by average Log2FC from EdgeR analysis from bottom to top.
E. QRT-PCR analysis of *CD1c and IL4I1* in primary CLL cells ($CD19^+$-positive selected) (n=8 and n=7 respectively) transfected with non-specific siRNA or ZAP-70 siRNA and further mono-cultured for 24 hours. Relative gene expression level is normalized to reference gene *GAPDH* and compared to NSC control.
F. QRT-PCR analysis of *CCL3 and CCL4* in primary CLL cells ($CD19^+$-positive selected) (n=8 and n=7, respectively) transfected with non-specific siRNA or ZAP-70 siRNA and further mono-cultured for 24 hours. Relative gene expression level is normalized to reference gene *GAPDH* and compared to NSC control.
G. QRT-PCR analysis of *CCL3 and CCL4* in primary CLL cells ($CD19^+$positive selected) (n=4) transfected with non-specific siRNA or ZAP-70 siRNA. Ibrutinib was treated at 0.5μM for 24 hours before cell harvesting. Relative gene expression level is normalized to reference gene *GAPDH* and compared to NSC control.
H. Gene ontology (GO) enrichment analysis of the DE-genes in (C). Background genes are the set of all genes that pass the noise threshold.

Figure 4: Constitutive binding of ZAP-70 to BCR-kinases and ribosomal proteins

A. Principal component analysis (PCA) of protein abundance, representing the variability between anti-ZAP-70 pull down -/+ anti-IgM stimulation for 20 minutes and IgG isotype control. Each dot represents one technical replicate with an individual pull-down of ZAP-70 or IgG.
B. Heat map of proteins annotated in KEGG 'B cell receptor signaling pathway', identified by mass spectrometry as ZAP-70 binding partners. Red and blue indicate relatively high and low protein abundance, respectively. Each condition analyzed depicts three (unstimulated) or four (IgM activated) technical replicates. Proteins were ranked from left to right by Log2FC ratio comparing IgM activated to unstimulated group.
C. Volcano plot showing proteins bound to ZAP-70 (Log2FC>0) vs. IgG isotype control (Log2FC<0) in unstimulated CLL cells.
D. Gene set enrichment analysis (GSEA) was applied to identify pathways that ZAP-70 associated proteins are associated with in unstimulated CLL cells. Pathways are listed in order of Normalized Enrichment Scores (NES) in columns (top x-axis). FDR-q values for each pathway set are indicated by the red dotted line (lower x-axis). Colors of column represent different sub-grouped cell functions.
E. Volcano plot showing proteins bound to ZAP-70 (Log2FC>0) vs. IgG isotype control (Log2FC<0) in anti-IgM activated CLL cells.
F. Gene set enrichment analysis (GSEA) to identify pathways associated with ZAP-70 in anti-IgM activated CLL cells.
G. Volcano plot showing proteins differentially bound to ZAP-70 (Log2FC>0) in unstimulated vs. anti-IgM activated states.
H. Gene set enrichment analysis (GSEA) to identify pathways associated with ZAP-70 after BCR-activation with in anti-IgM.

Figure 5. BCR-activation induces binding of ZAP-70 to ribosomal proteins and is associated with enhanced protein-biosynthesis

A. Venn diagram comparing ZAP-70-binding proteins identified by mass spectrometry (MS) in anti-IgM activated (20 minutes) primary CLL cells and BJAB cells (Log2FC>1, adj p<0.01).
B. GSEA gene ontology (GO) terms extracted from the unique 147 proteins identified in (A) from both primary CLL cells and BJAB cells. GO signatures are listed in order of Normalized Enrichment Scores (NES) in columns (top x-axis). FDR-q values for each signature set are indicated by the red dotted line (lower x-axis). Colors of column represent different sub-grouped cell functions.
C. The 147 unique proteins from (A) were plotted by the Log2 fold change (Log2FC). X-axis, identified in primary CLL cells MS; Y-axis, identified in BJAB cells. Colors indicate the sub-grouped functions of the proteins. Grey dots indicate proteins involved in other cellular functions.
D. Heat map of ZAP-70-associated ribosomal proteins from unstimulated or anti-IgM activated primary CLL cells. (adjusted p<0.05). Red and blue indicate relatively high and low protein abundance, respectively. Each row analyzed depicts a technical replicate.
E. Heat map of ZAP-70-associated ribosomal proteins from unstimulated or anti-IgM activated $BJAB^{ZAP-70}$ cells versus non-specifically biotinylated proteins from $BJAB^{control}$ cells (adjusted p<0.05). Red and blue indicate relatively high and low protein abundance, respectively. Each row analyzed depicts a technical replicate.
F. Whole cell lysates from primary CLL cells, unstimulated or stimulated with anti-IgM for 20 minutes, were immunoprecipitated (IP) with a ZAP-70 antibody or control IgG and immunoblotted (IB) with antibodies for ZAP-70, Syk and RPS17.
G. Quantification of RPS17 bound to ZAP-70. The levels of RPS17 co-immunoprecipitated with ZAP-70 were normalized to total ZAP-70 levels. Anti-IgM treated samples were then normalized to non-treated samples (n=4).
H. Comparison of polysome profiles between unstimulated (blue) and anti-IgM activated (20 minutes) (red) primary CLL samples. Vertical lines separating the curves indicate the fractions mainly extracted from 40s ribosome subunits, 60-80s ribosome subunits and polysomes.
I. Proteins from sucrose gradient fractions in primary CLL cells were precipitated by 20 % (w/v) trichloroacetic acid (TCA) and immunoblotted with ZAP-70, RPL29 and RPS17. Blue boxed graphs: unstimulated cells; Red boxed graphs: anti-IgM activated cells. Fractions 6-19 represent 40s subunits, 60-80s subunits, polysomes, separated by vertical lines.
J. Quantification of relative ZAP-70 protein levels (relative to RPL17) in indicated fractions in primary CLL samples (n=3). Anti-IgM activated samples were normalized to unstimulated samples.
K. Graph showing the relative (to untreated NSC) fold change in OPP labeling (30 minutes) in unstimulated or anti-IgM activated (20') primary CLL cells (n=11), previously transfected with NSC or ZAP-70 siRNA.
L. Representative MYC and β-actin immunoblots of primary CLL cells mono-cultured for 24 hours after non-specific siRNA or ZAP-70 siRNA transfection.
M. Quantification of c-MYC levels in primary CLL cells proficient or depleted of ZAP-70. Graphs were generated using ImageJ © (n=4).
N. Representative immunoblots for MYC, ZAP-70 and β-actin of primary CLL cells mono-cultured for 24 hours after non-specific siRNA or ZAP-70 siRNA transfection. To induce BCR-signaling, mono-cultured CLL cells were treated with bead-bound anti-IgM for 24 hours.

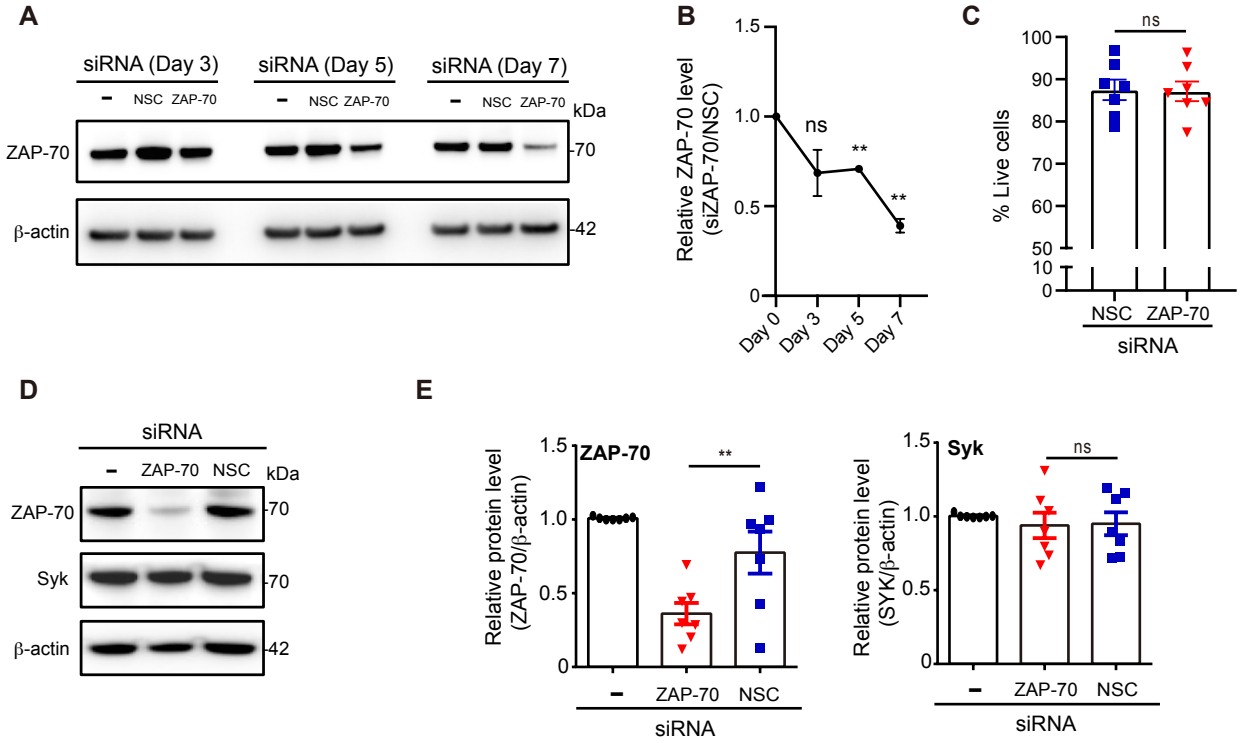

Figure 1
Chen et.al

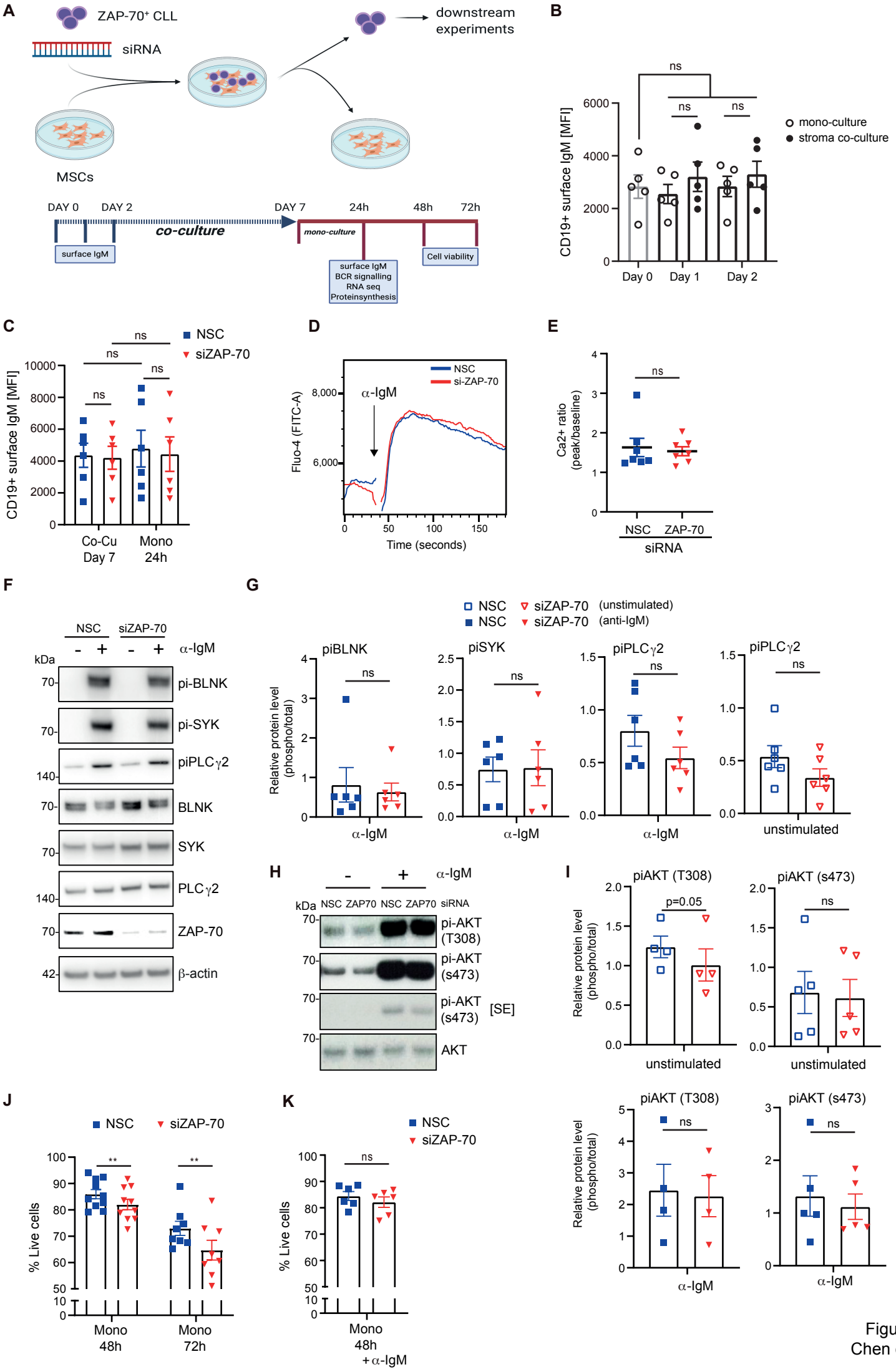

Figure 2
Chen et.al

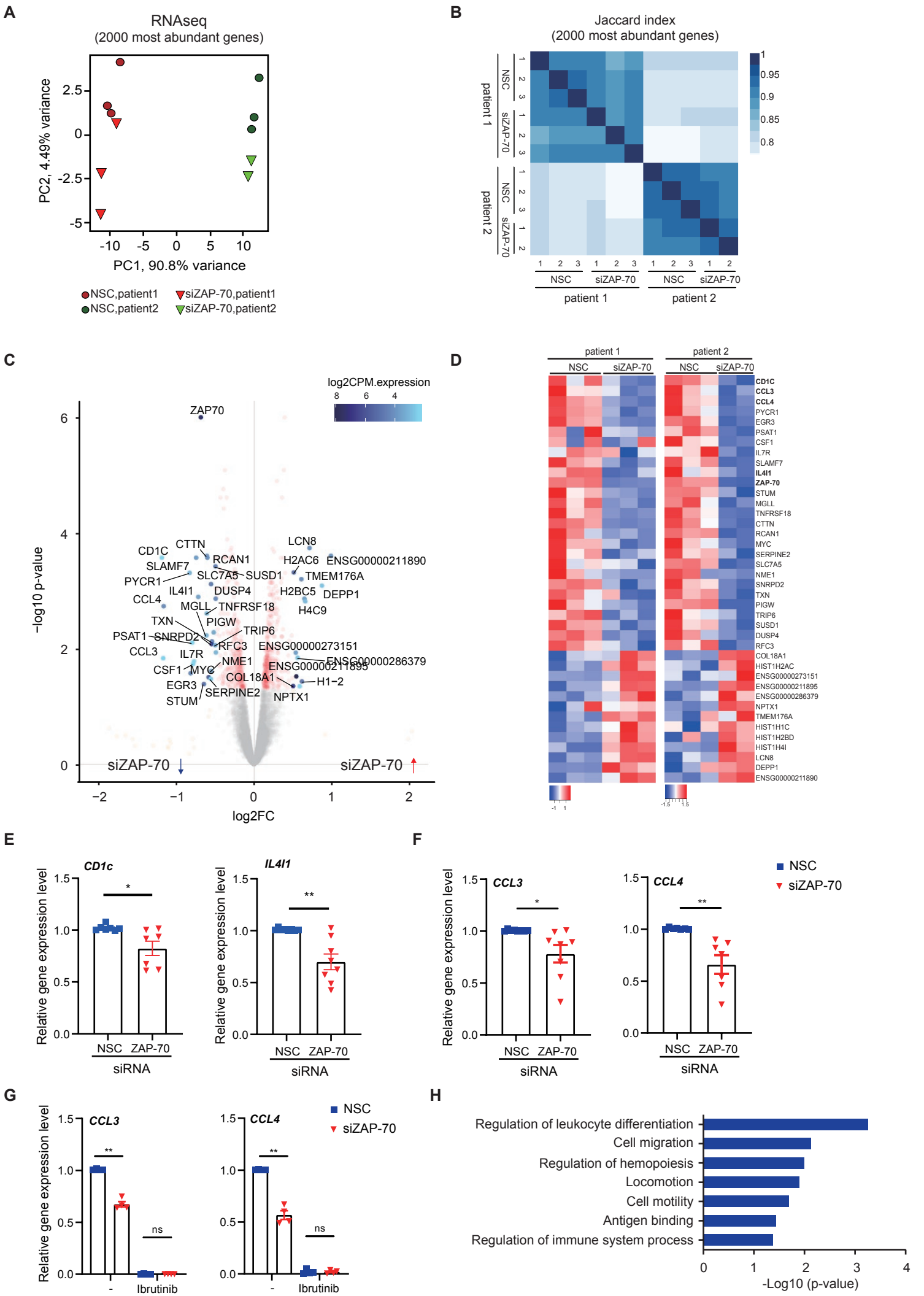

Figure 3
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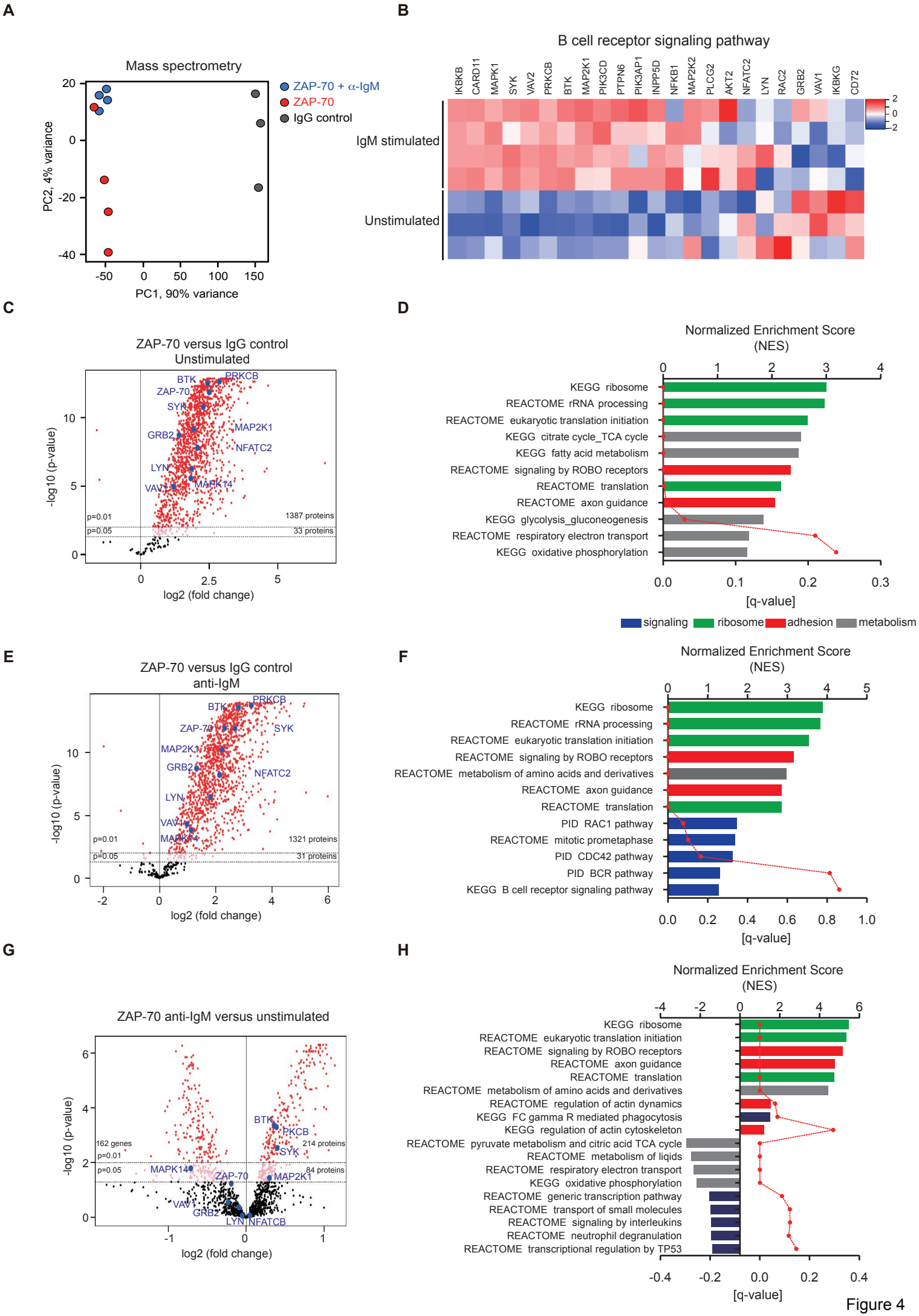

Figure 4
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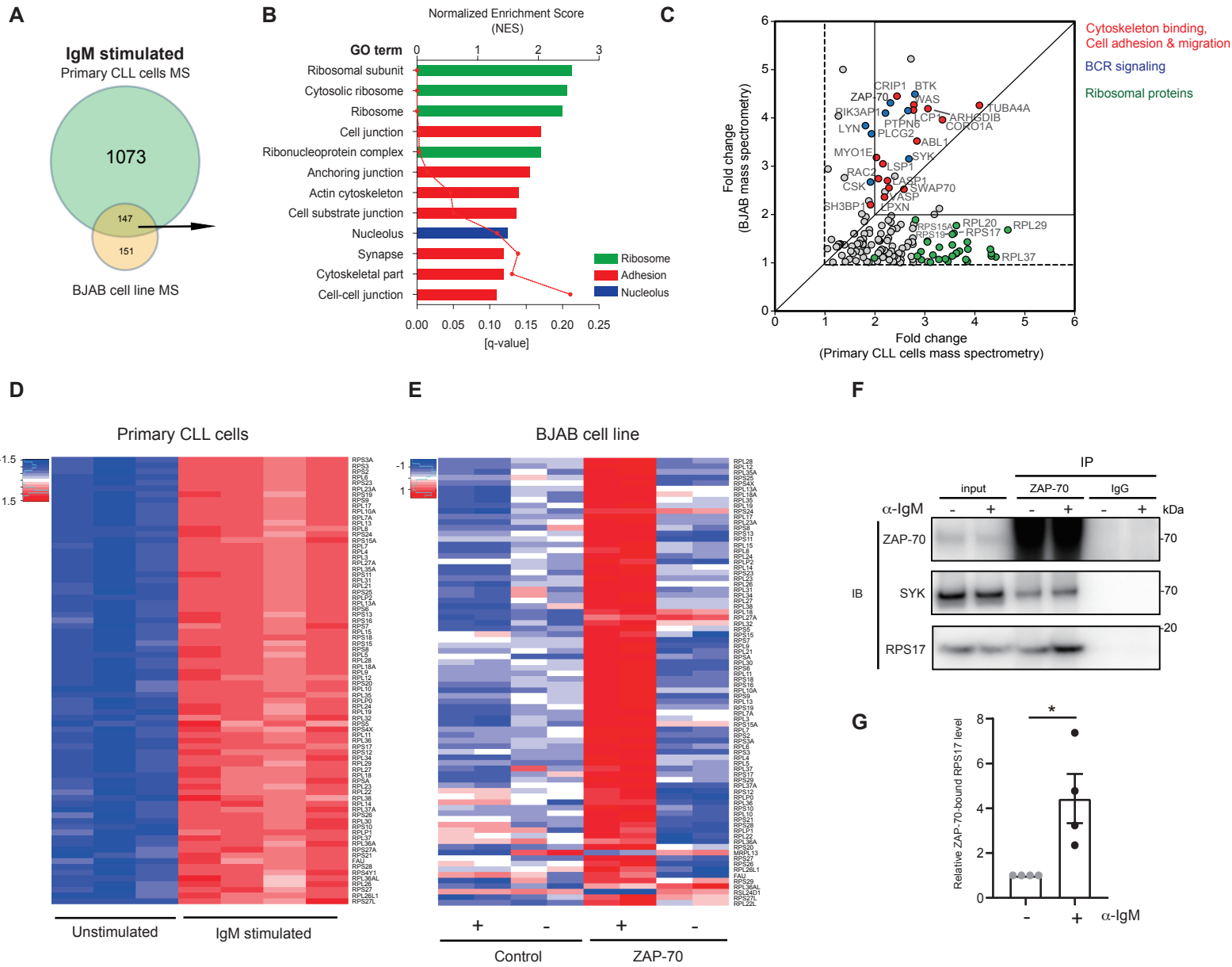

Figure 5
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Supplementary data for

ZAP-70 constitutively regulates gene expression and protein synthesis in chronic lymphocytic leukemia

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The following Supplementary data are available online:

Supplementary Table 1: Transcriptome data from CLL cells +/- ZAP-70 siRNA.
Supplementary Table 2: Mass spectrometry data from primary CLL cells.
Supplementary Table 3: Mass spectrometry data from BJAB cells.
Supplementary Table 4: Whole proteome analyses of $BJAB^{ZAP\text{-}70}$ +/- anti-IgM.
Supplementary Table 5: Common ZAP-70 binding partners in CLL and BJAB cells

The following Supplementary data are enclosed:

Supplementary Material and Methods
Supplementary Table 6: Primers used for qPCR
Supplementary Table 7: Antibodies used for FC, IB
Supplementary Table 8: Patient characteristics
Supplementary Figures 1-4

Supplementary Material and Methods

Reagents

Ibrutinib was obtained from SYNKinase, Australia (SYN-1171) and Ionomycin from Cayman Chemicals, USA.

Streptavidin pull down of ZAP-70^{BirA}

10% of total lysate from BJAB cell line was saved as "input". Remaining lysate was incubated for 4 hours with 10µl of MyOne streptavidin T1 (Dynabeads 65601) at 4°C with rotation brought up to 500µl using lysis buffer, the beads were washed five times with PBS prior to elution. Immunoblotting analysis was performed using 5% of each fraction.

Stroma-CLL co-culture conditions

Following siRNA transfection, primary CLL cells were cultured on murine stroma cells, either derived from embryonic livers (EL08-1D2 cells) or the bone marrow (selected by adhesion to plastic). Mouse stroma cells were cultured in MEM-GlutaMAX (Gibco), supplemented with 10% fetal bovine serum (FBS), 10% horse serum, penicillin/streptomycin 50 U/ml, 50µM β-mercaptoethanol. Stroma cells were seeded in 6-well plates at an initial confluency of 10%. After their adhesion and without subsequent irradiation, $5x10^6$ transfected CLL cells/ well were added on top and cultured for 7 days. CLL cells were physically removed from co-cultures and further purified as indicated in each experiment.

Generation of BJAB^{ZAP70} cell line

Cloning of FLAG tagged full-length ZAP70 fused to BirA recognition peptide.

ZAP-70 expression vectors were kindly provided by Arthur Weiss (University of California, San Francisco, USA). To generate BirA-tagged ZAP-70, ZAP70 was PCR amplified from a pcDNA4 ZAP70 WT vector using BAMHI ZAP70 forward primer (5'-3'):

GGATCCTCCCAGACCCCGCGGCGCACCTGCCCTTCTTCTACGGCAGCATCTCG
CGTGCCGAGGCCGAG

and BAMHI ZAP70 reverse primer (5'-3'):
CCACCACACTGGACTAGTGGATCCTCAGGCACAGGCAGCCTCAG.

The ZAP70 fragment was inserted into the pRetroCMV /T0/ Puro/GFP and pRetroCMV /T0/ Puro vector (kindly obtained from Dr. Daniel Hodson, University of Cambridge) by Gibson assembly using Flagbio BAMHI ZAP70 forward primer (5'-3'):
AGGCGCGCCGAGCTCGAGGATCCTCCCAGACCCCGCGGCGCACCT.

Transfections and Retroviral Transductions

Single suspensions of HEK293T cells were plated on a 6 well dish to achieve about

40% confluency in preparation for transfection. Transfection was performed 24 hours after seeding using room temperature TransIT-293 (Mirus) (vortexed well). In 200µl of 1x PBS (no FBS), 3.6µl of TransIT-293 was added, mixed well and incubated for 10 minutes at room temperature. After incubation, 200ng of packaging plasmid (DH28 and DH29 kindly obtained from Dr. Daniel Hodson at the University of Cambridge) and 800ng of retroviral plasmid were vortexed and added. This mixture was further incubated for 45 minutes at room temperature and then added drop wise onto the cells, which was swirled around gently to mix with the cell media. The cells were then incubated overnight at 37°C. Retroviral supernatant was harvested 48 hours post transfection and spun down at 2500rpm for 10minutes at 4°C. The supernatant had 25mM HEPES and 10µg/ml Polybrene added, which was then dispensed over the cells in a 24 well plate, plated with parental BJAB cells at 40% confluency, and mixed gently. The cells and supernatant were then spun at 2500g for 1 hour and 30 minutes at 30°C. After centrifugation, most of the media was aspirated and replaced with cell line appropriate media. After protein expression was seen (48-72 hours), antibiotic selection was carried out by adding 2µg/ml of puromycin and allowed to grow for a few more days.

Cell viability assay

Cells were harvested and stained by FITC-Annexin V (Biolegend) for 20 minutes at room temperature. DAPI was added prior to FACS analysis. Live cells were assessed as Annexin V^- and $DAPI^-$ populations.

Sucrose density gradient

1-2x10^8 of primary CLL cells were harvested or activated with 200µg Biotin-SP (long spacer) AffiniPure Fab Fragment Goat Anti-Human IgM (Jacksonimmuno Research) for 20 minutes. Cycloheximide was added at 100µg/ml in the media to prevent the protein synthesis process. Ribosomal subunits were separated by sucrose density gradients as described[1]. Proteins were precipitated from sucrose gradient fractions with 20 % (w/v) trichloroacetic acid, separated on SDS-PAGE gels and transferred to PVDF membranes for immunoblotting. Protein levels or intensity were measured using Fiji (Image J©).

Co-Immunoprecipitation (Co-IP)

1x10^8 of primary CLL cells were harvested or activated with 200µg Biotin-SP (long spacer) AffiniPure Fab Fragment Goat Anti-Human IgM (Jacksonimmuno Research) for 20 minutes. Cells were then harvested and double cross-linking was processed using Disuccinimidyl Glutarate (DSG 2mM) for 30 minutes and 1% formaldehyde for 15 minutes, followed by quenching with 125µM glycine for 5 minutes. Cells were then washed by cold 1xPBS for two times. Pellets were re-suspended in IP lysis buffer (Thermo Scientific) (supplemented with protease inhibitors) and went through three 'freeze and thaw' cycles on dry ice. Samples were then spun at 13000rpm for 6 minutes and supernatant containing the whole cell lysates were collected. 25 µl Dynabeads Protein A (Thermo Scientific) was added into lysates and gently span in 4°C to pre-

clear un-specific proteins that bind to the beads. Pre-cleared lysates were measured by Bradford assay to determine the protein concentration. An aliquot of lysates were taken and stored for further analysis as 'Input'. Total of 500μg-1mg protein lysates were mixed with 5μg ZAP70 antibodies (BD) or 5μg isotype IgG antibody (Abcam) and incubated at 4°C for overnight. 40μl Dynabeads Protein A was added into lysates to isolate protein-antibody complex. Beads-antibody-protein complex was then washed with 1xPBS for 5 times, followed by adding 1xSDS loading buffer to elute specific-bound proteins. Samples were then separated by SDS-polyacrylamide gel electrophoresis and followed by immunoblotting.

Immunoblotting

After the specified treatments, cells were harvested and lysed in RIPA buffer and a total of 10-20 μg protein was separated by SDS-polyacrylamide gel electrophoresis, followed by blotting to polyvinylidene difluoride (PVDF) membranes (Millipore), and probing with indicated primary antibodies. Protein signal intensity was measured using Fiji (Image J©) for quantification and statistical analysis.

qRT-PCR

Total RNA from CLL cells transfected with siRNA was extracted using RNeasy Mini Kit followed by DNase treatment (Qiagen) according to manufacturer instructions. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was used as template to amplify indicated genes by real time PCR. Relative gene expression level was assessed by $2^{-\Delta\Delta CT}$ and normalized to gene *GAPDH*.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) for mass spectrometry data was performed using the GSEA platform (GSEA_4.0.3) maintained by Broad Institute. The gene matrix(c2,cp.v7.1.symbols.gmt;c2,cp.kegg.v7.1.symbols.gmt; c5.all.v7.1.symbols.gmt) were selected as database for indicated analysis.

Proteomics analysis

Peptide intensities were normalized using median scaling and protein level quantification was obtained by the summation of the normalized peptide intensities. A statistical analysis of differentially-regulated proteins was carried out using the qPLEXanalyzer, R-bioconductor package[2]. Multiple testing correction of p-values was applied using the Benjamini-Hochberg method (https://www.jstor.org/stable/2346101?seq=1#page_scan_tab_contents) to control the false discovery rate (FDR).

Supplementary Table 6: Primers used for QRT-PCR

Gene	Sequence (5'-3')
CCL4, forward	CTGTGCTGATCCCAGTGAATC
CCL4, reverse	TCAGTTCAGTTCCAGGTCATACA
CCL3, forward	AGTTCTCTGCATCACTTGCTG
CCL3, reverse	CGGCTTCGCTTGGTTAGGAA
IL4I1 forward	ACTCGCCCGAAGACATCTAC
IL4I1, reverse	CATCCTCGGACATCACGTCTC
MYC, forward	GTCAAGAGGCGAACACACAAC
MYC reverse	TTGGACGGACAGGATGTATGC
GAPDH, forward	GTGAAGGTCGGAGTCAACG
GAPDH, reverse	TGAGGTCAATGAAGGGGTC

Supplementary Table 7: Antibodies used for FC, IB

Name	Source	Cat#	Fluorochrome
anti-ZAP70	BD	610240	
anti-SYK	Cell signaling	13198	
anti-RPS17	Abcam	ab128671	
anti-RPL29	Abcam	ab67196	
anti-BLNK	Cell signaling	36438	
anti-PLCγ2	Cell signaling	55512	
anti-SYK (Tyr525/526)	Cell signaling	2710	
anti-PLCγ2 (Tyr759)	Cell signaling	3874	
anti-BLNK (Tyr96)	Cell signaling	3601	
anti-AKT	Cell signaling	9272	
anti-AKT (Ser473)	Cell signaling	4060	
anti-AKT (Thr308)	Cell signaling	13038	
anti-PRS6	Cell signaling	2217	
β-actin	Cell signaling	5125	
anti-CD5	BD Biosciences	555352	FITC
anti-CD19	BD Biosciences	555415	APC
Annenxin V	Biolegend	640945	FITC

Supplementary Table 8: Patient characteristics

Patient ID	Age	Gender	% ZAP-70 expression*	IGHV status‡	FISH	P53 mutation
0067	81	female	28	M	n.d.	n.d.
5235	89	male	23	M	n.d.	n.d.
0991	55	male	20	UM	del13q	WT
6258	76	male	40	UM	del13q, del11q	WT
9941	69	male	29	M	del13q	WT
1081	82	female	20	UM	del17p	mutated
5148	63	male	27	M	del13q	WT
2847	93	female	36	UM	n.d.	n.d.
5296	68	male	22	UM	normal KT	WT
0336	64	female	26	UM	tri12	WT
9512	63	male	31	M	del13q	WT
3159	50	female	20	M	n.d.	n.d.
3414	63	male	16	UM	del13q	WT

* ZAP-70 expression was assessed by Flow cytometry (applying a cut-off of 20% positive cells[3]).
‡ IGHV sequences were considered as unmutated if their homology with the closest germline counterpart was equal or more than 98%; M=mutated, UM=unmutated.

n.d.=not done
WT=wild type; KT=karyotype
Patient marked in yellow were used for RNA-seq experiments (Figure 3)

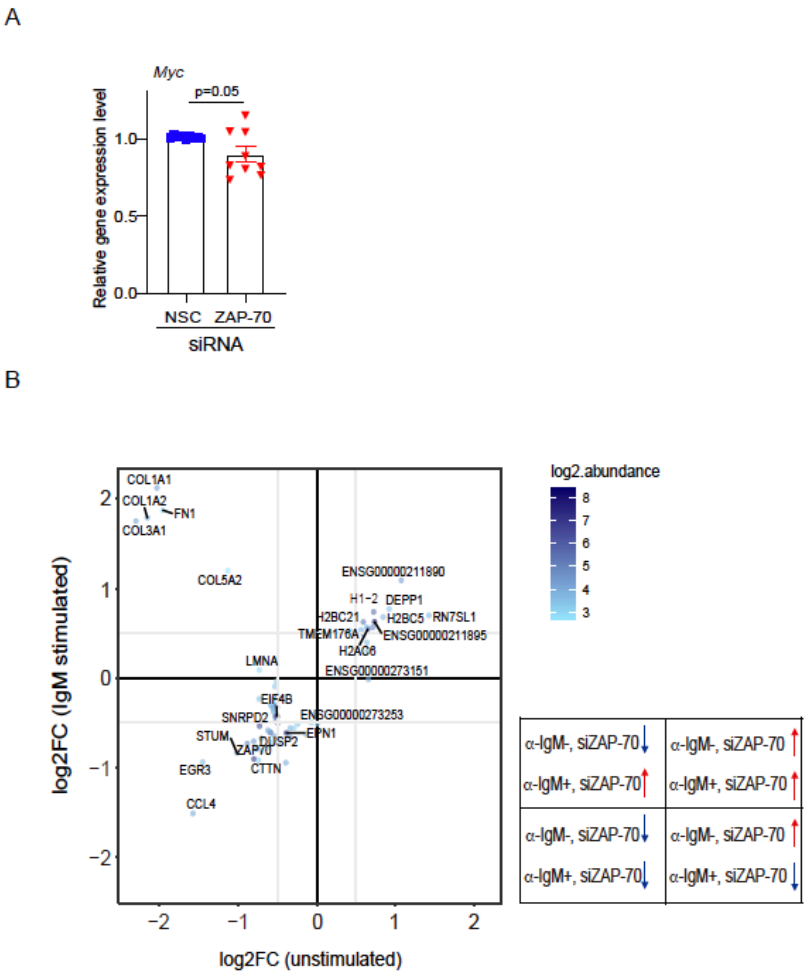
References

1 Tan, S. *et al.* EFL1 mutations impair eIF6 release to cause Shwachman-Diamond syndrome. *Blood* **134**, 277-290, doi:10.1182/blood.2018893404 (2019).

2 Papachristou, E. K. *et al.* A quantitative mass spectrometry-based approach to monitor the dynamics of endogenous chromatin-associated protein complexes. *Nature communications* **9**, 2311, doi:10.1038/s41467-018-04619-5 (2018).

3 Rassenti LZ, LZ, Huynh L, Toy TL, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *The New England journal of medicine*. 2004;351(9):893–901

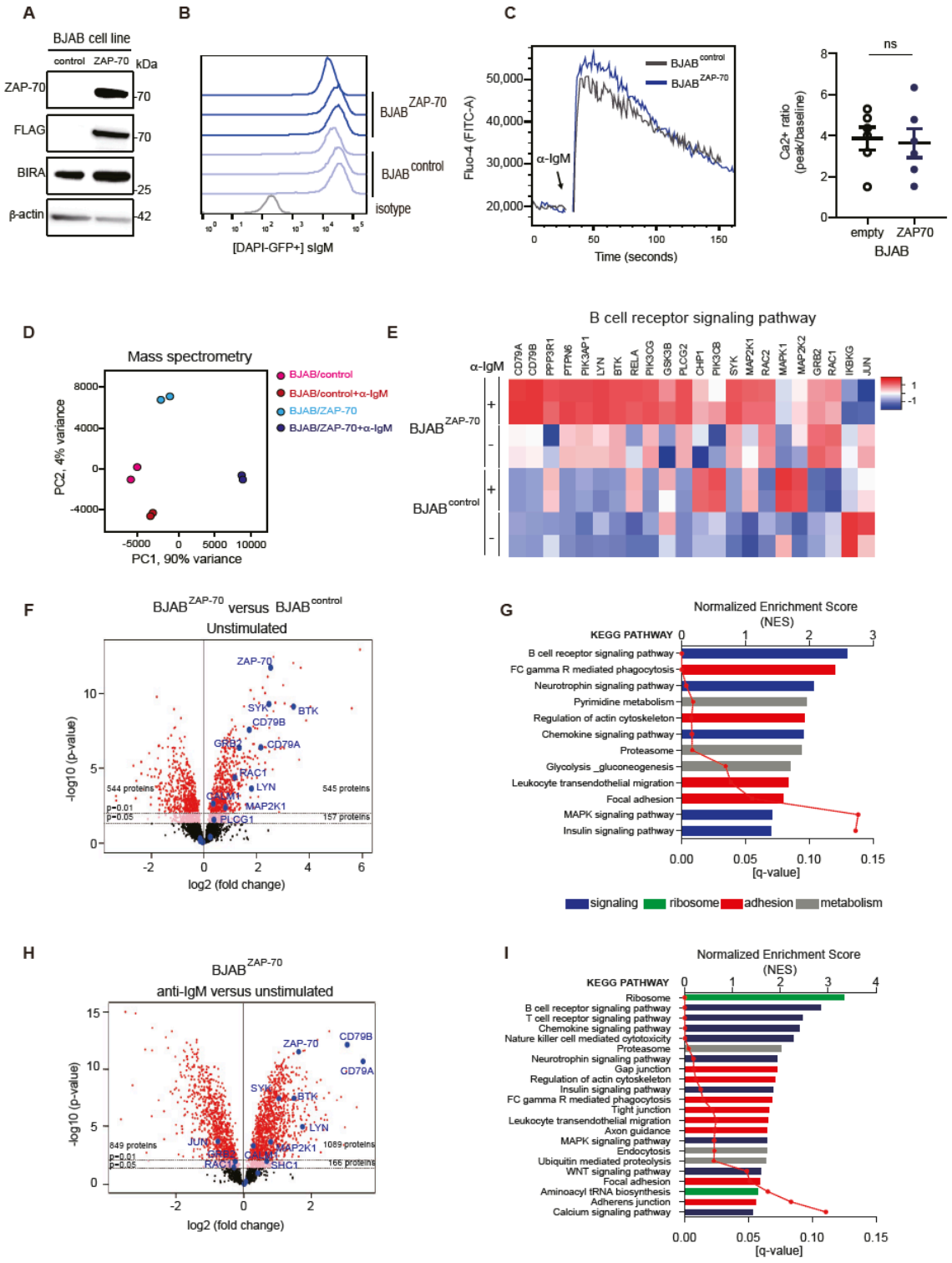
SUPPLEMENTARY FIGURES



Supplement Figure 1: ZAP-70 dependent gene expression in the absence or presence of a BCR-activating signal

(A) QRT-PCR analysis of *MYC* in primary CLL cells ($CD19^{+}$-positive selected) (n=9) transfected with non-specific siRNA or ZAP- 70 siRNA and further mono-cultured for 24 hours. Relative gene expression levels are normalized to reference gene GAPDH and compared to NSC control.

(B) The cross plot illustrates the variation in DE signatures with regard to anti-IgM treatment for 24h; the x-axis represents the log2(FC) of the Control vs siZAP-70 treatment in the unstimulated (IgM-) condition, the y-axis represents the analogous comparison in BCR-activated cells (IgM+). Each point represents a gene; the color-intensity is proportional to the average log2 abundance across the compared samples. The majority of points follow the secondary diagonal, suggesting a similar transcriptomic response for IgM both as directionality and amplitude of difference in expression. The few genes with opposite behavior (down-regulated in IgM- and up-regulated in IgM+, right upper quadrant) have lower abundance.



Supplement Figure 2: Mass spectrometry of BJAB cells expressing biotinylated ZAP-70

(A) ZAP-70, FLAG, BIRA and immunoblots of BJAB cells with control vector ($BJAB^{control}$) or ZAP-70-expression vector ($BJAB^{ZAP-70}$).

(B) Representative FACS histograms of surface IgM levels on DAPI-negative and GFP^+ $BJAB^{control}$ and $BJAB^{ZAP-70}$ cells.

(C) Representative kinetic plots show the calcium flux of $BJAB^{control}$ or $BJAB^{ZAP-70}$ cells. BJAB cells were harvested and labeled with Fluo-4 (FITC). Anti-IgM activation was triggered 20 seconds after flow cytometric measurement started. Quantification of Ca^{2+} flux ratios were calculated by using kinetic plots, dividing peak Fluo-4 (FITCA) intensity induced by anti-IgM activation by baseline Fluo-4 intensity. Statistical significance between samples was assessed by unpaired two tailed Student t-tests. ns, not significant (n=6).

(D) The principal component analysis (PCA) of the protein abundance was made to represent the variability between unstimulated or IgM-activated $BJAB^{control}$ and $BJAB^{ZAP-70}$ cells. BJAB cells were cross-linked with formaldehyde after treating with anti-IgM for 20 minutes. Biotinylated protein-complexes were then pulled-down by streptavidin, followed by on-bead digestion and mass spectrometry.

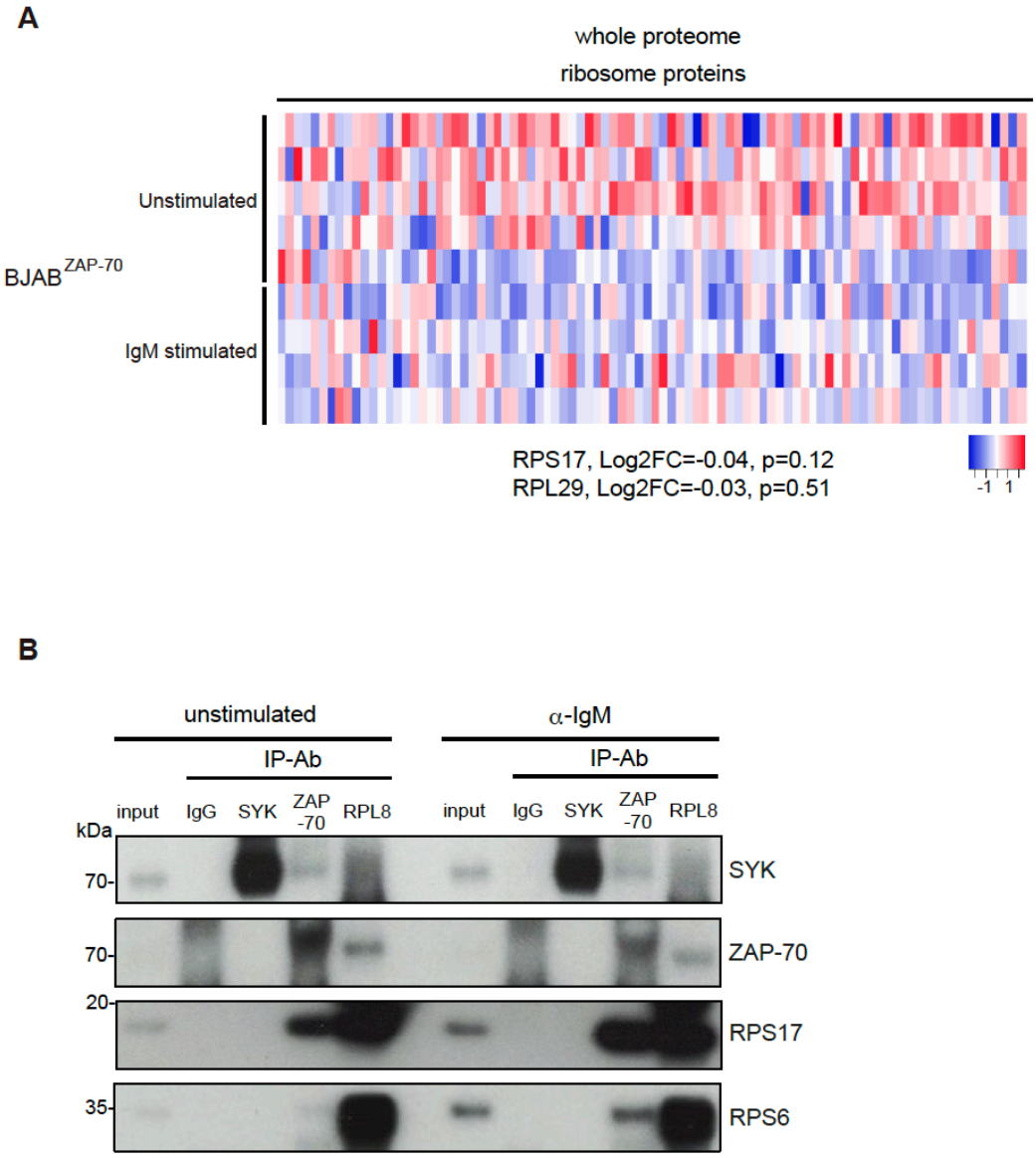
(E) Heat map of proteins annotated in KEGG B cell receptor signaling pathway identified in BJAB cells streptavidin-based pull down and mass spectrometry in unstimulated or IgM-activated $BJAB^{control}$ and $BJAB^{ZAP-70}$ cells. Red and blue indicate relatively high and low protein abundance, respectively. Each condition analyzed depicts two technical repeats. Proteins were ranked from left to right by Log2FC ratio compare IgM activated to unstimulated group in $BJAB^{ZAP-70}$ cells.

(F) Volcano plot showing the proteins specifically bind to biotinylated ZAP70 in unstimulated $BJAB^{ZAP-70}$ cells (Log2FC>0) or bind to unspecific biotinylated proteins in unstimulated $BJAB^{control}$ (Log2FC<0).

(G) Gene set enrichment analysis (GSEA) was applied to identify the pathways that ZAP-70-associated proteins are involved in unstimulated BJAB cells. Pathway sets are listed in order of Normalized Enrichment Scores (NES) in columns (top x-axis). FDR q values for each pathway set are indicated by the red dotted line (lower x-axis). Colors of column represent different sub-grouped cell functions.

(H) Volcano plot showing the proteins bind to ZAP-70 increased (Log2FC>0) or decreased (Log2FC<0) by anti-IgM treatment in $BJAB^{ZAP-70}$ cells.

(I) GSEA identifies the ZAP-70-associated proteins involved pathways in $BJAB^{ZAP-70}$ cells that are enhanced by anti-IgM treatment.



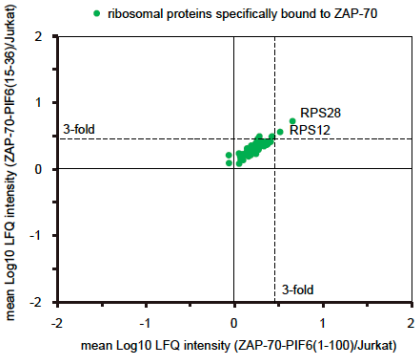
Supplement Figure 3: BCR-activation induces binding of ribosomal proteins to ZAP-70, but not SYK.

(A) Heat map of ribosomal proteins identified as ZAP-70 binding partners (Figure 5). Whole cells lysates were prepared from unstimulated and anti-IgM treated BJAB^{ZAP-70} cells and protein abundancy analyzed by mass spectrometry. Red and blue indicate relatively high and low protein abundance, respectively. Each row analyzed depicts a technical replicate. Log2 fold changes and adjusted-p value of RPS17 and RPL29 comparing anti-IgM activated to unstimulated cells are indicated below the heatmap.

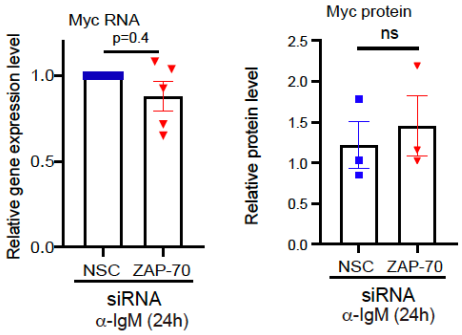
(B) Whole cell lysates from primary CLL cells unstimulated or stimulated with anti-IgM

for 20 minutes were immunoprecipitated (IP) with SYK antibody (mouse), ZAP-70 antibody (mouse), RPL8 antibody (rabbit) or control mouse IgG and immunoblotted (IB) with antibodies for SYK (rabbit), ZAP-70 (mouse), RPS17 (rabbit) and RPS6 (rabbit).

A



C



B

Protein name	Phosphorylation site	0 mins Fold change	0 mins Q value	2 mins Fold change	2 mins Q value	5 mins Fold change	5 mins Q value	10 mins Fold change	10 mins Q value
LCK	Y192	0.110703	**0.0011218**	0.0955827	**0.0110577**	0.2004166	**0.0070397**	0.1198575	**0.0053468**
RPL15	Y62	0.8653224	0.3366517	0.7730847	0.1684048	0.8025156	0.1394871	0.790822	0.3543279
RPL7a	Y230	0.8969173		0.5847143	0.2642519	0.9679515	0.5259707	0.8340175	0.3641149
RPL3	Y307*	0.9452259	0.2800032	0.9182762	0.4727244	0.8716227	0.2870189	0.9103368	0.276008
RPS10	Y12*	0.9475971	0.4107516	0.7813045	0.4903101	0.9653325	0.2255916	1.1554962	0.1871836
RPL15	Y59	0.9495386	0.3817648	0.979152	0.4811545	0.8701296	0.3114556	0.8840721	0.275818
RPS27a	Y148	0.9548	0.5226862	1.1324088	0.30486	1.0091583	0.4821449	1.4063006	0.1611096
RPL10a	Y11	0.9658509	0.4011662	0.9897053	0.3783685	0.8882235	0.2326466	0.8963135	0.2761529
RPL P0	Y24*	0.9722417	0.3569394	0.9539601	0.2806377	0.9691498	0.3234085	0.9827348	0.431715
RPS13	Y38*	1.2959862	0.2393159	1.0003273	0.488939	1.1498296	0.1244993	1.1941718	0.2880364
RPS3	Y120	1.3191067	0.3318686	2.1382458	**0.0439774**	2.195074	0.1132051	2.1881408	0.0508367
RPS29	Y7	1.4149873	0.1993443	0.8442997	0.4292265	1.4768822	0.372935	0.8838516	0.3864522
RPL35A	Y34	1.5913623	0.3268778	2.6117383	0.3053167	1.0675424	0.3377587	0.9489035	0.5182101
RPL27a	Y52	1.627726	0.191009	3.27925	0.3459988	4.3914446	0.2216934	11.831769	0.1888701
RPS16	Y82	3.3370006	0.1106356	9.079242	**0.0299897**	4.2798335	0.070967	2.9296483	0.1330099
RPL4	Y264	3.9546166	**0.0110577**	4.6978052	**0.0285582**	3.192	0.0598051	4.9813605	0.1379712
PTK2B	Y579	5.8669537	**0.027**	24.702393	**0.017**	34.307552	**0.001**	29.653439	**0.012**

TCR stimulation

Fold change (ZAP-70 inhibition/ZAP70) >1

Fold change (ZAP-70 inhibition/ZAP70) <1

Supplement Figure 4: Effects of ZAP-70 on MYC expression and modifications of ribosomal proteins

(A) Ribosomal proteins (green) specifically bound to ZAP-70 in Jurkat cells expressing ZAP-70-PIF6 (data derived from *Front Immunol. 2019 Feb 26;10:226*).

(B) Relative abundance of tyrosine phosphorylated ribosomal proteins in ZAP-70 inhibited, TCR-activated Jurkart cells (data derived from *Sci Signal. 2015 May 19;8(377):ra49*).

(C) Analysis of *MYC* mRNA level by qRT-PCR (n=5) and protein level by immunoblots (n=3) in purified primary CLL cells transfected with non-specific siRNA or ZAP-70 siRNA, further mono-cultured for 24h in the presence of bead-bound anti-IgM. Relative gene expression levels are normalized to reference gene GAPDH and compared to NSC control. Relative MYC protein levels are normalized to the expression of β-actin.