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Ofer Harel
Technion Israel Institute of Technology
Naythan Dcunha
Technion Israel Institute of Technology
Eli Benchetrit
Technion Israel Institute of Technology
Caterina Canetta
German Cancer Research Centre: Deutsches Krebsforschungszentrum
Eirini Sidiropoulou
German Cancer Research Centre: Deutsches Krebsforschungszentrum
Sandrine Sander
German Cancer Research Centre: Deutsches Krebsforschungszentrum
Doron Melamed
melamedd@technion.ac.il

Technion Israel Institute of Technology https://orcid.org/0009-0004-6032-6499

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miR29 imposes quiescence in mature B cells by repressing the SP1/MYC/miR17-92 activation circuit

Ofer Harel[1], Naythan Dcunha[1], Eli Benchetrit[1], Caterina Canetta[2], Eirini Sidiropoulou[2], Sandrine Sander[2,3] and Doron Melamed[1]*

[1] Technion-Israel Institute of Technology, The Ruth and Bruce Rappaport Faculty of Medicine, Department of Immunology, Technion–Israel Institute of Technology, Haifa, Israel.

[2] German Cancer Research Center (DKFZ), Adaptive Immunity and Lymphoma, Heidelberg, Germany.

[3] Comprehensive Cancer Center (CCC) Mecklenburg-Vorpommern, University Medicine Greifswald, Greifswald, Germany.

* Correspondence to: Doron Melamed, Technion, Rappaport Faculty of Medicine, Department of Immunology, Technion–Israel Institute of Technology, Bat Galim Haifa 31096, Israel. melamedd@technion.ac.il

Abstract

Cellular quiescence is a defining feature of mature immune cells, balancing long-term survival with the capacity for rapid activation while preserving tissue homeostasis. Despite its importance, the molecular mechanisms enforcing quiescence remain incompletely understood. In the B lineage, regulation of the PTEN/PI3K signaling axis is critical for the survival of quiescent cells. We previously demonstrated that miR29 replaces miR17-92 in controlling PTEN/PI3K signaling during B-cell maturation. Here, we show that miR29 expression in mature B cells is essential for imposing and maintenance of their quiescence stage. Using a miR29 targeting antagomiR and conditional ablation of miR29 in CD23-expressing B cells, we show that repression of miR29 in mature quiescent B cells enhances PI3K signaling and induces miR17-92 expression. This regulatory switch promotes increased survival and exit from cellular quiescence. Mechanistically, we found that miR29 represses SP1 in quiescent B cells, thereby limiting MYC expression and the downstream induction of miR17-92. Together, our results suggest that miR29 imposes cellular quiescence in mature B cells by repressing the SP1/MYC/miR17-92 activating axis, illustrating how dynamic microRNA networks remodel intracellular programs to control cellular states.

Introduction

B cell development is a highly regulated process, initiated in the bone marrow (BM) and continues to maturation in the periphery[1–3]. For most mature B cells, this developmental progression is accompanied by a shift from an active/proliferating phase to a resting/quiescent stage[4,5]. In the periphery, the majority of mature B cells are quiescent, a state that enables the mounting of specific antibody responses upon antigenic challenge[6,7]. The maintenance of cellular quiescence among resting B cells is essential for tissue homeostasis as unrestrained mature B cells can develop into cancer[8,9]. Although much is known about expression of specific gene programs controlling cellular quiescence, the mechanisms regulating this expression is not fully understood.

In the B lineage, the PTEN-PI3K signaling cascade is most important for survival during development in the BM and quiescence in the periphery[10–13]. We, and others, have shown that this PTEN-PI3K activity is regulated post-transcriptionally by microRNAs (miRNAs)[14,15]. miRNAs are short (20-30 bp), conserved, non-coding RNAs that regulate gene expression, mainly, through binding to the 3'UTR of the mRNA to facilitate its degradation. Biogenesis of microRNAs is vital for B cell development and activation[16,17]. Thus, deletion of enzymes responsible for biogenesis of miRNAs in proB cells aborts B cell development in the BM[18], and loss in transitional B cells alters the PTEN-PI3K activity and facilitates a consequential death[15,19].

The miR17-92 cluster is a key regulator of B cell development in the BM, mainly implicated with proliferation and survival of the cells[20–22]. In an early study, we showed that miR17-92 tunes the PTEN-PI3K activity for positive and negative selection of developing B cells through a MYC/miR17-92/PTEN axis[23]. However, while miR17-92 cluster is highly abundant in developing B cells, its expression declines with maturation[24]. More recently, we showed that miR29 replaces miR17-92 in regulating the PTEN-PI3K axis for B cell maturation and is highly expressed in mature quiescent B cells [19]. The miR29 family is abundantly expressed across different cell types, among which are B and T cells[25], and was shown to negatively regulate PTEN in cells of various origin[26–28]. The miR29

family consists of three main members, miR29a, miR29b and miR29c[29]. While conserved among mammals, miR29a appears to be the most abundant in expression[30]. We showed that loss of miR29 in proB cells impairs the PTEN-PI3K signals for B cell maturation to result in increased death of transitional B cells and formation of an abnormal peripheral B cell compartment with a defective response to immune challenge[19]. While this study revealed the mandatory function of miR29 in regulating PTEN-PI3K for B cell maturation, the role of miR29 in quiescent mature B cells in the periphery is still unknown.

To study this, we used in-vitro and in-vivo approaches to target miR29 specifically in mature B cells. We show that loss of miR29 in mature B cells elevates expression of miR17-92 and PI3K activity to increase survival and to terminate cellular quiescence. Furthermore, miR29 functions to repress miR17-92 through a MYC/SP1 circuit to impose cellular quiescence in mature B cells. Hence, we suggest that expression of miR29 is mandatory to tune PTEN-PI3K for B cell maturation, and its continuous expression in mature B cells is required to repress SP1/MYC/miR17-92 axis and to impose cellular quiescence.

Methods

Mice

The miR29ab1$^{fl/fl}$/miR29b2c$^{-/-}$ mice were described earlier[19] . The miR29ab1$^{fl/fl}$/miR29b2c$^{-/-}$ mice were crossed with CD23-Cre mice[31] to generate conditional CD23-Cre/ miR29ab1$^{fl/fl}$/miR29b2c$^{-/-}$ mice (termed CD23-Cre/miR29KO). All mice were on C57BL/6 genetic background (The Jackson Laboratory, Bar Harbor, ME). Littermates non transgenic for the CD23-Cre and carrying intact miR29b2c loci were used for control. Mice were bred and maintained in specific pathogen-free facility at Technion, Faculty of Medicine in accordance with protocols approved by the Technion ethic committee (IL-025-02-22).

Eu-MYC mice [32] (and wild-type littermates) were purchased from The Jackson Laboratory and housed under specific pathogen free status (SPF) condition in the central animal facility at the DKFZ Heidelberg, Germany, with approval of the Regierungspräsidium Karlsruhe (G119/19). MYC T58A mice were a gift from Dr. Ziv Schulman, Weizmann Institute, Israel [33].

Cells and treatments

Mature B cells were purified from spleen using CD43 magnetic beads (Miltenyi Biotec) and cultured in IMDM enriched with 10% FBS medium. Where indicated, the purified splenic B cells were cultured with or without 40uM phosphorothioate modified antagomir miR29 (IDT). The custom miR29 antagomirs were designed based on miRbase reported mature miRNA sequence, with phosphorothionate modifications marked in following sequence by *: TA*A*CCG ATT* TCA* GAT GGTG*C*T A. In some experiments the B cells were cultured with 10μM of MYC inhibitor (10058-F4, Sigma, Cat: F3680) or with 20nM of SP1 inhibitor (Mithramycin, Sigma, Cat: M6891). All inhibitors were used in non-toxic concentrations, based on working concentrations reported by the manufacturer, and verified by propidium iodine staining (PI stain) of splenic B cells incubated overnight at serial dilutions. Cells were used for staining or lysed for western blotting

analysis and for quantification of mRNAs and microRNAs. A20 cells[34] (ATCC), were cultured in RPMI medium supplemented with 10% FBS. HEK-293 cells (ATCC) were cultured in DMEM medium supplemented with 10% FBS. All cells were cultured at 37° in humidified incubators with 5% CO_2. In some experiments 10 μl of Presto Blue was added to the cultures for 10min and fluorescence at excitation and emission of 560/590 nm was determined.

Cell transfection and qPCR

To generate miR29-overexpression MGP plasmid, we cloned the miR29 stem-loop structure and flanking sequence from genome DNA isolated from C57BL/6 male mice. The PCR product was digested with XhoI and EcoRI and cloned into the retroviral vector MGP GFP/Puro[35] (Addgene). The miR29/MGP vector was then transfected to A20 cells to generate A20 miR29 overexpression as described[35].

Quantification of gene expression was determined by qPCR. Purified RNA samples were reverse transcribed (RT) to cDNA and subjected to qPCR using iScript PCR kit (Bio-Rad) or TaqMan predesigned RT primers (Thermo-Fisher), according to manufacturer instructions. Real-time quantitative PCR for gene expression was performed using SYBR Green PCR Master Mix (Luna Universal qPCR mix), based on oligoDNA primers (IDT) verified by PrimerBank. We normalized samples with HPRT or GAPDH gene amplification.

Real-time quantitative PCR for miRNA expression was performed using either Syber green mix (Luna Universal qPCR mix) with specific miRNA primer and 3' adaptor primer, or with TaqMan Master Mix (Thermo-Fisher) and TaqMan predesigned primers (Thermo-Fisher): hsa-miR29a (assay ID 2112), hsa-miR17 (assay ID 2308), hsa-miR19b (assay ID 396). SNO243, SNO202 and SNO142 (assay ID 1231) small nuclear RNA served as the endogenous reference gene. Reactions were performed on BioRad CFX connect Real-Time PCR and data was analyzed using the CFX Manager Software.

Luciferase target assay:

A 3kb sequence of the SP1 3'UTR (at loci 102341840-102344839) based on genomic DNA (Ensembl, ID number ENMUSG00000001280) was divided into 3 equal segments and each was cloned from genomic DNA by PCR (S1 at loci 102341840-102342839, S2 at loci 102342840-102343839 and S3 at loci 102343840-102344849, as shown in figure 7A). Each segment was ligated to pmiRglo plasmid (Addgene). For luciferase reporter assay, HEK-293 cells were transfected with the modified firefly luciferase vector (pmiRglo) and vector encoding miR29 (miR29MGP GFP/Puro) using Calfectin according to the manufacturer's instruction. The firefly and renilla luciferase activities were measured after 48h with the dual-luciferase reporter assay system (Promega). To control the transfection efficiency, firefly activity was normalized to renilla activity.

Flow Cytometry

Purified single B cell suspensions from mouse spleens were stained with antibodies specific to B220 (clone RA3-6B2), CD19 (clone 6D5), CD93 (clone AAA4.1), CD38 (clone 90), IgM (clone

RMM1), IgD (clone 11-26c.2a) (all from Biolegend). PI-Annexin V staining was performed according to manufacturer's protocol (eBioscience).
For phosphorylated intracellular staining, cells were fixed in 37% paraformaldehyde and permeabilized with 100% cold methanol. Cells were washed and stained for pAKT (Cell Signaling, clone SDRNR), pERK (clone 20A) or pIKb (BD PhosFlow, clone 25/IkBa/MAD-3). Samples were analyzed on an LSRFortessa (BD Pharmingen) cytometer and data were analyzed with FlowJo software (Tree Star).

PI Cell cycle Assay

Splenic B cells were cultured either untreated or treated with miR29 antagomiRs for 24-72h. Cells were collected, washed with PBS and incubated for 10 min in dark with propidium iodide (PI) Triton solution. Apoptotic cells were determined by their hypochromic sub-diploid staining profiles using LSRFortessa and the collected data were analyzed using FlowJo software.

Statistical analysis

The statistical significance of the difference between two experimental groups was determined using an unpaired two-tailed Student t test, with differences considered significant at $p<0.05$. Statistical significance between three or more groups was determined using ANOVA, with differences considered significant at $p<0.05$. Tukey's exam was used for ANOVA post-hoc analysis.

Results

Repression of miR29a enhances PI3K activity in mature quiescent B cells

We showed that a miR17-92-to-miR29 switch is mandatory for tuning PTEN-PI3K activity for B cell maturation [19]. Since miR29 is highly expressed in mature B cells[19], we further investigated its role in cellular quiescence of these cells. To do so, we used antagomiRs[36] targeting miR29a, which is the dominant miR29 isoform expressed in mature B cells (figure S1B), and has 90-95% sequence similarity with miR29b and miR29c, (figure S1A) to specifically repress its expression in purified splenic B cells from wild-type mice (figure 1A). This treatment had no effect on the abundance of unrelated miRNAs (miR150, miR181), nor the expression of surface molecules CD19, B220, IgM and IgD (figure 1B), and did not induce any detectable activation as measured by expression of activation and differentiation markers (figure S1C). Unexpectedly, we found that repression of miR29a led to a marked elevation in phosphorylated AKT (pAKT), a key indicator of PI3K activity (figure 1C). This contrasts with our previous findings showing that miR29 expression in immature/transitional B cells elevates PI3K activity for maturation and survival in the spleen [19]. The repression of miR29a in quiescent B cells did not have any effect on activation of the MAPK pathway nor the NFkb pathway (figure 1C). These results suggest that miR29 differentially modulates PI3K activity in mature quiescent B cells, relative to immature/transitional B cells.

Repression of miR29a promotes prolonged survival of B cells

Since PI3K controls mature B cell survival[10–13], we next explored whether repression of miR29a affects cell death in splenic B cells. To address this, purified wild-type (WT) B cells were cultured in vitro either untreated or treated with miR29a antagomiRs, and spontaneous cell death was quantified by PI staining after 24, 48, and 72 hours. The fraction of cells in sub-G0/G1 was significantly reduced following antagomiR treatment at all time-points (figure 2A). This finding was further confirmed by annexin V/7AAD stain which enabled specific quantification of apoptotic cells (annexin+/7AAD+, figure 2B). Complementary analyses of viable cells (annexin V-/7AAD-, figure 2B) and by Presto Blue viability assay (figure 2C) revealed a significantly increased viability of B cells upon miR29a repression. These results suggest that repression of miR29 enhances PI3K activity and promotes prolonged B cell survival.

Repression of miR29a induces miR17-92 expression and reduction in PTEN

The unexpected increase in PI3K activity following treatment with miR29a antagomiRs suggested a reduction in expression of PTEN. Indeed, we observed a 60% decrease in PTEN expression in splenic B cells treated with miR29a antagomiRs (figure 3A). Given the previously established role of the miR17-92 cluster in tuning PTEN-PI3K signaling during early B cell development[37], and because miR17-92 and miR29 are the major miRNAs regulating PTEN during B cell development[19], we next quantified miR17-92 expression in splenic B cells treated with miR29a antagomiRs. As shown in figure 3B, repression of miR29a resulted in a significant 4-5 folds increase in the expression of miR19b and miR17. These findings suggest that in mature quiescent B cells, miR29 functions to both tune PTEN-PI3K activity and to repress miR17-92.

Conditional ablation of miR29 in vivo elevates miR17-92 expression and PI3K activity, and prolongs survival of mature quiescent B cells.

To explore and validate the function of miR29 in mature quiescent B cells in a more physiological context, we generated a conditional mouse in which miR29 is specifically ablated in mature B cells. To do so, our miR29ab1$^{fl/fl}$/miR29b2c$^{-/-}$ mice[19] were crossed with CD23-Cre transgenic mice[38] to generate CD23-Cre/miR29ab1$^{fl/fl}$/miR29b2c$^{-/-}$ (hereafter referred to as CD23-Cre/miR29KO) mice. The CD23-Cre/miR29KO mice had no significant alterations in number, phenotype and death rate of B cells in spleens (figure 4 A-C). Consistent with the in vitro findings, mature miR29KO B cells had increased expression of miR17-92 (figure 4D) and significantly reduced PTEN (figure 4E). In agreement with these, miR29-defficient B cells showed elevated levels of pAKT (figure 4F) and prolonged survival when cultured in vitro for 24 hours (figure 4G). Collectively, our in vitro and in vivo results suggest that miR29 imposes cellular quiescence in mature B cells by repressing miR17-92, in addition to appropriately tuning PI3K signaling activity.

MYC represses miR29a and induces miR17-92 expression in B lineage cells

We next explored the mechanism by which miR29 and miR17-92 are interconnected. A key regulator of both microRNA clusters is MYC: Previous studies in lymphoma cells have shown that MYC represses miR29 expression[39], whereas other reports showed that MYC induces miR17-92 expression[40]. To investigate the miR29/miR17-92/MYC interplay, we quantified miR29 and miR17-92 in splenic B cells from two MYC-overexpressing mouse models: a MYC T58A mutant mouse, in which MYC degradation is halted by the gene mutation[33], and the Eμ-MYC mouse, in which MYC is constitutively expressed under the immunoglobulin heavy chain promoter[32]. In the T58A MYC model, splenic B cells exhibited elevated expression of MYC, which was reduced to levels comparable to those of normal B cells upon treatment with MYC inhibitor (figure 5A). We found that B cells from the T58A MYC mice displayed low miR29 expression and high miR17-92 expression. However, MYC inhibition reversed this pattern to result in elevated miR29 and repressed miR17-92 (figures 5B and 5C). Consistent with these findings, we observed that Eμ-MYC B cells, either at the pre-malignant or the tumor stage (sorted as shown in figure S3), exhibited low miR29 and high miR17-92 expression (figures 5D). These results suggest that in B lineage cells, MYC represses miR29 and induces miR17-92 to promote cell cycle and activation.

miR29 represses expression of MYC and cell growth

Expression of both MYC and miR17-92 is very low in mature resting B cells facilitating a state of quiescence. We next studied the role of miR29 in repression of MYC in quiescent B cells. Analysis of splenic B cells treated with miR29a antagomiRs revealed an approx. 10-folds increase in MYC expression (figure 6A). Similarly, CD23-Cre/miR29KO B cells express significantly increased MYC relative to WT B cells (figure 6B). In a reciprocal approach, we over-expressed miR29a in A20 lymphoma cells. We found that elevated miR29a repressed miR17-92 (figure 6C) and repressed MYC (figure 6F).

Further, the miR29a-mediated repression of miR17-92 and MYC inhibited proliferation of the A20/miR29-OE cells relative to control A20 cells when co-cultured in a 2:1 mixed ratio, as evident by the relative proportions of WT A20 cells and the A20/miR29-OE cells during 15 days of culture (figure 6E), and by quantification of cell viability (figure 6D). These findings are in agreement with the reduced expression of cell-cycle genes in A20/miR29-OE cells relative to the control A20 cells

(figure 6F). Collectively, these results suggest a reciprocal regulatory mechanism by which miR29 represses expression of MYC and miR17-92 to maintain quiescence in mature B cells.

miR29a regulates expression of the MYC co-factor SP1

We next investigated how miR29 represses MYC. Searching the MYC 3'UTR by TargetScan did not reveal predicted miR29 binding sites. Hence, we considered that miR29 may repress MYC indirectly, by regulating the expression of an important MYC co-factor. Database of known and predicted protein-protein interactions suggested SP1 is a strong MYC co-factor (figure S3) involved in the regulation of multiple cellular processes including cell growth, differentiation, and apoptosis[41–43]. SP1 binding sites are present on MYC gene promoter region mediating its activation and the expression of MYC[44,45]. SP1 and MYC jointly regulate miR17-92 expression[46]. A miR29a binding site was predicted by TargetScan on mouse SP1 3'UTR and using alignment tools (BLAST) identified it at positions 102343303-102343310 of the complete murine SP1 3'UTR sequence (ENSEMBL, Transcript ID ENSMUSG00000001280) (figure 7A). Luciferase reporter assay identified that this predicted miR29 binding site mediates repression of expression upon miR29 binding (figure 7B). Furthermore, treatment of splenic B cells with miR29a antagomiRs resulted in a significant increase in SP1 expression (figure 7C). Consistent with these findings, SP1 expression was elevated in splenic B cells from CD23-Cre/miR29KO (figure 7D), and splenic B cells from MYC T58A mice expressed high levels of SP1, which were significantly reduced upon treatment with a MYC inhibitor (figure 7E). Collectively, these findings suggest that miR29 represses SP1 expression in quiescent B cells.

miR29 represses miR17-92 through a SP1-MYC circuit

To test whether miR29 represses miR17-92 through a SP1-MYC circuit, WT splenic B cells were treated with miR29a antagomiRs with or without the SP1 inhibitor mithramycin[47]. While treatment with miR29a antagomiRs induces expression of miR17-92 (figure 8A and 8B, and figure 3), co-treatment with mithramycin blocked this induction (figure 8A and 8B). Further, inhibition of SP1 activity also hampered the induction of MYC following treatment with miR29a antagomiRs (figure 8C). Consistent with these findings, inhibition of SP1 in CD23-cre/miR29KO B cells repressed the expression of both miR17-92 and MYC (figure 8D). Lastly, using a spontaneous apoptosis assay, we examined whether inhibition of SP1 activity could prevent the extended survival resulting from miR29 repression. Indeed, the increased survival of splenic B cells induced following treatment with miR29a antagomiRs was abolished upon co-treatment with SP1 inhibitor (figure 8E). In agreement with these results, treatment of CD23-Cre/miR29KO B cells with SP1 inhibitor reduced their survival, as evidenced by the detection of over 70% cells in sub-G0/G1 phase. Collectively, these findings suggest that miR29 functions in mature B cell to impose cellular quiescence by repressing the SP1/MYC/miR17-92 transcriptional activation axis.

Discussion

Peripheral B cell maturation requires a decisive switch from an active/proliferative state to a tightly controlled quiescent program that enables stable follicular residency[4,7]. How active/proliferative pathways are silenced without compromising survival has remained largely unresolved. Here, we uncovered a microRNA-mediated regulatory mechanism governing both the establishment and maintenance of B cell quiescence. Specifically, we identified miR29 as a major regulator that imposes quiescence by repressing the SP1/MYC/miR17-92 activation axis, thereby restraining active/proliferative signaling while fine-tuning PTEN/PI3K signaling to support mature B cell survival during quiescence (Figure 9).

We have previously shown that PTEN/PI3K axis is regulated by miR17-92 during B cell development in the BM[23], and that miR29 replaces miR17-92 to establish appropriate PTEN-PI3K signaling during B cell maturation[19]. Therefore, it was unexpected to observe that knockdown of miR29 in mature B cells in vitro, or genetic ablation of miR29 in mature B cells in vivo, resulted in enhanced PI3K activation and extended cellular survival. We found that these effects were mediated by a compensatory increase in miR17-92 expression, indicating that, in quiescent mature B cells, miR29 functions to restrain miR17-92 expression. Notably, miR17-92 is widely implicated in cellular activation and proliferation across numerous normal and malignant tissues[48,49]. Consistent with this role, mice overexpressing miR17-92 display up to a two-folds expansion of the peripheral B cell compartment due to increased B cell numbers[50,51]. Hence, repression of miR17-92 must be enforced upon B cells maturation and entry into cellular quiescence. Here we show that this critical process is imposed by miR29 through a SP1/MYC/miR17-92 regulatory axis. Yet, in addition to repressing miR17-92, miR29 tunes PI3K activity in mature B cells by regulating the expression of PTEN[19]. These dual functions of miR29 are shown in Figure 9. Hence, the inverse expression of these two microRNA clusters ensures continuous, tightly controlled PTEN-PI3K activity to support survival, while simultaneously repressing the SP1/MYC/miR17-92 axis to facilitate transition from an active state to quiescence and to maintain long-term cellular quiescence.

Our results suggest that expression of miR29 is essential for developmental progression of transitional B cells into mature B cells. Although loss of miR29 in proB cells has no detectable effect on B cell development in the BM, it profoundly affects late stages of B cell differentiation. Failure of transitional B cells to express miR29, as observed in mb1-Cre/miR29KO mice, disrupts maturation and results in an abnormal peripheral B cell compartment[19]. In contrast, in CD23-Cre/miR29KO mice, in which miR29 is ablated in mature quiescent B cells, the peripheral B cell compartment is not significantly altered with respect to B cell numbers, phenotype and apoptosis. Instead, loss of miR29 at this stage relieves repression of the miR17-92 cluster and leads to termination of cellular quiescence. Hence, our results suggest that miR29 promotes B cell maturation at the transitional stage and enforces cellular quiescence in mature B cells.

miR29 and miR17-92 exert opposing biological functions. The miR17-92 cluster promotes cell-cycle and survival and is upregulated in many malignancies[21,40,46,48,50,52,53], whereas miR29 has been reported to function as tumor suppressor[27,54–56]. In normal B cells, expression of these microRNA clusters is mutually exclusive and tightly regulated along the differentiation pathway; miR17-92 is predominantly expressed during BM development, whereas miR29 is expressed in mature, quiescent peripheral B cells. The mechanisms governing this mutually exclusive expression are not completely understood. A central regulator balancing the expression of both

microRNA clusters is MYC. MYC induces expression of miR17-92[40], while represses expression of miR29[39,57,58]. In the BM, growth and differentiation of B cells is facilitated by increased expression of MYC and miR17-92[23,59], whereas in the periphery both are repressed [60,61], permitting expression of miR29 and subsequent entry into cellular quiescence. These contrasting functions of MYC are exemplified in Eμ-MYC and T58A MYC B cells, in which miR29 is repressed and miR17-92 is upregulated. This expression pattern, however, inverts upon inhibition of MYC (figure 5). In general, these opposing MYC-driven programs promote growth and development of normal tissue but, when dysregulated, facilitate tumorigenesis[62–65] . In agreement with this are earlier studies showing that MYC, through miR17-92 promotes oncogenesis[49]. Our results extend this model by suggesting that this oncogenic process is further facilitated by the concomitant repression of miR29.

Repression of MYC is essential for the maintenance of cellular quiescence[64–67]. Indeed, mature quiescent B cells express high level of miR29 and very low levels of MYC[60,61]. However, knockdown of miR29 in these cells induces MYC and miR17-92 (figures 3 and 4), thereby terminating cellular quiescence and resulting in extended survival. Conversely, overexpression of miR29 in lymphoma cells inhibits cell growth (figure 6), consistent with the established role of miR29 as a tumor-suppressor[55,58,68–70]. Our results further suggest that this tumor-suppressor function is mediated, at least in part, through the repression of MYC. Thus, miR29 and MYC sustain a reciprocal regulatory circuit that governs the transition between “active” and quiescent cellular states. During cell differentiation and cell-cycle progression, MYC is elevated and directly represses miR29 to promote an active state. In contrast, during cellular quiescence miR29 is upregulated and represses MYC expression and function (shown in figure 9).

While MYC binds the miR29 promoter and directly repress miR29 expression[57], we did not identify any putative conserved miR29 binding sites within the MYC gene transcript, including its 3’UTR. As MYC interacts with many cofactors and inhibitors to exert its transcriptional activity[63,71,72], we investigated potential intermediaries and identified SP1 as major cofactor containing a predicted miR29 binding site within its 3'UTR region (Figure 7B). SP1 is a zinc finger transcription factor that binds GC-rich DNA motifs and regulates multiple cellular pathways involved in cell growth, differentiation, and apoptosis[41–43]. MYC and SP1 regulate each other positively [44,45] and jointly control expression of miR17-92[73]. Here, we demonstrate that miR29 directly binds the SP1 3'UTR to repress SP1 expression, leading to reduced MYC and miR17-92 levels and induction of cellular quiescence. Accordingly, while knockdown of miR29 terminates quiescence, inhibition of SP1 abolishes this effect. Thus, in mature B cells, the inverse miR29/miR17-92 expression pattern is governed by a SP1/MYC regulatory circuit (illustrated in figure 9), facilitating the critical transition between active and quiescent stages during B cell development.

Yet, although we demonstrated that MYC and SP1 jointly regulate the transition from a miR29 to a miR17-92 expression pattern, additional factors are known to regulate miR17-92 in both normal and malignant cells[46]. One study reported that miR17-92 may be directly regulated by miR21, suggesting that miRNA-miRNA regulation may be mediated through direct binding of miRNA to a pri-miRNA transcript. While we did not identify conserved miR29 binding sites within the miR17-92 sequence, we cannot exclude possible contribution of non-conserved binding sites. Additionally, although miRNA binding sites may exist in other non-coding and coding regions of a gene, current studies suggest that synergistic regulation, concurrent with 3'UTR regulation, may

still be required[74,75]. Hence, other miR29-mediated pathways of regulation may also exist and are not mutually exclusive.

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Statements and Declarations

Ethics approval and consent to participate

Experiments with mice at Technion were in accordance with protocols approved by the Technion ethic committee (IL-025-02-22). Experiments with mice at DKFZ were in accordance of protocols approved by the Regierungspräsidium Karlsruhe (G119/19).

Consent for publication

Not applicable

Availability of data and material

The data generated during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Competing interest

The authors have no relevant financial or non-financial interests to disclose

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ofer Harel, Naythan Dcunha, Eli Benchetrit, Caterina Canetta, Eirini Sidiropoulou. Sandrine Sander and Doron Melamed conceived of the study and designed the experiments. Ofer Harel Sandrine Sander and Doron Melamed wrote and edited the manuscript

Authors' information

Not applicable

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Figure Legend:

Figure 1 - Repression of miR29a enhances PI3K activity in mature quiescent B cells

(A) Normalized relative expression of miR29a, miR150 and miR181 between antagomiR29a treated and untreated mouse splenic B cells. Each dot represents an independent experiment. Data are presented as the mean±SD (standard deviation).

(B) Representative flow cytometry analysis of surface markers on antagomiR29a treated and untreated (=control) mouse splenic B cells.

(C) Intracellular flow cytometry analysis of pAKT, p-ERK and p-IkB levels in mouse splenic B cells treated with antagomiR29a or scrambled antagomiR29a (scramble) or untreated for 24 hours.

Figure 2 - Repression of miR29a promotes prolonged survival of B cells

(A) Representative flow cytometry analysis of PI stained mouse splenic B cells treated with antagomiR29a or untreated for 24, 48 and 72 hours. Sub-G0/G1 phase cells are quantified.

(B) Representative 7AAD-Annexin V staining for cells as shown in (A).

(C) Presto Blue viability compared between antagomiR29a treated and untreated mouse splenic B cells. Each dot represents an independent experiment. Data are presented as the mean ± SD.

Figure 3 - Repression of miR29a induces miR17-92 expression and reduction in PTEN

(A) PTEN mRNA expression in antagomiR29a treated and untreated (=control) mouse B cells. Each dot represents an independent experiment. Data are presented as the mean ± SD.

(B) miR17-92 cluster expression in cells as shown in (A).

Figure 4 – Conditional ablation of miR29in vivo elevates miR17-92 expression and PI3K activity, and prolongs survival of mature quiescent B cells.

(A) Total spleen cells and splenic B-cell count in aged-matched WT and CD23-Cre/miR29KO mice. Each dot represents one mouse. Data are presented as the mean ± SD.

(B) Representative flow cytometry analysis of surface markers on splenocytes as shown in (A).

(C) 7AAD/Annexin V staining of freshly isolated splenic B cells from indicated genotypes.

(D) miR29a (left) and miR17 (right) expression in B cells as shown in (C). Each dot represents an animal. Data are presented as the mean ± SD.

(E) Relative PTEN mRNA expression in B cells as shown in (D).

(F) Intracellular flow cytometry analysis of pAKT in B cells as shown in (C).

(G) PI stain of in vitro cultivated (24h) mouse splenic B cells isolated from CD23-Cre/miR29KO and WT mice. Sub-G0/G1 phase cells are quantified.

Figure 5 - MYC represses miR29a and induces miR17-92 expression in B lineage cells

(A) Relative MYC mRNA expression in MYC T58A mutant splenic B cells compared to WT B cells. MYC T58A mutant cells treated with MYC inhibitor for 24h were included as well. Each dot represents an animal. Data are presented as the mean ± SD. Note that for these experiments only 2 MYC T58A were used due to the unfortunate loss of the colony.

(B) Relative expression of miR29 in B cells as shown in (A).

(C) Relative expression of miR17 in B cells as shown in (A).

(D) miR29 and miR17 relative expression in splenic B cells isolated from Eu-MYC and WT animals. Pre-malignant and tumor Eu-MYC mice were classified based on phenotypic parameters (including spleen weight, splenic B cell expansion). Each dot represents an animal. Data are presented as the mean ± SD.

Figure 6 - miR29 represses expression of MYC and cell growth

(A) Relative expression of MYC compared between miR29 antagomiR treated, scramble antagomiR and untreated splenic B cells.

(B) Relative expression of MYC compared between CD23-Cre/miR29KO and WT splenic B cells

(C) Relative expression of miR29 and miR17-92 cluster compared between A20 miR29 overexpressing cells and A20 WT.

(D) Presto blue cell viability comparison between A20 WT cells and miR-29a overexpressing A20 cells.

(E) Graph shows percentage of WT (green) and miR29a overexpressing cells (red) in cell mixture. Blue line indicates GFP expression level in cell culture containing only miR29a overexpressing cells.

(F) Relative expression of genes in A20 miR-29a overexpressing cells compared to A20 WT cells, mean ± SD, represents 4 repeats.

Figure 7 - miR29a regulates expression of the MYC co-factor SP1

(A) STRING Protein-protein interaction graph summarizing human c-MYC's (marked in blue) validated interactions with other proteins, including SP1 (marked in red). Interactions presented based on calculated confidence higher than 0.9.

(B) SP1 3'UTR map, indicating the locus of the 3'UTR, the three sections used for the luciferase assay and the location of the predicted miR29 binding site.

(C) Luciferase assay shows binding of miR29a to predicted 3'UTR binding sites of the SP1 gene.

(D) Relative expression of SP1 compared between miR29 antagomiR, scramble antagomir and untreated splenic B cells.

(E) Relative expression of SP1 compared between CD23-Cre/miR29KO and WT splenic B cells.

(F) Relative expression of SP1 compared between splenic B cells from a WT mouse and a T58A MYC mutated mouse model, either treated or untreated with a MYC inhibitor for 24 hours.

Figure 8 - miR29 represses miR17-92 through a SP1-MYC circuit

(A) Relative expression of miR29 compared between miR29 antagomiR, scramble antagomiR and untreated splenic B cells.

(B) Relative expression of miR17 compared between miR29 antagomiR, scramble antagomiR and untreated splenic B cells.

(C) Relative expression of miR19b compared between miR29 antagomiR, scramble antagomiR and untreated splenic B cells.

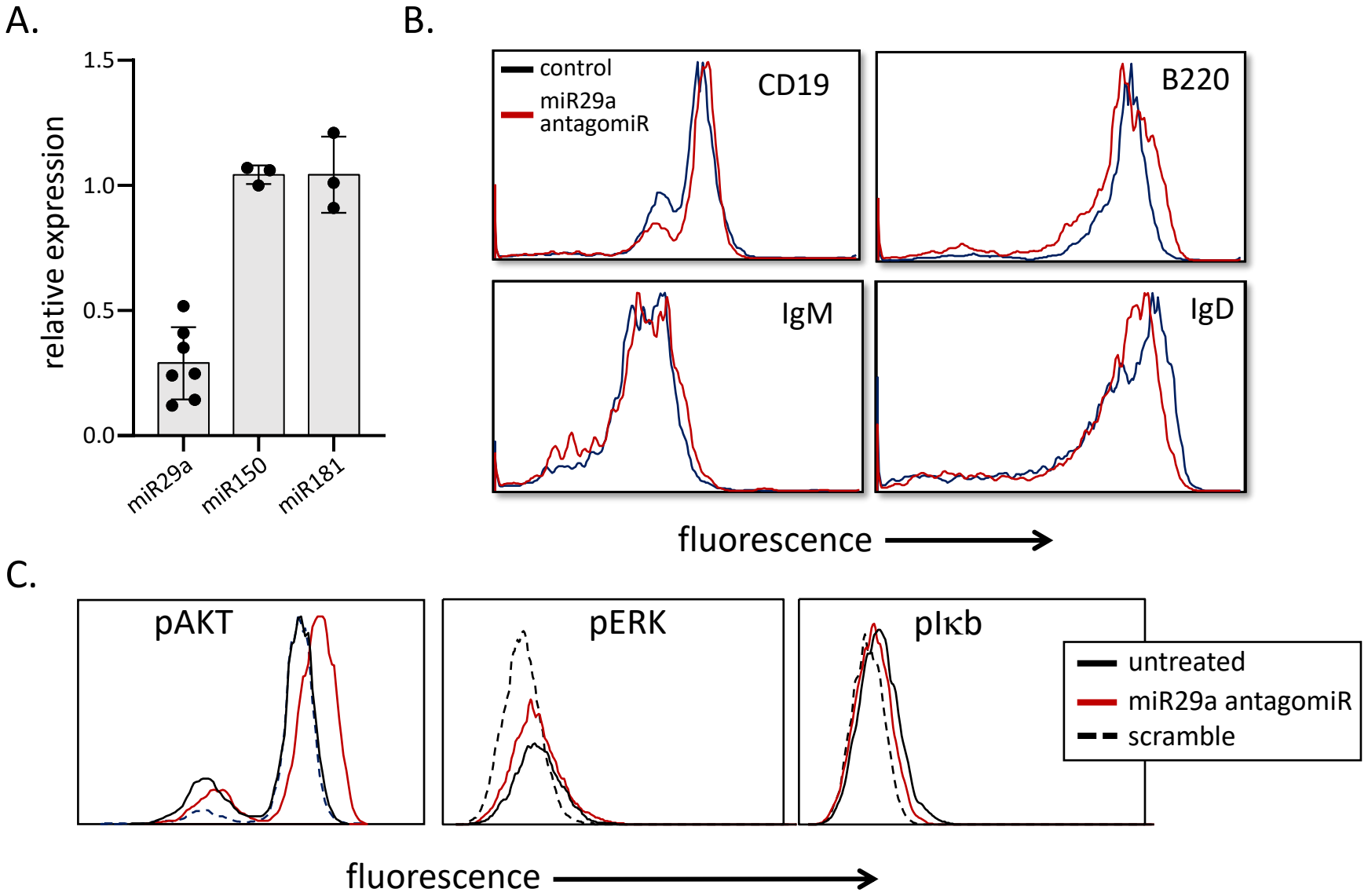
(D) Relative expression of MYC compared between miR29 antagomiR, scramble antagomiR and untreated splenic B cells.

(E) miR-29a, miR17b, miR-19b and MYC relative expression compared between WT splenic B cells and splenic B cells of CD23-Cre/miR29KO mouse, either treated or untreated with Mithramycin for 24 hours.

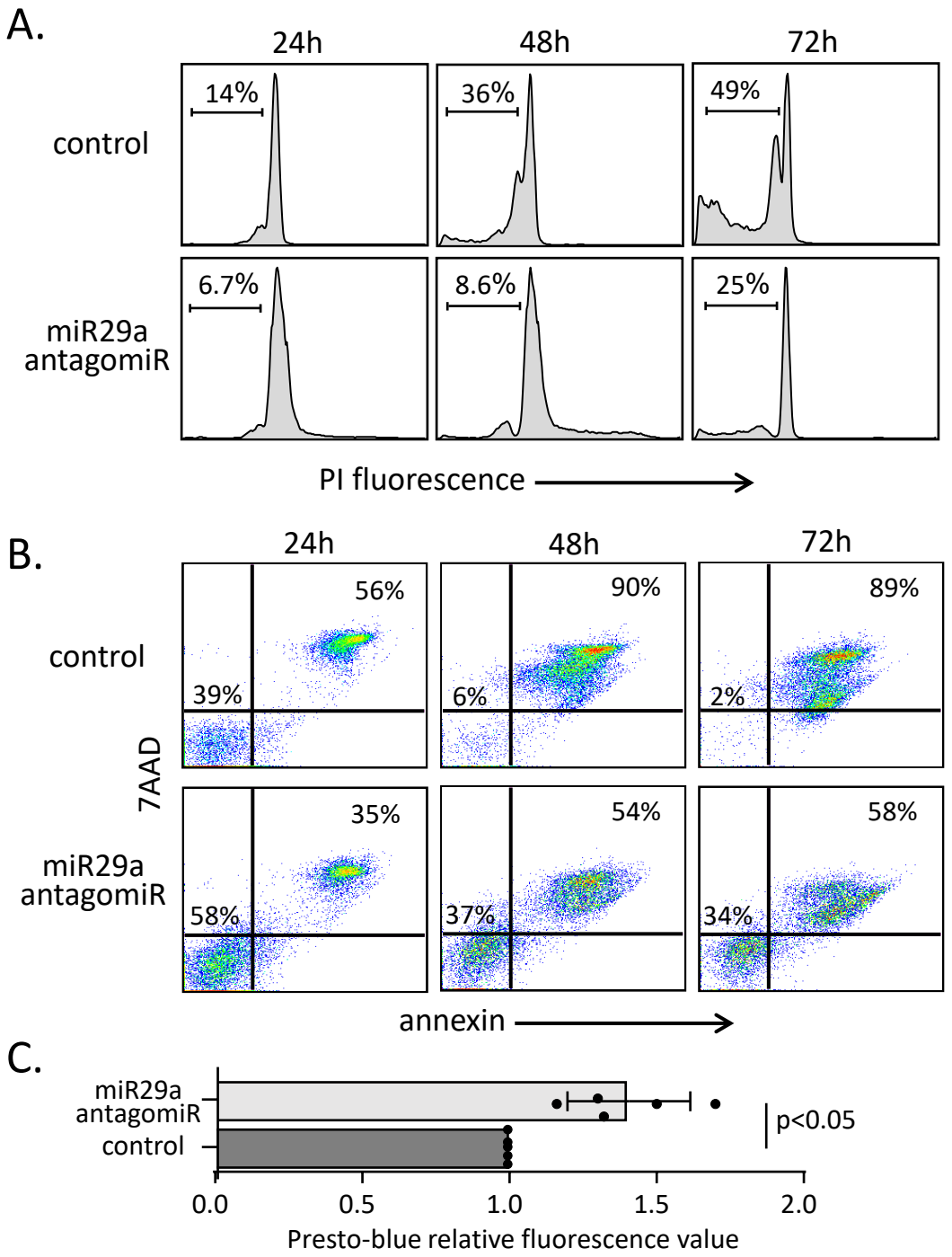
(F) PI stain of WT, CD23-Cre/miR29KO and anatgomiR29 treated splenic B cells after 24 hours, with or without treatment of Mithramycin. Bar marks Sub-G0 phase cells

Figure 9 – A schematic diagram modeling the miR29/SP1/MYC/miR17-92 regulatory circuit in quiescence state and in proliferating/active state. The miR29 expression is induced upon B cell maturation, resulting in repression of the SP1/MYC/miR17-92 activating axis and facilitating entering into a quiescent state. The high expression of miR29 is important to maintain cellular quiescence. Upon B-cell activation, the SP1/MYC/miR17-92 axis is induced. MYC represses miR29 to terminate cellular quiescence and to induce expression of miR17-92 to further activate the downstream proliferation/activation gene program.

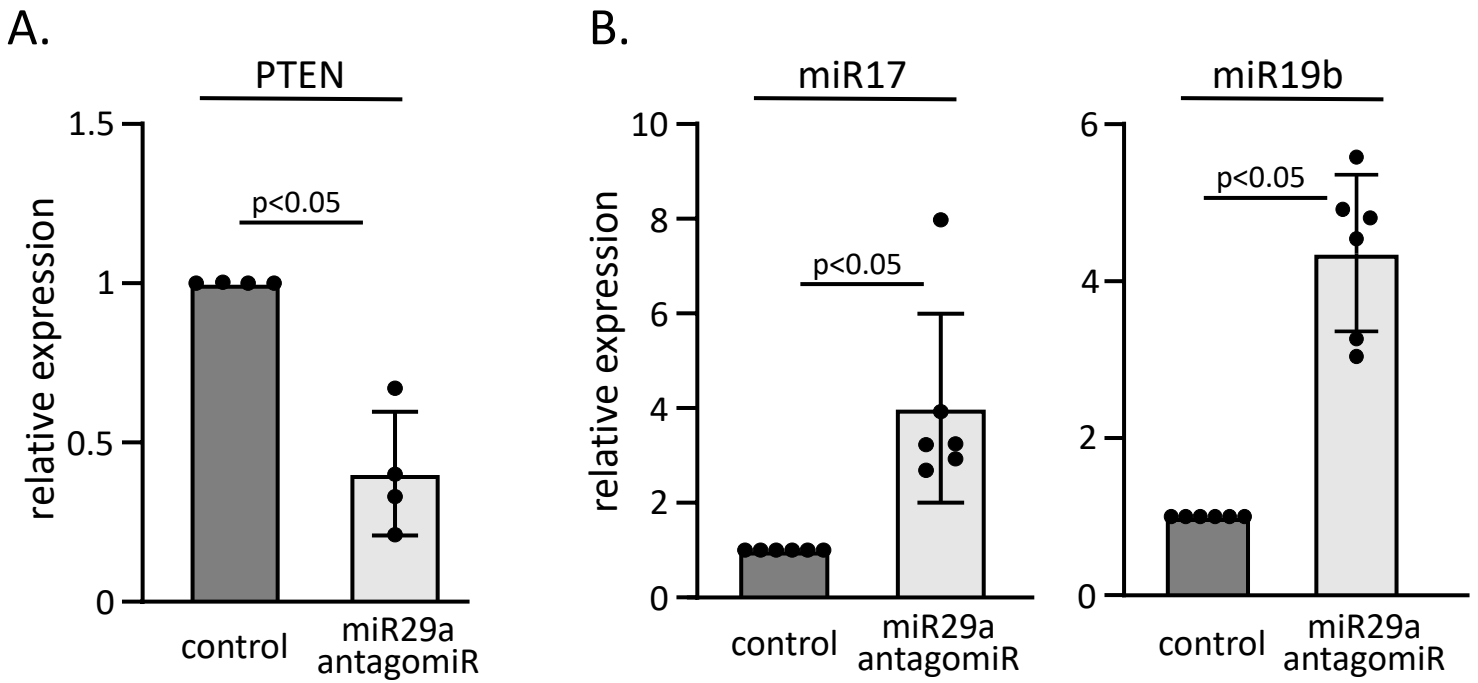
Harel et al figure 1



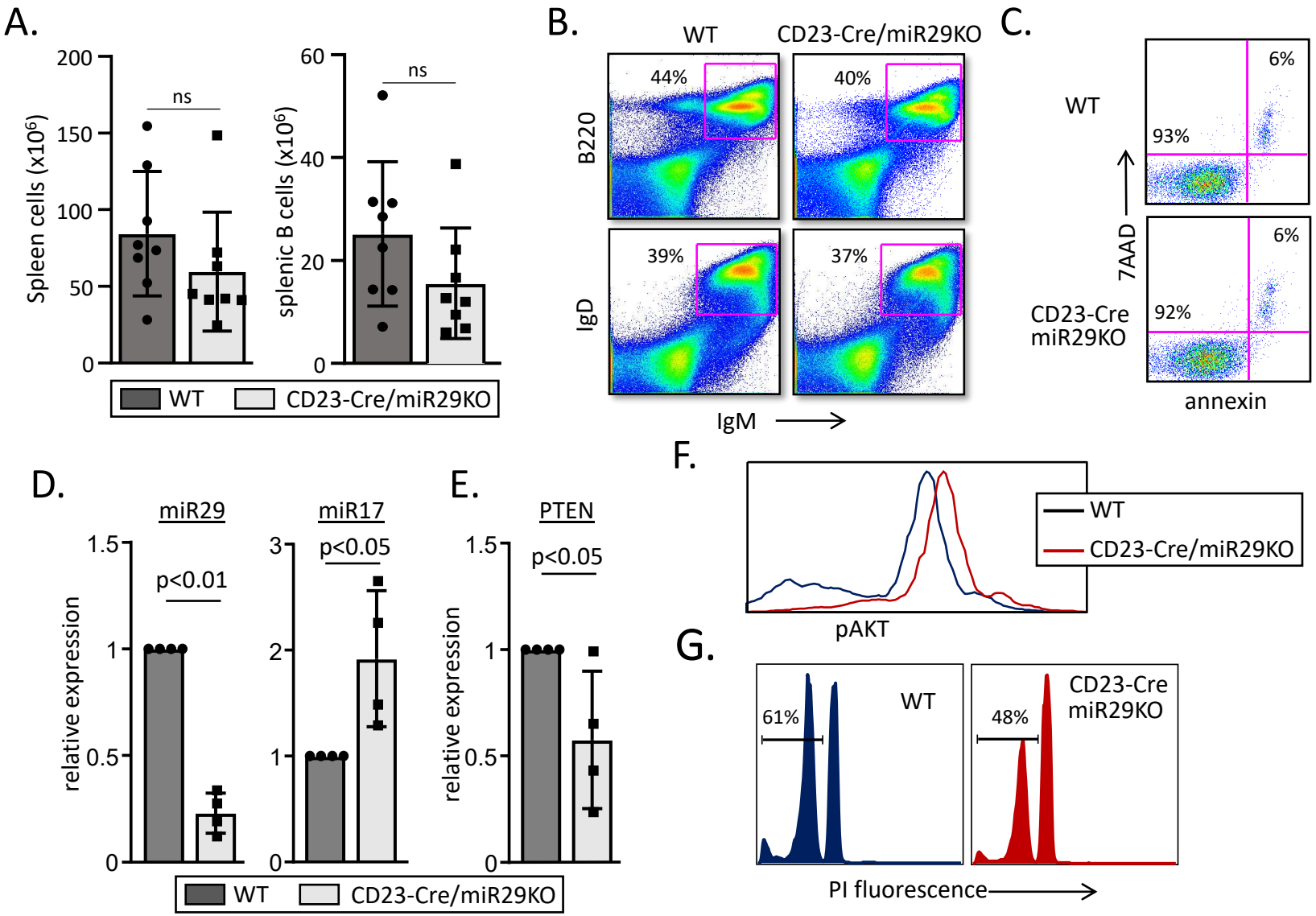
Harel et al figure 2



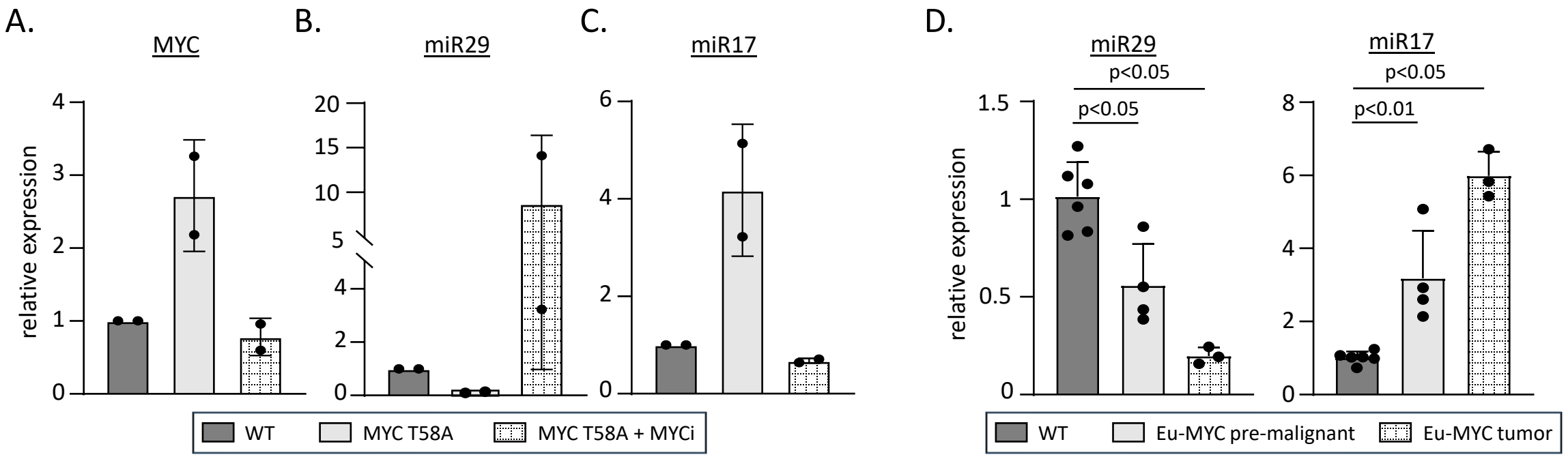
Harel et al figure 3



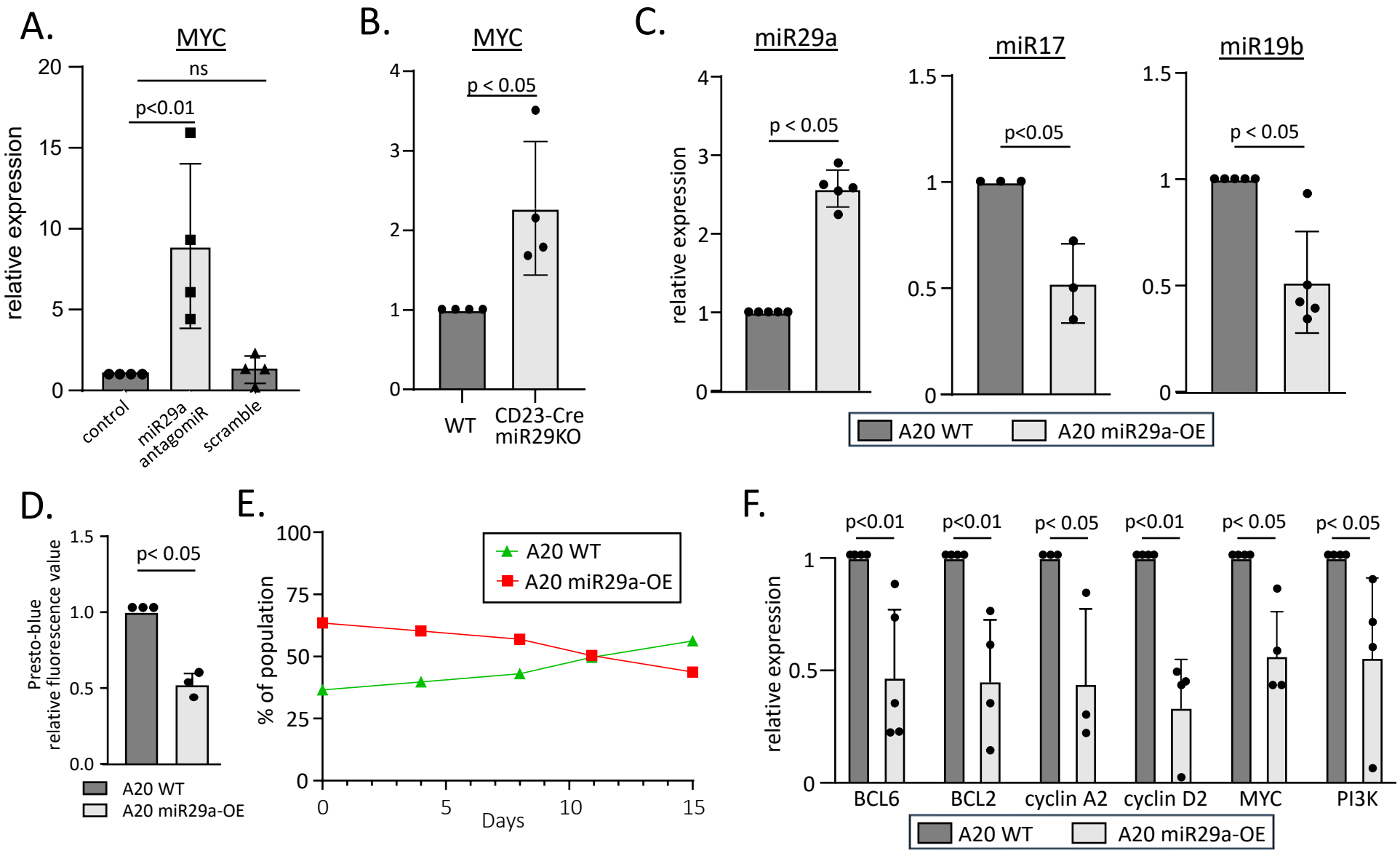
Harel et al figure 4



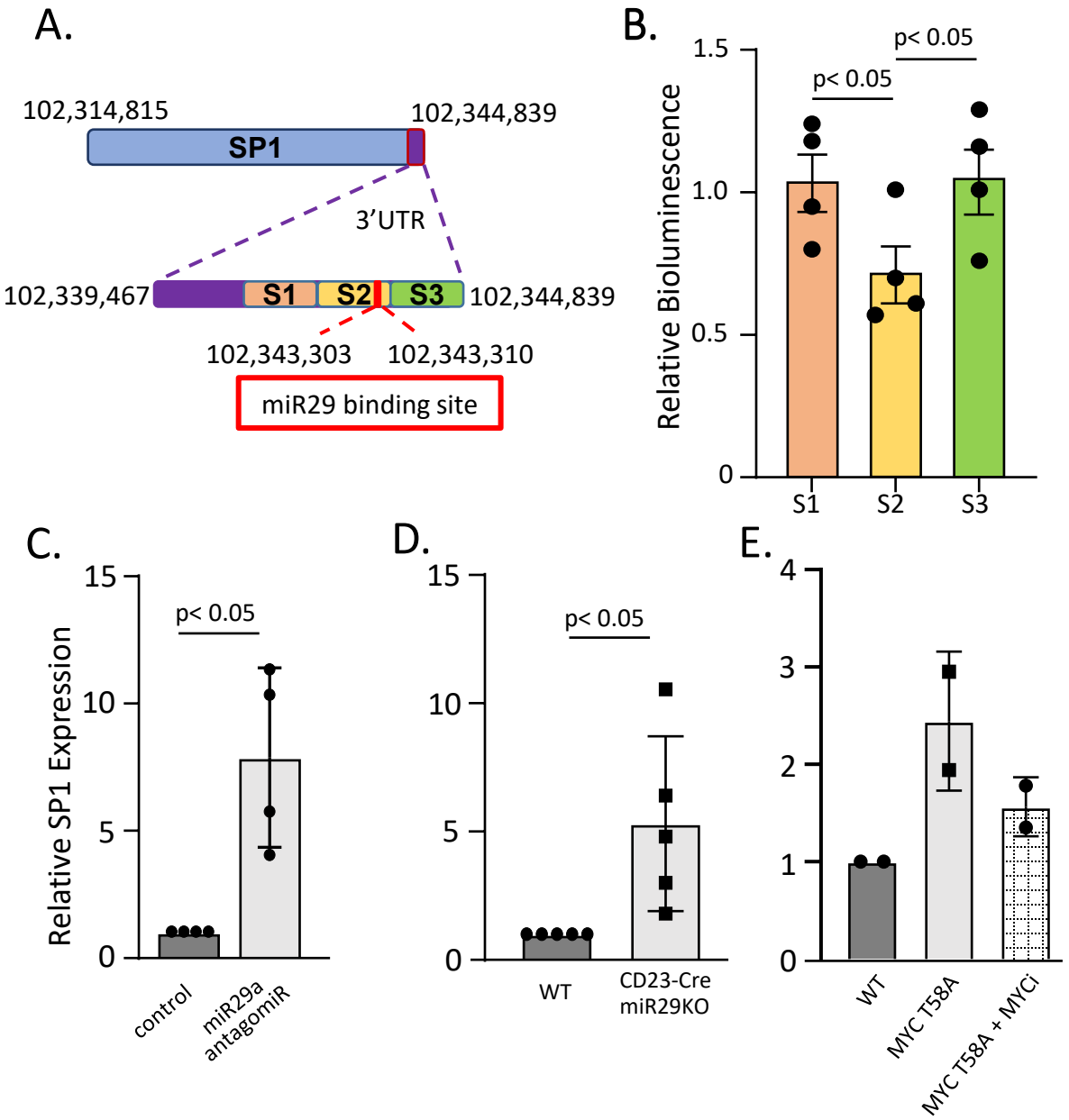
Harel et al figure 5



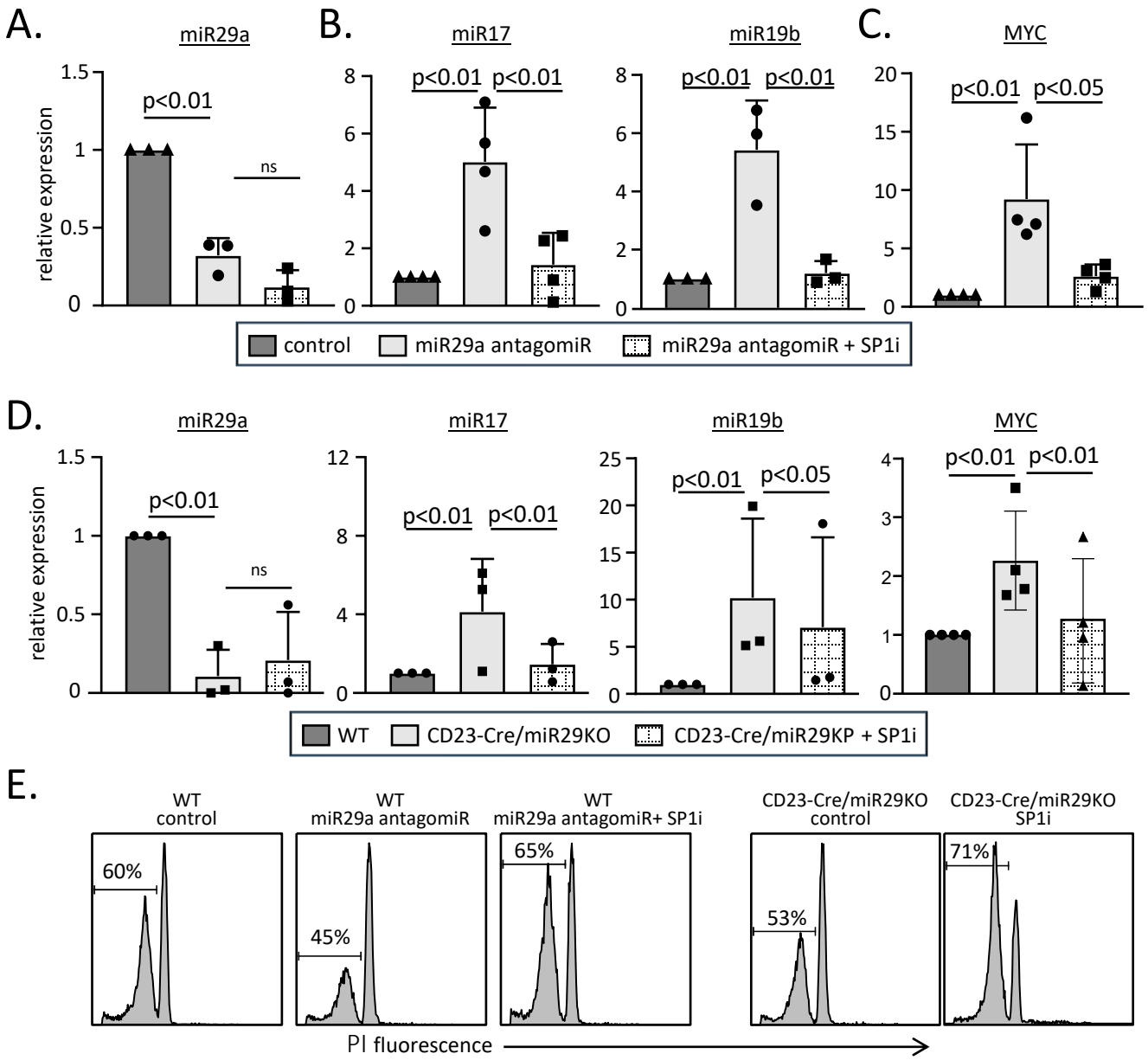
Harel et al figure 6



Harel et al figure 7

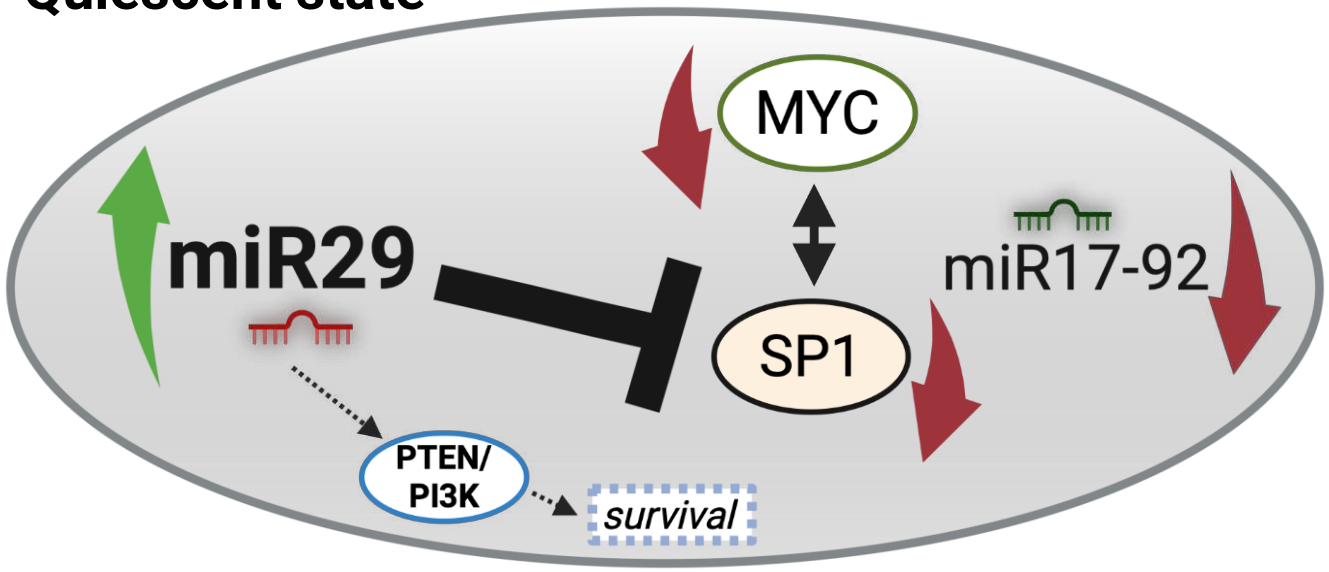


Harel et al figure 8

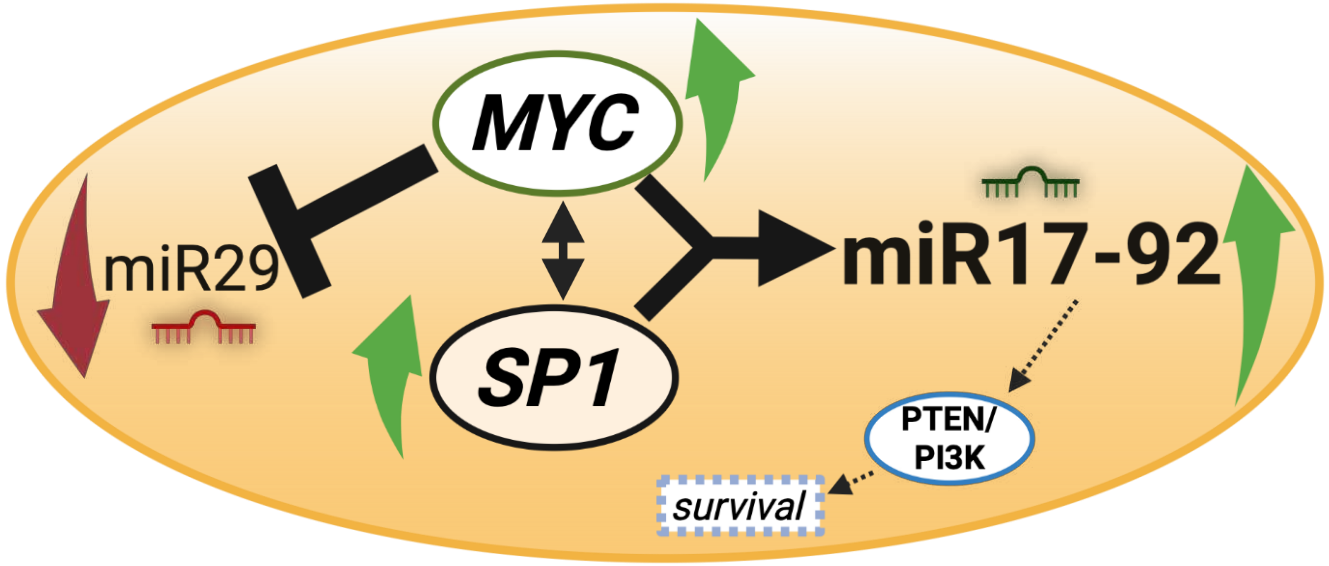


Harel et al figure 9

Quiescent state



Proliferating/active state



Supplementary Figures

Figure S1:

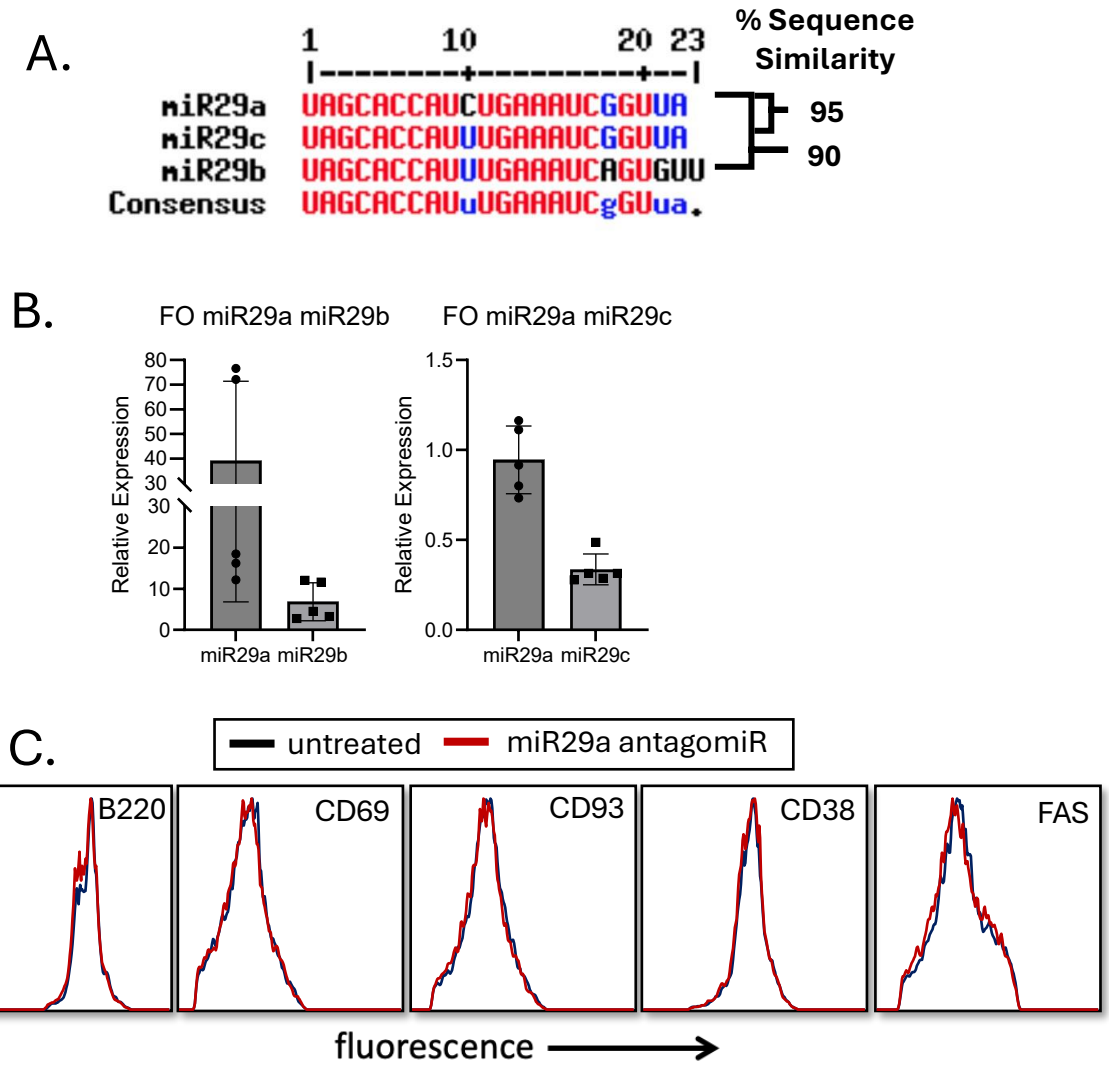


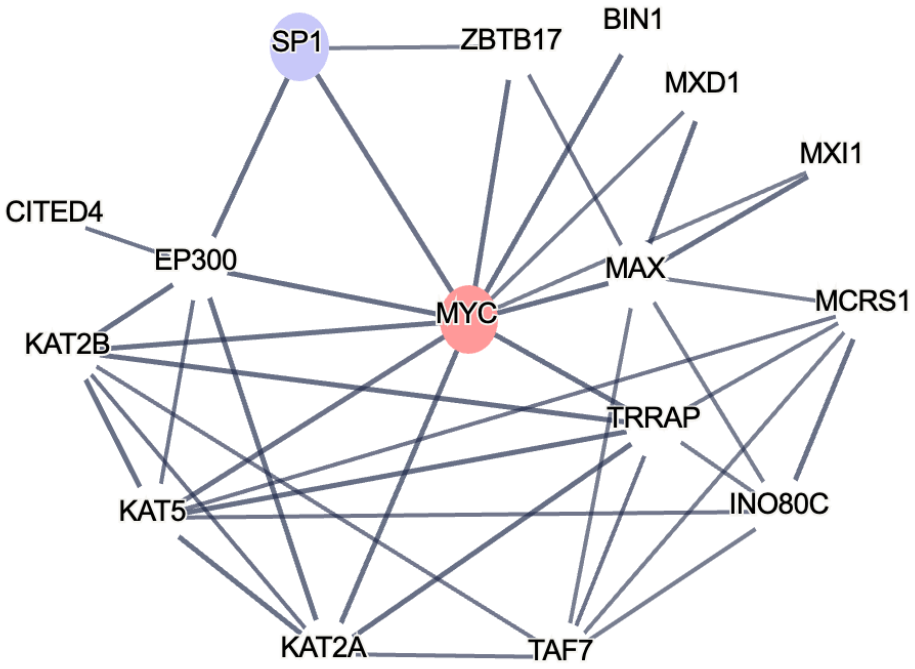
Figure S1 – Supplementary figures

(A) Homology comparison of miR29a, miR29b and miR29c sequences. Figure presents alignment of mature miRNA sequences, where high consensus areas are marked in red, low/neutral consensus areas are marked in blue or black and sequence length differences are marked as a dot ".". Sequence similarity percentage was calculated based on number of matching bases (red) between two sequences.

(B) Graph presents relative expression of miR29a compared to miR29b or miR29c in murine follicular B cells. Splenic murine B cells were stained and

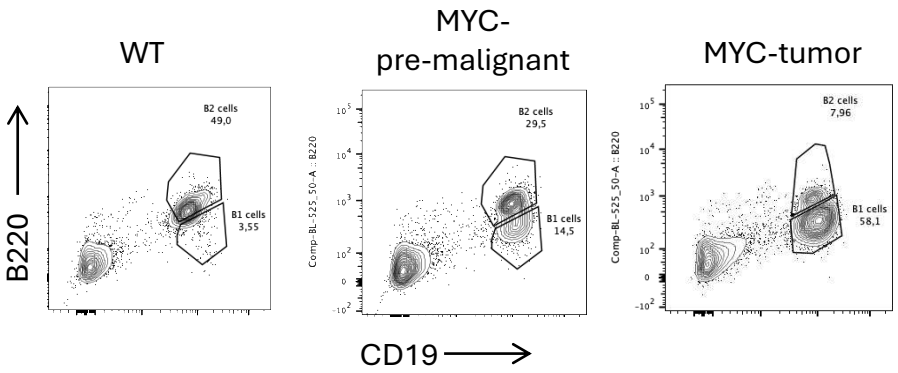
sorted for follicular B cells with FACS by gating $CD19^{pos}$, $B220^{pos}$, $CD38^{high}$, FAS^{low}, $reporter^{(double)neg}$. For normalization of miR29 levels, either miR15b (advanced TaqMan kit) or RNU6B (classical TaqMan kit) expression was used. Each symbol indicates an individual animal. Expression was measured in triplicates and average miRNA expression levels per mouse are depicted.
(C) Surface differentiation and activation markers comparison. Splenic murine B cells were isolated and incubated, either untreated or treated with a miR29a antagomiR. Following 24 hours, cells were stained for surface markers (B220, CD69, CD93, CD38, FAS) and analyzed by FACS. Graph presents no marked difference in fluorescence of markers between the two groups.

Figure S2:



STRING database Protein-protein interaction graph shows human c-MYC (marked in red) validated interaction with other proteins, including SP1 (marked in blue). Interactions presented based on STRING's calculated confidence higher than 0.9.

Figure S3:



Purified splenic B cells from WT and Eu-MYC mice were stained for CD19 (X-axis) B220 (Y-axis), then analyzed with flow cytometry. Graph marks B2-cell population ($CD19^{hi}B220^{hi}$) and B1-cell population ($CD19^{hi}B220^{lo\text{-}neg}$) within each sample.