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Original Article

Targetable vulnerability of deregulated FOXM1/PLK1 signaling axis in diffuse large B cell lymphoma

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Abstract: FOXM1 is a transcription factor that controls cell cycle regulation, cell proliferation, and differentiation. Overexpression of FOXM1 has been implicated in various cancer types. However, the activation status and functional significance of FOXM1 in diffuse large B cell lymphoma (DLBCL) have not been well investigated. Using proteomic approaches, we discovered that the protein expression levels of FOXM1 and PLK1 were positively correlated in DLBCL cell lines and primary DLBCL. Expression levels of *FOXM1* and *PLK1* mRNAs were also significantly higher in DLBCL than in normal human B cells and could predict poor prognosis of DLBCL, particularly in patients with germinal center B cell-like (GCB) DLBCL. Furthermore, proteomic studies defined a FOXM1-PLK1 signature that consisted of proteins upstream and downstream of that axis involved in the p38-MAPK-AKT pathway, cell cycle, and DNA damage/repair. Further studies demonstrated a mechanistic function of the FOXM1/PLK1 axis in connection with the DNA damage response pathways regulating the S/G2 checkpoint of the cell cycle. Therapeutic targeting of FOXM1/PLK1 using a FOXM1 or PLK1 inhibitor, as well as other clinically relevant small-molecule inhibitors targeting ATR-CHK1, was highly effective in DLBCL *in vitro* models. These findings are instrumental for lymphoma drug discovery aiming at the FOXM1/PLK1/ATR/CHK1 axis.

Keywords: FOXM1, PLK1, DLBCL, therapeutic potential

Introduction

Diffuse large B cell lymphoma (DLBCL) is the most frequent (~30,000 cases/year) aggressive non-Hodgkin lymphoma (NHL) in the Western world. Although often initially responsive to frontline chemoimmunotherapy, relapses of DLBCL portends poor prognosis and short survival [1]. While rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) has improved the rate of short-term initial responses (~80% partial or complete responses), almost half of DLBCL cases are still not cured with chemotherapy or stem cell transplant, often resulting in early relapse (within 2-3 years), with chemoresistance and an overall therapy-refractory state as the usual cause of treatment failure. Even with advances in cellular therapy, there is an unmet need for enhanced understanding the mechanisms underlying progression and resistance to DLBCL will be important in designing efficacious therapeutic approaches, critically needed for patients with relapsed/refractory DLBCL.

Forkhead box protein M1 (FOXM1) is a transcription factor of the Forkhead box protein superfamily highly expressed in various cancer types and involved in cell cycle regulation, cellular proliferation, self-renewal, DNA repair, tumorigenesis, and chemo-resistance [2]. FOXM1 is localized mainly in the nuclei in cancer cells

and functions as a transcription factor by binding to DNA and regulating the expression of target genes at the transcriptional level. The *FOXM1* gene at human chromosome 12p13.33 is amplified in 42% of cases of B-cell NHL, including DLBCL (50%), follicular lymphoma (39%), and chronic lymphocytic leukemia (33%) [3]. Further, amplification of *FOXM1* was found to be associated with an increased MYC oncogenic signaling signature, and siRNA-mediated knock-down of FOXM1 resulted in decreased MYC expression and G2 arrest. Comparative genome-wide expression profiling of malignant tumor counterparts across the human-mouse species barrier also identified FOXM1 as a potential therapeutic target in DLBCL [4]. Inhibition of FOXM1 in DLBCL cells using small-molecule inhibitors has been shown to induce apoptosis and decrease invasive and migratory capability [5]. These results clearly indicate the important role of FOXM1 expression in DLBCL and suggest that elucidating the expression and function of FOXM1 and connected signaling pathways could lead to the identification of therapeutic targets in the treatment of relapsed/refractory DLBCL, where there is an unmet need.

To explore the functional significance of FOXM1 in DLBCL, we used a panel of 38 DLBCL cell lines and proteomic approaches to examine pertinent oncogenic proteins linking to FOXM1 protein expression in DLBCL cells. We discovered a potential positive feedback mechanism between FOXM1 and polo-like kinase 1 (PLK1), a cell cycle regulatory enzyme, that controls DLBCL tumor cell growth and survival. It is intriguing to find out how the association between FOXM1 and PLK1 would affect cell growth and survival mechanisms in DLBCL. Indeed, their expression levels are positively correlated in both DLBCL cell lines (protein levels) and primary DLBCL samples (mRNA levels). In addition, *FOXM1* and *PLK1* mRNA levels were found to be significantly higher in primary DLBCL than in normal B cell counterparts and were predictive of poor clinical outcome in DLBCL patients. Moreover, the FOXM1-PLK1 axis was found to be associated with the ATR-CHK1 DNA damage response pathway and the CDK1/cyclin B1 S/G2/M cell cycle checkpoint. We further showed that targeting the FOXM1/PLK1/ATR/CHK1 axis with small-molecule inhibitors induced G2/M cell cycle arrest and subsequently activated DNA damage response and apoptosis in representative *in vitro* models of DLBCL.

Materials/subjects and methods

Cells and reagents

The DLBCL cell lines (MS, DS, DBr, McA, FN, HF, HB, MZ, LR, CJ, LP, WP, TJ, EJ, RC, RC-VR, GR, GR-VR, and JZ) were established in our laboratory and have been previously characterized and described [6-11]. The DLBCL cell lines U-2932, Pfeiffer, OCI-LY19, BJAB, Toledo, SUDHL4, SUDHL5, SUDHL6, SUDHL10, HBL-1, TMD-8, DB, HT, OCI-LY10, WILL-1, WILL-2, WSU-NHL, VAL, and OCI-LY3 were obtained from Drs. Michael Rosenblum and R. Eric Davis (The University of Texas MD Anderson Cancer Center) [12, 13]. All cell lines were routinely tested for *Mycoplasma* species using a MycoSEQ Mycoplasma Detection Kit (Invitrogen, Carlsbad, CA, USA) and were validated by short tandem repeat DNA fingerprinting at the Cytogenetics and Cell Authentication Core at The University of Texas MD Anderson Cancer Center. Stocks of authenticated cell lines were stored in liquid nitrogen for future use, and all cell lines used in the studies described here were obtained from these authenticated cell line stocks. Thiostrepton, volasertib, MK8776, BIRB-796 (doramapimod), and AZD-6738 (ceralasertib) were purchased from Selleckchem (Houston, TX, USA).

Viability assays

Cells from representative DLBCL cell lines were plated at 5,000 cells per well in 384-well plates. The assays were performed using the CellTiter-Glo Luminescent Cell Viability Assay according to the manufacturer's instructions (Promega, Madison, WI, USA).

Western blot analysis

Whole-cell or nuclear extracts were solubilized with 1% sodium dodecyl sulfate (SDS) buffer and subjected to SDS polyacrylamide gel electrophoresis on a 4% to 15% gel (Bio-Rad, Hercules, CA, USA). Proteins were transferred onto polyvinylidene difluoride membranes and probed them with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using the ECL system (Amersham, Little Chalfont, United Kingdom). Antibodies against

FOXM1, p-FOXM1 (Thr600, D9M6G, rabbit mAb, #14655).

Cyclin B1, CHK1, and c-MYC were purchased from Cell Signaling Technology (Danvers, MA, USA); PLK1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA).

Transient transfection and sgRNAs

Transient transfections in cultured lymphoma cells were conducted using the Neon Transfection System (Thermo Fisher Scientific, Waltham, MA) in representative DLBCL cells as previously described [14]. For CRISP knock-out of FOXM1 gene, pre-designed FOXM1 multi-guide sgRNA and control scrambled sgRNA were purchased from Synthego and its standard protocol (Menlo Park, CA, USA).

Reverse-phase protein array (RPPA) analysis

RPPA analysis and antibody validation were performed at The University of Texas MD Anderson Cancer Center Functional Proteomics RPPA Core Facility [15]. Specific details of the methods were previously described [16].

Statistical analysis

Spearman rank correlation coefficient with a paired *t*-test was used to evaluate correlative studies. Relative protein levels for each sample in the RPPA analysis were determined by interpolation of each dilution curve from the "standard curve" using the R package SuperCurve. All data points were normalized for protein loading and transformed to linear values. Normalized linear values were transformed to log2 values and then median-centered for hierarchical cluster analysis and heat map generation. The heat map was generated in Cluster 3.0 as a hierarchical cluster using Pearson correlation and a center metric. The resulting heat map was visualized in TreeView (https://www.java.com/en/) and presented in high-resolution bmp format.

GraphPad Prism 5.04 (GraphPad Software, Inc., La Jolla, CA) and R 3.1.2 with packages SuperCurve v1.4.4, BioNet v1.26.0, and nlme v3.1-120 were used for statistical analyses. *P* values less than 0.05 were considered significant.

Results

FOXM1 and PLK1 expression levels are positively correlated, highly expressed, and correlated with prognosis in DLBCL patients

Protein expression levels of both FOXM1 and PLK1 were analyzed in 38 representative DLBCL cell lines by RPPA (**Figure 1A**), and linear regression analysis revealed a positive correlation between FOXM1 and PLK1 protein expression ($r=0.5442$; $P<0.0004$) (**Figure 1B**). The positive correlation between FOXM1 and PLK1 could also be seen in primary DLBCL cells, at the mRNA level, in three separate DLBCL cohorts extracted from the Oncomine database (**Figure 1C**). In the same cohorts, the mRNA levels of *FOXM1* and *PLK1* were significantly higher in primary DLBCL samples in comparison to normal unstimulated naïve B lymphocytes (**Figure 1D**). Data from the Cancer Dependency Map (DepMap) portal showed a positive correlation between *FOXM1* and *PLK1* mRNA expression not only in B cell lymphoma cell lines (Supplementary Figure 1A), but also in all other cancer cell lines, including solid tumors (Supplementary Figure 1B), suggesting that the FOXM1/PLK1 association is common in all cancer types. Next, we examined the clinical significance of *FOXM1 and PLK1* mRNA expression levels in a cohort of de novo DLBCL patients treated with the standard R-CHOP regimen. This patient cohort also showed a similar pattern of positive correlation between *FOXM1* and *PLK1* (Supplementary Figure 2A). In the Lenz et al. data set [17] in Oncomine, patients with high mRNA expression levels of either *FOXM1* or *PLK1* had poor overall survival compared with those with low mRNA expression of *FOXM1* or *PLK1*, respectively (**Figure 2A**), and this difference was particularly noticeable in patients with the GCB subtype of DLBCL (**Figure 2B**), but not in the activated B cell-like subtype (ABC) of DLBCL (**Figure 2C**) or type 3 (DLBCL, unclassifiable) (**Figure 2D**), suggesting that these two transcription factors play a key role in the pathogenesis of the GCB-DLBCL subtype.

The FOXM1/PLK1 protein signature consists of cell cycle and DNA damage response pathways in DLBCL

To understand the pathways involved in FOXM1/PLK1 signaling, we first identified pro-

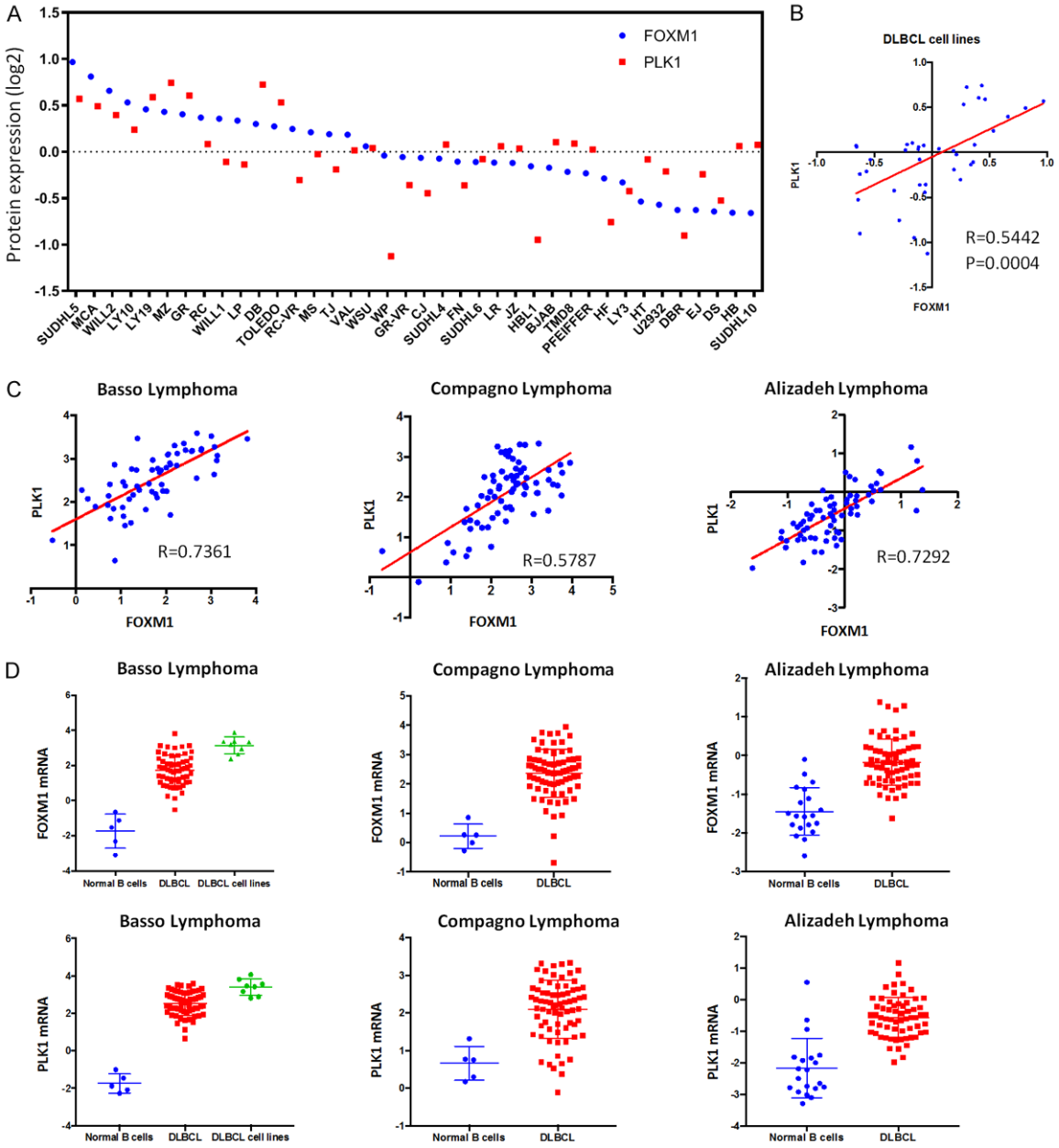


Figure 1. FOXM1 and PLK1 expression levels are positively correlated and highly expressed in DLBCL. A. FOXM1 and PLK1 protein expression levels in DLBCL cell lines (n=38) were measured by RPPA analysis, and quantitated protein expression levels were plotted. B. Linear regression analysis of FOXM1 and PLK1 protein expression in representative DLBCL cell lines. C. In three separate DLBCL cohorts extracted from the Oncomine database [30-33], the mRNA expression levels of FOXM1 and PLK1 were also positively correlated. D. In the same cohorts, the mRNA levels of FOXM1 (top) and PLK1 (bottom) were significantly higher in primary DLBCL cells compared to normal B cells.

teins positively associated with the FOXM1 and PLK1 axis using RPPA analysis in 38 representative DLBCL cell lines. Twenty-three proteins positively correlated with FOXM1, while 24 proteins positively correlated with PLK1. Out of these proteins, 10 were positively associated with both FOXM1 and PLK1 protein expression. The positively associated proteins are primarily involved in cell cycle (CDK1, p27, Cyclin B1, PCNA, CDC25c, and Aurora-B), cell signaling (c-Jun, PRAS40), and DNA damage response (CHK1 and NDRG1) pathways (**Figure 3A**). Examples of these positive correlations, between FOXM1 protein expression and cell

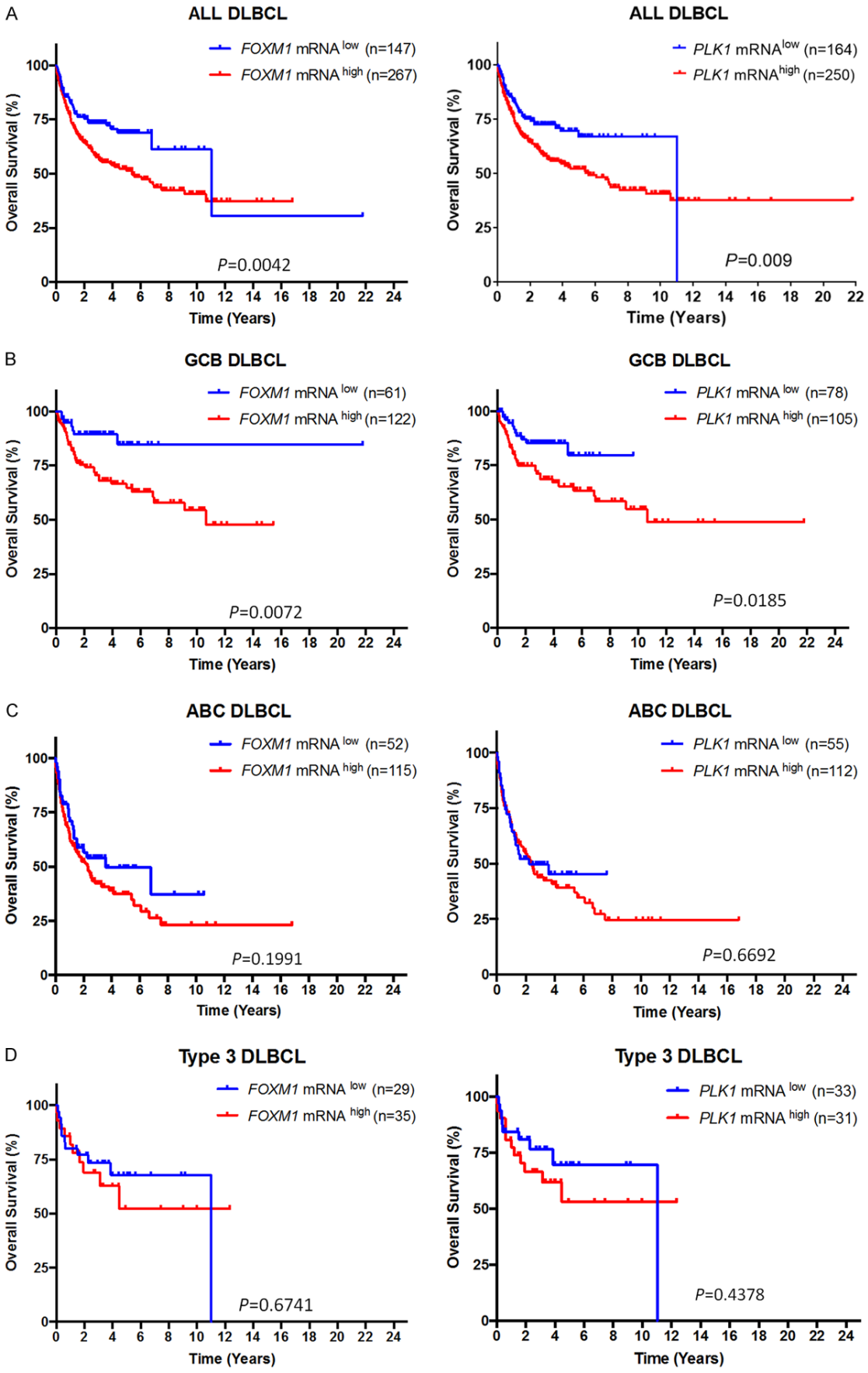

A
ALL DLBCL
FOXM1 mRNA low (n=147)
FOXM1 mRNA high (n=267)
P=0.0042
Overall Survival (%)
Time (Years)
ALL DLBCL
PLK1 mRNA low (n=164)
PLK1 mRNA high (n=250)
P=0.009
Overall Survival (%)
Time (Years)
B
GCB DLBCL
FOXM1 mRNA low (n=61)
FOXM1 mRNA high (n=122)
P=0.0072
Overall Survival (%)
Time (Years)
GCB DLBCL
PLK1 mRNA low (n=78)
PLK1 mRNA high (n=105)
P=0.0185
Overall Survival (%)
Time (Years)
C
ABC DLBCL
FOXM1 mRNA low (n=52)
FOXM1 mRNA high (n=115)
P=0.1991
Overall Survival (%)
Time (Years)
ABC DLBCL
PLK1 mRNA low (n=55)
PLK1 mRNA high (n=112)
P=0.6692
Overall Survival (%)
Time (Years)
D
Type 3 DLBCL
FOXM1 mRNA low (n=29)
FOXM1 mRNA high (n=35)
P=0.6741
Overall Survival (%)
Time (Years)
Type 3 DLBCL
PLK1 mRNA low (n=33)
PLK1 mRNA high (n=31)
P=0.4378
Overall Survival (%)
Time (Years)

Figure 2. Prognostic analysis of FOXM1 and PLK1 expression in de novo DLBCL treated with standard R-CHOP regimen. FOXM1 (left) or PLK1 (right) mRNA expressions were assessed in association with poorer overall survival in overall DLBCL patients (A), germinal center B cell-like (GCB) DLBCL patients (B), activated B cell-like (ABC) DLBCL patients (C), and Type 3 DLBCL patients (D). The cutoffs for *FOXM1* and *PLK1* mRNA mRNA are 2.4 and 2.0 for the normalized log2 values of the microarray data, respectively. The values above and below these are considered high and low, respectively.

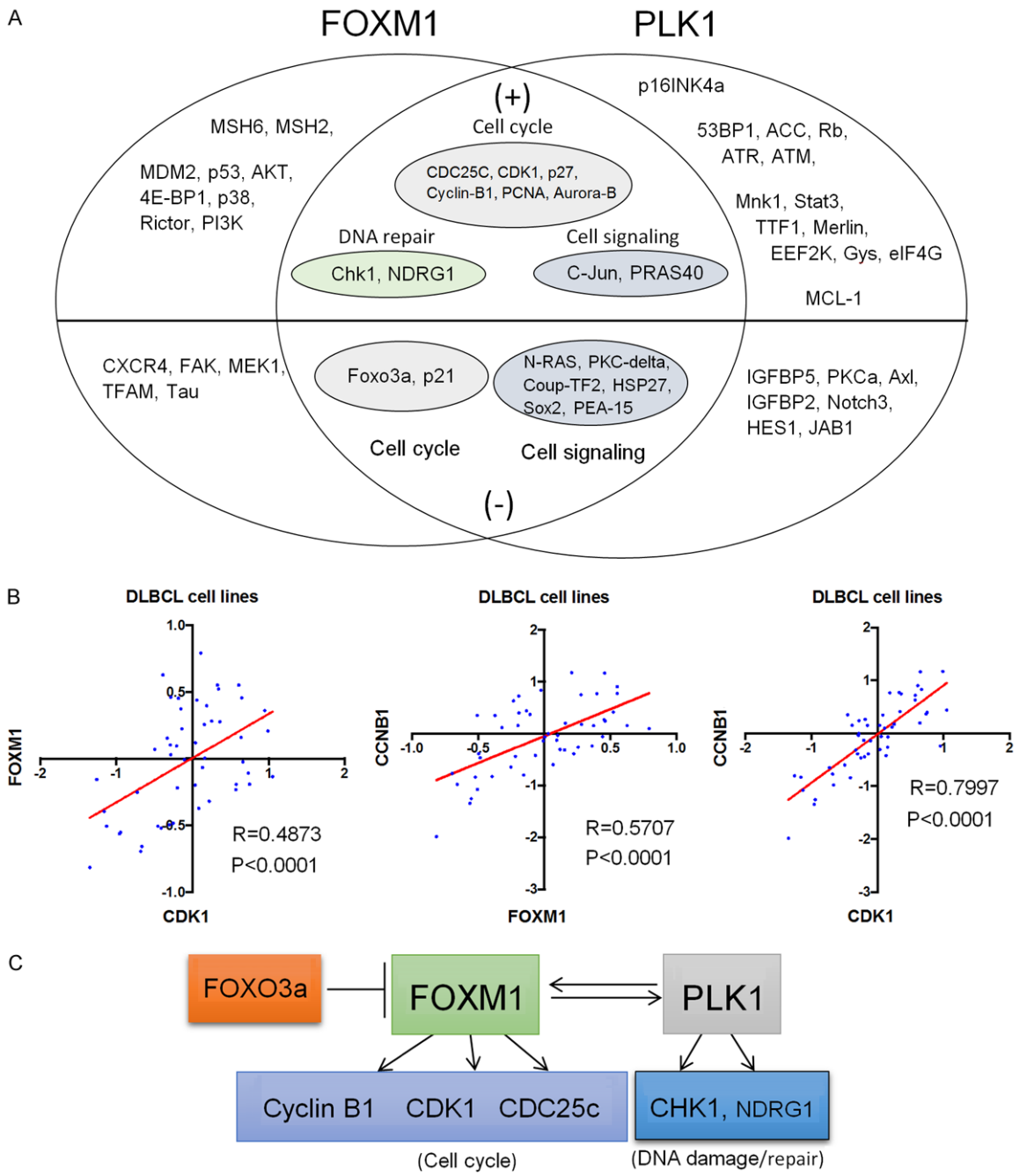


Figure 3. Downstream signaling pathways involved in FOXM1/PLK1 axis. A. Venn diagram showing proteins negatively or positively associated with FOXM1 or PLK1 and proteins shared between FOXM1 and PLK1 on RPPA analysis. B. Linear regression analysis of expression levels of FOXM1 and cell cycle-related protein CDK1 and cyclin B1 (CCNB1) was plotted in 38 DLBCL cell lines, and the Pearson correlation coefficient and *P* value were determined. C. Model depicting the proposed FOXM1/PLK1 pathway and potential mechanism of action, based on the RPPA data of 38 representative DLBCL cell lines.

cycle-related proteins CDK1 and Cyclin B1 (CCNB1), in representative DLBCL cell lines are shown in **Figure 3B** and Supplementary Figure 2A. **Figure 3B** also shows the correlation between CDK1 and CCNB1 themselves (rightmost panel). On the basis of these findings, we proposed a model in which FOXM1 and PLK1 FOXM1, possibly form a positive feedback pathway where PLK1 can regulate FOXM1 protein expression and vice versa. This model also depicts the cooperation between FOXM1 and PLK1 to regulate key downstream genes involved in both cell cycle regulation and DNA damage response pathways (**Figure 3C**).

FOXM1 inhibitor thiostrepton downregulates PLK1 and FOXM1/PLK1 axis downstream targets in DLBCL cells

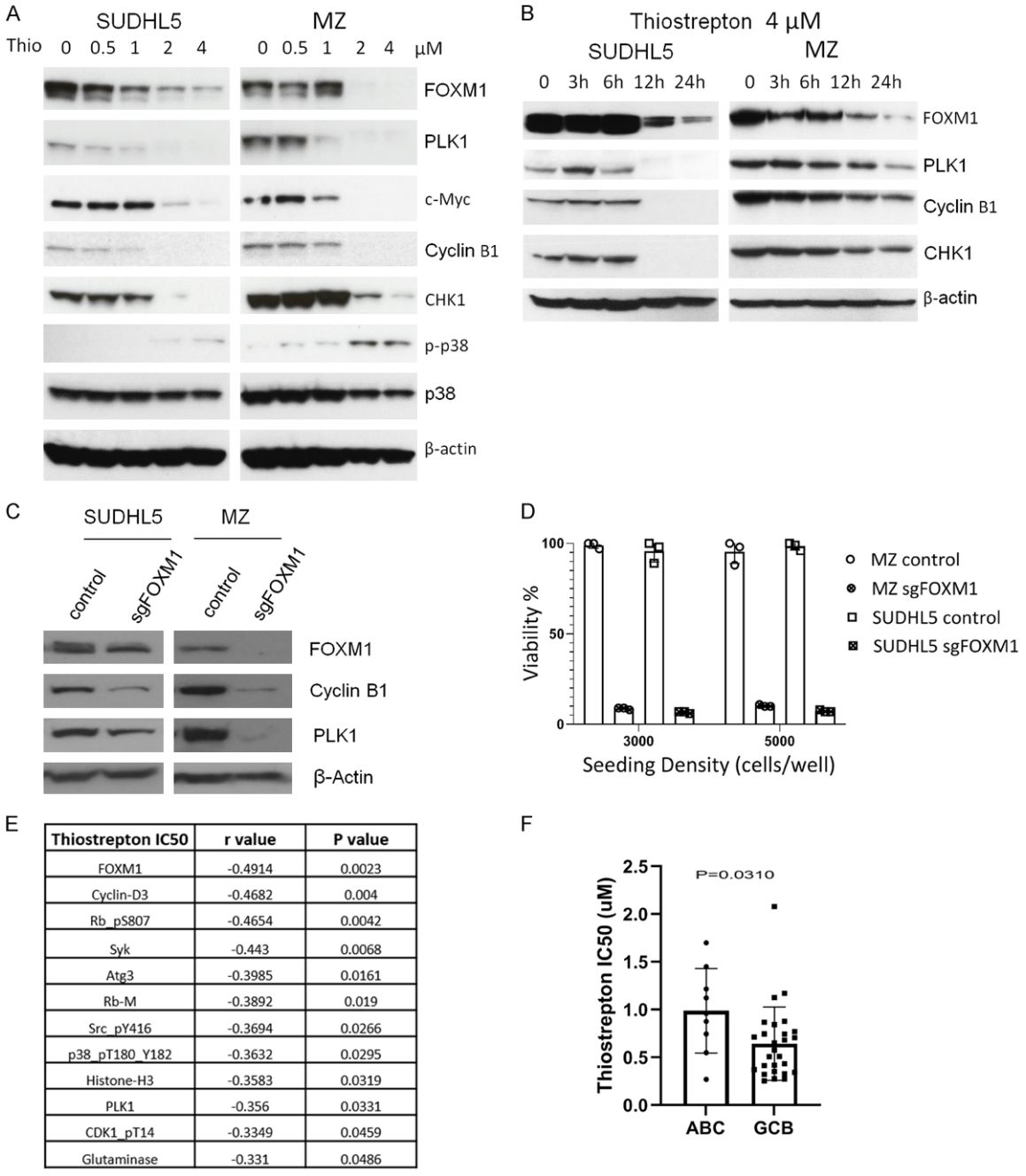
We next interrogated FOXM1 inhibition by using the FOXM1 inhibitor thiostrepton (FOXM1 transcriptional inactivator) [18] to examine whether inhibition of FOXM1 affects targets downstream of the FOXM1 and PLK1 axis. As shown in two representative DLBCL cell lines, thiostrepton treatment inhibited FOXM1, PLK1, Cyclin B1, and CHK1 in a dose-dependent (**Figure 4A**) and time-dependent (**Figure 4B**) manner. *MYC*, a known target gene of PLK1, was also downregulated in thiostrepton-treated cells, suggesting that PLK1 is a downstream target of FOXM1. Interestingly, the protein level of p-p38 was upregulated after thiostrepton treatment, suggesting that the p38 signaling pathway is either upstream of FOXM1 or a compensatory pathway of FOXM1 signaling (**Figure 4A**). To validate our findings, we evaluated whether knock-out of FOXM1 by the CRISPR method could recapitulate the drug inhibition effect of FOXM1. As shown in **Figure 4C**, FOXM1 multi-guide sgRNA-transfected cells showed lower abundance of FOXM1, PLK1, and Cyclin B1. More interestingly, FOXM1 multi-guide sgRNA-transfected cells showed lower cell viability, suggesting the function of FOXM1 and associated proteins in promoting tumor cell growth and survival (**Figure 4D**).

Thiostrepton inhibits cell growth and induces apoptosis and cell cycle arrest in DLBCL cells

To test our hypothesis that FOXM1 is required for cell growth and survival in DLBCL, we interrogated the effect of FOXM1 inhibition with thiostrepton in representative DLBCL cell lines. Cell viability assays were performed in 38 representative DLBCL cell lines and showed cell growth inhibition by thiostrepton in a dose-dependent manner. The IC50 for thiostrepton ranged from 0.2 μM to 2 μM in a 72-h assay (Supplementary Table 1). GCB subtype of DLBCL appears to be more sensitive to thiostrepton treatment in comparison to ABC subtype DLBCL (**Figure 4F** and Supplementary Table 1). Using RPPA protein expression profiles of DLBCL cell lines (n=38), linear regression analysis revealed a negative correlation between the basal protein level of FOXM1 and the thiostrepton IC50 values ($r=-0.4914$; $P<0.0023$) (**Figure 4E**), suggesting the on-target effect of thiostrepton in inhibiting FOXM1. Several FOXM1-associated proteins were also negatively correlated with thiostrepton IC50, including PLK1, Cyclin B1, and p38, suggesting that high expression levels of these proteins are also good predictive biomarkers for thiostrepton sensitivity. Next, we examined the apoptotic effect of thiostrepton on DLBCL cells. Thiostrepton treatments in four representative DLBCL cell lines showed increased apoptosis in a dose-dependent manner after 24 h and 48 h of treatment (**Figure 5A**). Thiostrepton-induced apoptosis in DLBCL cells was confirmed by Western blotting, showing increased cleaved caspase 3 and cleaved PARP (**Figure 5B**). The level of pH2AX, a marker for DNA damage, was increased in thiostrepton-treated DLBCL cells, suggesting that DNA damage response is also a potential mechanism of action of the thiostrepton. Cell cycle analysis demonstrated that thiostrepton induced G2/M cell cycle arrest in a dose-dependent manner (**Figure 5C**), as anticipated, since downstream targets of the FOXM1/PLK1 axis such as Cyclin B1 and CDK1 are key regulators of G2/M/S phase transition.

FOXM1 is a good predictive biomarker for therapies targeting FOXM1-associated signaling pathways

In addition to targeting FOXM1 with thiostrepton, we also examined whether targeting FOXM1-associated proteins, such as AKT and p38 (cell signaling upstream of FOXM1), PLK1 and CDK1/Cyclin B1 (cell cycle), and CHK1 and ATR (DNA damage response), would have similar *in vitro* therapeutic efficacy. Cell viability assays were performed in 38 representative DLBCL cell lines for the indicated inhibitors,



Thiostrepton IC50	r value	P value
FOXM1	-0.4914	0.0023
Cyclin-D3	-0.4682	0.004
Rb_pS807	-0.4654	0.0042
Syk	-0.443	0.0068
Atg3	-0.3985	0.0161
Rb-M	-0.3892	0.019
Src_pY416	-0.3694	0.0266
p38_pT180_Y182	-0.3632	0.0295
Histone-H3	-0.3583	0.0319
PLK1	-0.356	0.0331
CDK1_pT14	-0.3349	0.0459
Glutaminase	-0.331	0.0486

Figure 4. Thiostrepton downregulates the expression of PLK1 and other downstream targets in DLBCL cells. Two representative DLBCL cell lines, SUDHL5 and MZ, were treated with thiostrepton (Thio) at various doses (A) and schedules (B), and protein expression levels of FOXM1, PLK1, c-Myc, cyclin B1, CHK1, p-p38, p38, and β-actin (loading control) were assessed by Western blotting. For the varying durations of thiostrepton treatment, a 4 μM concentration was used. (C) Multi-guide sgFOXM1-transfected cells showed lower abundance of FOXM1, PLK1 and cyclin B1 by Western blotting. (D) Knock-down of FOXM1 by multi-guide sRNA inhibited DLBCL cell proliferation *in vitro*. (E) Linear regression analysis of RPPA protein expression profiles of DLBCL cell lines (n=38) and thiostrepton IC50 values. FOXM1 protein expression showed the highest correlation with thiostrepton treatment (r=-0.4914; P<0.0023). (F) IC50 of GCB or ABC subtypes of DLBCL with thiostrepton treatment (P=0.0310).

and their IC50 values were calculated (Supplementary Table 1) and compared to FOXM1 protein expression. For all drugs tested, FOXM1 protein expression showed a significantly negative correlation with drug IC50, indicating that FOXM1 is a good biomarker for pre-

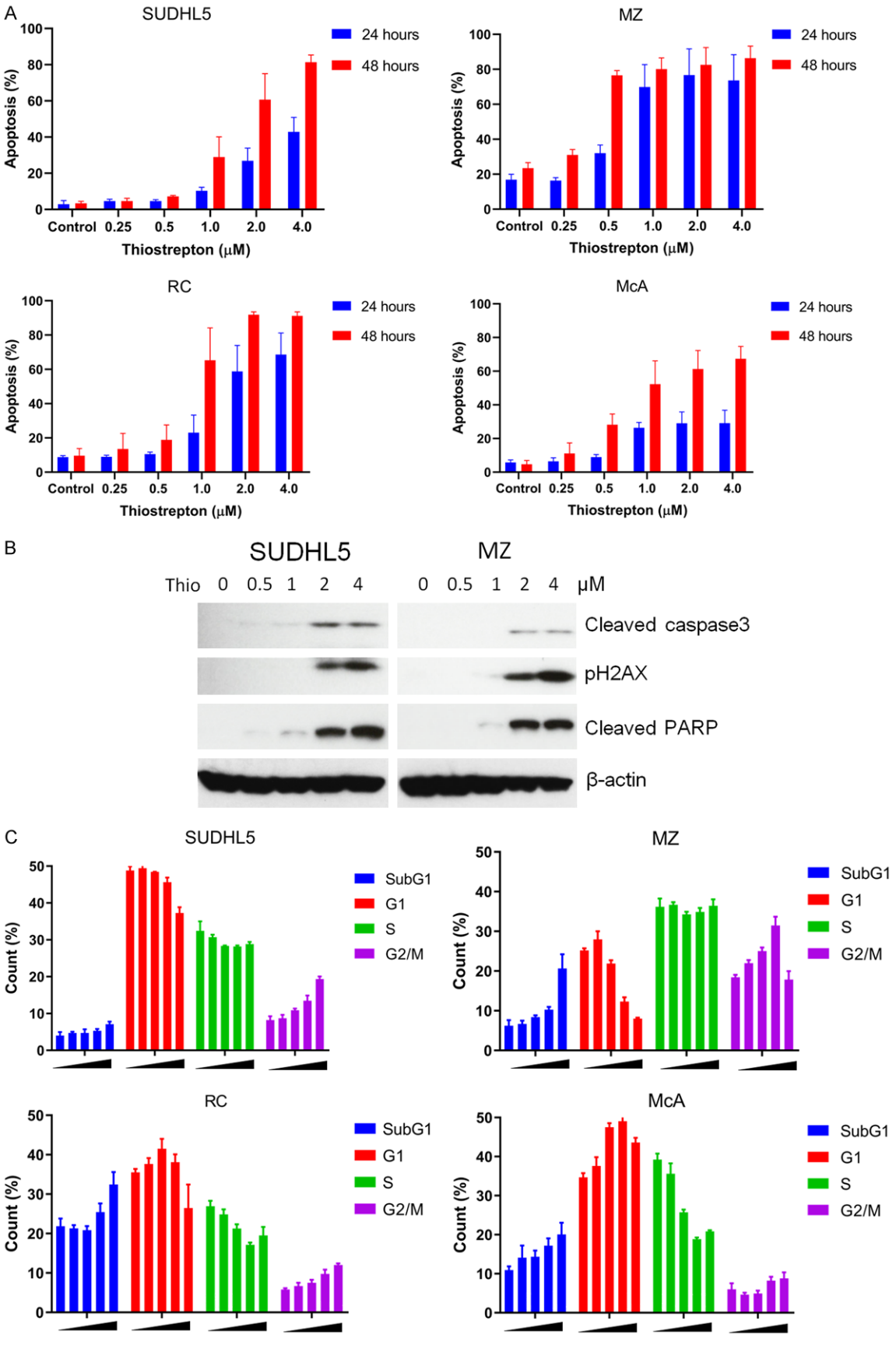

A
SUDHL5
MZ
RC
McA
24 hours
48 hours
Apoptosis (%)
Control 0.25 0.5 1.0 2.0 4.0
Thiostrepton (μM)
B
SUDHL5
MZ
Thio 0 0.5 1 2 4 0 0.5 1 2 4 μM
Cleaved caspase3
pH2AX
Cleaved PARP
β-actin
C
SUDHL5
MZ
RC
McA
SubG1
G1
S
G2/M
Count (%)

Figure 5. Thiostrepton inhibits cell growth and induces apoptosis and cell cycle arrest in DLBCL. A. Two representative DLBCL cell lines were treated with thiostrepton in a dose-dependent manner for 24 and 48 h, and apoptosis was measured using annexin V/PI staining and subsequently analyzed by flow cytometry. B. Two representative DLBCL cell lines were treated with thiostrepton in a dose-dependent manner for 24 h, and protein extracts were analyzed by Western blotting for apoptotic biomarkers caspase 3 and PARP cleavage and DNA damage biomarker pH2AX. C. Four representative DLBCL cell lines (SUDHL5, MZ, RC, and McA) were treated with thiostrepton in a dose-dependent manner (0, 0.25, 0.5, 0.75, and 1 μM) for 24 h, and cell cycle analysis was performed using PI staining and flow cytometry analysis.

dicting response to these inhibitors, at least *in vitro* (Supplementary Figure 2B).

Thiostrepton synergizes with key therapies targeting FOXM1-associated proteins

We have shown in **Figure 4A** that FOXM1 inhibition by thiostrepton increased phosphorylated-p38 in DLBCL cells. We then tested whether p38 inhibitor would inhibit thiostrepton-induced p-p38 activation, and subsequently result in synergistic cell growth inhibition. As shown in two representative DLBCL cell lines, p38 inhibitor BIRB-796 completely suppressed p-p38 activation induced by thiostrepton (**Figure 6A**). Combining the two drugs in treatment synergistically inhibited tumor cell growth (**Figure 6B**). Similar results were demonstrated for the PLK1 inhibitor in combination with the p38 inhibitor (data not shown), suggesting that p-p38 activation could be a compensatory pathway of the FOXM1/PLK1 axis and that inhibition of FOXM1/PLK1 pathway in combination with p38 inhibition could have therapeutic value in DLBCL. Interestingly, we also found that the PLK1 inhibitor induced FOXM1 activation in DLBCL cells, and this activity was further suppressed by the FOXM1 inhibitor thiostrepton (**Figure 6C**). This resulted indicated that that FOXM1 is a downstream target of PLK1. We discovered that the PLK1 inhibitor suppressed cell growth even at a very low drug concentration. However, the PLK1 inhibitor could not completely eradicate all the tumor cells, even at a very high drug concentration (**Figure 6D**). When the FOXM1 inhibitor was combined with the PLK1 inhibitor, complete cell growth inhibition was observed in the two representative DLBCL cell lines (**Figure 6D**) (see Supplementary Figure 3 for 3 additional DLBCL cell lines), suggesting that FOXM1 inhibition kills the remaining cell population spared by PLK1 inhibition. Such drug combinations show additive/synergistic effects in terms of cell growth inhibition and apoptosis induction.

Discussion

Using patient derived DLBCL cell lines as a model system and proteomic RPPA approaches, we have identified an association between FOXM1 and PLK1 that mediates cell growth and survival mechanisms in DLBCL. This association was further validated in primary human DLBCL specimens. Functional studies revealed key signaling pathways involving in cellular DNA damage response and G2/M cell cycle pathways. We also demonstrated the feasibility and efficacy of targeting the FOXM1/PLK1 axis in DLBCL *in vitro* models.

DLBCL is an aggressive, but also heterogeneous B cell lymphoma with various identified molecular genetic aberrancies that can lead to the activation of key transcription factors, including NF-κB and STAT3, and more recently NFATc1, for cell growth and survival mechanisms in DLBCL [19]. We showed here that the FOXM1 and PLK1 transcription factors also play a key role in the biology and pathophysiology of DLBCL. Previous studies have shown the individual functional roles of FOXM1 and PLK1 in the biology of DLBCL. However, the functional significance of both factors in DLBCL has not been demonstrated previously. FOXM1 has been shown to transcriptionally regulate the expression of PLK1 and vice versa [20, 21], demonstrating a positive feedback pathway, and these factors are co-expressed in various cancers, including gastric adenocarcinomas, esophageal adenocarcinoma, pancreatic cancer, and bladder cancer [22]. Our findings demonstrated the constitutive activation of FOXM1-PLK1 axis in DLBCL is an important component of the mitosis regulatory network, but also play a role in DNA damage regulatory mechanisms. In most cases, these two factors coordinate in cell mitosis, particularly during G2/M cell cycle transition, which is consistent with their functional role in regulating cell cycle target genes, such as CDK1, CDC25C, and CCNB1. Our data show that small-molecule inhibitors blocking

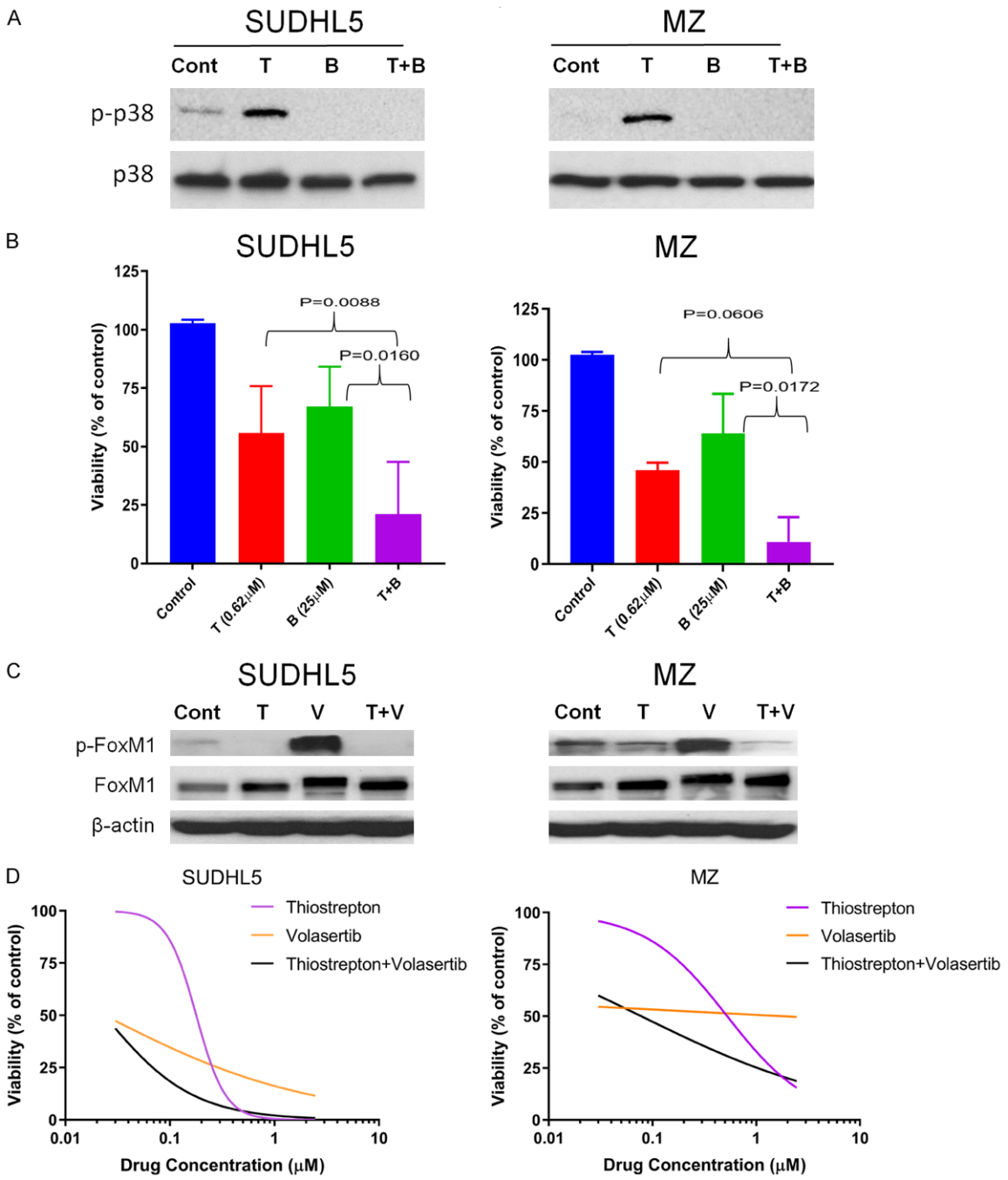


Figure 6. The FOXM1 inhibitor thiostrepton synergizes with key therapies targeting the FOXM1/PLK1 signaling axis. A. SUDHL5 and MZ cells were treated with a FOXM1 inhibitor thiostrepton (T; 0.5 μM), p38 inhibitor BIRB-796 (B; 25 μM), or the combination of both (T+B) for 24 h, protein extracts were used to detect for p-p38, p38, and actin (loading control) by Western blotting. B. Four-day CellTiter-Glo viability assays were performed in SUDHL5 and MZ cells treated with the FOXM1 inhibitor thiostrepton (T; 0.62 μM), p38 inhibitor BIRB-796 (B; 25 μM), or the combination of both. C. SUDHL5 and MZ cells were treated with thiostrepton (T; 0.5 μM), PLK1 inhibitor volasertib (V; 100 nM), or the combination of both (T+V) for 24 h, protein extracts were used to detect for FOXM1, PLK1, and actin (loading control) by Western blotting. D. Four-day CellTiter-Glo viability assays were performed in SUDHL5 and MZ cells treated with the FOXM1 inhibitor thiostrepton, PLK1 inhibitor volasertib, or the combination of both, at varying doses.

FOXM1 or PLK1 lead to G2/M cell cycle arrest, potentially through the downregulation of both CDK1 and Cyclin B1, two molecules that are part of the FOXM1/PLK1 protein signature. The

FOXM1-mediated S/G2/M cell cycle transition could also be controlled by the CHK1/ATR damage response pathway [23]. The central role of FOXM1 in DNA repair is underscored by the observation that increased DNA breaks were found in FOXM1-deficient cells [24]. Our studies showed that the FOXM1/PLK1 protein signature contains the DNA damage response proteins, including CHK1 and ATR, and that inhibition of FOXM1 activates the DNA damage marker pH2AX. These findings suggest that in DLBCL cells, an intrinsic S/G2 checkpoint is enforced by the DNA damage response pathway. This checkpoint could prevent a cellular "identity crisis" in which the S and G2 phases overlap, which would cause underreplication, early mitosis, and subsequent DNA damage. Given the frequent overexpression of FOXM1 in cancer, deregulation of this fundamental cell cycle transition could be a common event contributing to cancer genome instability.

In addition to cell cycle progression, the FOXM1/PLK1 axis could also play a role in chemoresistance in DLBCL since the mRNA levels of FOXM1 and PLK1 were associated with poor clinical response. The mechanism of chemoresistance could be due to the association of FOXM1 and PLK1 with p53 status, as their protein expression levels were highly correlated with p53 mutation in representative DLBCL cell lines (data not shown). As a major tumor suppressor, p53 is genetically altered in approximately 20%-25% of DLBCL, and cases with deregulated p53 often relapse or are refractory to front-line therapies [25]. *FOXM1* has been shown to be transcriptionally repressed by wild-type p53 [26] and was activated in its protein form via phosphorylation by the MEK-ERK pathway [27], suggesting that inactivated *TP53* and/or amplifications of the MEK-ERK pathway also have a role in promoting FOXM1 activity. However, in the absence of functional p53, genotoxic stress will lead to induction of FOXM1 expression through ATM and E2F1 [28]. Furthermore, the genotoxic agent epirubicin has been shown to induce *FOXM1* transcription via E2F1 activating the p38 MAPK-MK2 signaling axis [29]. FOXM1/PLK1 overexpression, as well as p53 mutation status, could be potential biomarkers predicting response to therapy targeting the FOXM1/PLK1 axis in DLBCL. Altogether, our study highlights the importance of the deregulated FOXM1/PLK1 signaling axis in DLBCL and shows that targeting this axis could have therapeutic potential in DLBCL. In addition, FOXM1 and PLK1 expression levels could represent potential biomarkers for risk stratification of DLBCL patients undergoing treatments with standard-of-care chemotherapies or targeted therapies such as FOXM1/PLK1 inhibitors.

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Disclosure of conflict of interest

None.

Abbreviations

DLBCL, Diffuse Large B Cell Lymphoma; GCB, Germinal Center B Cell-Like; NHL, Non-Hodgkin Lymphoma; R-CHOP, Rituximab Plus Cyclophosphamide, Doxorubicin, Vincristine, And Prednisone; FOXM1, Forkhead Box Protein M1; PLK1, Polo-Like Kinase 1; SDS, Sodium Dodecyl Sulfate; RPPA, Reverse-Phase Protein Array.

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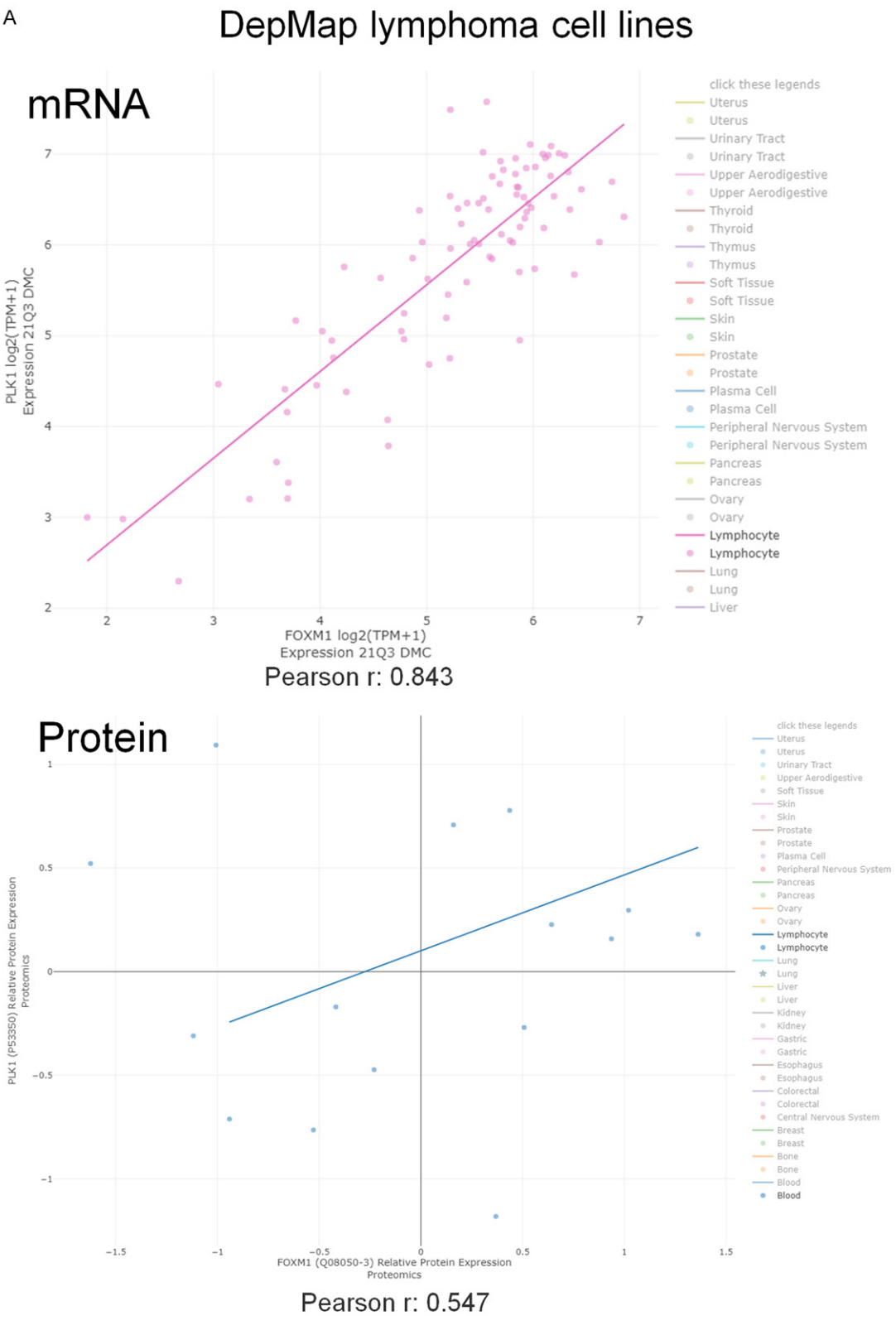
DepMap lymphoma cell lines
mRNA
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FOXM1 log2(TPM+1) Expression 21Q3 DMC
Pearson r: 0.843
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Uterus
Urinary Tract
Upper Aerodigestive
Thyroid
Thymus
Soft Tissue
Skin
Prostate
Plasma Cell
Peripheral Nervous System
Pancreas
Ovary
Lymphocyte
Lung
Liver
Protein
PLK1 (P53350) Relative Protein Expression Proteomics
FOXM1 (Q08050-3) Relative Protein Expression Proteomics
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Central Nervous System
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Blood

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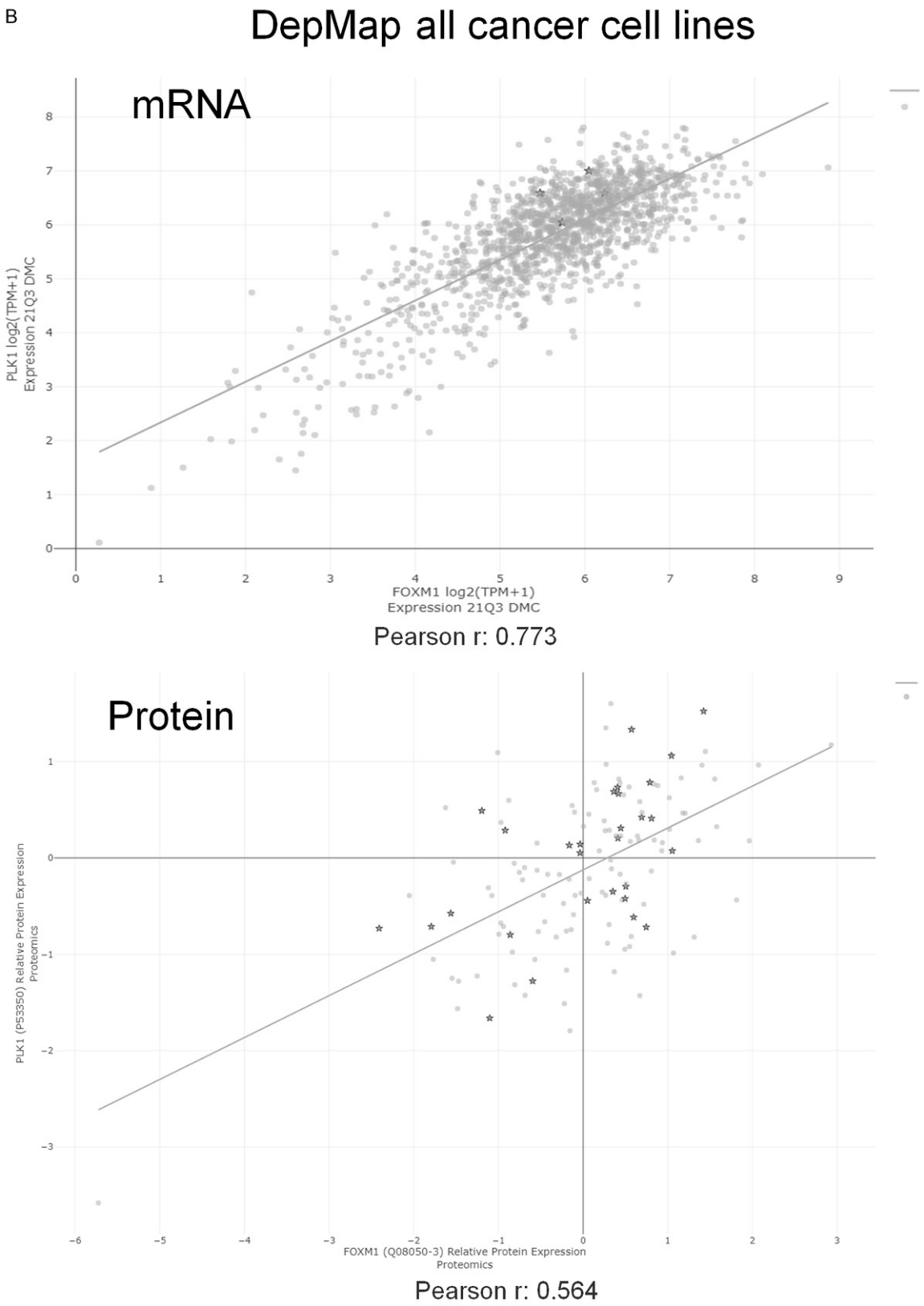

Supplementary Figure 1. Positive *FOXM1* and *PLK1* mRNA expression in DepMap cancer cell lines. A. Linear regression analysis of *FOXM1/PLK1* mRNA expression in all lymphoma cell lines found in the DepMap Consortium (DMC) portal. B. Linear regression analysis of FOXM1/PLK1 mRNA expression in all other cancer cell lines, including solid tumors found in the DMC portal.

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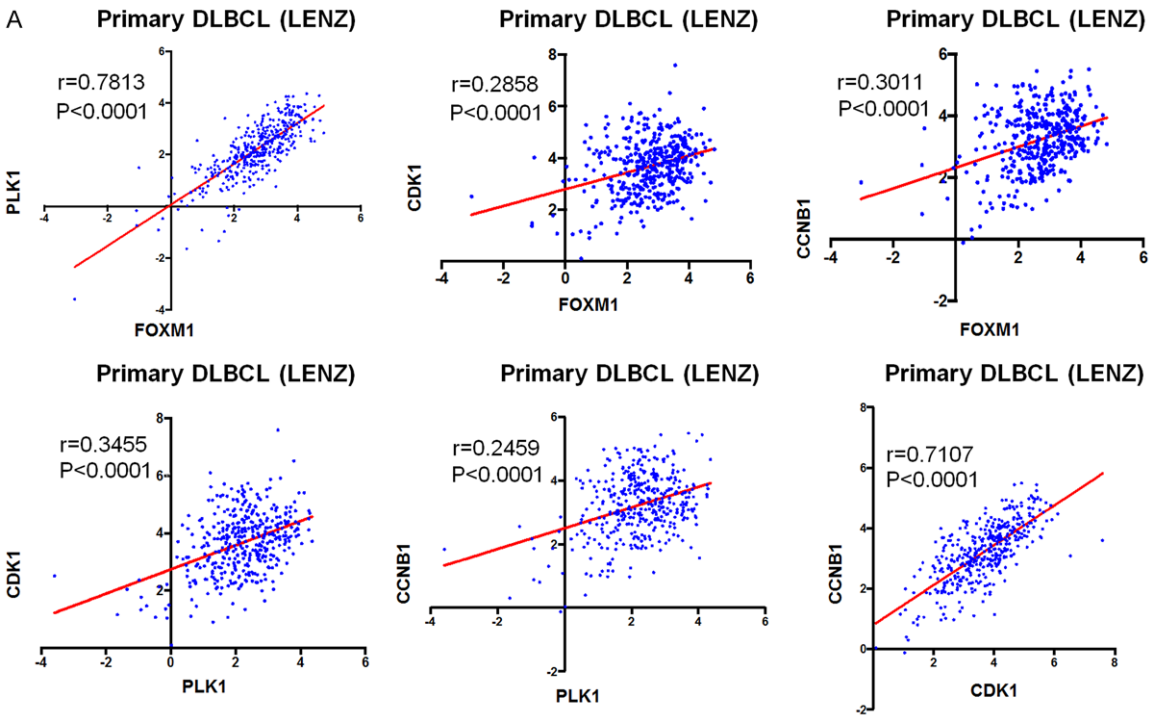


B

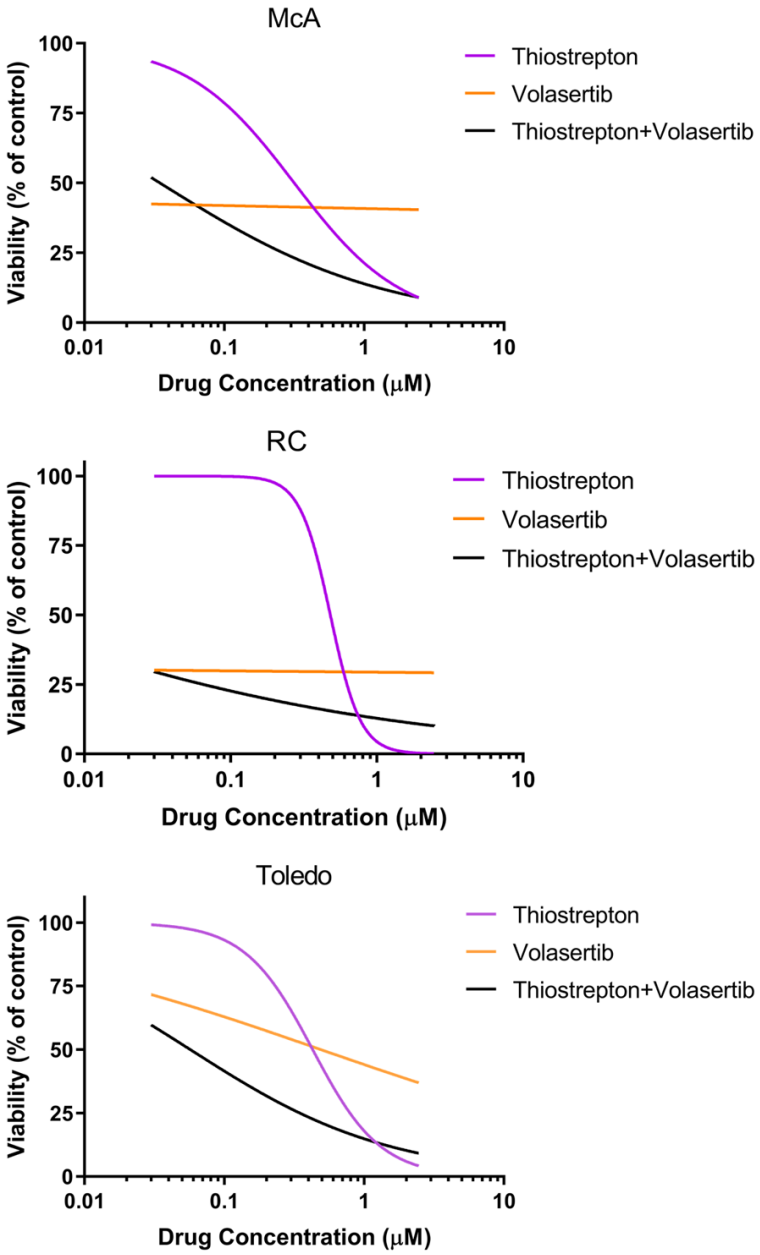
Inhibitors	Protein Inhibited	r value	P value
Volasertib	PLK1	-0.4659	0.0032
BIRB-796	p38 MAPK	-0.3992	0.0159
MK8776	CHK1	-0.3568	0.0279
AZD-6738	ATR	-0.3321	0.0729

Supplementary Figure 2. FOXM1 is a good predictive biomarker for therapies targeting FOXM1-associated signaling pathways. A. Linear regression analysis of FOXM1/PLK1 and cell cycle-related mRNA expression in primary DLBCL cases from Lenz et al. B. Linear regression analysis of FOXM1 protein expression and drug sensitivity to PLK1 inhibitor (volasertib), p38 MAPK inhibitor (BIRB-796), CHK1 inhibitor (MK8876), and ATR inhibitor (AZD-6738).

Supplementary Table 1. Drug sensitivity and the IC50 in DLBCL cell lines

Cell line	Subtype	Thiostrepton IC50 (μM)	BIRB-796 IC50 (μM)	MK8876 IC50 (μM)	AZD6738 IC50 (μM)	Volasertib IC50 (μM)
HB	ABC	1.7	44.31	110.74	7.45	100
HBL-1	ABC	0.55	169.3	99.38	31.96	6.88
LP	ABC	1.45	55.46	1.63	0.83	0.01
LR	ABC	0.27	13.95	12.68	2.9	0.91
OCI-LY10	ABC	1.22	68.19	18.73	47.88	9.11
OCI-LY3	ABC	0.75	270.05	17.11	2.06	2.41
TMD-8	ABC	0.88	31.44	12.83	0.86	0.69
WP	ABC	0.96	243.45	5.83	68.55	0.06
CJ	GCB	0.51	82.57	8.39	100	8.07
EJ	GCB	2.08	38.59	16.76	4.38	0
RC	GCB	0.37	38.37	11.41	9.01	0.99
U2932	ABC	1.12	182.81	0.1	51.25	49.95
WILL-2	GCB	0.88	46.64	3.2	0.43	0.24
VAL	GCB	0.65	80.66	3.54	1.05	3.23
BJAB	GCB	2	152.6	6.73	6.52	0.93
DB	GCB	0.58	60.16	57.32	2.5	9.61
DBR	GCB	0.51	141.3	38.71	15.48	11.01
DS	GCB	0.71	133.9	125.8	4.81	84.27
FN	GCB	0.25	77.96	23.08	3.09	0.01
GR	GCB	0.28	45.82	1.29	0.5	3.73
GR-VR	GCB	0.74	24.36	10	7.59	5.35
HF	GCB	0.33	37.44	14.12	4.3	2.63
HT	GCB	1.77	150	15.69	10.66	77
JZ	GCB	0.43	25.14	5.84	1.25	11.63
OCI-LY19	GCB	0.84	61.94	17.15	0.01	0
MCA	GCB	0.32	53.09	5.79	2.97	0.47
MS	GCB	1.17	70.27	29.92	18.63	0.01
MZ	GCB	0.34	14.17	11.79	1.63	0.08
Pfeiffer	GCB	1.71	102.3	5.96	15.16	3.87
RC-VR	GCB	0.42	22.59	22.48	90.32	6.56
SUDHL-10	GCB	0.87	28.85	130.26	2.37	45.18
SUDHL-4	GCB	0.68	29.76	13.74	5.44	1.25
SUDHL-5	GCB	0.15	9.87	10.26	0.01	0.21
SUDHL-6	GCB	0.42	18.9	8.31	0.01	3.16
TJ	GCB	1.13	27.85	1.12	0.89	0.02
Toledo	GCB	0.35	38.32	20.82	14.83	2.14
WILL-1	GCB	0.75	36.2	4.81	0.29	1.5
WSU-NHL	GCB	0.28	25.12	9.24	6.58	0.07

Representative DLBCL cell lines (n=38) were used to assess drug sensitivity. The IC50 values of FOXM1 inhibitor thiostrepton, PLK1 inhibitor volasertib, p38 MAPK inhibitor BIRB-796, CHK1 inhibitor MK8876, and ATR inhibitor AZD-6738 were calculated based on 4-day CellTiter-Glo assay.



Supplementary Figure 3. FOXM1 inhibitor thiostrepton synergizes with PLK1 inhibitor volasertib in DLBCL cells. Four-day CellTiter-Glo viability assays were assessed in McA, RC, and Toledo cells treated with FOXM1 inhibitor thiostrepton, PLK1 inhibitor volasertib, or the combination of both.