Response and resistance to CDK12 inhibition in aggressive B-cell lymphomas

Jing Gao,[1,*] Michelle Y. Wang,[1,*] Yuan Ren,[1] Tint Lwin,[1] Tao Li,[1] Joy C. Yan,[1] Eduardo M. Sotomayor,[2] Derek R. Duckett,[1] Bijal D. Shah,[3] Kenneth H. Shain,[3] Xiaohong Zhao[1] and Jianguo Tao[4]

[1]Chemical Biology and Molecular Medicine Program, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL; [2]Cancer Institute, Tampa General Hospital,Tampa, FL; [3]Departments of Malignant Hematology, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL and [4](Previous affiliation) Department of Hematopathology and Laboratory Medicine, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL, USA

**JG and MYW contributed equally as co-first authors.*

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Correspondence: *JIANGUO TAO* - dtep642021@gmail.com

Supplementary Information for Manuscript "Response and resistance to CDK12 inhibition in aggressive B-cell lymphomas"

Summary:

This supplement contains the Materials and Methods section as well as five supplementary figures with accompanying figure legends related to the six main figures submitted with this manuscript.

This PDF file contains:

Materials and Methods

Materials

Cell Lines

Mantle cell lymphoma (MCL) cell lines Z138, Jeko-1, Maver-1, HBL-2 and REC-1 and diffuse large B-cell lymphoma (DLBCL) cell line KARPAS-422 were purchased from ATCC. Double hit lymphoma (DHL) cell line DOHH2 (ACC47) and DLBCL cell line Val (ACC586) were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig. Germany). These cells and their THZ531-resistant derivatives were cultured in RPMI-1640 (Gibco-Invitrogen) with penicillin (100 U/mL) and streptomycin (100μg/mL) and maintained at 37°C in 5% CO2. Cell lines were routinely tested for Mycoplasma using the Universal Mycoplasma Detection Kit from ATCC and were confirmed for identity using STR analyses by the Molecular Genomics Core of the Moffitt Cancer Center.

Study Approval

The human specimen studies presented were approved by the Moffitt/University of South Florida Institutional Review Board and patients provided signed informed consent forms.

Antibodies

For Western blotting, the following antibodies were used: MYC (Abcam, catalog ab32072), β-actin (Santa Cruz Biotechnology Inc., catalog sc-47778HRP), RNAPII CTD phospho-Ser2 (Millipore, 04-1571-I), RNAPII CTD phospho-Ser5 (Millipore, 04-1572-I), RNAPII CTD phospho-Ser7 (Millipore, 04-1570-I), RNAPII CTD (Cell Signaling Technology, catalog 2629), p-p70S6K (Ser371) (Cell Signaling Technology, catalog 9208), p70S6K (Cell Signaling Technology, catalog 2708), p-4EBP1 (Thr37/46) (Cell Signaling Technology, catalog 2855), 4EBP1 (Cell Signaling Technology, catalog 9644), MCL-1 (Cell Signaling Technology, catalog 94296), BCL-2 (Cell Signaling Technology, catalog 2872), BCL-XL (Cell Signaling Technology, catalog 2762), cleaved PARP (Cell Signaling Technology, catalog 5625) and MDR1/ABCB1 (Cell Signaling Technology, catalog 12683).

Small Molecule Inhibitors

The following kinase and epigenetic inhibitors used in drug screening and cell-based drug screening assays: ABT-199 (Selleckchem, S8048), ABT263 (Selleckchem, S1001), A-1331852 (Selleckchem, S7801), ACP-196 (Selleckchem, S8116), Alisertib (Selleckchem, S1133), AZD7762 (Selleckchem, S1532), AZD8055 (Selleckchem, S1555), BEZ235 (Selleckchem, S1009), Bortezomib (Selleckchem, S1013), Carfizomib (Selleckchem, S2853), Dinaciclib (Selleckchem, S2768), Ibrutinib (Selleckchem, S2680), lenalidomide (Selleckchem, S1029), Lumpib (Selleckchem, S1069), MK-1775 (Selleckchem, S1525), Olaparib (Selleckchem, S1060), PIK-75 (Selleckchem, S1205), R406 (Selleckchem, S2194), SCH-772984 (Selleckchem, S7101), Trametinib

(Selleckchem, S2673), VE-821 (Selleckchem, S8007), Volasertib (Selleckchem, S2235), NVP2 (MedChemExpress, HY-12214A), Silvestrol (MedChemExpress, HY-13251), THZ1 (MedChemExpress, HY-80013), THZ531 (MedChemExpress, HY-103618), S63845 (ApexBio, A8737), INCB054329 and INCB059872 from Incyte Corporation (Wilmington, DE), A-366 (Cayman Chemical, 16081), BAY-598 (Cayman Chemical, 18238), BI-9564 (Cayman Chemical, 17897), GSK343 (Cayman Chemical, 14094), GSK484 (Cayman Chemical, 17488), GSK591 (Cayman Chemical, 18354), GSK864 (Cayman Chemical, 18762), GSK-J4 (Cayman Chemical, 12073), GSK-LSD1 (Cayman Chemical, 16439), I-CBP112 (Cayman Chemical, 14468), JQ1 (Cayman Chemical, 11187), MS049 (Cayman Chemical, 18348), OICR9429 (Cayman Chemical, 16095), PFI-2 (Cayman Chemical, 18119), PFI-3 (Cayman Chemical, 15267), PFI-4 (Cayman Chemical, 17663), SGC-1946 (Cayman Chemical, 13967), SGC707 (Cayman Chemical, 17017), UNC0642 (Cayman Chemical, 14604), UNC1215 (Cayman Chemical, 1398), UNC1999 (Cayman Chemical, 14621). Agents were dissolved in DMSO to a final concentration of 10 mM, aliquoted, and then stored at –20°C.

Methods

Generation of THZ531 Resistant Cell Lines

To establish the THZ531 resistant cell lines, DLBCL and MCL cells (KARPAS-422 and Maver-1) were grown in RPMI-1640 medium with 10% FBS and treated with vehicle control or escalating doses of THZ531 for approximately 3 months. Before exposing

cells to THZ531, cells were maintained in suspension. Trypan blue staining and CCK8 viability assay in suspension cells were used to determine viability every 2-3 weeks and THZ531 concentrations were increased if the viability of cells was >65%. If cell viability was <65%, populations were expanded in suspension in drug-free medium and then re-exposed to the same concentration of THZ531. After ~90 days, drug-resistant variants emerged, and IC50s were determined every two weeks. Following another 30 days of selection, stable variants emerged referred to as THZ-R. Drug-resistant variants were maintained in drug free medium for 3 days before being used in experiments. Cells exposed to DMSO were maintained in parallel and used for comparison with their respective drug-resistant cell lines. We used at least 10 × IC50 (ranging from 10 to 100 fold) as the threshold to define resistance.

Colony Formation Assays

For the colony formation assay, 2×10^3 HBL-2 parental and MCL1-overexpression cells (0.05ml) were added to 0.5ml MethoCult® (STEMCELL, 4034) per well in 24-well plates. Cells were treated with DMSO or THZ531 at either 50nM or 500nM. After 2 weeks in culture, the pictures of the colonies were taken.

MCL-1 Overexpression and EZH2 knockdown

Exogenous MCL-1 was overexpressed in HBL-2 cells by lentiviral transduction. Lentiviral particles were generated by transient transfection of HEK-293T cells with plenti-MCL-1 constructs and the packaging vectors pMD2.G (AddGene, 12259) and

psPAX2 (AddGene, 12260). 48 hours after transfection, culture supernatants were harvested and filtered. Cells were infected with the virus-containing supernatant and 8μg/mL polybrene. Three days after infection, cells were selected with 0.5μg/mL puromycin. Puromycin resistant cells were picked up and characterized by western blot to determine if MCL-1 was significantly overexpressed after transduction and selection.

Lentiviral particles were generated by transfection of HEK-293T cells with pLKO-shEZH2 constructs and the packaging vectors pMD2.G (AddGene, 12259) and psPAX2 (AddGene, 12260). 48 hours after transfection, culture supernatants containing virus were harvested and filtered. Cells were infected with the concentrated viral supernatants and 8μg/mL polybrene. For stable knockdown, 3 days after viral infection, cells were selected in 0.5μg/mL puromycin. Puromycin resistant cells were picked up and characterized by western blot to determine if EZH2 was significantly reduced after transduction and selection. The shRNA target sequences EZH2 were as follows: EZH2-sh-ATATTGCCTTCTCACCAGCTGC[1].

Cell Viability Assays

5000 cells were seeded in 96-well plates in 50μl medium. 50μl drug medium at five or nine serial diluted concentrations was added to the cell suspension in each well. Each condition on these plates was seeded in triplicate. After a 72-hour incubation, 20ul of Resazurin reagent (R&D Systems, AR002) was added into each well. After a 2-hour incubation at 37°C in 5% CO_2, the plates were read using 560/590 nm wavelengths to estimate cell proliferation. Relative cell viability was normalized to DMSO treated wells.

High-Throughput Small-Molecule Drug Screens

Using a semi-automated platform, we tested the potency of a 60 small molecule annotated library in cell lines. Cell viability was estimated by using Resazurin. In brief, cells were seeded in 384-well plates with 2,000 cells per well in 90μL medium. Cells were cultured in the presence of different compounds at serial threefold diluted concentrations, with each condition having duplicate wells. After 3 (kinase inhibitors) or 6 (epigenetic inhibitors) days of treatment, 10μL of Resazurin reagent was added into each well and incubated for at 37°C in 5% CO_2 for 2 hours. Plates were read at 560/590 nm wavelength to estimate cell proliferation.

cDNA Synthesis and Quantitative PCR

Total RNA was prepared as described above. cDNA was synthesized using the iScript cDNA Synthesis kit (BioRad Cat# 1708890) according to instructions of the manufacturer. Quantitative real-time PCR (qRT-PCR) was performed using SYBR™ Green PCR Master Mix (ThermoFisher Scientific) for indicated target genes and human β-actin was used as internal sample probe to correlate for inter-assay variability. Relative quantification of expression levels was performed according to the comparative threshold cycle (Ct) method assuming equal efficiency of target and housekeeping gene probes. Primer sequences: β-actin (Forward: 5'-CCTGTACGCCAACACAGTGC; Reverse: 5'-ATACTCCTGCTTGCTGATCC), MDR1(Forward: 5'-CAGGAACCTGTATTGTTTGCCACCAC-3'; Reverse: 5'-TGCTTCTGCCCACCACTCAACTG-3).

Gene Set Enrichment Analysis (GSEA)

Gene set enrichment analysis (GSEA) was performed as described[2]. The most differentially expressed genes ranked by Log2 fold change for each comparison were used to generate a signature for GSEA analysis. GSEA estimates if genes are specific to either parental or resistant cells, indicating they are associated with a specific phenotype, rather than being distributed uniformly or randomly across the list. An enrichment score (ES) is calculated to quantify the degree to which a gene set is over-represented at the top or bottom of the entire ranked list. After calculation of the scores for a collection of gene sets, an empirical phenotype-based permutation test procedure is used to estimate p values. GSEA normalizes the ES for each gene set to account for the variation in set sizes, yielding a normalized enrichment score (NES) and a false discovery rate (FDR). The FDR gives an estimate of the probability that a set with a given NES represents a false positive finding; it is computed by comparing the tails of the observed and permutation-computed null distributions for the NES.

Determination of P-gp Activity

The activity of P-gp was determined by a fluorimetric MDR Assay kit (Abcam, Cambridge, UK). Following the user protocol provided in the fluorometric MDR assay kit, cells (1.5×10^5 cells/well) were seeded into 96-well flat clear-bottom black-wall microplates and were treated with GSK343 (10μM and 30μM), UNC1999 (10μM and 30μM) and 0.1μM Tariquidar respectively for 12 hours. The P-gp substrate verapamil (30μM) was used as positive control. Then, 100μL of MDR dye-loading solution was

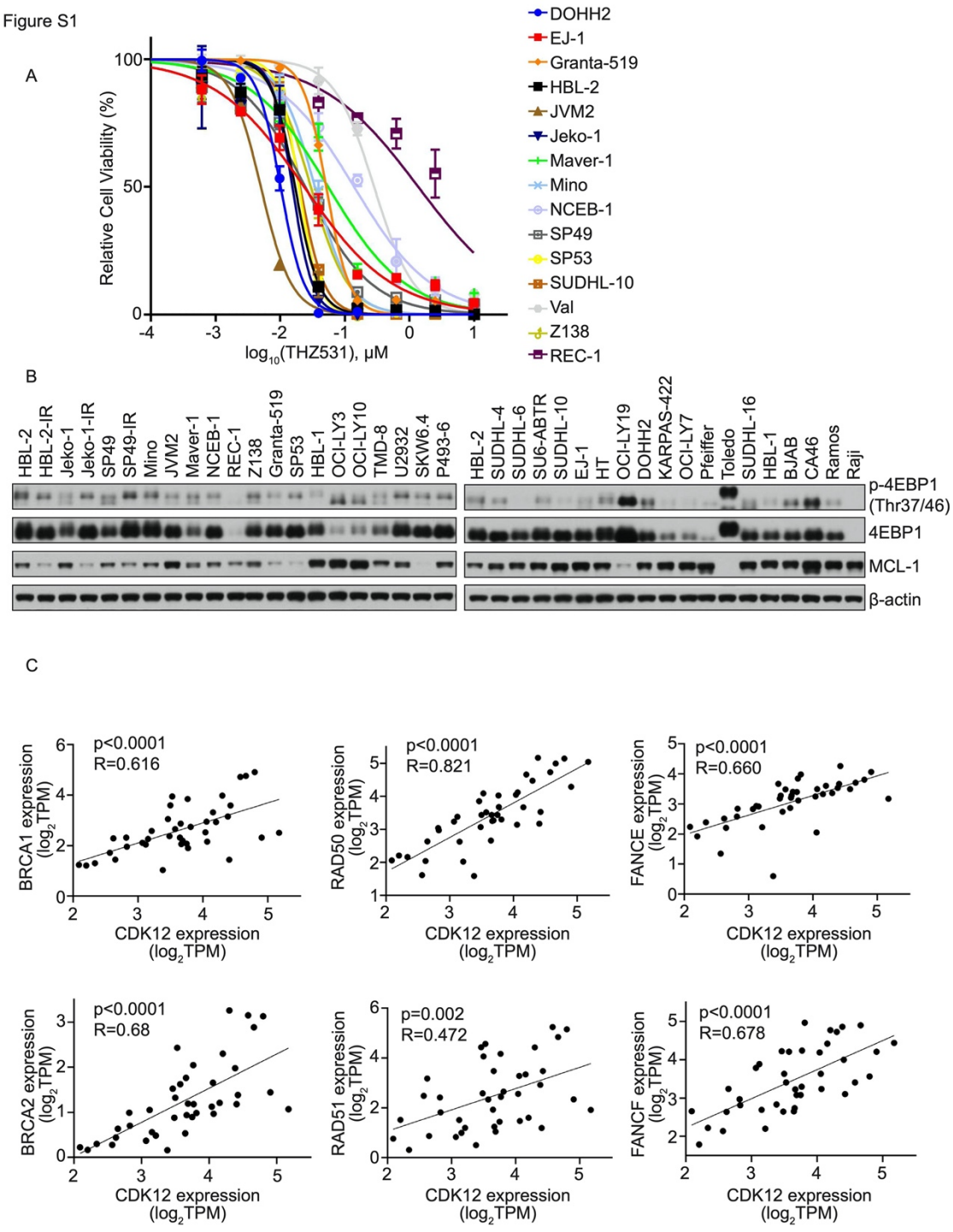
added to each well and the plate was incubated at room temperature for another hour isolated from light. Intracellular fluorescence was detected using a microplate reader (SpectraMax M5, Molecular Devices, USA) at an excitation wavelength of 490 nm and an emission wavelength of 525 nm. All experiments were performed in triplicate and compared to controls.

References

1 Xu K, Wu ZJ, Groner AC et al. EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycomb-independent. Science. 2012;338(6113):465-1469.

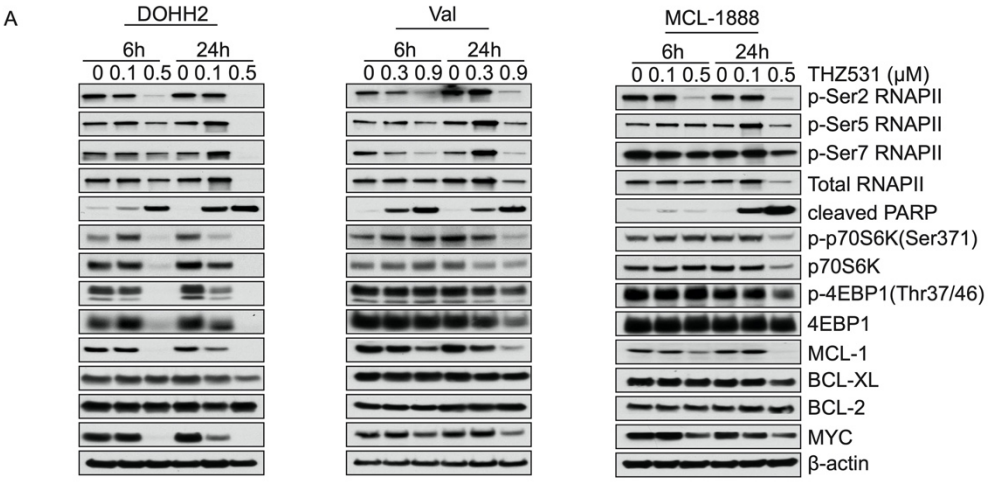
2 Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 2005;102(43):15545-15550.

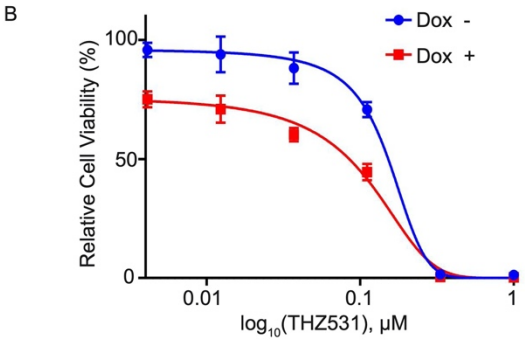
Figure S1

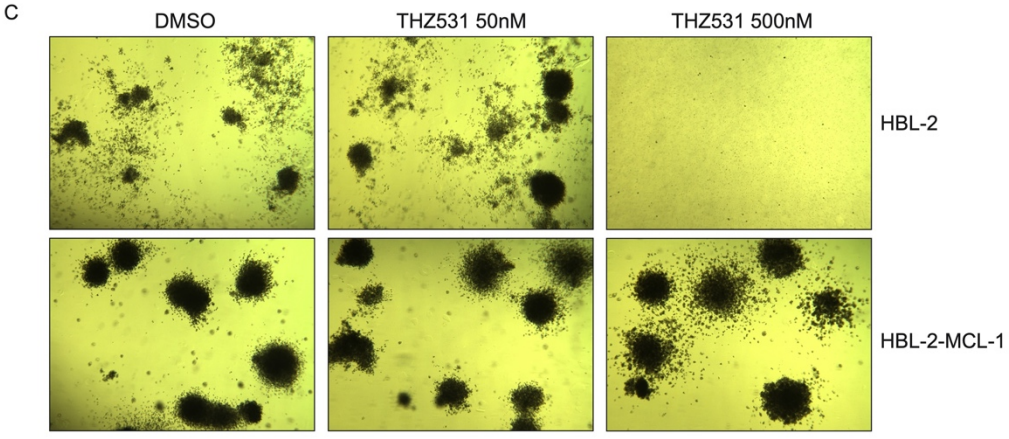

A
Relative Cell Viability (%)
100
50
0
-4
-3
-2
-1
0
1
log10(THZ531), μM
DOHH2
EJ-1
Granta-519
HBL-2
JVM2
Jeko-1
Maver-1
Mino
NCEB-1
SP49
SP53
SUDHL-10
Val
Z138
REC-1
B
HBL-2
HBL-2-IR
Jeko-1
Jeko-1-IR
SP49
SP49-IR
Mino
JVM2
Maver-1
NCEB-1
REC-1
Z138
Granta-519
SP53
HBL-1
OCI-LY3
OCI-LY10
TMD-8
U2932
SKW6.4
P493-6
HBL-2
SUDHL-4
SUDHL-6
SU6-ABTR
SUDHL-10
EJ-1
HT
OCI-LY19
DOHH2
KARPAS-422
OCI-LY7
Pfeiffer
Toledo
SUDHL-16
HBL-1
BJAB
CA46
Ramos
Raji
p-4EBP1 (Thr37/46)
4EBP1
MCL-1
β-actin
C
BRCA1 expression (log2TPM)
p<0.0001
R=0.616
RAD50 expression (log2TPM)
p<0.0001
R=0.821
FANCE expression (log2TPM)
p<0.0001
R=0.660
BRCA2 expression (log2TPM)
p<0.0001
R=0.68
RAD51 expression (log2TPM)
p=0.002
R=0.472
FANCF expression (log2TPM)
p<0.0001
R=0.678
CDK12 expression (log2TPM)

Supplementary Figure 1 (Related to Figure 1): Mantle cell lymphoma (MCL) and other B-cell lymphoma cell lines and primary samples are exquisitely sensitive to CDK12 inhibition with THZ531, regardless of genetic background and drug resistance status. A. Dose-response curves of MCL cell lines treated with THZ531 for 72 hours. Data is shown as mean ± SD, n = 3 technical replicates for each cell line. **B.** Western blot analysis of selected proteins in B-cell lymphoma cell lines. p-4EBP1: phosphorylated 4EBP1. **C.** Correlation of BRCA1 (top, left), BRCA2 (bottom, left), RAD50 (top, middle), RAD51 (bottom, middle), FANCE (top, right) and FANCF (bottom, right) gene expression (log_2TPM) with CDK12 gene expression (log_2TPM) in primary patient samples. Data shown in **A** is representative of at least 3 independent experiments.

Figure S2

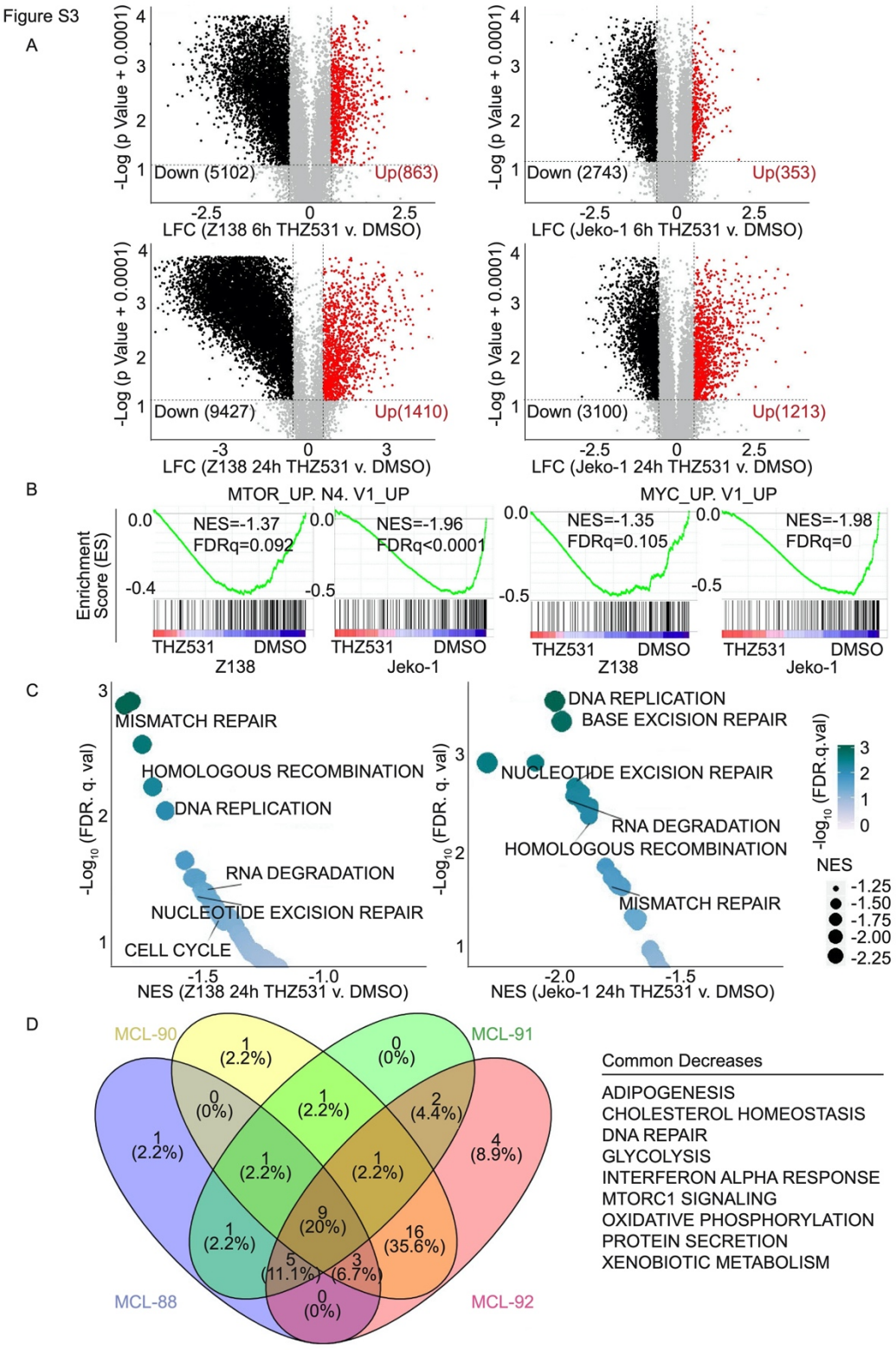

A
DOHH2
Val
MCL-1888
6h
24h
0 0.1 0.5 0 0.1 0.5
0 0.3 0.9 0 0.3 0.9
THZ531 (μM)
p-Ser2 RNAPII
p-Ser5 RNAPII
p-Ser7 RNAPII
Total RNAPII
cleaved PARP
p-p70S6K(Ser371)
p70S6K
p-4EBP1(Thr37/46)
4EBP1
MCL-1
BCL-XL
BCL-2
MYC
β-actin


B
Dox -
Dox +
Relative Cell Viability (%)
100
50
0
0.01
0.1
1
log10(THZ531), μM


C
DMSO
THZ531 50nM
THZ531 500nM
HBL-2
HBL-2-MCL-1

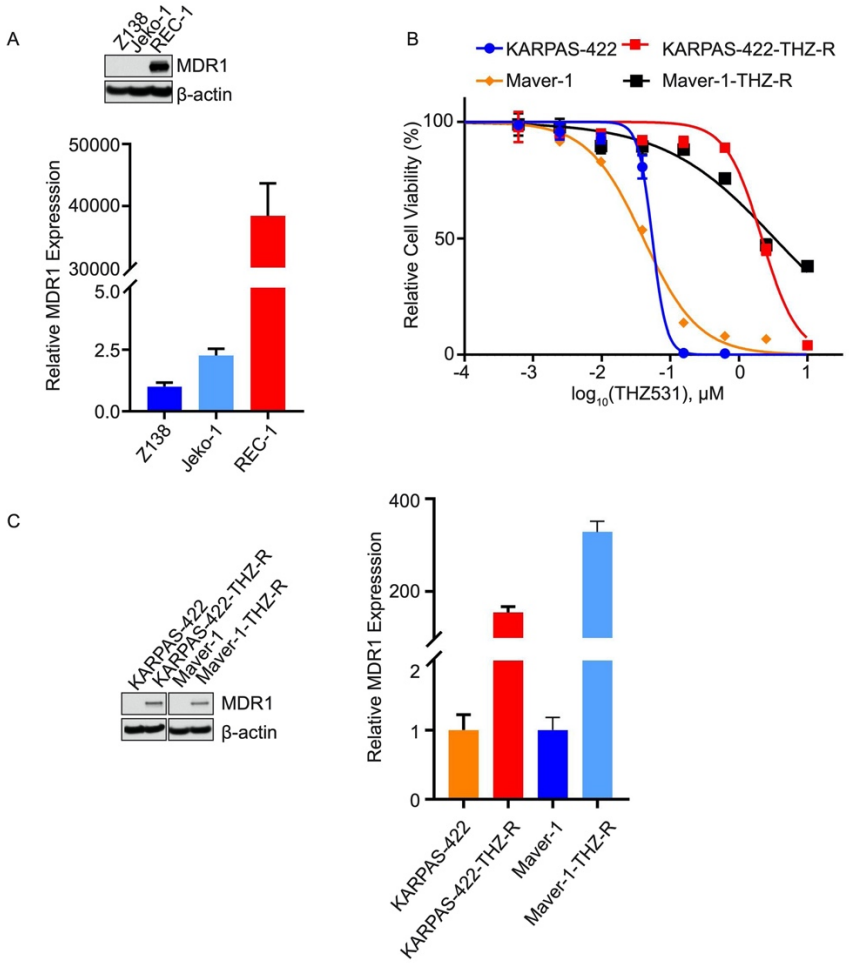
Supplementary Figure 2 (Related to Figure 2): CDK12 sustains cell growth and survival through transcriptional activation of MYC and the mTOR-4EBP1-MCL-1 axis in MCL and MYC-associated B-cell lymphomas. A. Western blot analysis of phosphorylation of RNAPII in Ser2, 5 and 7, RNAPII, cleaved PARP, phosphor-p70S6K, p70S6K, phosphor-4EBP1, 4EBP1, MCL-1, BCL-XL, BCL-2 and MYC in THZ531 sensitive cell lines and MCL primary sample treated with the indicated doses of THZ531 at different time points. **B**. Dose response curves of HBL-2 cells with and without doxycycline-induced overexpression of 4EBP1 after 72-hour treatment with THZ531. **C.** Clonogenic growth assay images of HBL-2 cells with and without MCL-1 overexpression treated with DMSO as vehicle control or THZ531 (50nM and 500nM, respectively) for 2 weeks. Data shown in **B** is representative of at least 3 independent experiments.

Figure S3


A
-Log (p Value + 0.0001)
Down (5102)
Up(863)
LFC (Z138 6h THZ531 v. DMSO)
Down (2743)
Up(353)
LFC (Jeko-1 6h THZ531 v. DMSO)
Down (9427)
Up(1410)
LFC (Z138 24h THZ531 v. DMSO)
Down (3100)
Up(1213)
LFC (Jeko-1 24h THZ531 v. DMSO)
B
MTOR_UP. N4. V1_UP
MYC_UP. V1_UP
Enrichment Score (ES)
NES=-1.37
FDRq=0.092
NES=-1.96
FDRq<0.0001
NES=-1.35
FDRq=0.105
NES=-1.98
FDRq=0
THZ531
DMSO
Z138
Jeko-1
C
-Log10 (FDR. q. val)
MISMATCH REPAIR
HOMOLOGOUS RECOMBINATION
DNA REPLICATION
RNA DEGRADATION
NUCLEOTIDE EXCISION REPAIR
CELL CYCLE
NES (Z138 24h THZ531 v. DMSO)
BASE EXCISION REPAIR
NES (Jeko-1 24h THZ531 v. DMSO)
-log10 (FDR.q.val)
NES
-1.25
-1.50
-1.75
-2.00
-2.25
D
MCL-90
MCL-91
MCL-88
MCL-92
1 (2.2%)
0 (0%)
0 (0%)
1 (2.2%)
2 (4.4%)
1 (2.2%)
4 (8.9%)
1 (2.2%)
1 (2.2%)
1 (2.2%)
9 (20%)
1 (2.2%)
16 (35.6%)
5 (11.1%)
3 (6.7%)
0 (0%)
Common Decreases
ADIPOGENESIS
CHOLESTEROL HOMEOSTASIS
DNA REPAIR
GLYCOLYSIS
INTERFERON ALPHA RESPONSE
MTORC1 SIGNALING
OXIDATIVE PHOSPHORYLATION
PROTEIN SECRETION
XENOBIOTIC METABOLISM

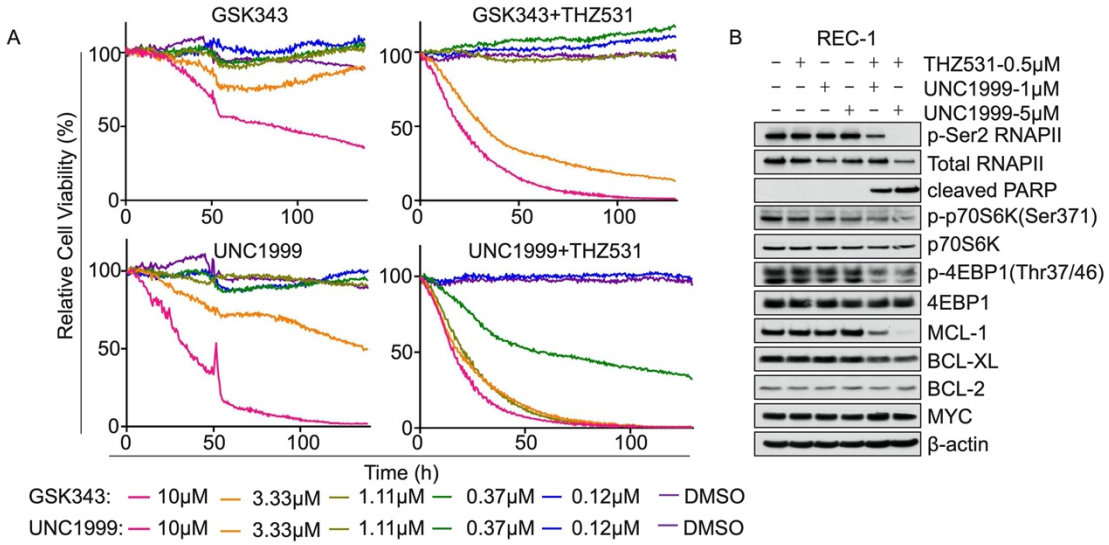
Supplementary Figure 3 (Related to Figure 3): Transcriptomic analysis of THZ531 sensitive cells. A. Volcano plots of differentially expressed genes in THZ531 treatment for 6 hours (top) and 24 hours (bottom) versus DMSO in Z138 (left) and Jeko-1 (right). Red: Upregulated genes, black: downregulated genes. LFC: log2 fold change cut-off of log2 (1.5), P-value cut-off of 0.05. n = 3 biologically independent samples. **B.** Enrichment score plots of pathways from Oncogenic Signatures gene set from MSigDB (C6) significantly negatively enriched in both Z138 and Jeko-1 after THZ531 treatment. **C.** Scatterplot of gene set enrichment analysis (GSEA) normalized enrichment scores (NES) of negatively enriched pathways in KEGG gene set from MSigDB (C2) in Z138 (left) and Jeko-1 (right) cells treated with THZ531 for 24 hours. Genes were ranked according to their expression fold change after treatment. FDR: false discovery rate. **D.** Venn diagram of significantly negatively enriched GSEA Hallmarks gene sets (NES < 0, FDR < 0.25) in MCL primary patient samples treated with 100nM THZ531 for 12 hours. Commonly negatively enriched pathways between all four samples are listed.

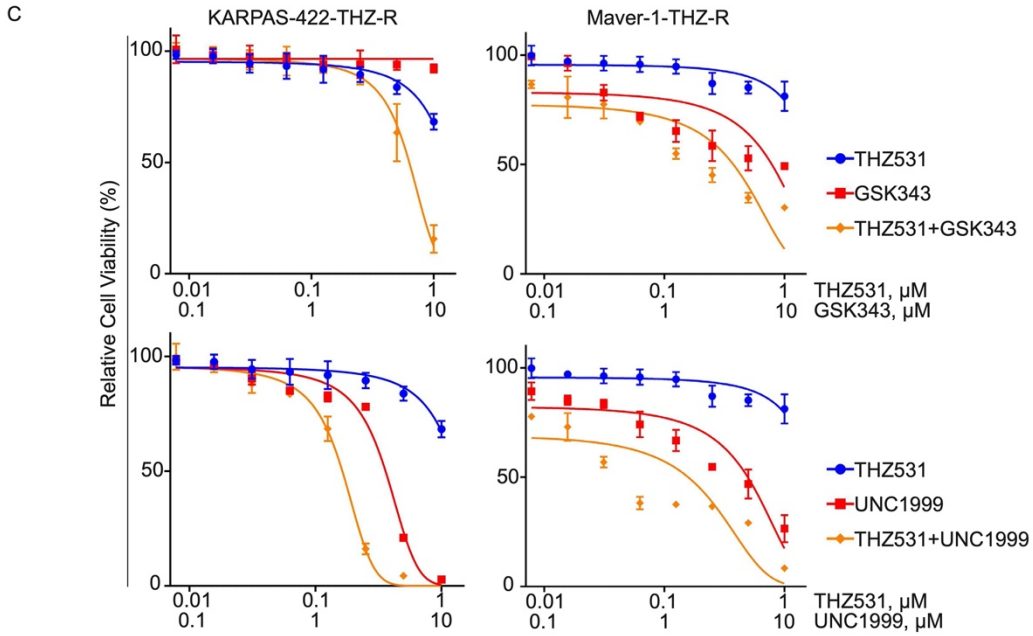
Figure S4

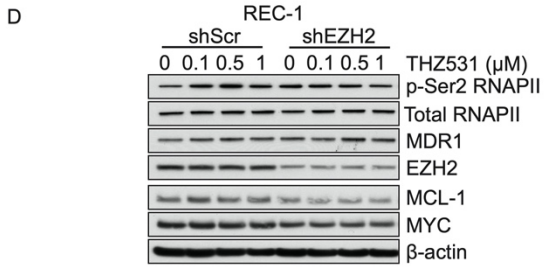


Supplementary Figure 4 (Related to Figure 4): MDR1 upregulation drives THZ531 resistance in MCL and other aggressive B-cell lymphomas. A. Western blot and qRT-PCR analysis of MDR1 expression in THZ531 sensitive (Z138 and Jeko-1) and resistant (REC-1) cell lines **B.** Dose-response curves of KARPAS-422 and Maver-1 parental and THZ-R cell lines after 72-hour treatment with THZ531. Data is shown as mean ± SD, n = 3 technical replicates for each cell line. **C.** Western blot and qRT-PCR analysis of MDR expression in KARPAS-422 and Maver-1 parental and THZ-R cell lines. Data shown in **B** is representative of at least 3 independent experiments.

Figure S5


A
GSK343
GSK343+THZ531
UNC1999
UNC1999+THZ531
Relative Cell Viability (%)
Time (h)
GSK343: 10μM 3.33μM 1.11μM 0.37μM 0.12μM DMSO
UNC1999: 10μM 3.33μM 1.11μM 0.37μM 0.12μM DMSO
B
REC-1
THZ531-0.5μM
UNC1999-1μM
UNC1999-5μM
p-Ser2 RNAPII
Total RNAPII
cleaved PARP
p-p70S6K(Ser371)
p70S6K
p-4EBP1(Thr37/46)
4EBP1
MCL-1
BCL-XL
BCL-2
MYC
β-actin


C
KARPAS-422-THZ-R
Maver-1-THZ-R
Relative Cell Viability (%)
THZ531
GSK343
THZ531+GSK343
THZ531, μM
GSK343, μM
THZ531
UNC1999
THZ531+UNC1999
THZ531, μM
UNC1999, μM


D
REC-1
shScr
shEZH2
0 0.1 0.5 1 0 0.1 0.5 1
THZ531 (μM)
p-Ser2 RNAPII
Total RNAPII
MDR1
EZH2
MCL-1
MYC
β-actin

Supplementary Figure 5 (Related to Figure 5): EZH2 inhibitors restored sensitivity to THZ531 in THZ531-resistant cells by competing with THZ531 for MDR1. A. Image-based cell-viability assays of REC-1 cells in response to treatment with GSK343 or UNC1999 (left) and treatment with GSK343 or UNC1999 combined with 500nM THZ531 (Right). 1 x 10^6 cells were seeded in a 384-well plate with extracellular matrix and lymphoma stromal cells. GSK343 or UNC1999 at five serial diluted concentrations was added to the medium, and plate was continuously imaged every 30 min for 96 hours. All images were analyzed using a digital imaging analysis algorithm to detect cell viability based on membrane motion, and changes in viability were quantified by area under curve (AUC). **B.** Western blot analysis of phosphorylation of RNAPII in Ser2, RNAPII, cleaved PARP, phosphor-p70S6K, p70S6K, phosphor-4EBP1, 4EBP1, MCL-1, BCL-XL, BCL-2 and MYC protein expression in REC-1 cells after 24-hour treatment with DMSO or indicated doses of THZ531 and/or UNC1999. **C.** Top: Dose-response curves of KARPAS-422-THZ-R (left) and Maver-1-THZ-R (right) cells after 72-hour treatment with THZ531, GSK343, or THZ531+GSK343 at different doses. Bottom: Dose-response curves of the KARPAS-422-THZ-R (left) and Maver-1-THZ-R (right) cells after 72-hour treatment with THZ531, UNC1999, or THZ531+UNC1999 at different doses. Data is shown as mean ± SD, n = 3 technical replicates for each cell line. **D.** Western blot analysis of phosphorylation of RNAPII in Ser2, RNAPII, MDR1, EZH2, MCL-1, and MYC protein expression in REC-1 scramble control and EZH2 knockdown cells after 24-hour treatment with the indicated doses of THZ531. Data shown in **C** is representative of at least 3 independent experiments.