

## Specific interactions of BCL-2 family proteins mediate sensitivity to BH3-mimetics in diffuse large B-cell lymphoma

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## **Supplementary Methods**

### **Immunoprecipitation and flow cytometry to detect active BAX and BAK**

For analysis of BAX and BAK activation, immunoprecipitation was performed with antibodies (mouse anti-BAX clone 6A7, Sigma; mouse anti-BAK Ab-1, Calbiochem) that recognize an N-terminal epitope which is usually buried within the protein and only becomes accessible for antibody binding upon the conformational change associated with activation of BAX and BAK. CHAPS-containing lysates were incubated with 2  $\mu$ l of antibody and 10  $\mu$ l of anti-mouse IgG Dynabeads (Invitrogen) overnight before washing of the precipitate and analysis of bound proteins by Western blotting. To quantify the amount of cells with active BAX or BAK, cells were fixed in 2% paraformaldehyde followed by gentle permeabilization in 0.1% saponin and staining with mouse anti-BAX (BD Biosciences, Cat 610983) or mouse anti-BAK Ab-1. Staining was visualized by addition of anti-mouse-IgG-PE and flow cytometry.

### **Knockdown of BMF**

For silencing of individual genes cells were electroporated using Neon transfection system (ThermoFisher) using two pulses of 20 ms at 1200 V. The following silencer select siRNAs (ThermoFisher) were used at 100 nM: BMF (#1 s40385, #2 s40386, #3 s40387).

### **TMRM staining**

For analysis of mitochondrial membrane potential, cells were stained with 50 nM tetramethylrhodamine methyl ester (TMRM) for 10 min at 37°C before analysis by flow cytometry.

### **Statistical analysis**

The half-maximal response ( $EC_{50}$ ) to BH3-mimetics was calculated in GraphPad Prism using a sigmoidal dose response curve (unequal variance). Correlation analysis of drug or BH3-profiling responses and protein expression was done in GraphPad Prism using linear regression analysis. For statistical significance, student's t-test or One-way ANOVA was used.

## Supplementary Figure Legends

### Supplementary Figure 1. Response of DLBCL cells to BH3-mimetics

A-B) DLBCL cell lines were exposed to different concentrations of A1155463 (A) or A1210477 (B) before analysis of cell viability using CellTiterGlo at 72h. Data shown are mean and S.D. (n=4-6).

### Supplementary Figure 2. Quantification of BCL-2 protein levels

A) Protein expression relative to GAPDH loading control was quantified using Licor Image Studio Lite from 5 independent Western blots. Heatmap was created using Microsoft Excel to visualize high (red) or low (green) protein expression. B) Genetic alterations of *BCL2* in this panel of DLBCL cell lines as outlined in Table 1 (mt) are associated with higher BCL-2 protein expression as compared to the expression in wildtype (wt) cells without genetic alteration of BCL-2. Scatter blot displays individual values and mean plus S.D. \* $p < 0.05$ . C) Genetic alterations of *BCL2* in this panel of DLBCL cell lines are not significantly associated with sensitivity to ABT-199. Scatter blot displays individual values and mean and S.D. (n.s.; not significant).

### Supplementary Figure 3. Correlation of BCL-2 protein expression and response to BH3-mimetics

BCL-2 protein expression relative to GAPDH loading control was correlated with the EC<sub>50</sub> values for ABT-199 (A), A1331852 (B) or S63845 (C). Sensitivity to ABT-199 correlated with expression of BCL-2 and the ratio of BCL-2 versus MCL-1 or combined BCL-X<sub>L</sub> and MCL-1 expression. B) Sensitivity to A1331852 did not significantly correlate with the expression of BCL-X<sub>L</sub> but the ratio of BCL-X<sub>L</sub> versus combined BCL-2 and MCL-1 expression. C) Sensitivity to S63845 did not significantly correlate with the expression of MCL-1 but did inversely correlate with BCL-X<sub>L</sub> expression or the ratio of MCL-1 to BIM expression (n.s.; not significant).

### Supplementary Figure 4. Molecular mechanism of BH3-mimetic induced cell death

A) DLBCL cells were exposed to BH3-mimetics (RIVA: 3 nM ABT-199, RCK8: 3 nM A1331852, SUDHL10: 100 nM S63845) for 2, 4, or 8h before analysis of caspase-3 cleavage by Western blotting. GAPDH was used as loading control. Representative Western blots of 3 independent experiments are shown. B) DLBCL cells were exposed to BH3-mimetics (RIVA: 3 nM ABT-199, RCK8: 3 nM A1331852, SUDHL10: 100 nM S63845) with or without the caspase inhibitor zVAD.fmk (50 μM) for 24h before analysis of apoptosis by staining with AnnexinV-FITC and flow cytometry. Mean and S.D. are shown (n=3). C) DLBCL cells were exposed to BH3-mimetics (RIVA: 3 nM ABT-199, RCK8: 3 nM A1331852, SUDHL10: 100 nM S63845) for the indicated time points before analysis of mitochondrial membrane potential using staining with TMRE and flow cytometry. Mean and S.D. are shown (n=3).

### Supplementary Figure 5. BAX and BAK activation in DLBCL

A-C). BAX and BAK conformational change was detected upon treatment of cells with ABT-199 (A) in RIVA (3 nM) or U2932 (10 nM) cells, upon treatment with A1331852 (B) in RCK8 (3 nM) or SUDHL8 (10 nM) cells, or upon treatment with S63845 (C) in SUDHL10 (100 nM) or U2946 (300 nM) for 4h. Input lanes show presence of overall BAX and BAK in the CHAPS-containing lysates. Immunoprecipitation (IP) samples show the presence of conformationally active BAX or BAK, which was detected using IP with active conformation specific antibodies. D) Conformationally active BAK was assessed in untreated CHAPS-

containing lysates of 18 DLBCL cells lines. Input blot (lower panel) shows presence of overall BAK in the CHAPS lysates. BAK Immunoprecipitation (IP) blot (upper panel) shows the presence of conformationally active BAK, which was detected using IP with active conformation specific antibody. RIVA, U2932 and OCI-LY1 input lysate was loaded in the first three lanes as control on the BAK IP blot. GAPDH is shown as loading control.

#### **Supplementary Figure 6. Silencing of BIM using distinct siRNA oligonucleotides has little effect on apoptosis**

A-C) Depletion of BIM using three distinct siRNAs (#1,2,3) was performed before treatment with ABT-199 (A) in RIVA (3 nM) or U2932 (10 nM) cells, upon treatment with A1331852 (B) in RCK8 (3 nM) or SUDHL8 (10 nM) cells, or upon treatment with S63845 (C) in SUDHL10 (100 nM) or U2946 (300 nM) for 4h and analysis of PS-exposure by staining with AnnexinV-FITC and flow cytometry. Untransfected cells and non-targeting siRNA (siCtrl) were used as controls. Mean and S.D. are shown (n=3-4). \*p< 0.05;\*\*p< 0.01; \*\*\*p< 0.001. D). Knockdown efficiency was controlled by Western blotting at 48h after transfection.

#### **Supplementary Figure 7. BMF does not play a role in ABT-199 induced cell death**

A) Depletion of BMF using siRNA mediated gene silencing was performed in RIVA cells before treatment with 3 nM ABT-199 and analysis of PS-externalization. Mean and S.D. are shown (n=3). B) Knockdown efficiency was assessed by Western blotting. GAPDH is shown as loading control.

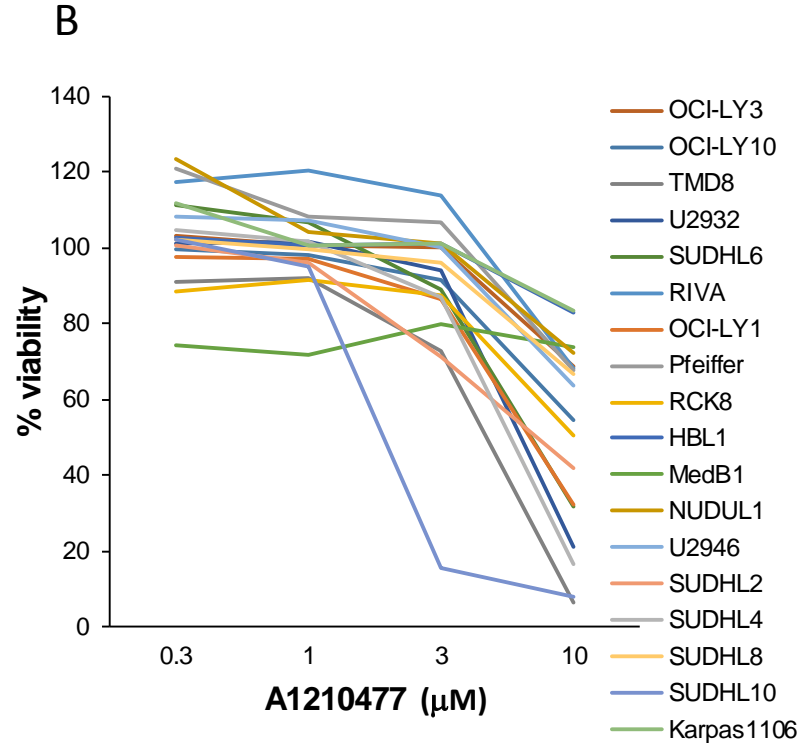
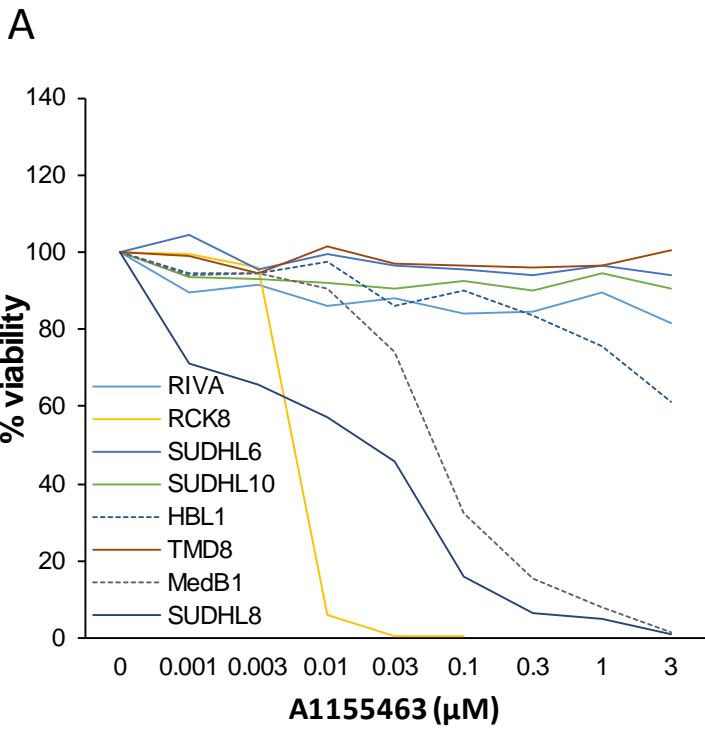
#### **Supplementary Figure 8. Knockdown of BAK is only able to prevent ABT-199 induced apoptosis, but does not inhibit A1331852 or S63845 induced apoptosis.**

A-D) Depletion of BAK using siRNA mediated gene silencing was performed before treatment with ABT-199 (A) in RIVA (3 nM) or U2932 (10 nM) cells, upon treatment with A1331852 (B) in RCK8 (3 nM) or SUDHL8 (10 nM) cells, or upon treatment with S63845 (C) in SUDHL10 (100 nM) or U2946 (300 nM) cells for 4h and analysis of PS-exposure by staining with AnnexinV-FITC and flow cytometry. Untransfected cells and non-targeting siRNA (siCtrl) were used as controls. Mean and S.D. are shown (n=3-4). \*p< 0.05;\*\*p< 0.01; \*\*\*p< 0.001. D) Knockdown efficiency was assessed by Western blotting. \*p< 0.05;\*\*p< 0.01; \*\*\*p< 0.001.

#### **Supplementary Figure 9. Knockout of BAK using CRISPR/Cas9 has little effect on A1331852 induced apoptosis.**

A) Deletion of BAK was performed in SUDHL8 using CRISPR/Cas9. Cells were transduced with pLentiCRISPRv2 either carrying non-human target (NHT) control gRNAs or BAK gRNAs (BAK) followed by selection of a stable clone with BAK deletion. Western blotting was performed to confirm deletion of BAK. B) NHT or BAK deleted cells were exposed to different concentrations of A1331852 for 4h before analysis of PS-exposure by staining with AnnexinV-FITC and flow cytometry. Mean and S.D. are shown (n=3).C) NHT or BAK deleted cells were treated with 100 nM A1331852 for 4h before analysis of BAX activation using intracellular staining with an active conformation specific BAX antibody and flow cytometry. Mean and S.D. are shown (n=3). \*p< 0.05;\*\*p< 0.01; \*\*\*p< 0.001.

# Supplementary Figure 1

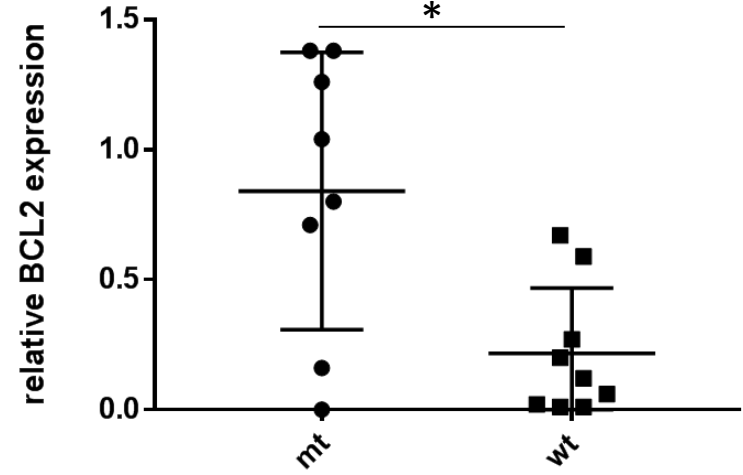


# Supplementary Figure 2

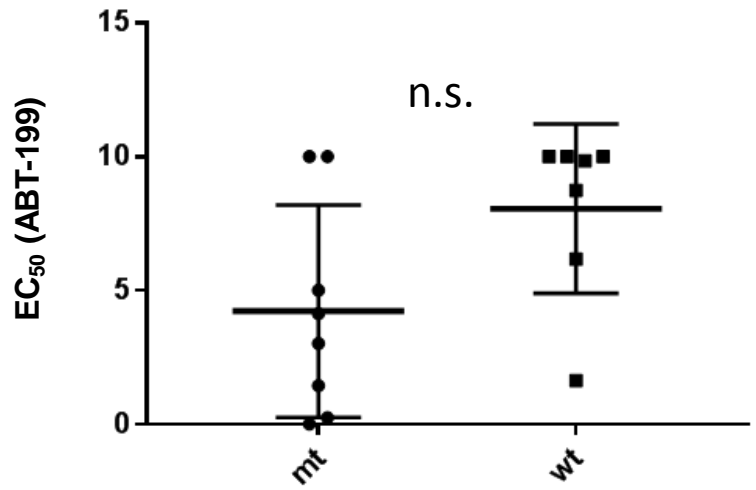
A

	BCL-2	BCL-XL	MCL-1	BCL2A1	BAX	BAK	BIM	NOXA	BMF	PUMA
RIVA	1.4	0.4	0.3	0.1	0.2	0.3	2.9	0.3	1.1	0.2
U2932	1.4	0.1	1.1	0.3	0.3	0.4	2.4	1.2	0.1	0.1
OCI-LY1	1.3	0.4	0.4	0.0	0.5	0.5	3.0	0.1	0.6	0.1
SUDHL4	0.7	0.2	0.2	0.1	0.3	0.3	1.5	0.1	0.0	0.0
OCI-LY10	0.9	0.1	0.5	0.4	0.8	0.4	0.1	0.5	0.0	0.5
RCK8	0.1	1.7	0.3	0.3	0.7	0.5	0.6	0.5	0.0	0.1
SUDHL8	0.0	1.1	0.3	0.0	0.3	0.3	1.9	0.1	0.0	0.0
MedB1	0.3	1.2	0.4	0.0	0.3	0.5	0.0	1.1	0.0	0.1
SUDHL6	0.8	0.0	0.6	0.2	0.5	0.6	0.7	0.3	0.5	0.0
TMD8	0.6	0.4	0.5	0.8	0.7	0.7	0.3	0.0	0.1	0.4
NUDUL1	0.1	0.4	0.6	0.0	0.4	0.3	0.8	0.3	0.0	0.0
U2946	0.0	0.1	1.4	0.1	0.4	0.3	2.6	0.5	0.0	0.0
SUDHL10	0.0	0.0	0.7	0.1	0.1	0.2	0.9	0.4	0.0	0.0
Karpas1106	0.0	0.7	0.6	0.4	0.4	0.6	1.2	0.0	0.0	0.0
SUDHL2	0.2	0.2	0.5	1.3	0.1	0.7	2.1	0.4	0.1	0.1
HBL1	0.7	0.7	0.3	0.2	0.5	0.3	2.0	0.3	0.6	0.0
OCI-LY3	1.0	0.4	1.0	0.9	0.6	0.7	2.5	0.5	0.6	0.4
Pfeiffer	0.2	0.8	0.5	1.0	0.2	0.3	3.5	0.0	0.0	0.1

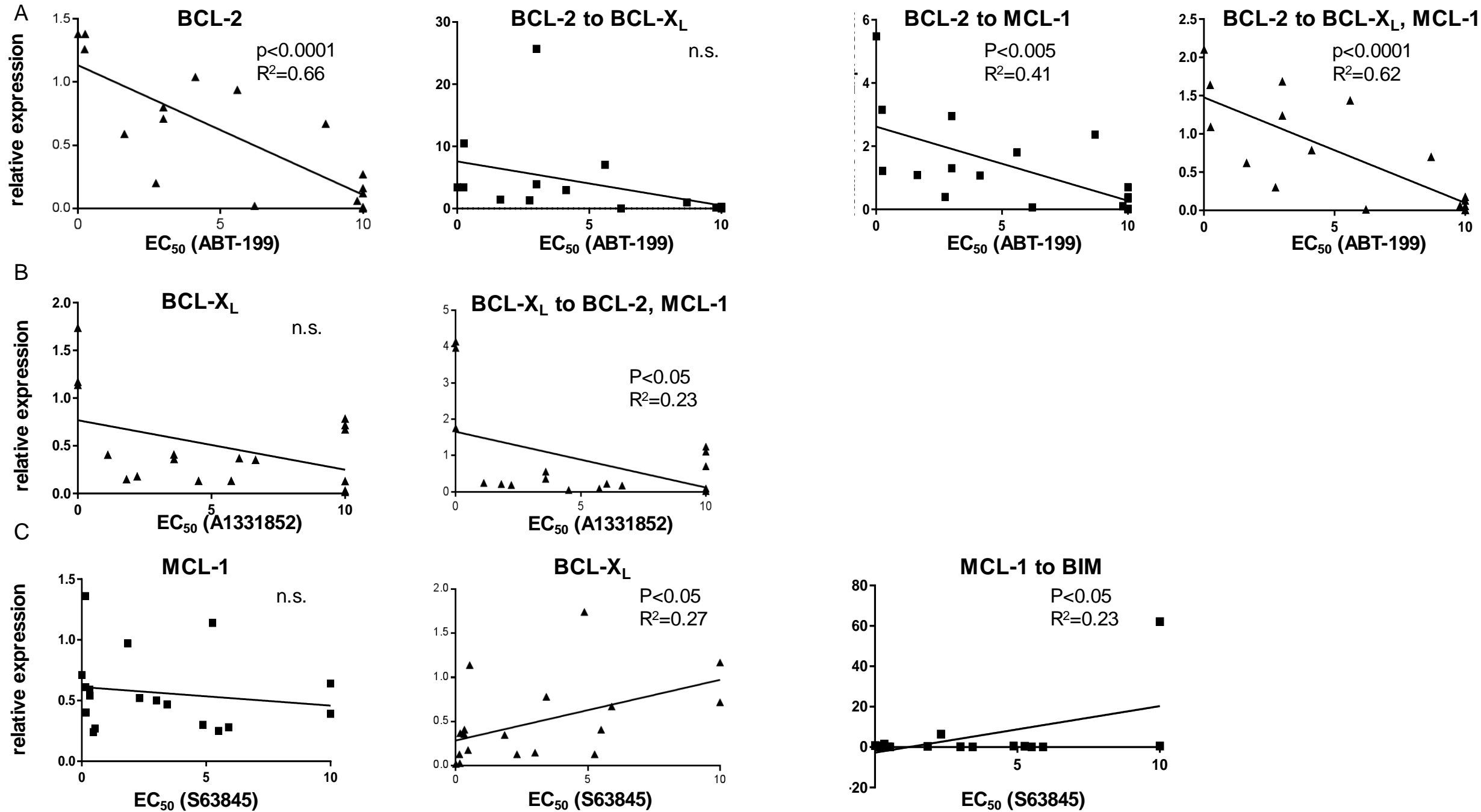
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C

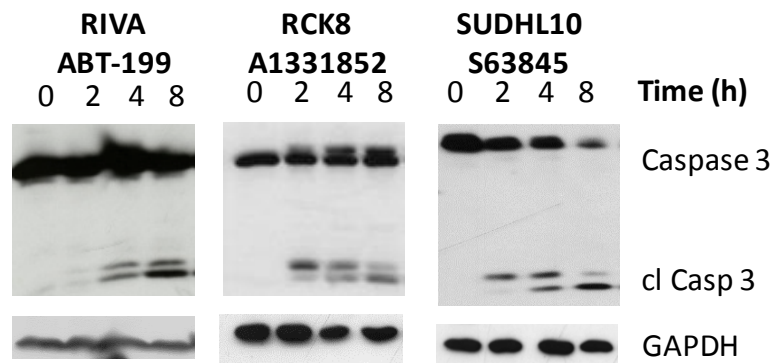


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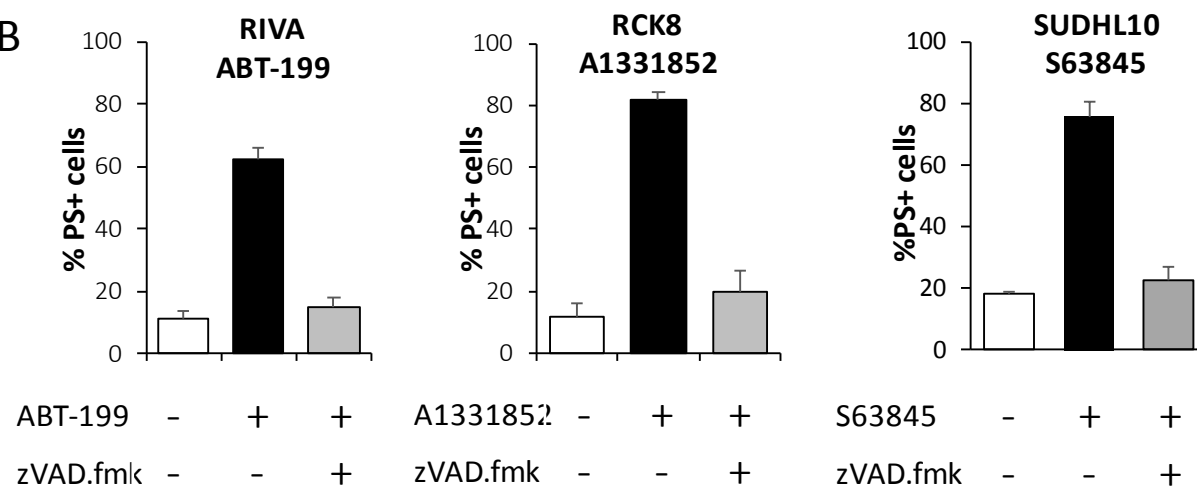


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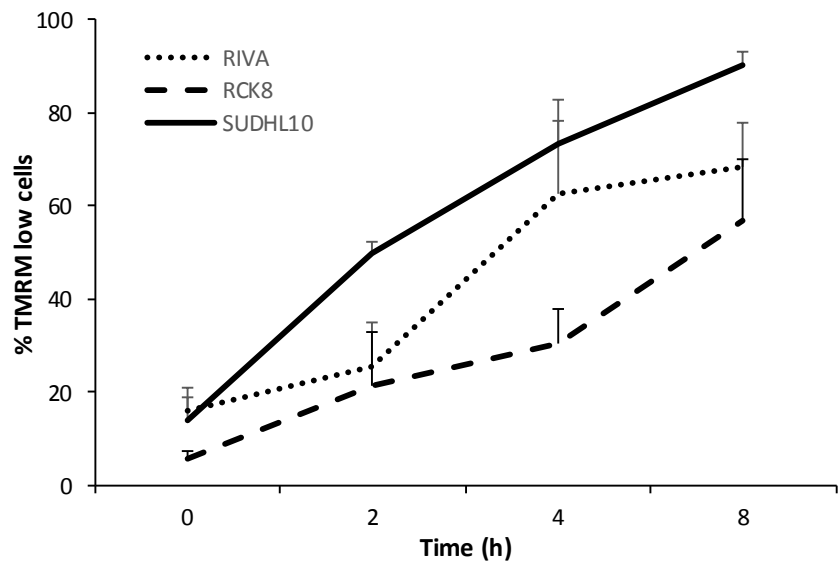
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**B**

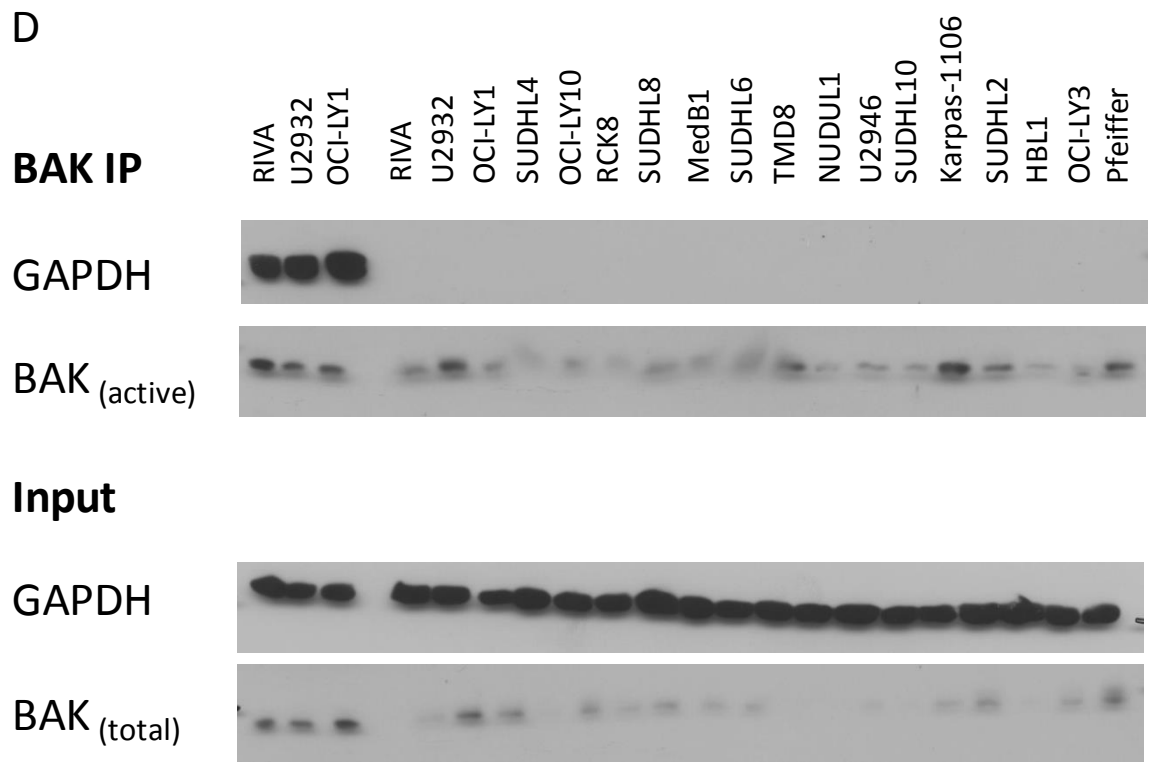
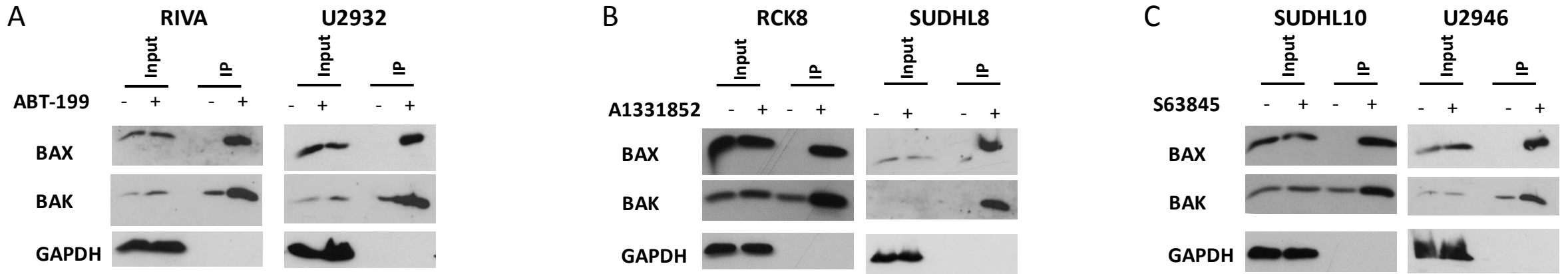


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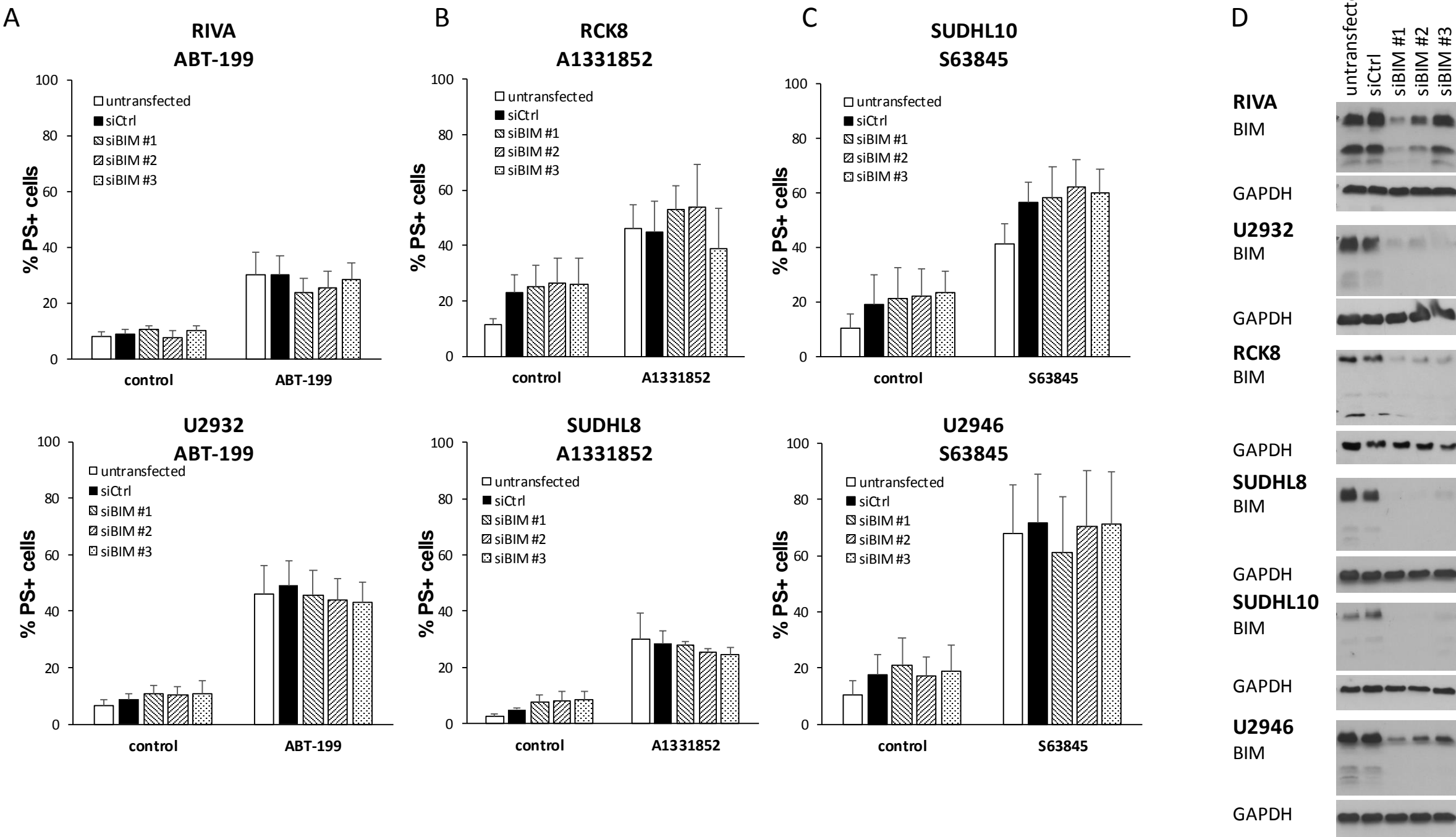




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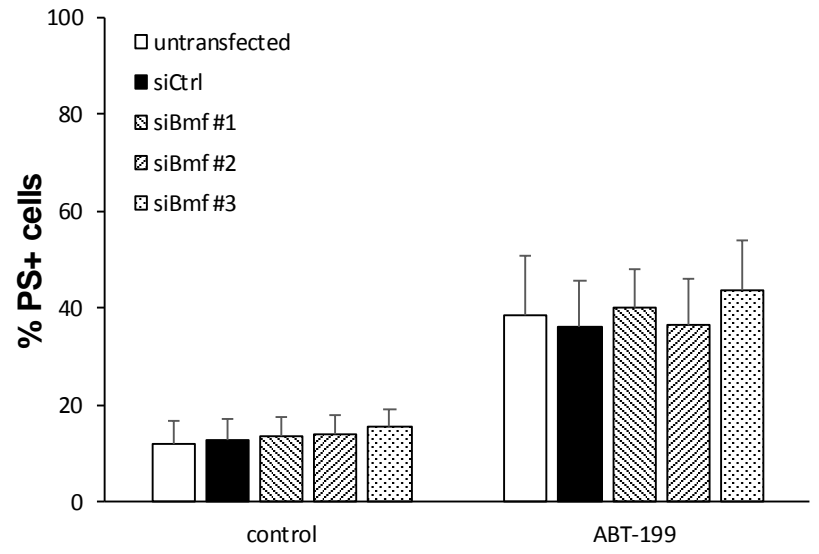


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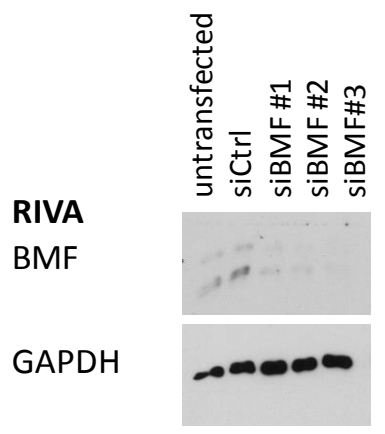


# Supplementary Figure 7

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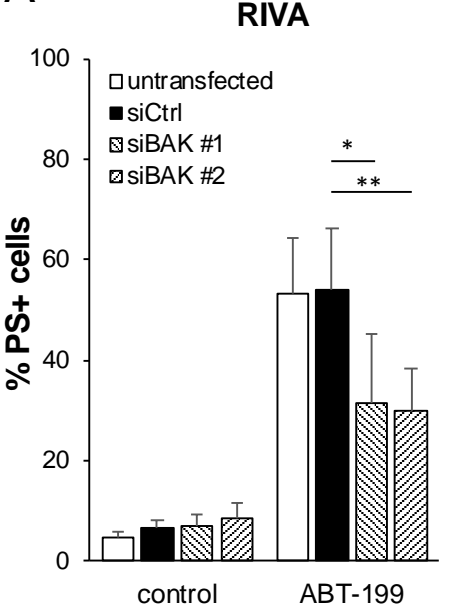


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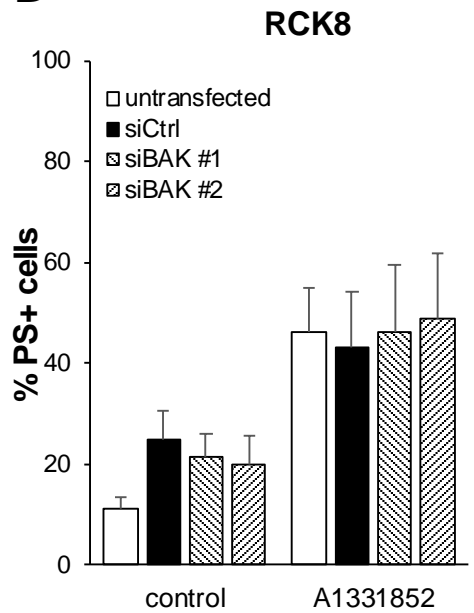


# Supplementary Figure 8

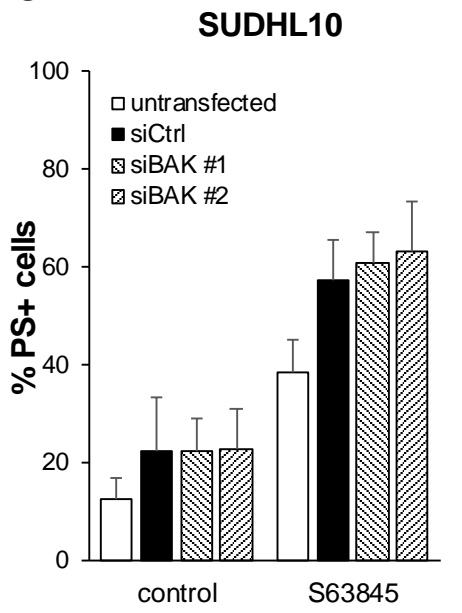
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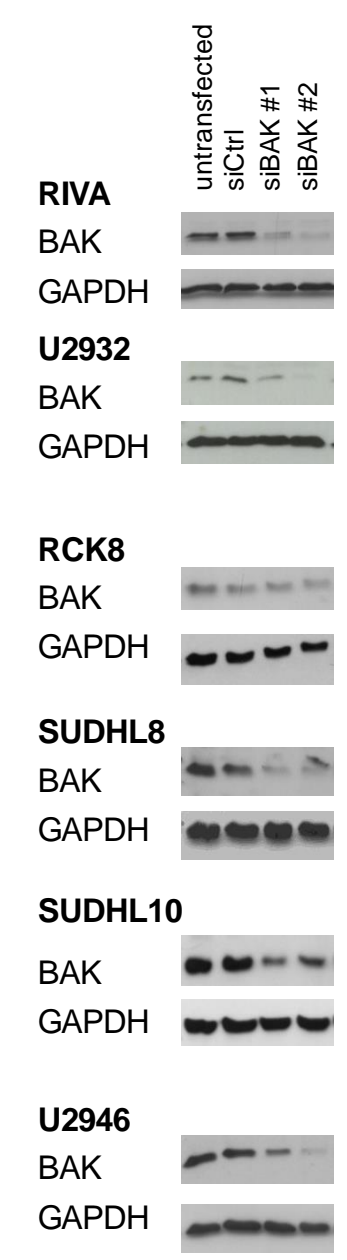
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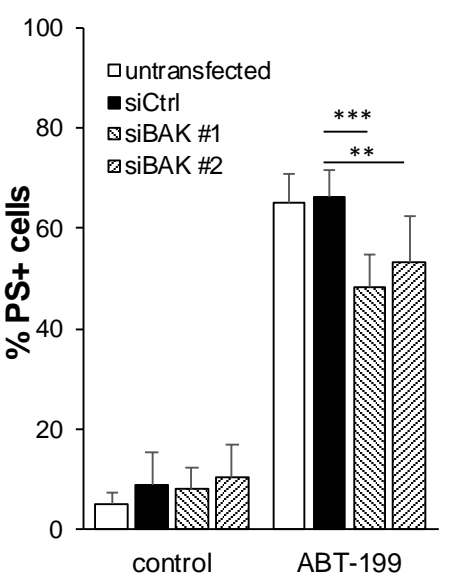
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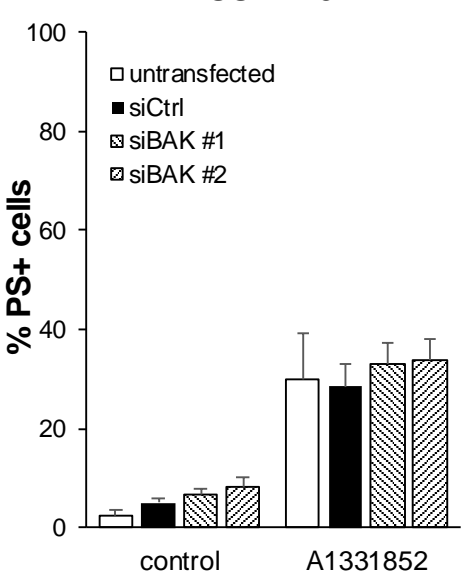
**D**



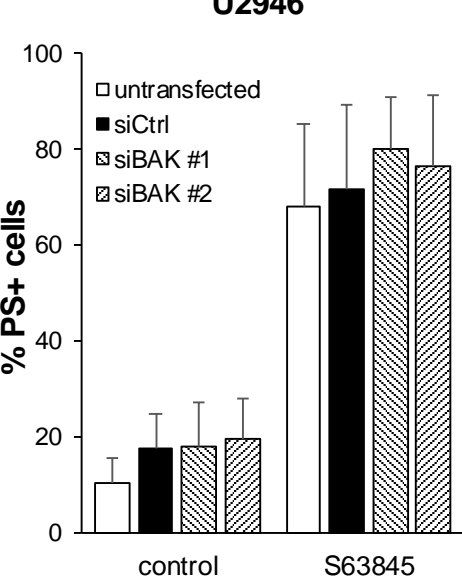
**U2932**



**SUDHL8**

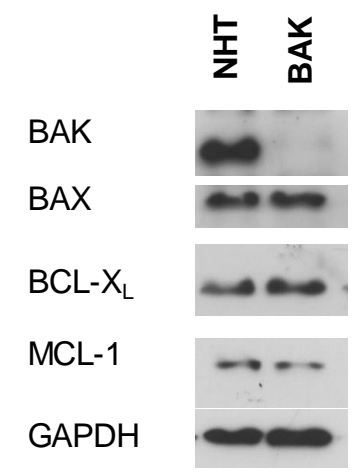


**U2946**

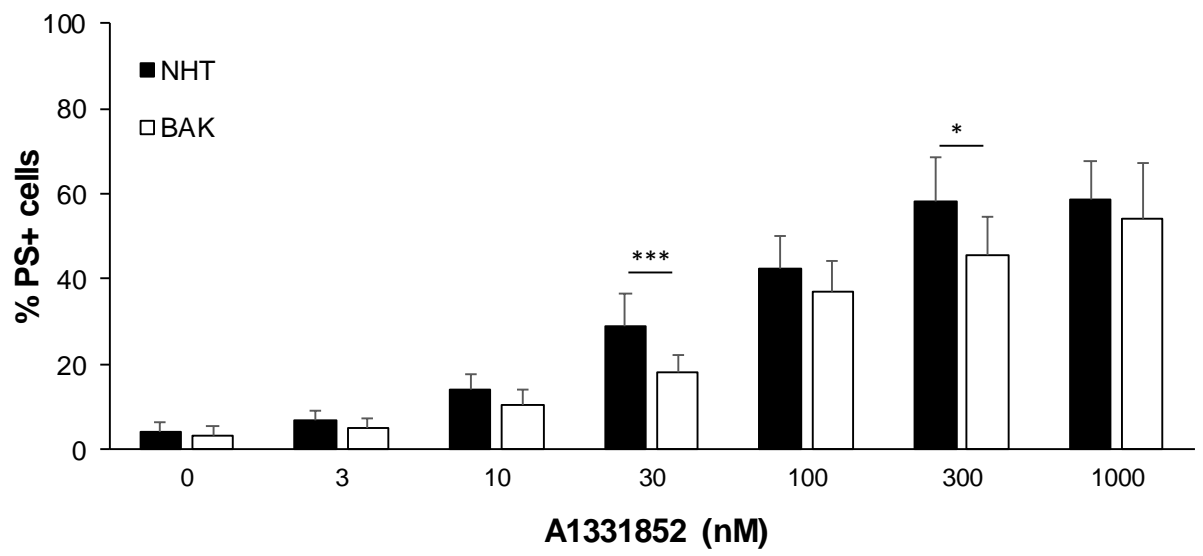


# Supplementary Figure 9

A



B



C

