Modulation of Navitoclax Sensitivity by Dihydroartemisinin-Mediated MCL-1 Repression in BCR-ABL$^+$ B-Lineage Acute Lymphoblastic Leukemia

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Supplemental Methods

MCL-1 half-life and stability experiments. BCR-ABL$^+$ B-ALL cells were pretreated for 4 hrs with DHA (1250 nM) and pulsed with cycloheximide for the indicated times before immunoblotting. To determine if MCL-1 is degraded through proteasome, cells were treated with DHA (1250 nM) alone, bortezomib (50 nM) alone or co-treated for six hours and then immunoblotted.

^{35}S Pulse Labeling of Cells. BCR-ABL$^+$ B-ALL cells were treated with DHA or cycloheximide (CHX) for 8 hours and then pulse-labeled with 100 µCi/ml of ^{35}S methionine-^{35}S cysteine (PerkinElmer, Waltham, MA) for 1 hr as described previously (1). After washing with PBS, cells were lysed in lysis buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane before subjected to autoradiography to determine new protein synthesis. Total amounts of proteins on the membranes were detected by Ponceau S solution (Sigma) and MCL-1 protein was detected by immunoblotting.

Measurement of Reactive Oxygen Species (ROS) and Treatment with ROS Scavengers. BCR-ABL$^+$ B-ALL cells were seeded at density of 5x10^5 cells in six-well plates and pretreated with MnTMPyP (Calbiochem, MA) and Diphenyleneiodonium chloride (DPI, Sigma) for one hour before treatment with DHA. To detect ROS, the cells were stained with dihydroethidium (DHE,

Sigma) at a final concentration of 5 μM for 30 minutes, washed twice with ice-cold PBS and analyzed by flow cytometry.

Supplementary Figure Legends

Sup. Fig. 1. MCL-1 is repressed by DHA and Ectopic MCL-1 expression renders BCR-ABL$^+$ B-ALL cells resistant to DHA-induced killing. (**A**) BCR-ABL$^+$ B-ALL cells were treated with either 1250 nM DHA or vehicle (DMSO) for indicated time points. The cell viability was analyzed by staining the cells with Annexin-V and quantifying apoptotic cells (Annexin-V$^+$) by flow cytometry. Data displayed are the average of 3 independent experiments and error bars indicate the SEM. One-way ANOVA with Bonferroni multiple comparison indicates significance $p<0.01$** and $p<0.001$***. (**B**) BCR-ABL$^+$ B-ALL cells were exposed to 1250 nM DHA for indicated times. Total cellular extracts were subjected to immunoblotting for expression of indicated proteins. Cleaved PARP indicates the induction of apoptosis and Actin serves as the control for loading. (**C**) BCR-ABL$^+$ B-ALL (Parentals) and BCR-ABL$^+$ B-ALL DKO leukemic cells were treated with indicated doses of DHA for 24 hours and analyzed by immunoblotting for indicated proteins. **(D)** BCR-ABL$^+$ B-ALL cells (vector or *Mcl-1* over expression) were seeded in 96-well round bottom plates ($6x10^4$ cells/well). DHA or DMSO vehicle controls were added at indicated concentrations and cells were in complete RPMI with 10% fetal calf serum. After 24 hours, the plates were centrifuged and cell death was determined by Annexin-V staining and measured by flow cytometry. (**E)** Vector or MCL-1 overexpression BCR-ABL$^+$ B-ALL cells were treated with DHA 1250 nM for 24 hours and whole cell lysates were subjected to immunoblotting with indicated antibodies.

Sup. Fig. 2. DHA treatment abrogates MCL-1 protein expression by post-transcriptional mechanism. (A) BCR-ABL$^+$ B-ALL cells were pre-treated for 4 hours with either vehicle (DMSO), 1250 nM DHA, or cycloheximide (10 µg/ml as positive control to block synthesis) and then pulsed with ^{35}S-labeled methionine and cysteine media. After 1 hour, the cells were lysed and resolved by SDS-PAGE and transferred to PVDF membrane. The membrane was assessed by autoradiography for new protein synthesis, stained with Ponceau S to assess steady state protein levels, and immunoblotted for MCL-1 expression. This experiment is representative of 3 independently-performed assays. (**B**) BCR-ABL$^+$ B-ALL cells were pre-treated for 4 hours with either vehicle (DMSO) or 1250 nM DHA after which cycloheximide (10 µg/ml) was added to block new protein synthesis. Protein lysates were collected at indicated time points for immunoblotting for MCL-1 and Actin expression. Experiment is representative of 3 independent experiments. (**C**) BCR-ABL$^+$ B-ALL cells were either treated with vehicle or 1250 nM DHA in the presence of absence of the proteasome inhibitor bortezomib (50 nM) for 6 hours after which total cellular lysates were subjected to immunoblot analysis for MCL-1 and Actin expression. Experiment is representative of 3 independent experiments. **(D)** Detection of DHA induced ROS in BCR-ABL$^+$ B-ALL cells: Cells were pretreated with MnTMPyP (25 µM) and DPI with indicated doses for one hour before exposing them to DHA 1250 nM for 8 hours. ROS was detected by staining cells with DHE and analyzed by flow cytometry. **(E)** BCR-ABL$^+$ B-ALL cells were pretreated with anti-oxidants as described in **D** and whole cell lysates were subjected to immunoblotting using indicated antibodies.

Sup. Fig. 3. Loss of NOXA expression does not prevent DHA-induced repression of MCL-1. (**A**) Volcano plot from microarray analysis of genes induced when BCR-ABL$^+$ B-ALL cells were

treated for 4 hours with vehicle (control) versus 312 nM DHA. Experiments were performed in triplicate on the Affymetrix Mouse Gene 2.0st platform. Genes marked with green dots are those that correlate with an ER stress pathway. The red dot indicates *Mcl-1*. **(B)** BCR-ABL^{+} B-ALL were treated with 1250 nM DHA for indicated time points and the expression of ER stress family members identified from the microarray were validated by quantitative PCR. Each measurement was performed from three different biological replicates performed in triplicate. The average fold change is indicated and the error bars indicate the S.E.M. **(C)** Wild-type and *Pmaip1*-deficient (encodes NOXA) KO MEFs were treated with DHA (10 μM) for 24 hours and whole cell lysates were subjected to immunoblotting with indicated antibodies. **(D)** BCR-ABL (p185)$^{+}$ *Arf*-null and p185^{+} *p53*-null B-ALL cells were treated with DHA 1250 nM for 24 hours and whole cell lysates were subjected to immunoblotting with indicated antibodies. **(E)** p185^{+} *Arf*-null and p185^{+} *p53*-null B-ALL cells were treated with indicated DHA doses and cell viability was analyzed by staining with Annexin-V and PI and analyzed by flow cytometry.

Sup. Fig. 4. DHA represses MCL-1 in TKI-resistant BCR-ABL^{T315I} B-ALL cells.

(A) BCR-ABL^{+} and BCR-ABL^{T315I} B-ALL cells were cultured with indicated doses of imatinib for 24 hours, stained with Annexin-V, and analyzed by flow cytometry. Graph depicts average of three independent experiments each performed in triplicate and error bars indicate the S.E.M. One-way ANOVA with Bonferroni multiple comparison indicates significance $p<0.01$** **(B)** BCR-ABL^{+} and BCR-ABL^{T315I} B-ALL cells were cultured with indicated doses of DHA for 24 hours, stained with Annexin-V and analyzed by flow cytometry. Graph depicts average of three independent experiments each performed in triplicate and error bars indicate the S.E.M. There was no significant difference in the response of the two leukemic cell lines to DHA. **(C)** BCR-

ABL^{+} and $BCR\text{-}ABL^{T315I}$ B-ALL cells were cultured with indicated doses of DHA for 24 hours, lysed, and subjected to immunoblot analysis for the indicated proteins. This experiment is representative of 3 independently performed experiments.

Sup. Fig. 5. Synergistic effect of Ph^{+} human cell lines to combined treatment with DHA and ABT-263. (**A-C**) The following human Ph^{+} ALL cell lines **(A)** BV-173, **(B)** OP-1, and **(C)** SUP-B15 were treated with indicated doses of DHA and/or with the caspase inhibitor Q-VD (10 μM) to inhibit apoptosis. After 24 hours, the cells were lysed and immunoblotted for expression of indicated proteins. Cleaved PARP indicates the induction of apoptosis. (**D-F**) The human Ph^{+} cell lines **(D)** BV-173, **(E)** OP-1, and **(F)** SUP-B15 were cultured with ABT-263 (0 nM, 20 nM, or 40 nM concentrations) and combined with DHA at indicated doses in culture. Following 24 hours of incubation, the cells were harvested, stained with Annexin-V, and viable cells were detected by flow cytometry. Each experiment was performed at least 3 times in triplicate and the average viable cells are plotted with error bars indicating the S.E.M. One-way ANOVA with Bonferroni multiple comparison indicates significance $p<0.001$**** between the DHA alone (0 nM ABT-263) and both combined treated arms (20 nM and 40 nM) at indicated doses of DHA. Response surface modeling of the combination of DHA and ABT-263 showed a synergistic interaction in **(D)** BV-173 ($\alpha=75$; $p=7.11\times10^{-7}$) **(E)** OP-1 ($\alpha=902$; $p=2.82\times10^{-15}$) and **(F)** SUP-B15 ($\alpha=23602$; $p=1.91\times10^{-44}$).

Sup. Fig. 6. Pathology of DHA and ABT-263 treatment in mice. (**A**) Bioluminescence measurements for the indicated cohorts of mice from analysis on day 13 after leukemia transplant. **(B)** Total white blood cell count (WBC) count in peripheral blood of mice treated with the

indicated groups on day 13. Each bar represents the average WBC count of the indicated group. Error bars indicate the standard error of mean at least 8 mice per group. One-way ANOVA was performed to test the statistical significance among the groups. **(C)** 40x images from H&E stained sections of BCR-ABL$^+$ B-ALL bearing mice at sacrifice: **i**) sternum bone marrow shows tumor infiltration and replacement of normal bone marrow cells, **ii**) vertebral bone marrow shows tumor infiltration and replacement of normal bone marrow cells, **iii**) brain with tumor cells above the dura and in the epidural space, **iv**) cerebellum shows tumor cells above the dura in the epidural space. **(D)** Sections from the spleen of BCR-ABL$^+$ B-ALL leukemic mouse at time of sacrifice stained as indicated and presented at indicated magnifications. Size bars indicate 50 µm on 40x images and 20 µm on 50x images.

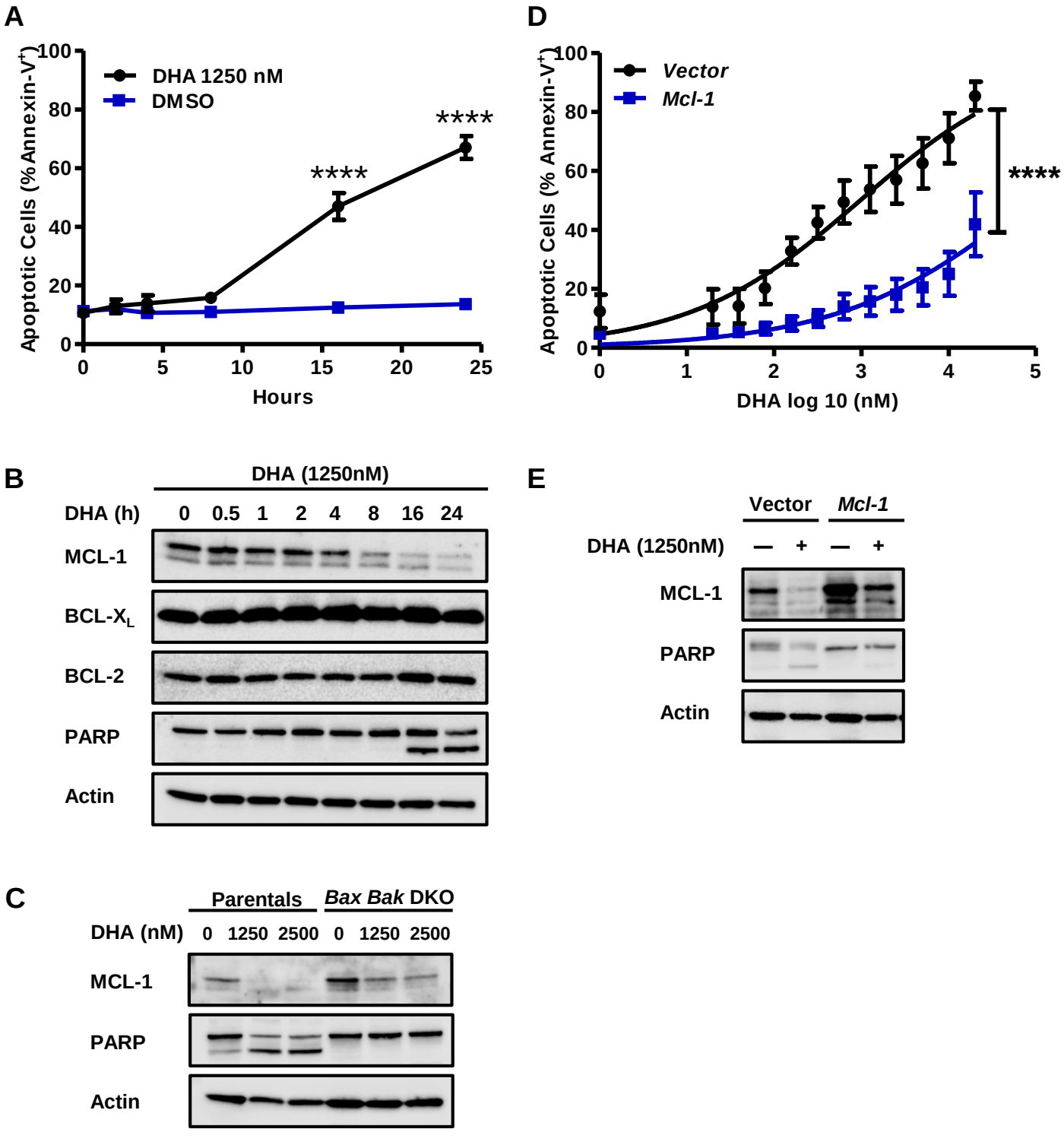
Sup. Fig. 7. Densitometry Data for Immunoblots. Immunoblots were developed using Odyssey Fc imaging system from LI-COR Biosciences. Image Studio Lite Ver 5.2 software was used to select each band and quantify signal intensities by subtracting background and analyzed by GraphPad Prism. Each sub-panel in Sup. Fig.7 corresponds to the immunoblot panel in the main text (Figures 1-5) or supplementary figures (S1-S6). All experiments are representative of 3 independently-performed assays. **(1B)** DHA cleaved PARP and represses MCL-1 protein expression in BCR-ABL$^+$ B-ALL cells in a dose-dependent manner without significantly affecting BCL-X_L and BCL-2 protein levels. Two-way ANOVA with Bonferroni multiple comparison indicates significance $p<0.001$*** **(1C)** Loss of MCL-1 protein expression is independent of caspase activation in BCR-ABL$^+$ B-ALL cells. Two-way ANOVA with Bonferroni multiple comparison indicates significance $p<0.01$** or not significant (ns). **(2B)** DHA and thapsigargin treatment represses MCL-1, cleaved PARP, induces NOXA and CHOP in BCR-ABL$^+$ B-ALL cells in a time-dependent manner. **(2C)** DHA and positive controls of ER stress (thapsigargin and

tunicamycin) represses MCL-1 and induces NOXA and CHOP in dose-dependent manners in mouse embryonic fibroblasts (MEFs). **(2D)** *Ddit3*-deficient MEFs exhibits less repression of MCL-1 protein in response to DHA treatment. One-way ANOVA with Bonferroni multiple comparison indicates significance p<0.0001****. **(2E)** *Ddit3*-deficient BCR-ABL$^+$ B-ALL cells exhibits less repression of MCL-1 protein in response to DHA treatment. Two-way ANOVA with Bonferroni multiple comparison indicates significance p<0.01**. **(4A)** DHA treatment represses MCL-1 protein expression in human Ph$^+$ ALL cell line TOM-1 in a dose-dependent manner. Two-way ANOVA with Bonferroni multiple comparison indicates significance p<0.0001**** or not significant (ns). **(5C)** DHA significantly represses MCL-1 protein levels *in vivo*. Unpaired *t*-test indicate significance p<0.001*** between vehicle and DHA 200 mg/kg at 8 hour time point. **(S1B)** DHA represses MCL-1 protein expression in BCR-ABL$^+$ B-ALL cells in a time-dependent manner without significantly affecting BCL-X_L and BCL-2 protein levels. Two-way ANOVA with Bonferroni multiple comparison indicates significance p<0.05*. **(S1C)** Loss of MCL-1 protein expression is independent of BAX/BAK-dependent mitochondrial permeabilization in BCR-ABL$^+$ B-ALL cells. Two-way ANOVA with Bonferroni multiple comparison indicates significance p<0.01**. **(S1E)** Overexpression of MCL-1 rendered BCR-ABL$^+$ B-ALL cells more resistant to DHA induced MCL-1 repression and apoptosis. Two-way ANOVA with Bonferroni multiple comparison indicates significance p<0.01**. **(S2A)** DHA treatment represses new protein synthesis in BCR-ABL$^+$ B-ALL cells. Unpaired *t*-test indicates significance between DMSO vs DHA p<0.05* and DMSO vs CHX p<0.0001****. **(S2B)** DHA and CHX treatment. There are no significant differences in the rate of MCL-1 elimination. **(S2C)** DHA induced MCL-1 repression could be rescued by bortezomib. Unpaired *t*-test indicates significance between DHA alone vs DHA with bortezomib p<0.05*. **(S2E)** ROS scavengers were unable to block the attenuation of

MCL-1 by DHA treatment. Unpaired *t*-test indicates significance between Con. Vs DHA p<0.0001**** DPI vs DPI + DHA p<0.01** and MnTMPyP vs MnTMPyP + DHA p<0.05*. **(S3C)** DHA induced NOXA is not required to repress MCL-1 expression in MEFs. One-way ANOVA indicates significance between DMSO vs DHA in *Pmaip1* wt and ko p<0.001*** and p<0.01** respectively. **(S3D)** DHA induced NOXA is not required to repress MCL-1 expression in BCR-ABL^{+} *p53*-null B-ALL cells. The difference between *Arf*-null and *p53*-null B-ALL cells treated with DHA is not significant (ns). **(S4C)** DHA represses MCL-1 expression similarly in BCR-ABL^{+} and BCR-ABL^{T315I} leukemic cells. **(S5A-C)** DHA represses MCL-1 expression in human Ph^{+} BV-173, OP-1, and SUP-B15 cell lines. Two-way ANOVA with Bonferroni multiple comparison indicates significance p<0.05*, p<0.01** and p<0.001***.

References to Supplement

1. Rahmani M, Davis EM, Crabtree TR, Habibi JR, Nguyen TK, Dent P, et al. The kinase inhibitor sorafenib induces cell death through a process involving induction of endoplasmic reticulum stress. Mol Cell Biol. 2007;27(15):5499-513.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****
****
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D
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****
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B
DHA (1250nM)
DHA (h) 0 0.5 1 2 4 8 16 24
MCL-1
BCL-XL
BCL-2
PARP
Actin
E
Vector
Mcl-1
DHA (1250nM) — + — +
MCL-1
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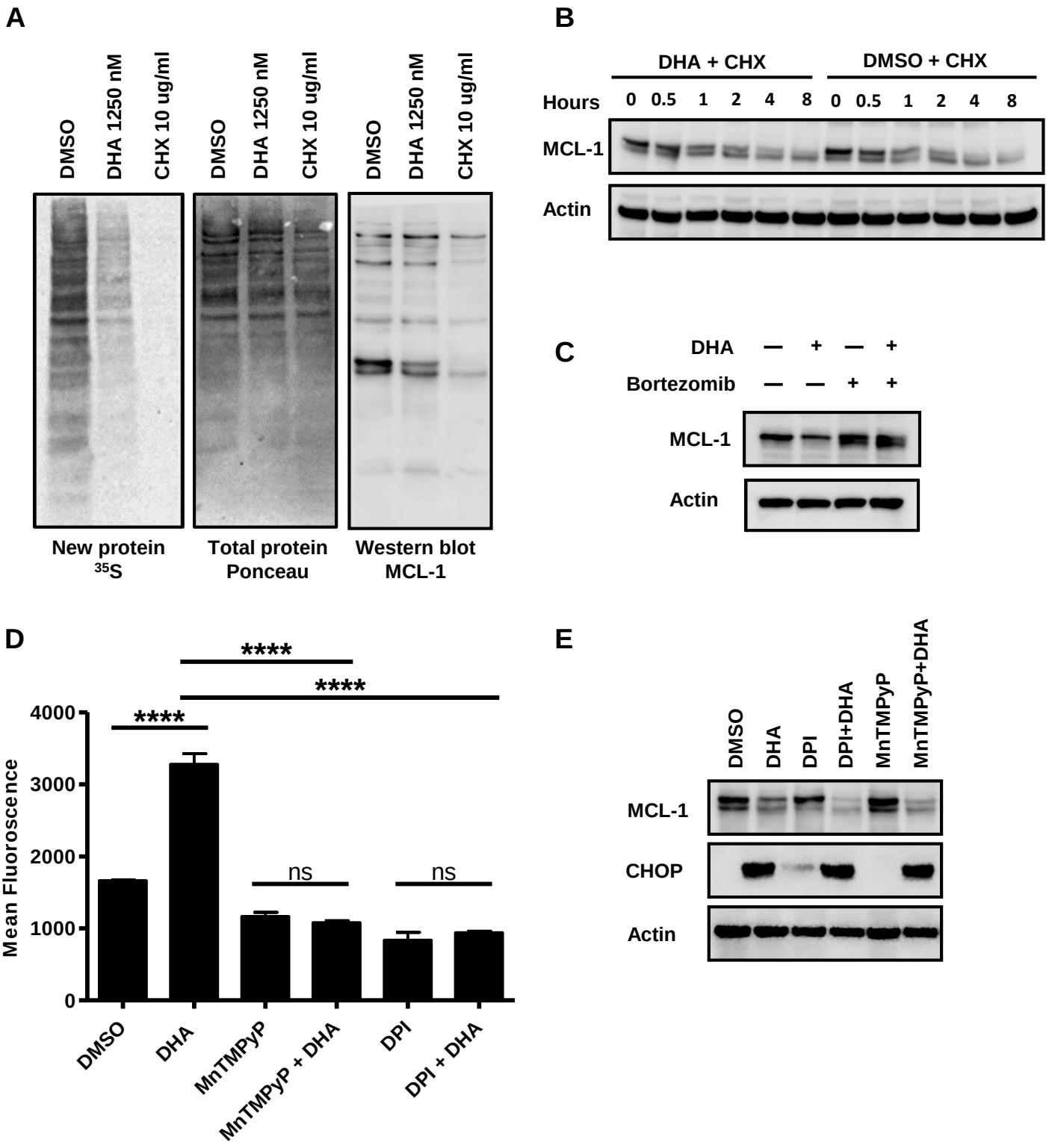
MCL-1
PARP
Actin

Sup. Fig. 1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35S
Total protein
Ponceau
Western blot
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B
DHA + CHX
DMSO + CHX
Hours
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Actin
C
DHA — + — +
Bortezomib — — + +
MCL-1
Actin
D
****
****
****
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3000
2000
1000
0
Mean Fluorescence
ns
ns
DMSO
DHA
MnTMPyP
MnTMPyP + DHA
DPI
DPI + DHA
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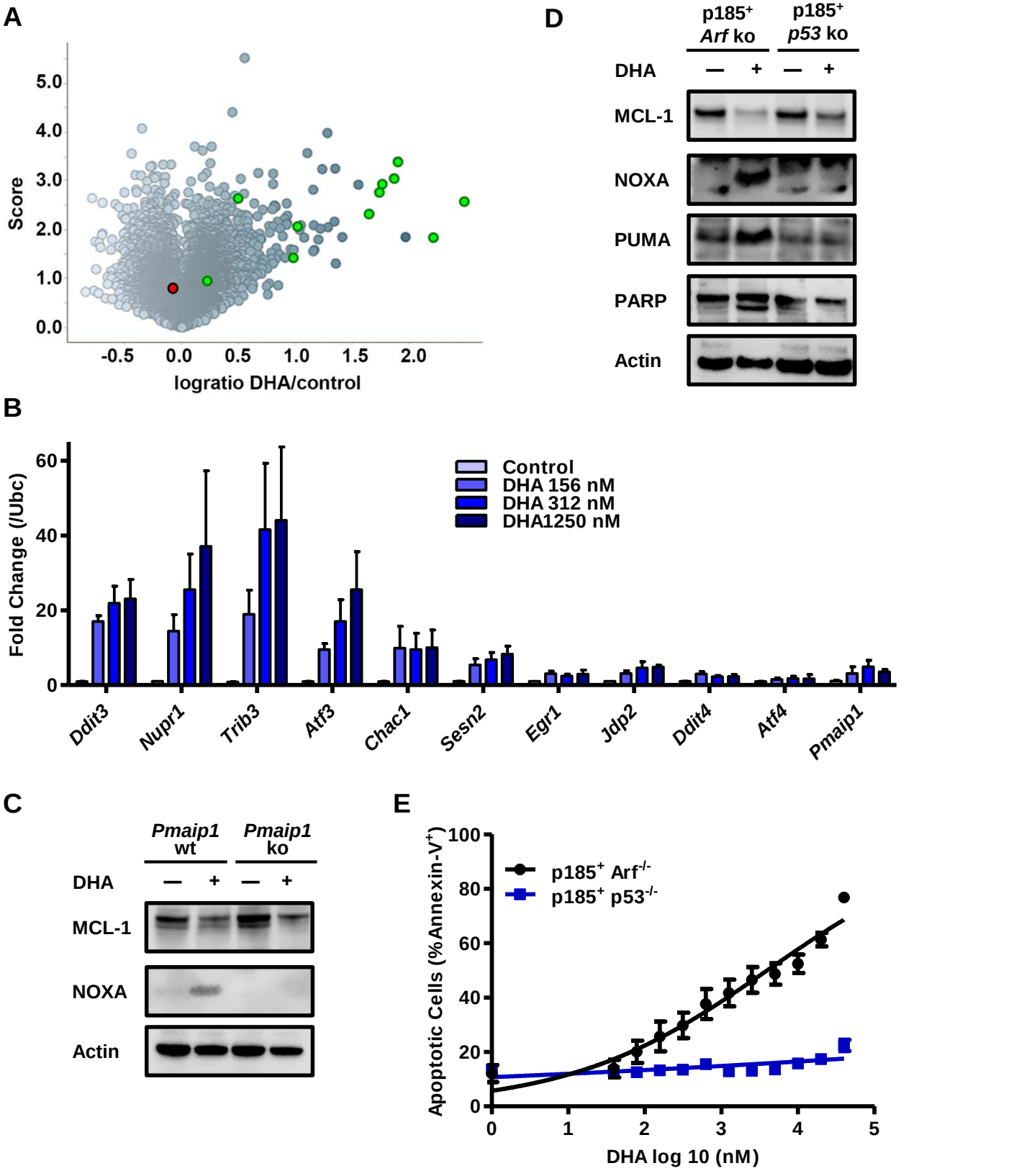
CHOP
Actin

Sup. Fig. 2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D
p185+ Arf ko
p185+ p53 ko
DHA
—
+
—
+
MCL-1
NOXA
PUMA
PARP
Actin
B
Fold Change (/Ubc)
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40
20
0
Control
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DHA 312 nM
DHA1250 nM
Ddit3
Nupr1
Trib3
Atf3
Chac1
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C
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—
+
—
+
MCL-1
NOXA
Actin
E
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80
60
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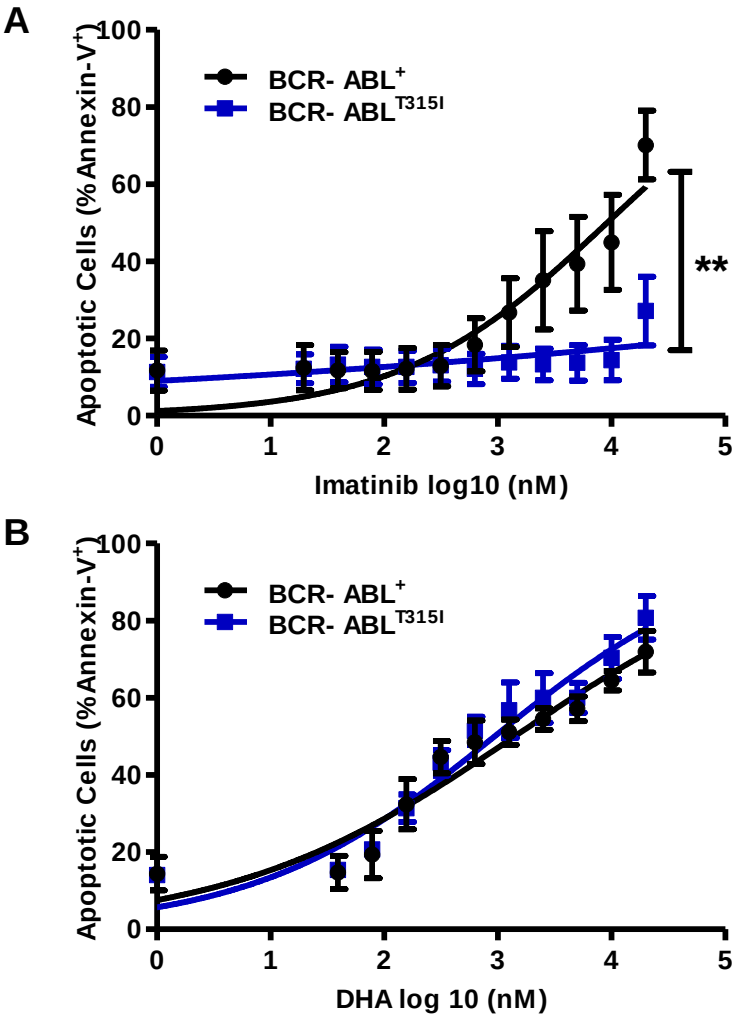
2
3
4
5
DHA log 10 (nM)

Sup. Fig. 3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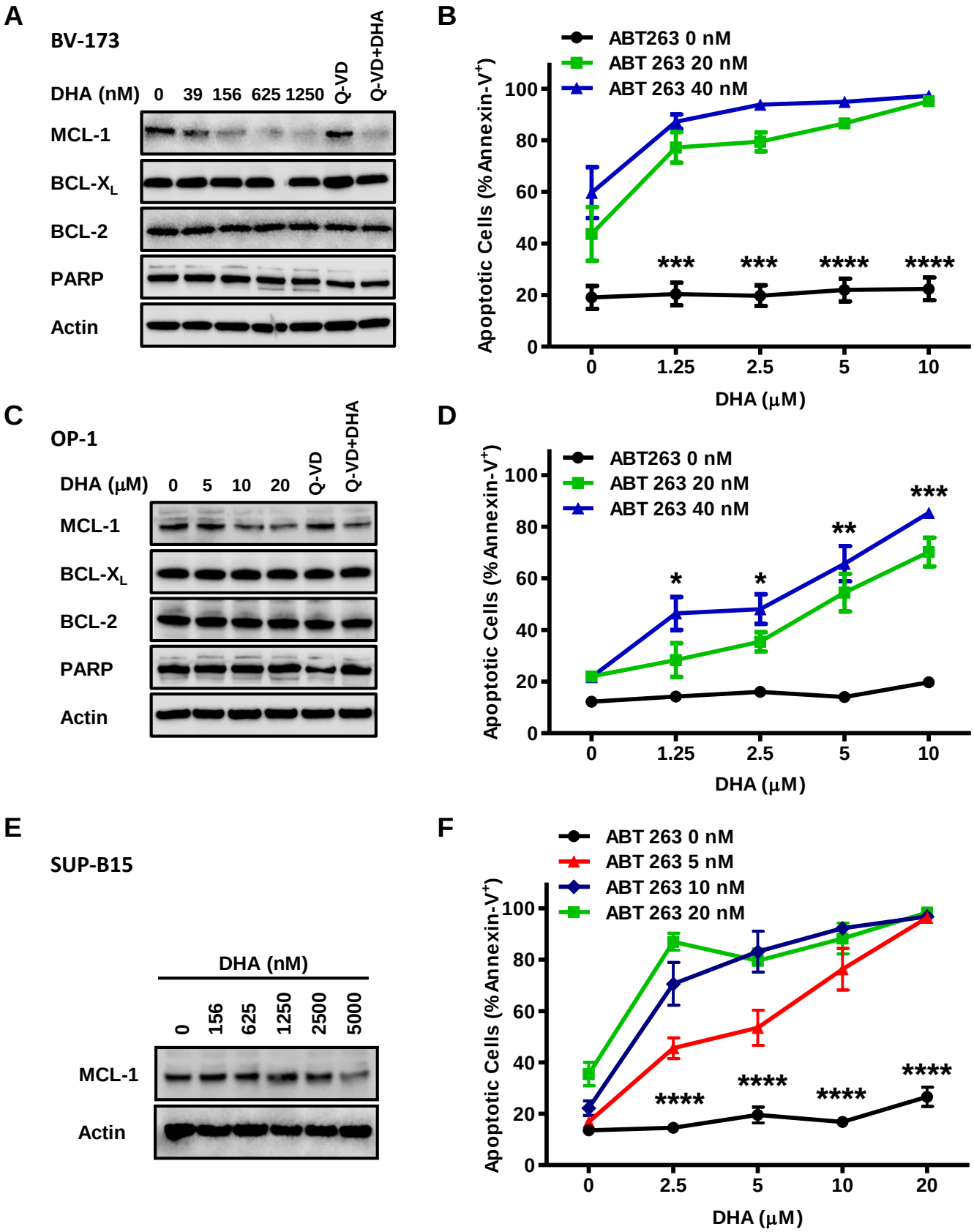
Actin

Sup. Fig. 4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ABT 263 20 nM
ABT 263 40 nM
Apoptotic Cells (%Annexin-V+)
***
***
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****
DHA (μM)
C
OP-1
DHA (μM) 0 5 10 20 Q-VD Q-VD+DHA
MCL-1
BCL-XL
BCL-2
PARP
Actin
D
ABT263 0 nM
ABT 263 20 nM
ABT 263 40 nM
Apoptotic Cells (%Annexin-V+)
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DHA (μM)
E
SUP-B15
DHA (nM)
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F
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Apoptotic Cells (%Annexin-V+)
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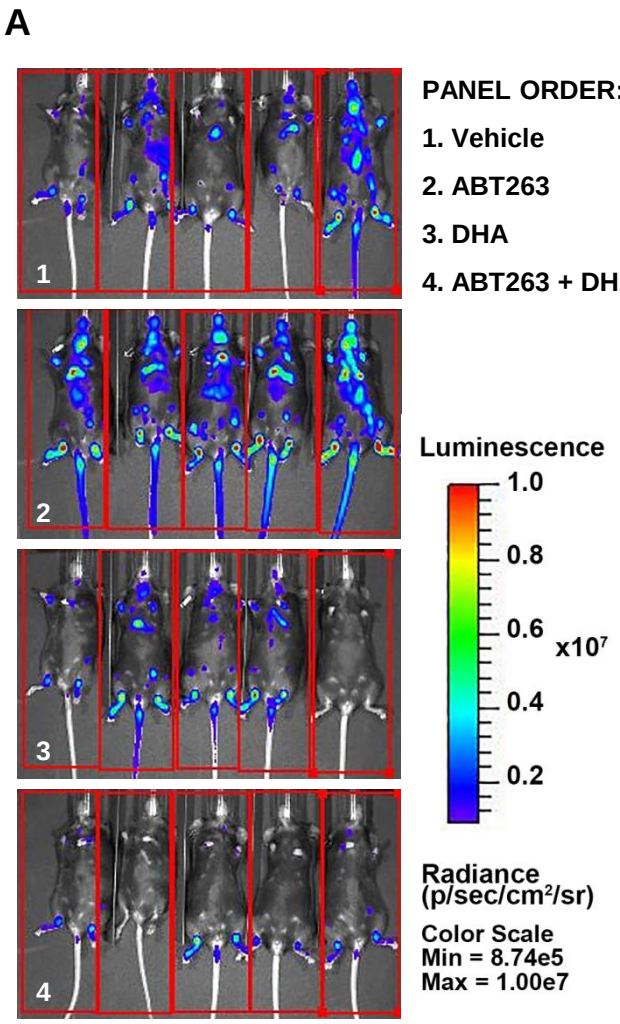
DHA (μM)

Sup. Fig. 5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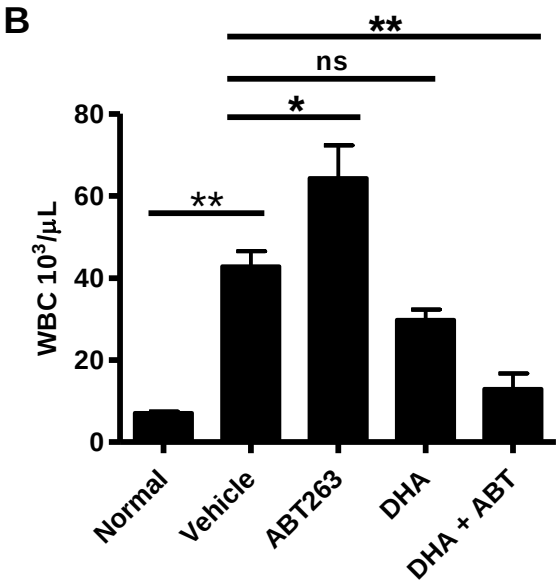
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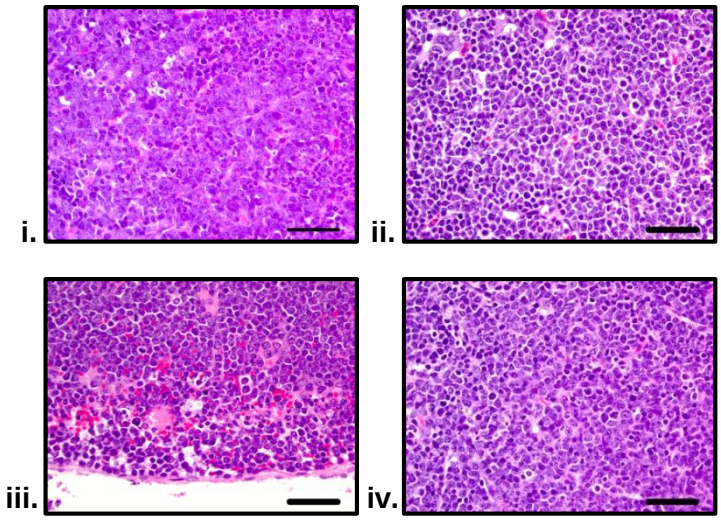
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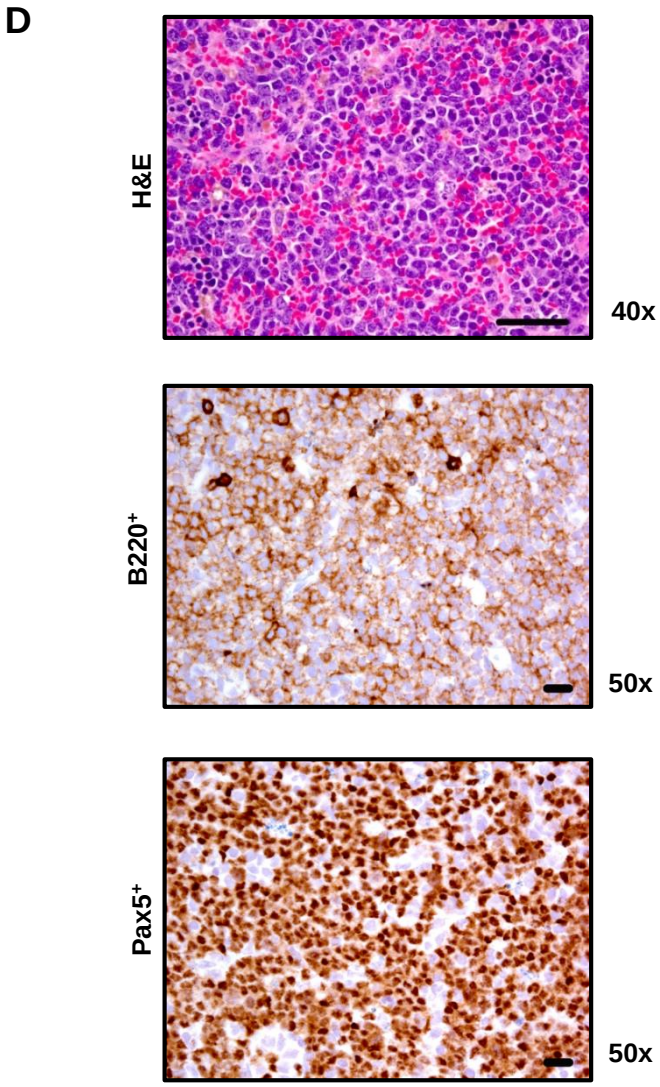
ii.
iii.
iv.


D
H&E
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B220⁺
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Sup. Fig. 6