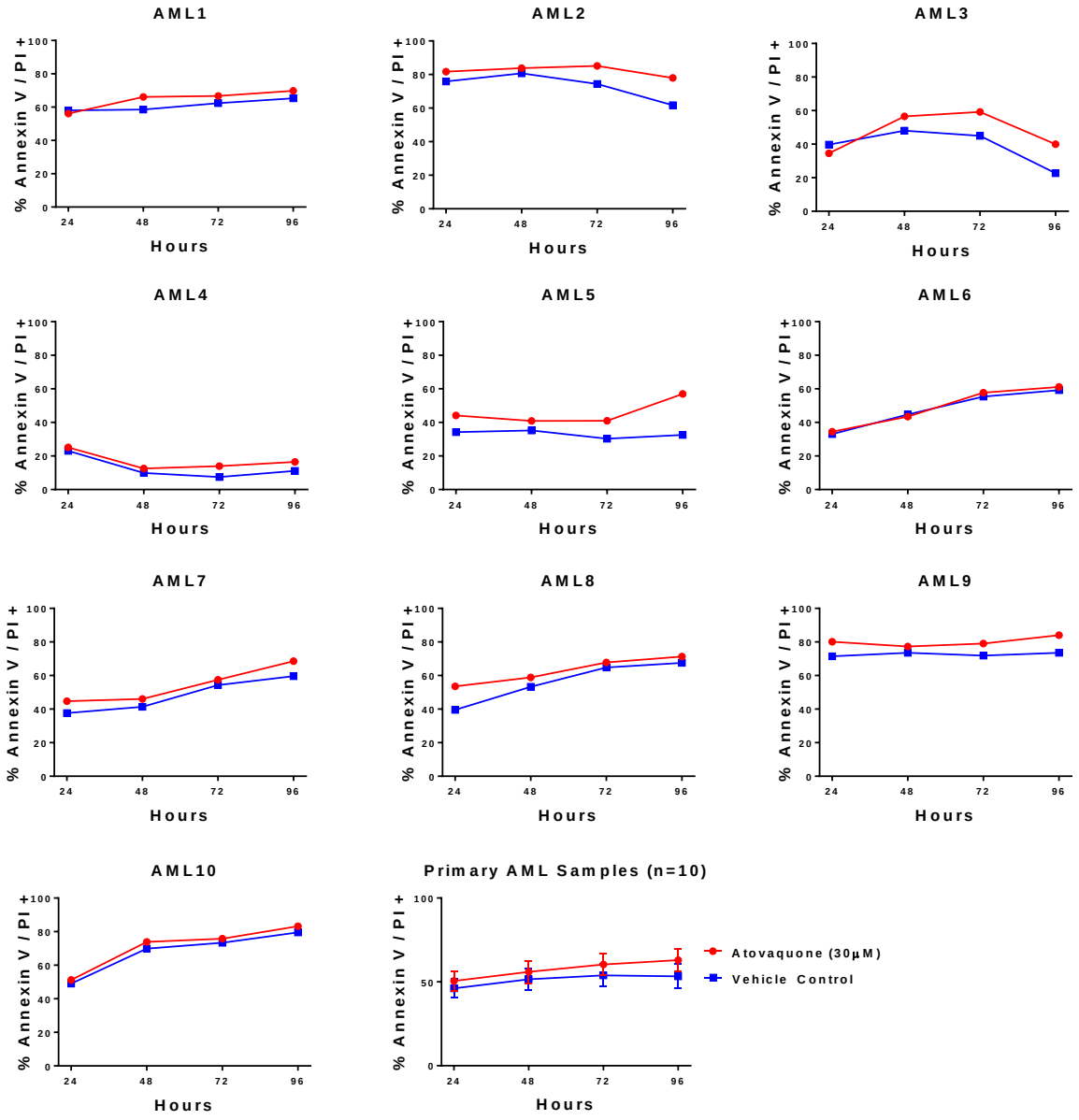
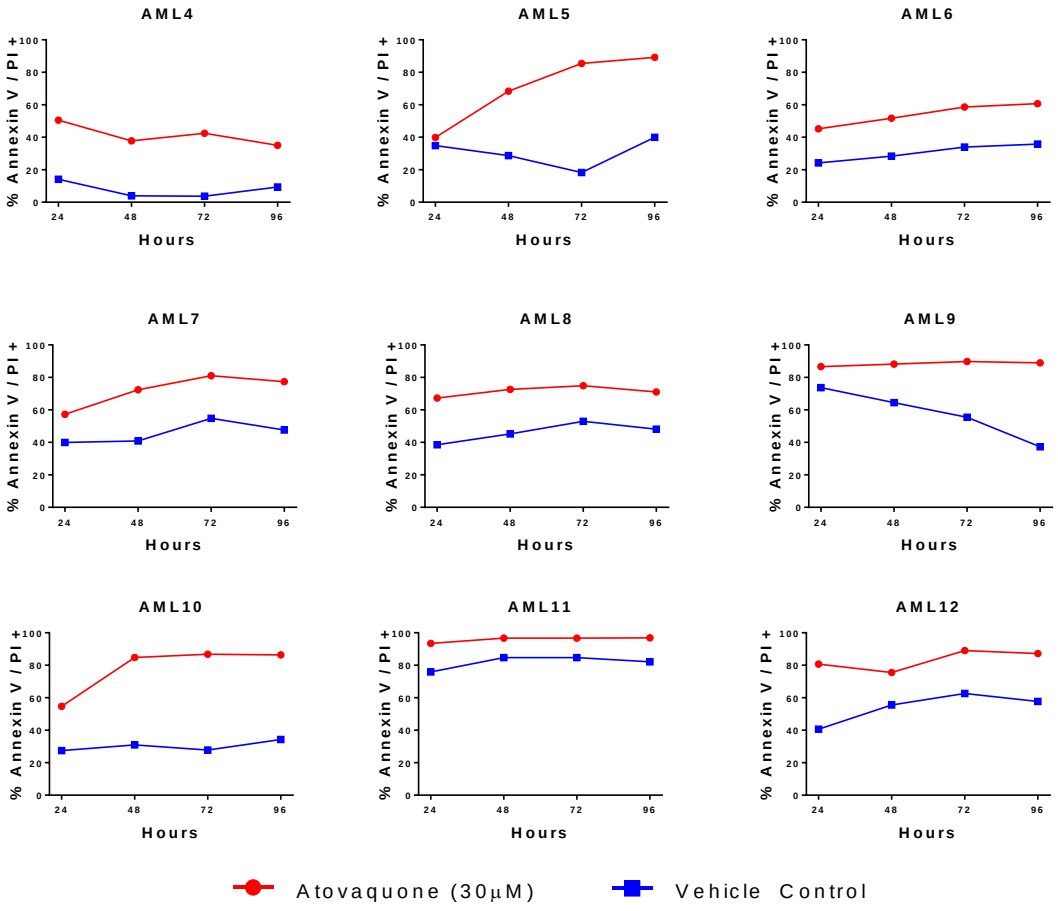
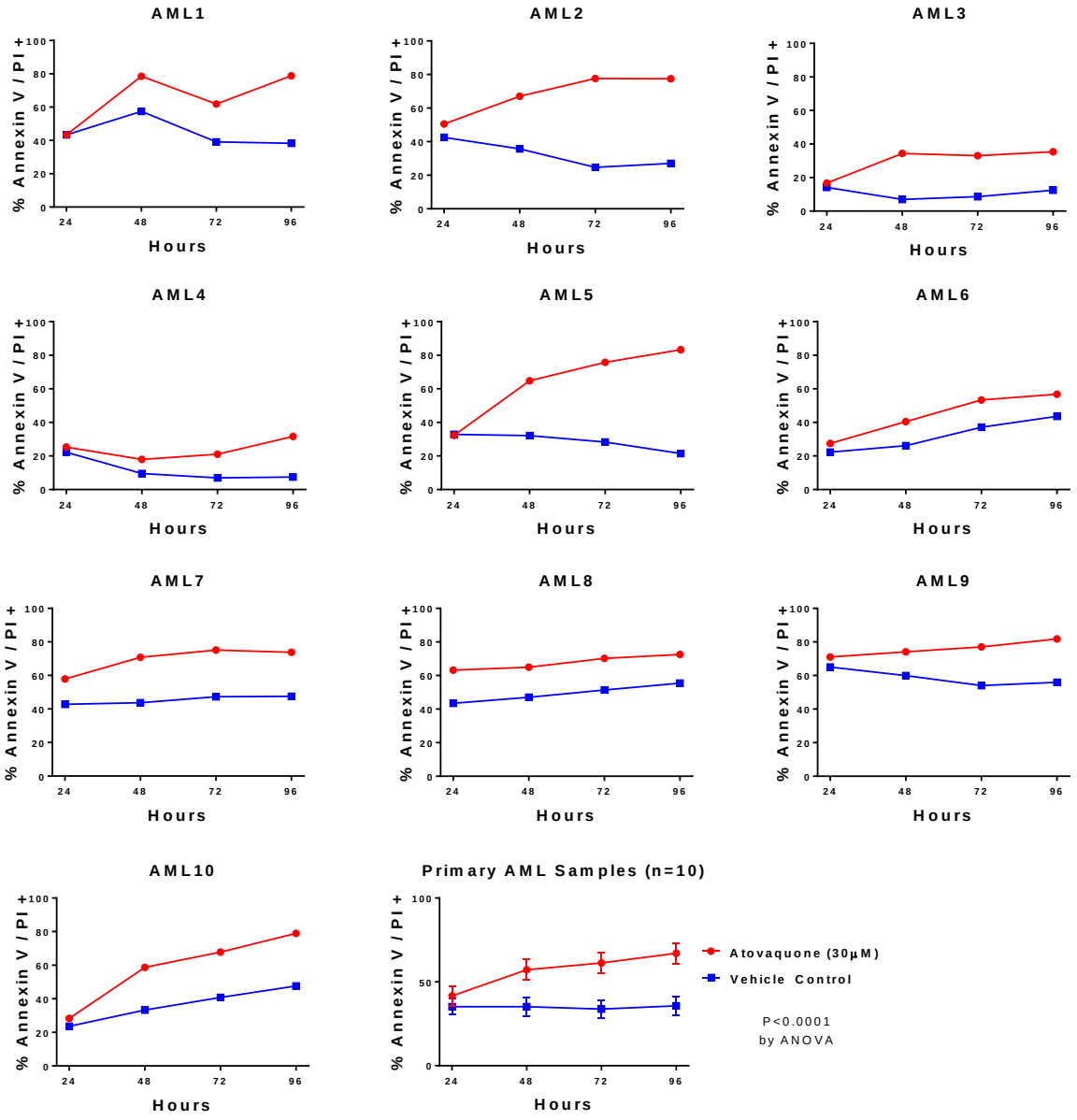


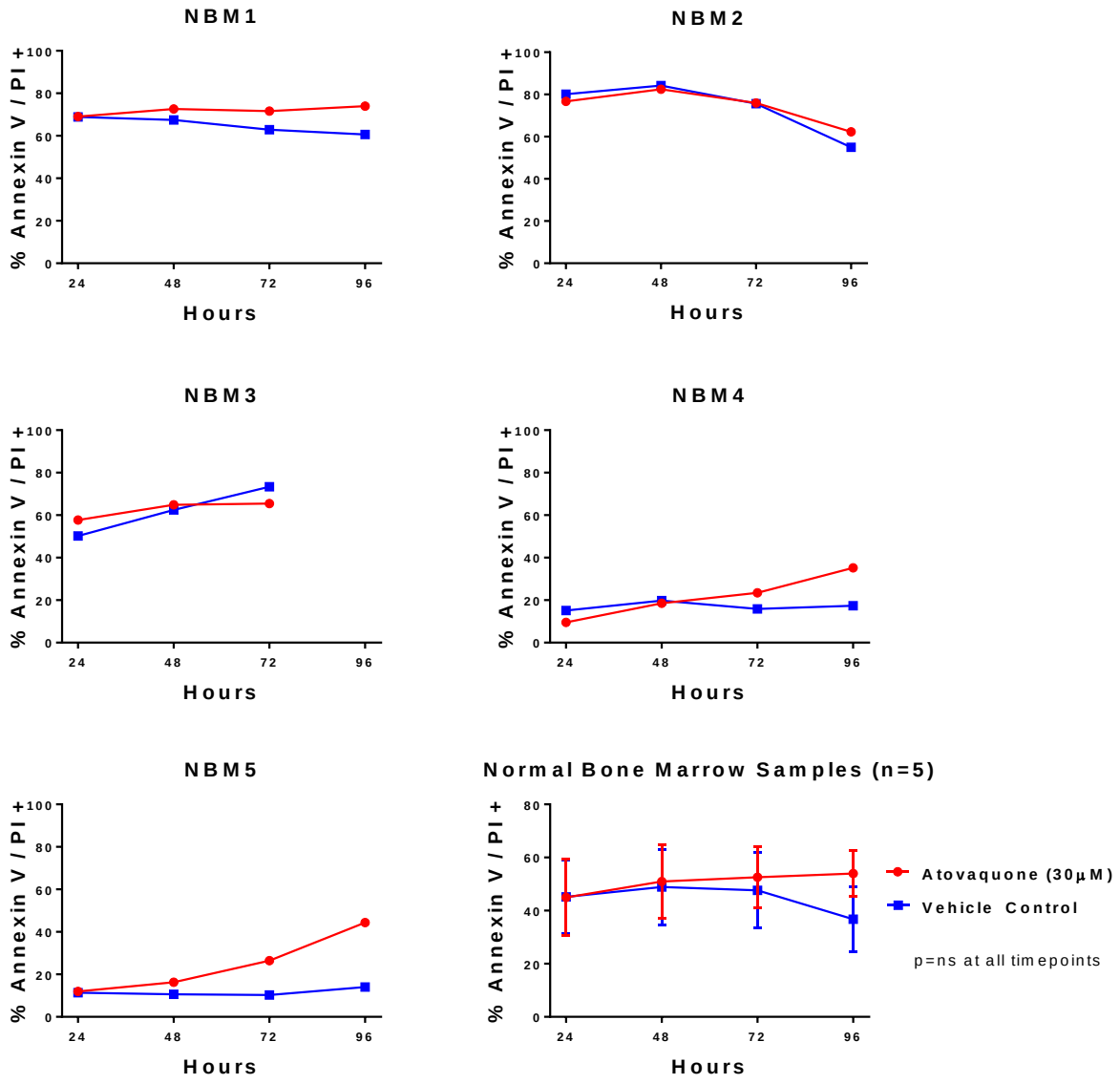
Supplemental Figure 1. Primary samples without supportive conditions demonstrated little atovaquone-induced apoptosis. Ten of 12 primary pediatric AML samples had sufficient cell numbers to evaluate the effect of 30µM atovaquone on cell viability by Annexin V and PI positivity when cells were suspended in standard IMDM with 20% FBS, 1% pen/strep, and 1% L-glutamine. The bottom right panel depicts means and SEM for all 10 samples. In the absence of HS5-derived soluble factors, apoptosis rates were not significantly different in primary samples exposed to atovaquone when compared to vehicle.



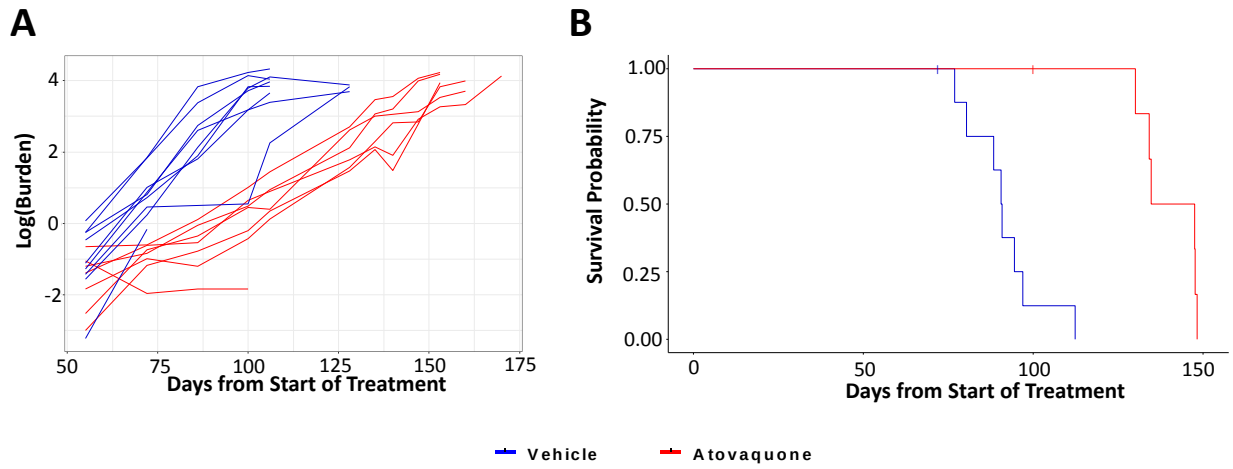
Supplemental Figure 2. Individual sample data depicting the effect of atovaquone on AML patient samples supported by HS5 stromal cells. For patient samples AML4-AML12, cells co-cultured with HS5 cells, separated by a transwell, demonstrated increased apoptosis over time when treated with 30µM atovaquone, compared to vehicle-treated cells.



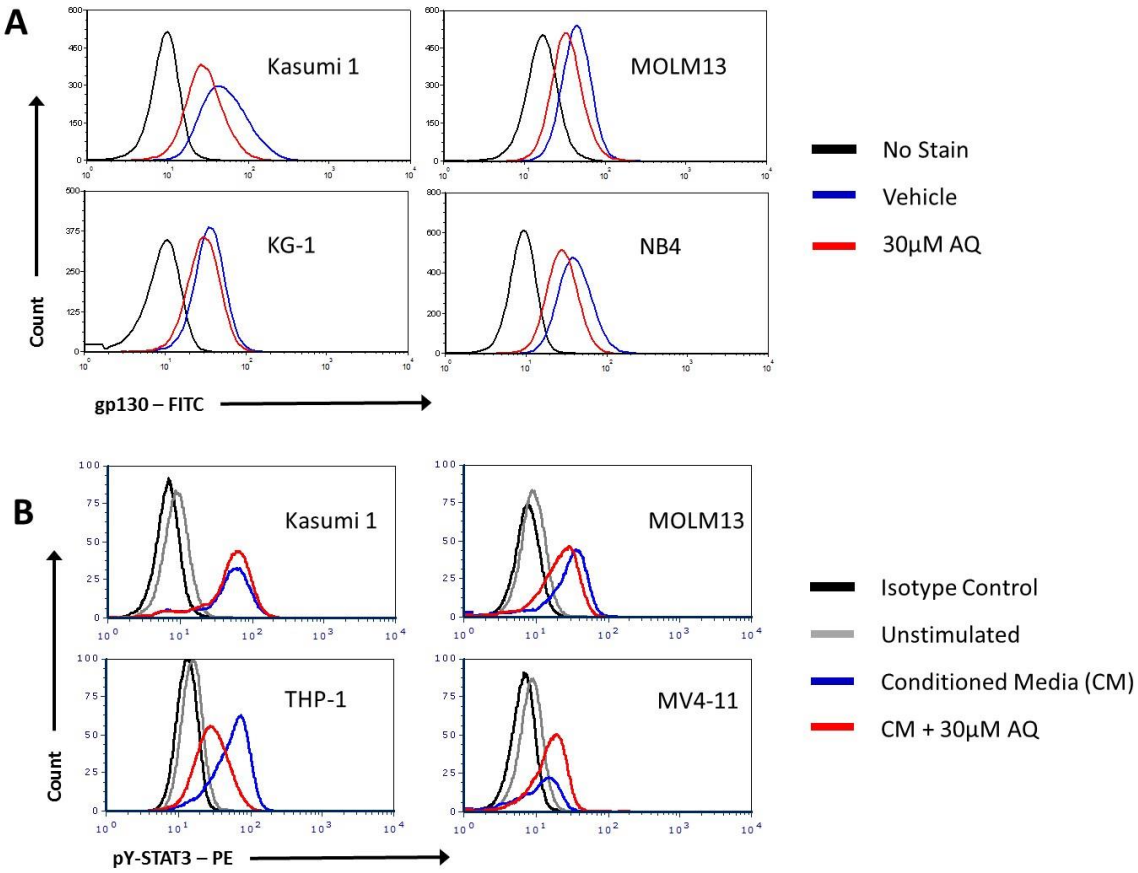
Supplemental Figure 3. Primary samples supported by HS5 conditioned medium demonstrate atovaquone-induced apoptosis. Ten of 12 primary pediatric AML samples had sufficient cell numbers to evaluate the effect of 30µM atovaquone on cell viability by annexin V and PI positivity when cells were suspended in HS5 conditioned medium. The bottom right panel depicts means and SEM for all 10 samples. Apoptosis rates are significantly different in primary samples exposed to atovaquone compared to vehicle, by ANOVA.



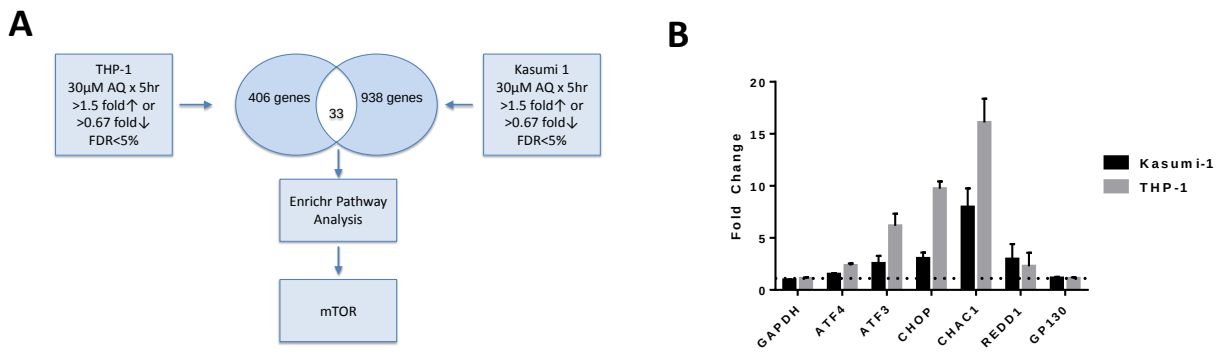
Supplemental Figure 4. Normal bone marrow samples supported by HS-5 conditioned medium demonstrate no significant effect of atovaquone. Five primary pediatric bone marrow samples supported with HS5 conditioned medium demonstrate no significant increase in apoptosis over time when treated with 30µM atovaquone compared to vehicle. The percent Annexin V+/ PI+ is shown for vehicle control and atovaquone-treated wells at each time point to demonstrate the difference between spontaneous apoptosis and atovaquone-induced apoptosis. The bottom right panel depicts means and SEM for all 5 samples. NBM= normal bone marrow, ns=not significant.



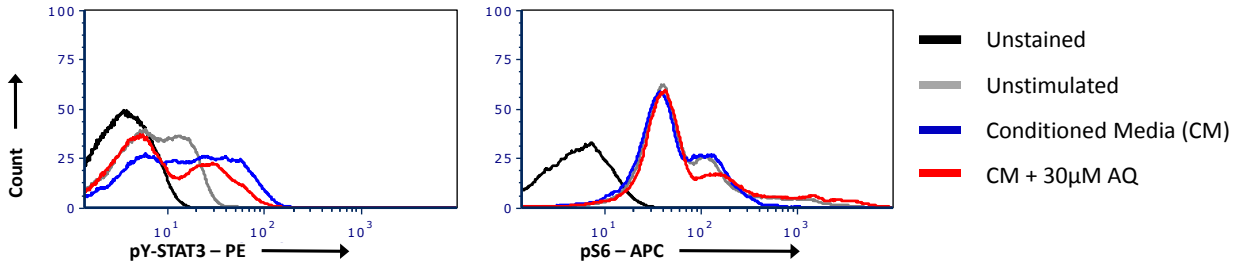
Supplemental Figure 5. Treatment with atovaquone as a single agent delays disease progression and prolongs time to reaching 20% blasts in peripheral blood in a patient derived xenograft model. **(A)** Additional analysis of the NSG mice injected by tail vein with primary patient cells from sample AML3 utilizing a mixed-effect model with time and treatment as fixed events, mouse as random effect, and disease burden (log-transformed) as the response. The interaction between treatment and time was found to be significantly different ($P < 0.0001$). (**B)** Additional survival analysis of the same cohort of mice (with an event of reaching a disease burden of 20% hCD45/hCD33+ cells in the peripheral blood) demonstrated improved survival in atovaquone-treated mice compared to vehicle by the generalized Wilcoxon test ($P<0.001$)



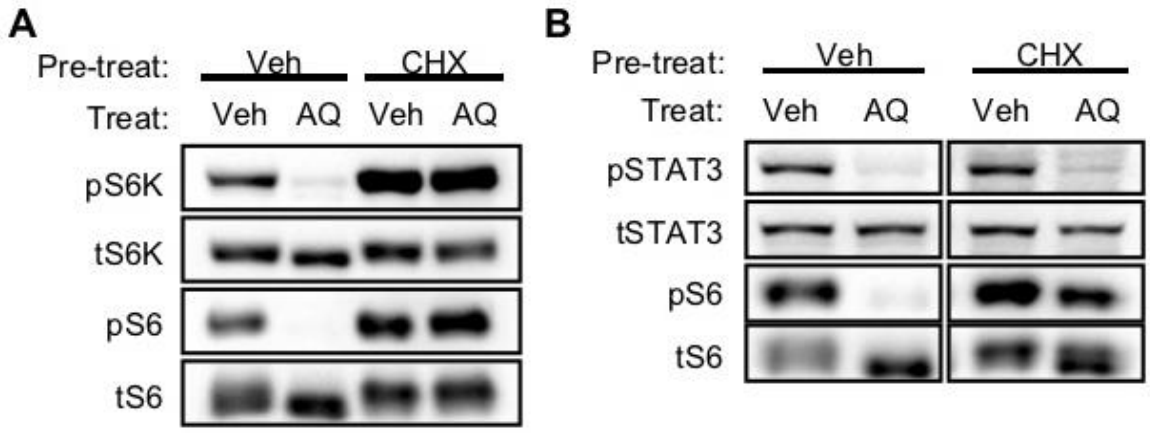
Supplemental Figure 6. Atovaquone exposure results in variable reductions in gp130 surface expression and pY-STAT3 activation in AML cell lines. (A) Using flow cytometry, gp130 surface expression was quantified at baseline and after exposure to 30µM of atovaquone for 24 hours. Despite induction of apoptosis at this dose, little effect was seen on gp130 surface expression after atovaquone exposure. Similar results were seen at 6, 10 and 48 hour timepoints (data not shown). **(B)** Reduction in conditioned media (CM) stimulated pY-STAT3 by atovaquone is minimal in several AML cell lines. Data shown is representative of at least two independent experiments.



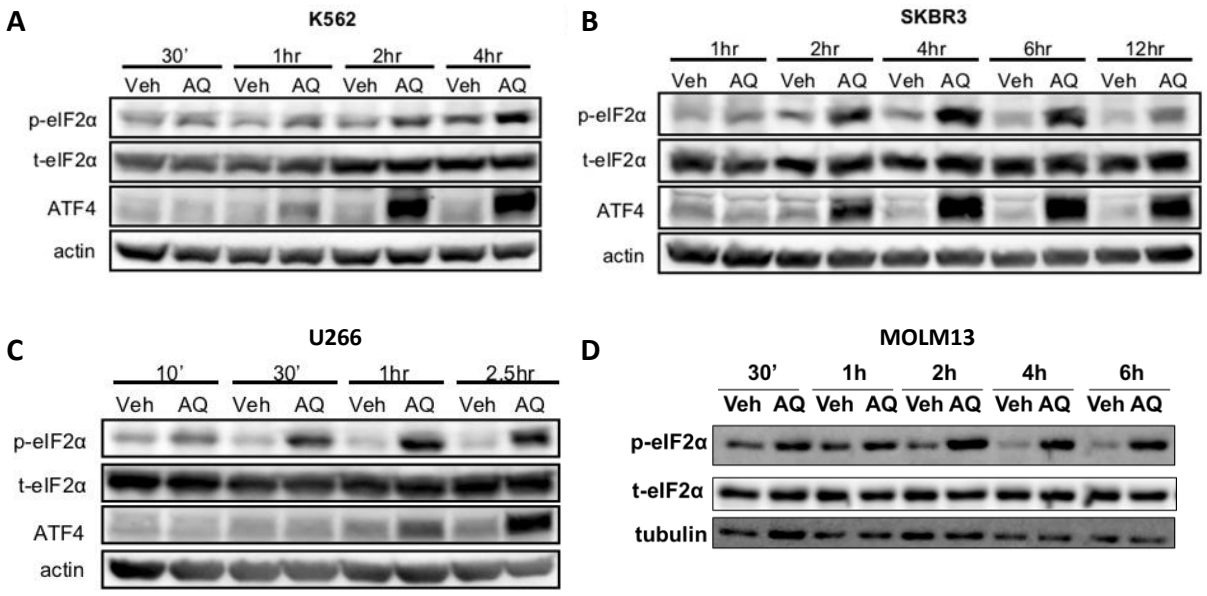
Supplemental Figure 7. ATF4 target genes are upregulated in THP1 and Kasumi-1 cells after atovaquone exposure. (A) Schematic of gene expression changes. **(B)** Differences in fold change generated from RNAseq data of genes related to ATF4, its target genes, and GP130. Means and SEM for each cell line are shown. Dotted line indicates fold change of 1.



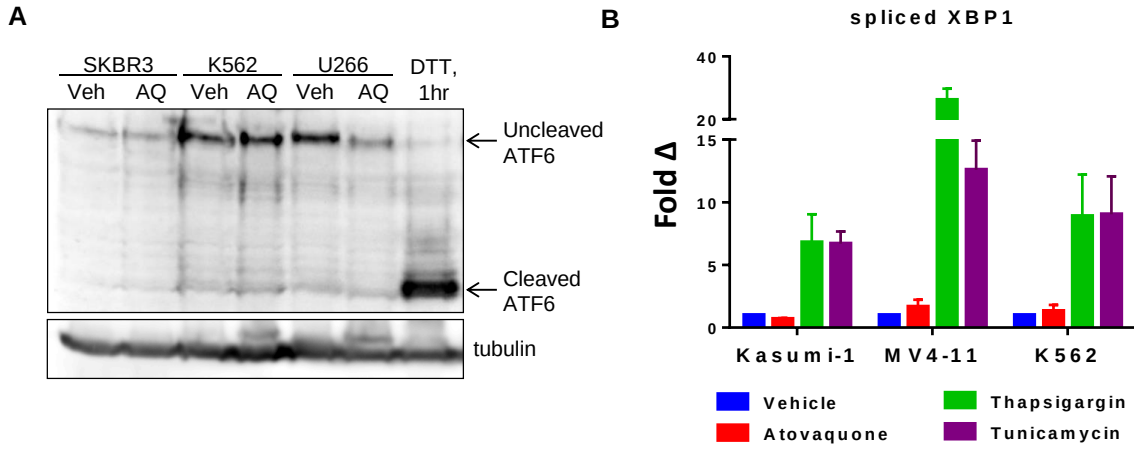
Supplemental Figure 8. Atovaquone slightly decreases pY-STAT3 and does not reduce pS6 in a normal bone marrow sample. Phosphoflow evaluation of a normal pediatric bone marrow sample (NBM8) after a 5 hour incubation with atovaquone (AQ) or vehicle followed by a 15 minute exposure to conditioned media revealed a small reduction in phospho tyrosine STAT3 activation but no effect of pS6 activity.



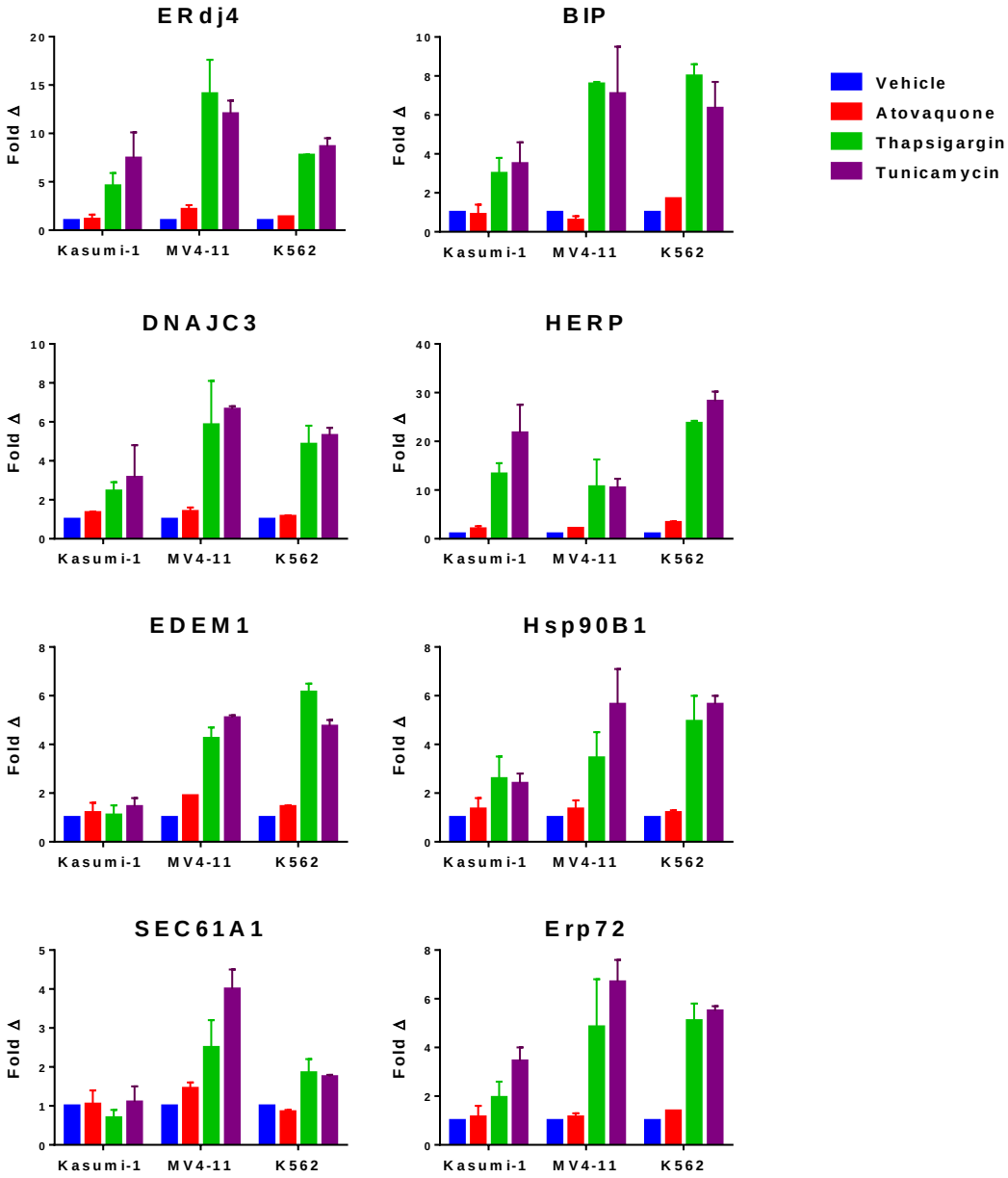
Supplemental Figure 9. Inhibition of protein synthesis with cycloheximide abrogates atovaquone-mediated decrease of phosphorylation of S6 and S6 kinase, but not of STAT3. (A) K562 cells were pre-treated with vehicle or cycloheximide (2 μg/ml) for 1 hour, then treated with vehicle or atovaquone (20 μM) for 5 hours prior to immunoblot for the depicted proteins. **(B)** U266 cells were pre-treated with vehicle or cycloheximide (2 μg/ml) for 1 hour, then treated with vehicle or atovaquone (15 μM) for 2.5 hours. Immunoblots are representative of two independent experiments.



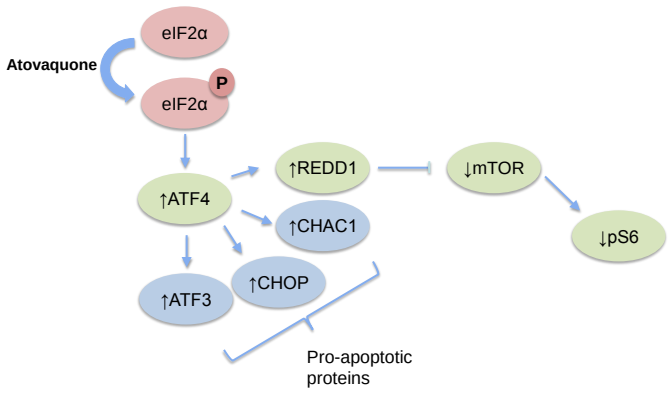
Supplemental Figure 10. Atovaquone induces phosphorylation of eIF2α and increases ATF4 levels. (A) K562 cells were treated with atovaquone (20 μM) for the indicated time periods, followed by western blotting. Data are representative of two independent experiments. **(B)** SKBR3 cells were treated with atovaquone (25 μM) for the indicated time periods, followed by western blotting. Data are representative of two independent experiments. **(C)** U266 cells were treated with atovaquone (20 μM) for the indicated time periods, followed by western blotting. Data are representative of two independent experiments. **(B)** MOLM13 cells were treated with atovaquone (25 μM) for the indicated time periods, followed by western blotting. Data are representative of two independent experiments.



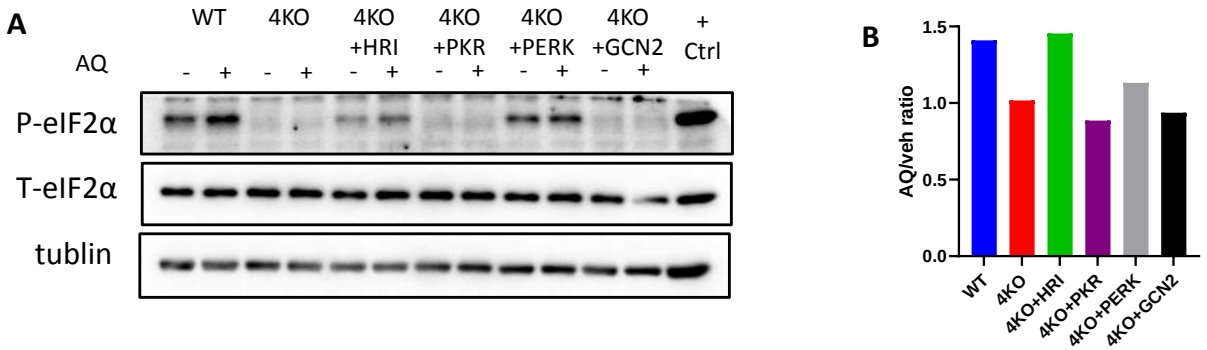
Supplemental Figure 11. Atovaquone does not trigger cleavage of ATF6 or splicing of XBP1. **(A)** SKBR3, K562, and U266 were treated with vehicle (Veh) or atovaquone (AQ), followed by western blotting to assay for ATF6 cleavage. U266 cells treated with 5 mM DTT for 1 hour served as a positive control. Due to the insolubility of ATF6, all of these samples were prepared by boiling in sample buffer. **(B)** Kasumi-1, MV4-11, and K562 cells were treated with atovaquone (20 μM), thapsigargin (1 μM), or tunicamycin (5 μg/ml) for 6 hours. Afterwards, qRT-PCR was performed using primers specific to spliced XBP1. Data are means ± SEM from three independent experiments.



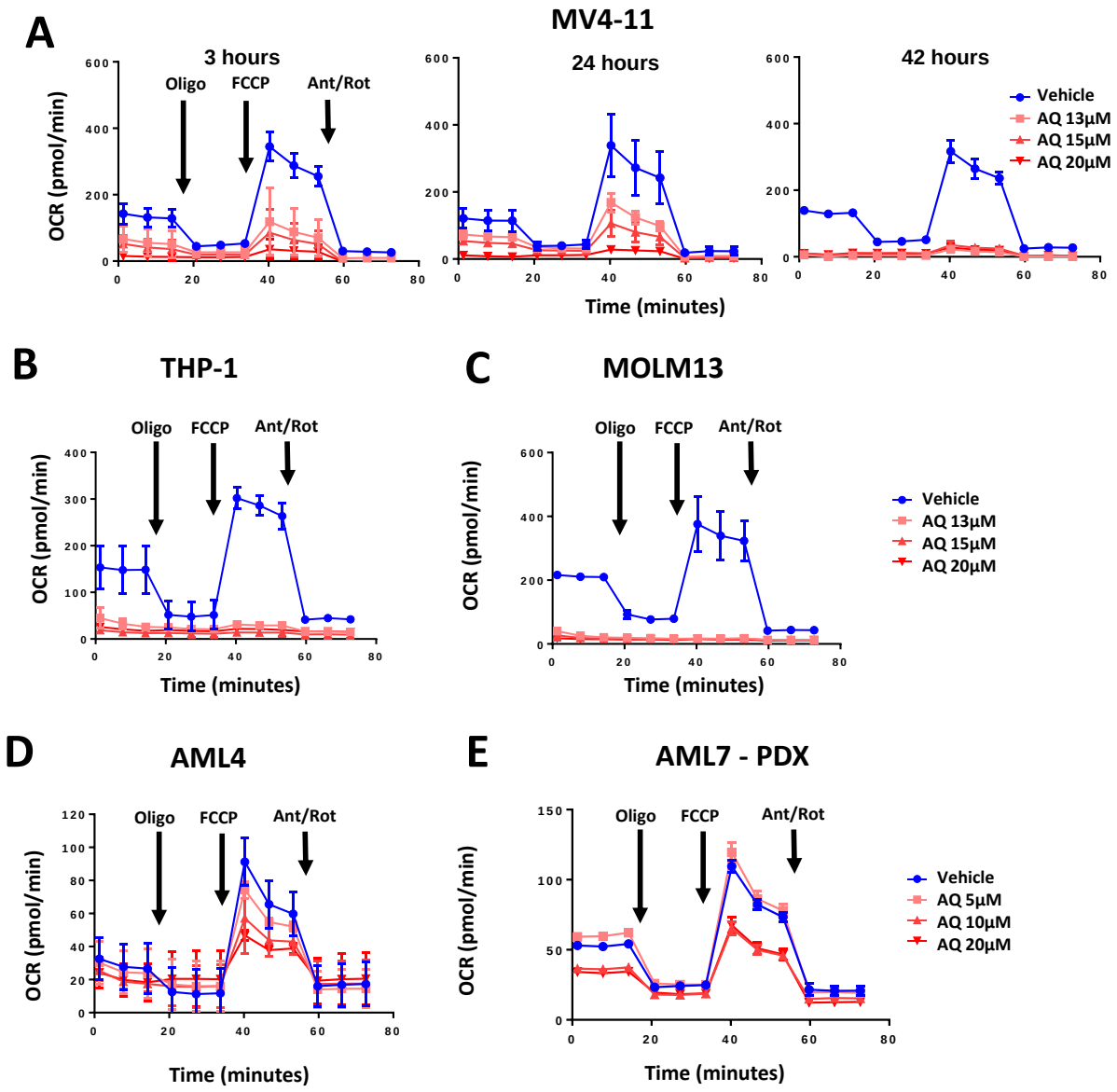
Supplemental Figure 12. Atovaquone does not induce downstream components of the unfolded protein response other than ATF4-specific target genes (ATF3, CHAC1, CHOP, REDD1). Kasumi-1, MV4-11, and K562 cells were treated with atovaquone (20 μM), thapsigargin (1 μM), or tunicamycin (5 μg/ml) for six hours, followed by qRT-PCR using the indicated primers. Data are means ± SEM of two independent experiments.



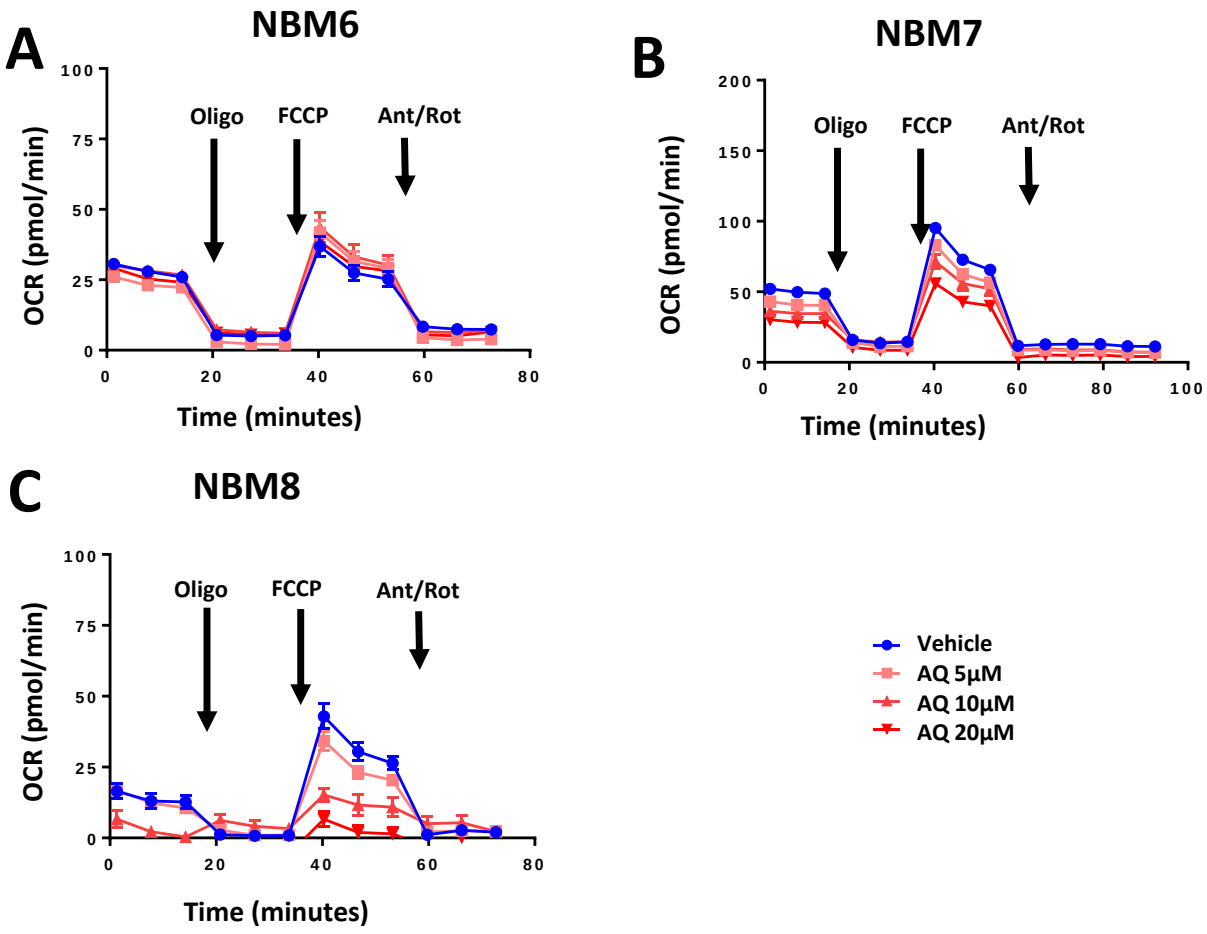
Supplemental Figure 13. Cartoon of mechanism by which atovaquone suppresses the mTOR pathway through activation of phospho-eIF2α/ATF4.



Supplemental Figure 14. The eIF2α kinases HRI and PERK can mediate atovaquone-induced phosphorylation of eIF2α. (A) MEFs with all four known eIF2α kinases (heme-regulated inhibitor, HRI; protein kinase R, PKR; PKR-like endoplasmic reticulum (ER) kinase, PERK; and, general control non-depressible 2, GCN2) deleted were used. Each kinase was added back individually and cells were treated with vehicle or 25 mM atovaquone for four hours. As a positive control, wildtype cells were treated with thapsigargin (200 nM) for one hour. Lysates were then analyzed by western blotting. Data are representative of two independent experiments. **(B)** Relative eIF2a band intensities (normalized to total eIF2α) is shown.



Supplemental Figure 15. Atovaquone induces a time- and dose-dependent suppression of basal and maximal mitochondrial respiration of AML cell lines and primary samples. (A-C) Dose-dependent reductions in oxygen consumption rate (OCR) of AML cells after 3 hour treatment with increasing doses of atovaquone (AQ). Doses of AQ for MV4-11, THP-1, and MOLM13 were 0, 13, 15, and 20 μM. **(A)** MV4-11 cells also demonstrated a time-dependent effect of AQ. **(D, E)** Doses of AQ for patient samples were 0, 5, 10, 20 μM. Patient samples were rested in HS5 conditioned medium for 24 hrs prior to 3 hour incubation with indicated doses of AQ. Data for each cell line and patient sample are means ± SEM of two independent experiments with the exception of AML7 which is a single independent experiment. The cells used to quantify OCR in response to AQ for AML7 were obtained after harvest of bone marrow cells from a patient derived xenograft established from this sample. Oligo= oligomycin, FCCP=carbonyl cyanide-4-(trifluromethoxy) phenylhydrazone, and Ant/Rot= antimycin A/ rotenone, AQ=atovaquone.



Supplemental Figure 16. Atovaquone has minimal impact on basal and maximal mitochondrial respiration of healthy patient samples. (A-C) Dose-dependent reductions in oxygen consumption rate (OCR) of normal pediatric hematopoietic cells after 3 hour treatment with increasing doses of atovaquone (AQ). Doses of AQ were 0, 5, 10, and 20 µM. Patient samples were rested in HS5 conditioned medium for 24 hrs prior to 3 hour incubation with indicated doses of AQ. Data are single independent experiments for each fresh bone marrow sample. Oligo= oligomycin, FCCP=carbonyl cyanide-4-(trifluromethoxy) phenylhydrazone, and Ant/Rot= antimycin A/ rotenone, AQ=atovaquone.

Supplemental Table 1. Cells Used and Sources

Cell Line Name	Source
THP-1	ATCC
THP-1.ffluc	gift from Dr. Sarah Tasian (Children's Hospital of Philadelphia, Philadelphia, PA)
Kasumi-1	ATCC
MV-4-11	ATCC
HL-60	ATCC
NB4	gift from Dr. Shuo Dong (Baylor College of Medicine, Houston, TX)
KG-1	ATCC
MOLM13	gift from Dr. Stephen Gottschalk (St. Jude Children's Research Hospital, Memphis, TN)
K562	ATCC
U266	ATCC
SK-BR-3	ATCC
MDA-MB-468	ATCC
INA-6	gift from Dr. Renate Burger (University of Kiel, Kiel, Germany)
RPMI 8226	ATCC
HS-5	ATCC
TSC2-null mouse embryonic fibroblasts and littermate controls	gift from Dr. John Blenis (Weill Cornell Medical School, New York, NY)
REDD1-null mouse embryonic fibroblasts and littermate controls	gift from Dr. Leif Ellisen (Massachusetts General Hospital, Boston, MA)
eIF2α kinase-null mouse embryonic fibroblasts and littermate controls	gift from Dr. Seiichi Oyadomari (Tokushima University, Tokushima, Japan)

Cells were grown in a humidified 37^{o}C incubator with 5% CO2, in medium recommended by the supplier and supplemented with FBS and penicillin/streptomycin and L-glutamine. INA-6 cells were supplemented with interleukin 6 (1 ng/mL, Peprotech, Rocky Hill, NJ)

Supplemental Table 2. Individual mouse information for patient derived xenografts

Sex	Age at tail vein injection (weeks)	Treatment	Weight on day of AML injection (g)	% PB blasts at engraftment check prior to death	Day of euthanasia/death after AML injection	Censored (Y/N)	% PB blasts at euthanasia	% BM blasts at euthanasia	% Spleen blasts at euthanasia	Spleen weight at euthanasia (mg)	Plasma AQ concentration at euthanasia
F	7.3	AQ	20.5	51.59	153	N	51.59	98.41	83.28	220	16.0µM
F	7.3	AQ	20.7	N/A	5	Y	unknown	unknown	unknown	unknown	unknown
F	8.3	AQ	17.9	68.3	155	N	75.34	98.96	85.21	558	27.9µM
F	8.3	AQ	18.3	65.32	155	N	66.65	98.73	85.65	437	unknown
F	8.3	AQ	19.9	62.33	174	N	81.83	97.85	61.75	unknown	unknown
M	8.3	AQ	21.5	54.31	163	N	69.32	99.36	86.45	462	unknown
M	8.3	AQ	21.9	N/A	19	Y	unknown	unknown	unknown	unknown	unknown
M	8.3	AQ	23.0	40.48	163	N	63.49	98.75	88.12	401	unknown
M	9.0	AQ	22.1	0.16	102	Y	unknown	unknown	unknown	unknown	unknown
F	7.3	VC	18.3	75.49	116	N	unknown	unknown	unknown	205	unknown
F	7.3	VC	20.0	57.05	121	N	71.68	97.57	85.53	306	unknown
F	7.3	VC	22.2	46.32	121	N	31.52	98.68	77.66	269	unknown
F	8.0	VC	18.4	38.58	120	N	63.56	98.71	83.78	265	unknown
F	8.0	VC	22.9	52.47	120	N	84.36	97.95	78.03	248	unknown
M	8.0	VC	22.0	40.35	134	N	47.33	98.47	76.1	354	unknown
M	8.3	VC	22.0	45.96	134	N	35.93	96.38	71.11	336	0µM
M	9.0	VC	22.5	0.85	72	Y	unknown	unknown	unknown	222	unknown
M	9.0	VC	23.5	48.17	132	N	unknown	unknown	unknown	287	unknown

Supplemental Table 1: AQ= atovaquone (200mg/kg/day by oral gavage). VC= vehicle control daily oral gavage. Shaded rows indicate atovaquone-treated mice.

Supplemental Table 3: primers used for real-time PCR

Gene	**Forward (5'→3')**	**Reverse (5'→3')**
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
ATF4	CCAACAACAGCAAGGAGGAT	GTGTCATCCAACGTGGTCAG
BIP	CACAGTGGTGCCTACCAAGA	CAGTCAGATCAAATGTACCCAGA
Hsp90B1	AACGGGCAAGGACATCTCTA	CGTCGAAGCATGTCTCTGAT
DNAJC3	CATCTTGAATTGGGCAAGAAA	AGCCCTCCGATAATAAGCAA
HERP	GCGACTTGGAGCTGAGTGG	CCAACAACAGCTTCCCAGAAT
Erp72	AGCAGGTTTGATGTGAGTGG	TTCTCTGACCTTGGCAACAA
EDEM1	GTGAAAGCCCTTTGGAACCT	AGGCCACTCTGCTTTCCAAC
Spliced XBP1	CTGAGTCCGCAGCAGGTG	ACTGGGTCCAAGTTGTCCAG
ERdj4	TTTCACAAGTTGGCCATGAA	AAGCACTGTGTCCAAGTGTATCA
SEC61A1	AGCAGCAGATGGTGATGAGA	CCTAGGAAGTCAGCCAGGAC