Supplementary Materials and Methods:

Supplemental Methods

Coefficient of Variability Measurements

Robust coefficient of variability measurements were determined using the BD Diva software. To generate a priming heterogeneity metric independent of average priming, we calculated the log-transformed area under the curve of the robust coefficient of variability at different Bim concentrations. For cell lines, we used a 2 fold Bim titration. Therefore the for each peptide concentration doubling, we essentially measure how the dispersion in apoptotic priming variability changes. This results in a metric that is independent of average priming which would not hold true for area under the curves of linear Bim concentration. For primary AML tumors, we used approximately a half-log Bim titration owing to limited sample quantity. Alternatively, the maximum dispersion at any Bim peptide concentration correlates with the area under the curve measurement, however the area under the curve measurement presents less sampling bias compared to choosing the maximum dispersion.

Live Cell Imaging

Live cell imaging of Patu8902 and SU86.86 pancreatic cells was performed in glass bottom MatTek dishes. Tubes were connected through artificially made holes in the top of MatTek dishes to facilitate washes. For the duration of live cell imaging, cells were exposed to 5% CO2 and kept at 37 C using chamber encapsulating the entire microscopy stage. Cells were imaged every 15 minutes. Multiple fields were imaged, and after a suitable number of observed divisions (generally 6 hours for both cell lines), cells were washed with PBS, then with BH3 profiling buffer, and then BH3 profiling buffer with digitonin to permeabilize cells, and synthetic BH3 peptides and TMRE to measure MOMP. After peptide addition, the imaging frequency was increased to once every 2-3 minutes. MOMP was complete across the entire dish within 90 minutes. We empirically found that 0.5 uM of synthetic Bim peptide produced complete MOMP in SU86.86 cells within 90 minutes, and 1.5 uM of synthetic Bim peptide was required for Patu8902 cells.

Images were exported and analyzed in Metamorph. Sister cell pairs were visually determined. TMRE traces were obtained by quantifying loss of TMRE in the whole cell. In some instances, the whole dish needed to be re-focused after washing potentially owing to physical perturbations associated with washes and peptide addition, thus delaying the initial imaging of TMRE accumulation in mitochondria. TMRE traces were fit in Prism, and half-times were quantified using a dose response curve. In instances where we could visualize an increase in TMRE fluorescence right after TMRE addition to cells, these points were excluded from the dose response curve fit. Sister cell videos were produced using Image J.

Cell Viability Measurements

AML cell lines were treated with increasing concentrations of Etoposide (Sigma). Cell viability measurements of AML cell lines was performed using TMRE (a measurement of MOMP) at 50 nM for and Hoechst 33342. Cells were stained for 30 minutes prior to flow cytometry. Since all cells that were TMRE positive were also and Hoechst 33342positive, and since MOMP precedes

DNA degradation during apoptosis, we used the proportion of TMRE positive cells as a measurement of cell death. The robust coefficient of variation provided a reasonable measurement of the dispersion of the population (i.e. whether the population was mostly TMRE positive, TMRE negative, or split), we used this as a measurement of frank apoptotic heterogeneity.

FACS Sorting and Imaging of Intermediate Cells

BH3 Profiles of Panc8902 was performed at 5 uM of Bim peptide for 1 hour. Cells were fixed and stained for cytochrome c, and cells that had intermediate levels of priming were sorted. Cells were pre-stained with Mitotracker Orange (1 uM for 30 minutes, Life Technologies) to facilitate marking of mitochondria within cells. Similar protocols were followed for staining of primary AML cells. Sorted cells were then placed on glass slides and immediately imaged using a Yokogawa Spinning Disk Confocal Microscope with a 60x objective. In some instances, 3D stacks of cells were imaged. Z-stack movies were imaged at 200nm, and interpolated in Image J.

AML Clinical Specimens

Complete remission was defined by <5% blasts and recovery of neutrophil and platelet counts after standard induction chemotherapy. Samples were collected, ficolled and frozen in 10% DMSO and 90% FBS in liquid nitrogen from between a few months to several years.

Calculation of Effective Concentrations

Using Bim titrations of AML primary samples, we fit data points to a 4-parameter dose respose curve in GraphPad Prism (nonlinear regression). Using the hill slope and the calculated EC-50 we calculated the EC-X (where X is any other response cutoff).

BH3 Profiling and Intracellular Stains

Following conventional FACS based BH3 profiling as described in the main text, we added antibodies against internal mitochondrial proteins. Antibodies are listed below. Cytochrome c was stained along with a single Bcl-2 protein. For Bax, Bak, Bcl-xl, Mcl-2, we used primary antibodies followed by a rabbit secondary. This does not label our cytochrome c primary conjugated antibody. Bcl-2 and MnSOD were primary conjugated antibodies. Owing to limited sample, we only performed BH3 profiles at 0.25, 0.5 and 1 uM of the Bim peptide, and selected the concentration with the maximum cytochrome c dispersion for analysis. In 2 additional samples, we did not see substantial MOMP at these concentrations, and these were obviously not analyzed. Data was analyzed on BD Diva software, FCS Express and Excel. The ratio of immunofluorescence in unprimed and primed subpopulations was quantified as described in the main text.

Antibodies: Bcl-XL (2764S, Cell Signalling), anti-Rabbit Alexa 555 (A21430, Life Technologies), CD45-V450 (642275, BD), Cytochrome c Alexa 488/647 (560263, 558709, BD), Bcl-2 PE (556537, BD), MnSOD (S1450, Sigma)

FACS Sorting and BH3 Profiles

BH3 profiles for primary AML samples was performed as described above and in the main text using cytochrome c to measure MOMP. Owing to limiting sample of primary tumors, we only performed BH3 profiles at 2 concentrations (0.5 and 1 uM Bim). We sought to sort 10-15% of

the most unprimed cells and 40-50% of the most primed cells. These numbers were chosen to meet the requirements for whole exome sequencing (approximately 100 ng of genomic DNA). Isolated DNA was quantified using pico Green. Samples were submitted for sequencing at the Broad Institute using Exome Express. Briefly, libraries were constructed and sequenced on an Illumina Hi Seq 2000 using 76 bp paired end reads. Output from Illumina software was processed by the PICARD data-processing pipeline to yield BAM files (*1*). In addition to the three samples analyzed in the paper, we also analysed two other samples. One sample did not undergo MOMP even at 1 uM of Bim. A second sample produced low amounts of genomic DNA, had poor mean coverage and could not be sufficiently analyzed.

Genomic DNA was submitted for Whole Exome Sequencing (Exome Express – Broad Institute). Coverage ranged from (122X-200X). Fastq files were aligned to hg19 version of the human genome with BWA 0.6.2. Single nucleotide and small insertion and deletion calling was performed with samtools-0.1.18 mpileup and Varscan 2.2.3. Annovar was used to annotate variants, including population allele frequency in 1000 Genomes and the Exome Sequencing Project, and presence in COSMIC. Variants were filtered out if they had less than 15 reads, were more than 50 bp outside of the defined exome capture baits, had excessive strand bias, or excessive number of calls in the local region. Variants that were known common sterotyped mutations in MDS and AML were prioritized in the calling.

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Supplemental Methods

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Supplementary Fig. S6: Apoptotic Heterogeneity in AML Cells in Response to Etoposide.

Supplementary Fig. S7: Examples of Priming Heterogeneity in AML Patient Samples.

Supplementary Fig. S8: Comparison of Bim EC-50 in Treatment Naïve and Relapsed Patients**.**

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Supplementary Fig. S10: ROC Curves for Different Effective Concentrations of Bim.

Supplementary Fig. S11: Comparison of Bim EC-90 to clinical indicators of outcome in treatment naïve patients.

Supplementary Fig. S12: Scatter plots of Bcl-2 family immunofluorescence and apoptotic priming.

Supplementary Fig. S13. Gating controls and sorted AML subpopulations**.**

Supplementary Table S1. Characteristics of patients used in the study

Supplementary Movie S1: Confocal Z-stack of Patu8902 cells that have undergone intermediate cell-wide MOMP.

Supplementary Movie S2: Time lapse of a mother cell dividing into two sister cells followed by a BH3 profile.

Supplemental Movie Legends

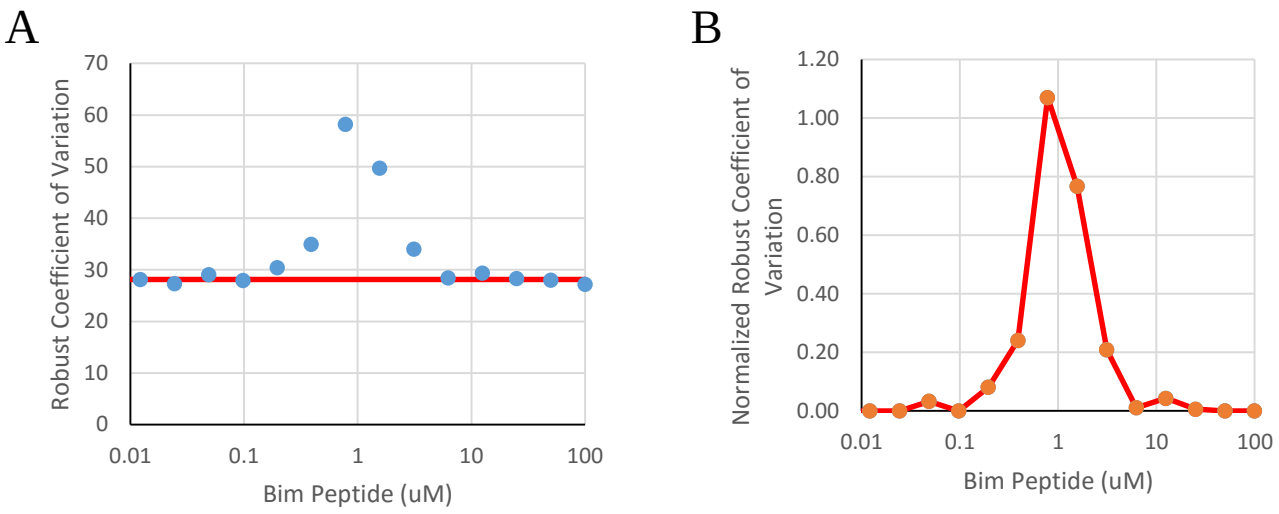
Movie S1: Confocal Z-stack of Patu8902 cells that have undergone intermediate cell-wide MOMP. Cytochrome c – Alexa 488 is shown in green and mitotracker Orange a marker of all mitochondria is shown in orange. Note that some mitochondria in this cell appear to have cytochrome c, while others do not.

Movie S2: Time lapse of a mother cell dividing into two sister cells followed by a BH3 profile. TMRE is added at the time that BH3 profile reagents are added creating an increase in TMRE fluorescence. Over time MOMP occurs in all cells and TMRE is lost. In the two sister cells at the center of this video, MOMP occurs at similar time and much later than other cells. Brightfield image in gray and TMRE is shown in orange.

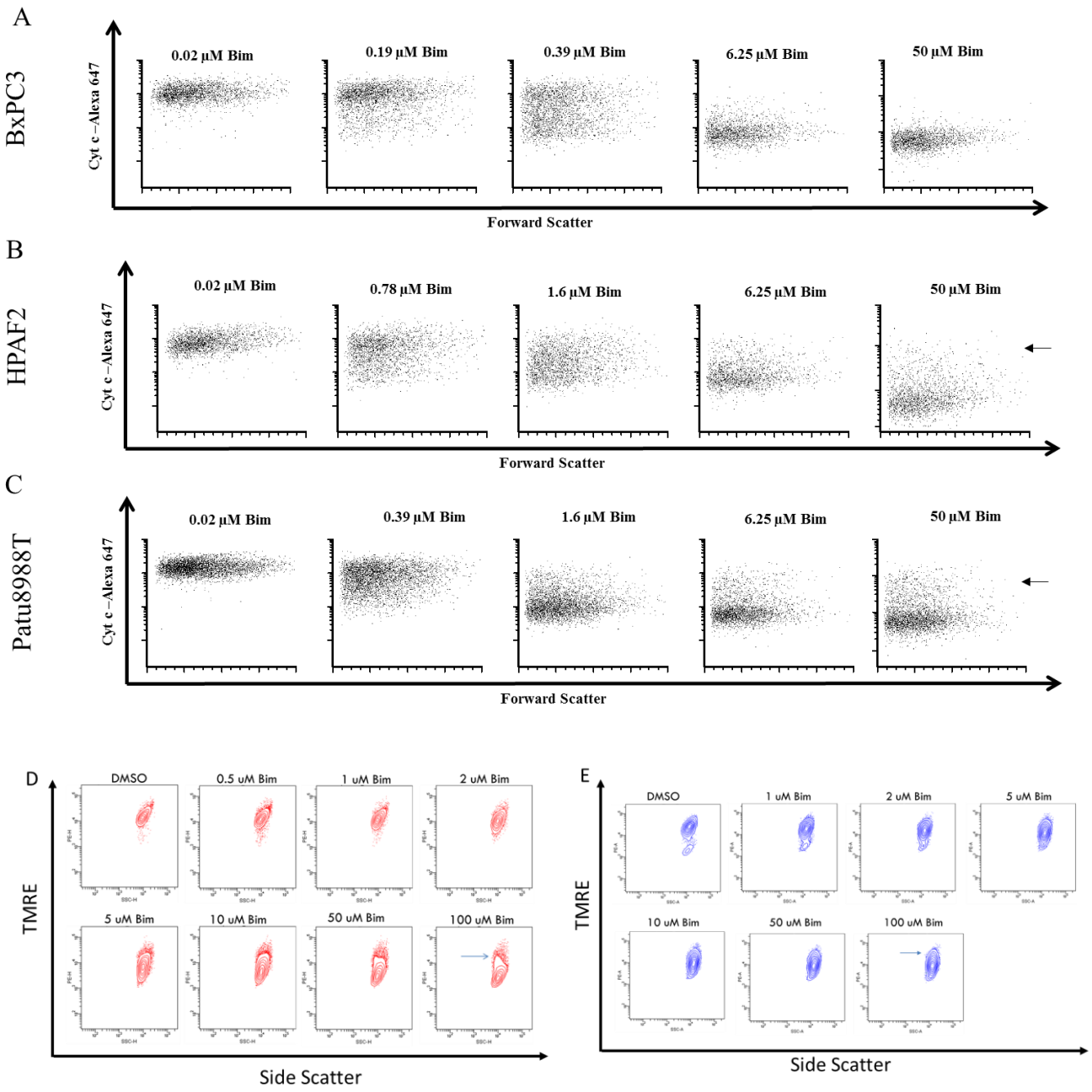
Supplemental References and Notes:

1. D. A. Landau, S. L. Carter, P. Stojanov, A. McKenna, K. Stevenson, M. S. Lawrence, C. Sougnez, C. Stewart, A. Sivachenko, L. Wang, Y. Wan, W. Zhang, S. A. Shukla, A. Vartanov, S. M. Fernandes, G. Saksena, K. Cibulskis, B. Tesar, S. Gabriel, N. Hacohen, M. Meyerson, E. S. Lander, D. Neuberg, J. R. Brown, G. Getz, C. J. Wu, Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* **152**, 714-726 (2013).

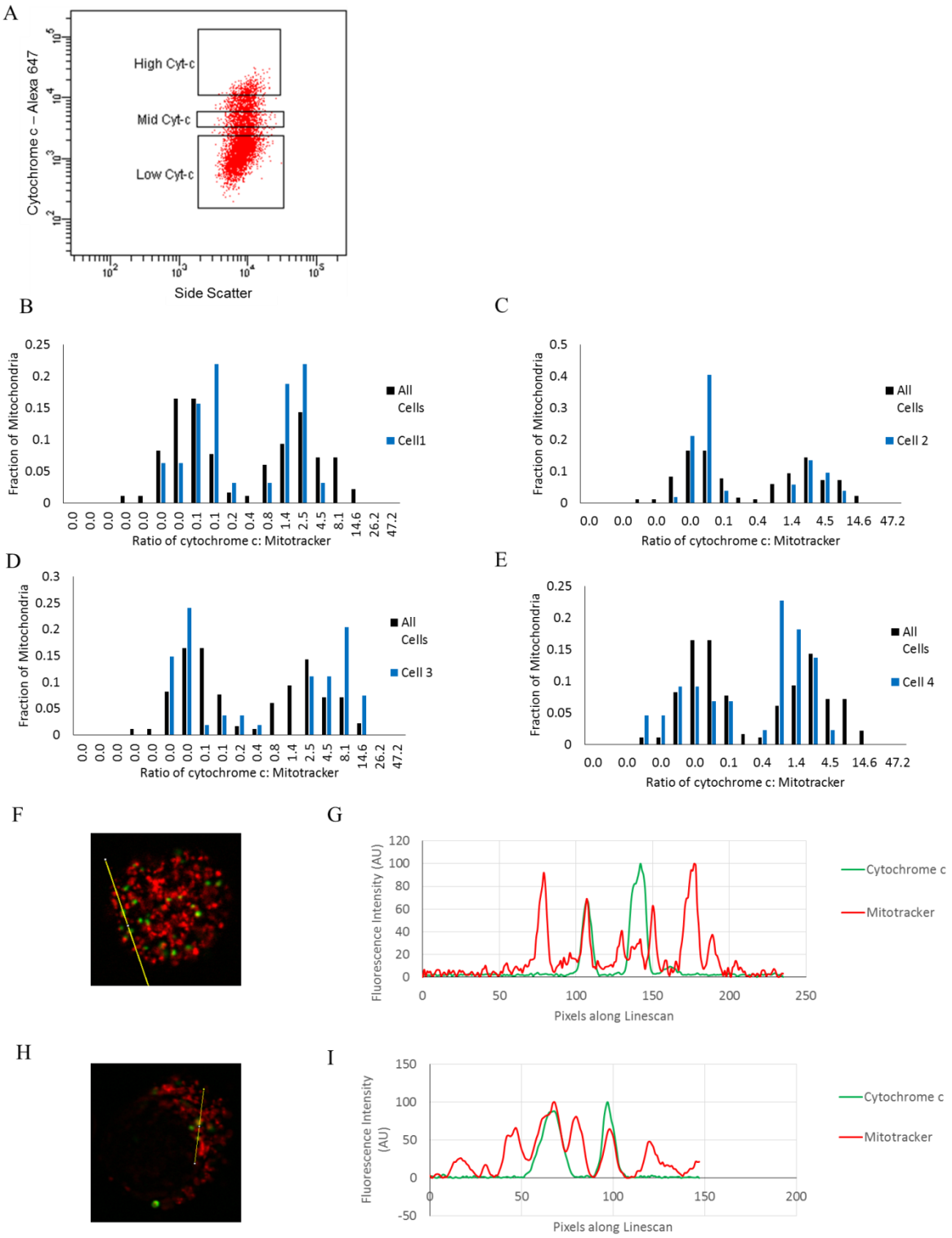
Supplemental Figures



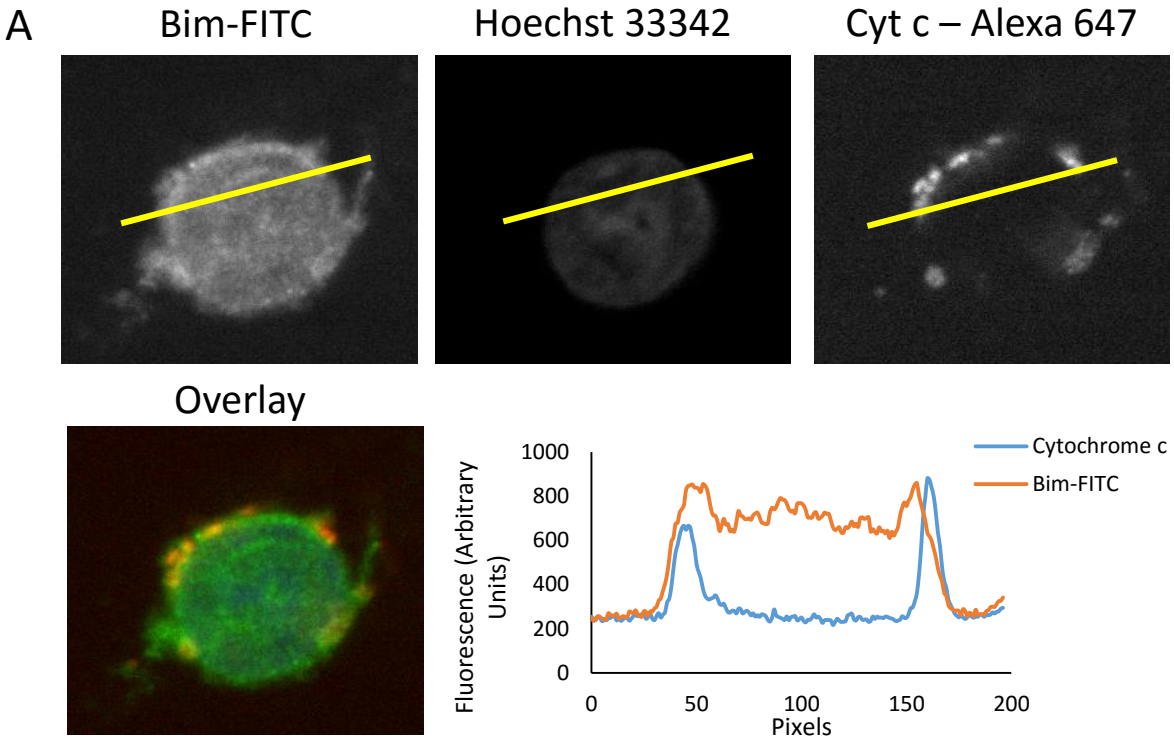
Supplemental Figure 1: Calculation of Mitochondrial Priming Heterogeneity. **(A)** The robust coefficient of variation is used as a measure of cytochrome c dispersion for increasing concentrations of the synthetic Bim peptide. The robust coefficient of variation is a better measurement of dispersion for distributions as in Fig. 1 that are not normally distributed. **(B)** To eliminated quantitative biases caused by differing baseline cytochrome c dispersion, we normalize the robust coefficient of variation at all peptide concentrations to its value without any peptide. Finally, to eliminate biases caused by the fact that different cell lines are differentially primed for apoptosis, we calculated the log transformed area under the curve of the normalized robust coefficient of variation.

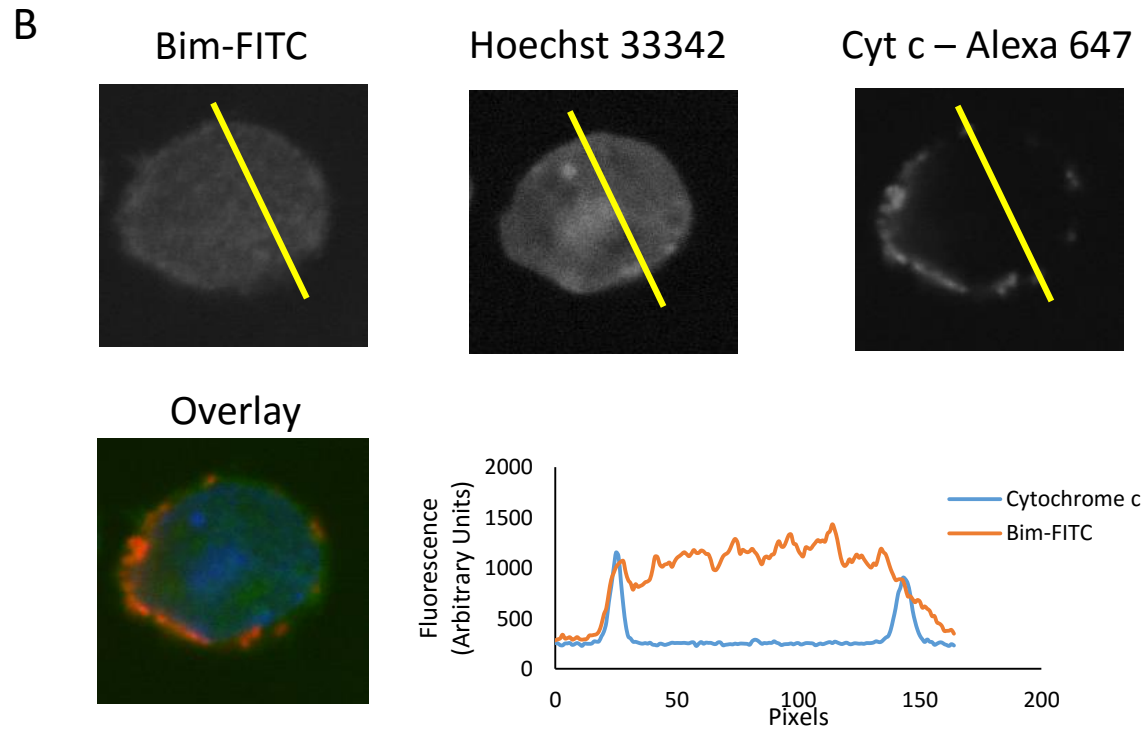


Supplemental Figure 2: Persistently Unprimed Apoptotic Subpopulations. In several solid tumor cell lines we observe that some cells within the cell line retain cytochrome c even at very high synthetic Bim peptide concentrations. These cells are potentially ultra-chemoresistant cells. **(A)** Example of the BxPC3 cancer cell line that lacks persistently unprimed cells. **(B & C)** The HPAF2 and PATU8988T pancreatic cell lines show priming heterogeneity at intermediate peptide concentrations, but also have several individual cells in the population that do not release cytochrome c even a high concentrations. These cells are marked by the arrows. (**D & E**) are different ovarian ascites samples that were BH3 profiled with increasing amounts of the Bim peptide. Note that in both tumors at high Bim concentrations, there are cells that are poorly primed for apoptosis. Ovarian tumor cells were identified based on EpCam staining. Experiments in cell lines were performed in duplicate.

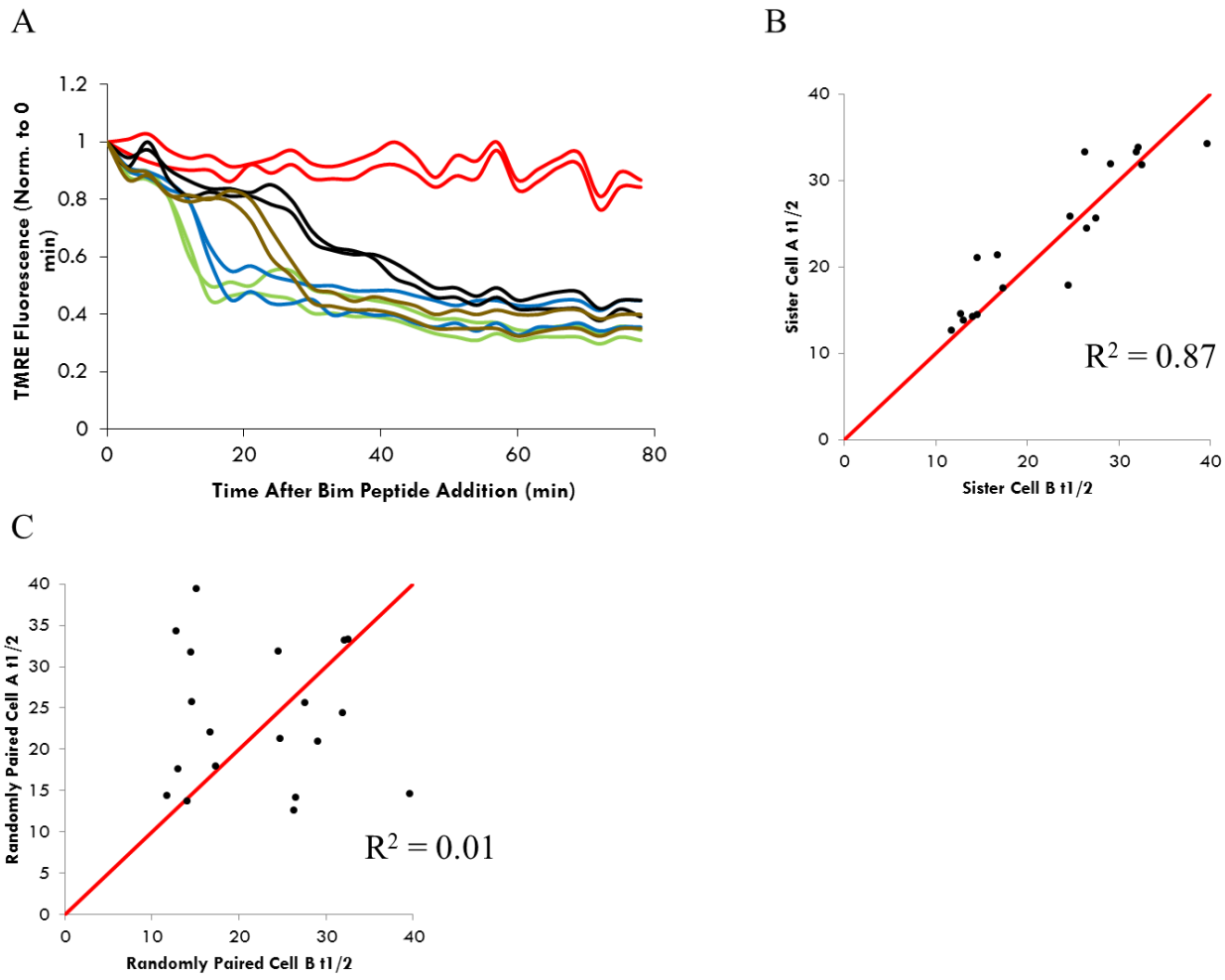


Supplemental Figure 3: Subcellular heterogeneity in apoptotic priming. (A) FACS scatter plot of cytochrome c loss during BH3 profiling from a cell line. (**B-E**) Histogram of cytochrome c/mitotracker intensity loss across multiple individual Patu8902 cells showing a bimodal distribution of cytochrome c loss. The composite histogram of all cells is shown in black, and the histogram for individual cells is shown in blue. (F,H) Examples of images showing intermediate cytochrome c release Cytochrome c in green and mitoctracker in red. Images were acquired using confocal microscopy and a 60X objective. (G,I) Examples of line scans corresponding to lines shown in F and H.

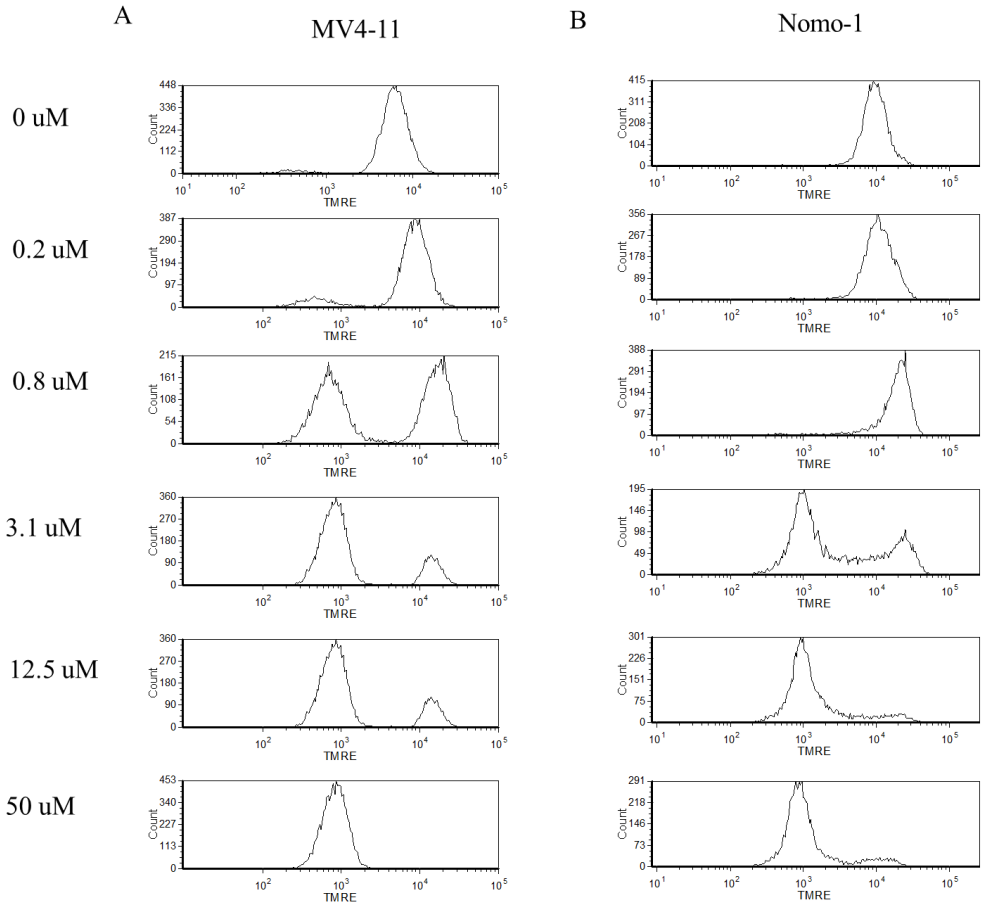




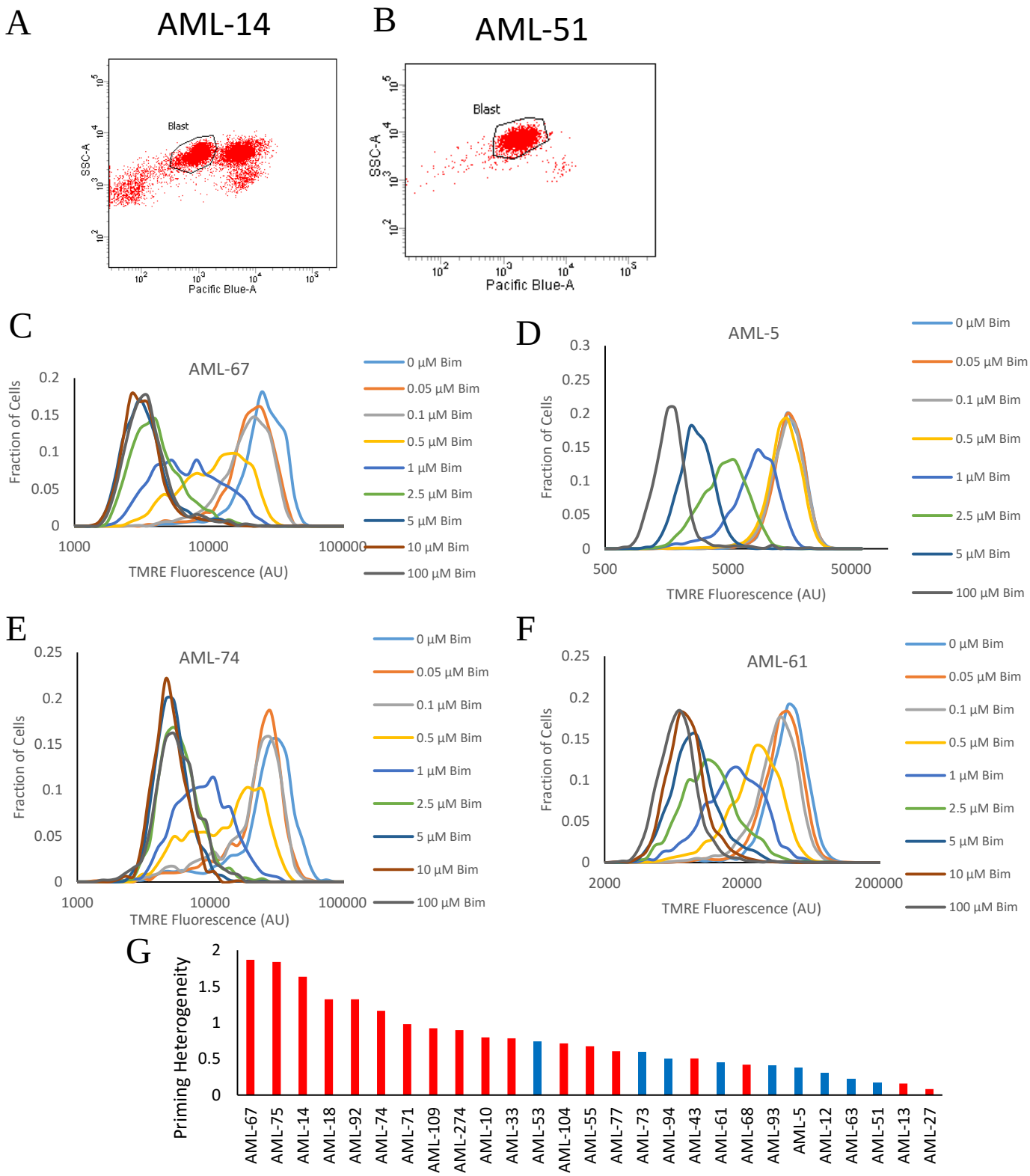
Supplemental Figure 4: Examples of peptide distribution in cells after permeabilization. BH3 profiles were performed using fluorescently conjugated peptides. Cells were fixed and stained with Hoechst 33342 and anti-cytochrome c – Alexa647, 5 minutes after permeabilization. Figures A and B represent different examples of quantified line scans across the cell. Images were acquired using confocal microscopy and a 60X objective. Images and line scans are representative of 10 cells.



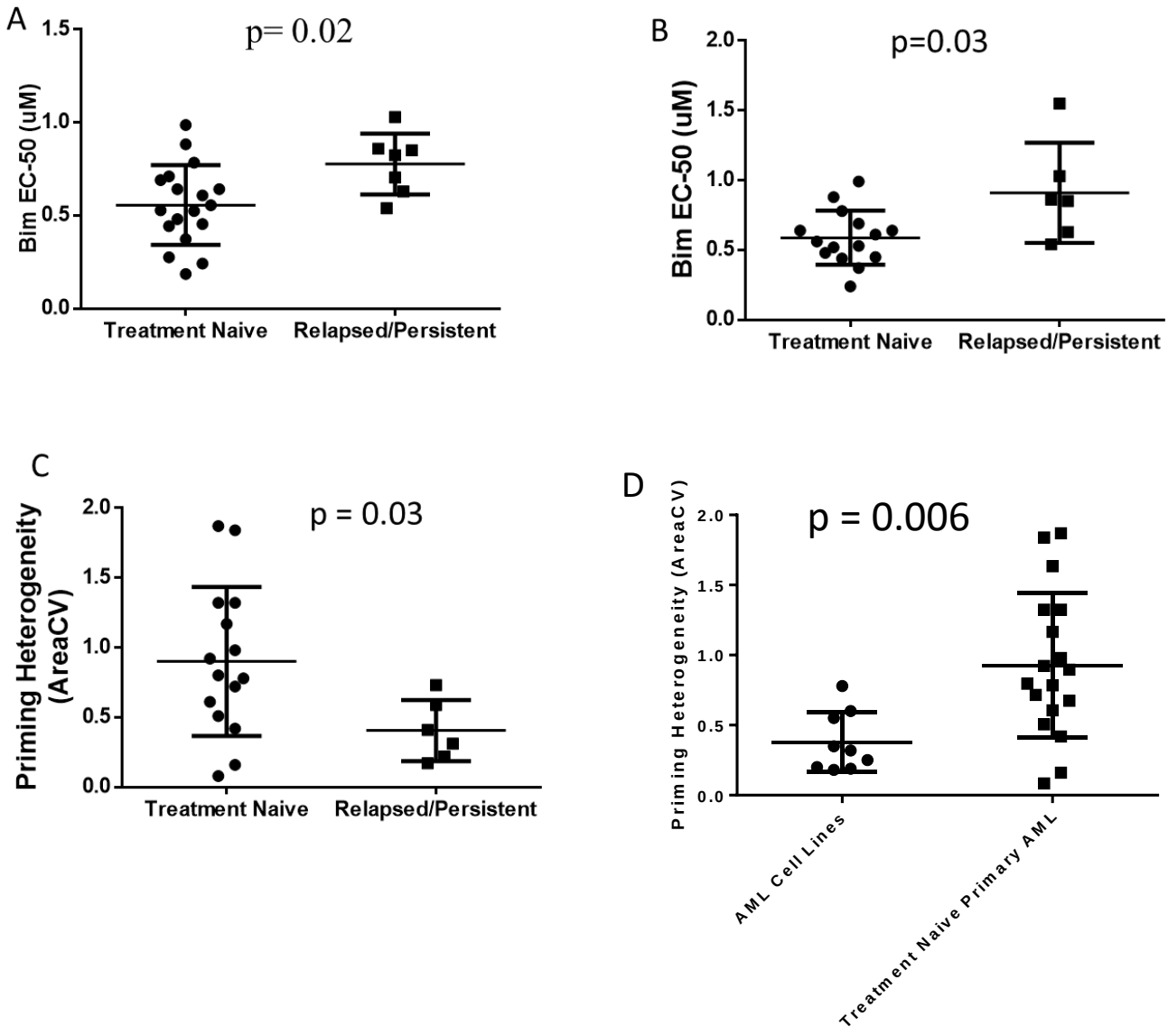
Supplemental Figure 5: Sister Cell Priming Heterogeneity in SU86.86 Pancreatic Cell Lines. A) Traces of TMRE loss in SU86.86 cells. Sister cells shown in similar colors. B) Correlation of half-times of sister cell TMRE loss ($R^2 = 0.87$). (C) Correlation of TMRE loss between randomly paired cells ($R^2 = 0.01$).



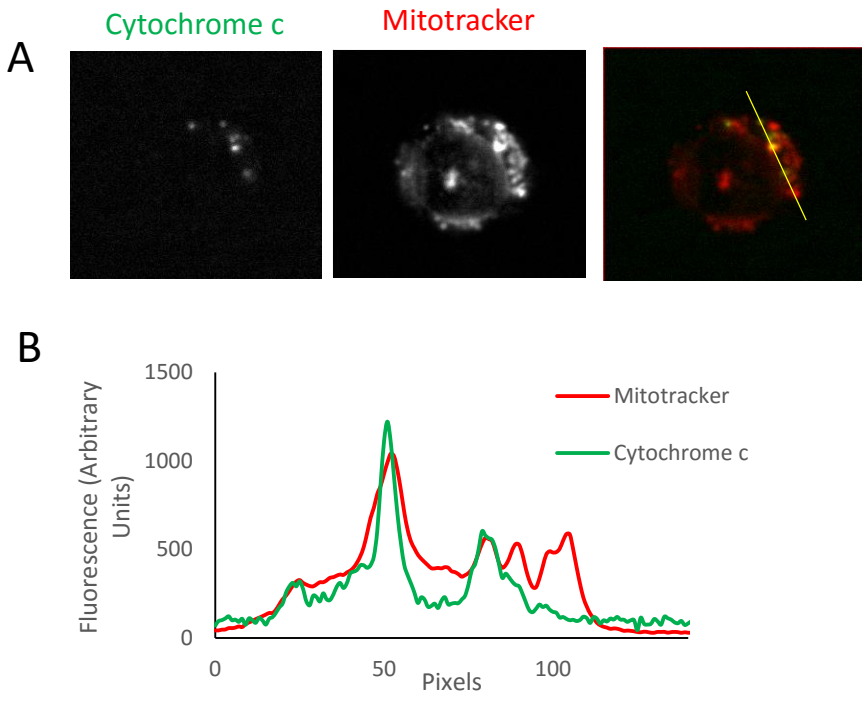
Supplemental Figure 6: Apoptotic Heterogeneity in AML Cells in Response to Etoposide. A) MV4-11 cells treated with increasing concentrations of Etoposide for 48 hours. Cell viability determined by retention of TMRE and flow cytometry. B) Cell viability of Nomo-1 AML cells. Data is representative of three experiments.



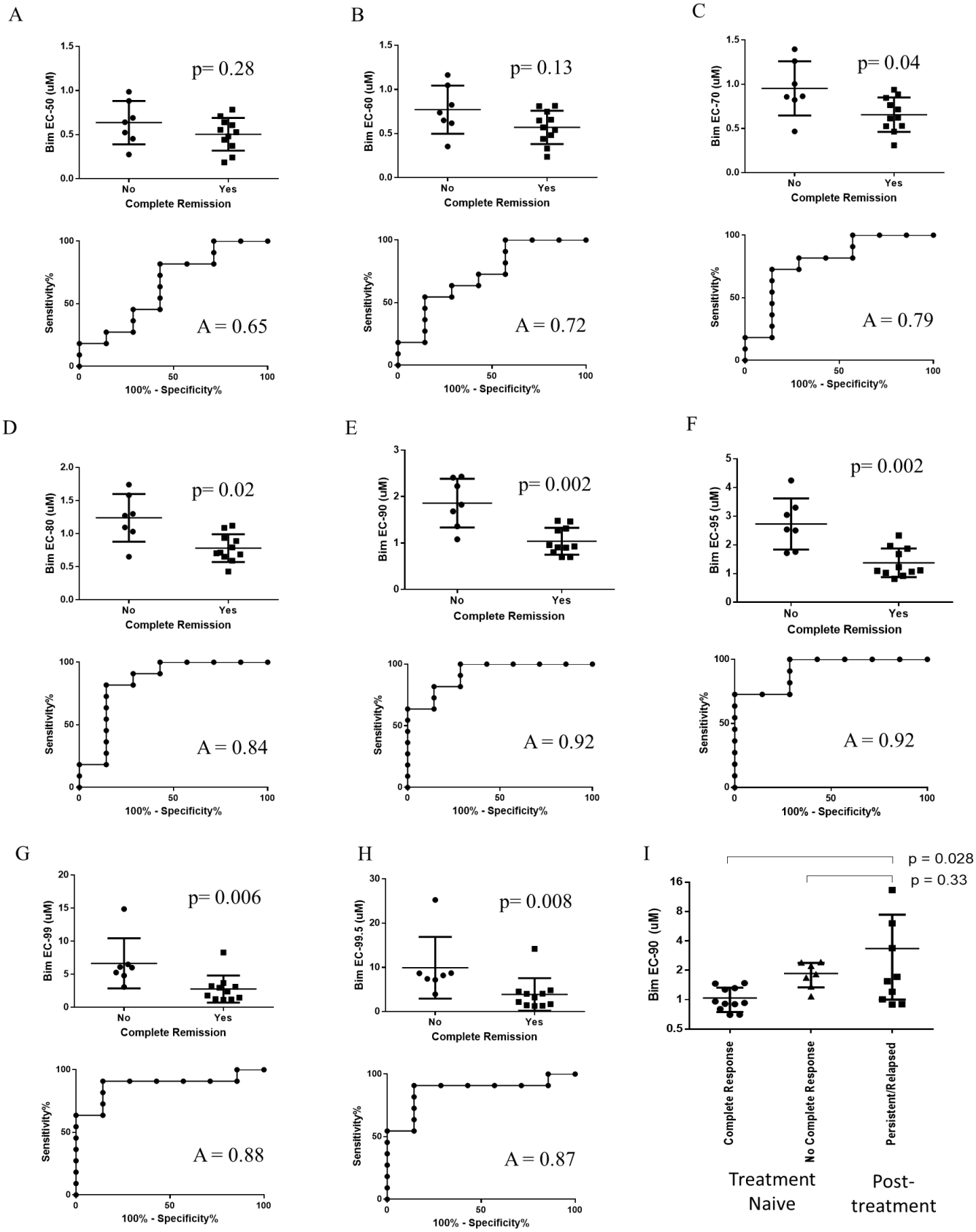
Supplemental Figure 7: Examples of Priming Heterogeneity in AML Patient Samples. (A,B) Example of gating strategy used to identify myeloblasts. CD-45-V450 staining is measured using thte Pacific Blue filter sets and plotted along the x-axis. Side scatter is plotted along the Y-axis. These tumors correspond to the tumors used in Figure 4A and B. C-F) Examples of histograms of TMRE loss in response to increasing concentrations of the synthetic Bim peptide . C,E are from treatment naïve patients, and D,F are from pre-treated relapsed or persistent patients. G) Priming heterogeneity metric from all samples. Treatment naïve patients in red and pre-treated relapsed or persistent patients in blue.



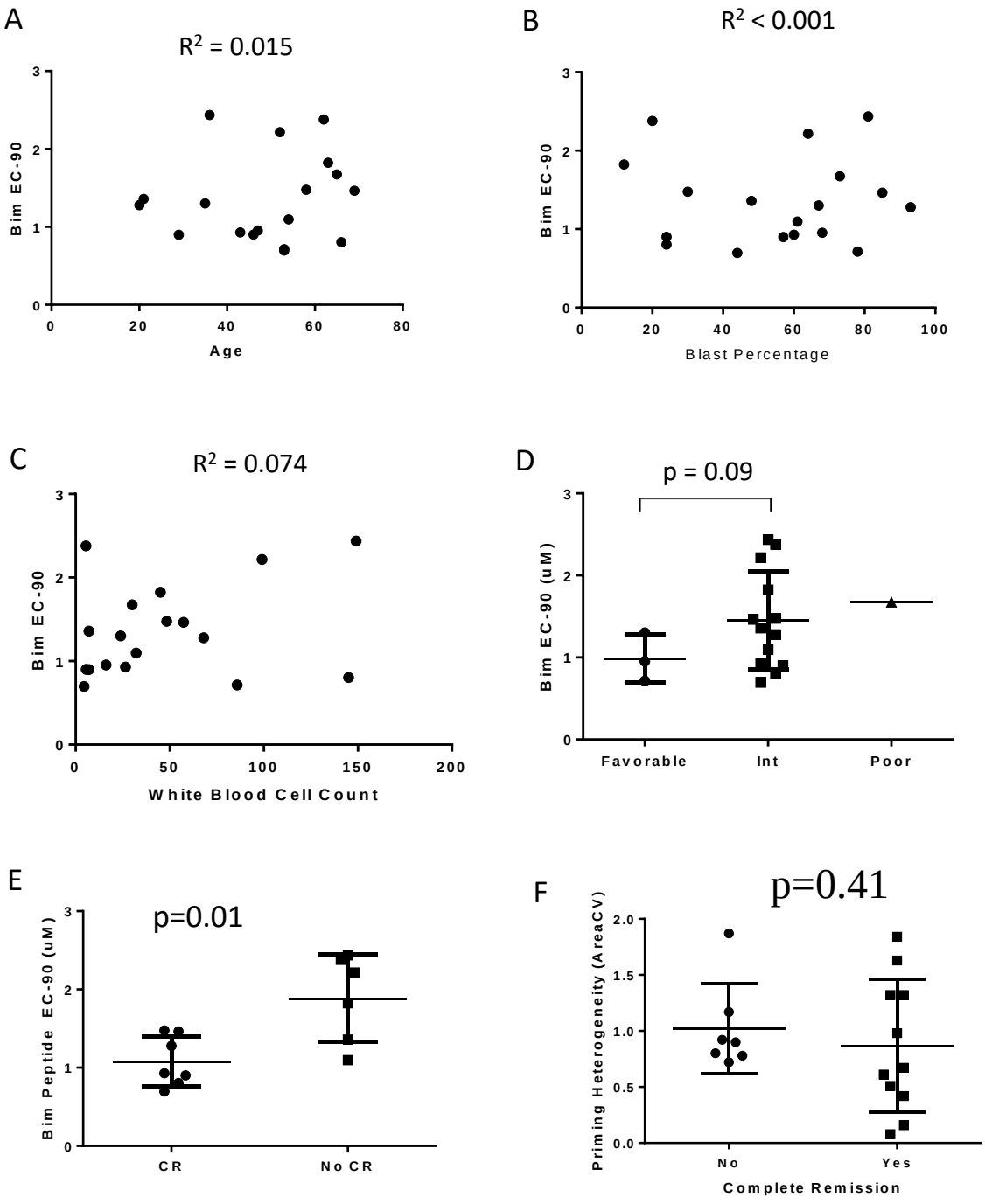
Supplemental Figure 8: Comparison of Bim EC-50 in Treatment Naïve and Relapsed Patients. **(A)** In Figure 4C, two relapsed/persistent patients have much higher EC-50 compared to all other tumors. After removing outlier relapsed patients from Figure 4, we still observe a significant difference in the EC-50 or treatment naïve and relapsed patients (p=0.02, Mann-Whitney test). (**B,C**) Most AML tumors in Figure 4 were extracted from the bone marrow. Conversely a few were extracted from the peripheral blood (3 of 18 treatment naïve tumors, and 3 of 9 relapsed/persistent tumors). To determine whether the disproportionate number of peripheral blood samples in relapsed/persistent tumors was not skewing our results, we compared Bim EC-50 and priming heterogeneity plots using only bone marrow cells finding significant difference in Bim EC-50 (p=0.03 , Mann-Whitney test), and in priming heterogeneity (p=0.03 , Mann-Whitney test) in treatment naïve and treated tumors. **(D)** Comparison of priming heterogeneity between treatment naïve AML primary tumors and AML cell lines indicates that there is more priming heterogeneity in primary tumors (p=0.06, Mann-Whitney test).



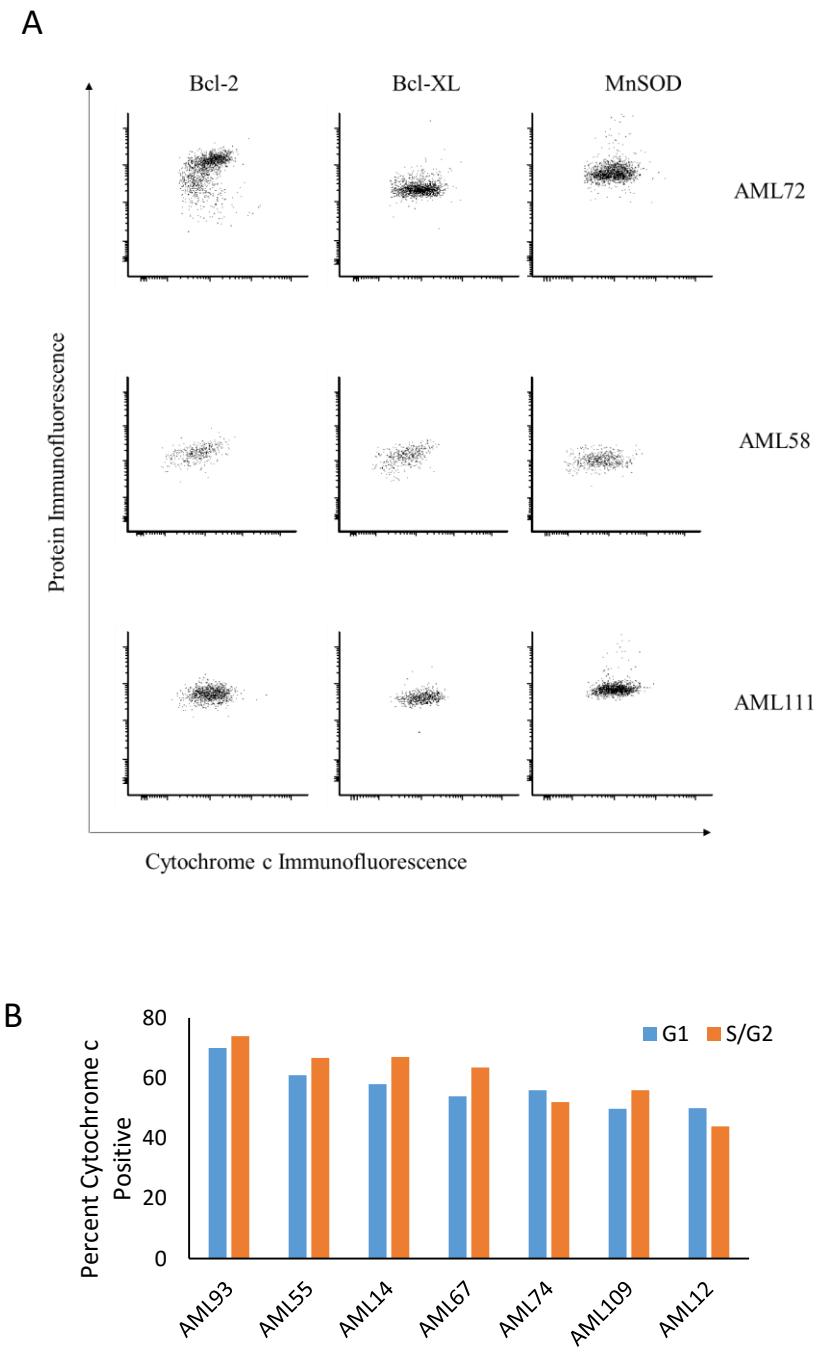
Supplemental Figure 9: Intracellular apoptotic priming heterogeneity in primary AML cells. Primary AML tumors were treated with 1 uM of the Bim peptide. Cells were sorted based on CD45 and side scatter. Images were acquired using confocal microscopy and a 60X objective. (A) Representative cell where there is cytochrome c release from some but not all mitochondria in the same cell in response to the Bim peptide (representative of 10 different cells). Cells were stained with cytochrome c – Alexa 488 (green), and cells were prestained with the mitochondrial dye Mitotracker Orange. (B) Quantification of the line in A indicates that cytochrome c is lost from some but not all mitochondria. (C) Quantification of the cytochrome c intensity to mitotracker orange intensity in 4 different cells shows a bimodal distribution indicating that cytochrome c loss is all or none.



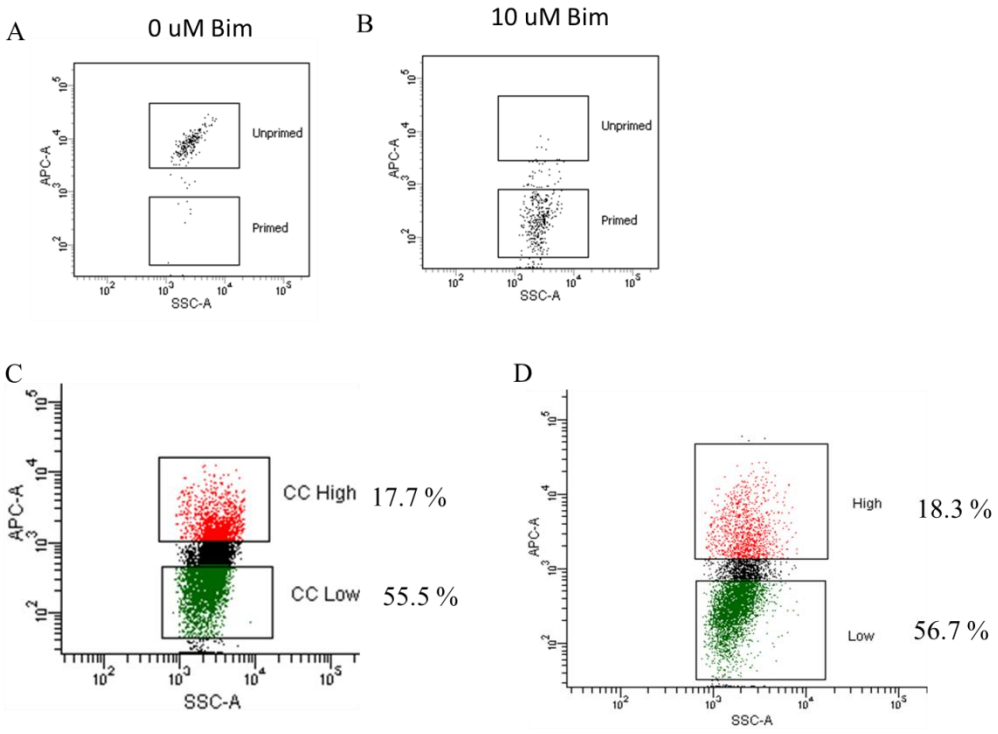
Supplemental Figure 10:ROC Curves for Different Effective Concentrations of Bim. (A) Top- Bim EC-50 values stratified according to patients with no response or complete response. Bottom – ROC Curve for the Bim EC-50 as a predictor of response. (B-H) Similar data for different Bim effective concentrations. (I) Comparison of Bim EC-90 in treatment naïve patients that undergo complete response, treatment naïve patients that undergo no complete response, and treated tumors from persistent or relapsed patients. Axis of panel I is in a log2 scale. All statistical tests are two-tailed Mann-Whitney t-tests.



Supplemental Figure S11: Comparison of Priming Heterogeneity and the Bim EC-90 to clinical indicators of outcome in treatment naïve patients. (A) Comparison of Bim EC-90 to age. (B) Comparison of Bim EC-90 to Blast percentage. (C) Comparison of Bim EC-90 with white blood cell count. (D) Comparison of Bim EC-90 to European Leukemia Net cytogenetic classifications. (E) Comparison of the Bim peptide EC-90 in treatment naïve patients with intermediate cytogenetic prognosis (p=0.01, Mann-whitney test). (F) Lack of correlation between priming heterogeneity and patient response. The overall heterogeneity in the treatment naïve patients doesn't correlate with complete remission in AML (p=0.41, Mann-Whitney). This is not surprising if only the most unprimed cells contribute to persistence



Supplemental Fig. S12: Comparison of apoptotic priming with molecular features. (A) Scatter plots of Bcl-2 family immunofluorescence and apoptotic priming. Cells were BH3 profiled and stained with cytochrome c to measure MOMP as well as BCL-2 and BCL-XL. Scatter plots in each row corresponds to the same patient. Quantification of protein expression in the most primed and unprimed cells is shown in Figure 6. (B) Comparison of priming (measured as cytochrome c positivity) to cell cycle status. Using the Hoechst 33342 DNA stain, cells were segregated into G1 and S/G2 phase. Apoptotic priming (as measured by cytochrome c positivity) was calculated for several samples indicating little or no difference between the phases of the cell cycle.



Supplemental Fig. S13. Gating controls and sorted AML subpopulations. (A,B) Gating controls used for AML567 (from Figure 6). Cytochrome c is plotted along the y-axis and measured using the APC filter set. Side scatter is plotted along the x-axis. When cells are treated with 0uM of the synthetic Bim peptide most cells are in the unprimed or cytochrome c + gate. Conversely when cells are treated with 10 uM of peptide, most cells are in the cytochrome c – or primed gate. (C) FACS sorting plot of AML 453. Cytochrome c staining measured using the APC channel on the y-axis, with side scatter on the x-axis. Cytochrome c high cells (unprimed) and cytochrome c low (primed) cells were sorted. (D) Similar sorting plot for AML 157.

AML #	Source	Diagnosis	Status	Complete Remission	Cytogenetics	FLT-3	NPM1	Priming Heterogeneity Area CV	Bim EC-50	Hill Slope
10	BM	AML	Pre-treatment	N	normal	+	+	0.80	0.88	-2.38
74	BM	AML	Pre-treatment	N	normal	-	-	1.17	0.45	-1.32
67	BM	AML	Pre-treatment	N	complex	+	-	1.87	0.52	-1.88
109	BM	AML	Pre-treatment	N	normal	unknown	unknown	0.92	0.99	-2.44
104	BM	MDS/AML	Pre-treatment	N	trisomy 21	unknown	unknown	0.72	0.69	-2.26
33	BM	AML	Pre-treatment	N	normal	-	-	0.78	0.64	-2.92
274	PB	AML	Pre-treatment	N	normal	-	-	0.90	0.28	-1.61
14	PB	AML	Pre-treatment	Y	normal	-	+	1.63	0.19	-1.66
71	BM	AML	Pre-treatment	Y	inv(16), del(7q)	unknown	unknown	0.98	0.48	-3.50
55	PB	AML	Pre-treatment	Y	normal	+	+	0.67	0.71	-3.04
18	BM	AML	Pre-treatment	Y	normal	+	+	1.32	0.56	-2.66
68	BM	AML	Pre-treatment	Y	normal	-	-	0.42	0.37	-2.47
43	BM	AML	Pre-treatment	Y	(8;21)(q22;q22)	-	unknown	0.51	0.24	-1.30
77	BM	AML	Pre-treatment	Y	t(8;21), AML1-ETO	-	-	0.61	0.78	-10.98
18	BM	AML	Pre-treatment	Y	normal	-	unknown	1.32	0.53	-5.26
13	BM	AML	Pre-treatment	Y	normal	-	-	0.16	0.61	-5.22
75	BM	AML	Pre-treatment	Y	normal	+	+	1.84	0.44	-4.80
27	BM	AML	Pre-treatment	Y	normal	-	-	0.08	0.64	-2.63
51	BM	AML	Post Treatment - Primary Resistant	NA	t(11;19)(q23;p13.1)	+	-	0.17	0.54	-5.71
63	BM	AML	Post Treatment - Relapsed	NA	del(7)	-	-	0.22	1.55	-2.82
5	PB	AML	Post-Treatment Relapsed	NA	trisomy 11	-	-	0.38	0.82	-3.01
53	BM	AML	Post Treatment - Primary Resistant	NA	complex	unknown	unknown	0.73	1.03	-0.86
61	PB	AML	Post-Treatment Relapsed	NA	unknown	unknown	unknown	0.45	1.99	-1.97
12	BM	AML	Post Treatment - Relapsed	NA	inv(9)	+	+	0.31	0.86	-3.75
93	BM	AML	Post Treatment - Primary Resistant	NA	inv(6)(p13q22)	unknown	unknown	0.41	0.63	-4.67
73	BM	AML	Post Treatment - Relapsed	NA	normal	-	unknown	0.59	0.85	-6.28
94	PB	AML	Post Treatment - Primary Resistant	NA	trisomy 21	unknown	unknown	0.51	0.70	-8.99

Supplemental Table S1. Characteristics of patients used in the study

		CD3+	Unprimed	Primed
AML567	BCL 455nonfs del			0.06
	DNMT3A N879D	0.28	0.53	0.39
	IDH2 R140Q	0.32	0.51	0.43
	NRAS G12D		0.2	0.17
	PTPN11 E76K		0.06	
	PTPN11 S502L	0.05	0.12	0.18
AML453	DNMT3A R792fs(del)		0.44	0.43
	NPM1 L287fs(ins)		0.3	0.35
	TET2 Y1128X(stop)	0.04	0.44	0.42
AL157	BCORL1 L1361X		0.02	
	KIT D816Y		0.02	0.05
	NRASQ61K		0.32	0.35
	NRASQ12D		0.02	0.03

Supplemental Table S2. Variant Allele Fraction of Primed and Poorly Primed Cells.