Supplemental information

Supplemental Methods

Quantitative PCR

RNA was extracted from samples using TRIzol (Invitrogen) and cDNA synthesised using random hexamers and the MMLV-Reverse Transcriptase RNase H Minus, Point Mutant enzyme (Promega). Real time TaqMan Gene Expression Assays for AEP and β2 microglobulin (β2m) were performed using on-demand reagents and the Applied Biosystems 7900HT Sequence Detection System (Applera). β2m expression was used as endogenous reference to normalise the data. The comparative cycle threshold ($\Delta\Delta C_T$) method was used to calculate fold differences in AEP expression using the HRC57 cell line as calibrator.

Microscopy

Active AEP was labeled by incubating SD1, REH and SupB15 cells (1-2 × 10^5) with a fluorescent cell-permeable activity-based probe (ABP) (gift from Dr. Matthew Bogyo, Stanford University, California) at a concentration of 1μM for 2 hours at 37^0C. In other experiments, SD1 cells were incubated with 100nM of the lysosomal probe, LysoTracker Red DND-99 (Molecular Probes, Invitrogen) for 45 minutes at 37^0C. Following live cell labeling, cells were resuspended in fresh media, cytospins (Shandon Cytospin 3 Cytocentrifuge) were prepared on microscope slides and cells were fixed with 10% neutral buffered formalin (Sigma-Aldrich) for 15 minutes. After two washes with PBS, fixed cells were mounted in fluorescence mounting medium (Dako) containing DAPI (1:1000, Roche). In some experiments, ABP-labeled formalin-fixed cell cytospins were permeabilized with 0.25% Triton X-100 (Sigma-Aldrich) in PBS for 15 minutes and serially incubated for 1 hour at room temperature with 10% goat serum (Vector Laboratories Ltd.) in 1% BSA, 1:100 dilution of a primary antibody to lysosome-associated membrane protein 1

(LAMP1) (H4A3 clone, BD Biosciences, Oxford, UK) and 1:1000 dilution of a secondary AlexaFluor488-conjugated goat anti-mouse IgG (Molecular Probes, Invitrogen) with intervening PBS washes (three times per wash cycle) and in parallel with appropriate controls. Lysotracker Red labeled formalin-fixed cell cytospins were permeabilised and similarly serially incubated with 10% rabbit serum (Vector), 1:100 dilution of an anti-human AEP primary antibody (R&D Systems) and 1:1000 dilution of an Alexa-Fluor488-conjugated rabbit anti-goat IgG (Molecular Probes, Invitrogen). Using a Zeiss 7D Axiovert 200M microscope, high-resolution images were acquired with oil immersion objectives FLUAR 40×/1.30NA and alphaPLAN-FLUAR 100×/1.45NA (with a 1 – 1.6× optivar) and a cooled EMCCD low-light camera (Photometrics Cascade II:1024 Megapixel) operated by the Metamorph FRAP-Advanced Imaging software (v7.5.2.0, Molecular Devices). In some instances, images were acquired on an Olympus BX51 microscope using UPlan Apo 40×/1.00NA and 100×/1.35NA oil immersion objectives and a cooled CCD camera (Olympus Colour View 12) operated by the AnalySIS image acquisition software (Soft Imaging Systems GmbH). Acquired images were later processed using the Adobe Photoshop CS3 software (v10.0.1).

Supplemental Figure Legends

Supplemental Figure 1

A. AEP is overexpressed by the pre-B lymphoblast cell line SD1 and the normal mature B-lymphoblastoid cell line, HRC57, while the two pre-B lymphoblast cell lines, REH and SupB15 show low AEP expression.

Representative immunoblot showing AEP expression in whole cell lysates. Relative AEP RNA expression using the $\Delta\Delta C_T$ approximation method is shown in the right panel.

B-D. Serial inhibition of cellular proteases using a panel of protease inhibitors identifies the cysteine proteases, AEP and CTSB, as the enzymes responsible for degrading ASNase.

Figure 1B in the main text is derived from these panels. Immunoblotting of representative digests following incubation of whole cell lysates from the pre-B lymphoblast cell lines, SD1 (Supplemental Figure 1B) and REH (Supplemental Figure 1C), with ASNase alone or together with a protease inhibitor cocktail, its individual components (Leupeptin, E-64, Pepstatin, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride [AEBSF], Bestatin, Aprotinin), with escalating concentrations of the metalloprotease inhibitor, EDTA (Supplemental Figure 1D) and with the AEP-specific inhibitor MV026630 (AEPi) alone or in combination with either a non-selective (Leupeptin or E-64) or selective (CA074Me [CTSBi]) CTSB inhibitor. DMSO served as vehicle control.

Supplemental Figure 2

AEP expression is variable in primary pre-B lymphoblasts while CTSB expression is relatively uniform. Figure 2B in the main text is derived from these panels. Representative immunoblots for AEP (upper panel), CTSB (middle panel) and β-actin (loading control, lower panel) in whole cell lysates of primary lymphoblasts (UPN 1-12, see Supplemental Table 3) are shown. The 56kDa and 36kDa bands represent the inactive pre-pro and mature active forms of AEP. The 25kDa and 20kDa bands represent the heavy chains of mature active CTSB dimers while the 50kDa bands represent the inactive propeptide. Also shown are representative immunoblots of AEP and CTSB expression in the four cell lines, SD1, REH, SupB15 and HRC57 (extreme right, upper and middle panels respectively).

Supplemental Figure 3

ASNase is cleaved when incubated with whole cell lysates of primary lymphoblasts (UPN 1-12). The pattern of cleavage inhibition is determined by the combined expression of AEP and CTSB by blast cells.

Supplemental Figure S4

Figure 6 in the main text is derived from these blots. Immunoblots of recombinant WT and mutant ASNase proteins incubated with (+) or without (-) recombinant human AEP (upper panel) and HRC57 whole cell lysate (lower panel). The N24G mutant with the conserved D124 residue resists AEP cleavage, suggesting that the N24 residue in ASNase represents the primary AEP cleavage site ('* denotes a Vector-only control).

Supplemental Figure 5

A. The cell permeable fluorescent activity-based probe (AEP-ABP) is relatively specific for active AEP.

Upper panel: Live cell labeling of active AEP by AEP-ABP in SD1 cells. Cytospins were formalin-fixed and also incubated with control non-specific mouse IgG (5μg/ml, equivalent to the anti-LAMP1 antibody dilution) to demonstrate specificity of LAMP1 antibody staining (Magnification ×40, Scale bar 5μm).

Middle panel: Probe specificity was examined by incubating SD1 cells with AEP-ABP alone (left) or after prior incubation with cell-permeable AEPi (1μM, 8 hours) (right) (Magnification ×40).

Lower panel: Probe specificity is also demonstrated by observing minimal labeling in the low AEP-expressing cell lines, REH and SupB15 (images acquired with a 20×/0.5NA dry objective on an Olympus BX51 microscope).

B-C. SD1 cells show distinct intracellular AEP staining patterns with a relative excess of mature active AEP towards the cell periphery.

Immunostaining for AEP (Supplemental Figure 4B) and AEP-ABP labeling (Supplemental Figure 4C) in SD1 cells suggest distinct patterns of intracellular AEP localization with diffuse perinuclear granular staining and bright peripheral punctate staining (Magnification ×40, scale bar 5μm).

D. Active AEP in SD1 cells is localised in a LAMP1-positive compartment. Immunostaining for LAMP-1 in SD1 cells labelled with AEP-ABP (RFP) (Magnification ×100 with optivar magnification 1.6).

Supplemental Table Legends

Supplemental Table 1

Quantification of ASNase activity using the Indooxine method (1) in plasma samples collected 7-10 days post ASNase administration in 44 children with acute lymphoblastic leukaemia treated on regimens A, B (for Standard and Intermediate risk disease) or C (for High risk disease) on the MRC ALL2003 treatment protocol. Four distinct trends were observed in plasma ASNase activity between the Induction and post-Induction phases of treatment. Plasma ASNase activity ≥ 100U/L was designated adequate.

Supplemental Table 2

Both AEP and CTSB inactivate ASNase. Loss of ASNase activity (MAAT activity assay) following digestion with either activated recombinant human AEP or CTSB. Experiments were performed in triplicates.

Supplemental Table 3

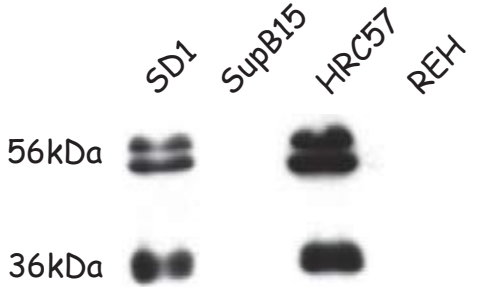
Karyotype data and relative AEP RNA expression levels in patients with ALL and in pre-B lymphoblast cell lines. Relative AEP RNA quantitation was performed using on-demand Taqman assays and calculated using the $\Delta\Delta C_T$ approximation method as described above.

Reference

(1) Lanvers C, Pinheiro JPV, Hempel G, Wuerthwein G, Boos J. Analytical validation of a microplate reader based method for the therapeutic drug monitoring of L-Asparaginase in human serum. Anal Biochem 2002;309: 117-126.

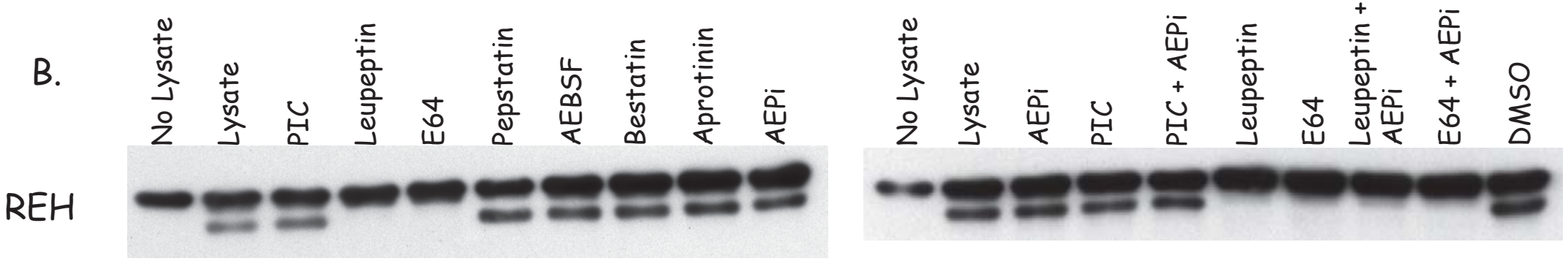
Supplemental Figure 1. Expression of AEP and degradation of ASNase in ALL cell lines

A.



Cell lines	Relative AEP Expression $2^{-\Delta\Delta CT}$
REH	0.19
SD1	1.62
SupB15	0.02
HRC57	1.00

B.



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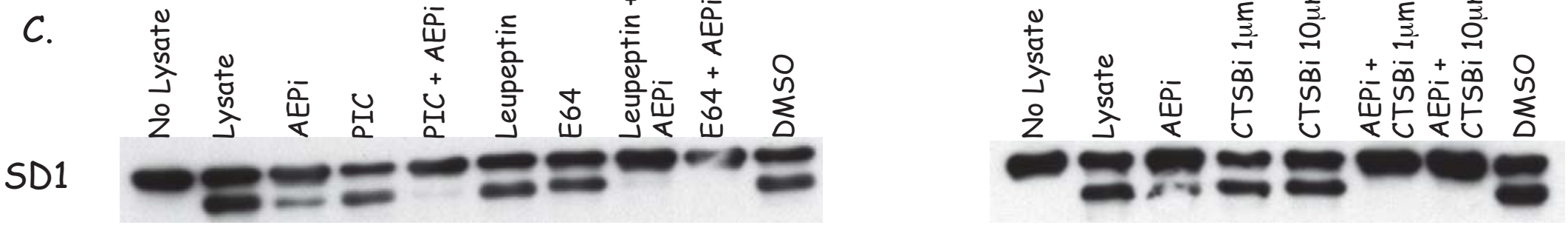
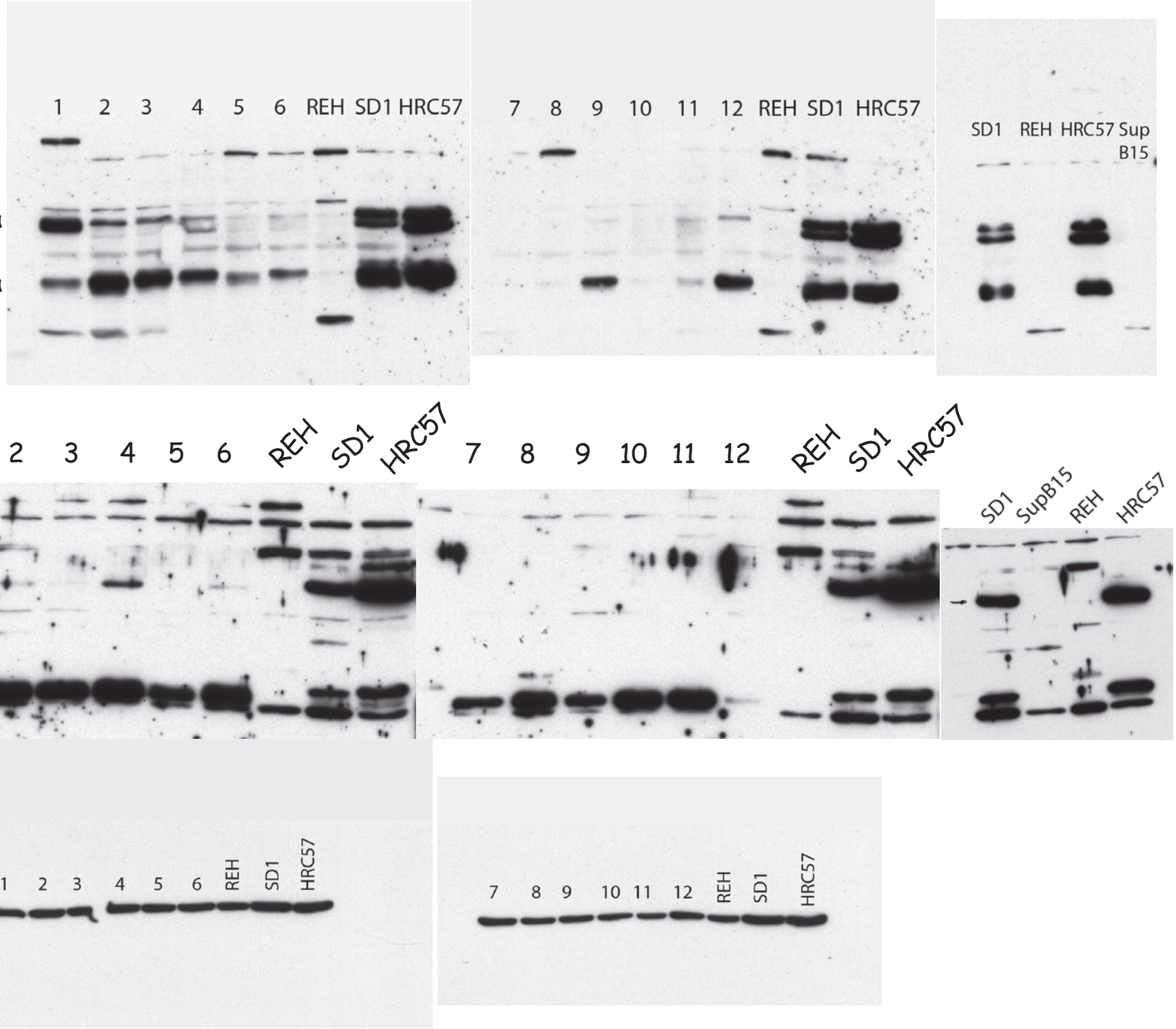
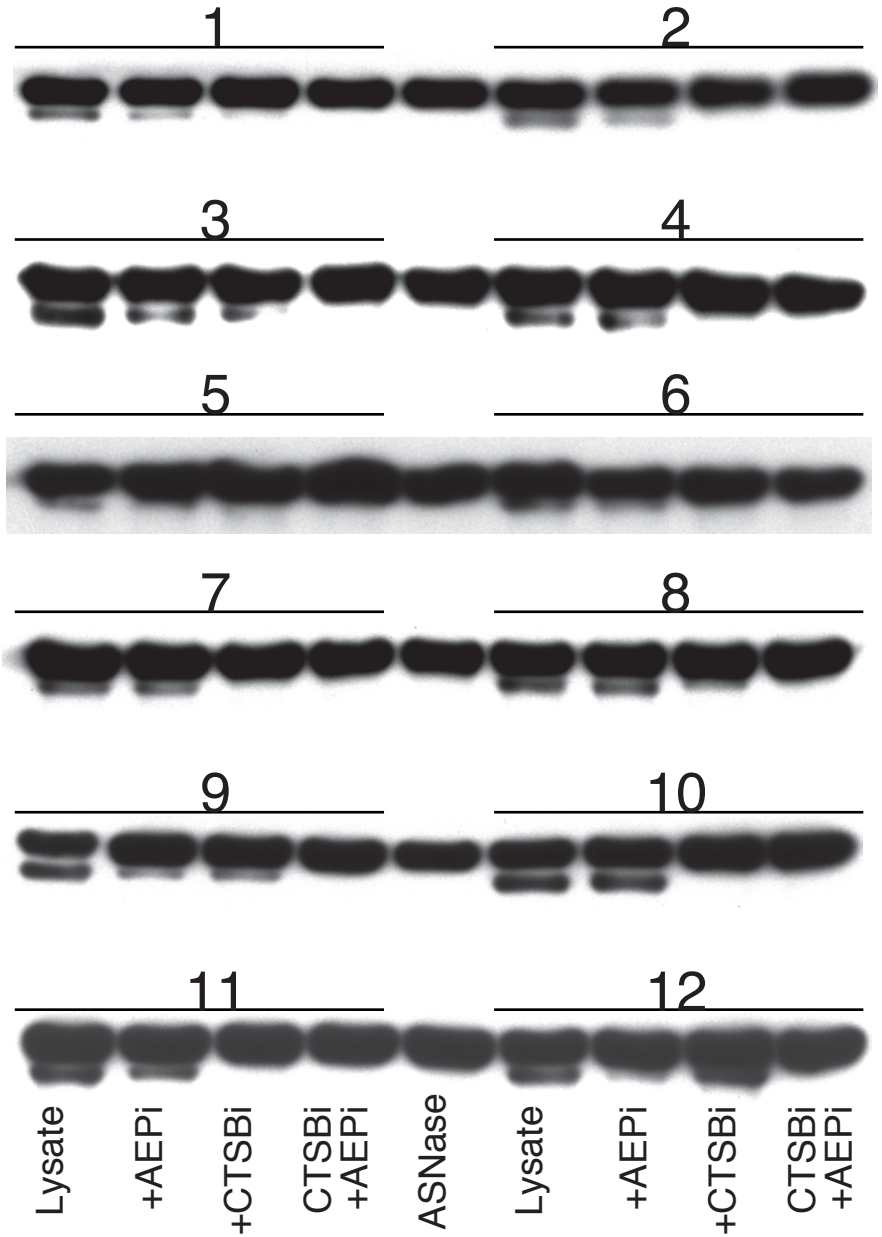


D.

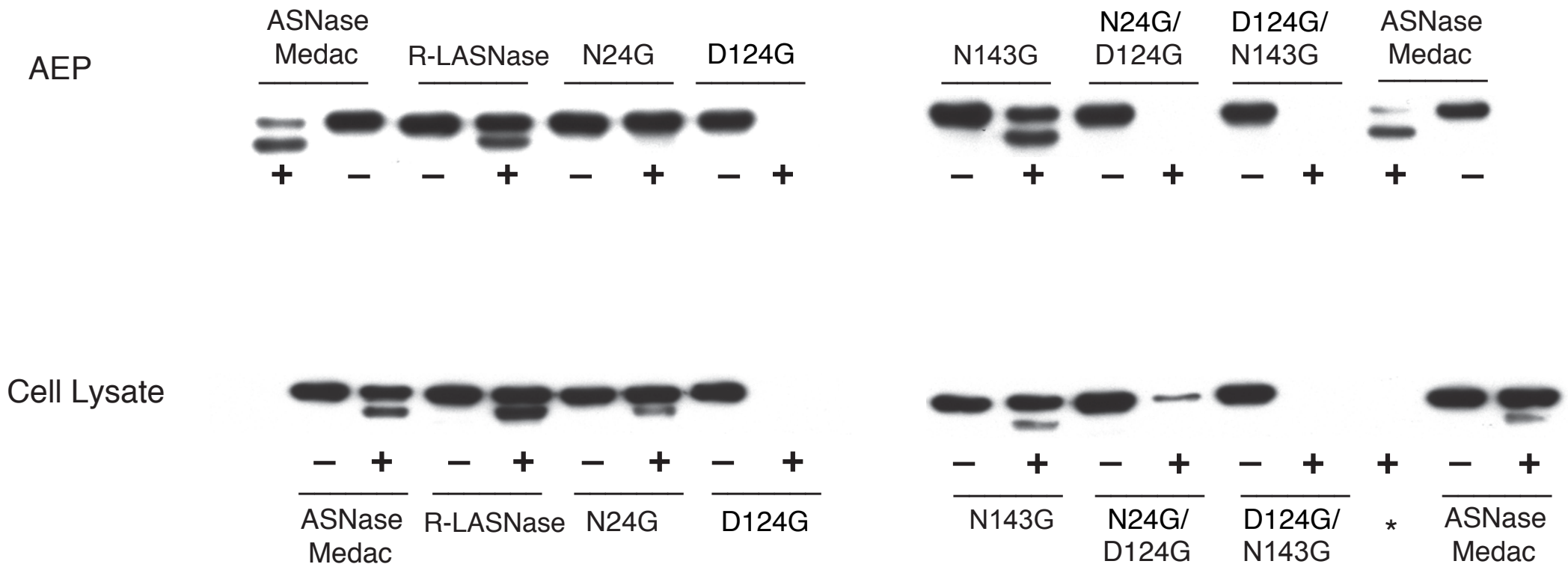
Supplemental Figure 2



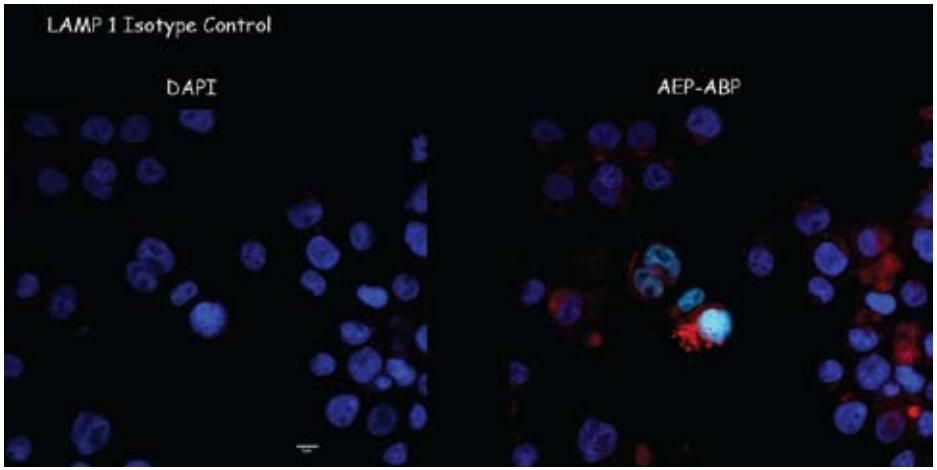
Supplemental Figure 3

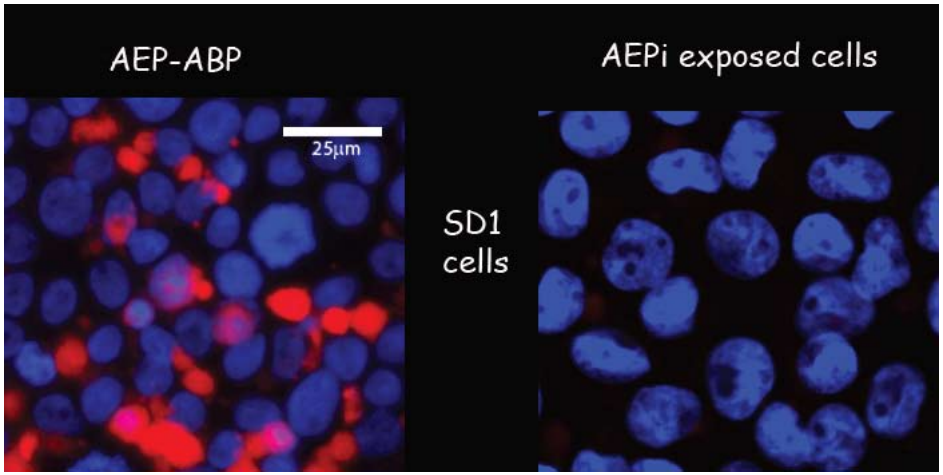


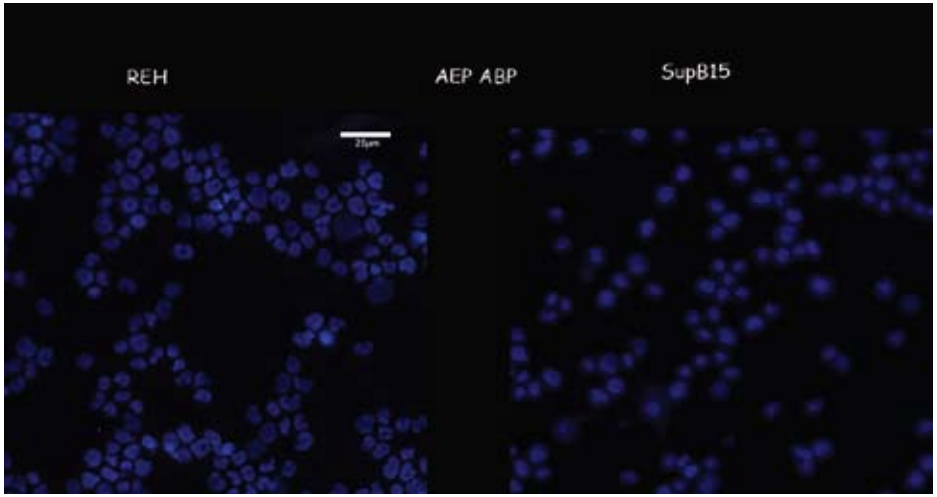
Supplemental Figure 4



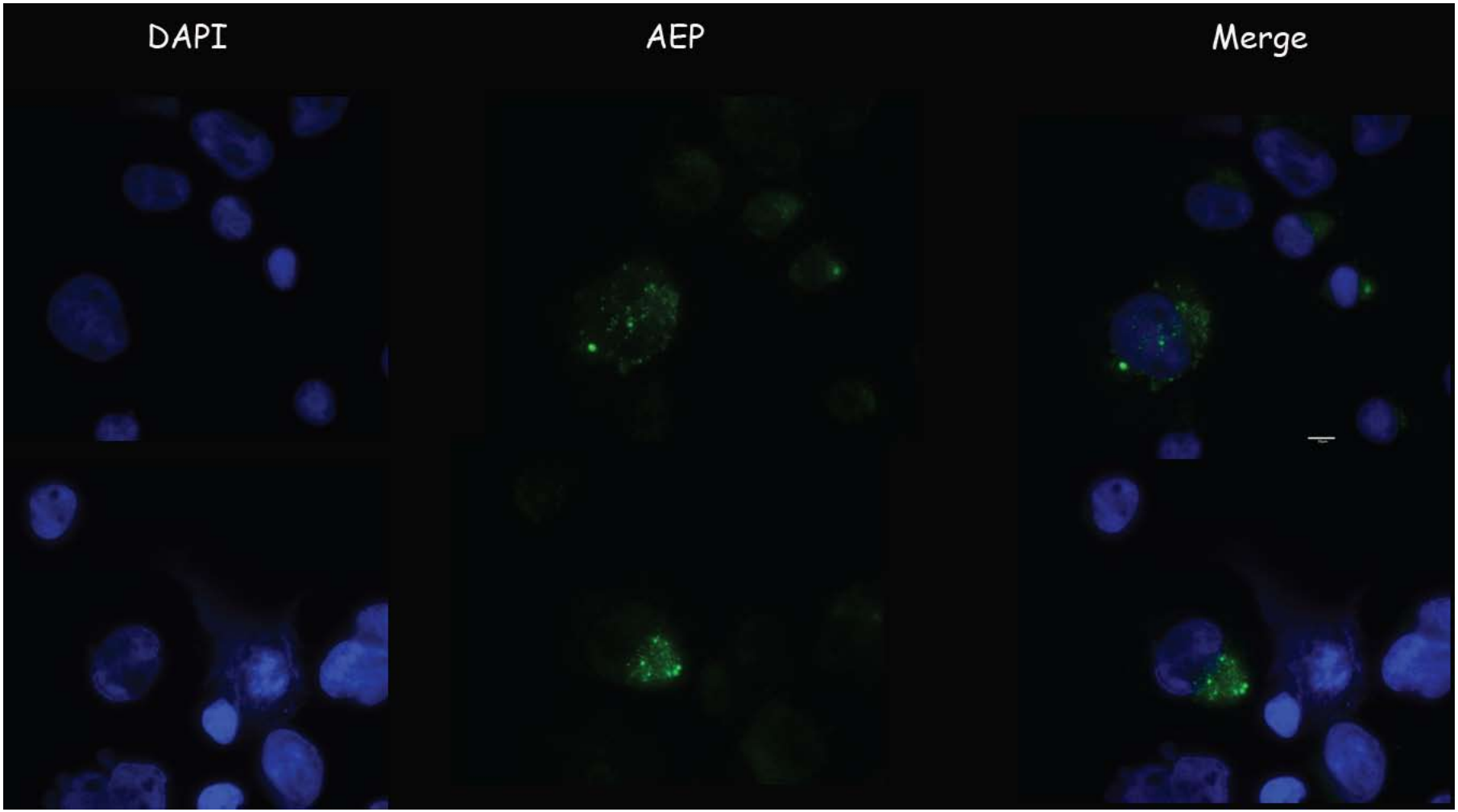
Supplemental Figure 5A



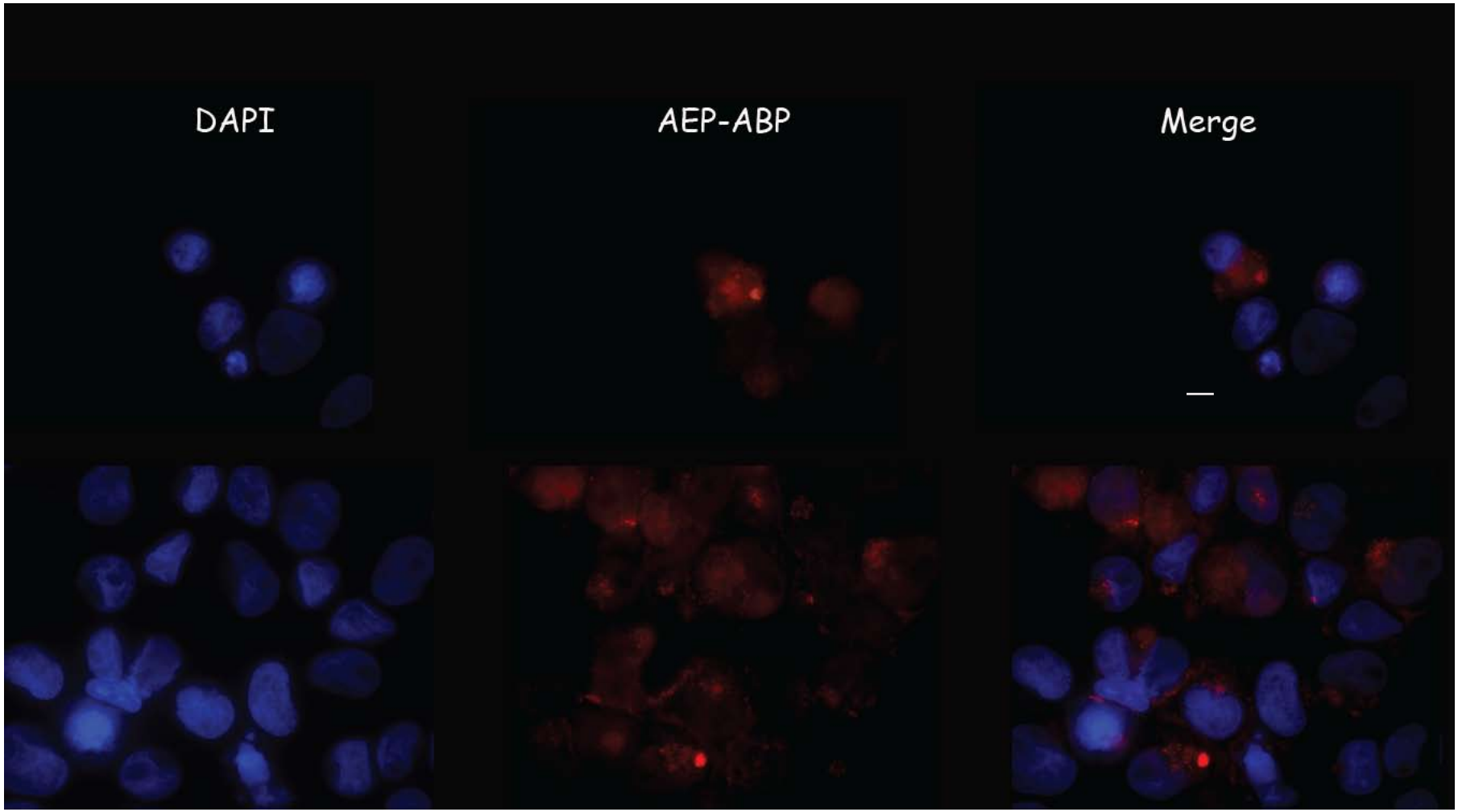




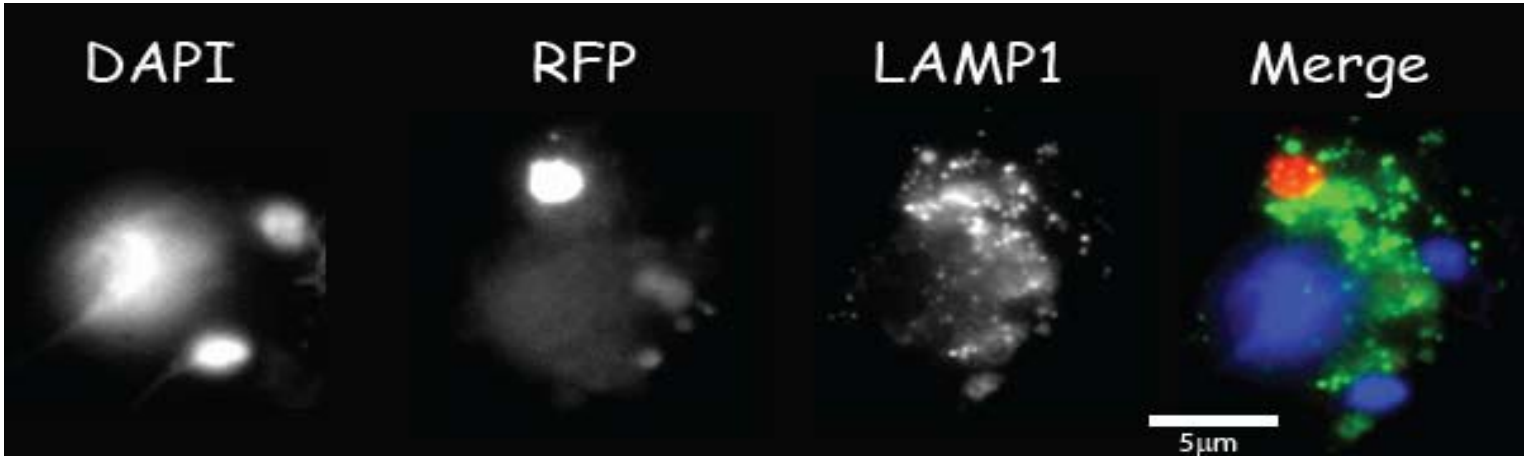
Supplemental Figure 5B



Supplemental Figure 5C



Supplemental Figure 5D



Supplemental Table 1

Plasma ASNase activity trends in children with ALL treated on MRC ALL2003 protocols (n=44)

ASNase activity		
Induction phase	Post-induction phase	No. of patients
Adequate	Adequate	27
Adequate	Inadequate	4
Inadequate	Adequate	8
Inadequate	Inadequate	5

Supplemental Table 2

Residual ASNase activity after digestion with activated recombinant human AEP or CTSB

ASNase incubated for 3 hours with	Replicates	Residual ASNase activity (%)	Mean residual ASNase activity (%)
AEP	Digest 1	39	44
	Digest 2	42	
	Digest 3	51	
CTSB	Digest 1	23	26
	Digest 2	25	
	Digest 3	30	

Supplemental Table 3

Karyotype information and relative AEP RNA expression data in patients with ALL and in experimental cell lines

Patient Samples			
UPN	**Cytogenetic category**	**Complete cytogenetics data**	**$2^{-\Delta\Delta CT}$**
1	ETV6-RUNX1	46, XY, interphase FISH ETV6-RUNX1 fusion	1.06
2	High Hyperdiploid	57, XY, +X, +4, +. +6, +9, +10, +15, +17, +18, +21, +21,	0.54
3	High Hyperdiploid	56, XY, +X, +4, +5, +6, +8, +14, +17, +18, +21, +21/46XY	2.12
4	E2A-PBX1	46, XX, der(19) t(1;19)(q23;p13)/ 50, idem, +5, +8, +8, +14	0.30
5	E2A-PBX1	46, XY, t(1:19)(q23;p13)/45, idem, -Y, del(9)(p13-21p24)/45, XY	0.37
6	E2A-PBX1	46, XX, der(19)t(1;19)(q23;p13), inc[cp3]/46XX(5)	0.20
7	Hypodiploid	26, X, +Y, +14, +21/26, idem, -14, +mar/52, idem x2/52, idem x2, -14, -14, +mar, +mar/46, XY	0.09
8	BCR-ABL	49, XX, +8, t(9;22)(q34;q11.2), +der(22)t(9;22), +mar/46, XX	0.49
9	iAMP21	46, XX, der(21) iAMP(21q)/46, XX	2.83
10	MLL-AF4	46, XY, t(4;11)(q21;q23)/46, XY	0.28
11	Other	46, XX, interphase FISH del(12p-[ETV6]) in 90% cells	0.51
12	Other	46, XX, t(1;9)(q21;p21), +5q3(10)	0.94
Cell lines			
HRC57	Normal	Human diploid karyotype	1.00
REH	ETV6-RUNX1	46(44-47), -X, +16, del(3)(p22), t(4;12;21;16)(q32;p13;q22;q24.3)-inv(12)(p13q22), t(5;12)(q31-q32;p12), der(16)t(16;21)(q24.3;q22)	0.19
SupB15	BCR-ABL	46XY, der(1)t(1;1)(p11;q31), add(3)(q2?7), der(4)t(1;4)(p11;q35), t(9;22)(q34;q11), +(10)(q25), ?del(14)(q23q31), der(16)t(9;16)(q11;p13)	0.02
SD1	BCR-ABL	92(88-92) XXXX, t(9;22)(q34;q11)x2	1.62