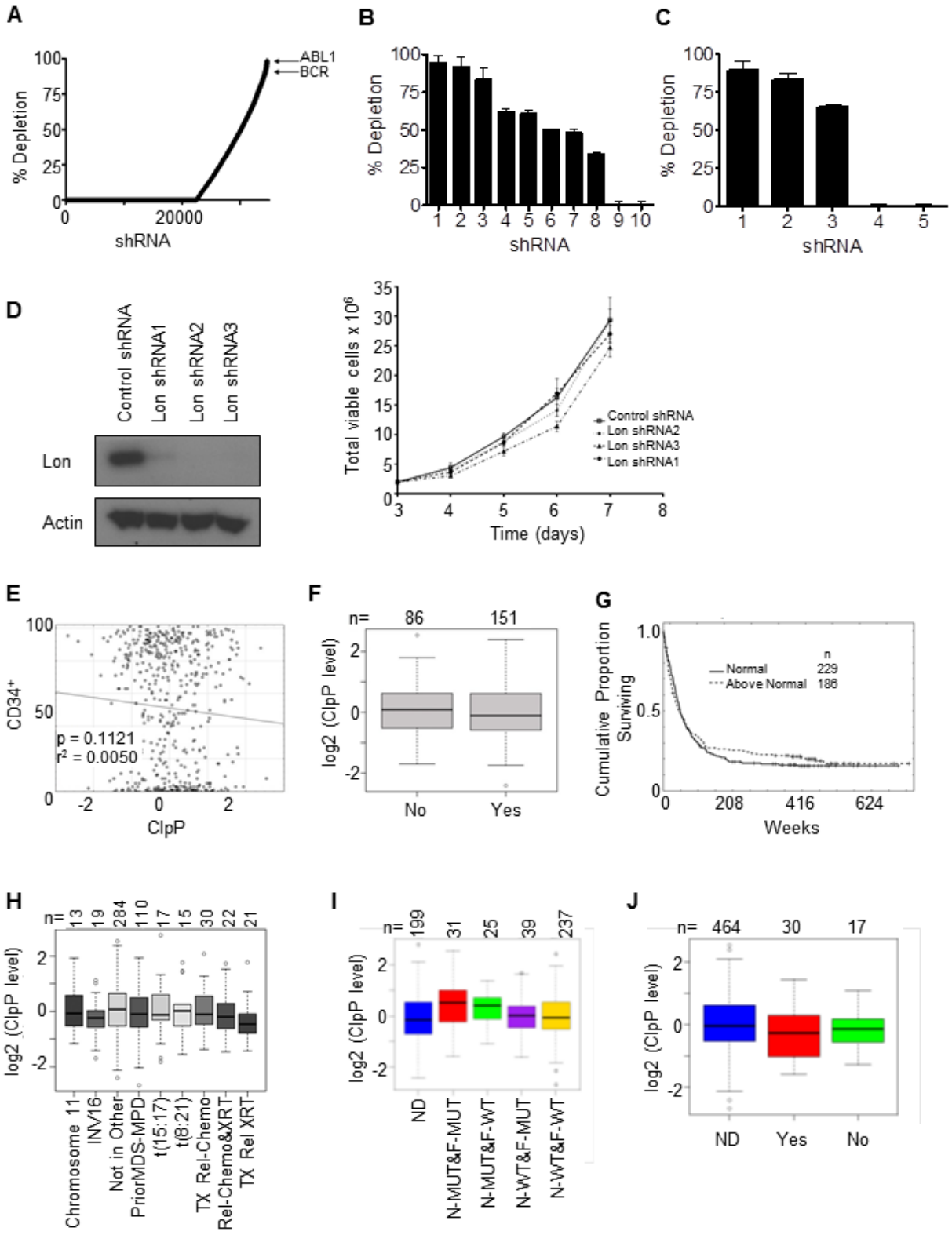
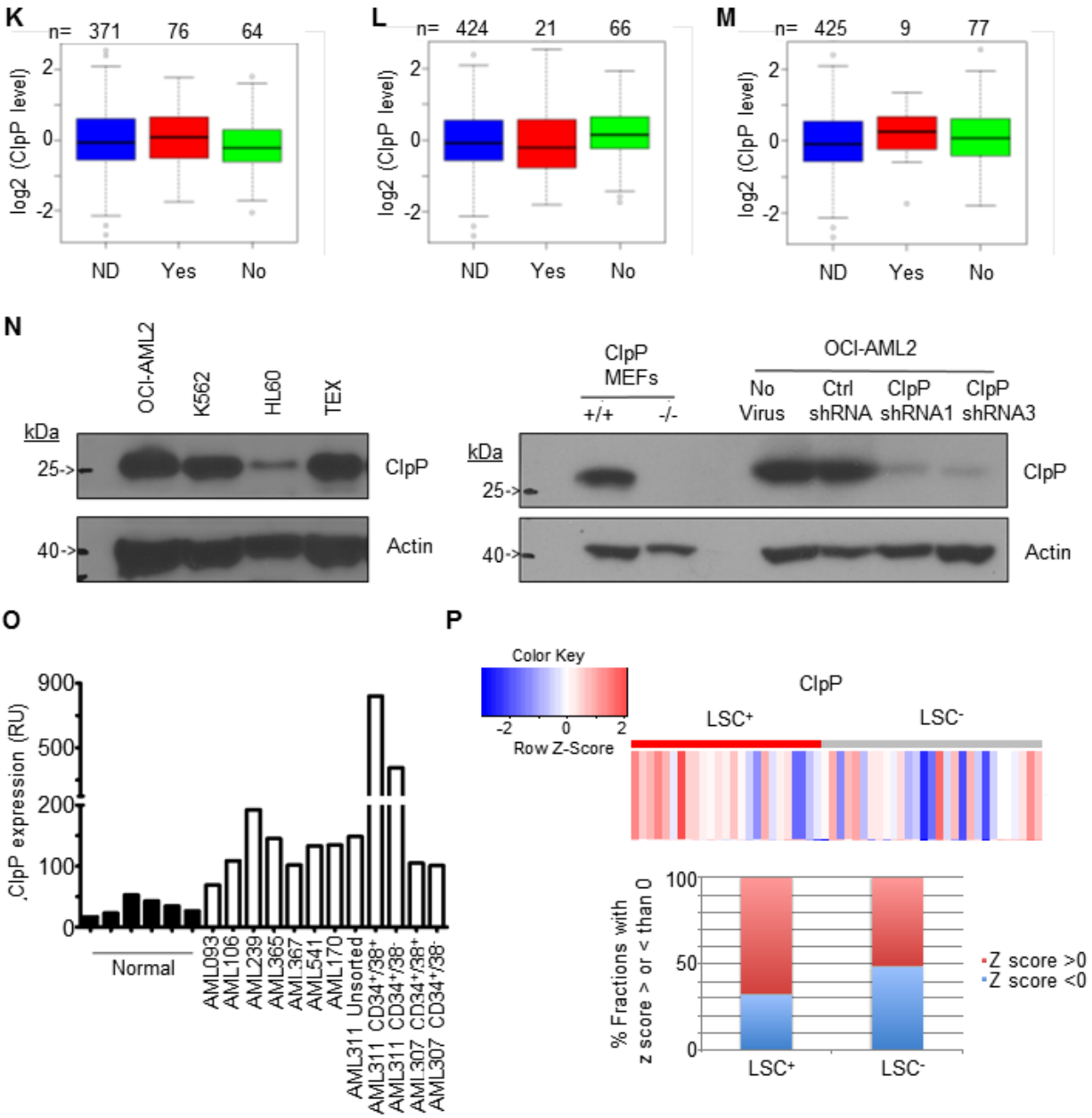
# Supplemental Data

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ND Yes No
L
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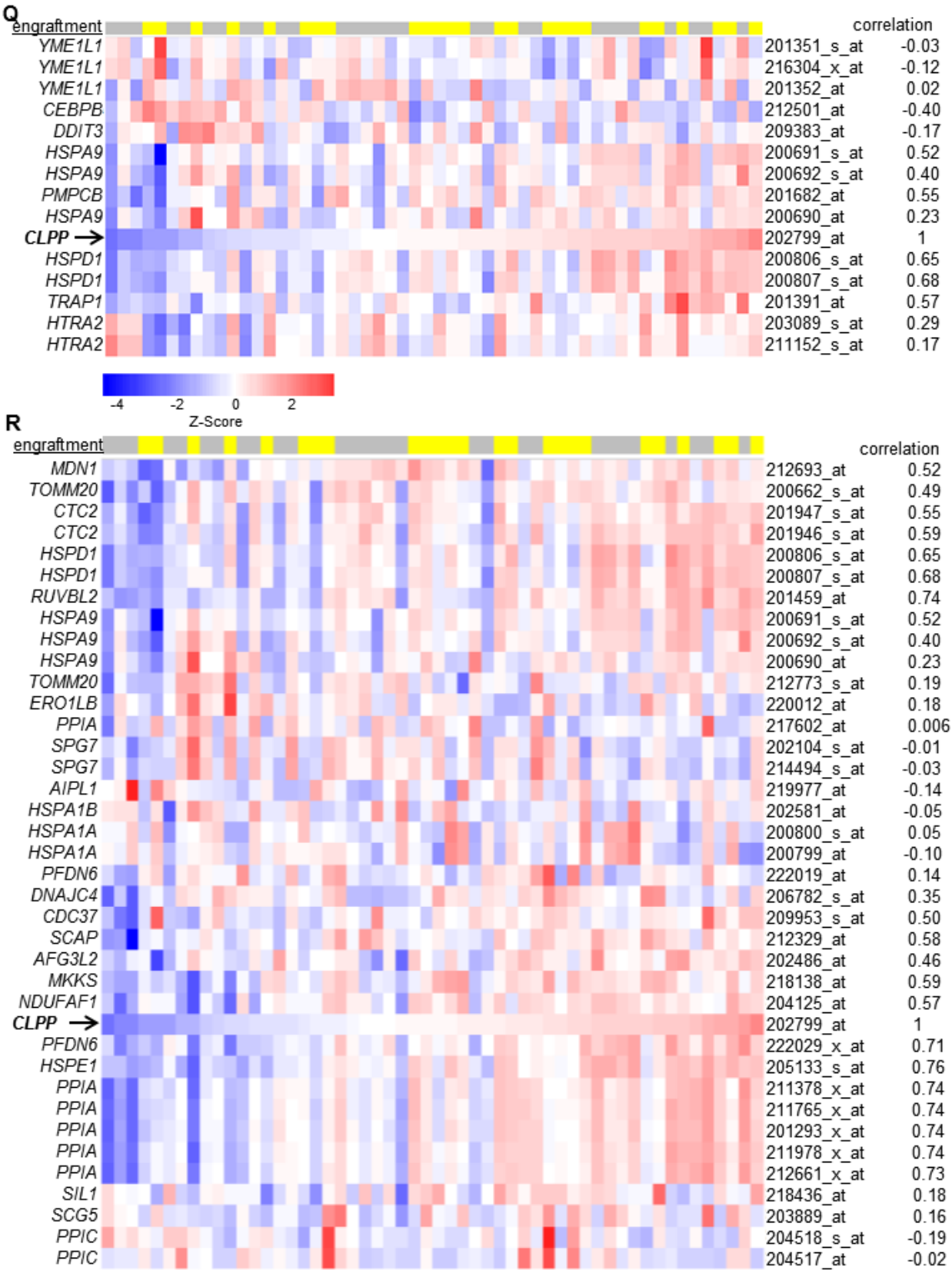
log2 (ClpP level)
ND Yes No
M
n= 425 9 77
log2 (ClpP level)
ND Yes No
N
OCI-AML2
K562
HL60
TEX
kDa
25->
ClpP
40->
Actin
ClpP MEFs
+/+ -/-
OCI-AML2
No Virus
Ctrl shRNA
ClpP shRNA1
ClpP shRNA3
kDa
25->
ClpP
40->
Actin
O
ClpP expression (RU)
900
500
200
100
0
Normal
AML093
AML106
AML239
AML365
AML367
AML541
AML170
AML311 Unsorted
AML311 CD34+/38+
AML311 CD34+/38-
AML307 CD34+/38+
AML307 CD34+/38-
P
Color Key
-2 0 2
Row Z-Score
ClpP
LSC+
LSC-
% Fractions with z score > or < than 0
100
50
0
LSC+
LSC-
Z score >0
Z score <0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PFDN6 222019_at 0.14
DNAJC4 206782_s_at 0.35
CDC37 209953_s_at 0.50
SCAP 212329_at 0.58
AFG3L2 202486_at 0.46
MKKS 218138_at 0.59
NDUFAF1 204125_at 0.57
CLPP 202799_at 1
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HSPE1 205133_s_at 0.76
PPIA 211378_x_at 0.74
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PPIC 204517_at -0.02

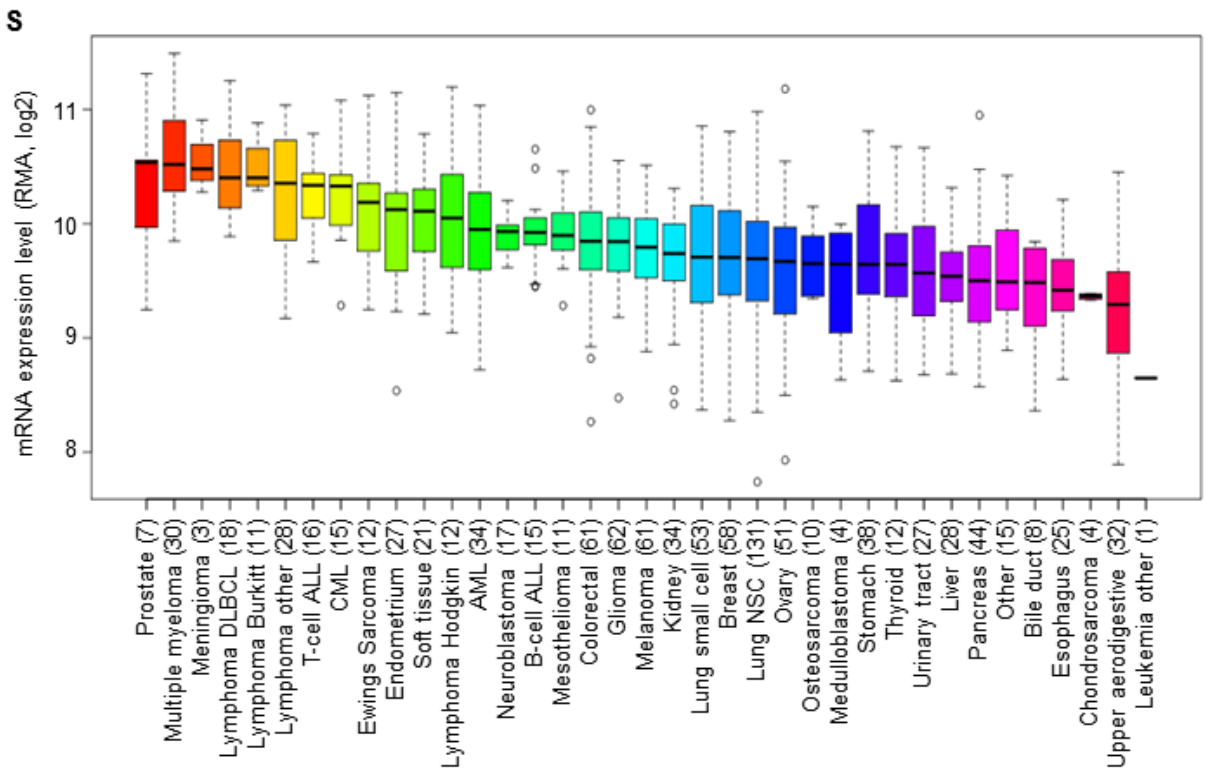



**Figure S1 related to Figure 1: ClpP in AML.**

**(A)** K562 cells were transduced with a pooled library of 54,021 shRNAs in lentiviral vectors targeting 11,255 nuclear encoded genes. Of these, 34,600 associated with known genes and transcripts were considered in the analysis. The percent depletion of shRNA sequences from the surviving cells was determined by microarray analysis 21 days after transduction.

**(B)** The mean ± SD percent depletion of individual clones in the library targeting ABL1.

**(C)** The mean ± SD percent depletion of individual clones in the library targeting BCR.

**(D)** TEX leukemia cells were transduced with shRNA targeting Lon or control sequences in lentiviral vectors containing puromycin selection markers. The day after transduction, cells were treated with puromycin to select for transduced cells for 2 days. Cells were then seeded into fresh media. Expression of Lon and Tubulin was tested by immunoblotting (left panel). The

mean ± SD number of viable cells after Lon knockdown was determined by trypan blue staining (right panel).

**(E)** Correlation of ClpP expression and $CD34^+$ expression in the primary AML cells. Expression of ClpP was measured in primary AML cells by reverse phase protein array.

**(F)** Expression of ClpP in primary AML cells by reverse phase protein array classified by response to induction chemotherapy. The box represents the first and third quartiles, whiskers represent the range, the center line represents the median, and the circles outliers.

**(G)** Kaplan-Meir plot of overall survival categorized by ClpP expression. ClpP expression was measured by by reverse phase protein array and dichotomized above or below the mean expression of ClpP in normal $CD34^+$ cells. $p = 0.76$.

**(H-M)** Expression of ClpP in primary AML by reverse phase protein array cells classified by WHO classification **(H)**, NPM and FLT3 mutations **(I)**, p53 mutations (ND = not done) **(J)**, RAS mutations **(K)**, IDH1 mutations **(L)**, and IDH2 mutations **(M)**. The box represents the first and third quartiles, whiskers represent the range, the center line represents the median, and the circles outliers.

**(N)** Expression of ClpP and Actin in AML cell lines (left panel from Figure 1), WT and *Clpp*$^{-/-}$ MEFs, and OCI-AML2 cells untransduced or transduced with ClpP shRNA or control sequences.

**(O)** Expression of *CLPP* mRNA. mRNA was isolated from primary normal hematopoietic cells, primary AML cells, and fractions of primary AML cells sorted by CD34 and CD38 expression. *CLPP* mRNA expression was measured by nanostring and normalized to $\beta_2 M$ expression in each sample.

**(P)** *CLPP* mRNA expression was analyzed in fractions of AML samples sorted based on the presence of leukemia stem cells (LSC). Microarray data from 16 AML patients sorted into 29

$LSC^-$ and 25 $LSC^+$ fractions was analyzed (array dataset archived online in the NCBI Gene Expression Omnibus database, accession #: GSE30375). LSC fractions were determined based on engraftment into immune deficient mice. Heatmaps represent expression of *CLPP* in the $LSC^+$ and $LSC^-$ fractions. The percent of $LSC^+$ and $LSC^-$ samples with expression of *CLPP* higher (z score >0) and lower (z score <0) than the average *CLPP* expression for all AML fractions is shown.

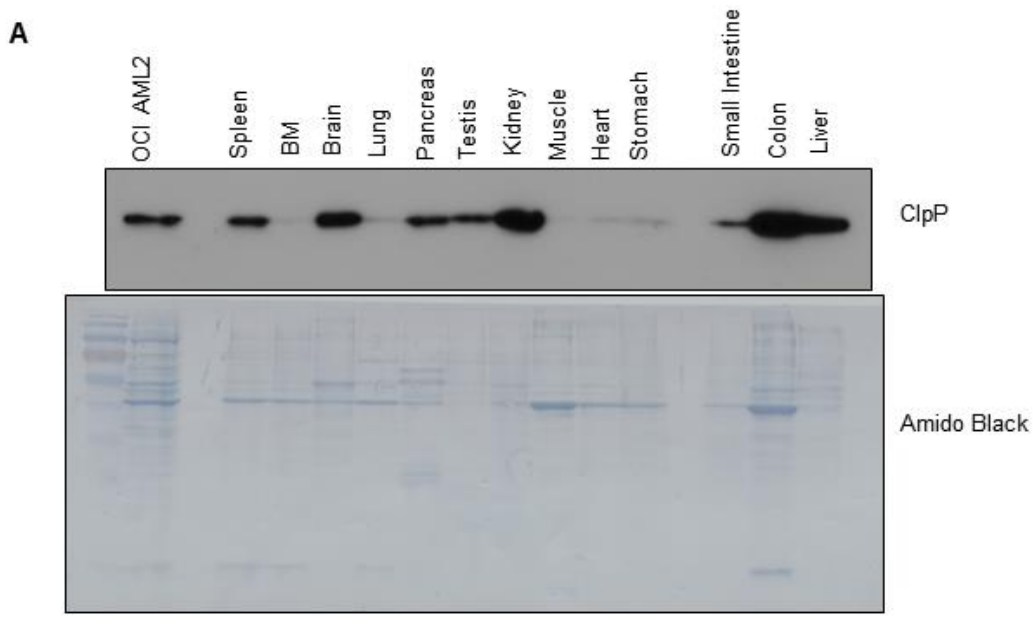
**(Q-R)** Expression of *CLPP* and genes associated with the mitochondrial unfolded protein response (mtUPR) in $LSC^+$ (yellow; n = 25) and $LSC^-$ (gray; n = 29) fractions from patients with AML (n = 16). Gene expression data was derived from the dataset #: GSE30375. Gene names appear more than once as more than one probe targeting the gene was present in the array. Genes representing the mtUPR were derived from the current literature on mtUPR signaling (Aldridge et al., 2007; Haynes et al., 2013; Pellegrino et al., 2013) **(Q)** and the Gene Ontology database (GO:0051082) representing unfolded protein binding **(R)**. **(R)** uses the same color key shown in **(Q)**.

**(S)** Expression of *CLPP* mRNA in different malignant cell lines from the Cancer Cell Line Encyclopedia. (http://www.broadinstitute.org/ccle/home). The box represents the first and third quartiles, whiskers represent the range, the center line represents the median, and the circles outliers.

**Table S1 related to Figure 1: Correlation of ClpP expression with genetic mutations in AML.**

| Gene | WT | Mutant | t-test p value | KS test p value |
|---|---|---|---|---|
| *DNMT3A* | 146 | 51 | 0.2 | 0.3 |
| *FLT3* | 142 | 55 | 0.2 | 0.2 |
| *NPM1* | 143 | 54 | 0.7 | 0.7 |
| *TET2* | 180 | 17 | 0.06 | 0.2 |
| *RUNX1* | 180 | 17 | 0.3 | 0.2 |
| *IDH2* | 178 | 19 | 0.6 | 0.3 |
| *TP53* | 182 | 15 | 0.3 | 0.4 |
| *CEBPA* | 184 | 13 | 0.5 | 0.3 |
| *IDH1* | 178 | 19 | 0.1 | 0.2 |
| *NRAS* | 182 | 15 | 0.2 | 0.7 |
| *WT1* | 185 | 12 | 0.8 | 0.9 |
| *KIT* | 189 | 8 | 0.8 | 0.1 |

Footnote: The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov) data was analyzed to relate expression of *CLPP* RNA (TCGA - LAML RNA-hiseq v2 3.1.1.0 normalized) to genetic mutations (IlluminaGA_DNASeq level_2.2.13.0somatic) in patients with AML. The number of patients with WT or mutated genes is shown. Differences in expression were compared with t-tests and Kolmogorov-Smirnov (KS) tests. The KS test was used to compare the distributions of gene expressions in the two groups (WT and mutant).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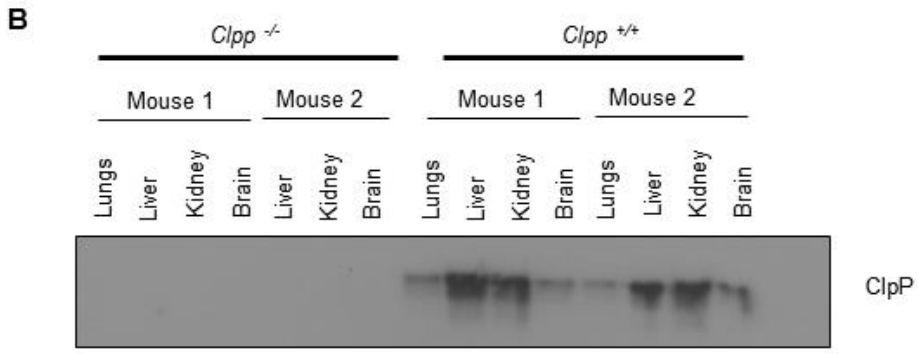
OCI AML2
Spleen
BM
Brain
Lung
Pancreas
Testis
Kidney
Muscle
Heart
Stomach
Small Intestine
Colon
Liver
ClpP
Amido Black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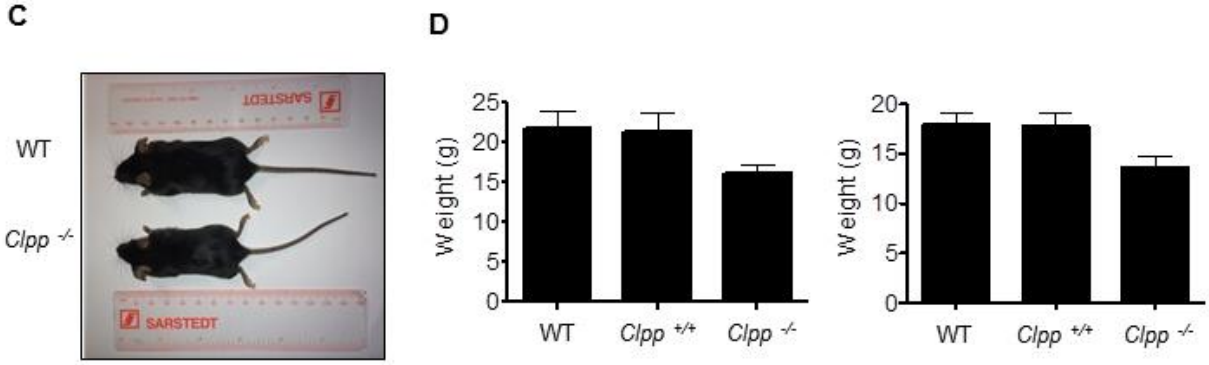
Clpp -/-
Clpp +/+
Mouse 1
Mouse 2
Mouse 1
Mouse 2
Lungs
Liver
Kidney
Brain
Liver
Kidney
Brain
Lungs
Liver
Kidney
Brain
Lungs
Liver
Kidney
Brain
ClpP


C
WT
Clpp -/-
SARSTEDT
SARSTEDT
D
Weight (g)
25
20
15
10
5
0
WT
Clpp +/+
Clpp -/-
Weight (g)
20
15
10
5
0
WT
Clpp +/+
Clpp -/-

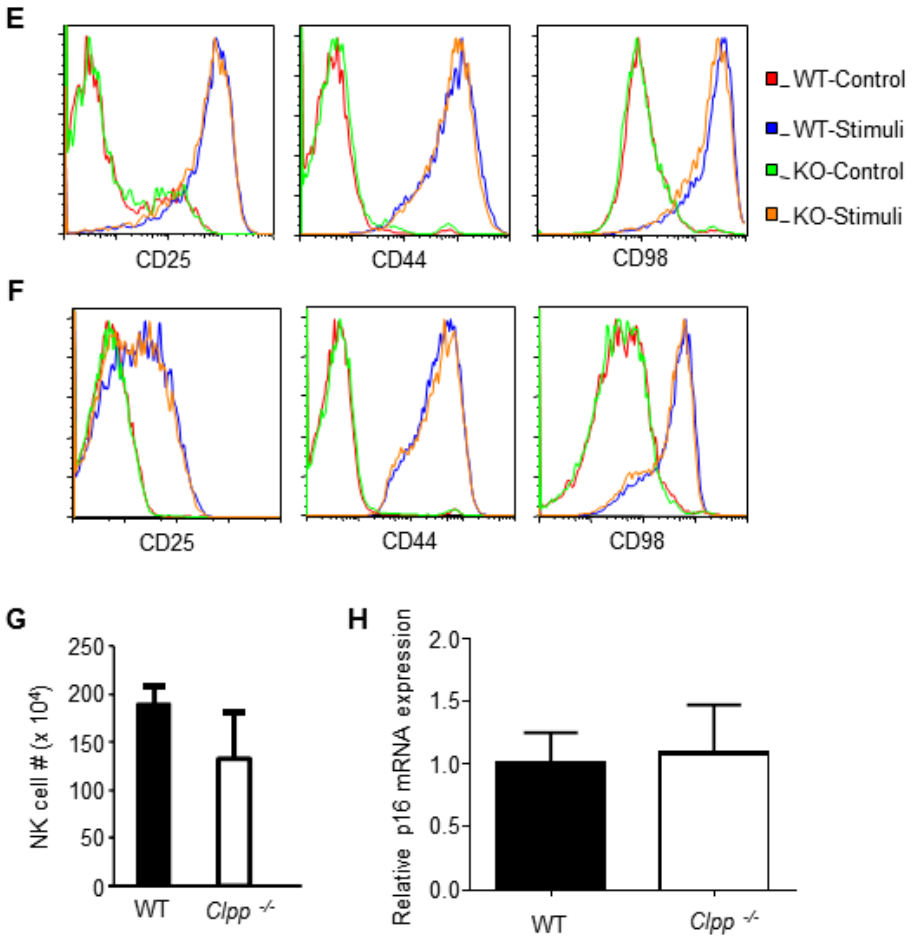



**Figure S2 related to Figure 3: ClpP knockout mice are viable with normal hematopoiesis.**

**(A)** Organs were harvested from WT mice and total proteins were isolated. Expression of ClpP in the organs was determined by immunoblotting. Equal protein loading was confirmed by staining the blots with Amino Black.

**(B)** Expression of ClpP in the organs of *Clpp* $^{-/-}$ and *Clpp* $^{+/+}$ mice was determined by immunoblotting.

**(C)** Representative image of WT and *Clpp* $^{-/-}$ mice.

**(D)** The mean ± SD weight of 10 male and 18 female WT mice, and 6 male and 7 female *Clpp* $^{-/-}$ mice, all at 6 weeks of age. Left panel: male mice; right panel: female mice.

**(E-F)** Levels of activation markers on unstimulated and stimulated splenocytes from 3-4 month old WT and *Clpp* $^{-/-}$ mice were determined by flow cytometry. Splenocytes were stimulated for 24 hr with plate-bound anti-CD3 (3 μg/ml) and anti-CD28 (1 μg/ml) to activate T cells **(E)**. Splenocytes were left untreated or cultured for 24 hr with 10 μg/ml goat anti-mouse IgM (F(ab')2 fragment to activate B cells. Cells were immunostained to detect the level of CD25, CD44 and CD98 expression **(F)**. Representative histograms are shown.

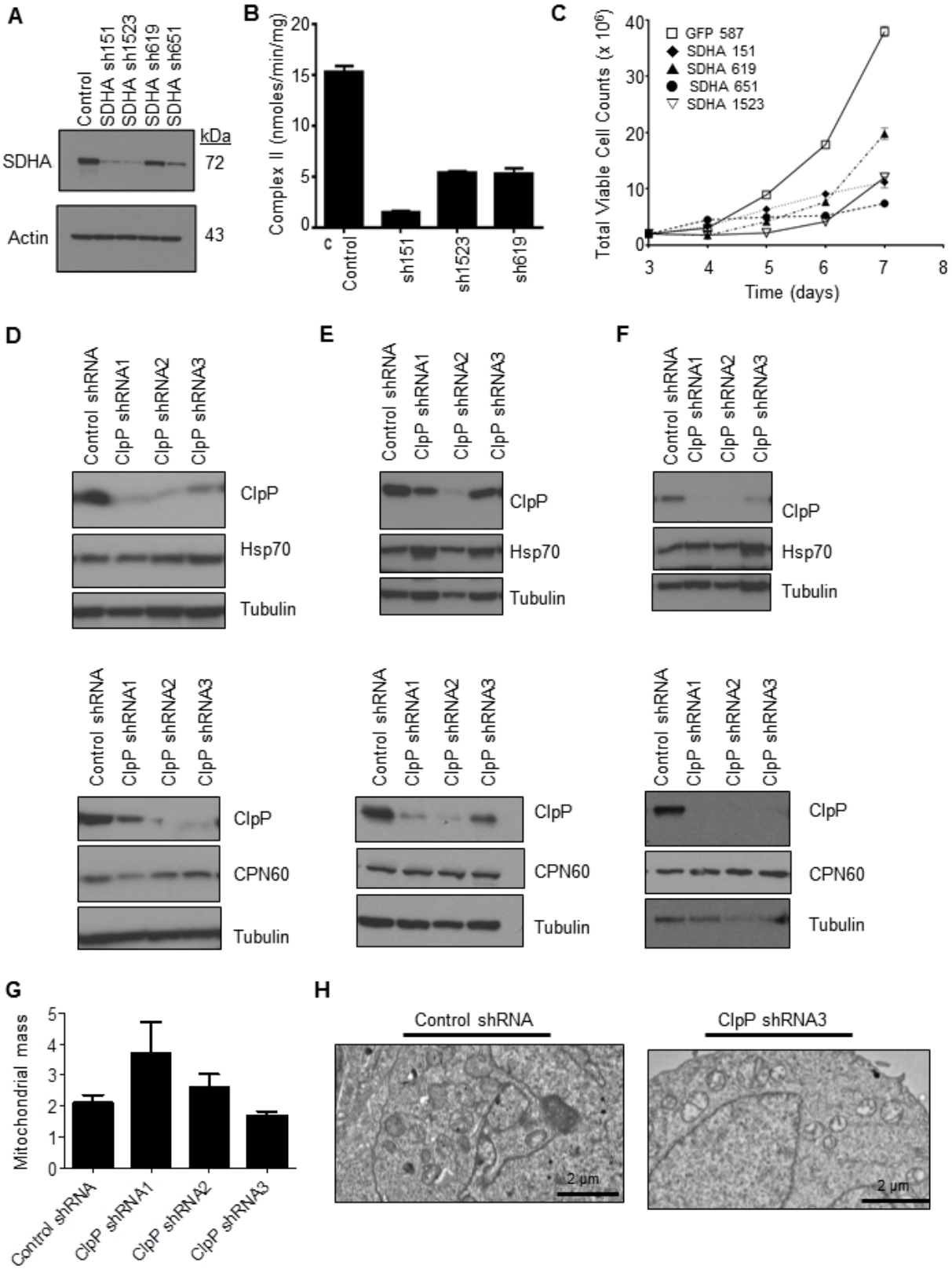
**(G)** Splenic NK cell number (mean ± SD, n=3; p = 0.081, Student's t-test of WT versus *Clpp* $^{-/-}$).

**(H)** p16 mRNA expression in thymocytes from WT and *Clpp* $^{-/-}$ mice. Data represent mean ± SD from 3 mice per group. p = 0.8 by t-test.

**Table S2 related to Figure 4: List of mitochondrial ClpP-interacting proteins, 49 of which preferentially interact with wild-type or mutant ClpP over the unrelated mitochondrial matrix enzyme ornithine transcarbamoylase (OTC).**

| Gene Name | Protein Name | OTC | WT ClpP | Mutant ClpP |
|---|---|---|---|---|
| *CLPP* | ClpP caseinolytic peptidase, ATP-dependent, proteolytic subunit homolog (E. coli) | 14.5 | 1730.75 | 1759.5 |
| *CLPX* | ClpX caseinolytic peptidase X homolog (E. coli) | 24.5 | 51 | 80.75 |
| *CS* | citrate synthase | 7.5 | 31 | 34.75 |
| *CARS2* | cysteinyl-tRNA synthetase 2, mitochondrial (putative) | 7.75 | 29.25 | 43.25 |
| *SDHA* | succinate dehydrogenase complex, subunit A, flavoprotein (Fp) | 8.25 | 25.75 | 30.75 |
| *NFS1* | NFS1 nitrogen fixation 1 homolog (S. cerevisiae) | 1.75 | 19.25 | 26.5 |
| *ALDH4A1* | aldehyde dehydrogenase 4 family, member A1 | 2.5 | 18.25 | 19 |
| *QRSL1* | glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1 | 4.25 | 16 | 24.25 |
| *SSBP1* | single-stranded DNA binding protein 1 | 2.5 | 13.25 | 13 |
| *NIPSNAP1* | nipsnap homolog 1 (C. elegans) | 7.75 | 12.25 | 21 |
| *WARS2* | tryptophanyl tRNA synthetase 2, mitochondrial | 3.25 | 11 | 12 |
| *COX5A* | cytochrome c oxidase subunit Va | 2.75 | 9.75 | 15.25 |
| *ACADSB* | acyl-Coenzyme A dehydrogenase, short/branched chain | 4 | 9.5 | 12.75 |
| *AK3* | adenylate kinase 3 | 1.5 | 8.75 | 8.25 |
| *OXCT1* | 3-oxoacid CoA transferase 1 | 2.25 | 8.5 | 8 |
| *GSTK1* | glutathione S-transferase kappa 1 | 0 | 8.5 | 6 |
| *FECH* | ferrochelatase (protoporphyria) | 2 | 8.25 | 11 |
| *POLDIP2* | polymerase (DNA-directed), delta interacting protein 2 | 7.25 | 7.5 | 22.25 |
| *COX5B* | cytochrome c oxidase subunit Vb | 0 | 6.75 | 6.25 |
| *NARS2* | asparaginyl-tRNA synthetase 2, mitochondrial (putative) | 0 | 6.25 | 6.75 |
| *IDH3B* | isocitrate dehydrogenase 3 (NAD+) beta | 0.5 | 6 | 6.25 |
| *TFAM* | transcription factor A, mitochondrial | 3.25 | 5.75 | 10 |
| *ACADM* | acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain | 0 | 5.75 | 5.25 |
| *ACSS1* | acyl-CoA synthetase short-chain family member 1 | 0 | 4.75 | 4.75 |
| *FDX1* | ferredoxin 1 | 0 | 4.25 | 7.75 |
| *PMPCB* | peptidase (mitochondrial processing) beta | 0.75 | 4.25 | 3.25 |
| *PET112* | PET112-like (yeast) | 0.5 | 4 | 8 |
| *HSD17B4* | hydroxysteroid (17-beta) dehydrogenase 4 | 0 | 4 | 1.5 |
| *ATP8* | Cytochrome c oxidase subunit 3 | 0 | 3.75 | 3.5 |
| *TST* | thiosulfate sulfurtransferase (rhodanese) | 0 | 3 | 2.25 |

| | | | | |
|---|---|---|---|---|
| *SDHC* | succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa | 0 | 3 | 1.5 |
| *ND1* | NADH-ubiquinone oxidoreductase chain 1 | 0 | 2.75 | 3.75 |
| *CDS2* | CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2 | 0 | 2 | 1.5 |
| *TMLHE* | trimethyllysine hydroxylase, epsilon | 0 | 2 | 0.75 |
| *NDUFA4* | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9kDa | 1 | 1.5 | 3 |
| *CBR4* | carbonyl reductase 4 | 0 | 1.5 | 0.5 |
| *ALAS1* | aminolevulinate, delta-, synthase 1 | 0 | 1.25 | 9.5 |
| *UQCR10* | ubiquinol-cytochrome c reductase complex (7.2 kD) | 0 | 1.25 | 1.25 |
| *PDK3* | pyruvate dehydrogenase kinase, isozyme 3 | 0.5 | 1 | 8.5 |
| *COX6A1* | cytochrome c oxidase subunit VIa polypeptide 1 | 0 | 0.75 | 0 |
| *GCDH* | glutaryl-Coenzyme A dehydrogenase | 0 | 0.5 | 2.75 |
| *MTRF1* | mitochondrial translational release factor 1 | 0 | 0.5 | 1.5 |
| *COX7C* | cytochrome c oxidase subunit VIIc | 0 | 0.5 | 0.5 |
| *COQ3* | coenzyme Q3 homolog, methyltransferase (S. cerevisiae) | 0 | 0.5 | 0.5 |
| *POLG2* | polymerase (DNA directed), gamma 2, accessory subunit | 0 | 0.5 | 0 |
| *GLUD2* | glutamate dehydrogenase 2 | 0 | 0 | 6.25 |
| *NDUFAF1* | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1 | 1 | 0 | 4.25 |
| *USMG5* | up-regulated during skeletal muscle growth 5 homolog (mouse) | 0 | 0 | 1.75 |
| *MRRF* | mitochondrial ribosome recycling factor | 0 | 0 | 1.25 |
| *ACO2* | aconitase 2, mitochondrial | 0 | 0 | 1.25 |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Control
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SDHA sh1523
SDHA sh619
SDHA sh651
kDa
SDHA
72
Actin
43
B
Complex II (nmoles/min/mg)
20
15
10
5
0
c
Control
sh151
sh1523
sh619
C
Total Viable Cell Counts (x 10^6)
40
30
20
10
0
GFP 587
SDHA 151
SDHA 619
SDHA 651
SDHA 1523
3
4
5
6
7
8
Time (days)
D
Control shRNA
ClpP shRNA1
ClpP shRNA2
ClpP shRNA3
ClpP
Hsp70
Tubulin
E
Control shRNA
ClpP shRNA1
ClpP shRNA2
ClpP shRNA3
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Hsp70
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ClpP shRNA3
ClpP
CPN60
Tubulin
G
Mitochondrial mass
5
4
3
2
1
0
Control shRNA
ClpP shRNA1
ClpP shRNA2
ClpP shRNA3
H
Control shRNA
ClpP shRNA3
2 µm
2 µm

**Figure S3 related to Figure 4: ClpP regulates mitochondrial metabolism.**

**(A-C)** OCI-AML2 cells were transduced with shRNA targeting SDHA or control sequences lentiviral vectors with a puromycin selection marker. The day after transduction, cells were treated with puromycin for 3 days to select tranduced cells. Then, equal numbers of cells were seeded into fresh medium. 7 days after transduction, cells were harvested and total lysates were prepared. The expression of SDHA and Actin were detected by immunoblotting **(A)**. Mean ± SD complex II activity was measured. Of note, there were not enough cells available for SDHA shRNA clone 651 to conduct complex activity studies **(B)**. The mean ± SD number of viable cells was measured over time by trypan blue staining **(C)**.

**(D-G)** OCI-AML2 **(D)**, TEX **(E)**, and K562 **(F)** cells were transduced with shRNA targeting ClpP or control sequences. 3 days after transduction, cells were harvested, total cell lysates were prepared and the expression of ClpP, Hsp70, CPN60, and Tubulin was measured by immunoblotting. 4 days after transduction, mitochondrial mass was determined by flow cytometric analysis of cells stained with Mitotracker Green FM. Data represent the mean ± SD mitochondrial mass from a representative experiment **(G).**

**(H)** Mitochondrial structure was analyzed by electron microscopy 3 days after knockdown of ClpP in OCI-AML2 cells.

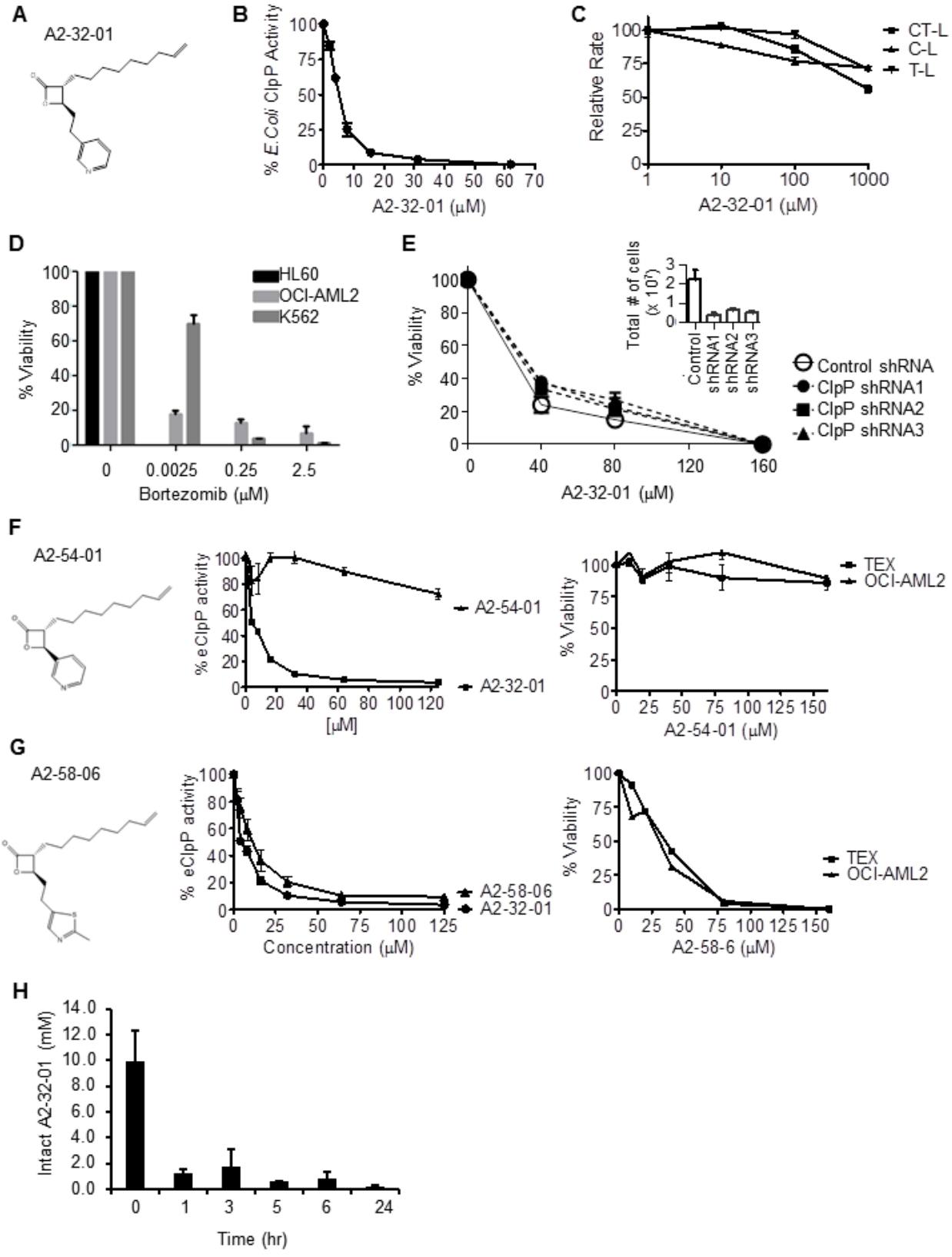

A
A2-32-01
B
% E.Coli ClpP Activity
A2-32-01 (μM)
C
Relative Rate
CT-L
C-L
T-L
A2-32-01 (μM)
D
% Viability
HL60
OCI-AML2
K562
Bortezomib (μM)
E
% Viability
Total # of cells (x 10⁵)
Control
shRNA1
shRNA2
shRNA3
Control shRNA
ClpP shRNA1
ClpP shRNA2
ClpP shRNA3
A2-32-01 (μM)
F
A2-54-01
% eClpP activity
A2-54-01
A2-32-01
[μM]
% Viability
TEX
OCI-AML2
A2-54-01 (μM)
G
A2-58-06
% eClpP activity
A2-58-06
A2-32-01
Concentration (μM)
% Viability
TEX
OCI-AML2
A2-58-6 (μM)
H
Intact A2-32-01 (mM)
Time (hr)

**Figure S4 related to Figure 5: A2-32-01 inhibits ClpP and is cytotoxic to AML cells.**

**(A)** Chemical structure of A2-32-01.

**(B)** Effects of increasing concentrations of A2-32-01 on the activity of purified recombinant bacterial ClpP to cleave the fluorogenic substrate Suc-LY-AMC.

**(C)** Lysates of red blood cells were treated with increasing concentrations of A2-32-01 for 2 hours. The chymotrypsin (CT-L), trypsin (T-L), and caspase-like(C-L) enzymatic activity was measured by cleavage of their preferential fluorogenic substrates (Suc-Leu-Leu-Val-Tyr-AMC, Boc-Leu-Arg-Arg-AMC, and Z-Leu-Leu-Glu-AMC). Data represent mean ± SD from one of three representative experiments.

**(D)** HL60, OCI-AML2, and K562 cells were treated with increasing concentrations of bortezomib. 72 hours after incubation, cell growth and viability was measured by MTS assay. Data represent mean ± SD from a representative experiment.

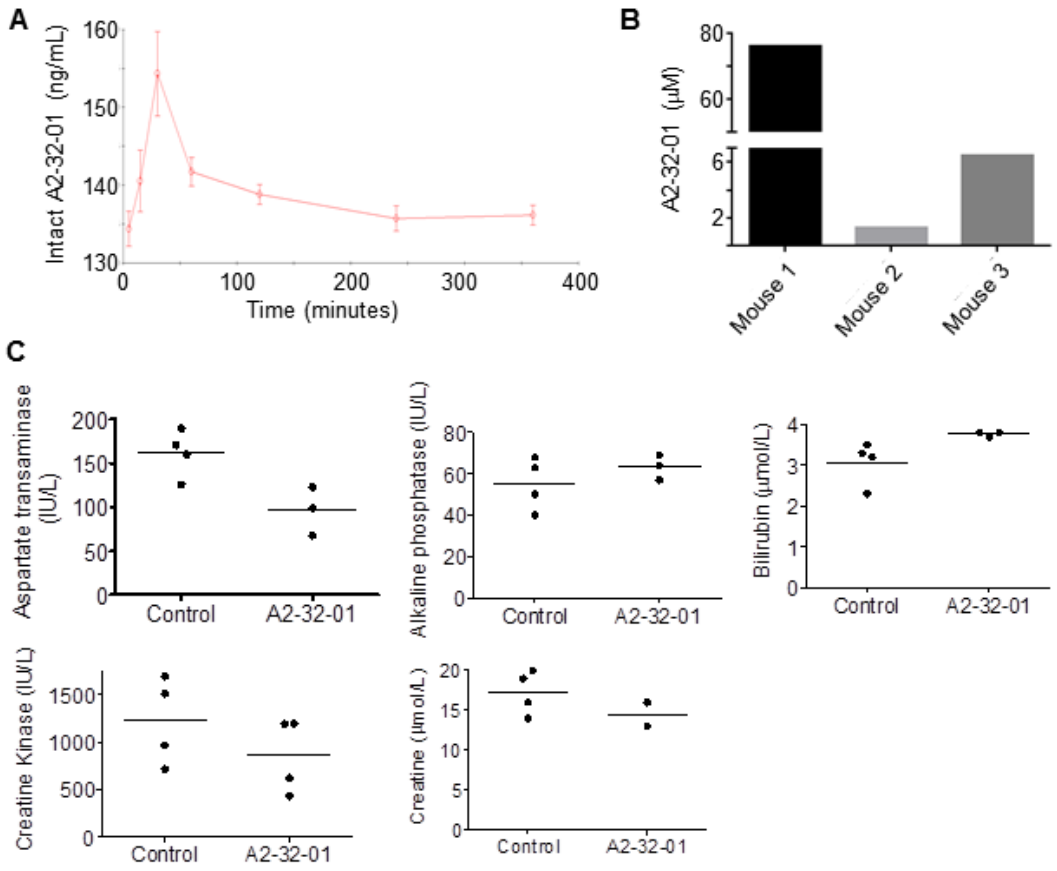
**(E)** OCI-AML2 cells were transduced with shRNA targeting ClpP or control sequences. Increasing concentrations of A2-32-01 were added and the number of viable cells after 72 hours of treatment was measured by trypan blue staining as described in the material and methods. Each shRNA is normalized to 100% and reflects the cell number without A2-32-01 treatment. Insert: The effect of ClpP knockdown on the growth and viability of OCI-AML2 cells is shown. The data represents mean ± SD.

**(F)** Chemical structure of A2-54-01 (left panel). Effects of increasing concentrations of A2-54-01 and A2-32-01 on the activity of purified bacterial ClpP (*E. coli* ClpP) to cleave the fluorogenic substrate Suc-LY-AMC (middle panel). TEX and OCI-AML2 cells were treated with increasing concentrations of A2-54-01 and A2-32-01 as described in the material and

methods. Forty-eight hours after incubation, the mean ± SD percent of viable cells was measured by Trypan Blue staining (right panel).

**(G)** Chemical structure of A2-58-06 (left panel). Effects of increasing concentrations of A2-58-06 and A2-32-01 on the activity of purified bacterial ClpP (*E. coli* ClpP) to cleave the fluorogenic substrate Suc-LY-AMC (middle panel). TEX and OCI-AML2 cells were treated with increasing concentrations of A2-54-01 and A2-32-01 as described in the Material and Methods. Forty-eight hours after incubation, the mean ± SD percent of viable cells was measured by Trypan Blue staining (left panel).

**(H)** Degradation of A2-32-01 over time. A2-32-01 was dissolved in IMDM medium (10 μM final concentration) and incubated at room temperature. The stability of A2-32-01 was measured over time using a Waters Xevo Quadruple Time-of-flight (Q-Tof) hybrid mass spectrometer (MS) system coupled with ACQUITY ultra-performance liquid chromatography (UPLC). Data represent mean ± SD concentration of intact A2-32-01.


A
Intact A2-32-01 (ng/mL)
160
150
140
130
0
100
200
300
400
Time (minutes)
B
A2-32-01 (μM)
80
60
6
2
Mouse 1
Mouse 2
Mouse 3
C
Aspartate transaminase (IU/L)
200
150
100
50
0
Control
A2-32-01
Alkaline phosphatase (IU/L)
80
60
40
20
0
Control
A2-32-01
Bilirubin (μmol/L)
4
3
2
1
0
Control
A2-32-01
Creatine Kinase (IU/L)
1500
1000
500
0
Control
A2-32-01
Creatine (μmol/L)
20
15
10
5
0
Control
A2-32-01

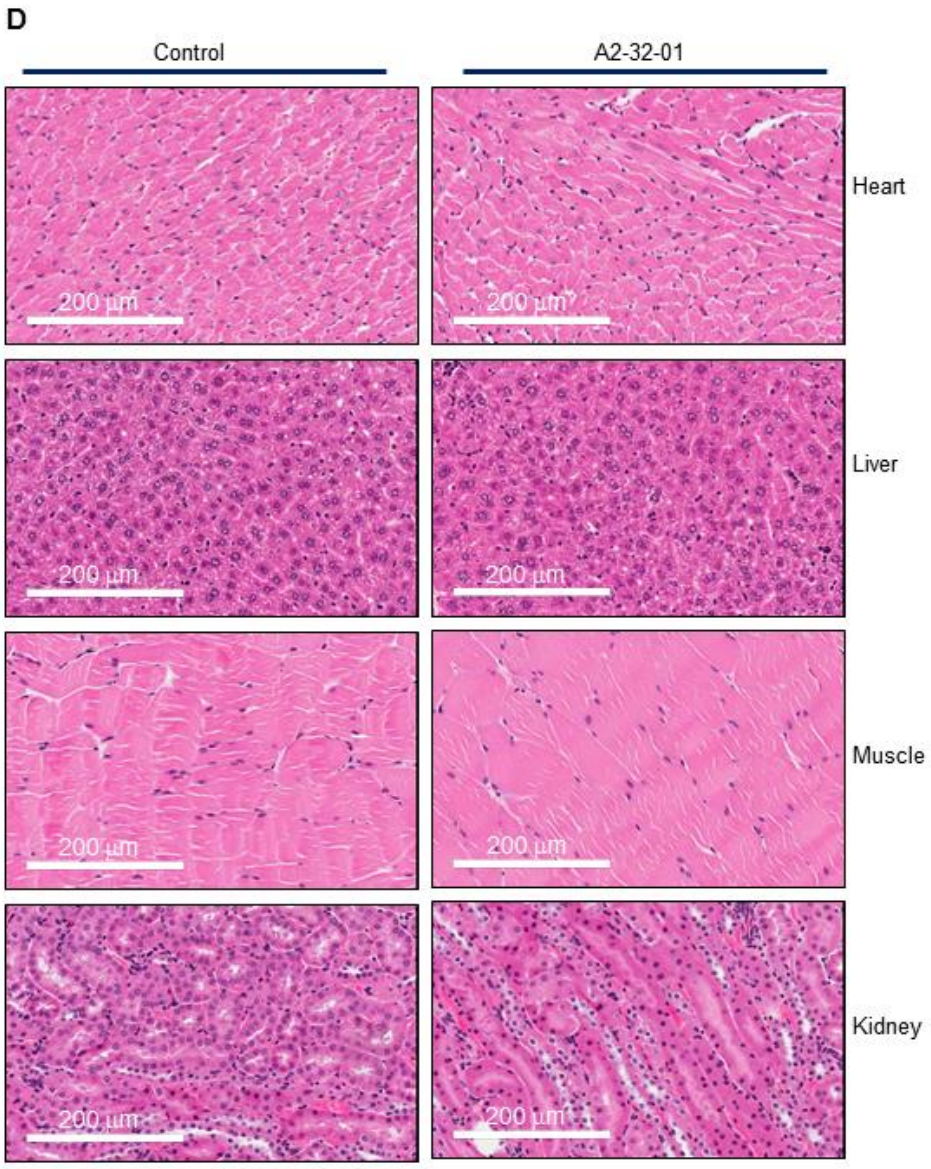



**Figure S5 related to Figure 7: A2-32-01 shows anti-AML activity in xenograft models of human leukemia**

**(A)** SCID mice were treated with a single dose of A2-32-01 (50 mg/kg) dissolved in corn oil via intraperitoneal injection. Blood samples were collected from three mice at 5, 15, 30 min, 1, 2, 4, 6, and 24h. Plasma was separated from blood by centrifugation and the integrity of A2-32-01 was measured using LCMS. Data represent the mean ± SD plasma concentration of intact A2-32-01.

**(B)** SCID mice were treated with A2-32-01 (300 mg/kg) dissolved in corn oil via intraperitoneal injection daily x 3 days. One hour after the third injection, mice were sacrificed, the tumors excided, lysates prepared, and levels of A2-32-01 were measured by LCMS.

**(C)** SCID mice were xenografted with OCI-AML2 cells. Once tumors were palpable, mice were treated with A2-32-01 (300mg/kg) daily for 5 of 7 days by i.p. injection or corn oil vehicle control for 9 days. At the end of the experiment, mice were sacrificed. Peripheral blood was collected (n = 3 per group) and biochemical markers of liver (aspartate transaminase, alkaline phosphatase, bilirubin), muscle (creatine kinase), and renal (creatine) toxicity were measured. Horizontal lines indicate the mean value.

**(D)** Hematoxylin and eosin stained sections of mouse heart, liver, muscle, and kidney from one representative control and one representative A2-32-01-treated mouse.

## Supplemental Experimental Procedures

### Cell lines

TEX leukemia cells (Warner et al., 2005) were maintained in IMDM (Iscove's modified Dulbecco's medium), with 15% FBS, 2 mM L-glutamine, 20 ng/mL SCF (stem cell factor), 2 ng/mL IL-3. OCI-AML2, K562 and HL60 leukemia cells were maintained in IMDM and NB4 were grown in RPMI. 143B cells were maintained in Dulbecco's medium. Rho (0) 143B cells were maintained in Dulbecco's medium with the addition of 50 µg/mL of uridine and 100 µg/mL of sodium pyruvate. All cells were supplemented with 10% FBS and penicillin-streptomycin except where noted above. All cells were incubated at 37 °C in a humidified air atmosphere supplemented with 5% $CO_2$.

### Gene expression analysis

Affymetrix HT_HG-U133A CEL files were downloaded from GSE30375 (NCBI Gene Expression Omnibus repository). This dataset contains FACS sorted AML fractions that were functionally determined to be enriched for LSC or not (25 and 29 respectively). Data were processed using the RMA method for background correction /summarization and quantile normalized (R 3.0.1, affy_1.38.1). Heatmaps were generated with parameter 'scale' set to row to calculate Z scores (ggplot2_0.9.3.1, heatmap.2).

### Immunoblotting

Total cell lysates were prepared from cells similar to previously described (Xu et al., 2010). Briefly, cells were washed twice with phosphate buffered saline pH 7.4 and suspended in RIPA buffer. Protein concentration was measured by the Bradford assay (Bio Rad, Hercules, CA) Equal amounts of protein were subjected to 10% SDS-PAGE (dodecyl sulphate -polyacrylamide gels) followed by transfer to nitrocellulose membranes. Membranes were probed with anti-tubulin 1:3000 (Santa Cruz Biotechnology SC-9104), anti-actin 1:20,000 (Santa Cruz Biotechnology SC-69879), anti-CPN60 (Millipore 3514), anti-HSP70 1:2000 (AKA GRP75, Cell Signaling 3593s), anti-SDHA (Abcam 14715), or anti-ClpP 1:300 (Abcam 56455), and secondary antibodies from GE Health (IgG peroxidase linked species-specific whole antibody).

For native gel electrophoresis and immunoblotting, cells were lysed in the presence of n-dodecyl-beta-D-maltoside and protein concentration estimated by the BCA method using manufacturer reagents and procedures (Pierce/Thermo Scientific, Rockford, IL, USA). Mitochondria lysates were loaded onto NativePAGE Novex Bis-Tris gels and ran in the presence of dark blue followed by light blue cathode buffer using materials and protocols from the manufacturer (Life Technologies, Carlsbad, CA, USA). After electrophoresis, the molecular weight protein ladder was excised and stained with GelCode (Pierce/Thermo Scientific, Rockford, IL, USA) and the rest of the gel transferred onto PVDF. The membrane was blocked in 5% milk PBST. Anti SDHA (Abcam) was incubated in 5% milk PBST overnight, washed and

blotted with anti- mouse HRP (GE Healthcare) and finally developed with ECL (Pierce/Thermo Scientific, Rockford, IL, USA).

ClpP expression was measured in primary AML cells by immunoblotting and expressed relative to ClpP expression in OCI-AML2 cells as measured by densitometry. Primary AML cells were treated with 80 μM A2-32-01 and cell viability was measured Annexin-V/PI staining 48 hours after treatment.

**Quantitative real-time polymerase chain reaction**

cDNAs encoding ClpP and 18s were amplified using the following primer pairs: (ClpP-Forward) 5'-CCATCTACGACACGATGCAG-3', (ClpP-Reverse) 5'-CATGATCTCCTCTGCCTGGA-3', (18sForward) 5'-AGGAATTGACGGAAGGGCAC-3', (18sReverse) 5'-GGACATCTAAGGGCATCACA-3'. Equal amounts of cDNA for each sample were added to a prepared master mix (SYBR Green PCR Master mix; Applied Biosystems, Foster City, CA). Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) reactions were performed on an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) as described previously (Schimmer et al., 2006; Skrtic et al., 2011). The relative abundance of a transcript was represented by the threshold cycle of amplification (CT), which is inversely correlated to the amount of target RNA/first-strand cDNA being amplified. To normalize for equal amounts of the latter, we assayed the transcript levels of 18s gene. The comparative CT method was calculated per the manufacturer's instructions. The expression level of ClpP relative to the baseline level was calculated as $2^{-\Delta CT(mClpP-1)}$, where ΔCT is (average mClpP CT – average 18s CT) and is CT (average CT-treated sample – average CT-untreated sample).

**Measurement of basal oxygen consumption**

Measurement of oxygen consumption was performed using a Seahorse XF96 analyzer (Seahorse Bioscience, North Billerica, MA, USA) as previously described (Skrtic et al., 2011). After shRNA knockdown of ClpP, cells were resuspended with alpha MEM supplemented with 2% FCS and penicillin-streptomycin . Cells were seeded at 1 x $10^5$ cells/well in XF96 plates. Cells were equilibrated to the unbuffered medium for 45 min at 37 ºC in a $CO_2$-free incubator before being transferred to the XF96 analyzer.

**Mitochondrial isolation**

Intact mitochondria from cell lines and primary samples were isolated as previously described (Skrtic et al., 2011). Cells were harvested, washed and frozen in dry ice. Pellets were thawed on ice and lysed with sterile distilled water for 15 min at 4 ºC. Osmolarity was restored by adding equal volume of 2× ice-cold buffer A (500 mM sucrose, 20 mM Tris-HCl pH 7.0, 0.4 mM EGTA). Nuclei and unbroken cells were removed by centrifugation at 600 ×*g* at 4 ºC for 10

minutes. Mitochondria-containing supernatants were collected and centrifuged at 12,000 ×*g* for 15 min at 4 ºC. The mitochondrial-enriched pellets were resuspended in 1× buffer A (250 mM sucrose, 10 mM Tris-HCl pH 7.0 and 0.2 mM EDTA pH 8.0) containing 0.5 mM PMSF and aliquots stored at -80 ºC. Mitochondrial protein concentration was determined using the Bradford Protein Assay (BioRad).

**Respiratory chain complex II enzymatic assays**

Complex II activity was measured in isolated mitochondria by monitoring malonate sensitive reduction of 2,6-dichloroindophenol when coupled to complex II-catalyzed reduction of decylubiquinol (Jung et al., 2000).

**Mitochondrial ROS measurement**

Cells (2 x $10^4$) were centrifuged, resuspended in 0.2 mL of MitoSox (Molecular Probes/Life Technologies, Eugene, OR, USA) and incubated in the dark for 30 minutes at 37 ºC and 5% $CO_2$ in humidified atmosphere. Cells were then centrifuged to remove the dye, resuspended in 0.2 mL binding buffer containing Annexin V-FITC (BioVision, Milpitas, CA, USA) and analyzed by flow cytometry on a Canto II 96 well cytometer (Becton Dickinson, San Jose, CA, USA). 50 µM of antimycin A (Sigma, Saint Louis, MO, USA) treatment was used as positive control for increased ROS production. The percentage of Annexin V negative and MitoSox positive cells was determined and the fold increase of ROS production was calculated.

**Mitochondrial mass measurements**

Mitochondrial mass was measured by staining OCI-AML2 cells with 100 nM MitoTracker Deep Red FM (Molecular Probes, Eugene, OR) in Phenol Red-free Hanks Buffer for 30 minutes at 37 $^{o}$C. After incubation cells were centrifuged and stained with Annexin V-FITC and flow cytometry performed in a Canto II 96 wells cytometer (Becton Dickinson). Data were analyzed with FlowJo version 7.7.1 (TreeStar).

**Transmission electron microscopy**

Transmission electron microscopy (TEM) was used to assess the mitochondrial morphology of OCI-AML2 cells which had been genetically depleted of ClpP using shRNA's in lentiviral vectors as described above. Cells (5 x $10^6$) were harvested, and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 1 hour at room temperature. Cells were washed with cacodylate buffer 3 times for 30 minutes. Next, cells were post-fixed with 1% osmium tetroxide for 1 hour and washed again using distilled water twice for 30 minutes, dehydrated with ethanol, washed with propylene oxide, and infiltrated and embedded with epoxy resin, which was polymerized at 55 $^{o}$C for 48 h. The solid epoxy blocks were sectioned on a Leica UC 6 microtome to 90 nm thickness, collected on 300 mesh copper grids and counterstained with

uranyl acetate and lead citrate. Sections were examined with a Hitachi H7650 (Hitachi, Tokyo, Japan) transmission electron microscope at an accelerating voltage of 80 kV.

**Cell proliferation and viability assays**

Cell lines or primary samples were seeded at 1 x $10^5$ cells/mL in 20 mL of medium in T75 flasks. Cells were then treated with increasing concentrations of A2-32-01 or DMSO control. Fresh A2-32-01 or vehicle control was added once daily during the incubation period. Cells were harvested 6 hours after the last treatment with A2-32-01. Cell growth and viability was measured using trypan blue staining.

Primary AML and normal hematopoietic stem cells were treated as above. Cell death was measured by Annexin V fluoroscein isothiocyanate (FITC) and Propidium Iodide (PI) (Biovision Research Products, Mountain View, CA) staining using flow cytometry according to the manufacturer's instructions. Clonogenic growth assays with primary AML mononuclear cells were performed as previously described (Spagnuolo et al., 2010). AML mononuclear cells (2 x$10^6$ cells) were treated with A2-32-01 or with DMSO daily for 48 hours. After treatment, cells were washed and plated by volume in duplicate 35 mm dishes (Nunclon, Rochester, USA) in a final volume of 1mL/dish in MethoCult GF H4434 medium (StemCell Technologies, Vancouver, Canada) and incubated at 37 $^{o}$C, 5% $CO_2$ with 95% humidity for 7 days. AML colonies containing 10 or more cells were counted as previously described (Spagnuolo et al., 2010).

In vitro hematopoiesis in ClpP WT and $^{-/-}$ mice was evaluated by culturing bone marrow cells (5 x $10^4$) in MethoCult™ GF M3434 (StemCell Technologies) in duplicate in 35 mm dishes (Nunclon, Rochester, USA) in a final volume of 1mL/dish. Cultures were incubated at 37 $^{o}$C, 5% $CO_2$ with 95% humidity for 8 days and BFU-E and CFU-GM counted.

**Stability and pharmacokinetics of A2-32-01**

The concentration of intact A2-32-01 in IMDM medium or blood was measured using Waters Xevo Quadruple Time-of-flight (Q-Tof) hybrid mass spectrometer (MS) system coupled with ACQUITY ultra-performance liquid chromatography (UPLC). Chromatographic separations were carried out on an ACQUITY UPLC BEH C18 (2.1 X 50 mm, 1.7 µm) column. The mobile phase was 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Compound concentrations were measured relative to a freshly prepared calibration curve.

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