Confocal immunofluorescence microscopy

HL-60 and OCI-AML3 cells were cultured and cytospun onto glass slides. The cells were double immunofluorescence stained for Mt NPM1 and fibrillarin (nucleolar marker). Briefly, the cells were fixed with 4% paraformaldehyde in PBS for 10 minutes, permeabilized with 0.5% Triton X-100 in PBS for 5 minutes, and blocked with blocking buffer (3% BSA and 0.05% Tween-20 in PBS) for one hour. Following this, cells were incubated for one hour with anti-mut-NPM1 antibodies. After 3 washes with PBS, the slides were stained with anti-fibrillarin (nucleolar marker) and incubated for 1 hour at room temperature. The slides were washed three times with 1× PBS and incubated with Alexa Fluor 488 and Alexa Fluor 594-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) for 1 hour at a 1:2000 dilution and washed with 1× PBS. Cells were counterstained with Vectashield mountant containing DAPI (4′,6-diamidino-2-phenylindole) and imaged using Zeiss LSM510 confocal microscope using a 63×/1.2 NA oil immersion lens (Carl Zeiss, Heidelberg, Germany).

Primer sequences for reverse transcription polymerase chain reaction and quantitative polymerase chain reaction

The following primers were used for semi-quantitative or quantitative polymerase chain reaction against cDNA from cultured and primary AML cells.

NPM-wt	Forward	5′-GGTGGTTCTCTTCCCAAAGTGGA-3′
NPM1-wt	Reverse	5′-GACTTCCTCCACTGCCAGAGATC-3′;
NPM1-mut	Reverse	5′-GAGACTTCCTCCACTGCCAGAC-3′.
HoxA9:	Forward	5′-TGCAGCTTCCAGTCCAAGG-3′
	Reverse	5′-GGACAAAGTGTGAGTGTCA-3′
Meis1:	Forward	5′-GATCAGCAAATCTAACTGACCAG-3′
	Reverse	5′-TCTTCAGAAGGGTAAGGGTG-3′
FLT3:	Forward	5′-TGTCGAGCAGTACTCTAAACA-3′
	Reverse	5′-ATCCTAGTACCTTCCCAAACTC-3′
β-actin:	Forward	5′-CTACAATGAGCTGCGTGTGG-3′
	Reverse	5′-AAGGAAGGCTGGAAGAGTGC-3′.

Figure S1. Detection and localization of nucleophosmin in HL-60 and OCI-AML3 cells
(A) RNA was extracted from HL-60 and OCI-AML3 cells, reverse transcribed. The resulting cDNAs were PCR amplified with NPM1 wild-type (wt) and mutant (mut) detecting primers. PCR amplicons were resolved on a 2% agarose gel and documented with a UV transilluminator. (B) DNA (top panel) and protein (bottom panel) sequences of wild-type and mutant nucleophosmin (NPM1) are as depicted. In the DNA sequence, the mutant insert sequence is in bold letters and stop codon for each sequence is underlined. (C) Immunoblot analyses of mutant and wild type NPM1 were performed on total cell lysates and sub-cellular fractions (nuclear and cytosolic) from HL-60 and OCI-AML3 cells. The expression of EZH2 and β-actin served as the nuclear and cytosolic fraction controls, respectively. (D) HL-60 and OCI-AML3 cells were cytospun onto glass slides then fixed, permeabilized and stained for Mt NPM1, the nucleolar marker protein fibrillarin, and DAPI. Images were acquired with an LSM510 Meta confocal microscope using a 63×/1.2 oil immersion lens. Representative images for HL-60 and OCI-AML3 are displayed.

Figure S2. NPM1 knockdown in HL-60 cells does not induce sensitivity to ATRA induced apoptosis
HL-60 cells were transfected with control and NPM1 siRNA and incubated for 24 hours. Then cells were treated with the indicated concentrations of ATRA for 48 hours. At the end of treatment, cells were washed with 1× PBS and stained with annexin V and propidium iodide. The percentages of apoptotic cells were determined by flow cytometry. Columns represent the mean of three independent experiments; Bars represent the standard error of the mean.

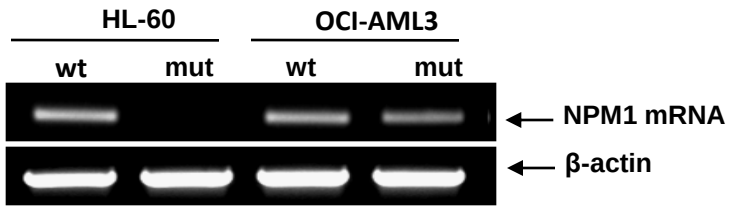
Figure S3. NPM1 knockdown does not abrogate the apoptotic effects of NSC348884 treatment in AML cells
OCI-AML3 cells were transfected with control and NPM1 siRNA and incubated for 48 hours. Cells were washed with complete media and then treated with the indicated concentrations of NSC348884 for 24 hours. Cells were stained with annexin V and propidium iodide and the percentages of apoptotic cells were determined by flow cytometry. Columns represent the mean of three independent experiments; Bars represent the standard error of the mean.

Figure S4. Detection of mutant NPM1 and FLT3-ITD in primary AML samples
Of the 20 primary AML samples analyzed, 9 are shown as positive for Mt NPM1 determined by qPCR described in Methods. Total RNA was isolated from 20 primary AML cell samples and reverse transcribed. The resulting cDNAs were PCR amplified with FLT3-specific primers (Sequences are listed in Supplemental Methods). Amplified PCR products were resolved on a 2% agarose gel and documented with a UV transilluminator. Samples exhibiting amplicons greater than 366 base pairs are considered to be positive for FLT3-ITD.

Figure S5. Effect of treatment with NSC348884 and/or Ara-C in primary AML with WT NPM1 and Mt-NPM1 cells
Primary AML cells with WT-NPM1 (n=3), Mt-NPM1 (n=3) or normal CD34+ cells (n=3) were treated with the indicated concentrations of NSC348884 and/or Ara-C for 48 hours. At the end of treatment, cells were washed with 1× PBS and stained with annexin V and propidium iodide. The percentages of apoptotic cells were determined by flow cytometry. Columns represent the mean of three independent experiments; Bars represent the standard error of the mean.

Figure S1

A



B

DNA

Wild-type: GATCTCTG----GCAGTGGAGGAAGTCTCTTTAA

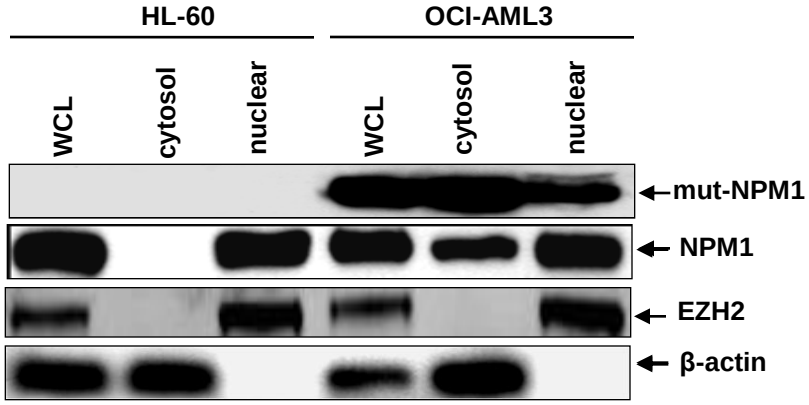
Mutant: GATCTCTG**TCTG**GCAGTGGAGGAAGTCTCTTTAAGAAAATAG

Protein

Wild-type: D L W Q W R K S L Stop

Mutant: D L **C L A V E E V S L R K** Stop

C



D

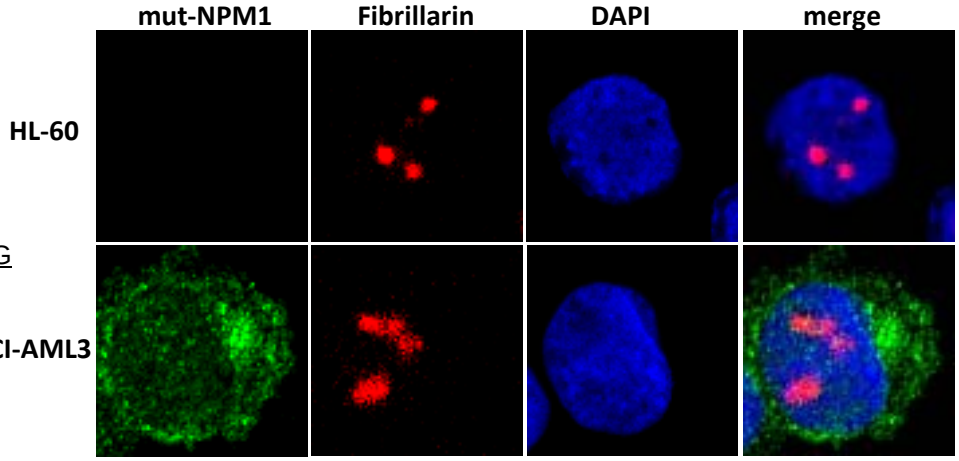


Figure S2

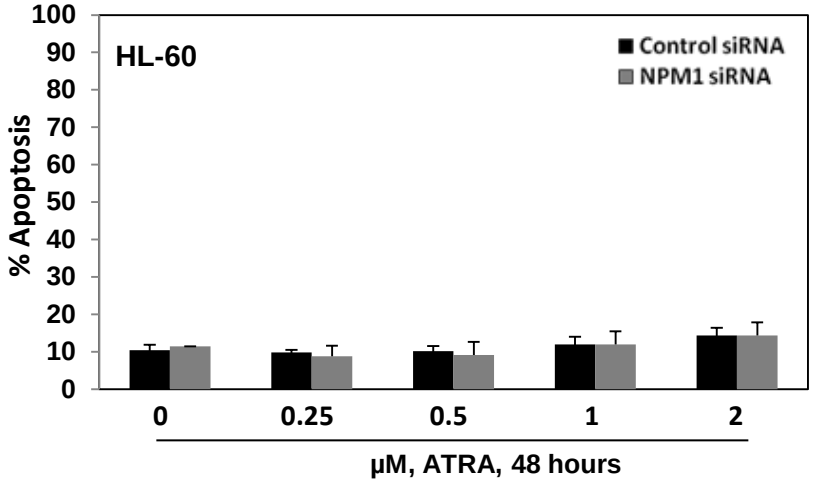

HL-60
Control siRNA
NPM1 siRNA
% Apoptosis
100
90
80
70
60
50
40
30
20
10
0
0
0.25
0.5
1
2
μM, ATRA, 48 hours

Figure S3

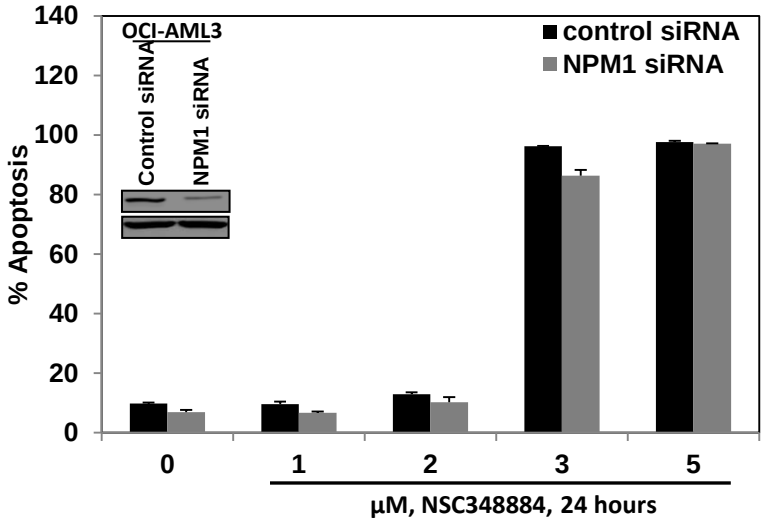

OCI-AML3
Control siRNA
NPM1 siRNA
control siRNA
NPM1 siRNA
% Apoptosis
140
120
100
80
60
40
20
0
0
1
2
3
5
µM, NSC348884, 24 hours

Figure S4

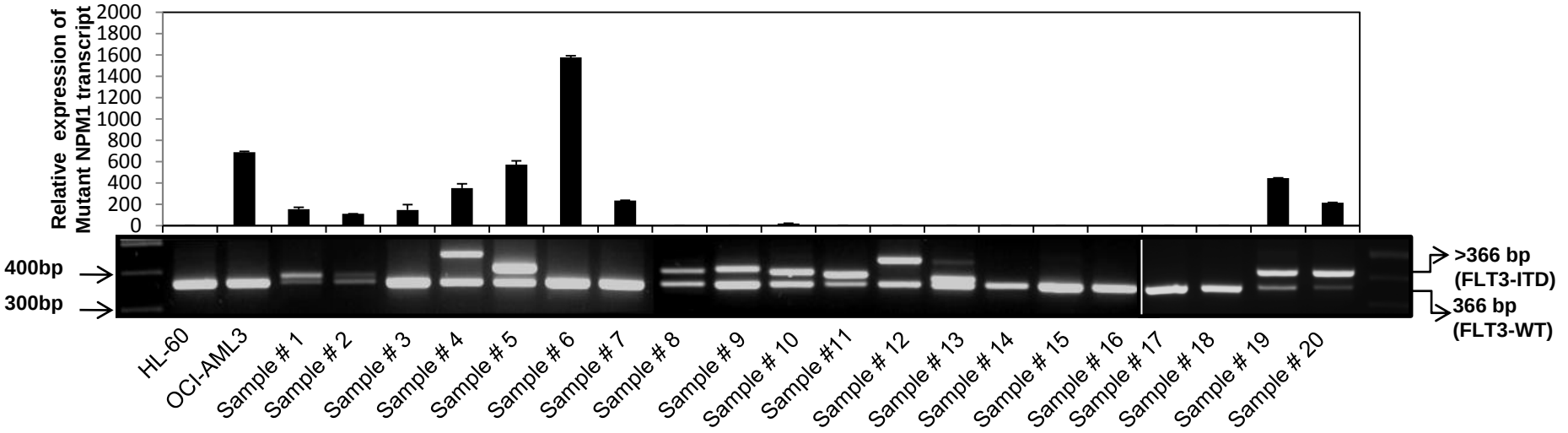
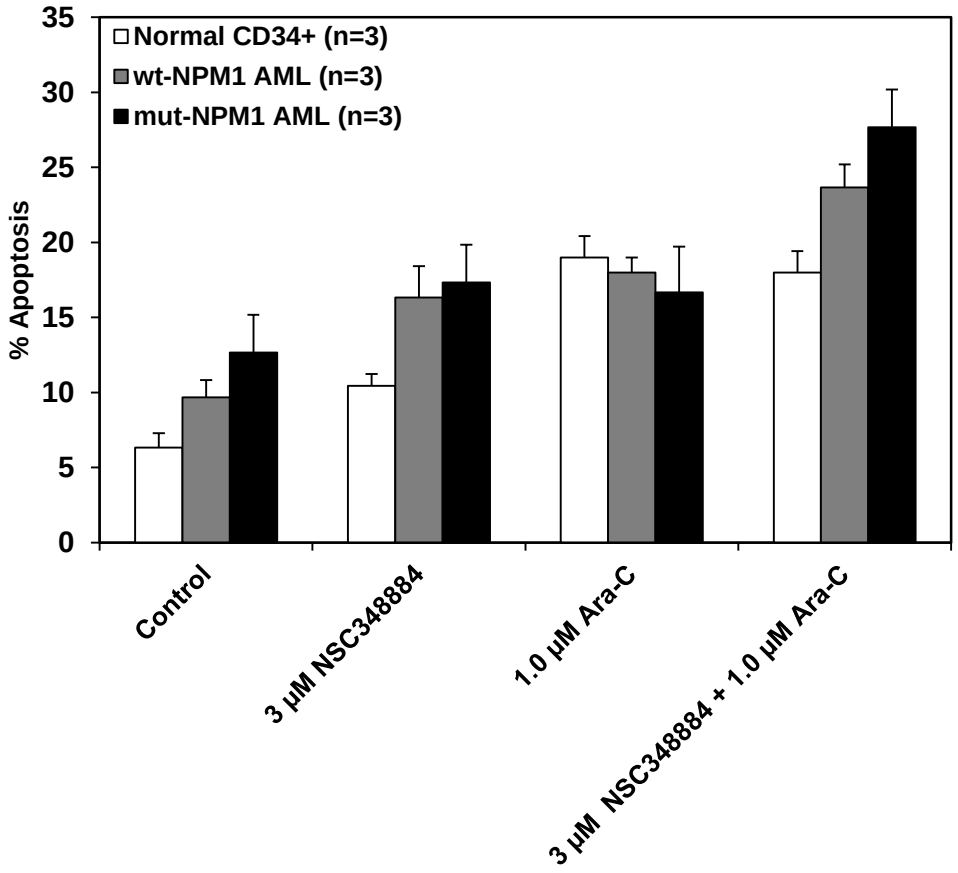

Relative expression of Mutant NPM1 transcript
2000
1800
1600
1400
1200
1000
800
600
400
200
0
400bp
300bp
>366 bp (FLT3-ITD)
366 bp (FLT3-WT)
HL-60
OCI-AML3
Sample # 1
Sample # 2
Sample # 3
Sample # 4
Sample # 5
Sample # 6
Sample # 7
Sample # 8
Sample # 9
Sample # 10
Sample #11
Sample # 12
Sample # 13
Sample # 14
Sample # 15
Sample # 16
Sample # 17
Sample # 18
Sample # 19
Sample # 20

Figure S5


Normal CD34+ (n=3)
wt-NPM1 AML (n=3)
mut-NPM1 AML (n=3)
% Apoptosis
35
30
25
20
15
10
5
0
Control
3 µM NSC348884
1.0 µM Ara-C
3 µM NSC348884 + 1.0 µM Ara-C