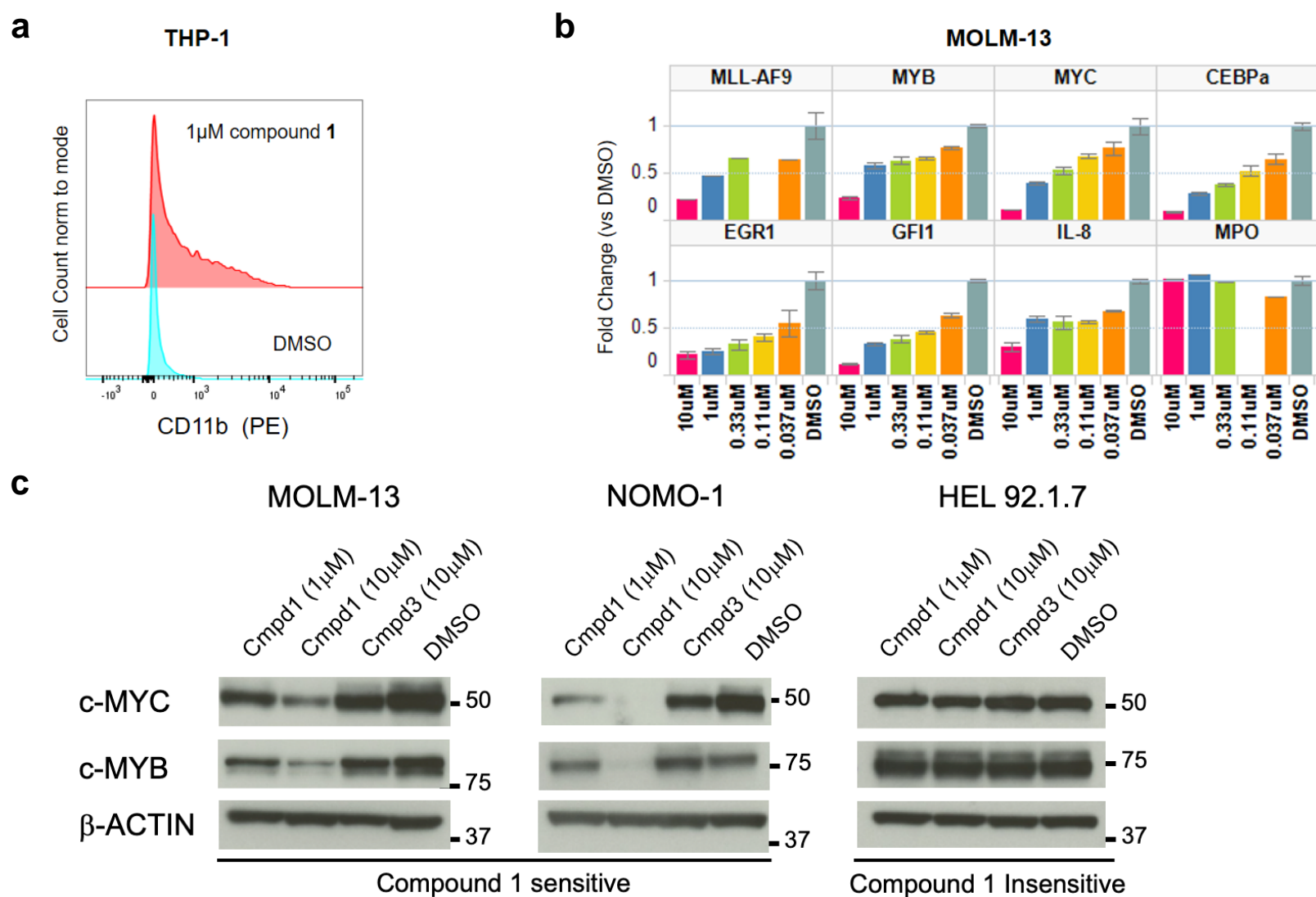


Supplementary Figure 1: a, FACS read-out for cell surface differentiation markers in A-673 cells, following compound **1** treatment (left panels) or upon knock-down of EWS-FLI1 in A-673 cells stably transduced with a vector containing published shRNA 1917 against FLI1 (right panels) (n=2). **b,c**, Analysis of EWS-FLI1 target gene NKX2-2 expression in response to treatment of A-673 cells with compound **1** by **(b)** RT-qPCR, with doxycycline (dox) inducible shRNA-mediated knock-down of EWS-FLI1 for comparison (relative quantification by standard curve was applied for all qPCR reactions, normalizing transcript quantities per target gene to those of endogenous control PPIA per sample and setting the DMSO-treated condition per target gene equal to 1. Center represents mean and error bars represent standard deviation, with three biological replicates per condition), or **(c)** by western blot with β-actin as loading control (repeated two independent times).



Supplementary Figure 2: **a**, FACS read-out for cell surface differentiation marker CD11b in THP-1 cells following compound 1 treatment for 72 h (n=1). **b**, Expression analysis by RT-qPCR of MLL-AF9 and a panel of known target genes in MOLM-13 cells treated with compound 1 in dose-response for 4 h (relative quantification by standard curve was applied for all qPCR reactions, normalizing transcript quantities per target gene to those of endogenous control PPIA per sample and setting the DMSO-treated condition per target gene equal to 1. Center value represents mean, error bars represent standard deviation, with three biological replicates, except for MLL-AF9 and MPO, which were processed as three technical replicates of one biological replicate). **c**, Expression analysis by western blot of c-MYC and c-MYB in response to compound 1 treatment comparing compound-sensitive AML cell lines with MLL-fusion (MOLM-13 and NOMO-1) to compound-insensitive and non-translocated AML cell line HEL 92.1.7 (repeated 3 independent times). An uncropped scan of this blot is provided in Supplementary Figure 20.

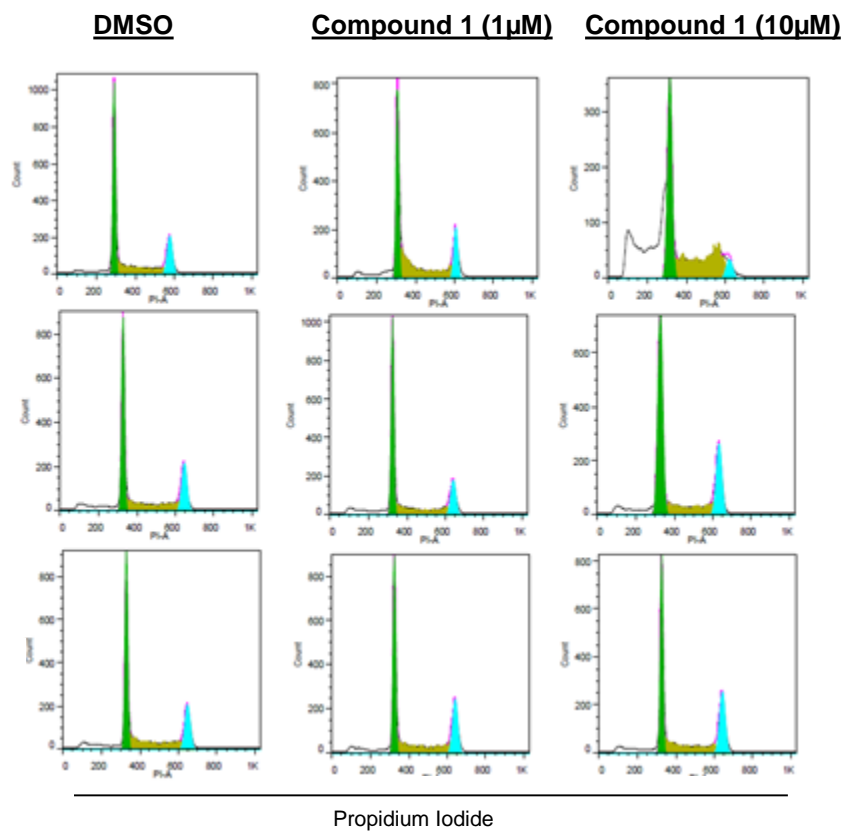
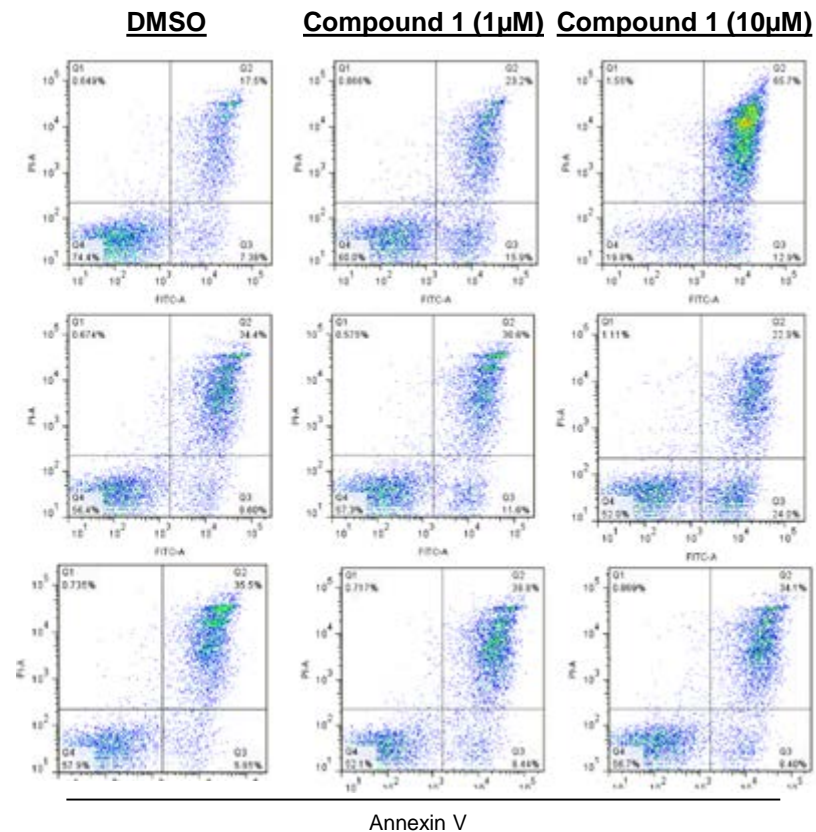
a**Cell Cycle**

■ sub-G1 (%)
 ■ G1 (%)
 ■ S (%)
 ■ G2 (%)

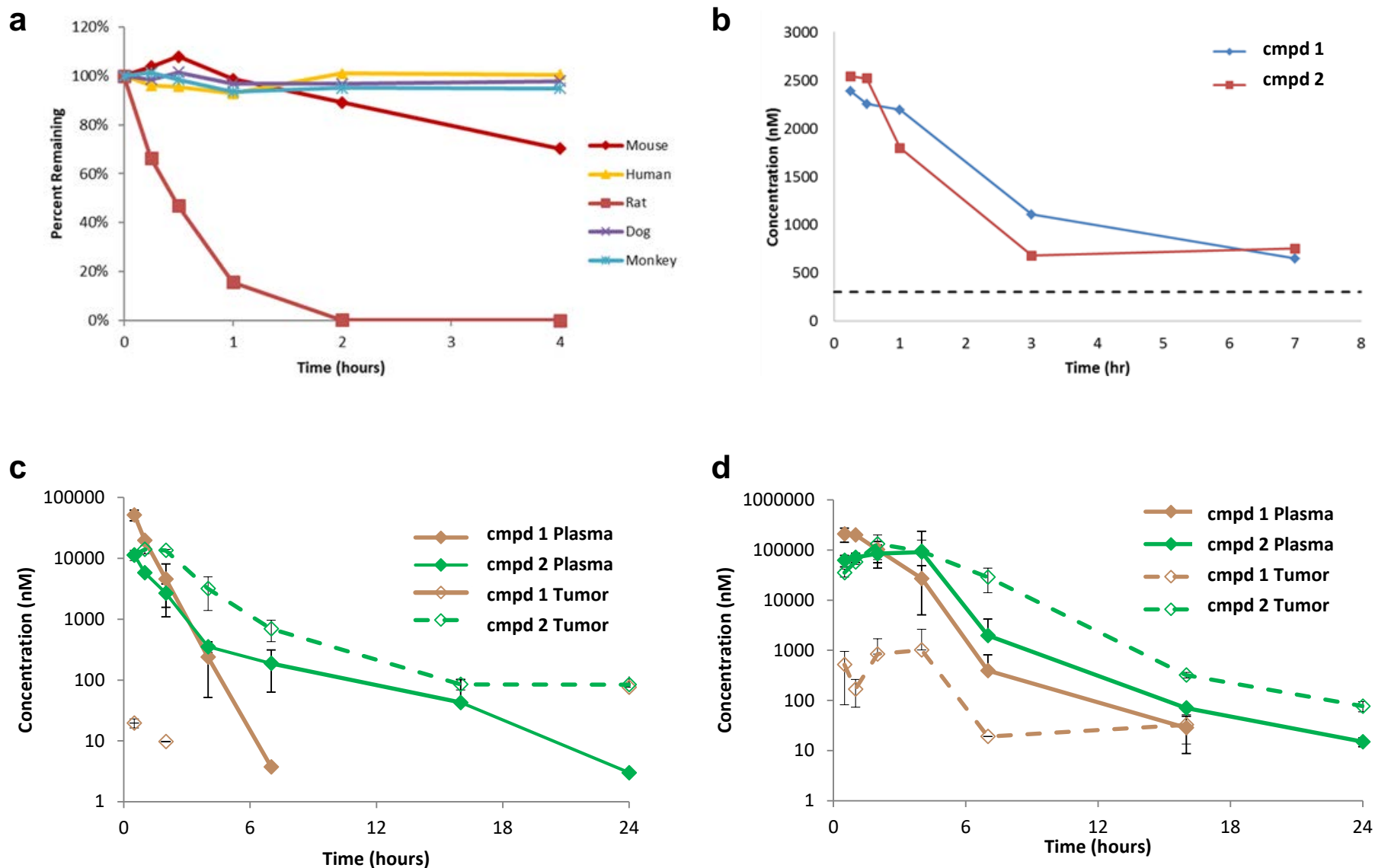
24 hours

4 hours

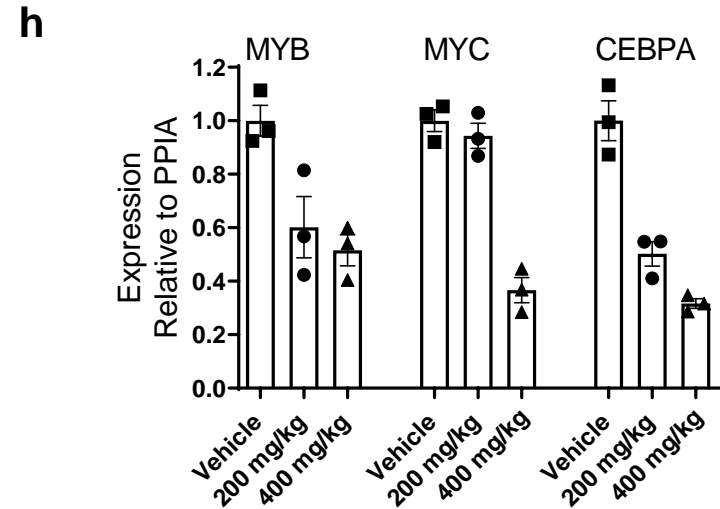
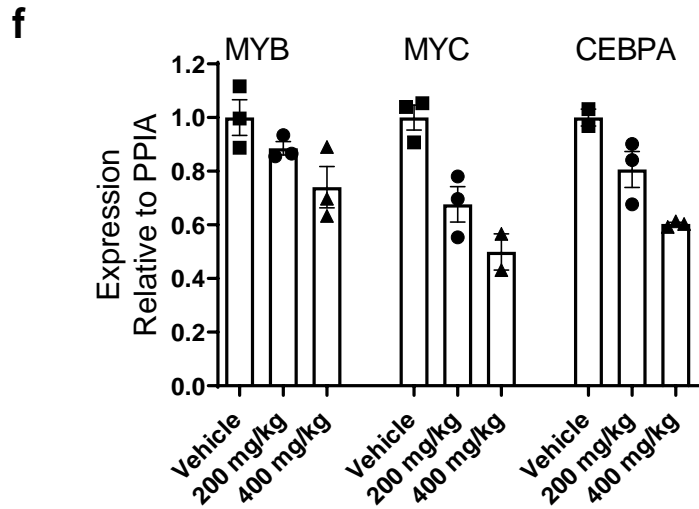
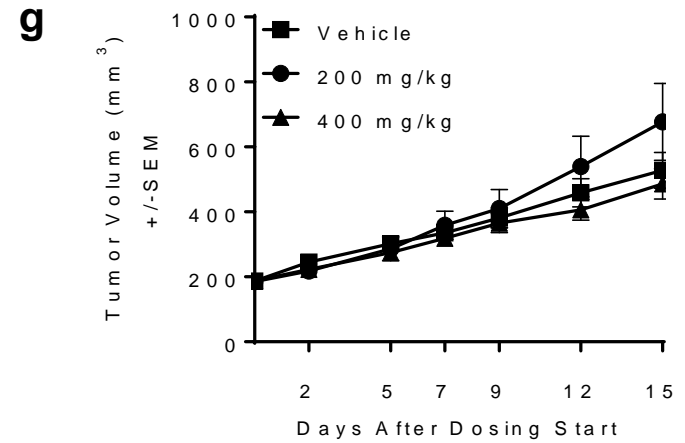
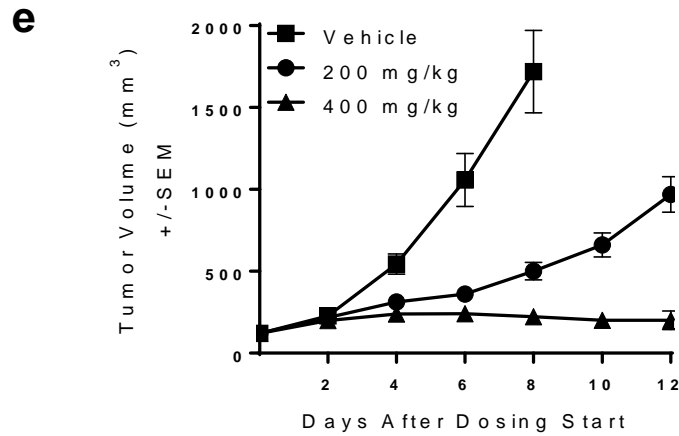
1.5 hours

**b****Apoptosis**

Supplementary Figure 3: a, Cell cycle analysis by propidium iodide staining or **b**, apoptosis assessment by Annexin V staining and FACS read-out in NOMO-1 cells treated with compound **1** as indicated (repeated independently two times).



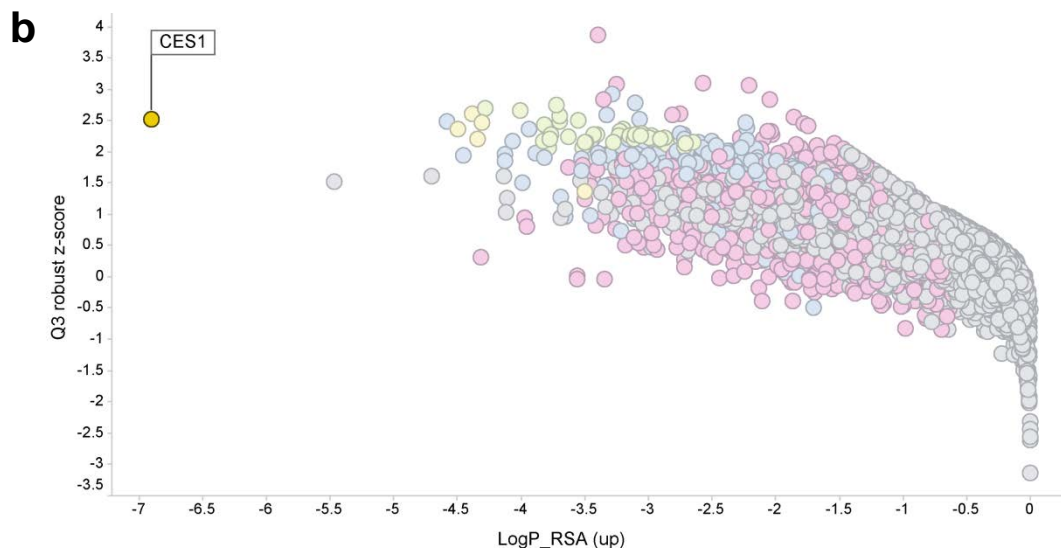
Supplementary Figure 4: MOLM-13 xenograft mouse *in vivo* study, including plasma stability and pharmacokinetics. **a**, Assessment of compound **1** stability in plasma per species as indicated, incubating 2.5 μ g/ml of **1** at 37°C for 4.5h in presence of K2 EDTA as anti-coagulant. **b**, Plasma concentration over time of compound **1** and **2** when dosing **1** subcutaneously in mice at 30mg/kg (n = 2). Black dashed line represents compound **1** IC₅₀ value in MOLM-13 cell line. **c,d**, Plasma and tumor concentration over time of compound **1** and **2** when dosing **1** subcutaneously at **(c)** 100 mg/kg or **(d)** at 400 mg/kg in mouse xenograft model (n = 3, presented as mean plus/minus standard deviation).



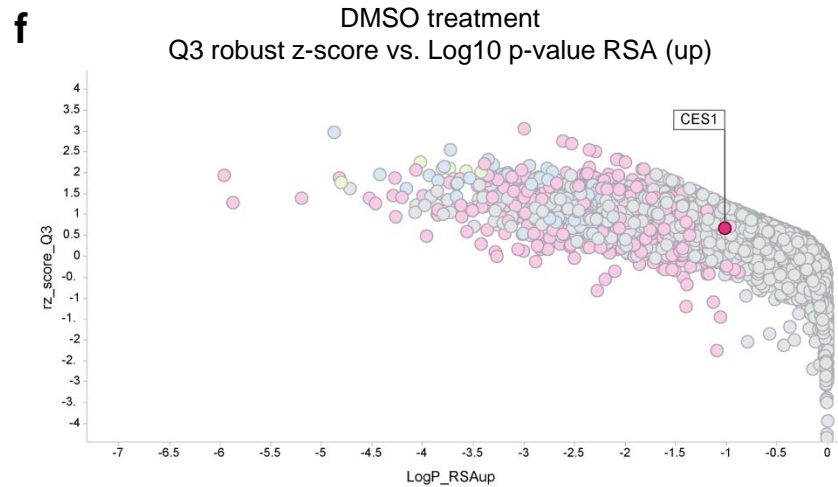
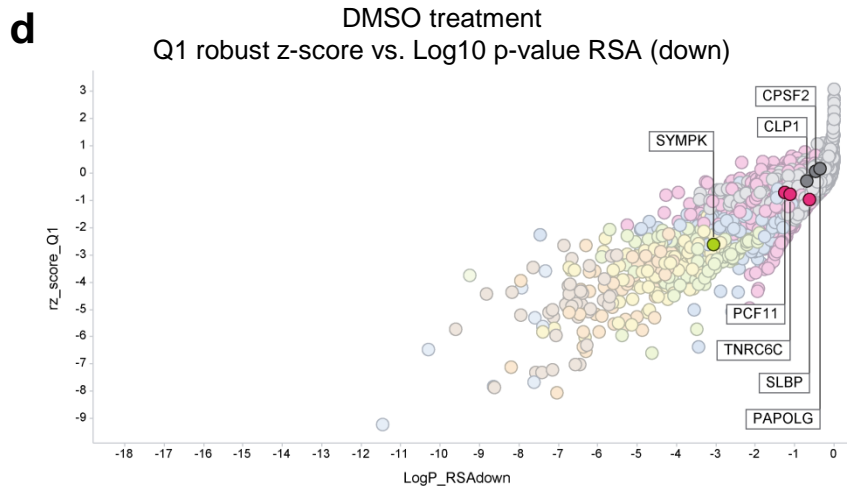
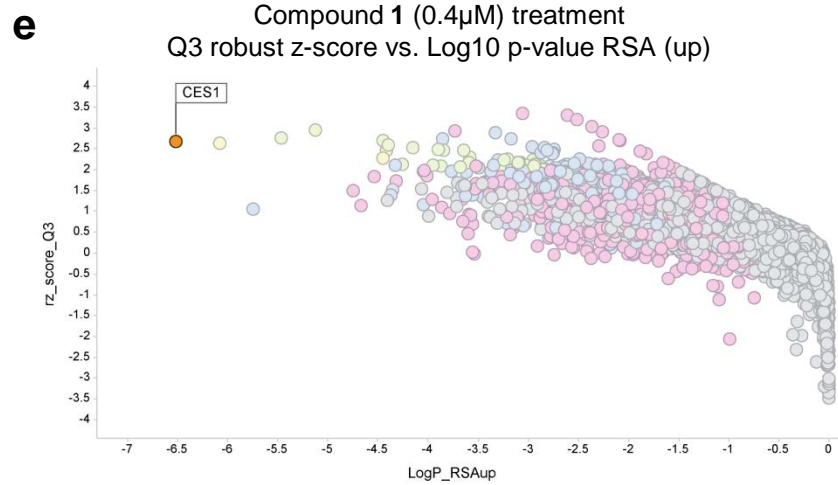
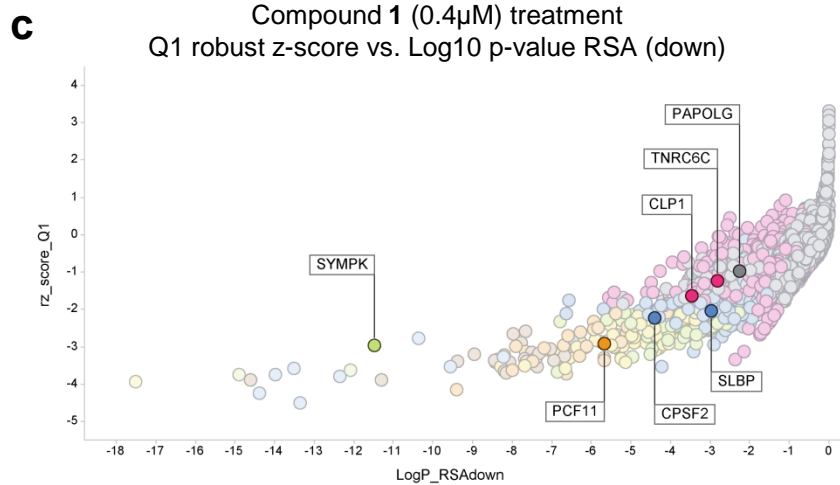
Supplementary Figure 4 continued: **e**, Tumor volume and **f**, biomarker expression by RT-qPCR in MOLM-13 mouse xenografts treated with compound **1**. **g**, Tumor volume and **h**, biomarker expression by RT-qPCR in mouse xenografts of A549 lung adenocarcinoma cells (compound insensitive, high CES1 expression) treated with compound **1**. For efficacy experiments, 2×10^6 cells were implanted subcutaneously and allowed to grow until tumor volume reached 150-200 mm³ (8 days for MOLM-13, 21 days for A549). **1** was dosed twice per day by subcutaneous injection. Relative quantification by comparative CT method was applied for all qPCR reactions, normalizing transcript quantities per target gene to those of endogenous control PPIA per sample and setting the vehicle-treated condition per target gene equal to 1. Center value presented as mean plus/minus standard error of mean, with eight animals per treatment group.

a

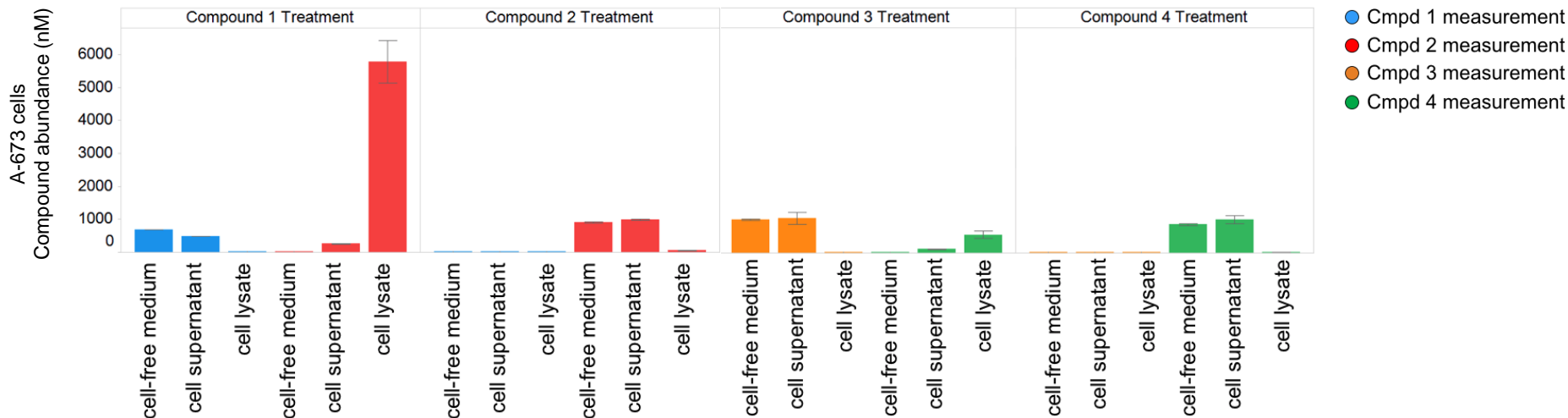
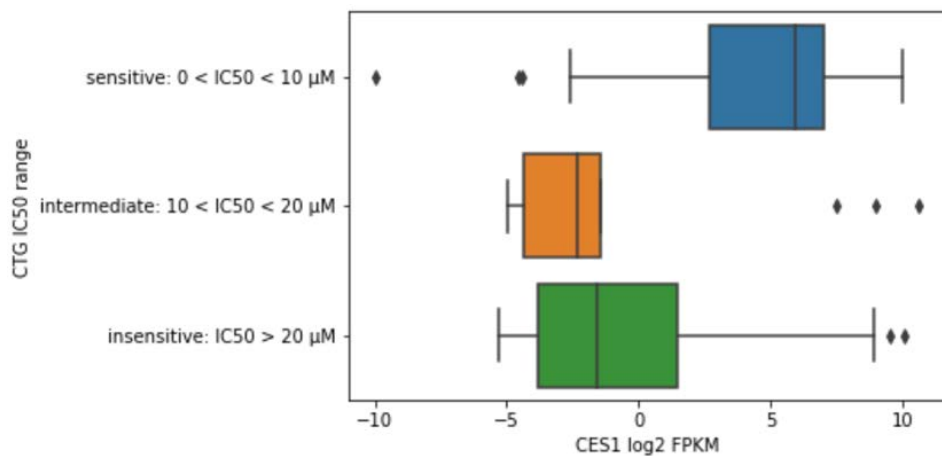
GO term	FDR	Genes in network	Genes in genome	GO term type	GO accession
termination of RNA polymerase II transcription	4.21E-47	25	45	biological_process	GO:0006369
mRNA 3'-end processing	2.06E-40	26	88	biological_process	GO:0031124
DNA-templated transcription, termination	6.25E-39	25	84	biological_process	GO:0006353
RNA 3'-end processing	6.47E-39	26	101	biological_process	GO:0031123
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	1.97E-28	25	208	biological_process	GO:0000377
mRNA splicing, via spliceosome	1.97E-28	25	208	biological_process	GO:0000398
RNA splicing, via transesterification reactions	3.47E-28	25	214	biological_process	GO:0000375
mRNA processing	3.47E-28	27	288	biological_process	GO:0006397
RNA splicing	1.61E-25	25	274	biological_process	GO:0008380
nucleic acid transport	1.08E-18	15	85	biological_process	GO:0050657
RNA transport	1.08E-18	15	85	biological_process	GO:0050658
RNA localization	1.08E-18	15	84	biological_process	GO:0006403
establishment of RNA localization	1.08E-18	15	85	biological_process	GO:0051236
mRNA export from nucleus	2.46E-18	14	68	biological_process	GO:0006406
mRNA transport	2.87E-18	14	69	biological_process	GO:0051028
RNA export from nucleus	1.18E-17	14	76	biological_process	GO:0006405
nucleobase-containing compound transport	2.81E-17	15	106	biological_process	GO:0015931
nuclear export	3.77E-15	14	113	biological_process	GO:0051168
mRNA cleavage factor complex	4.78E-12	7	12	cellular_component	GO:0005849
RNA polyadenylation	1.33E-11	8	24	biological_process	GO:0043631



Supplementary Figure 5: Genome-wide siRNA plus compound synergy screen. The siRNA library was tested as one replicate with each gene represented by $n=8$ siRNAs on average per condition. **a**, 20 most highly enriched GO terms from GeneMANIA analysis of top 50 gene candidates (compound **1** minus DMSO analysis). **b**, Full genome siRNA plus compound **1** synergy screen in A-673 cells, with cell viability read-out via CellTiter Glo assay, as shown in Figure 2a, but here plotting of redundant siRNA activity (RSA statistical model, one-sided, up) versus Q3 Z-score to identify siRNA-targeted genes whose knock-down desensitized cells to compound **1** treatment. Top screening hit CES1 labeled.

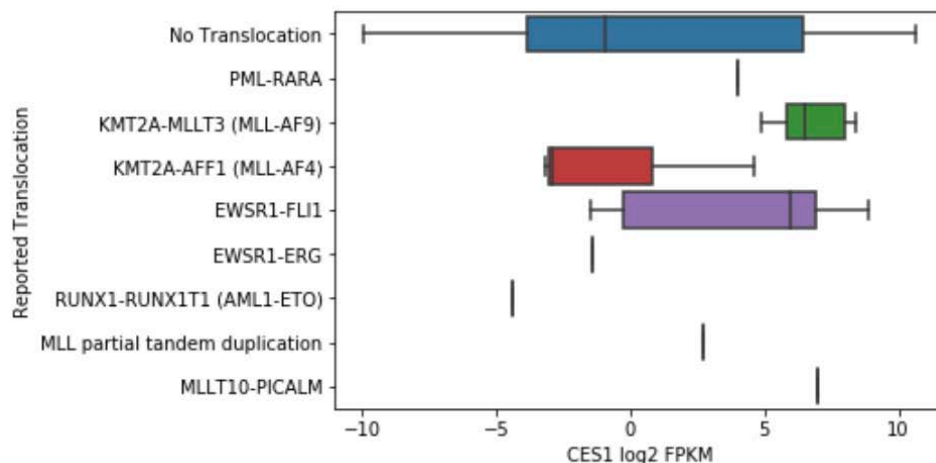


Supplementary Figure 5 continued: c-f, Gene-level activity in individual screening conditions as indicated. Known regulators of mRNA processing (RSA statistical model, one-sided, down versus Q1) as well as CES1 (RSA statistical model, one-sided, up versus Q3) are highlighted and named.

a**b**

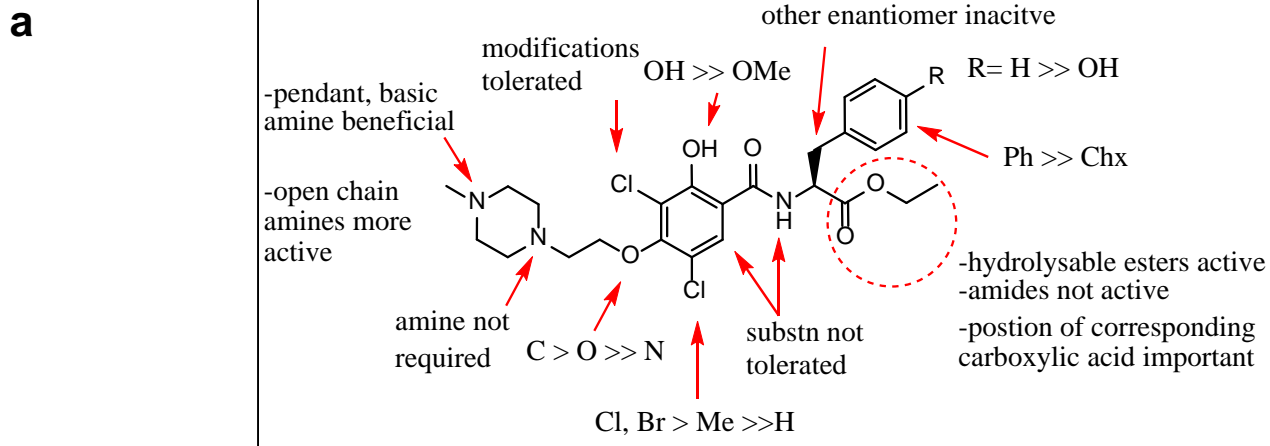
cell_viability_ctg_assay_ic50_range	count	mean	std	min	25%	50%	75%	max
insensitive: IC ₅₀ > 20 -μM	33	-0.256780663	4.705832014	-5.288398479	-3.841009155	-1.602478555	1.489078884	10.05994969
intermediate: 10 < IC ₅₀ < 20 -μM	13	-0.383578782	5.548321975	-4.971039387	-4.33566764	-2.342195007	-1.422455742	10.63364717
sensitive: 0 < IC ₅₀ < 10 -μM	29	4.362697636	4.829708908	-9.965784285	2.688829832	5.965276354	6.988612272	10.01360107

Supplementary Figure 6:

c

transloc	count	mean	std	min	25%	50%	75%	max
EWSR1-ERG	1	-1.422455742		-1.422455742	-1.422455742	-1.422455742	-1.422455742	-1.422455742
EWSR1-FLI1	5	3.99409302	4.578493264	-1.485170226	-0.248255271	5.965276354	6.891957358	8.846656884
KMT2A-AFF1 (MLL-AF4)	3	-0.511452519	4.410127831	-3.193600633	-3.056408698	-2.919216763	0.829621539	4.57845984
KMT2A-MLLT3 (MLL-AF9)	5	6.722386443	1.492343251	4.85184068	5.83895357	6.482208176	8.008769571	8.430160218
MLL partial tandem duplication	1	2.688829832		2.688829832	2.688829832	2.688829832	2.688829832	2.688829832
MLLT10-PICALM	1	6.988612272		6.988612272	6.988612272	6.988612272	6.988612272	6.988612272
No Translocation	57	0.933014101	5.519291564	-9.965784285	-3.841009155	-0.915516826	6.422854329	10.63364717
PML-RARA	1	3.991000923		3.991000923	3.991000923	3.991000923	3.991000923	3.991000923
RUNX1-RUNX1T1 (AML1-ETO)	1	-4.417885379		-4.417885379	-4.417885379	-4.417885379	-4.417885379	-4.417885379

Supplementary Figure 6: a, A-673 compound sensitive cells treated with compound **1**, **2**, **3**, or **4** for 4h. The presence or appearance of each compound were measured by mass spectrometry in media without cells (cell-free media), media from wells containing cells (cell supernatant), or from lysates of washed cell pellets (cell lysate). Center value represents mean and error bars represent standard deviation (n=3). **b,c**, Comparison of CES1 mRNA expression to (**b**) sensitivity to compound **1** across 92 cell line panel or to (**c**) reported translocation status among AML and Ewing's sarcoma cell lines tested. Boxplots in (**b,c**) represent Median bounded by Q1 and A3, with bars extended to cover the standard deviation.

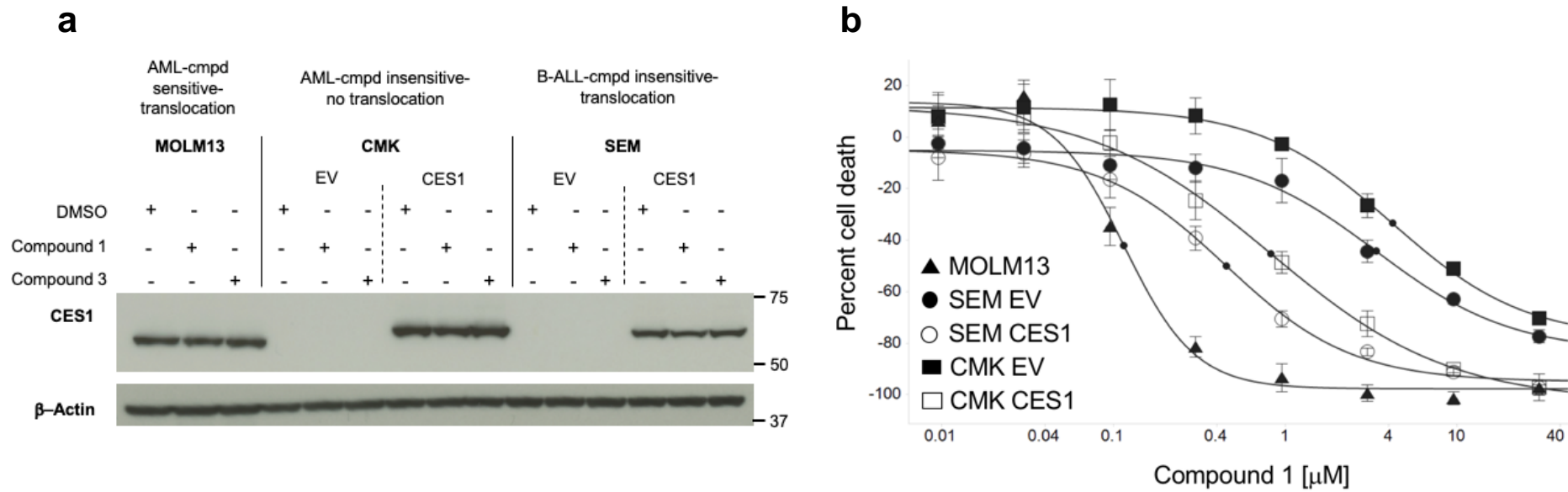


b

	1	2	3	5
AML cell lines sensitive to compound 1	MOLM-13: 0.27 μ M NOMO-1: 0.48 μ M	MOLM-13: 0.43 μ M* NOMO-1: >30 μ M	MOLM-13: >30 μ M NOMO-1: >30 μ M	MOLM-13: 0.18 μ M NOMO-1: 0.32 μ M
AML cell lines not sensitive to compound 1	CMK: >30 μ M HEL92.1.7: >30 μ M	CMK: >30 μ M HEL92.1.7: >30 μ M	CMK: >30 μ M HEL92.1.7: >30 μ M	CMK: >30 μ M HEL92.1.7: >30 μ M
Ewing's sarcoma sensitive to compound 1	A-673: 0.55 μ M SK-ES-1: 0.60 μ M	A-673: >30 μ M SK-ES-1: >30 μ M	A-673: >30 μ M SK-ES-1: >30 μ M	A-673: 2.3 μ M SK-ES-1: 2.5 μ M

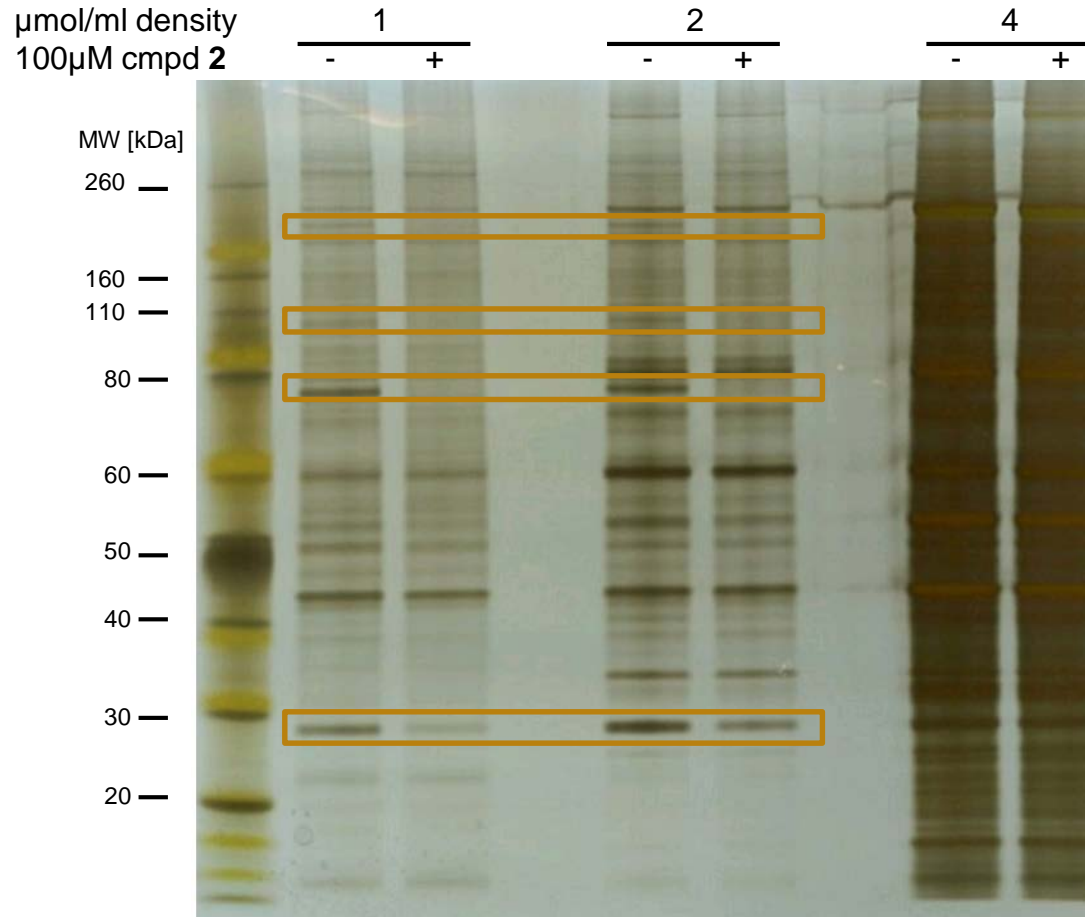
* partial activity

Supplementary Figure 7: a, Structure-activity relationships for compound 1. **b**, IC₅₀ values for compounds as indicated, derived from cell viability (CellTiter Glo) experiments across six cell lines used for compound optimization and SAR.

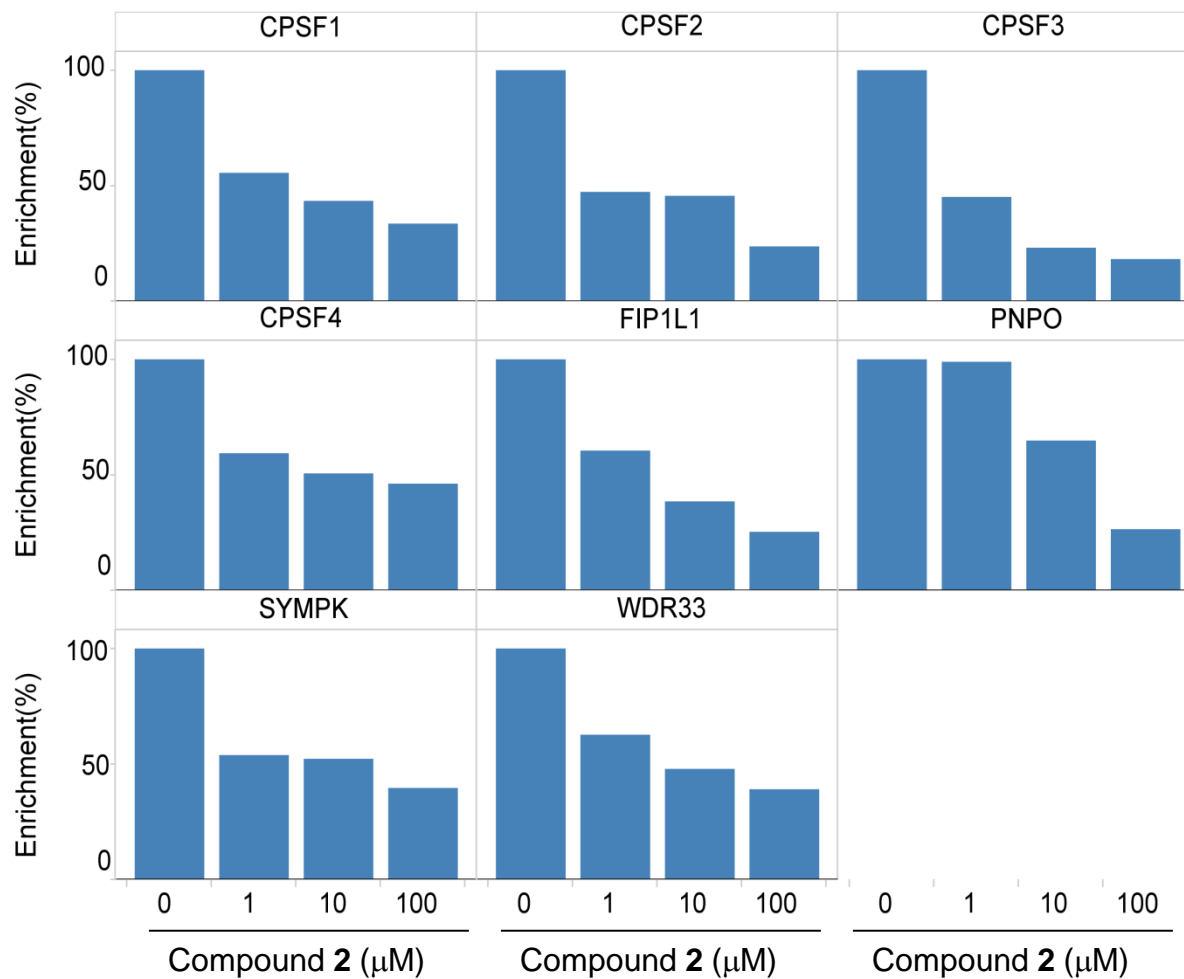


Supplementary Figure 8: a, Western blot assay to assess CES1 protein expression following stable viral transduction of human CES1 cDNA into CMK or SEM cell lines, with empty vector (EV) transduction as control. Treatment with 10μM compound 1 or 3 or DMSO as indicated. Non-transfected MOLM13 cell line shown for comparison (n=2). An uncropped scan of this blot is provided in Supplementary Figure 21a. **b**, Cell viability assessment of CES1 versus EV transduced CMK and SEM cell lines as indicated in response to compound 1 treatment in 8-point dose-response, with non-transfected MOLM13 cell line assayed in parallel (n=6, center point represents mean, error bars represent standard deviation, small black dots on fitted curves represent IC₅₀ values).

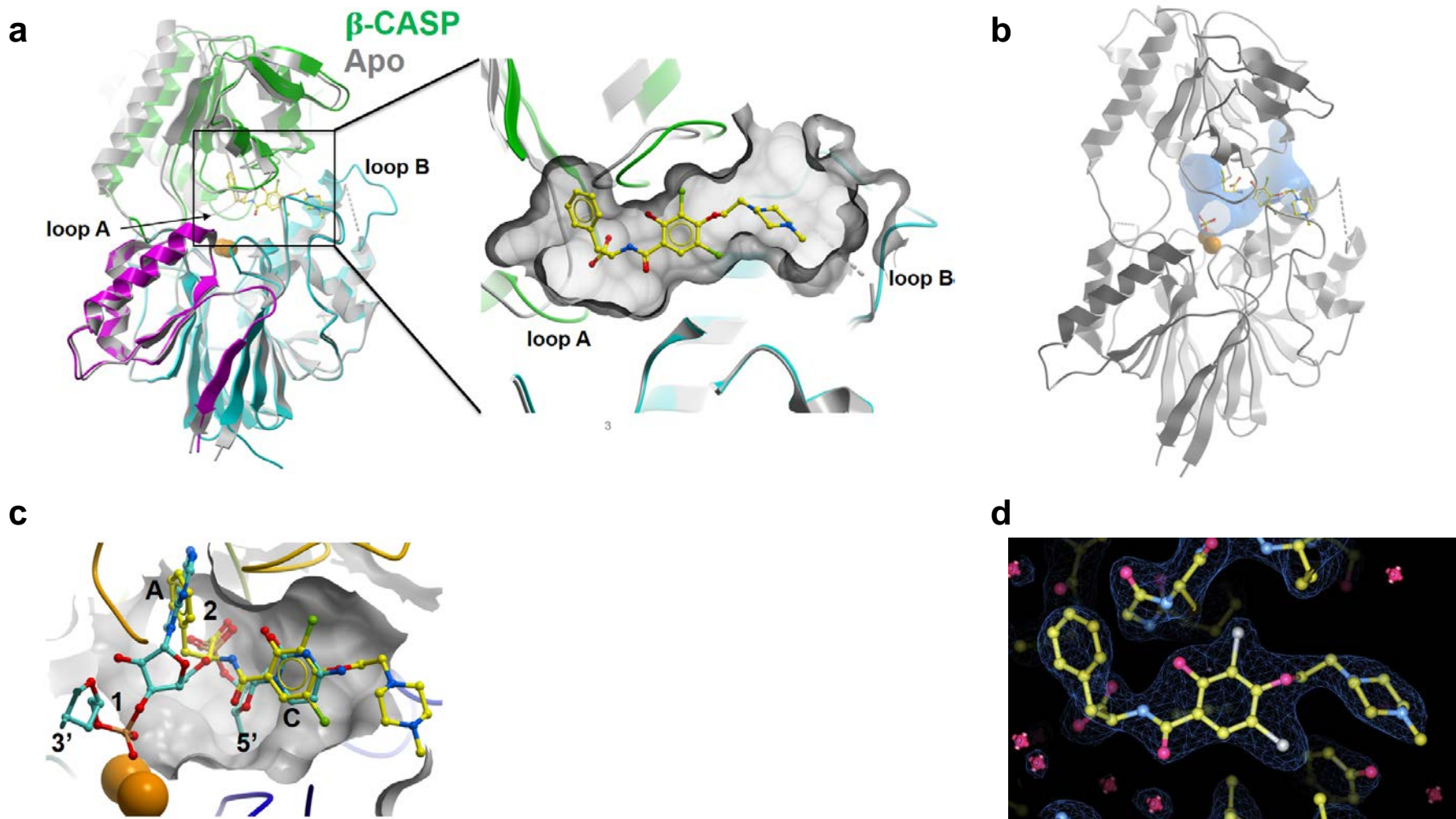
Input material: 5 mg Nomo-1 (9:1 S0.8+P0.8; no benzonase treatment)
Affinity matrix: Compound **6**
Competition compound: 0 (-) or 100 μ M (+) compound **2**



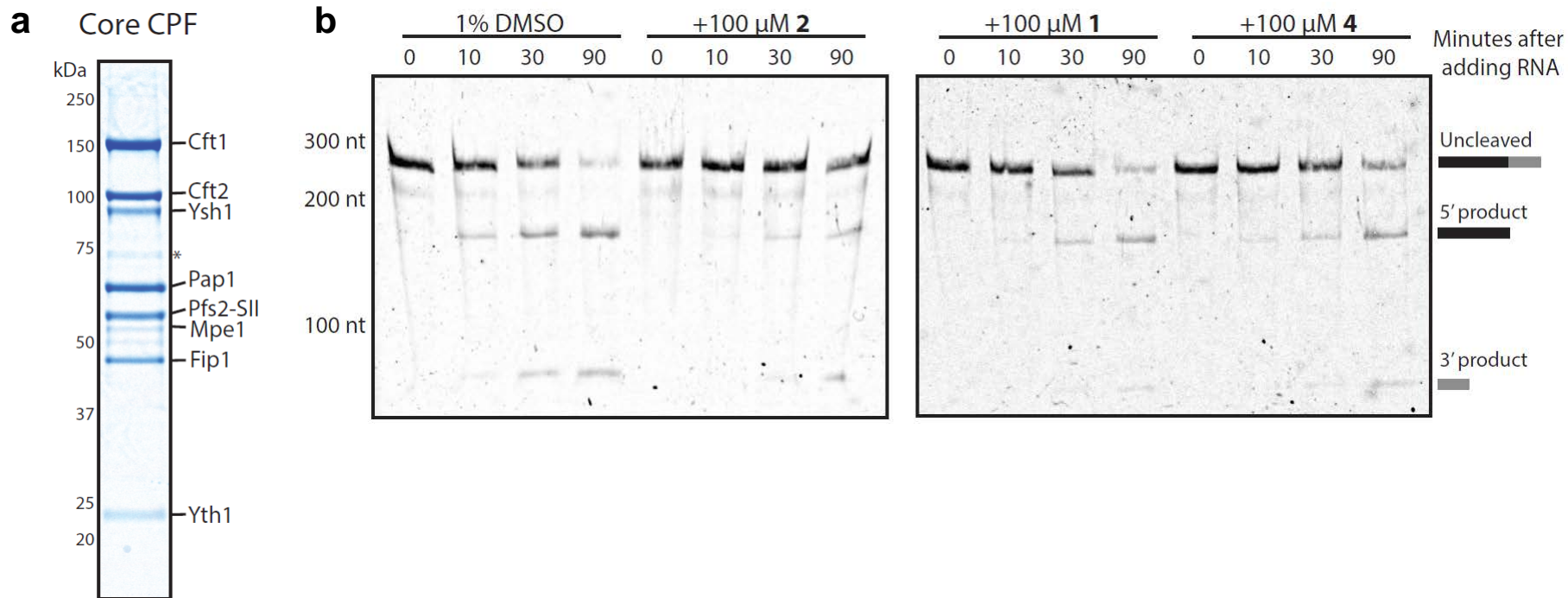
Supplementary Figure 9: Bioactive linker analog coupled to sepharose resin to give affinity matrix **6** incubated with NOMO-1 cell lysate. 1 and 2 μ mol/ml coupling densities allow for protein pull-down and competition by free compound **2** as observed by silver stain SDS-PAGE (n=1). Boxed bands indicate proteins specifically competed with free compound.



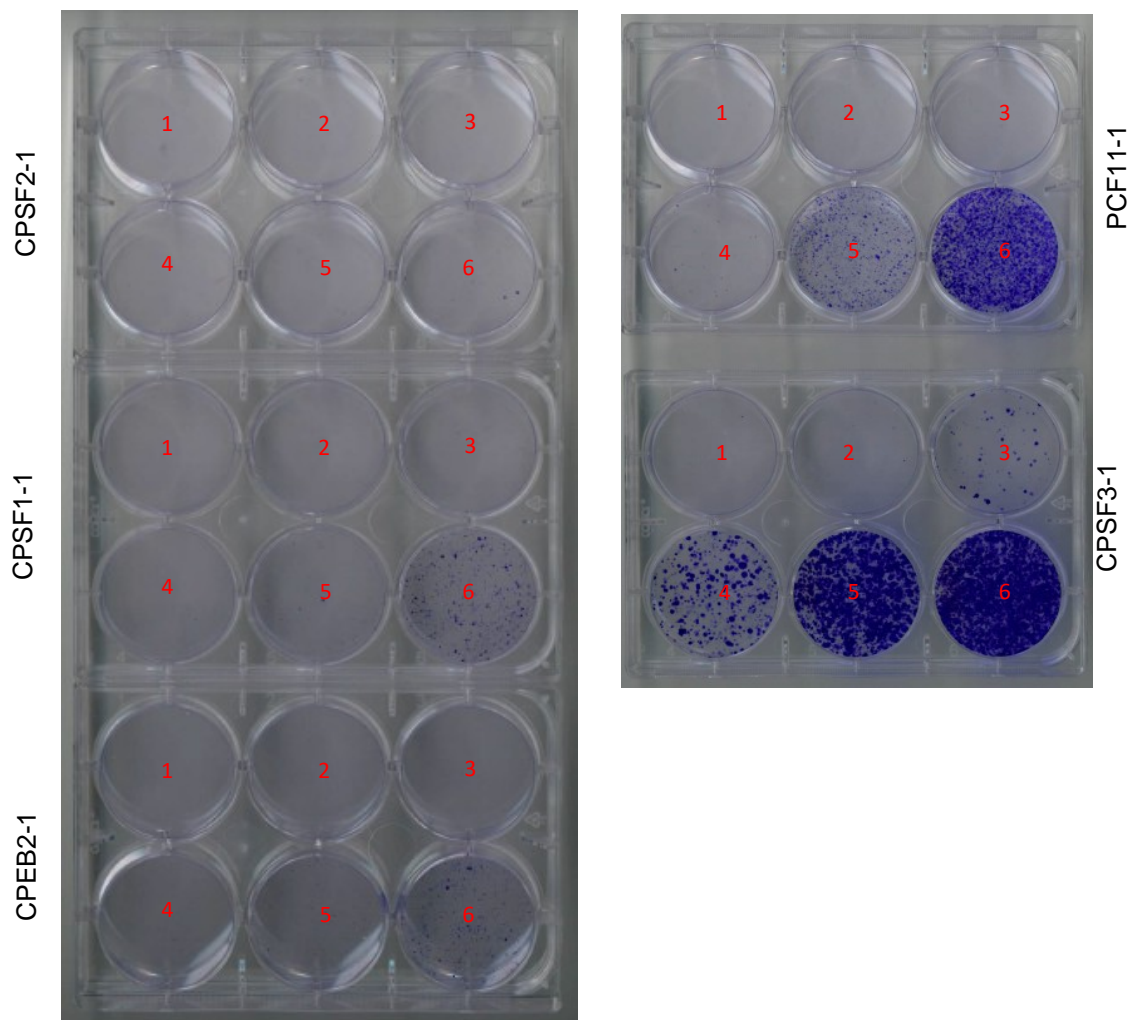
Supplementary Figure 10: Top hits from affinity pulldown experiment in NOMO-1 cells are concentration-dependent binders as determined by compound **2** competition with the affinity matrix **6**.



Supplementary Figure 11: CPSF3 co-crystal structure with compound **2**. **a**, Domain rotation and conformational changes of CPSF3 upon binding of **2** (yellow). Apo CPSF3 (pdb code 2I7T) is shown in ribbon representation in grey and CPSF3 bound to **2** is colored according to Figure 3a. The large metallo- β -lactamase domains of both structures were aligned (grey and blue) and the β -CASP domains were allowed to move freely. Overall, the β -CASP domain (green) shows a 7.3 degree rotation upon binding of **2**. Loop regions lining the domain interfacial cavity show structural rearrangements upon ligand binding, the most significant changes were observed for loop A and B. **b**, Active site cavity (blue volume) in apo CPSF3. Position of compound **2** shown for comparison. A sulfate coordinates the two zinc ions. **c**, Model of a CA RNA fragment (cyan) bound to the active site of CPSF3. Phosphodiester 1 was overlaid with the zinc-coordinating phosphate observed in crystal structures of **2**-bound CPSF3. Phosphodiester 2 was placed at the carboxylate position of **2**. Adenine and cytosine were overlaid with the phenyl and hydroxy-dibromo-phenyl groups of **2**, respectively. **d**, $2F_o - F_c$ electron density for compound **2** contoured at 1.4σ .



Supplementary Figure 12: Compound 2 inhibits the endonuclease activity of yeast core CPF. **a**, Recombinant 8-subunit core CPF complex was purified from insect cells. 5 μ l were resolved by SDS-PAGE and stained with Coomassie. **b**, 6% TBE-Urea PAGE gels of 259 nt *Cyc1* RNA substrate cleavage reactions treated with 100 μ M compound 2, 1, 4 or 1% DMSO. Gels were stained with SYBR Green II. Shown are one set of representative gels from three independent experiments.

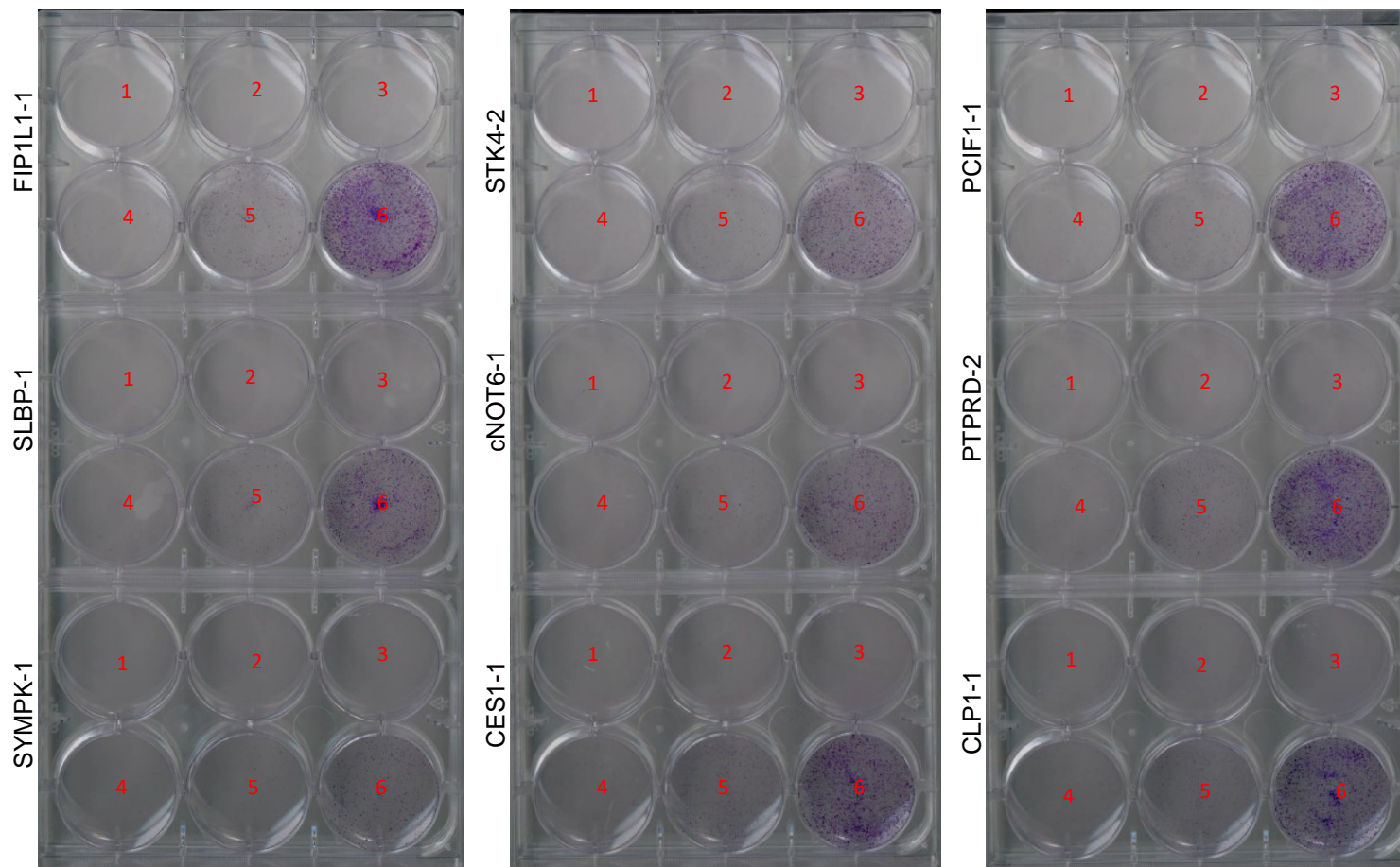
a

- Day 1: Seeded 8,000/well stably infected A-673 cells.
- Day 3: Added compound **1** in dose-response as indicated.
- Day 10: Fixed and stained cells.

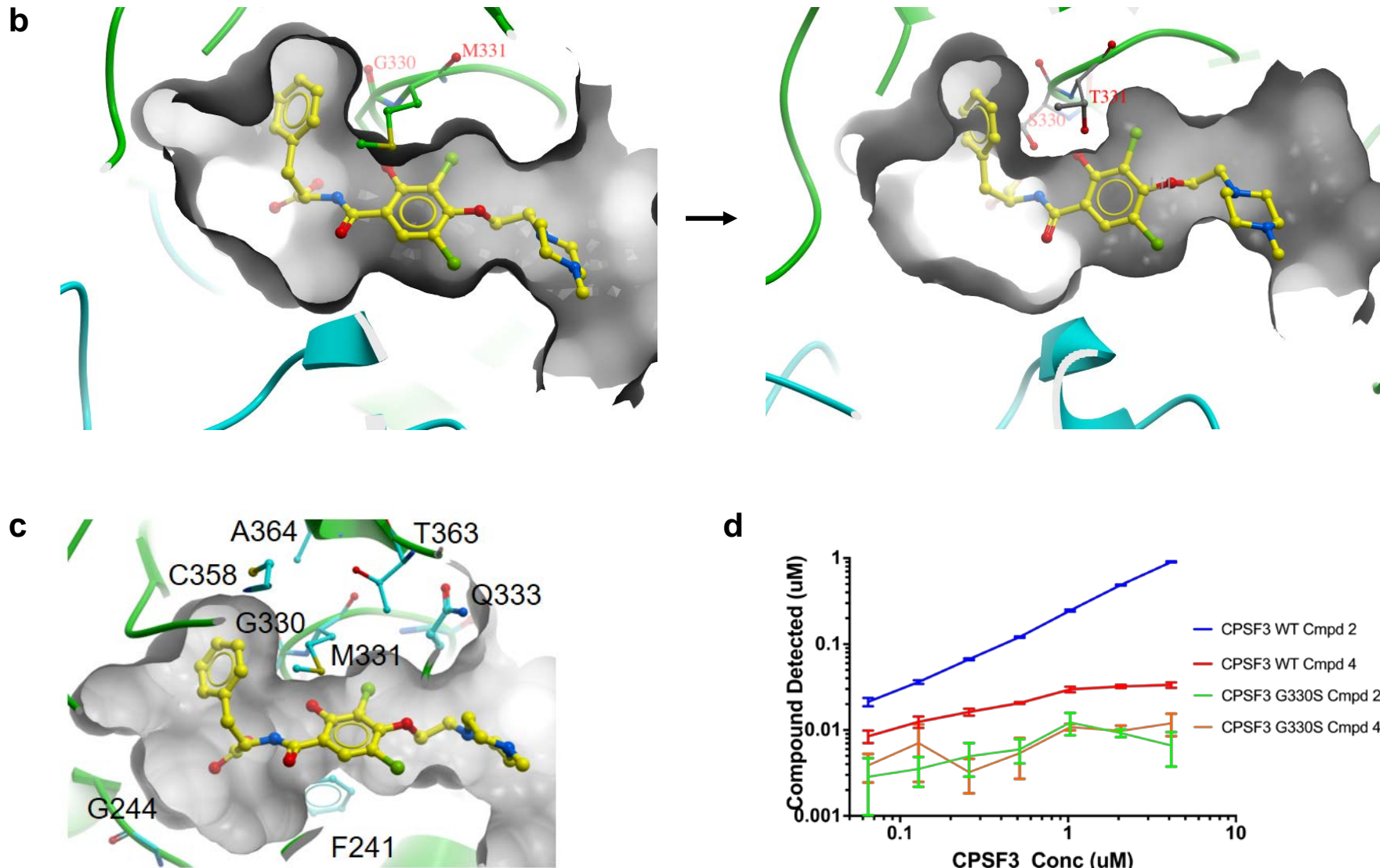
6w-plate well number	Compound 1 (μM)
1	50
2	25
3	12.5
4	6.25
5	3.125
6	1.5625

Supplementary Figure 13: a, Functional variomics of top 14 candidate compound targets in colony formation assays *in vitro* using A-673 cells treated with compound **1** as indicated (repeated independently two times).

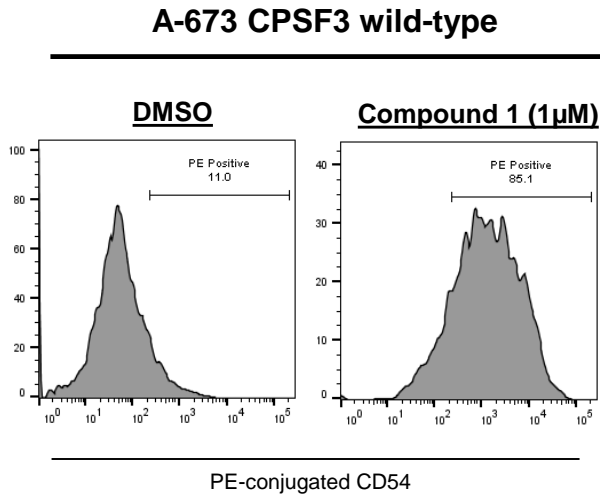
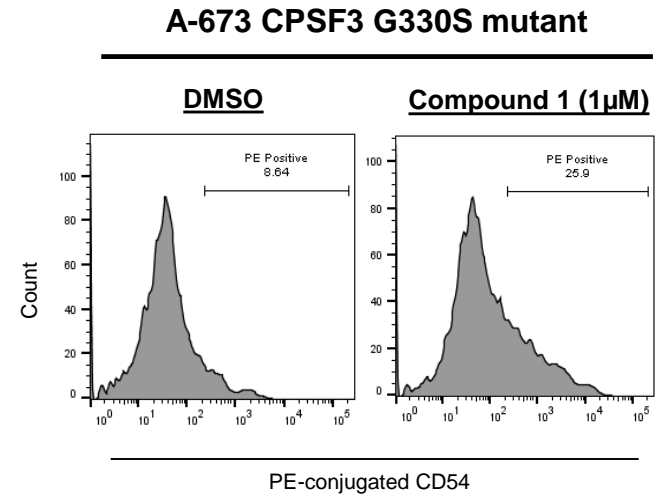
a



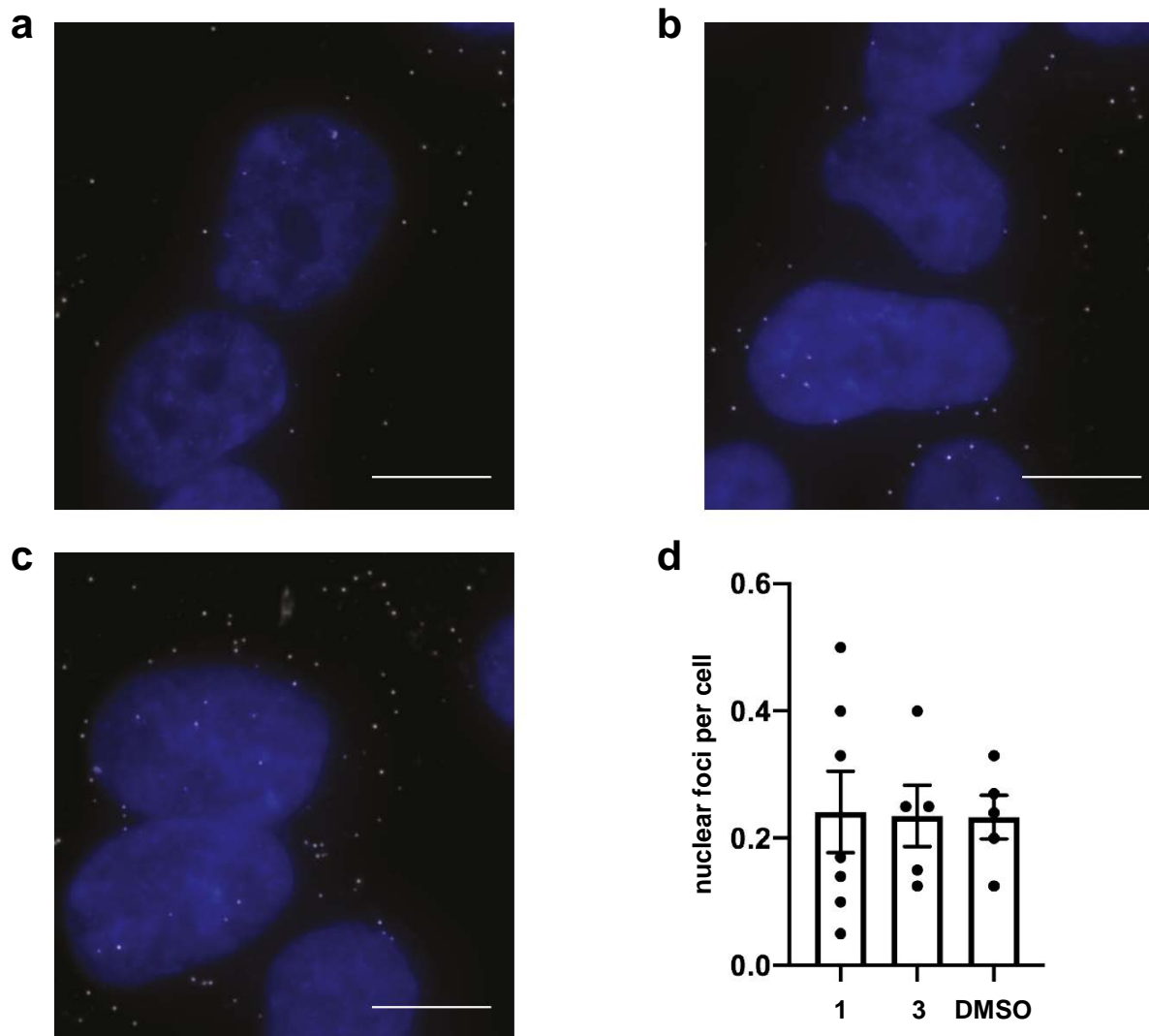
Supplementary Figure 13 continued: a, Functional variomics of top 14 candidate compound targets in colony formation assays *in vitro* using A-673 cells treated with compound **1** as indicated.



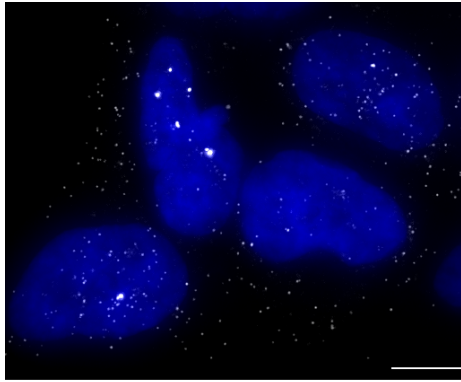
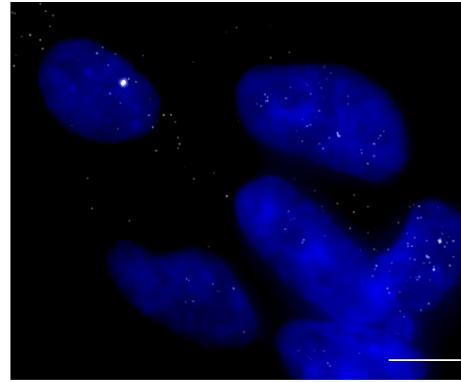
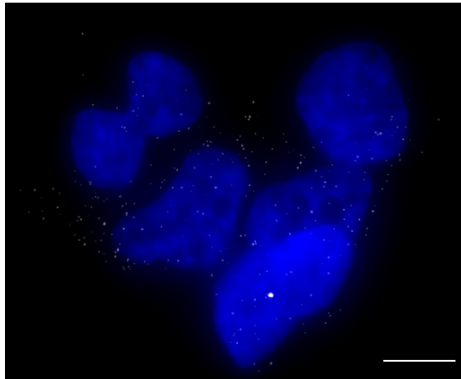
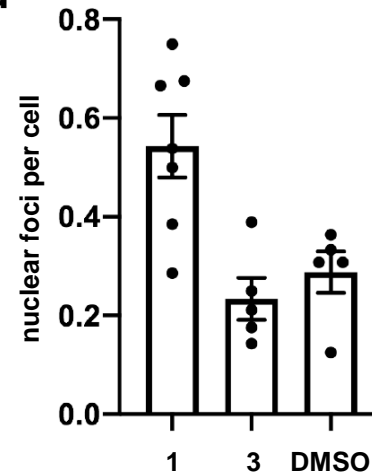
Supplementary Figure 13 continued: **b**, The two most prevalent missense mutations Gly330Ser, Met331Thr/Val modeled onto X-ray structure are predicted to constrict the compound **2** binding pocket while retaining catalytic activity. **c**, Positions of most frequently mutated residues (count > 4) cluster in the small β -CASP domain of CPSF3 (top), and are in close proximity to the binding site of **2**. **d**, Affinity binding measurement of compound **2** or **4** to human CPSF3 wild-type (WT) or G330S mutant protein as determined by SEC-TID. Error bars represent standard deviation of the mean (n=3).

e**f**

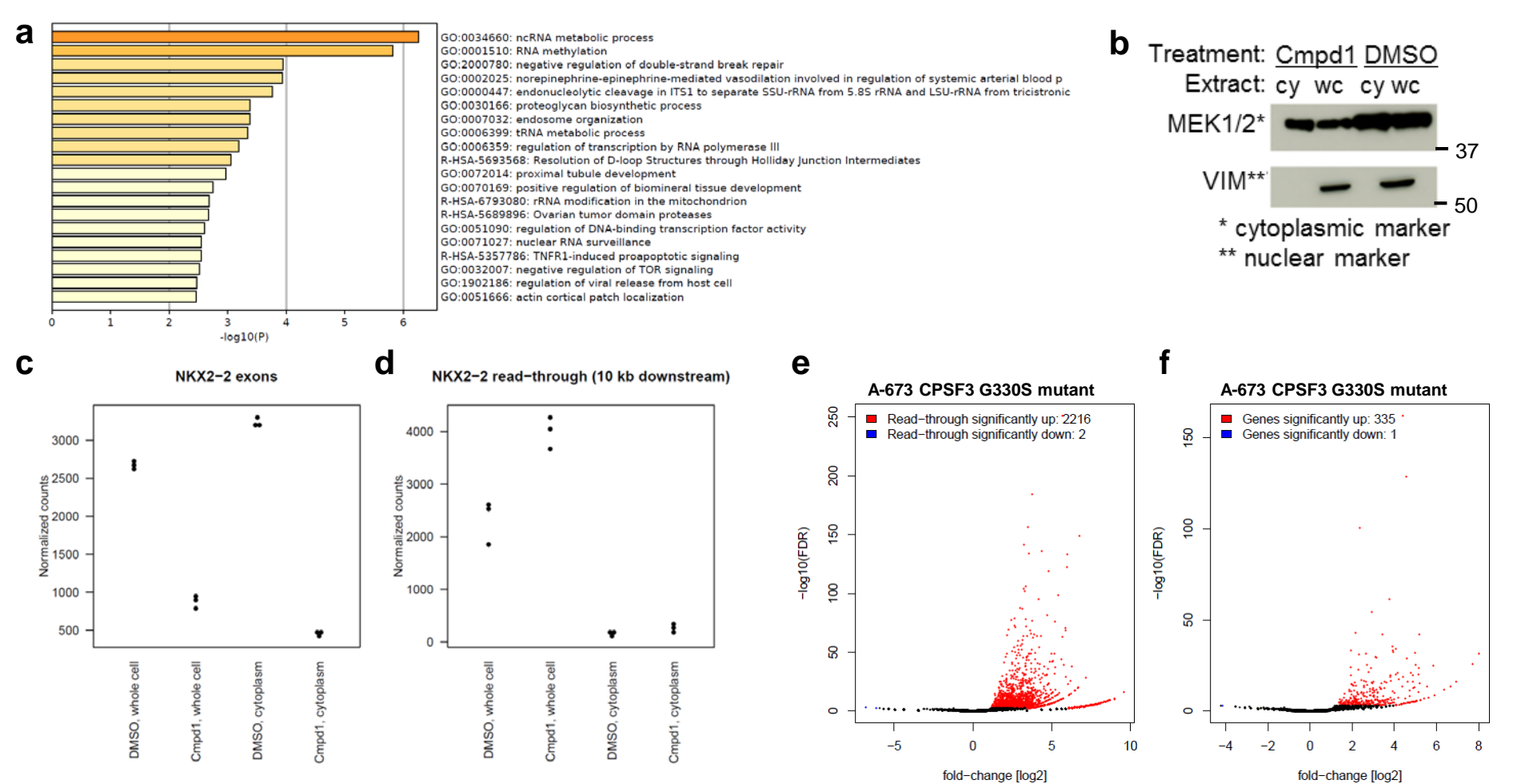
Supplementary Figure 13 continued: e,f, FACS analysis of cell surface CD54 expression in response to compound **1** treatment comparing (e) A-673 cells with CPSF3 wild-type to (f) variomics-derived A-673 cells expressing the CPSF3 G330S mutant (repeated independently two times).



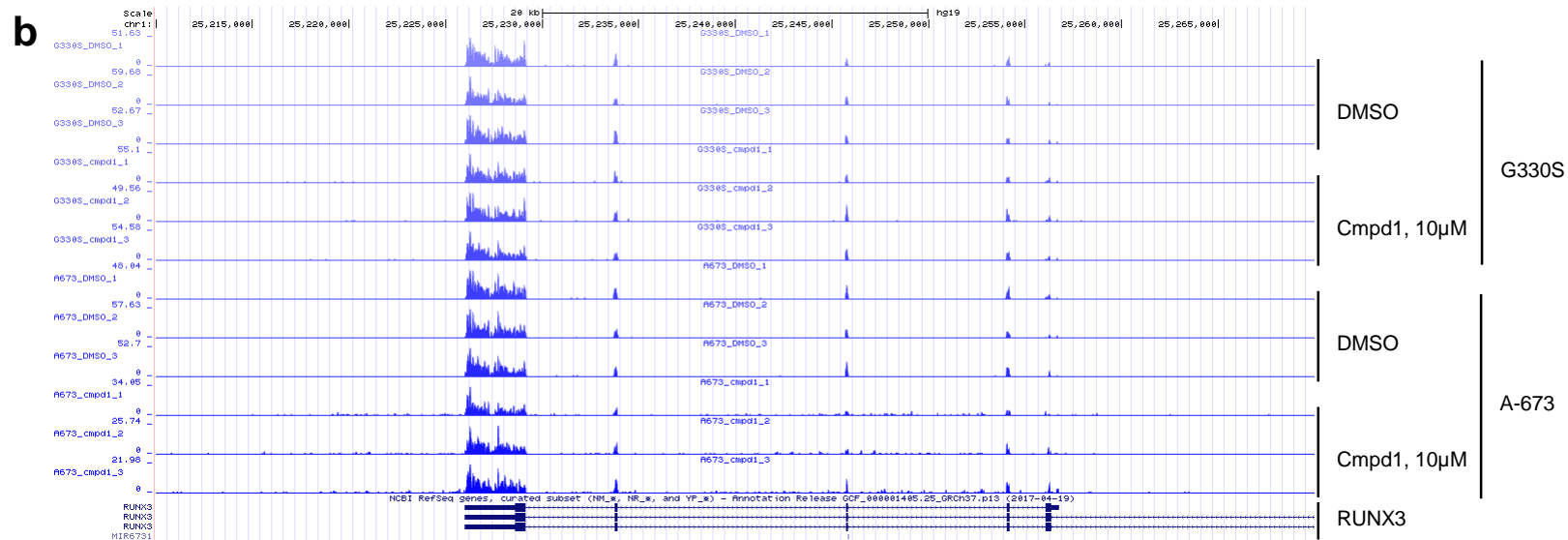
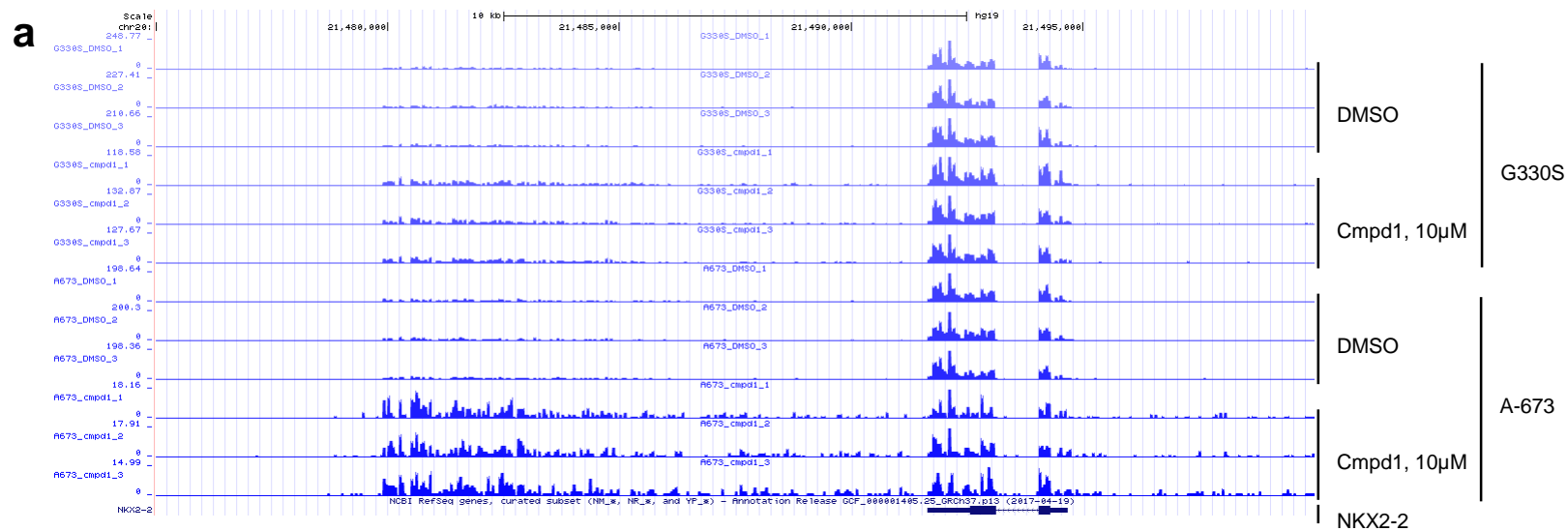
Supplementary Figure 14: a,b,c, Images of A-673 cells that were treated with compound **1** (a), compound **3** (b) or DMSO (c) for four hours prior to fixation and probed for RUNX-3 mRNAs (white) using FISH probes targeted to the coding sequence of the transcript. Nuclei (blue) were stained with DAPI. Images are representative from three independent experiments performed in duplicate. Scale bar = 10 μm. **d**, Similar numbers of nuclear foci of RUNX-3 transcripts were detected in cells treated with DMSO (52 cells), compound **1** (41 cells) or compound **3** (44 cells) (p-value 0.98, one-way ANOVA). Data are presented as mean plus/minus standard error of mean for nuclear foci counted in each experiment.

a**b****c****d**

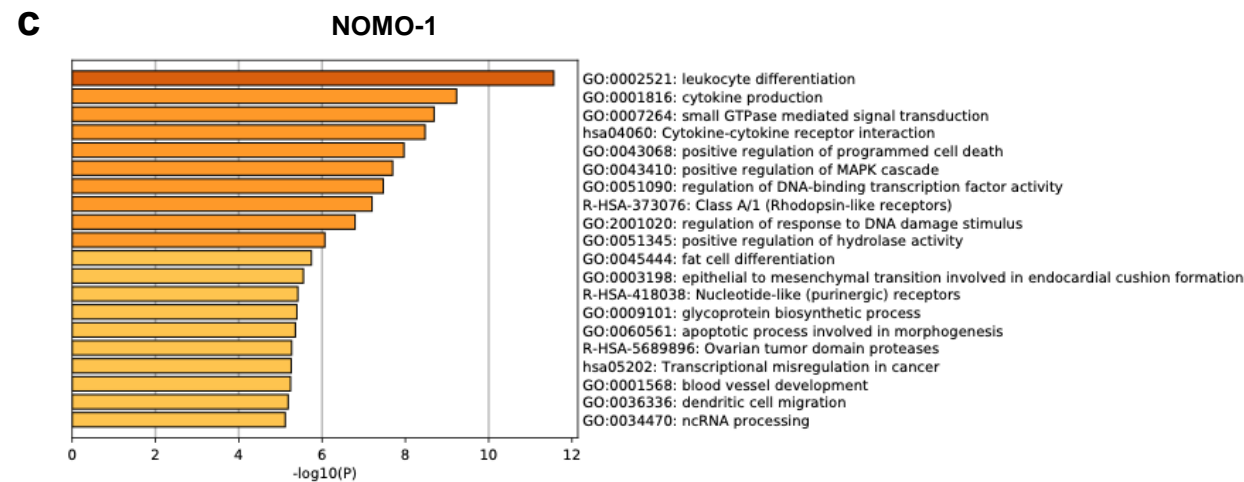
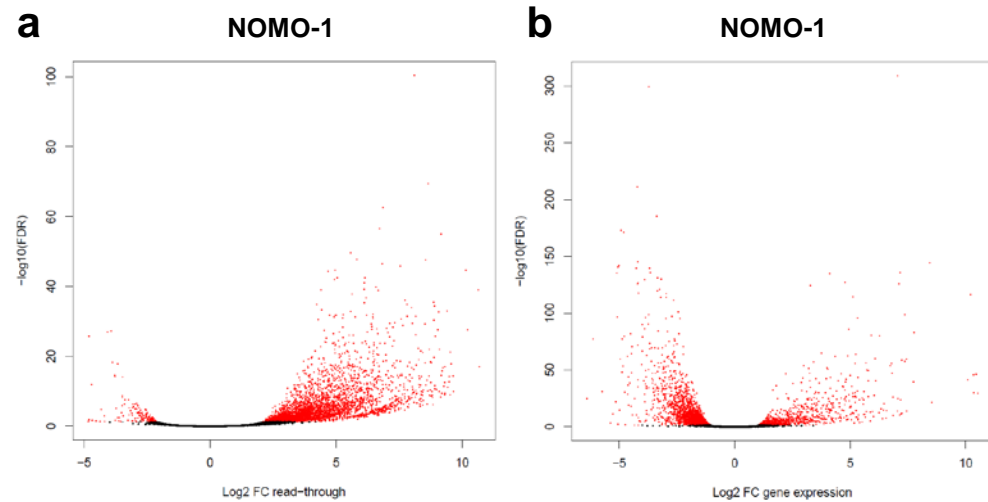
Supplementary Figure 15: **a,b,c**, Images of A-673 CPSF3 G330S cells that were treated with compound 1 (**a**), compound 3 (**b**) or DMSO (**c**) for 4 h prior to fixation and probed for NKX2-2 mRNAs (white) using FISH probes targeted to the coding sequence of the transcript. Nuclei (blue) were stained with DAPI. Images are representative from two independent experiments performed in duplicate. Scale bar = 10 μ m. **d**, Quantification of the number of NKX2-2 nuclear foci in cells treated with compound 1 (85 cells), compound 3 (80 cells) and DMSO (54 cells). Data are presented as mean plus/minus standard error of mean for nuclear foci counted in each experiment.



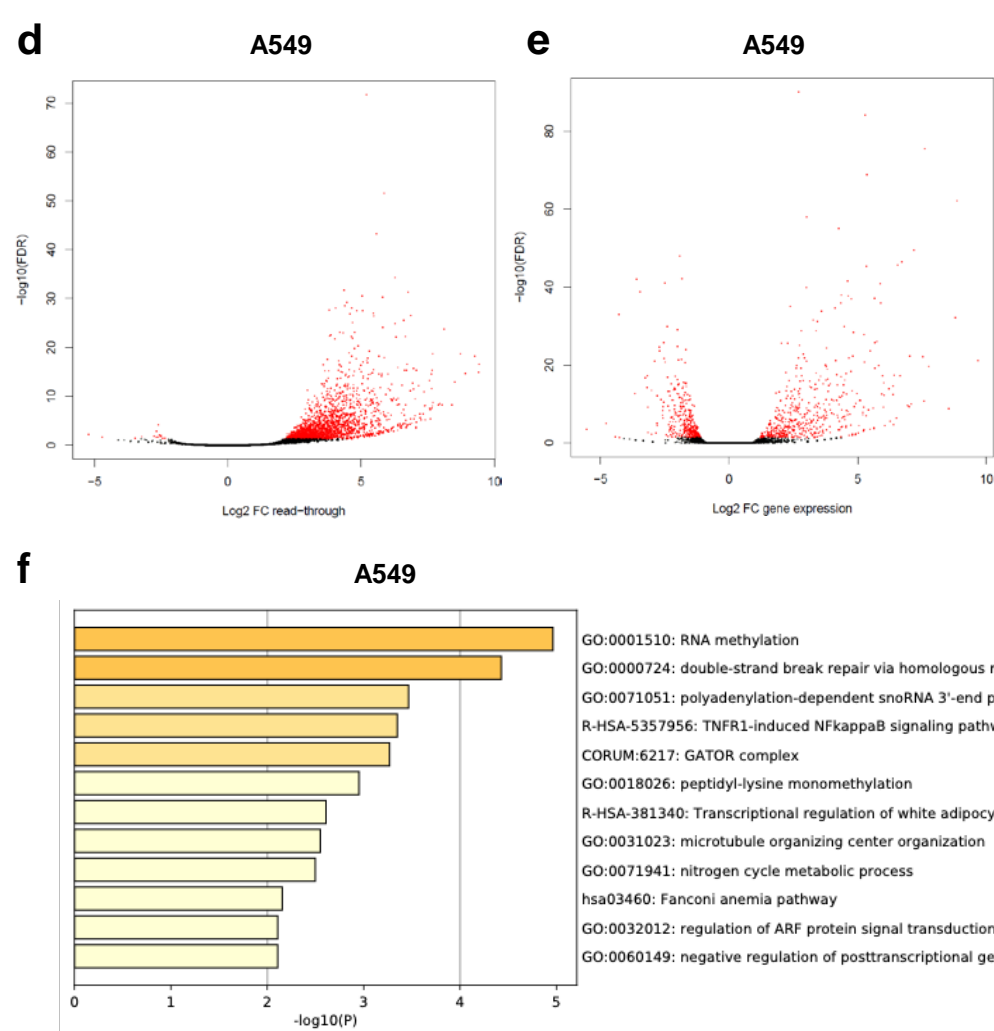
Supplementary Figure 16: **a**, Pathway and process enrichment using Metascape among genes significantly downregulated with 10 μ M compound **1** in A-673 cells based on RNA-seq expression profiling from whole cell extract (as shown in Figure 4g). Geneset enrichments were calculated using the hypergeometric test and Benjamini-Hochberg multiple comparison adjustment via Metascape. **b**, Protein expression analysis by Western blot against MEK1/2 as a cytoplasmic marker and Vimentin as a nuclear marker in cytoplasmic (cy) extracts or whole cell (wc) extracts to assess purity of subcellular fractionation (n=1). An uncropped scan of this blot is provided in Supplemental Figure 21b. **c,d**, NKX2-2 exonic (**c**) versus read-through (**d**) expression levels quantified by RNA-seq in A-673 cells treated with **1** or DMSO for 4 h prior to RNA purification from either whole cell or cytoplasmic extracts, with three biological replicates per condition. **e,f**, RNA-seq based quantification of (**e**) read-through expression genome-wide and of (**f**) changes in global gene expression in A-673 Variomics-derived CPSF3 G330S mutant cells, upon 4 h treatment with 10 μ M **1** versus DMSO analyzing RNA purified from whole cell extracts of three biological replicates. Normalized read counts were analyzed using a negative binomial generalized log-linear model with two-sided comparisons and false discovery rate controlled using Benjamini-Hochberg multiple comparisons adjustment via edgeR. Significance cut-offs defined as absolute value fold-change [log₂] > 1 and adjusted p-val/FDR < 0.05.



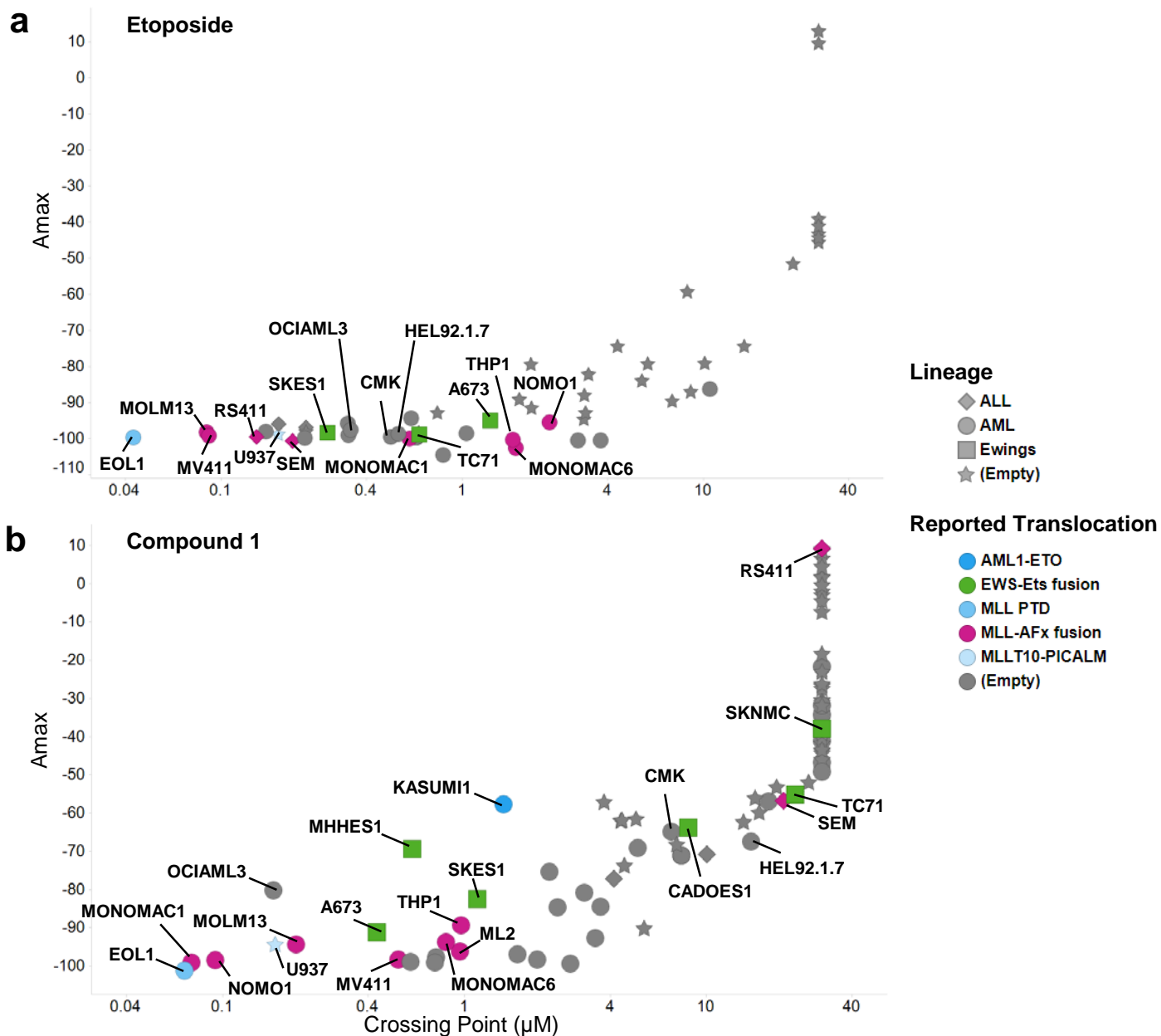
Supplementary Figure 17: RNA-seq traces comparing 4h compound **1** treatment (10µM) to DMSO control in either A-673 or CPSF3 G330S mutant expressing cells. Dynamic scaling of y-axis to visualize degree of transcript read-through at 3'-UTR and beyond in relation to read coverage at exons. **a**, NKX2-2 locus, hg19 chr20:chr20:21,475,000-21,500,000 and **b**, RUNX3 locus, hg19 chr1:25,210,000-25,270,000. Three separate tracks are shown per condition reflecting replicate samples.



Supplementary Figure 18: **a,b**, RNA-seq based quantification in NOMO-1 cells of read-through expression genome-wide (**a**) and of changes in global gene expression in (**b**), both upon 4 h treatment with 10 μ M of **1** versus DMSO. Normalized read counts were analyzed using a negative binomial generalized log-linear model with two-sided comparisons and false discovery rate controlled using Benjamini-Hochberg multiple comparisons adjustment via edgeR. Significance cut-offs marked in red defined as absolute value fold-change [\log_2] > 1 and adjusted p-val/FDR < 0.05. **c**, Pathway and process enrichment using Metascape among genes significantly downregulated with 10 μ M compound 1 in NOMO-1 cells. Geneset enrichments were calculated using the hypergeometric test and Benjamini-Hochberg multiple comparison adjustment via Metascape. All analyses utilized RNA purified from whole cell extracts of three biological replicates.



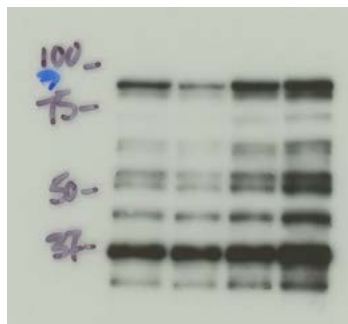
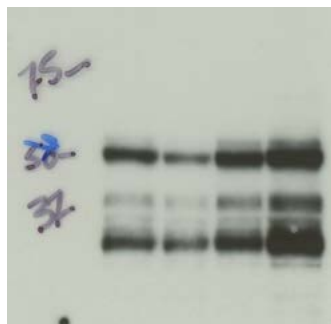
Supplementary Figure 18 continued: d,e, RNA-seq based quantification in A549 cells of read-through expression genome-wide (**d**) and of changes in global gene expression in (**e**), both upon 4 h treatment with 10 μ M of **1** versus DMSO. Normalized read counts were analyzed using a negative binomial generalized log-linear model with two-sided comparisons and false discovery rate controlled using Benjamini-Hochberg multiple comparisons adjustment via edgeR. Significance cut-offs marked in red defined as absolute value fold-change [\log_2] > 1 and adjusted p-value/FDR < 0.05. **f**, Pathway and process enrichment using Metascape among genes significantly downregulated with 10 μ M compound **1** in A549 cells. Geneset enrichments were calculated using the hypergeometric test and Benjamini-Hochberg multiple comparison adjustment via Metascape. All analyses utilized RNA purified from whole cell extracts of three biological replicates



Supplementary Figure 19: a,b, Cell viability profile for (a) etoposide, analogous to experiment shown in Figure 1b for compound 1 and replotted here as (b). Note, the etoposide cell line panel was overlapping but did not include all 92 lines tested for compound 1. Cell lines are shape-coded by lineage and color-coded by reported translocation. Lineage is given as ALL, AML, Ewing's or Other (Empty). Note: EWS-FLI1 translocated Ewing's lines TC71 and SKNMC have low CES1 expression.

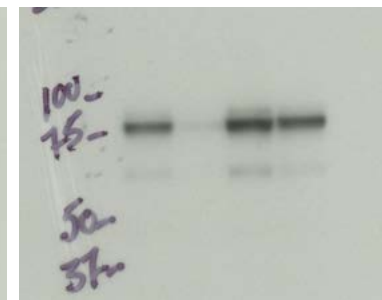
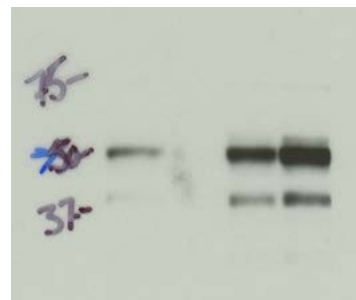
MOLM-13 α -cMYC

MOLM-13 α -cMYB



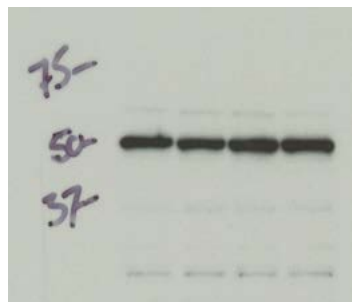
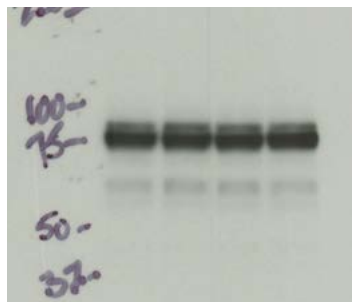
NOMO-1 α -cMYC

NOMO-1 α -cMYB

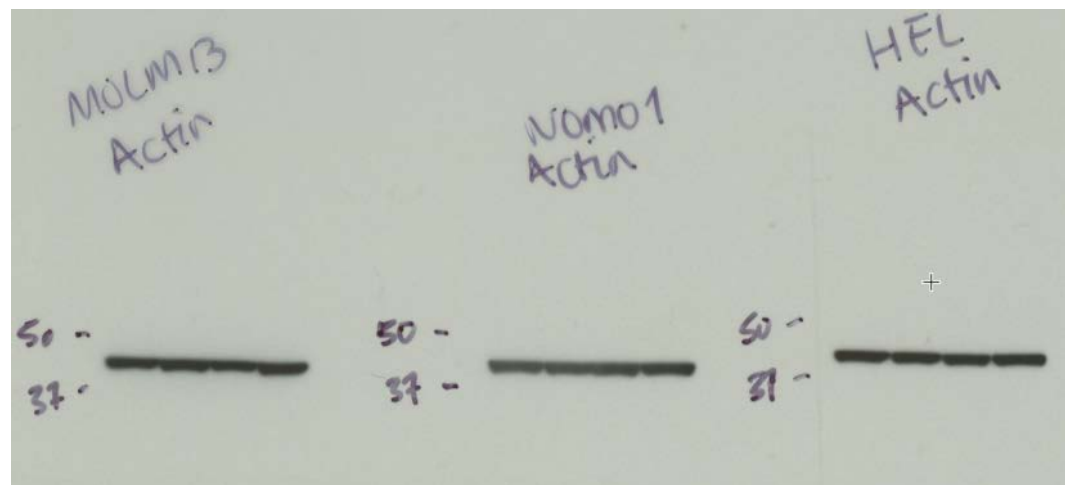


HEL 92.1.7 α -cMYB

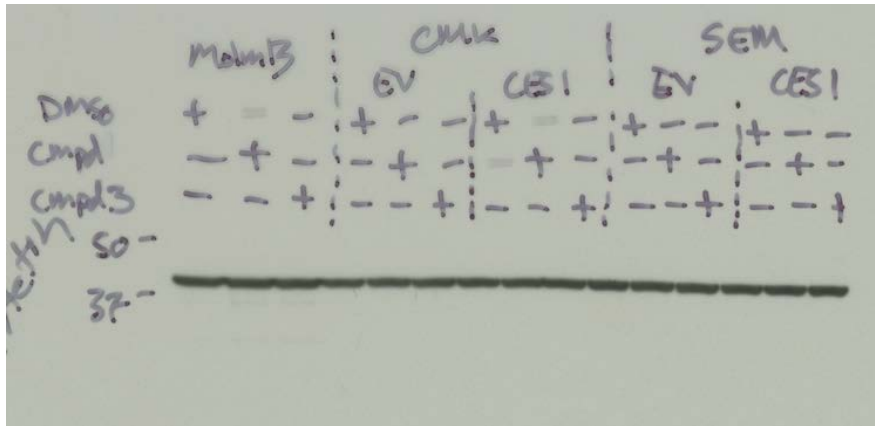
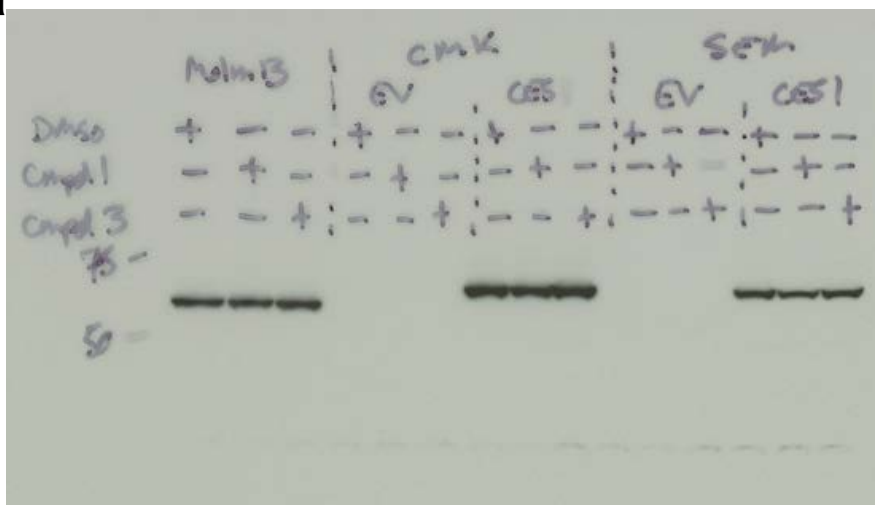
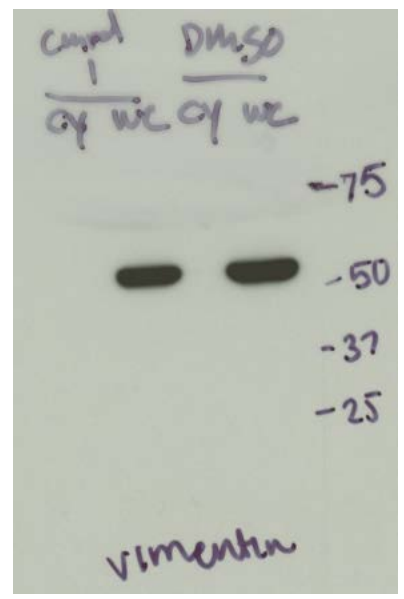
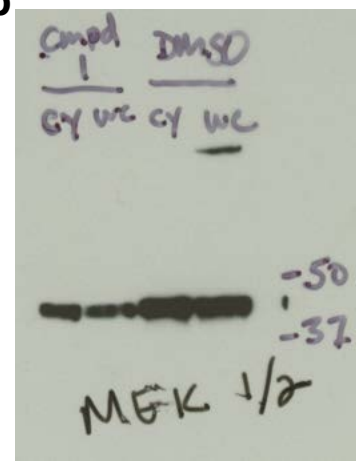
HEL92.1.7 α -cMYC



MOLM-13, Nomo-1, HEL 92.1.7, all with α - β -ACTIN



Supplementary Figure 20: Uncropped western blots for Supplementary Figure 2c. Sample order for all blots: Compound 1 1 μ M, Compound 1 10 μ M, Compound 3 10 μ M; DMSO

a**b**

Supplementary Figure 21: **a**, Uncropped western blots for Supplementary Figure 8a (top: anti-CES1; bottom: anti- β -ACTIN). **b**, uncropped western blot for Supplementary Figure 16b (right: anti-MEK1/2; left: anti-VIMENTIN)

List of Supplementary Tables:

Supplementary Table 1: Panel of 92 cancer cell lines used in this study.

Supplementary Table 2: X-ray data collection and refinement statistics.

Supplementary Table 3: Antibodies used in this study.

Supplementary Table 4: smFISH probes used in this study.

Supplementary Notes

Supplementary Note- Synthetic Procedures: Chemical Synthesis Materials, General Spectroscopic Methods and Preparation of Compounds & Proton and Carbon NMR spectra compound 2, 3, 4, 5 and 7

List of Supplementary Data Sets provided as individual Excel files

Supplementary Data Set 1: siRNA library.

Supplementary Data Set 2: siRNA plus cmpd 1 synergy screen gene level activity per compound 1, or DMSO, or differential.

Supplementary Data Set 3: Chemoproteomics data.

Supplementary Data Set 4: PAL data.

Supplementary Table 2 X-ray data collection and refinement statistics for CPSF3-compound **2** co-crystal structure

	CPSF3/compound 2 (pdb code: 6M8Q)
Data collection	
Space group	P4 ₃ 2 ₁ 2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	106.2, 106.2, 206.0
α , β , γ (°)	90, 90, 90
Resolution (Å)	2.49 – 90 (2.49 – 2.51)
<i>R</i> _{merge}	0.138 (1.27)
<i>R</i> _{pim}	0.056 (0.507)
CC _{1/2}	0.996 (0.652)
<i>I</i> / σ <i>I</i>	12.6 (2.2)
Completeness (%)	99.7 (99.7)
Redundancy	7.1 (7.1)
Refinement	
Resolution (Å)	2.49
No. reflections	41,852
<i>R</i> _{work} / <i>R</i> _{free}	0.175 / 0.221
No. atoms	
Protein	7,255
Ligand/ion	84
Water	495
<i>B</i> -factors	
Protein	51.7
Ligand/ion	50.5
Water	56.1
R.m.s. deviations	
Bond lengths (Å)	0.010
Bond angles (°)	1.18

Only one crystal was needed to obtain the structure. Highest-resolution shell is shown in parentheses.

Supplementary Table 3: Antibodies used in this study

Antibody (epitope)	Manufacturer	Catalog Number	Application	Lot Number	Dilution	Link to manufacturer's validation data
PE-conjugated CD11b	BioLegend	301306	FACS	B183220	5ul/1E6 cells	https://www.biolegend.com/en-us/products/pe-anti-human-cd11b-antibody-768
PE-conjugated CD44	BioLegend	338808	FACS	B189534	5ul/1E6 cells	https://www.biolegend.com/en-us/products/pe-anti-human-cd44-antibody-5745
PE-conjugated CD54	BioLegend	353106	FACS	B169127	5ul/1E6 cells	https://www.biolegend.com/en-us/products/pe-anti-human-cd54-antibody-7447
PE-conjugated CD73	BioLegend	344004	FACS	B189876	5ul/1E6 cells	https://www.biolegend.com/en-us/products/pe-anti-human-cd73-ecto-5-nucleotidase-antibody-6092
Alexa 568 goat anti mouse	ThermoFisher Scientific	A11031	R-loop staining	2026148	1:1000	https://www.thermofisher.com/antibody/product/Goat-anti-Mouse-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11031
Alexa 647 goat anti rabbit	ThermoFisher Scientific	A21245	R-loop staining	2051068	1:1000	https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21245
Nucleolin	Abcam	ab22758	R-loop staining	GR303746-3	1:1000	https://www.abcam.com/nucleolin-antibody-ab22758.html
DNA-RNA Hybrid clone S9.6	Millipore	MABE1095	R-loop staining	06131_4	1:200	http://www.merckmillipore.com/CH/de/product/Anti-DNA-RNA-Hybrid-Antibody-clone-S9.6_MM_NF-MABE1095
CPSF3	Abgent	AT1610a	WB	11175	1:1000	http://www.abgent.com/products/AT1610a-CPSF3-Antibody-monoclonal-M01
FLI1	Abcam	ab124791	WB	YH112404C	1:1000	https://www.abcam.com/fli1-antibody-epr4645-ab124791.html
MEK1/2	Cell Signaling Technologies	8727	WB	5	1:1000	https://www.cellsignal.com/products/primary-antibodies/mek1-2-d1a5-rabbit-mab/8727
MYB	Cell Signaling Technologies	12319	WB	1	1:1000	https://www.cellsignal.com/products/primary-antibodies/c-myb-d2r4y-rabbit-mab/12319
MYC	Cell Signaling Technologies	13987	WB	5	1:1000	https://www.cellsignal.com/products/primary-antibodies/c-myc-d3n8f-rabbit-mab/13987
NKX2.2	Abcam	ab187375	WB	GR299445-4	1:1000	https://www.abcam.com/nkx22-antibody-nx2294-ab187375.html
VIMENTIN	Cell Signaling Technologies	5741	WB	1	1:1000	https://www.cellsignal.com/products/primary-antibodies/vimentin-d21h3-xp-rabbit-mab/5741
BETA-ACTIN	Sigma	A5441	WB	127M4866V	1:10000	https://www.sigmaaldrich.com/catalog/product/sigma/a5441

Supplementary Table 4: smFISH probes used in this study.

FISH probe	Accession Number(s)	Sequence	Sequence Name	Three Modification	Three Lambda Max
NKX2-2 mRNA	NM_002509.3	tagtttctaactccaggagg	NKX2-2 mRNA_1	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	aaagcacgcggaaatggacg	NKX2-2 mRNA_2	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	ttgtagcttcacttggtcaa	NKX2-2 mRNA_3	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	ccccaaaattatgtcgcaa	NKX2-2 mRNA_4	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	ttgtgttggtcagcgacatg	NKX2-2 mRNA_5	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	caggtctaagatgctctga	NKX2-2 mRNA_6	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	tcgtagaaggggttctcag	NKX2-2 mRNA_7	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	cttgagcttgagctcgag	NKX2-2 mRNA_8	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	ttgtcattgtccggtgactc	NKX2-2 mRNA_9	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	ttggagaaaagcactcgccg	NKX2-2 mRNA_10	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	tggaaccagatctgactcg	NKX2-2 mRNA_11	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	cttcactctgtagcgggtgt	NKX2-2 mRNA_12	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	tgagcgcgtgacatggttg	NKX2-2 mRNA_13	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	ttgtactgcatgtctgcag	NKX2-2 mRNA_14	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	tagtggagccgagagtcac	NKX2-2 mRNA_15	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	aatctgccactccaaggaga	NKX2-2 mRNA_16	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	ctgtaaacacggcgtagagt	NKX2-2 mRNA_17	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	agaagcgaagctgcgcaaac	NKX2-2 mRNA_18	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	gacgacattaacctgggac	NKX2-2 mRNA_19	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	ggctttttctctgtttcaa	NKX2-2 mRNA_20	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	gctgacaatcgtctactca	NKX2-2 mRNA_21	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	cagaactgtttacatggccat	NKX2-2 mRNA_22	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	aaagcgaatctgccaccag	NKX2-2 mRNA_23	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	tcaccaccgatattacaac	NKX2-2 mRNA_24	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	cctgaaggtcattttggcaa	NKX2-2 mRNA_25	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	agaagagagttggaccacaga	NKX2-2 mRNA_26	Quasar 570	550 nm
NKX2-2 mRNA	NM_002509.3	aatagctgagctccaagttc	NKX2-2 mRNA_27	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	cggagttagttctctcattg	RUNX3_Var1_Var2_1	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	gaagcgaagtcgttgaacc	RUNX3_Var1_Var2_2	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	atggtcagggtgaaactctt	RUNX3_Var1_Var2_3	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	tcggagaatgggttcagttc	RUNX3_Var1_Var2_4	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ggaagagcgggtcaactgg	RUNX3_Var1_Var2_5	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	catgagaactgtaggagc	RUNX3_Var1_Var2_6	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	catcactggtcttgaagtt	RUNX3_Var1_Var2_7	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	taacctatgcctctgtacaa	RUNX3_Var1_Var2_8	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	gaagtatgggatgagacggc	RUNX3_Var1_Var2_9	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	catagctggagacagtgagg	RUNX3_Var1_Var2_10	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ctctctggaagagatggc	RUNX3_Var1_Var2_11	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ccaacagtttaggaacggagg	RUNX3_Var1_Var2_12	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	tgatgccatgactcactt	RUNX3_Var1_Var2_13	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	tgcttctgggtttaagaa	RUNX3_Var1_Var2_14	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ctggtaagtgcatggagga	RUNX3_Var1_Var2_15	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	gaacagagatggtgagcgtt	RUNX3_Var1_Var2_16	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	aaaatgatccctcactcaa	RUNX3_Var1_Var2_17	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	cagggacattgatgtctgac	RUNX3_Var1_Var2_18	Quasar 570	550 nm

RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	tcgcaagattggctggatc	RUNX3_Var1_Var2_19	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ctatttgccttcagagcaca	RUNX3_Var1_Var2_20	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	tacctgtaagagactctgtg	RUNX3_Var1_Var2_21	Quasar 570	550 nm
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RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	tagcccttgagaaagtatt	RUNX3_Var1_Var2_23	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	aattcagggcgaagactca	RUNX3_Var1_Var2_24	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ctgatgtgagaatccatgca	RUNX3_Var1_Var2_25	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	tttctaagccttttaggg	RUNX3_Var1_Var2_26	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	gttaaaataccgcactgctc	RUNX3_Var1_Var2_27	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	agcatttttagggcagatt	RUNX3_Var1_Var2_28	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	gcctcacagagacaaccaa	RUNX3_Var1_Var2_29	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	taggatcaccagaagactg	RUNX3_Var1_Var2_30	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	aagagaaccgcagcaggag	RUNX3_Var1_Var2_31	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	caggagctcagcaactatt	RUNX3_Var1_Var2_32	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ctaagaagcatgagagggc	RUNX3_Var1_Var2_33	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	acaatggattcatagctgct	RUNX3_Var1_Var2_34	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ccagagaacaggagggaaga	RUNX3_Var1_Var2_35	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	gggttagtaatctgggatga	RUNX3_Var1_Var2_36	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ttacagactcagtcagctg	RUNX3_Var1_Var2_37	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	aaggacctactttaccagc	RUNX3_Var1_Var2_38	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	acaacttaacgcagccttg	RUNX3_Var1_Var2_39	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ttctacatcagtggtttg	RUNX3_Var1_Var2_40	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	caaaactcttctctctc	RUNX3_Var1_Var2_41	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	caggtcacagtaaacactat	RUNX3_Var1_Var2_42	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	aaagattgttaccactact	RUNX3_Var1_Var2_43	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	cacacctcagcatgacaata	RUNX3_Var1_Var2_44	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	tacatcagatgagtcagca	RUNX3_Var1_Var2_45	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	atagtcaagcagtttcca	RUNX3_Var1_Var2_46	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	tgtacaagcaagttgtgcgt	RUNX3_Var1_Var2_47	Quasar 570	550 nm
RUNX3_Var1_Var2	NM_001031680.2; NM_004350.2	ttcacacatctcagagtta	RUNX3_Var1_Var2_48	Quasar 570	550 nm