1. Supplementary Methods

1.1. siRNA transfection in A549 cells. All siRNAs were purchased from Qiagen and the sequences are provided in Supplementary Table 1. For siRNA transfection in 12-well plates, 1 µl of 20 µM siRNA stock was diluted in 99 µl RPMI (Gibco) supplemented with 25 mM Hepes (Gibco) and incubated at room temperature for 5 minutes, then 4 µl Hiperfect (Qiagen) was added and incubated for 25 minutes at room temperature. This solution was then added to 900 µl A549 cell suspension containing $5x10^4$cells and incubated at 37 ^oC and 5% CO_2. At 24 hours post siRNA transfection, culture medium was replaced and cells incubated at 37 ^oC and 5% CO_2 for another 24 hours. For transfection in 384-well plates, 4 µl of 200 nM siRNA per well was mixed with 8 µl RPMI supplemented with 25 mM HEPES containing 0.35 µl Hiperfect and incubated at room temperature for 10 minutes. A suspension of 2000 A549 cells in 30 µl medium was then added to the siRNA-lipid mixture in each well and incubated at 37^oC with 5% CO_2 for 48 hours.

1.2. Virus infection. Influenza virus stock was diluted in cold infection buffer (PBS supplemented with 0.2% bovine serum albumin, 100 mM $CaCl_2$ and 100 mM $MgCl_2$) according to the specified multiplicity of infection (MOI). Cells used for infection were washed with 1x PBS, then virus solution was added to the cells and incubated at room temperature for 1 hour. Virus solution was then replaced with infection medium (DMEM supplemented with 0.2% bovine serum albumin, 4 mM L-glutamine and 100 U/ml penicillin/streptomycin) and cells incubated at 37 ^oC, 5% CO_2 for the specified infection time. For influenza virus replication assay, A549 cells were infected at MOI 0.01 with infection medium supplemented with 10 µg/ml TPCK-treated trypsin (Sigma). At the specified time post-infection, the medium was collected and titer of virus progeny in the supernatant was quantified by infection of MDCK cells for 6 hours followed by indirect immunofluorescence staining.

1.3. Indirect immunofluorescence staining for virus titer determination. Cells were fixed with 3.7% formaldehyde overnight at 4°C, washed twice with PBS and blocked with PBS/0.3% Triton X-100/10% FCS for 10 minutes at room temperature. Antibody against viral nucleoprotein (NP, clone AA5H, AbD Serotec), diluted at 1:10,000 in PBS/10% FCS/0.1% Tween 20, was then added to cells and incubated at room temperature for 1 hour. Cells were washed once with PBS/0.1% Tween 20 for 5 minutes, followed by incubation with secondary Cy3-labelled anti-mouse IgG (1:100, Jackson Immuno Research Lab) containing 10 µg/ml Hoechst (Sigma) in PBS/10% FCS/0.1% Tween 20 for 1 hour at room temperature. Cells were washed 3 x with PBS + 0.1% Tween 20 and kept in PBS. Fluorescence images were acquired and analyzed using the ScanR software (Olympus) as described previously (Karlas et al., 2010). The normalization was performed by dividing the infection rate (%) of the inhibitor by the average of infection rate (%) from all DMSO controls. The infection rate was obtained from the immunofluorescence data of MDCK cells infected with the supernatant of A549 cells treated with inhibitor and infected with A/WSN/H1N1 virus. The final virus titer can be extrapolated from the infection rate based on the following formula: multiplicity of infection = -ln(1- infection rate) as described recently (Lesch et al., 2019).

1.4. Isolation of primary cells from murine lungs. Whole lungs of mice with equal weights were removed under sterile conditions from sacrificed mice and kept in DMEM-F12 supplemented with penicillin-streptomycin. The lungs were washed in PBS supplemented with antibiotics and cleaned from any fat tissue, then cut into small pieces and incubated in solution containing 1mg/ml Collagenase I and 2.5mg/ml dispase II (Sigma) at 37°C for 2 hours with constant stirring. Cells were then separated from the tissue by passing through a cell strainer and collected by centrifuging at 1200 rpm, 4°C for 5 min. Cells were washed once with PBS and resuspended in Quantum 286 (PAA) medium supplemented with antibiotics. Cells were

cultured in Matrigel™-coated plates at 37°C with 5% CO_2. Plates were prepared by adding a solution of 20 µl Matrigel™/ml cold PBS, followed by polymerization at 37°C for 2 hours. The solution was then removed and the plate was dried. 24 hours after cell preparation cells were washed with PBS supplemented with antibiotics to remove erythrocytes and fresh culture medium added. After incubation at 37°C with 5% CO_2 for 4-5 days, cells were detached using Accutase (Sigma) at 37 °C for 10-15 min and resuspended in Quantum 286 medium supplemented with antibiotics and seeded in 24-well plates at $4x10^4$ cells/well for influenza virus infection.

1.5. Host cell viability determination by WST-1 assay. Determination of host cell viability upon siRNA transfection was performed using cell proliferation assay WST-1 (Roche). WST-1 reagent was added to the cells 48 h after siRNA transfection or chemical inhibitor treatment and incubated at 37 °C for 1.5 h. Absorbance was measured at 460nm and at the reference wavelength 590 nm. Non-targeting siRNA Allstars or non-treated cells and siPLK1 (known to induce cytotoxicity due to knockdown of the endogenous gene polo-like kinase 1 (PLK1) were used as a positive and negative control, respectively.

1.6. RNA analyses. Cells were lysed using Trizol (Invitrogen) and RNA was isolated according to manufacturer's instruction. For quantification of influenza M and NS mRNA, reverse transcription of total RNA was performed using Oligo-dT(18) primer and SuperScript III Reverse Transcription Kit (Invitrogen). cDNA was then subjected to quantitative PCR using Power SYBR® Green mix (Applied Biosystems) and primer pairs specific for M1 (5'-GACCAATCCTGTCACCTC-3' and 5'-GATCTCCGTTCCCATTAAGAG-3'), M2 (5'-GAGGTCGAAACGCCTAT-3' and 5'-CTCCAGCTCTATGTTGACAAA-3') (Cheung et al., 2005), NS1 (5'-TGACCGGCTGGAGACTCTAA-3' and 5'-TGTCTCACTTCTTCAATCAACCA-3'), NS2 (5'-GTGTCAAGCTTTCAGGACATAC-3' and 5'-TGTCTCACTTCTTCAATCAACCA-3') and GAPDH (5'-

GGTATCGTGGAAGGACTCATGAC-3' and 5'-ATGCCAGTGAGCTTCCCGTTCAG-3'). The relative mRNA level was calculated using the ΔΔCt method normalized to control treated cells with GAPDH as internal standard.

Validation of RNAi experiment by quantitative PCR was performed as previously described (Machuy et al., 2005). Total RNA was isolated at 48 hours post siRNA transfection and relative mRNA level was quantified using qRT-PCR using Power SYBR® Green One-Step Kit (Applied Biosystems) and gene-specific primer as listed in Supplementary Table 2. The relative mRNA level was normalized to control transfected cells with GAPDH as internal standard.

1.7. Western blotting. Protein samples were collected from A549 cells using 1xSDS sample buffer (75 mM Tris HCl (pH 6.8), 25% glycerol, 0.6% SDS, 7.5% β-mercaptoethanol and 0.001% bromophenol blue) followed by incubation at 95°C for 10 minutes. Samples were then separated in 10-12% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was then blocked for 1 hour in Tris-buffered saline containing 3% bovine serum albumin and 0.1% Tween 20. Viral proteins were detected using antibodies against viral matrix protein (M1, 1:100, AbD Serotec), viral ion-channel protein (M2, 1:1000, Santa Cruz) or viral non-structural protein 1 (NS1, 1:1000, Santa Cruz). As loading control, antibody against cellular β-actin (1:2500, Sigma) was used. Membranes were developed with ECL western blotting detection reagent (Amersham).

Supplementary References

Cheung, T.K., Guan, Y., Ng, S.S., Chen, H., Wong, C.H., Peiris, J.S., Poon, L.L., 2005. Generation of recombinant influenza A virus without M2 ion-channel protein by introduction of a point mutation at the 5' end of the viral intron. J. Gen. Virol. 86, 1447-1454.

Karlas, A., Machuy, N., Shin, Y., Pleissner, K.P., Artarini, A., Heuer, D., Becker, D., Khalil, H., Ogilvie, L.A., Hess, S., Maurer, A.P., Muller, E., Wolff, T., Rudel, T., Meyer, T.F., 2010. Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. Nature 463, 818-822.

Lesch, M., Luckner, M., Meyer, M., Weege, F., Gravenstein, I., Raftery, M., Sieben, C., Martin-Sancho, L., Imai-Matsushima, A., Welke, R.W., Frise, R., Barclay, W., Schonrich, G., Herrmann, A., Meyer, T.F., Karlas, A., 2019. RNAi-based small molecule repositioning reveals clinically approved urea-based kinase inhibitors as broadly active antivirals. PLoS Pathog. 15, e1007601.

Machuy, N., Thiede, B., Rajalingam, K., Dimmler, C., Thieck, O., Meyer, T.F., Rudel, T., 2005. A global approach combining proteome analysis and phenotypic screening with RNA interference yields novel apoptosis regulators. Mol. Cell. Proteomics 4, 44-55.

2. Supplementary Figure Legends

Figure S1

Characterization of used siRNAs used in this study. (A) Viability of A549 cells transfected with the indicated siRNAs determined by WST-1 assay. Unlike PLK1, knockdown of CLK, SRPK or DYRK isoforms did not reduce cell viability significantly; calculated by one-way ANOVA with Dunnett´s post hoc test. (B) Knockdown levels on RNA level of indicated target genes in A549 cells upon siRNA transfection. (C) Expression profile of murine CLK1 expression in lung cells isolated of the indicated mouse strains.

Figure S2

Influence of indicated chemical compounds on cell viability. A549 cells were cultivated in the presence of compounds at indicated concentrations for 48 h, and the viability was determined by WST-1 assay.

Figure S3

The influence of the CLK1 inhibitors on the splicing of the M and NS gene segment of influenza viruses (A/WSN/33) on mRNA and protein levels. (A-D) A549 cells were pre-treated with inhibitors for 2 h, then infected with influenza A/WSN/33 at MOI 4 for 5 h. The levels of M1, M2, NS1 and NS2 were determined by quantitative RT-PCR. (E-F) Quantification of M1, M2 and NS1 protein level from immunoblots using ImageJ. The relative intensities were normalized to the loading control β-actin.

Figure S4

Characterization of siRNAs targeting SR proteins used in this study. (A) Viability of A549 cells transfected with the indicated siRNAs determined by WST-1 assay. (B) Knockdown levels on RNA level of indicated target genes in A549 cells upon siRNA transfection. Gene expression was determined by quantitative RT-PCR. (C) Knockdown levels of siRNA targeting SRSF3. A549 cells were transfected with siRNA targeting SRSF3 or Allstars control in 12-well plate. The levels of SRSF3 were determined by quantitative RT-PCR. (D, E) Influence of the knockdown of SRSF3 on the splicing of the M and NS gene segment. A549 cells were transfected with siRNA targeting SRSF3 or Allstars control in 12-well plates. After 48 h, cells were infected with

influenza A/WSN/33 at MOI 4 for 5 h. The levels of M1, M2, NS1 and NS2 were determined by quantitative RT-PCR. (F) Quantification of M1, M2 and NS1 protein level from immunoblots using ImageJ. The relative intensities were normalized to the loading control β-actin.

Figure S5

Structures of CLK inhibitors used in this study: KH-CB19, NIH-39, VCC080174, VCC463764.

Figure S6

Quantification of GFP positive cells with speckled and non-speckled pattern of SC35 staining.

Figure S7

Prediction of SRSF1, SRSF2, SRSF5, SRSF6 binding sites using ESE Finder (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) and SRSF1 and SRSF3 binding sites using SFMap (http://sfmap.technion.ac.il/) using influenza A/WSN/33 M and NS mRNA sequences. Depicted binding sites were chosen for these SR proteins and the sites with highest prediction score. Data input sequences: Influenza A virus (A/WSN/1933(H1N1)) segment 7 matrix protein 2 (M2) and matrix protein 1 (M1) genes, complete cds (https://www.ncbi.nlm.nih.gov/nuccore/mf039638) and influenza A virus (A/WSN/1933(H1N1)) segment 8, complete sequence (https://www.ncbi.nlm.nih.gov/nuccore/cy034136).

Table S1

List of siRNA target sequences used

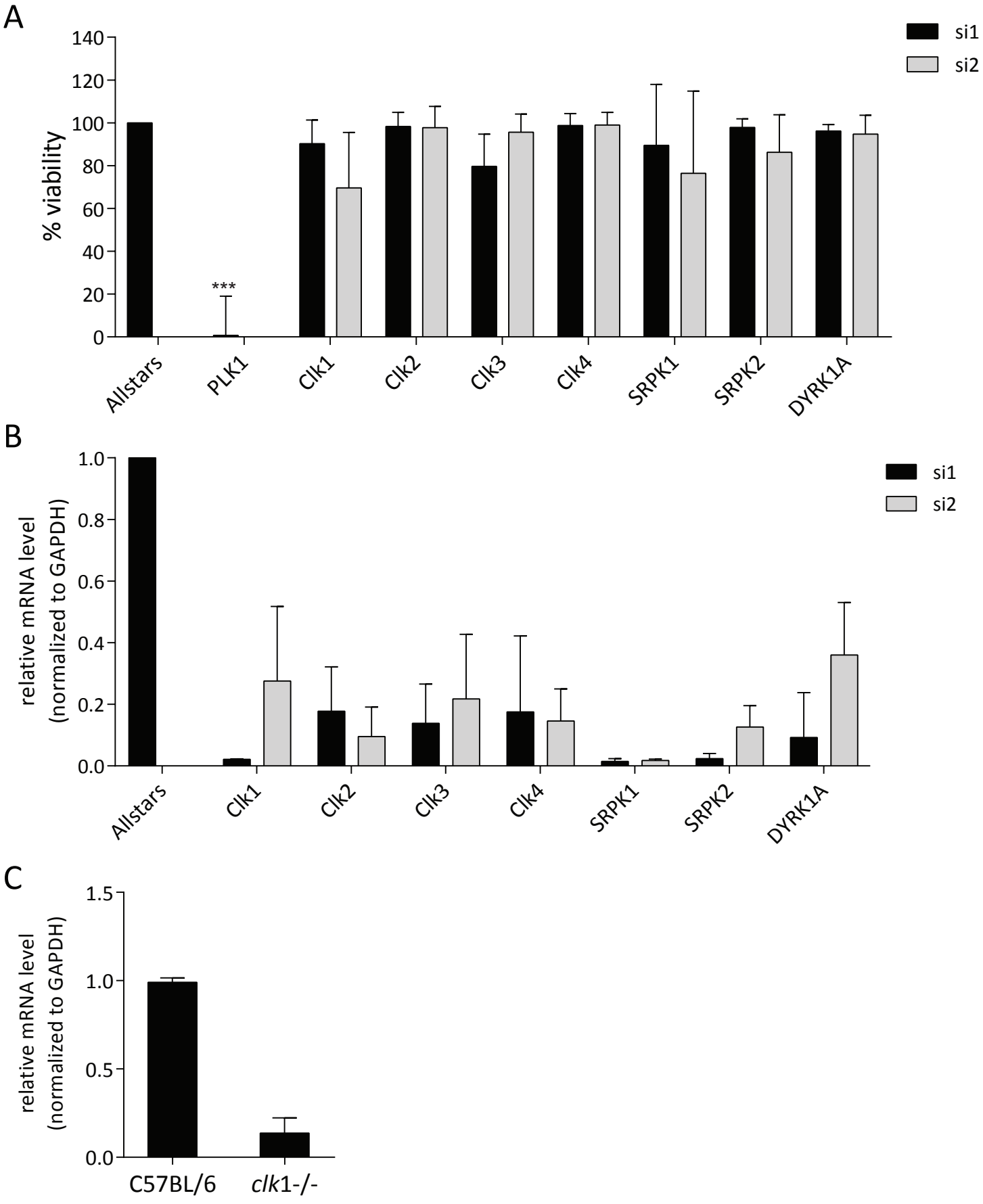
Table S2

List of primer sequences used

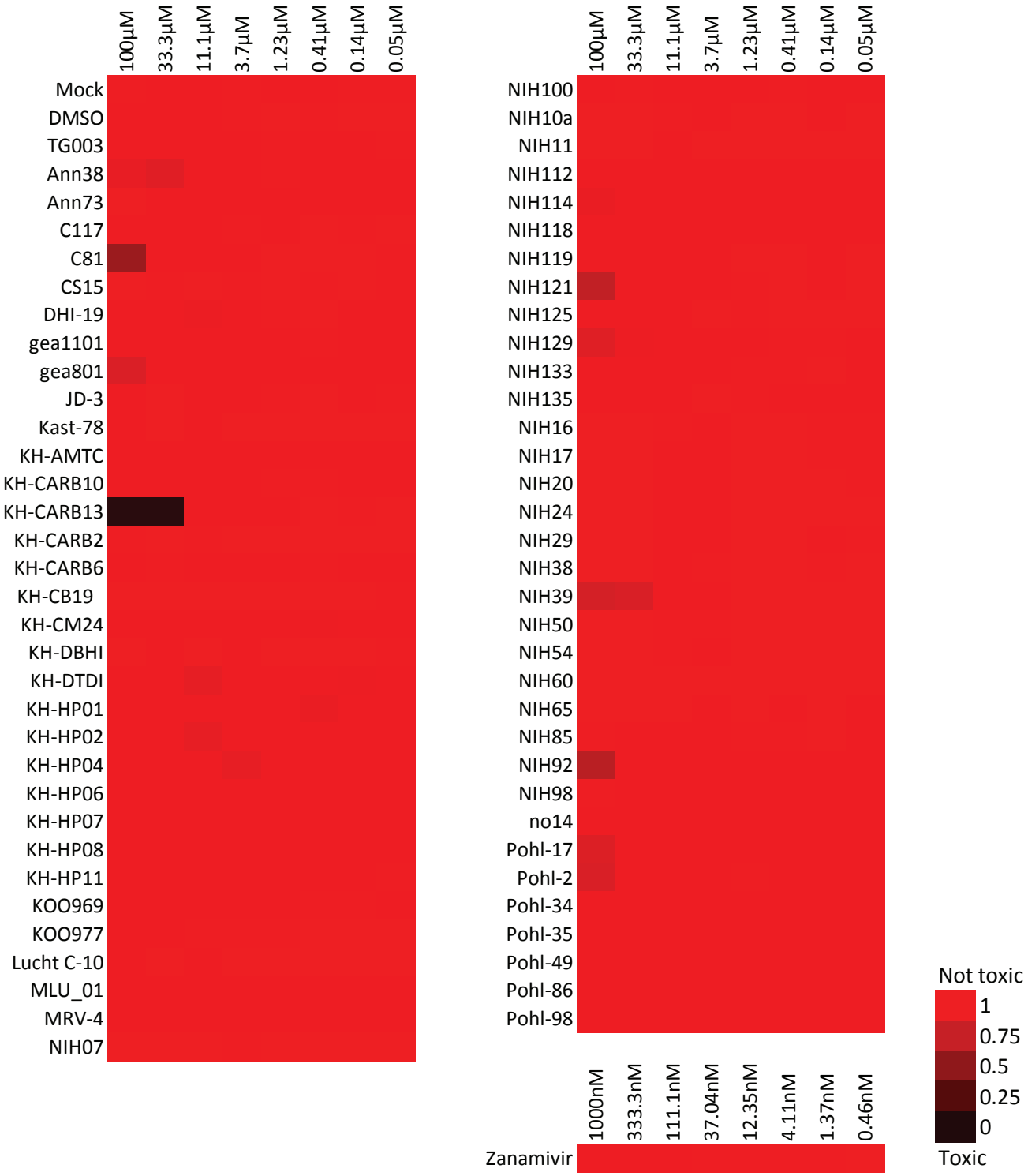
Table S3

List of compounds found to be effective against CLK1 by enzymatic screening. Shown are IC50 values determined by the Kinase-Glo Luminescent Kinase and the Caliper mobility shift assay.

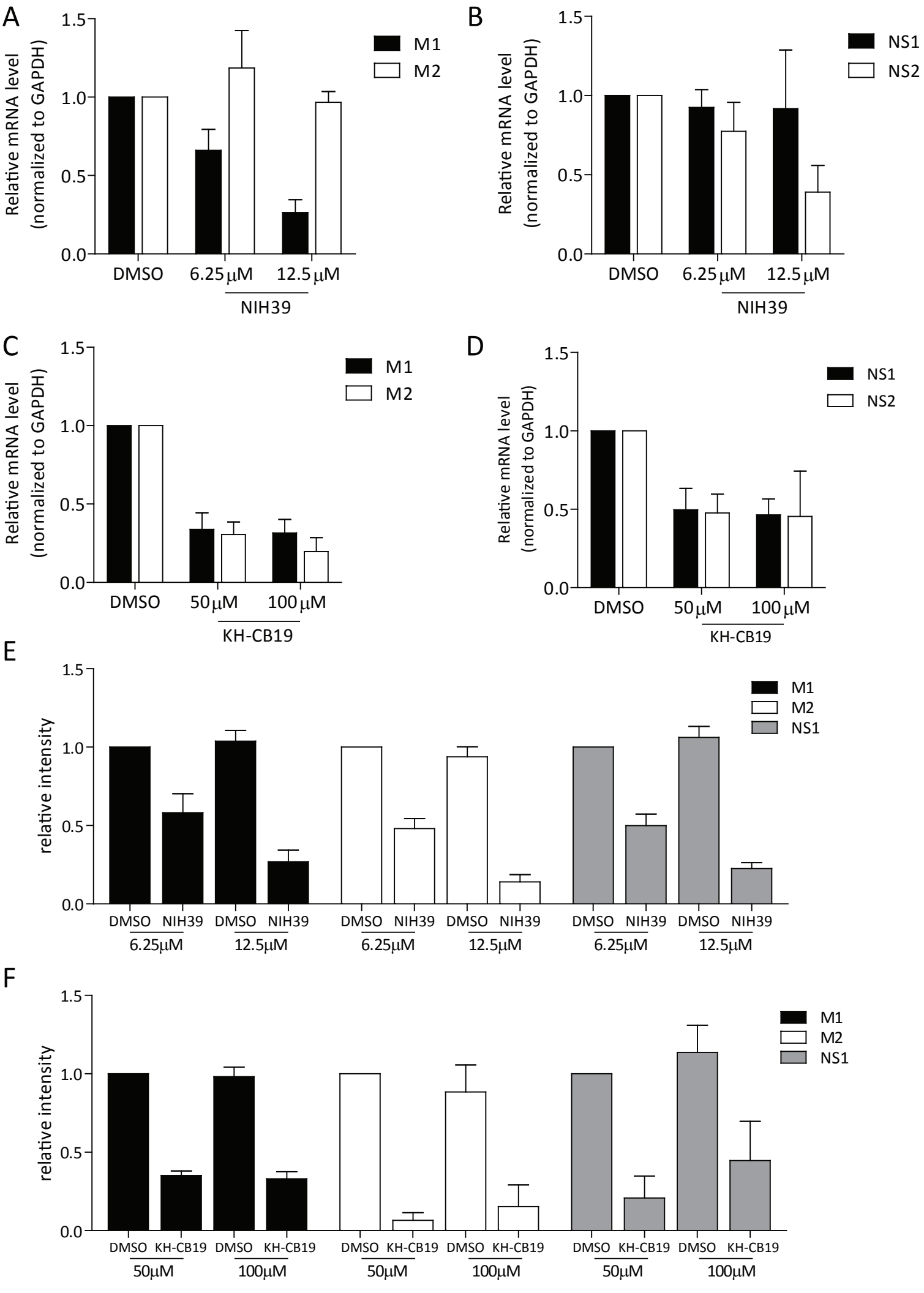
Supplementary Figure 1



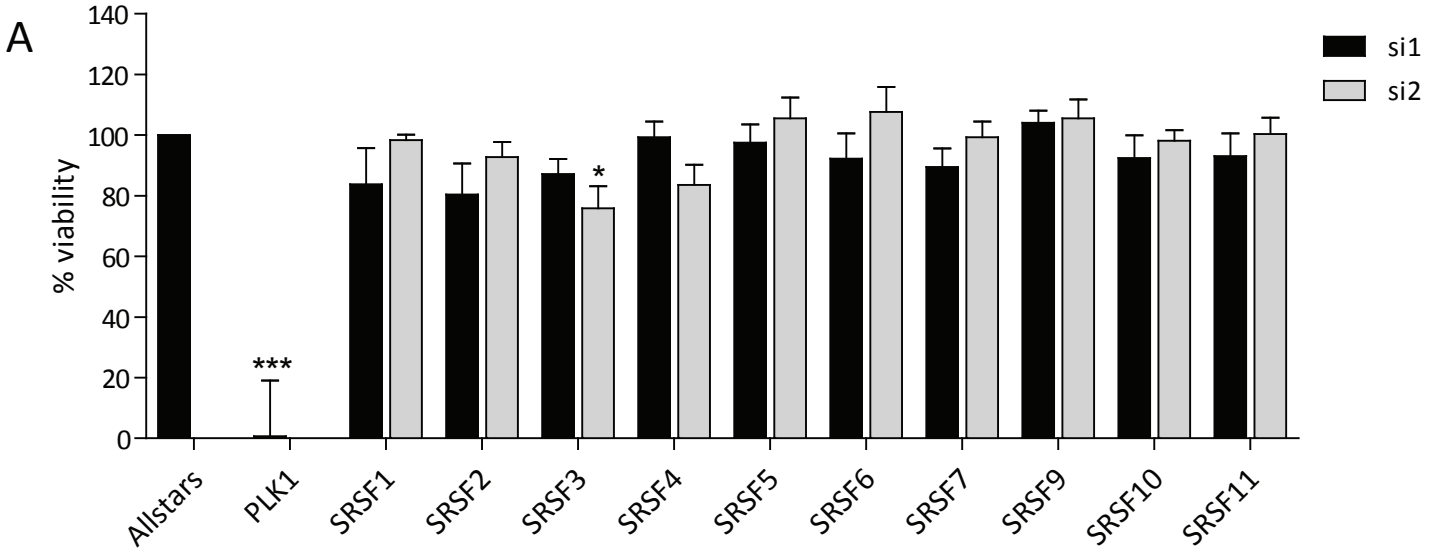
Supplementary Figure 2

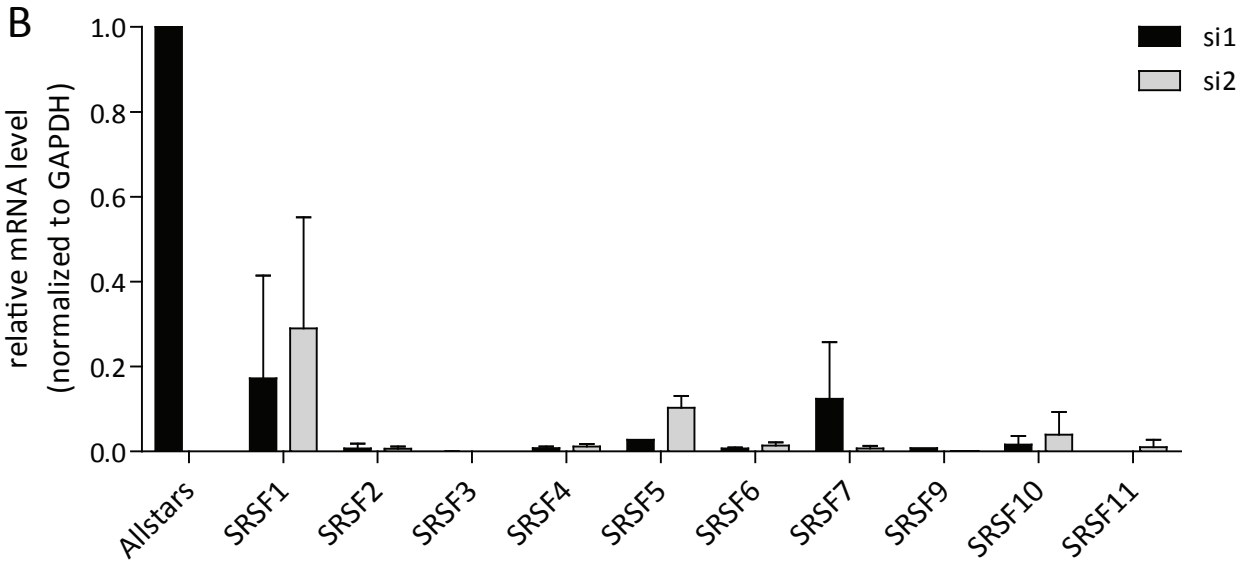


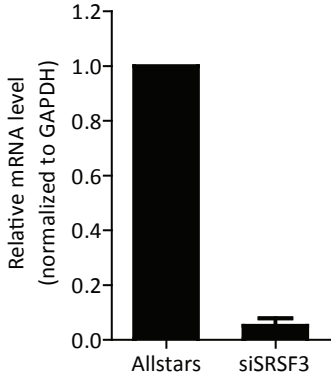
Supplementary Figure 3

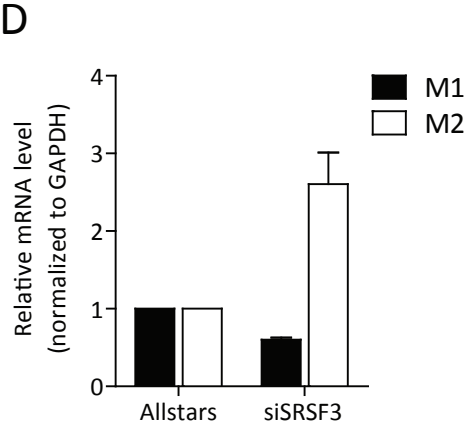


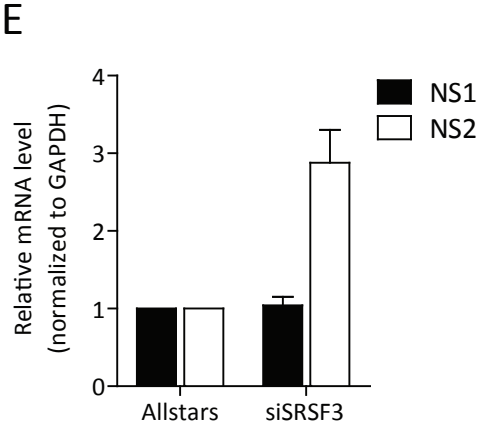
Supplementary Figure 4

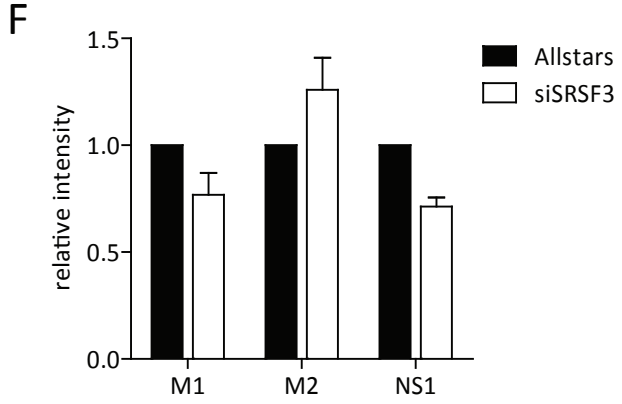




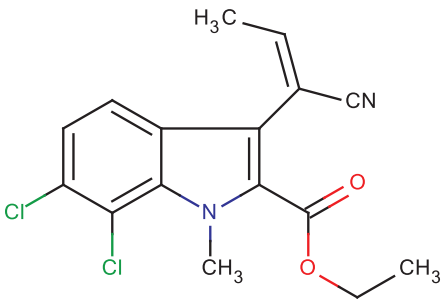




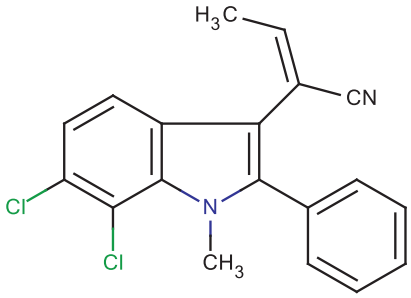




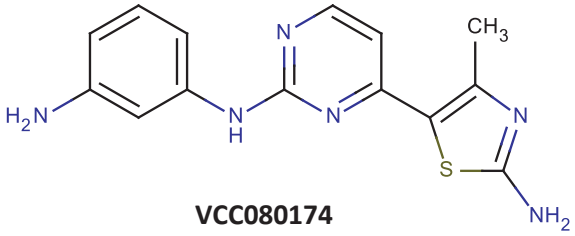
Supplementary Figure 5



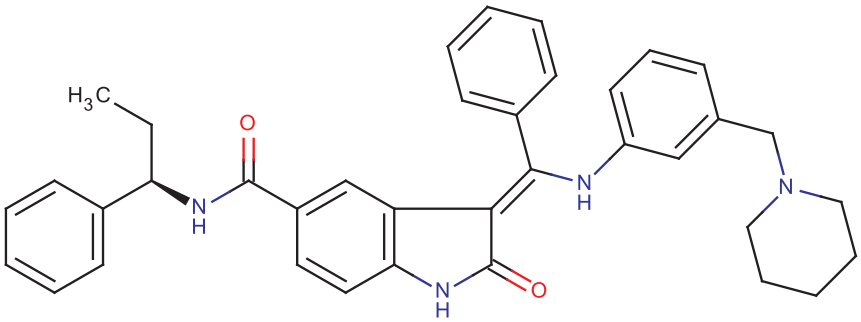
KH-CB19



NIH39

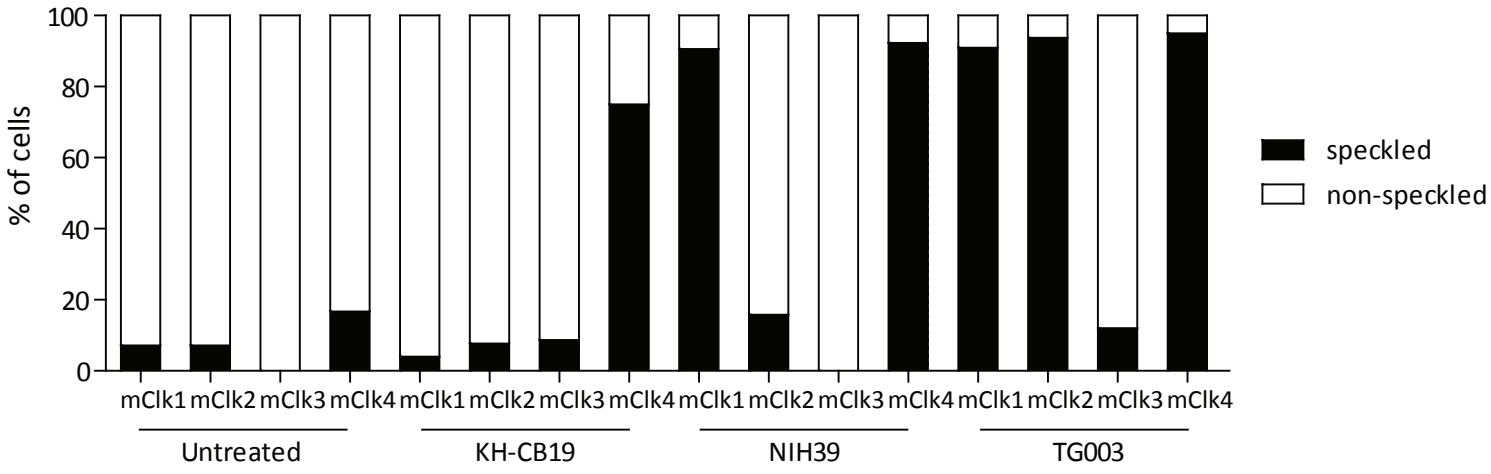


VCC080174

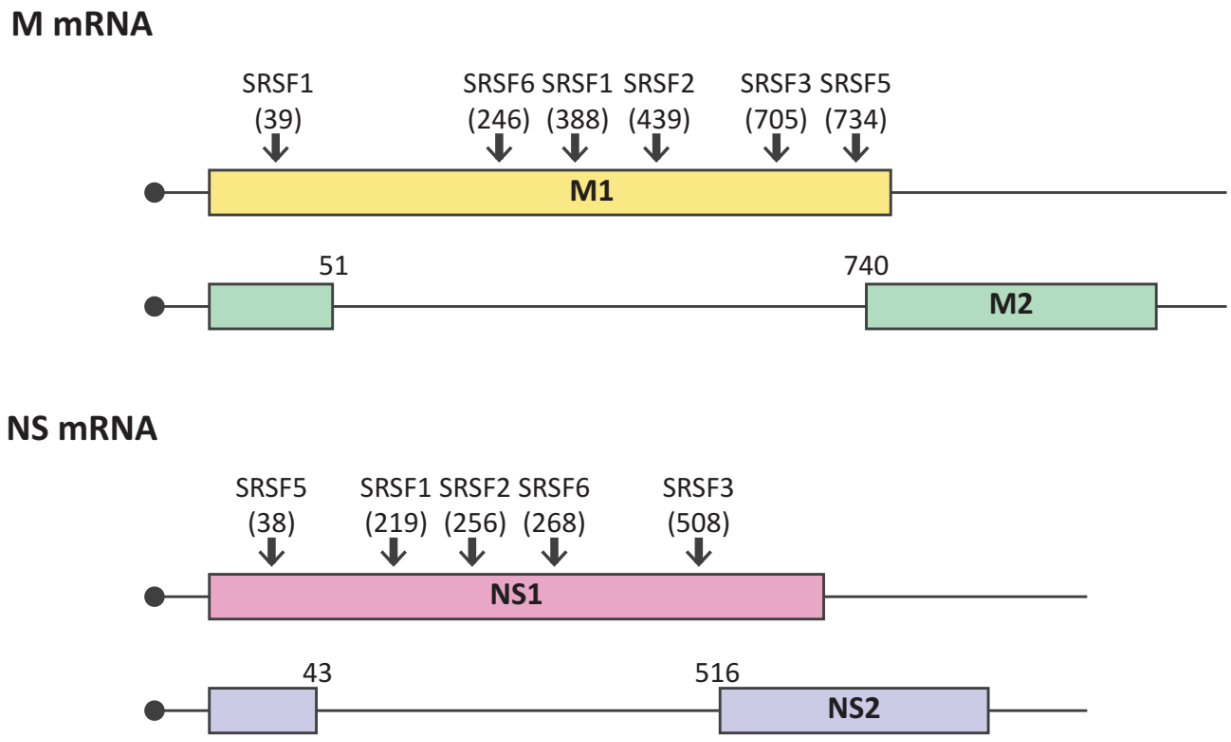


VCC463764

Supplementary Figure 6



Supplementary Figure 7



Supplementary Table 1

Target gene	siRNA	siRNA target sequence
Clk1	si1/ siClk1	CACGATAGTAAGGAGCATTTA
	si2	AACGTGATGAACGCACCTTAA
Clk2	si1	GTGAAATAGTGTAAATATGAA
	si2	TCGCCTGGATTGGGATGAGAA
Clk3	si1	CTGGTTCAACTTCCACGGTCA
	si2	CACGAAGATCTCGGTCCAGAA
Clk4	si1	CATAATTAACTTGTTAAGCAA
	si2	TACGTAAACCTATAAGAATTA
SRPK1	si1	ACGCTTATGGAACGTGATACA
	si2	CAGGAATTCTGGTGATATTAA
SRPK2	si1	CAGAAAGTGATTACACATATA
	si2	AAGGGTTAGATTACTTACACA
DYRK1A	si1	CTCTTTGAACCTAACACGAAA
	si2	CCTGCTCATATTCTTGACCAA
SRSF1	si1	TTGGCAGGATTTAAAGGATCA
	si2	CATCATAGTGCTTGCGTTTAA
SRSF2	si1	CTGCGGTCTCCTGTTTGATAA
	si2	CCGGGCCGCCACTCAGAGCTA
SRSF3	si1/siSRSF3	CAGACTGATAATAAACCTCTA
	si2	AACCCTAGATCTCGAAATGCA
SRSF4	si1	CAGGTCGAGATCCAATTCCAA
	si2	CAGAATCACGCTCCAGATCAA
SRSF5	si1	CAGAATTAGTTTAATGCCTTA
	si2	TTGCCTCTTATGGTGACTTAA
SRSF6	si1	GAGCATAGGGTTGACTGATAA
	si2	TAGCCACGACCAATTTATTAA
SRSF7	si1	CTGGATGATCTTTATACTAAT
	si2	CCCGACGTCCCTTTGATCCAA
SRSF9	si1	CAGGGCCATATTAGCAGTGAA
	si2	AGGATTTAGTGTCTTAGGAAA
SRSF10	si1	CGGGACTACTATAGCAGATCA
	si2	AACCGGGTGCTTCAAAGTACA
SRSF11	si1	AGGGAACTGGTGATTCACTAA
	si2	TTCGTTGACAGAGCTTTGATA
PLK1	siPLK1	CCGGATCAAGAAGAATGAATA

Supplementary Table 2

Target gene	Primer	Sequence (5’ – 3’)
Clk1	Forward	TAGGGTGGTCCCAACCATGT
	Reverse	AGGTCCAAGAATCCTTTCCATCA
GAPDH	Forward	GGTATCGTGGAAGGACTCATGAC
	Reverse	ATGCCAGTGAGCTTCCCGTTCAG
Clk1 (mouse)	Forward	ACTGTGAAGCTGCTCAATCGGAAA
	Reverse	ACCTCGATGCTCAAACCACTCCA
GAPDH (mouse)	Forward	TCACCATCTTCCAGGAGCG
	Reverse	AAGCAGTTGGTGGTGCAGG
Clk2	Forward	TCGCCGCCAGAACGATGCCG
	Reverse	CGCCGGTCATACACCCTCCGGT
Clk3	Forward	GGACCTTCACGTTCTCGTCAT
	Reverse	CGCTGCTACAAGACCTGGTG
Clk4	Forward	GAGAACAGGCATTGTAAACCACA
	Reverse	CCGATAATCTCGCTCATTCAAGG
SRPK1	Forward	CCATGTGATCCGAAAGTTAGGC
	Reverse	AGGGTCTGAATTGCGAACTGA
SRPK2	Forward	CCGCGTCGGAATGAGCTCCC
	Reverse	TGAGGCTCCGGCTTTTTCGGA
DYRK1A	Forward	AAGAAGCGAAGACACCAACAG
	Reverse	TTTCGTAACGATCCATCCACTTT
SRSF1	Forward	CTCCAAGTGGAAGTTGGCAGGATT
	Reverse	ACACCAGTGCCATCTCGGTAAACA
SRSF2	Forward	TCGCTACAGCCGCTCGAAG
	Reverse	ACTCTTCGATCGCGACCTG
SRSF3	Forward	ACTTCATAAGCTTGGTGCAT
	Reverse	ACACCTTTGTGTCACTGTTT
SRSF4	Forward	TTAAGGGCTACGGGAAGATCC
	Reverse	ATGCTCAACAATTACTCGCTCA
SRSF5	Forward	GATTCTTCAAGGGATATGGACGG
	Reverse	GACCGAGCCCTAGCATGTTC
SRSF6	Forward	GGTACGGCTTCGTGGAGTTC
	Reverse	GGGCGTGCTCTACGATCAC
SRSF7	Forward	CGGTACGGAGGAGAAACCAAG
	Reverse	AGCCACAAATCACCTTTCCATC
SRSF9	Forward	GCTTCGTGTGGAGTTCCCC
	Reverse	TCTTCTTGTAGGAGGCCCATT
SRSF10	Forward	CAGAACTACGGCGAGCGGGAAT
	Reverse	CGCCTGGAATCTTCCTTGGAGCG
SRSF11	Forward	ATGAGCAACACTACCGTCGTC
	Reverse	GGGAGACATTAGTCACCTGGAT

Supplementary Table 3

ID	Clk1_Luciferase_IC50 (μM)	Clk1_IC50 Labchip (μM)
KH-CB19T	N/A	< 0.05
VCC185059	0.027	0.187
VCC463764	0.206	0.025
VCC005651	0.035	0.021
VCC080174	0.062	0.936
VCC889564	0.063	0.381
VCC460649	0.154	0.059
VCC240536	0.114	0.347
VCC424351	0.107	0.690
VCC090131	0.005	0.000
VCC316310	0.837	0.018
VCC323745	0.039	0.050
VCC352333	0.017	0.047
VCC275426	0.276	0.911
VCC209800	0.160	0.105
VCC783456	0.301	0.078
VCC440300	0.078	0.041
VCC237658	0.018	0.626
VCC322466	0.131	0.161
VCC581887	0.032	0.245
VCC185767	0.406	0.119
VCC599621	0.054	0.032
VCC000902	0.043	0.682
VCC027029	0.406	0.033
VCC084109	0.017	0.751
VCC116601	0.020	0.022
VCC230913	0.032	0.311
VCC303142	0.005	0.037
VCC348917	0.021	0.026
VCC369679	0.042	0.019
VCC386531	0.003	0.577
VCC448924	0.005	0.016
VCC497245	0.065	0.838
VCC558180	0.012	0.114
VCC815262	0.405	0.300
VCC885878	0.565	0.011
VCC892742	0.239	0.676
VCC976022	0.176	0.014
VCC372525	0.004	0.744
VCC077038	0.034	0.017
VCC936586	0.027	0.052
VCC180519	0.046	0.014
VCC140861	0.016	0.285
VCC133766	0.023	0.096

VCC445786	0.025	0.013
VCC609838	0.084	0.055
VCC754620	0.017	0.110
VCC092207	0.201	0.073
VCC283994	0.157	0.016
VCC716806	0.364	0.136
VCC378503	0.040	0.026
VCC842416	0.255	0.201
VCC303508	0.065	0.070
VCC229760	0.022	0.021
VCC657622	0.009	0.016
VCC890061	0.020	0.217
VCC762355	0.044	0.214