

Supplementary Methods

Cell lines

A549 (ATCC, CCL-185), Mv4;11 (ATCC, CRL-9591), NOMO-1 (DSMZ, ACC 542), MOLM1-3 (DSMZ, ACC 554), SHI-1 (DSMZ, ACC 645), KG-1 (DSMZ, ACC 14), OCI-AML3 (DSMZ, ACC 582) were grown according to supplier instructions. MDM2 WT and MDM2 C305F were established from Mdm2^{tm1.1Ypz} mice¹ (kindly provided by Prof Yanping Zhang (University of North Carolina, Chapel Hill, USA) and cultured as described² under ANU Animal Ethics Committee-approved protocol A2018/56. Cells were maintained below 80% confluency (adherent) or 2x10⁶ cells/ml (suspension) in a humidified 5% CO₂ incubator at 37°C. Cells were seeded 24 hours prior to treatment to ensure an exponentially growing phase at the start of the treatment.

High-throughput compound screening and analysis

Compound screening protocol: This screening approach was performed as previously described³ with some adaptations. At the commencement of screening (Day 1), A549 cells (750/well, duplicate technical replicates) were reverse transfected with 10 nM siGENOME human siRPS19 SMARTpool siRNA (GE Dharmacon, M-003771) in 384-well black-walled optical microplates (Corning Costar 3571) and incubated for 24 hours (37°C, 5% CO₂, humidified). On Day 2, growth medium (DMEM/F12, 2 mM L-glutamine, 10% FBS) was replaced, and DMSO or compounds (prepared in growth medium) were added to wells to a final treatment concentration of 5 µM, 1.04 µM, 210 nM and 40 nM for 48 hours. The compound library (4169 FDA-approved compounds) was obtained from the Walter and Eliza Hall Institute Drug Discovery Centre (stored and plated by Compounds Australia, Griffith University, Australia), curated from a combination of sources and/or suppliers (details provided in Supplementary screen data). On Day 4 (48 hours post-drug treatment), plates were fixed with 4% Paraformaldehyde (Proscitech C0042B), stained with 0.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI, D9542, Sigma-Aldrich) and permeabilised with 0.5% Triton X-100 (Sigma-Aldrich, #X-100). Cells were incubated with anti-p53 and anti-mouse Alexa Fluor 594 (**Supplementary Table 2**) before being imaged on the Cellomics ArrayScan VTi (Thermo Fisher Scientific) using the following parameters: image acquisition was carried out using the 20X objective in standard mode (1024x1024; 2x2 binning), capturing 25 fields/well at a fixed exposure time.

Image analysis and data processing: Images were processed using a Cellomics Target Activation Bioapplication (described in ³ Supplementary File 8), which was developed to identify DAPI-stained

nuclei as a surrogate measure of cell number. The resulting nuclear mask was used to quantify the average nuclear intensity of p53 staining on a per-cell basis; these values were then aggregated to generate an average per well as the primary screening readout. Data were normalized to the mean of siRPS19 DMSO-treated control wells to calculate fold changes for two parameters: average cell count and average p53 intensity.

Assay quality metrics: Assay quality was assessed by calculating the Z'-factor for siRPS19-transfected cells on each screened plate. DMSO served as the negative control, while BEZ-235 (Dactolisib; SelleckChem #S1009; 1 μ M) was used as a positive control, having been previously identified as an agent that suppresses p53 stabilization (i.e. reduces average p53 intensity when RPS19 is depleted) without rescuing cell number. Z'-factor calculations were performed as previously described⁴, using the average p53 intensity readout. Across the screen, the assay demonstrated an average Z'-factor of 0.42 ± 0.12 . Average cell number was not included in the Z'-factor analysis due to the limited dynamic range observed for cell number rescue in this screen.

Evaluation and ranking of screening data: Compounds were evaluated based on their effects on p53 expression and cell number, with priority given to those increasing p53 intensity while reducing cell count (p53 up, cell count down). To rank compounds within each response quadrant, Mahalanobis distance⁵ was used as a multivariate scoring metric, calculated for each compound using p53 intensity and cell count relative to the joint distribution of all compounds, thereby accounting for covariance between the two parameters. Compounds were ranked by decreasing Mahalanobis distance within each quadrant, with larger distances indicating stronger combined effects; no formal outlier test was applied. Distances and ranks were determined independently for each screened dose (5 μ M, 1.04 μ M, 210 nM, and 40 nM). To prioritise compounds with consistent effects across doses, dose-specific ranks were combined using a rank-product metric, defined as the geometric mean of ranks across the four doses. Compounds absent from a quadrant at a given dose were assigned the lowest observed rank for that quadrant, penalising inconsistent responses. This approach produced a complete ranking of compounds based on their overall product rank metric in the p53-up/cell-count-down quadrant (Top 40 ranked compounds presented in **Table 1**).

All statistical analyses and visualisations were performed in R. A description of the Mahalanobis analysis approach and data are available at: https://github.com/mevers/compoundscreen_RPS19-KD. Screening data is provided in the **Supplementary screen data** file.

siRNA transfection

A549 cells were reverse transfected with 5nM NT (L-013549), RPS19 (L-003771), RPL5 (L-013611) or RPL11 (L-013703) SMARTpool siRNA and DharmaFECT1 (T-2001) as per manufacturer instructions (Dharmacon). After 24 hours of transfection, media was replaced with A549 growth media containing either vehicle, single drug or drug combination. Cells were incubated for a further 48 hours prior to harvest for Protein or RNA expression analysis.

Inhibitors

Flavopiridol (Selleckchem S1230), Dinaciclib (Selleckchem S2768), AZD-4573 (MedChemExpress, HY-112088) and Tambiciclib (MedChemExpress, HY-X0009) were dissolved in 100% DMSO and CX-5461 was dissolved in 50mM NaH₂PO₄ pH 4.5 and stored at -20°C until experimental use. Inhibitor stocks were diluted to the required concentrations in corresponding vehicles: 50µM NaH₂PO₄ or 0.1% DMSO for single drug assays, or 50 µM NaH₂PO₄ + 0.1% DMSO for combination assays.

Target inhibitory concentration (TIC₅₀) determination

Cell lines were seeded 24 prior to drug treatment and treated with a range of drug concentrations for 3 hrs. For Pol I transcription inhibition IC₅₀ determination, RNA expression was analysed as described in “RNA expression analysis by qPCR” using primers for 5’ETS, cFOS and B2M (see Table 2). For CDK inhibition, protein extracts were prepared and Phospho-RPB1 (Ser2) and p53 expression (see Table 3) were analysed as described in “Protein expression analysis by WB”. The expression changes were further normalised to the vehicle and the dose response curve obtained with GraphPad Prism 10 using non-linear regression ([inhibitor] versus response – variable slope (four parameters)) and the target IC₅₀ (TIC₅₀) calculated.

Growth inhibitory concentration (GIC₅₀) determination

Cell lines were treated with a range of drug concentrations and grown until vehicle treated culture reached 100% confluency. Images were collected every 6 hours using Incucyte Zoom (Essen BioScience 2016A) life cell imaging system. Images were analysed with Incucyte Control software (Essen BioScience) to determine percentage confluency at different time points. Confluency values at the time point when vehicle-treated cells reached confluency were plotted, and the dose response curve

obtained with GraphPad Prism 10 using non-linear regression ([inhibitor] versus response – variable slope (four parameters)) and the growth IC₅₀ (IC₅₀) calculated.

Cell proliferation assays

A549 cells treated with vehicle, CX-5461, Dinaciclib/Flavopiridol or combination were imaged every 6 hours and confluency calculated as described in “GIC₅₀ determination”. Confluency values at the time point vehicle-treated cell reach confluency were used for analysis. Mv4;11 and KG1 cells treated with vehicle, CX-5461, Dinaciclib or combination were counted every 24h from the time treatment started using Z series CoulterCounter (Beckman Coulter). Cell counts/confluency values were normalized to the time when treatment was started (T=0h). For drug synergy proliferation assays 4 concentrations of drug were tested based on the determined GIC₅₀. In Mv4;11 0, 30, 60 and 120nM CX-5461; 0, 5, 10 and 15nM Dinaciclib and AZF4573 and 0, 15, 30 and 60nM Tambiciclib were tested. In KG-1 0, 35, 70 and 100nM CX-5461 and 0, 5, 10 and 15nM Dinaciclib were tested.

RNA expression analysis by quantitative polymerase chain reaction (qPCR)

After treatment with vehicle, single drug or drug combinations, total RNA was extracted using a Nucleospin RNA kit (Macherey-Nagel 740955), cDNA was prepared using SuperScript IV Reverse Transcriptase (Invitrogen) and qPCR performed on the StepOne Plus Real-Time PCR System (Applied Biosystems) using a SYBRGreen Fast Master Mix (Applied Biosystems) and target specific primers (see **Supplementary Table 1**). For each replicate, RNA expression was normalised the housekeeping gene B2M via the $2^{-\Delta C_t}$ method. Expression changes were further normalised to vehicle (treatment/vehicle).

Protein expression analysis by immunoblotting

After 3 hours treatment with a single drug or drug combinations, whole cell protein extracts were prepared in 20mM HEPES, 0.5mM EDTA, 2% SDS, 0.1M DTT buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis using 4-20% Mini-PROTEAN TGX Precast Protein gels (Bio-Rad 4561093) and transferred to Immobilon-P PVDF membrane (Millipore, IPVH00010) using Trans-Blot Turbo Transfer System (BioRad) or using iBlot Transfer Stack with PVDF membrane (IB24001) using iBlot2 Gel Transfer Device (Invitrogen IB21001). Membranes were probed with target-specific antibodies (see **Supplementary Table 2**) and visualised by using Clarity Western ECL Substrate (BioRad 1705061) in the ChemiDoc System (BioRad). Integrated volume for antibody-

specific bands were calculated with Image Lab software (version 6.0.1, 2017) and values were normalized to housekeeping protein β -actin (ACTB) or Vinculin (VCL).

Protein expression analysis by immunofluorescence (IF)

After the required incubation with the different single drug or drug combinations, cells were fixed with 4% Paraformaldehyde (PFA; Electron Microscopy Sciences 15714S), permeabilized with 0.5% Triton X100, blocked with 5% bovine serum albumin (BSA) in phosphate buffer saline (PBS). Primary antibodies were incubated either 1h at RT or 16h at 4°C and secondary antibodies were incubated for 1h at RT (except for gH2AX staining where secondary was incubated at 37°C) and stained with 5 μ g/ml DAPI (Sigma D-9542). See **Supplementary Table 2** for antibodies list. A549 cells were cultured, treated and stained on glass coverslip or Phenoplate 96 well microplates (Revvity, 6055302). Mv4;11 were cultured and treated in multi-well plates and cytopun onto PLL coated glass slides (Sigma-Aldrich P0425) or Poly-L-lysine (PLL)-coated Phenoplate 96 well microplates (Revvity, 6055302). Slides were mounted using Fluorescence Mounting Media (Agilent S3023) and imaged using LSM800 confocal microscope (Zeiss) with a 63X (1.4) lens. Cells in 96 well plates were kept in PBS plate were sealed and imaged in Opera Phenix High-Content Screening System (PerkinElmer) with a 40X lens. Images were processed using Harmony Software (PerkinElmer).

Viability analysis

Cells were treated with vehicle, 0.1 to 10X IC₅₀ of CX-5461 and Dinaciclib alone or in combination for 72h. To assess cell viability, cells were stained with 1 μ g/ml Propidium Iodine (PI; Sigma-Aldrich 287075), data was acquired with FACSVerse (BD Life Sciences) and analysed using FlowJo™ Software. Synergy was evaluated using Chou-Talalay CI values, calculated with the CalcuSyn software 2.1 (Biosoft) as previously described⁶. The lowest CI value obtained corresponding to Fa value greater than 0.5 was used to assess the degree of synergy (CI<1), additivity (CI=1) or antagonism (CI>1). Chou-Talalay was calculated for effect levels between 50 and 90% (fraction affected 0.5-0.9) and a combinatorial effect was considered synergistic if a single CI was lower than 1.

Apoptosis analysis

Cells were stained at 3, 6 and 24h post-treatment with vehicle, CX-5461, Dinaciclib or in combination using APC Annexin V Apoptosis Detection Kit (BioLegend 640920). Data was acquired with BD LSR II flow cytometer (BD Life Sciences) and analysed using FlowJo™ v10.8 Software. Synergy was

assessed using Bliss independent model and synergy defined by Excess over Bliss Combination Index (CI) > 0. See **Supplementary Table 2** for antibodies list.

Cell cycle analysis

Cells were analysed at 12 and 24h post-treatment with vehicle, CX-5461, Dinaciclib or in combination. Thirty minutes prior to harvest, cells were pulse-labelled with 10uM BrDU. After harvesting, cells were fixed with ice cold 80% ethanol, treated with 2N HCl/ 0.5% (v/v) Triton X100 and neutralized with 0.1M Na₂B₄O₇·10H₂O. BrdU incorporation was probed with anti-BrDU antibody and anti-IgG-A488 and DNA was stained with 10µg/ml Propidium Iodine. Data was acquired with APF-LSR II flow cytometer (BD Life Sciences) and analysed using FlowJo™ v10.8 Software.

Synergy analysis

Except when stated otherwise (viability and apoptosis analysis), synergy scores were calculated using SynergyFinder V3 (<https://synergyfinder.fimm.fi/>) using Bliss independence model. Synergy was defined by Bliss score > 10. Concentrations with the most significant synergy score are presented.

MLL-ENL Nras^{G12D} and MLL-AF9 Nras^{G12D} and AML1-ETO9a Nras^{G12D} AML combinatorial treatment

Eight to 10 weeks old female C57BL/6 mice were transplanted with 5e⁵ MLL-ENL Nras^{G12D}, MLL-AF9 Nras^{G12D} or AML1-ETO9a Nras^{G12D} AML cells (p53 WT) via tail vein injection. Seven days post-transplant, the mice were randomized into four groups.

For survival experiments MLL-ENL Nras^{G12D}, MLL-AF9 Nras^{G12D} mice were dosed continuously with either CX-5461 vehicle (CX-5461 - 50mM Phosphate buffer in PBS (pH 4.5)) or 30mg/kg CX-5461 (3 days/week) by oral gavage; CDK inhibitor vehicle (20% W/V 2-Hydroxypropyl-β-cyclodextrin in PBS (Sigma-Aldrich C0926), 20mg/kg Dinaciclib or 7.5mg/kg Flavopiridol by interperitoneally injection (2 day/week) or a combination of both CX-5461 and CDK9 drugs until an ethical endpoint was reached. Engraftment and disease progression were monitored once a week (starting at day of treatment) by bioluminescence imaging using the IVIS Xenogen after injection of mice with luciferin (200µl per mouse of a 1mg/ml stock solution). Mice were weight at least three times a week.

For short term therapy experiments, mice were treated with a single dose of vehicles, CX-5461, Dinaciclib or combination of both. Peripheral blood was collected 10h post-treatment, whole blood

cell counts were performed with ADVIA 120 Hematology System (Siemens) and circulating tumour burden was evaluated by flow cytometry. Bone marrow and spleen tissues were harvest at 3, 10, 24 or 48 hours post-treatment for RNA and protein expression analysis, tumour burden, apoptosis and cell cycle analysis. Experiments were conducted under institutionally approved animal ethics protocols (ANU: A2015/12 & A2018/48; Peter MacCallum Cancer Centre: E462).

Primary human AML colony formation assay

Mutation analysis was performed in de-identified AML patient biopsy samples to identify AML driver mutations as described previously^{8,9}. Colony formation capacity of primary human AML cells was analysed in methylcellulose semi-solid media (Stem Cell Technologies M4435) as described previously¹⁰. Bone marrow cells (10^5) were plated and treated with vehicles, 25nM CX-5461, 15nM Dinaciclib or combination of both drugs. Patient consent was obtained under Central Adelaide Local Health Network human research ethics HREC/18/CALHN/588.

Statistical analysis

Except when otherwise stated all data was collected, presented and analysed as described below. Any variations are described in figure legends.

All *in vitro* data was obtained from a minimum of 3 biological experiments. Assuming an effect size of 25%, and a variability of 10%, we estimate the need of 3 samples per treatment group to obtain statistical significance. All statistical analysis assumed normal distribution and equal variance between study groups. Data is presented as mean and error bars as standard deviation (SD). Statistical test results are shown as p-values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$). Significance was calculated using ordinary one-way ANOVA with multiple comparison test (see figure legends for specific test).

Assuming an effect size of 25%, and a variability of 20%, we estimate the need of 5 to 10 mice per treatment group to obtain statistical significance. Consequently, 5 (short-term therapy) or 10 mice (survival) were used per treatment group. Animals were allocated to experimental groups based on their disease burden determined by chemiluminescence or peripheral blood analysis so that disease burden is balanced between all groups. Animals were excluded from analysis if they failed to engraft or reached the ethical endpoint prior to the experimental endpoint. No blinding was used. Animal models survival data, presented in Kaplan-Meier plots, was analyzed by log-rank (Mantel-Cox) test with Benjamini and Hochberg correction.

Supplementary Table 1. Primers

Primer pair	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Mouse 5'Ets	CCAAGTGTTTCATGCCACGTG	CGAGCGACTGCCACAAAAA
Mouse B2m	TTCACCCCCACTGAGACT	GTCTTGGGCTCGGCCATA
Human 5'ETS	GCTCTTCGATCGATGTGGTGACG	CGGGCGGAGCGAGAAGGAC
Human cFOS	GGGATAGCCTCTCTTACTACCA	GAGAAGTCCTGCGCGTT
Human B2M	CCGTGGCCTTAGCTGTGCTCGC	CCCACTTAACTATCTTGGGCTG
Human cMYC	CAGCTGCTTAGACGCTGGATT	GTAGAAATACGGCTGCACCGA
Human TP53	GAGCGTGCTTTCCACGAC	GCTCGACGCTAGGATCTGAC
Human CDKN1	GGACCTGGAGACTCTCA	GGATTAGGGCTTCCTCTTG
Human BAX	TTCTGACGGCAACTTCAACT	CAGCCCATGATGGTTCTGAT
Human BBC3	CGACCTCAACGCACAGTA	GGAGTCCCATGATGAGATTGTA
Human NOXA	ACTGTTCGTGTTTCAGCTC	GTAGCACACTCGACTTCC
Human MDM2	TGCCAAGCTTCTCTGTGAAA	AGATGTACCTGAGTCCGATGA

Supplementary Table 2. Antibodies

Antibody ID	Reactivity	Cat number	Supplier
RPB1 phospho-CTD (S2)	human, mouse	13499S	Cell Signalling
p53	human	sc-126	Santa Cruz Biotech
P21	human	2947S	Cell Signalling
MCL1	human, mouse	ab32087	Abcam
Cleaved CASPASE 3	human, mouse	9661	Cell Signalling Technologies
PARP	human, mouse	9541	Cell Signalling Technologies
FIBRILLARIN	human, mouse	ab5821	Abcam
NUCLEOLIN	human, mouse	ab136649	Abcam
phospho-γH2AX	human, mouse	05-636	Merk
BETA ACTIN HRP (ACTB)	human, mouse	sc-47778	Santa Cruz Biotech
VINCULIN-HRP (VCL)	Human, mouse	18799	Cell Signalling Technologies
BrdU	N/A	347580	BD Biosciences
IgG-Alexa Fluor 488	rabbit	A11008	Life Technologies
IgG- Alexa Fluor 488	mouse	A11001	Life Technologies
IgG- Alexa Fluor 594	mouse	A21203	Life Technologies
IgG- Alexa Fluor 647	mouse	A21235	Life Technologies

Supplementary Material References

1. Macias E, Jin A, Deisenroth C, Bhat K, Mao H, Lindstrom MS, *et al.* An ARF-independent c-MYC-activated tumor suppression pathway mediated by ribosomal protein-Mdm2 Interaction. *Cancer Cell* 2010 Sep 14; **18**(3): 231-243.
2. Ferreira R, Hein N. Purification of Mouse Embryonic Fibroblasts (MEFs). *Curr Protoc* 2023 Nov; **3**(11): e921.
3. Hannan KM, Soo P, Wong MS, Lee JK, Hein N, Poh P, *et al.* Nuclear stabilization of p53 requires a functional nucleolar surveillance pathway. *Cell Rep* 2022 Nov 1; **41**(5): 111571.
4. Birmingham A, Selfors LM, Forster T, Wrobel D, Kennedy CJ, Shanks E, *et al.* Statistical methods for analysis of high-throughput RNA interference screens. *Nat Methods* 2009 Aug; **6**(8): 569-575.
5. Wilks SS. Multivariate Statistical Outliers. *The Indian Journal of Statistics* 1963; **25**(4): 407-426.
6. Bijnsdorp IV, Giovannetti E, Peters GJ. Analysis of drug interactions. *Methods Mol Biol* 2011; **731**: 421-434.
7. Lanevski A, Giri AK, Aittokallio T. SynergyFinder 3.0: an interactive analysis and consensus interpretation of multi-drug synergies across multiple samples. *Nucleic Acids Res* 2022 Jul 5; **50**(W1): W739-W743.
8. Murphy KM, Levis M, Hafez MJ, Geiger T, Cooper LC, Smith BD, *et al.* Detection of FLT3 internal tandem duplication and D835 mutations by a multiplex polymerase chain reaction and capillary electrophoresis assay. *J Mol Diagn* 2003 May; **5**(2): 96-102.
9. Li G, Shen J, Cao J, Zhou G, Lei T, Sun Y, *et al.* Alternative splicing of human telomerase reverse transcriptase in gliomas and its modulation mediated by CX-5461. *J Exp Clin Cancer Res* 2018 Apr 10; **37**(1): 78.
10. Hein N, Cameron DP, Hannan KM, Nguyen N-YN, Fong CY, Sornkom J, *et al.* Inhibition of Pol I transcription treats murine and human AML by targeting the leukemia-initiating cell population. *Blood* 2017: blood-2016-2005-718171.