Supplemental materials

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1: diMF induces polyploidization and expression of differentiation markers of multiple megakaryocytic cell lines. (Related to Figure 2)

(A) diMF increased mean polyploidy and expression of CD41 and CD42 cell surface markers in human CMS, CHRF, Meg01 and K562 cell lines 72 hours after treatment, and mouse G1ME and Y-10 cells 48 hours after treatment. Bar graphs depict mean ± SD of 2 independent experiments conducted in triplicate. Representative flow plots are shown. ** $p<0.01$. (B) diMF inhibited proliferation and induced megakaryocyte polyploidization and CD41 expression of primary AMKL cells derived from Tg-ERG mice, which express ERG under the control of the vav promoter. Vav-ERG mice develop AMKL with high penetrance and short latency (L.G., Y.B, and S.I, unpublished data).

Figure S2: diMF selectively induces megakaryocyte polyploidization and differentiation of primary murine and human cells. (Related to Figure 2)

(A-D) Murine bone marrow or fetal liver cells were cultured in presence of 10ng/ml TPO for 72h to differentiate megakaryocytes. diMF dose dependently increased mean polyploidy (A) and expression of CD41 (B) and CD42 (C) of wild type bone marrow megakaryocytes. (D) diMF dose dependently increased the mean polyploidy of GATA1s knock-in mutant fetal liver megakaryocytes. n=4 animals per group. Mean polyploidy of the CD41+ population is shown. (E) diMF dose dependently increased the mean polyploidy of CD41+, but not CD41- cells derived from human bone marrow mononuclear cells cultured in the presence of 10ng/ml TPO for 5 days. Bar graphs depict mean ± SD of 2 independent experiments. * $p<0.05$, ** $p<0.01$.

Figure S3: Effect of diMF on normal and malignant hematopoiesis. (Related to Figure 3)

(A-E) diMF is well tolerated in vivo. C57Bl/6 mice were given vehicle or diMF twice a day for 7 days by oral gavage. The animals were sacrificed on day 14. diMF did not significantly affect mouse body weight (A). (B-E) Furthermore, diMF did not alter the numbers of platelets (B), red blood cells (C), neutrophils (D), or lymphocytes (E) in peripheral blood. (F-K) diMF reduces tumor burden of mice transplanted with 6133/MPL cells. Forty-eight hours after transplantation of 6133/MPL cells, mice were given vehicle or diMF at 66 mg/kg by oral gavage twice a day for 3 days. diMF induced CD41 expression (F) and apoptosis (G) of transplanted 6133/MPL cells in bone marrow in vivo. (H-K) diMF reduced GPF positive cells in peripheral blood (H), spleen (I), liver (J) and bone marrow (K) of recipient mice. Line graphs and bar graphs depict mean ± SD of 2 independent experiments. n=3 animals per group. * $p<0.05$.

Figure S4: Identification of hits from RNAi screen. (Related to Figure 4)

(A) shRNAs targeting 775 human genes, 650 of which are kinases, were screened for their effect on DNA content of CMK cells in comparison to vector. Five to ten shRNAs were screened for each gene. To identify the hits, three types of comparisons were performed, including the significant increase of polyploidy with DMSO, the significant increase of polyploidy with 1μM diMF and the significant difference between these two conditions. Data were analyzed using weighted-sum metric of the GENE-E software, which requires at least two scoring shRNAs. Genes with p-value <0.05 were considered to be hits. (B) Rank order of shRNAs according to their effect on DNA content of CMK cells either in the presence of DMSO or 1 μM diMF. The percentages of cells with DNA content of 8N or greater are shown. Only a small fraction of shRNAs caused a significant increase of DNA content. Genes targeted by these shRNAs may be targets of diMF.

Figure S5: Aurora kinase inhibitors induce polyploidization, expression of differentiation markers, proliferation arrest and apoptosis of mouse megakaryocytic cell line and primary megakaryocytes. (**Related to Figure 5)**

(A-D) MLN8237 and AZD1152-HQPA dose-dependently increased mean polyploidy (A), apoptosis (B) and expression of CD41 and CD42 (C,D) cell surface markers in murine 6133/MPL cells 48h after treatment. (E-G) Murine bone marrow cells were cultured in the presence of 10ng/ml TPO for 72h to differentiate megakaryocytes. MLN8237 induced expression of CD41 (E) and CD42 (F) and polyploidization of CD41+ megakaryocytes (G) in murine bone marrow. (H) MLN8237 dose dependently increased the mean polyploidy of CD41+, but not CD41- cells derived from human CD34+ cells cultured in the presence of 10ng/ml TPO for 8 days. Bar graphs and line graphs depict mean ± SD of 2 independent experiments conducted in triplicate. * $p<0.05$, ** $p<0.01$.

Figure S6: MLN8237 is well tolerated in vivo in wild-type mice. (**Related to Figure 6)**

C57Bl/6 mice were given water, arginine or MLN8237 twice a day for 2 weeks by oral gavage and various parameters were measured. (A) diMF did not significantly affect mouse body weight. (B-E) diMF did not alter the numbers of platelets (B), red blood cells (C), neutrophils (D), or lymphocytes (E) in peripheral blood. Data are representative of 2 independent experiments and shown as mean ± SD. N=4 animals per group.

EXTENDED EXPERIMENTAL PROCEDURES

Compounds

JAK3 inhibitor VI, latrunculin B, K252A, PLK1 inhibitor, CDK1 inhibitor, CDK2 inhibitor and SU6656 were purchased from EMD Chemicals (Gibbstown, NJ). diMF, MLN8237 and AZD1152 and AZD 1152-HQPA were prepared according to literature methods and characterization by ^{1}H NMR (and optical rotation for diMF) was consistent with literature reports.

Cell culture

CMK, CMS, CHRF, K562, Meg-01, Y-10, 6133/MPL and tg-ERG cell lines were grown in RPMI Media 1640 supplemented with 10% FBS and antibiotics (100 mg/ml penicillin/streptomycin mix). G1ME cells were grown in α -MEM media supplemented with 20% FBS and 1% TPO conditioned medium as described previously (Stachura et al., 2006). To culture murine primary megakaryocytes, progenitor cells were enriched from bone marrow of untreated mice by negative selection using a progenitor cell enrichment kit (Stem Cell Technologies). Megakaryocytes were grown in a serum-free system as described previously (Huang et al., 2007).

Human primary mononuclear cell and CD34+ cells were purchased from AllCells, LLC (Emeryville, CA). Both human primary mononuclear cell and CD34+ cells were cultured in serum-free medium in the presence of recombinant 10ng/mL human TPO. Ingredients used to prepare the serum-free medium were as previously described (Gilles et al., 2009). Human CD41+ non-DS AMKL blasts from primary NSG recipients were purified on a BD Aria III cell sorter (BD Biosciences) and cultured in RPMI1640 supplemented with 20% FBS, 10ng/ml hIL3, 10ng/ml hIL6, 10ng/ml hSCF, 10ng/ml hTPO, 10ng/ml hEPO, 10ng/ml hIL11. The cultured cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2.

Cell-based high-content chemical screening

For screening, 4,000 CMK cells per well were seeded into black 384-well optical-bottom plates (Nunc, Rochester, NY) at 50 μl per well using an automated plate filler (MicroFill, Bio-Tek; Winsooki, VT). Fifty nL of each compound was pin-transferred in duplicate into each well with a steel pin array, using the CyBi-Well robot (CyBio, Woburn, MA). Cells were cultured at 37 °C for 72 hours. Cells were fixed and stained with 1 μg/ml Hoechst 33342 (Invitrogen) in 3.7% formaldehyde in phosphate buffered saline (PBS) for 30 minutes at room temperature followed by a wash with 50 μl PBS and replenishment of 50 μl PBS into each well. A robot (CRS; Thermo, Ontario, Canada) was used to feed the prepared plates into ImageXpress Micro (Molecular Devices), where cells were imaged with a 20X objective at 9 sites within each well. CellProfiler was employed to correct illumination anomalies in the images, identify isolated nuclei, and measure the integrated intensity of the DNA stain within each nucleus (Carpenter et al., 2006).

For each plate, the nuclear intensity corresponding to 2N was determined from the peak of the nuclear intensity distribution of cells in the DMSO wells. Different values for an intensity cutoff were explored and it was found that using an intensity cutoff of 2N multiplied by $2^{1.5}$ was optimal. This cutoff (midway between the 4N and 8N peaks) allows inclusion of the full 8N peak. Binomial models under-estimated the variance in the data by approximately an order of magnitude, suggesting that inter-well variability was the dominant source of variance. Variance estimates were developed using a beta-binomial fit to the distribution of the DMSO control wells on each plate. These data were analyzed using R scripts and a compound was scored as a hit if the fraction of nuclear DNA content greater than the cutoff for the compound was significantly greater than that induced by DMSO ($p<0.001$ calculated relative to the beta-binomial model). Confirmatory assays were conducted using 8 concentrations (0.16, 0.31, 0.63, 1.25, 2.5, 5, 10 and 20μM) for each hit compound under the same imaging and data analysis conditions.

Fluorescence-activated cell sorting

Cells were fixed in 2% paraformaldehyde at room temperature for 10 minutes and stained with 4, 6-diamidino-2-phenylindole (DAPI; Sigma; St Louis, MO)/saponin solution containing 0.1% saponin and 1 μg/mL DAPI (freshly prepared). Surface marker staining for human or mouse CD41 (GPIIb; BD Pharmingen, San Diego, CA) and CD42b (GPIb; BD Pharmingen or Emfret Analytics, Wurzburg, Germany) was performed by incubation for 30 minutes in Ca^{2+} free, Mg^{2+} free phosphate-buffered saline (PBS). Other mouse surface makers, such Ter119 (BD Pharmingen), CD3 (BD Pharmingen), CD19 (BD Pharmingen), and Gr-1 and Mac1 (BD Pharmingen) were stained in a similar fashion. For analysis of phosphorylation of histone H3, the cell was fixed with 2% paraformaldehyde and permeablized with 90% methanol before incubation with anti-phospho Histone H3 antibody (Cell signaling). For annexin V staining, cells were incubated with an annexin V antibody (BioVision) in staining buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) for 15 min. DNA content, surface marker expression, apoptosis and phosphorylation of Histone H3 were determined using flow cytometry on an LSR II (BD Biosciences). Data were analyzed using FlowJo software (Treestar, Ashland, OR).

Western blot

Cells were lysed in RIPA buffer (Tris-HCl, 50 mM, pH 7.4; NP-40, 1%; Na-deoxycholate, 0.25%; NaCl, 150 mM; EDTA, 1 mM) supplemented with protease inhibitors (pepstatin, leupeptin, aprotinin, each 10 μ g/ml; PMSF, 1 mM) and phosphatase inhibitor (Na3Vo4, 1 mM). Protein was separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blotted with antibodies detecting phospho-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198) (Cell Signaling Catalouge No. 2914) and HSC70 (Santa Cruz, CA).

Retroviral transduction

For knockdown of RPS6KA4 and MYLK2 in CMK cells, cells were infected with luciferase lentivirus (PLKO1 luciferase; Broad Institute, RNAi platform; clone ID, TRCN0000072261) or a virus that contained an shRNA against RPS6KA4 (PLKO1 RPS6KA4; Broad Institute, RNAi Platform; clone ID, TRCN0000199015) or MYLK2 (PLKO1 MYLK2; Broad Institute, RNAi Platform; clone ID, TRCN0000219654).

For knockdown of Aurkb in 6133/MPL cells, cells were infected with control vector lentivirus (PLKO1; Open Biosystems) or a virus that contained a shRNA against Aurkb (PLKO1 Aurkb; Open Biosystems; clone ID, TRCN0000028774 and TRCN0000028756).

Both lentiviruses for transduction of CMK cells and 6133/MPL cells contain a puromycin resistant cassette. The infected cells were selected in 2 μg/ml puromycin for 48 hours and cultured in RPMI Media 1640 supplemented with 10% FBS and antibiotics (100 mg/ml penicillin/streptomycin mix).

Pharmacokinetic study

The pharmacokinetic studies of diMF and MLN8237 on C57Bl/6 mice were approved by the Institutional Animal Care and Use Committee of National University of Singapore. The mice (n=3 mice for each time point) were given a single dose of diMF at dose of 66 mg/kg or MLN8237 at a dose of 15 mg/kg through oral gavage. Serial blood samples were collected before dosing and at different time points post-dose via retro-orbital vein or by cardiac puncture immediately after sacrifice. After centrifuging at 3000×g (4 °C) for 8 min, the plasma was collected and stored at −80 °C. The plasma concentrations of diMF and MLN8237 were determined by validated LC-MS/MS assays based on FDA guidelines. The LC-MS/MS system consisted of an Agilent 1100 binary pump connected to an API 4000 triple-quadrupole mass spectrometer. Chromatographic separations were achieved on an Alltima C18 column (150 mm×2.1 mm, 5μ) for diMF and a ZORBAX Eclipse XDB-C8 column (50 mm×2.1 mm, 5μ) for

MLN8237. Mobile phases comprised 0.1% formic acid and acetonitrile with gradient elution mode. The pharmacokinetic parameters were calculated using mean concentrations from pooled data at each time point and analyzed using non-compartmental analysis. Good linearity ($r^2 \geqslant 0.999$) was achieved within the range of 1-400 nmol/L for diMF and 5-2000 nmol/L for MLN8237. Pharmacokinetic calculations were performed using WinNonLin version 6.1.

Animal experiments

Animal tumor studies were approved by the Animal Care and Use Committee of Northwestern University and Institut Gustave Roussy Institutional Committee. Unless otherwise specified, all the mice used in this study were six- to 8-week-old C57Bl/6 female mice. For drug treatment of non-transplanted mice, vehicle or test compound was fed to mice by oral gavage twice a day for 7 or 14 days. Mice were monitored 4 times a week for welfare and health status. Mice were sacrificed on day 14 after initiation of treatment to evaluate the effect of compounds. For the drug pretreatment experiment, 6133/MPL cells were treated with vehicle or compound for 24h. Live cells were separated by ficoll-hypaque density gradient centrifugation(Bain and Pshyk, 1972). Recipient mice were sub-lethally irradiated at 600 cGy, and then 10^6 live 6133/MPL cells in a volume of 200 μl in sterile Hank's Balanced Saline Solution were injected into the tail vein of each mouse. Mice were monitored 4 times a week after transplantation for the development of leukemia. Symptoms used to assess the presence of leukemia included body weight loss of $\geqslant$ 20%, scruffy hair, hunched posture and reduced mobility. For drug treatment after transplantation, a million 6133/MPL cells were transplanted into sub-lethally irradiated (600 cGy) mice. Forty-eight hours later, vehicle or compound was fed to mice by oral gavage twice a day. For drug treatment of mice transplanted with human AMKL blasts, bone marrow cells from an non DS-AMKL patient were injected into sub-lethally irradiated primary NSG (NOD/LtSz-scid IL2Rgc null) recipients at 300cGy. Twelve weeks after transplantation, bone marrow cells were

collected from primary NSG recipients and injected into sub-lethally irradiated secondary recipients that were then used for the in vivo drug treatment five weeks after transplantation. Prior to treatment initiation, animals were randomized into 3 groups of 7 animals. Then treatment was performed with vehicle, 30 or 60 mg/kg of diMF by oral gavage twice a day for 10 days and analyzed at the end of the treatment.

ROCK1 knockout mice were obtained from Dr. Reuben Kapur of Indiana University (Vemula et al., 2010). Aurora kinase A conditional knockout (flox/flox) mice were supplied by Dr. Terry Van Dyke of University of North Carolina at Chapel Hill (Cowley et al., 2009).

Complete blood counts

Blood (50 μL) was collected from the tail vein in EDTA-coated tubes and analyzed by a Hemavet 850 complete blood counter (Oxford, CT).

CFU assay

Colony forming unit megakaryocyte (CFU-MK) assays were performed on blasts from AMKL patients with Down syndrome. Ten thousand bone marrow mononuclear cells were seeded in Megacult-C medium supplemented with 10ng/ml interleukin-3 (IL-3), 10ng/ml of interleukin-6 (IL-6), and 50 ng/ml of thrombopoietin (TPO), and cultured for 10-12 days. Slides of Megacult cultures were fixed with a mixture of acetone and methanol and stained with anti-human CD41 antibody according to the manufacturer's instructions (StemCell Technologies). CD41 antibody stained colonies were then enumerated microscopically.

Ambit KinomeScan

The Ambit KinomeScan was performed by Ambit Bioscience (San Diego, CA). For most assays, kinase-tagged T7 phage strains were grown in parallel in 24-well blocks in an E. coli host derived from the BL21 strain. E. coli were grown to log-phase and infected with T7 phage from a

frozen stock (multiplicity of infection = 0.4) and incubated with shaking at 32° C until lysis (90-150 minutes). The lysates were centrifuged (6,000 x g) and filtered (0.2 μm) to remove cell debris. The remaining kinases were produced in HEK-293 cells and subsequently tagged with DNA for qPCR detection. Streptavidin-coated magnetic beads were treated with biotinylated small molecule ligands for 30 minutes at room temperature to generate affinity resins for kinase assays. The liganded beads were blocked with excess biotin and washed with blocking buffer (SeaBlock (Pierce), 1 % BSA, 0.05 % Tween 20, 1 mM DTT) to remove unbound ligand and to reduce non-specific phage binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1x binding buffer (20 % SeaBlock, 0.17x PBS, 0.05 % Tween 20, 6 mM DTT). Test compounds were prepared as 40x stocks in 100% DMSO and directly diluted into the assay. All reactions were performed in polypropylene 384-well plates in a final volume of 0.04 ml. The assay plates were incubated at room temperature with shaking for 1 hour and the affinity beads were washed with wash buffer (1x PBS, 0.05 % Tween 20). The beads were then re-suspended in elution buffer (1x PBS, 0.05 % Tween 20, 0.5 μm non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 minutes. The kinase concentration in the eluates was measured by qPCR. diMF and fasudil were screened at 5μM, and results for primary screen binding interactions are reported as '% Ctrl', where lower numbers indicate stronger hits.

%Ctrl Calculation

$$\left(\frac{\text{Test compound signal - positive control signal}}{\text{Negative control signal - positive control signal}} \right) \text{X } 100$$

test compound = diMF or fasudil

negative control = DMSO (100%Ctrl)

positive control = control compound (0%Ctrl)

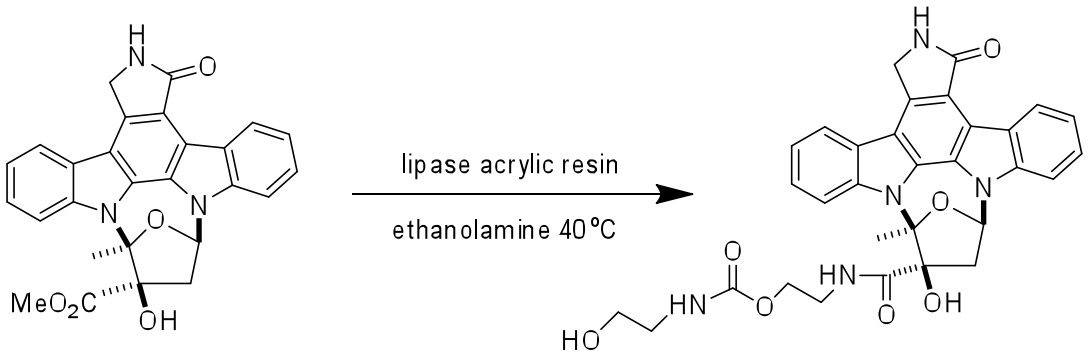
Affinity enrichment using SILAC (Stable isotope labeled amino acid in cell culture) mediated quantitative proteomics

Preparation of affinity matrices

All chemicals were purchased from Sigma-Aldrich and common solvents were purchased from Fisher (J.T.Baker) at HPLC grade unless otherwise noted. Automated flash chromatography was performed on Teledyne ISCO CombiFlashRf systems. LC and mass spectra were collected on either a WATERSMicromass ZQ or a WATERS Alliance 3100 system. 1H and 13C-NMR spectra were collected on a Bruker 300 MHz NMR spectrometer.

Abbreviations: CDI, *N,N'*-carbonyl dimidazole; DMAP, 4-Dimethylaminopyridine; DMF, *N,N'*-dimethylforamide; DSC, di(*N*succimidyl)carbonate; EDC, 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimidehydrochloride; EtOAc, ethyl acetate; PBS, phosphate buffered saline; TEA,triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

Generation of the (+)-K252a alcohol derivative, (*)



The starting material (+)-K252a (*) was purchased from BioMol International L.P. (catalog#: EI152)

(+)-K252a (25.0 mg, 0.043 mmol, 1.0 eq.) and lipase acrylic resin (66 mg) from

Candida antarctica (Sigma cat# L4777) were mixed in ethanolamine (4.0 mL,1545 eq.) at 40 ºC under nitrogen. The reaction was stirred overnight, filtered, washed with MeOH, concentrated and taken up with saturated NH4Cl, and then extracted with EtOAc. The organic layer was dried over Na2SO4, concentrated and purified by automated flash chromatography to afford the pure product (19.5mg, 92 %). 1H NMR (300 MHz, THF-*d8*) 1H NMR (300 MHz, THF) δ 8.46 (d, 1H, *J* = 7.9), 7.11 (m, 2H), 6.78 (d, 1H, *J* = 8.2), 6.53 (t, 2H, *J* = 7.7), 6.42 (t, 1H, *J* = 7.4), 6.33 (t, 1H, *J* = 7.5), 6.17 (dd, 1H, *J* = 4.8, 7.3), 4.64 (s, 4H), 4.08 (q, 2H, *J* = 17.2), 2.81 (d, 2H, *J* = 5.0), 2.55 (m, 2H), 2.38 (s, 1H), 1.35 (s, 3H); 13C NMR 19 (300 MHz, CDCl3) 171.19, 170.90, 139.57, 136.43, 131.50, 128.09, 125.59, 124.32, 123.47, 122.44, 119.79, 115.86, 113.85, 106.8, 99.31, 84.44, 84.40, 64.88, 46.90, 44.61, 41.86, 23.64; MS (calcd. 496.1747, ES+): 497.1027 $(M+H)^+$.

Solid-phase (bead) preparation: The solid-phase beads used were Affigel 102 (Bio-Rad) with a loading level of 12 μmol/mL suspension. The bead suspension (1.0mL) was transferred to a 2.0 mL eppendorf tube and washed with H2O (3 x 1.5mL) and DMF (3 x 1.5 mL). The beads were then suspended in anhydrous DMF(0.5 mL).

K252a activation: K252a (10 μmol) was dissolved in 200 μL of anhydrous acetonitrile. DSC (7.69 mg, 30 μmol,3 eq.) was dissolved in 400 μL of anhydrous acetonitrile and was added to the K252a solution before TEA (5.55 μL, 4.05 mg, 40 μmol, 4 eq.) was added. The reaction solution was stirred at 50 ºC for 1 h and the activation efficiency was monitored by LC-MS.

K252a immobilization: LC-MS indicated 85% of K252a molecules were activated so for 12.5% loading level, 106.8 μL of the activation solution were added to the beads. After adding the activation solution, the suspension was vortexed at room temperature for 1 h and the depletion of free activated bait molecule was monitored by LC-MS. After the immobilization, the vials were centrifuged, the supernatant was removed and the beads were washed with DMF (3 x 2 mL)and

H2O (3 x 2mL). The beads were subsequently suspended in 1x PBS (0.8 mL) and stored at4 ºC before use.

SILAC media preparation and cell culture conditions

We followed all standard SILAC media preparation and labeling steps as previously described (Ong and Mann, 2006) with the addition of light proline to prevent the conversion of arginine to proline (Bendall et al., 2008). Briefly, 30 mg/L of L-methionine and 200mg/L of L-Proline were added to base media according to standard formulations for RPMI. This base media was divided into two and either 'light' forms of arginine and lysine or 'heavy' L-arginine-U-13C6 (28 mg/L) and L-lysine-13C615N2 (146mg/L) was added to generate the two SILAC labeling mediums. Each medium with the full complement of amino acids was sterile filtered through a 0.22 μ filter (Milipore). CMK cells were grown in RPMI labeling media, prepared as described above, supplemented with 2mM L-glutamine, and 5% dialyzed fetal bovine serum plus antibiotics, in a humidified atmosphere with 5% CO2 in air. Cells were grown for at least six cell divisions in labeling media.

Biochemical purification with small molecule affinity matrices

Separate cultures of CMK cells SILAC labeled either with L-arginine and L-lysine (light) or L-arginine-13C6 and L-lysine-13C6-15N2 (heavy) were lysed in ice-chilled ModRIPA buffer containing 1% NP-40, 0.1% Na deoxycholate, 150mM NaCl, 1mM EDTA, 50mM Tris, pH 7.5, and protease inhibitors (CompleteTM tablets, RocheApplied Science, Indianapolis, IN). Lysates were vortexed intermittently while chilled on ice for 10 min and clarified by spinning at 14,000 x g. Protein concentrations of light and heavy lysates were estimated with the Protein Assay Dye Reagent Concentrate (Biorad, Hercules CA) and equalized. The protein concentrations of lysates varied between 1.7 to 2.2 mg/mL, affinity enrichments were performed in lysate volumes

of 1.4 mL in a 1.5 mL microcentrifuge tube. diMF (in DMSO) at 10 and 50-fold excess over the amount of K252a on beads was added to 2mg of light lysate. An equal volume of DMSO was then added to 2 mg of heavy lysate as a control. Thirty microliters of a 50% slurry in phosphate buffered saline (PBS) of K252a-bead was added to both light and heavy lysates. Affinity enrichments were incubated overnight (approx. 16 hr) on an end-over-end rotator at 4° C. Following incubation, the tubes were spun at 1000 g on a benchtop centrifuge to pellet the beads. The supernatant was aspirated, taking care to avoid disturbing the beads. Each tube in a set was washed with ModRIPA buffer twice to remove excess soluble small molecule competitor. Beads from the two tubes were then combined for an extra washing step in ModRIPA. After the third and final wash, beads were collected by spinning at 1000 x g and the wash aspirated leaving approximately 20 μL of buffer in the tube.

1D-SDS-PAGE and MS analysis

Proteins enriched in SILAC affinity pull-downs were reduced and alkylated, on bead, in 2mM DTT and 10mM iodoacetamide. One part LDS buffer (Invitrogen) was added to three parts sample (including beads) and tubes heated to 70°C for 10 minutes. Proteins were resolved on a 4-12% gradient 1.5 mm thick Bis-Tris gel with MES running buffer (Nupage, Invitrogen) and Coomassie stained (Simply Blue, Invitrogen). Gel lanes were excised into six pieces and then further cut into 1.5 mm cubes. The gel pieces were further destained in a solution containing 50% ethanol and 50% 50mM ammonium bicarbonate, then dehydrated in 100% ethanol before addition of sufficient trypsin (12.5 ng/μL) to swell the gel pieces completely. An additional 100μL of 50mM ammonium bicarbonate was added before incubating at 37° C overnight on a thermomixer (Eppendorf). Enzymatic digestion was stopped by the addition of 100μL of 1% TFA to tubes. A second extraction with 300 μL of 0.1% TFA was combined with the first extract and the peptides from each gel slice cleaned up on C18 StageTips (Rappsilber et al., 2007).

Peptides were eluted in 50μL of 80% acetonitrile/0.1% TFA and dried down in an evaporative centrifuge to remove organic solvents. The peptides were then resuspended by vortexing in 7μL of 0.1% TFA and analyzed by nanoflow-LCMS with an Agilent 1100 with autosampler and a LTQOrbitrap. Peptides were resolved on a 10 cm column, made in-house by packing a self pulled 75 μm I.D. capillary, 15 μ m tip (P-2000 laser based puller, Sutter Instruments) column with 3μm Reprosil-C18-AQ beads (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) with an analytical flow rate of 200nL/min and a 58min linear gradient (~ 0.57 %B/min) from 0.1% formic acid in water to 0.1% formic acid/90% acetonitrile. The run time was 108 min for a single sample, including sample loading and column reconditioning. We used a MS method with a master Orbitrap full scan (60,000 resolution) and data dependent LTQ MS/MS scans for the top five precursors (excluding z=1) from the Orbitrap scan. Each cycle was approximately 2 secs long. MS raw files were processed for protein identification and quantitation using MaxQuant, an open source academic software MSQuant (CEBI, open-source http://maxquant.org) IPI human ver.7.0 (http://ebi.ac.uk) with a concatenated decoy database containing randomized sequences from the same database. Common contaminants like bovine serum albumin and trypsin were also added to the database. Variable modifications used were oxidized methionine, arginine-13C6, lysine-13C6 15N2, and carbamidomethyl-cysteine was a fixed modification. The precursor mass tolerance used in the search was 7 ppm and fragment mass tolerance was 0.5 Da. Only proteins with a minimum of two quantifiable peptides were included in our dataset.

Statistical analysis of SILAC experiments

To model log2 protein ratio values, we adapted the empirical Bayes framework developed by Efron (Rappsilber et al., 2007) to compute the posterior probability that a ratio value arises from the null distribution. Briefly, by application of Bayes' theorem, this quantity is computed as $Pr(Z = 0 \mid X) = [Pr(X \mid Z = 0)Pr(Z = 0)]/Pr(X)$, where Z is a binary variable taking the value of one if the

protein is bound by the compound, and X is a measured log2 SILAC ratio. We model Pr(X) using a Gaussian kernel estimator with pareto distributions fit to 5% of the data at either tail, to avoid unreliable estimation in regions of data sparcity. The distribution for $Pr(X \mid Z = 0)Pr(Z = 0)$ is then inferred by fitting a Gaussian distribution using only the portion of Pr(X) arising from the central two thirds of the data.

RNAi screen

For RNAi screen, 1,000 CMK cells per well were seeded on day 1 similarly as described in the chemical screen. Puromycin (3μg/ml) selection was initiated the next day and continued for 3 days. On day 4, DMSO or 1μM diMF was added to each well. On day 7, the images were acquired by ImageXpress Micro and analyzed by CellProfiler for nuclear intensity and as done in the chemical screen. These data were further analyzed using R scripts and an shRNA was scored as a hit if the fraction of nuclear DNA content greater than the cutoff for the shRNA was significantly greater than that induced by vector DNA ($p<0.001$ calculated relative to the beta-binomial model) in presence of DMSO or 1μM diMF. A comparison was also made between these two conditions. To reduce the off-target effect of shRNA, a gene is determined as a hit only when two or more shRNAs of the gene score. Genes with a p-value < 0.05, using the Weighted-Sum metric in the GENE-E analysis, in any one of three category of comparison was considered to be hits. In total, we screened 775 genes, 650 of which are kinases, with 5-10 shRNAs for each gene.

Protein network analysis using Reactome

We have two different RNAi experimental designs producing three different p-values – the first one for shRNA alone, the second for shRNA with sub-maximal concentration of dimethyl fasudil, and the third for the difference between the first two modes. Since any of these modes can indicate a potential target, we combined them by taking the smallest of the three scores. For log

SILAC ratios, we computed mean μ = -0.0080979 and standard deviation σ = 1.3229, which allowed us to compute z-scores, $z = (x - \mu)/\sigma$, and p-values of z-scores using the inverse normal cumulative distribution function. We computed p-values for Kinome profiling data in a similar manner assuming half-normal distribution with mean μ = 100 and σ = 54.94. The combined p-value score was computed as geometric average of RNAi, SILAC, and Kinome profile p-values with p-value = 1 used in case of absent measurements.

To assist with the interpretation of 'hits' we turned to network analysis using the protein-protein interaction database Reactome (Vastrik et al., 2007). The decision of which proteins to include was made for each component (SILAC, RNAi, Kinome) separately. Proteins detected by SILAC were analyzed using an empirical Bayesian method (Margolin et al., 2009) and we included those with false discovery rates below 0.05. For RNAi, we included genes with p-value below 0.05 in any of the three modes (shRNA alone, shRNA with sub-maximal dose of diMF, and difference between the two). For kinases profiled by Ambit KinomeScan we included those that had % activations reduced by diMF below 35%. The three approaches yielded 117 proteins that were mapped to 116 nodes and 194 connections in the Reactome database (Figure 7). We used random graphs with given expected degrees (Pradines et al., 2005) to assess the statistical significance and obtained a p-value $7.1X10^{-64}$.

Molecular docking study

The ligands diMF and MLN8237 are subjected to LigPrep module implemented in Schrodinger software which converts 2D structures to 3D and uses the ionization engine to protonate and de-protonate at pH =7.0 ± 1. The appropriate stereoisomers/tautomers have been generated along with the low energetic conformers for all the ligands.

The crystal structures 3P9J, 1OL7 and 3D9V are imported from pdb databank. The protein preparation module has been utilized to fix up the hydrogens (in the hydrogen bonding orientation to all the residues), bond orders, charges, fill in missing side chain atoms, and fill in missing loop using Prime module and cap termini. Then the waters beyond 5å from the bound ligands have been deleted from the active site pockets. The first step in ligand docking is to generate the receptor grid. A grid box of 10å has been generated considering the centroids of the bound ligands with a van der Waals scaling factor of 1.0 and partial charge cutoff of 0.25 for the residues in the active site pockets. No other constraints have been used to define the grids to allow the flexible docking of the ligands. The ligand docking has been performed using the Glide XP (extra precision) option for all the ligands allowing nitrogen inversion and ring flexibility. The scaling factor for the van der Waals atomic radii for the ligands has been fixed to 0.8 with a partial charge cutoff of 0.15. The docking of all the ligands was performed with the above protocols with some special output options such as the post docking minimization of the ligands, XP descriptors evaluation and strain energy correction of the docked poses of the different ligands in order to eliminate the high energetic docking poses of the ligands. After obtaining the docked poses for various ligands, we computed the binding free energy of the ligands using Molecular Mechanics Generalized Born Surface Area (MMGBSA) approach. Along with the binding energy, the ligand strain energy is also computed. We specified a 5å region of the receptor from the centroid of the ligand to be flexible so that the protein structure will be relaxed in the computation of the binding energy of the ligands.

Aurora A Kinase Assay

The Aurora A kinase assay was performed using a kit from CycLex (Tera-Sawaoka, Japan). The assay plates are pre-coated with a substrate corresponding to recombinant Lats2, which contains a serine residue that can be phosphorylated by Aurora-A. Briefly, DMSO or different concentration of diMF or MLN8237, along with 0.04 Units of Aurora kinase A positive was added

to each well and incubated for 30 min at 30°C. Each well was washed five times with a washing buffer containing 2%Tween-20. The anti-phospho-Lats2-S83 Monoclonal Antibody ST-3B11 was added into each well and incubated at room temperature for 60 minutes. After washing, HRP-conjugated anti-mouse IgG was added into each well and incubated at room temperature for 60 minutes. Color reaction was developed by adding the chromogenic substrate, tetra-methylbenzidine (TMB). After 10 min, the reaction was terminated by adding the stop solution containing 0.5 N H_2SO_4. The absorbance in each well was measured using a spectrophotometric plate reader at 450nm.

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Figure S1

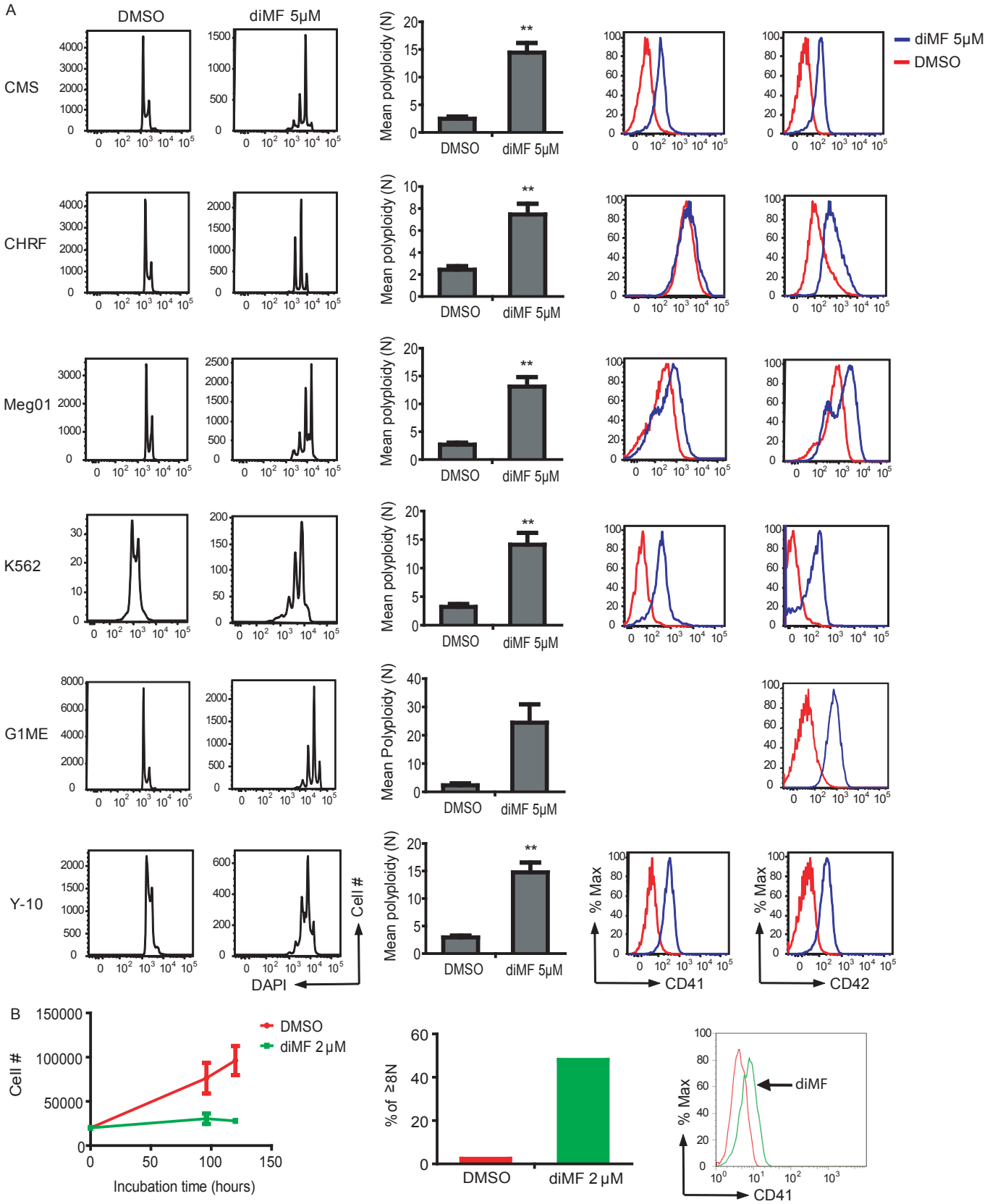

A
DMSO
diMF 5μM
CMS
CHRF
Meg01
K562
G1ME
Y-10
Mean polyploidy (N)
Mean Polyploidy (N)
**
DMSO
diMF 5μM
DAPI
Cell #
% Max
CD41
CD42
diMF 5μM
DMSO
B
Cell #
DMSO
diMF 2 μM
Incubation time (hours)
% of ≥8N
diMF

Figure S2

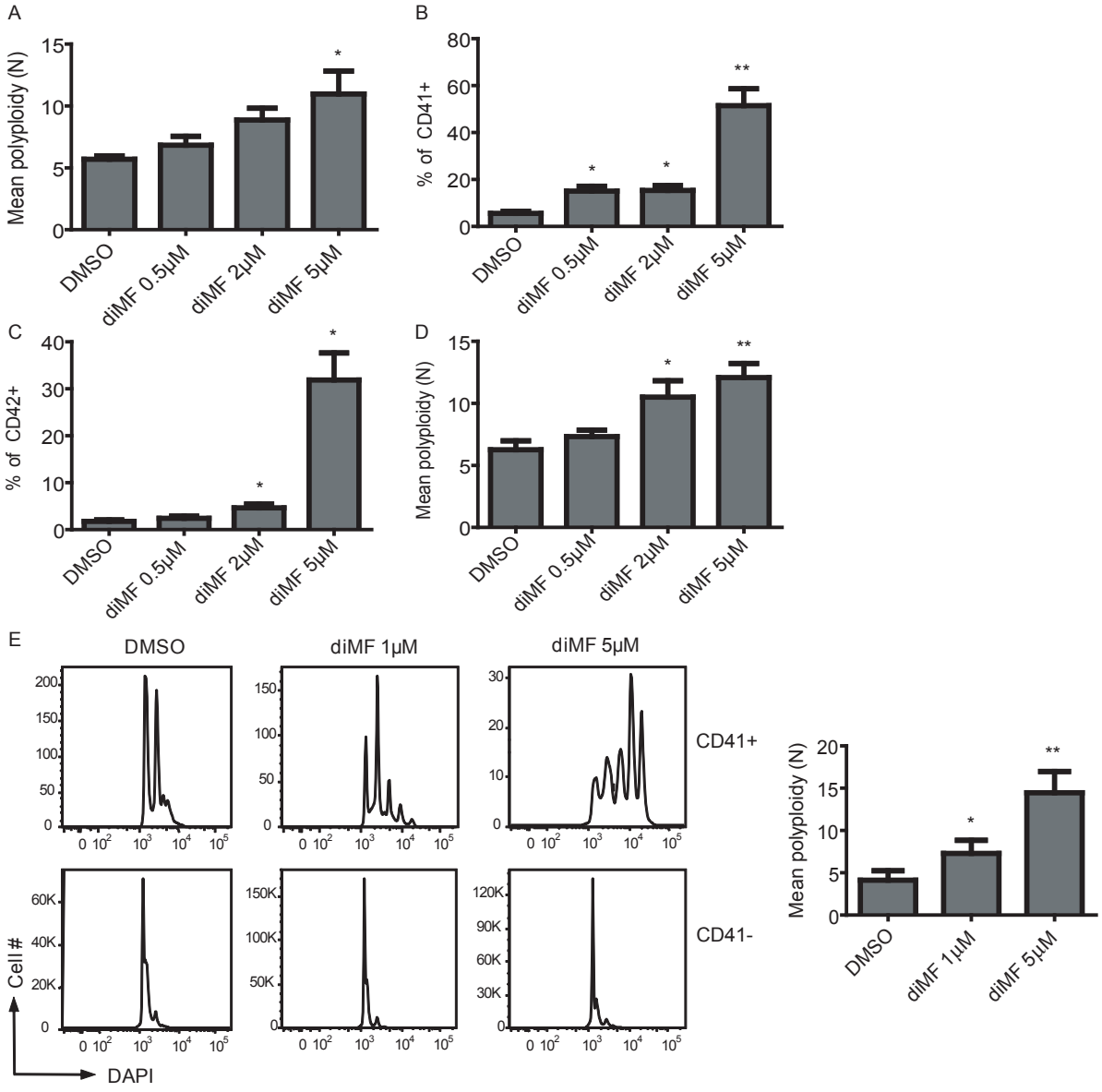

A
Mean polyploidy (N)
DMSO
diMF 0.5μM
diMF 2μM
diMF 5μM
B
% of CD41+
C
% of CD42+
D
Mean polyploidy (N)
E
DMSO
diMF 1μM
diMF 5μM
CD41+
CD41-
Cell #
DAPI
Mean polyploidy (N)

Figure S3

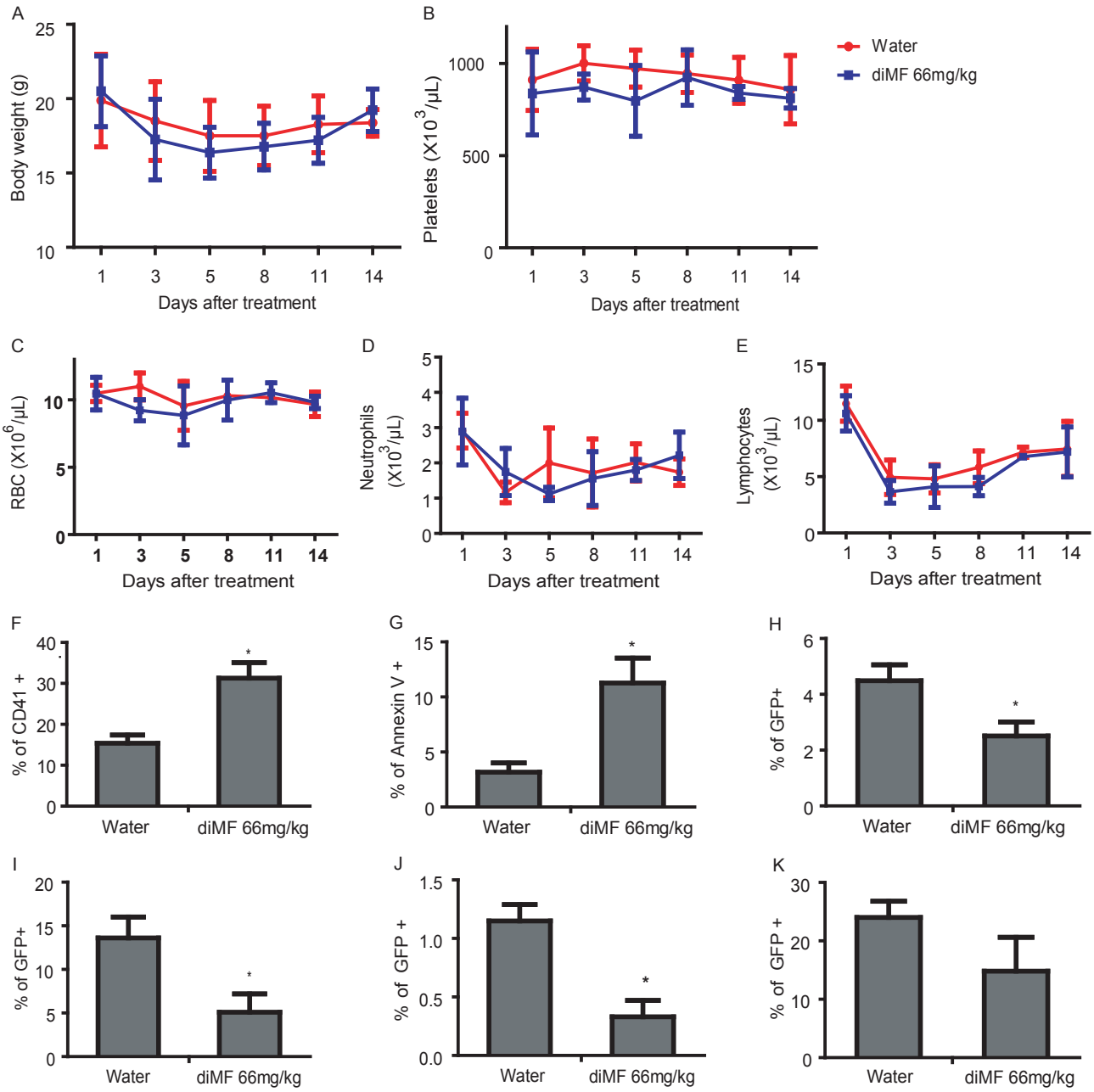

A
Body weight (g)
25
20
15
10
1 3 5 8 11 14
Days after treatment
B
Platelets (X10 3/µL)
1000
500
0
1 3 5 8 11 14
Days after treatment
Water
diMF 66mg/kg
C
RBC (X10 6/µL)
10
5
0
1 3 5 8 11 14
Days after treatment
D
Neutrophils (X10 3/µL)
5
4
3
2
1
0
1 3 5 8 11 14
Days after treatment
E
Lymphocytes (X10 3/µL)
15
10
5
0
1 3 5 8 11 14
Days after treatment
F
% of CD41 +
40
30
20
10
0
*
Water
diMF 66mg/kg
G
% of Annexin V +
15
10
5
0
*
Water
diMF 66mg/kg
H
% of GFP+
6
4
2
0
*
Water
diMF 66mg/kg
I
% of GFP+
20
15
10
5
0
*
Water
diMF 66mg/kg
J
% of GFP +
1.5
1.0
0.5
0.0
*
Water
diMF 66mg/kg
K
% of GFP +
30
20
10
0
Water
diMF 66mg/kg

Figure S4

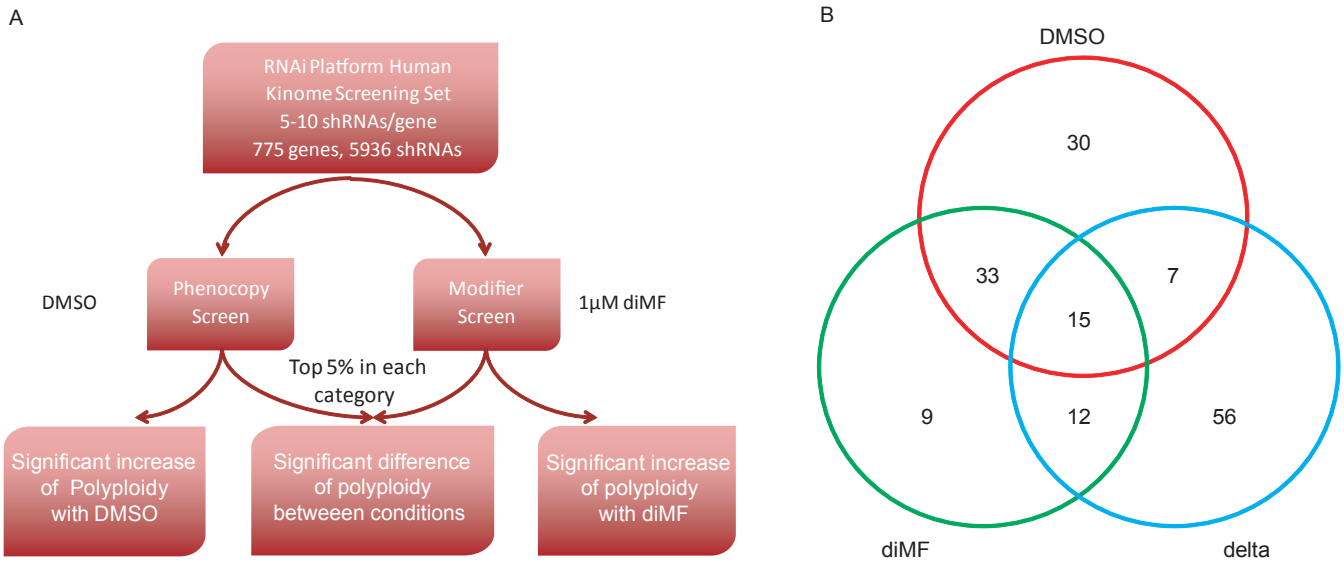

A
RNAi Platform Human Kinome Screening Set
5-10 shRNAs/gene
775 genes, 5936 shRNAs
DMSO
Phenocopy Screen
Modifier Screen
1μM diMF
Top 5% in each category
Significant increase of Polyploidy with DMSO
Significant difference of polyploidy betweeen conditions
Significant increase of polyploidy with diMF
B
DMSO
30
33
7
15
9
12
56
diMF
delta

Figure S5

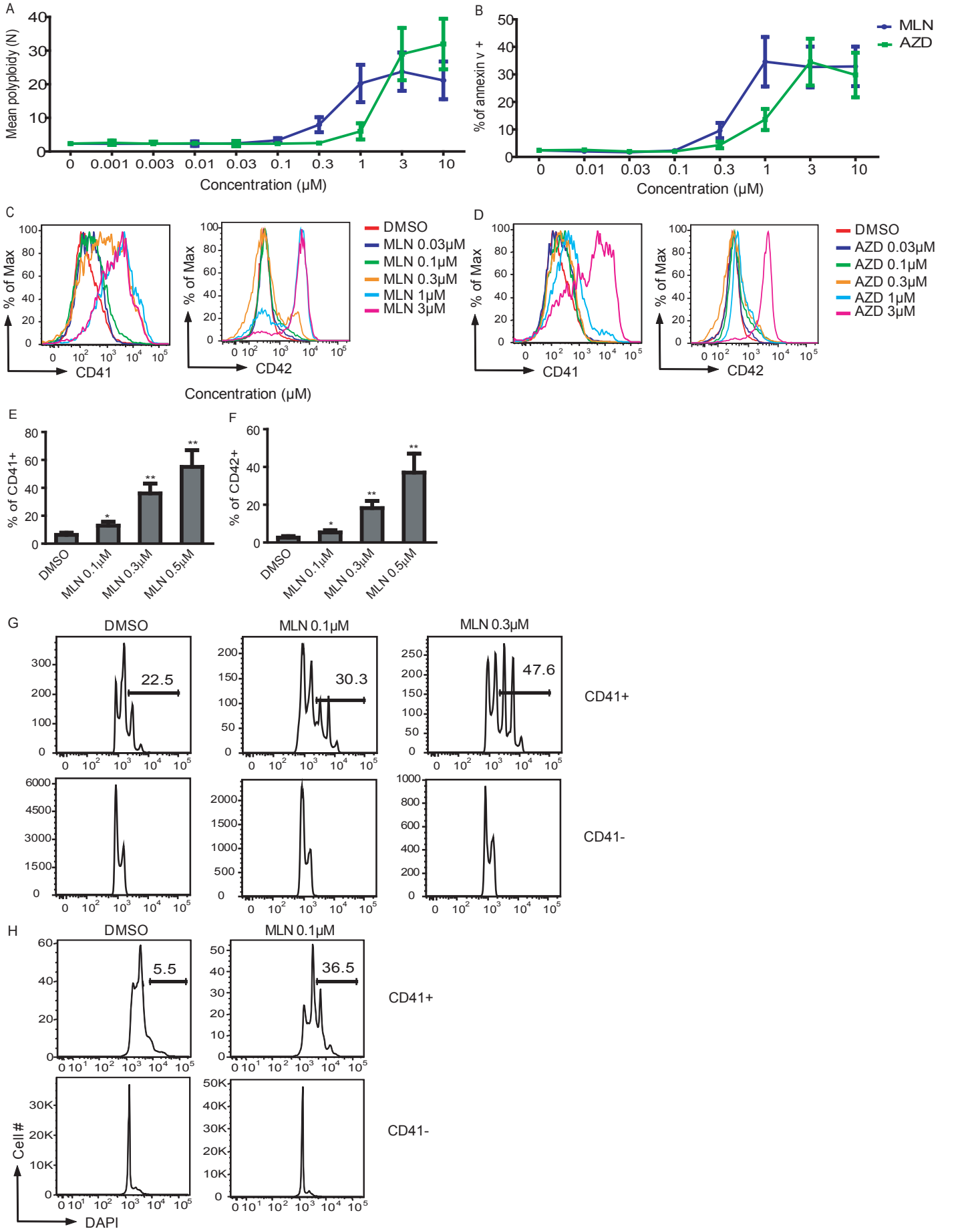
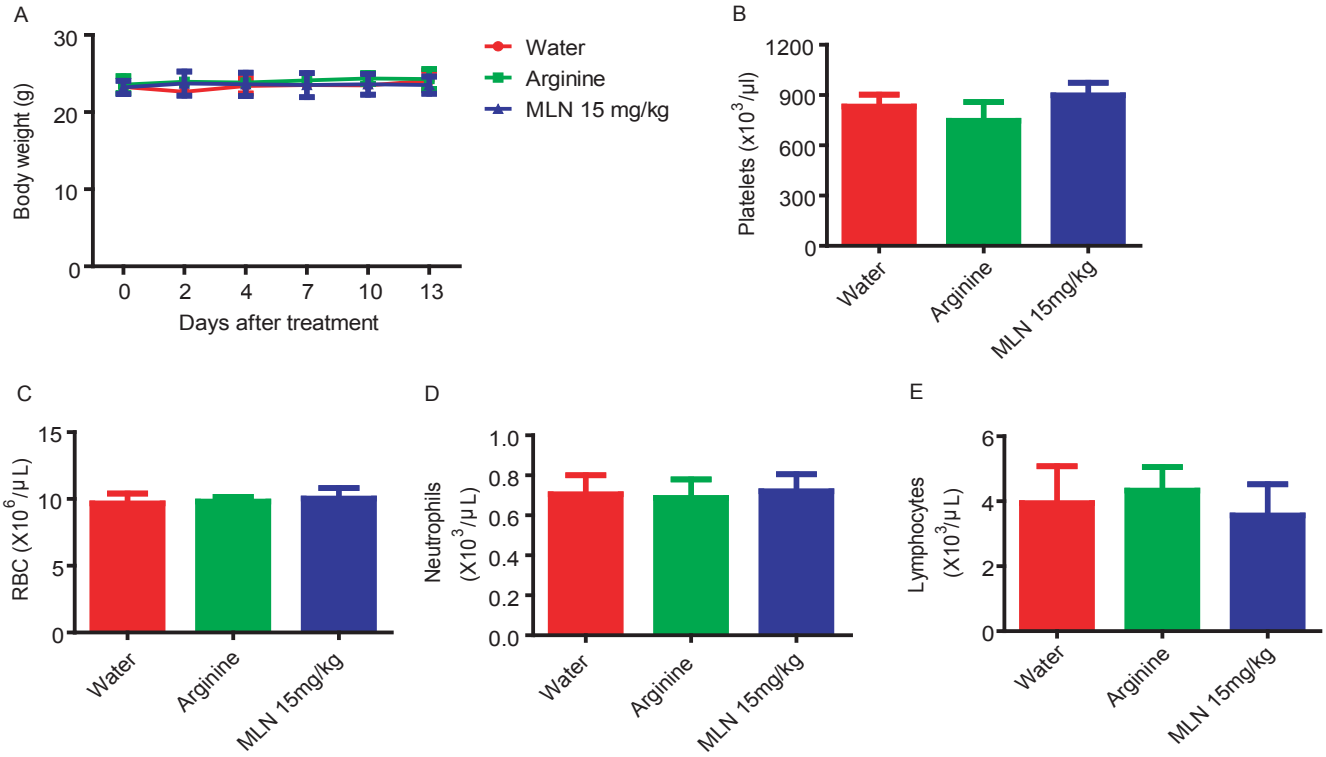

A
Mean polyploidy (N)
Concentration (μM)
B
% of annexin v +
MLN
AZD
Concentration (μM)
C
% of Max
CD41
CD42
DMSO
MLN 0.03μM
MLN 0.1μM
MLN 0.3μM
MLN 1μM
MLN 3μM
D
AZD 0.03μM
AZD 0.1μM
AZD 0.3μM
AZD 1μM
AZD 3μM
E
% of CD41+
F
% of CD42+
DMSO
MLN 0.1μM
MLN 0.3μM
MLN 0.5μM
G
22.5
30.3
47.6
CD41+
CD41-
H
5.5
36.5
Cell #
DAPI

Figure S6


A
Body weight (g)
30
20
10
0
0
2
4
7
10
13
Days after treatment
Water
Arginine
MLN 15 mg/kg
B
Platelets (x10^3/µl)
1200
900
600
300
0
Water
Arginine
MLN 15mg/kg
C
RBC (X10^6/µL)
15
10
5
0
Water
Arginine
MLN 15mg/kg
D
Neutrophils (X10^3/µL)
1.0
0.8
0.6
0.4
0.2
0.0
Water
Arginine
MLN 15mg/kg
E
Lymphocytes (X10^3/µL)
6
4
2
0
Water
Arginine
MLN 15mg/kg