Supplementary Methods

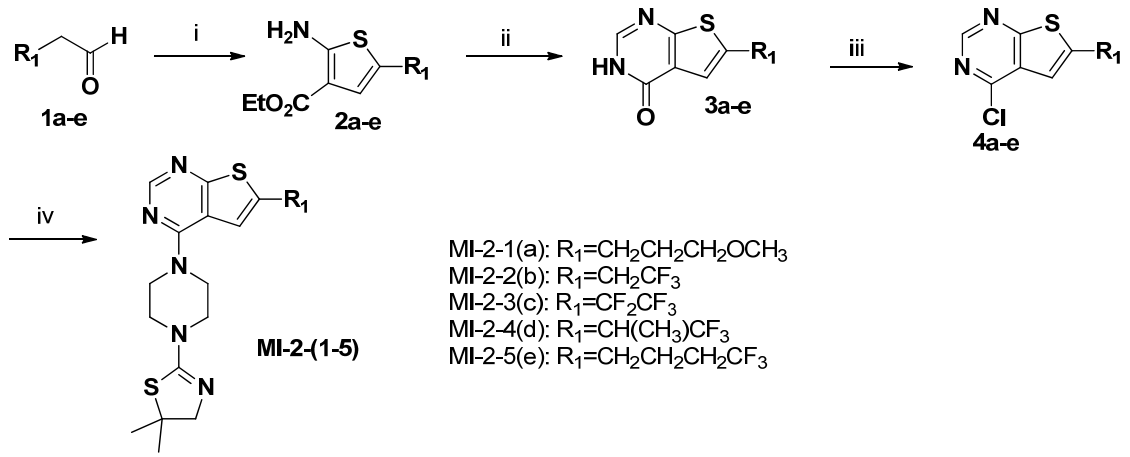
Thermal shift assays

Thermal shift binding experiments were carried out using Thermofluor 384 ELS system. Protein unfolding was examined by monitoring the fluorescence of ANS (1-anilinonaphthalene-8-sulfonic acid) by increasing the temperature from 20 to 60 °C. All samples were prepared in quadruplicates and contained proteins at 5 μM concentrations in 50 mM TRIS, 50 mM NaCl, 1 mM DTT, pH = 7.5; 50 μM ligand (2.5% final concentration of DMSO) and 50 μM ANS. To limit evaporation, the samples were covered with mineral oil.

Chemistry

MI compounds were synthesized in four steps according to our previously developed synthetic route in Scheme 1. Aldehydes **1a-e** were reacted respectively with ethyl cycnoacetate and sulfur in DMF in the presence of triethylamine to yield thiophenes **2a-e**. Cyclization of substituted thiophenes in heated formamide afforded compound **3a-e** in good yields which were then chlorinated to chloro-thienopyrimidines **4a-e** using $POCl_3$. **MI** compounds were obtained by coupling of compound **4a-e** with 5,5-dimethyl-2-(piperazin-1-yl)-4,5-dihydrothiazole in the presence of diisopropylethylamine in THF, which was then converted to their corresponding HCl salts by adding 1 equivalent of HCl in ether.

Scheme 1. Synthesis of Thienopyrimidines from aldehydes[a]



[a]Reagents and conditions: (i) ethyl cyanoacetate, sulfur, DMF, Et_3N 24h; (ii) formamide, 140°C, 24h; (iii) $POCl_3$, reflux, 6h; (iv) 5,5-dimethyl-2-(piperazin-1-yl)-4,5-dihydrothiazole, DIEA, THF, reflux, 12h.

Experimental

General. The NMR spectra were recorded on a Bruker instrument at 600 MHz for ^1H and 150 MHz for ^{13}C spectra or on Varian instrument at 400 MHz for ^1H and 100 MHz for ^{13}C spectra. Chemical shift values are recorded in δ units (ppm). Mass spectra were recorded on a Micromass TofSpec-2E Matrix-Assisted, Laser-Desorption, Time-of-Flight Mass Spectrometer in positive ESI mode unless otherwise noted. TLC was performed on Analtech silica gel HLF plates, 250 micron w/uv254. All reagents were commercially available and used as received.

General procedure for compounds 2a-e

To a solution of ethyl cyanoacetate (1.13 g, 10 mmol) and triethylamine (1.01 g, 10 mmol) in DMF (10 ml), aldehyde (10 mmol) was added, and the mixture was stirred for 10 minutes. Element sulfur (320 mg, 10 mmol) was added to the mixture. After it had stirred for 24 hours,

the suspension was partitioned between ethyl acetate and H_2O. The organic extracts were washed with brine, dried with $MgSO_4$ and concentrated to give orange oil. Silica gel chromatography using hexane and ethyl acetate (9:1) as eluent gave **2** as orange solid.

ethyl 2-amino-5-(2,2,2-trifluoroethyl)thiophene-3-carboxylate (2b) (90.4%)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.35 (3H, t, J=7Hz), 3.30 (2H, q, J=11Hz), 4.25 (2H, q, J=7Hz), 6.10 (2H, broad s), 6.89 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 14.41, 34.46 ($\underline{C}CF_3$, q, J=32Hz), 59.83, 106.64, 111.47, 123.81 (CF_3, q, J=277Hz), 127.42, 162.92, 165.10; *m/z* (ES^+) 254.2 (M+1).

ethyl 2-amino-5-(1,1,1-trifluoropropan-2-yl)thiophene-3-carboxylate (2d) (95%)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.34 (3H, J=7Hz), 1.45 (3H, d, J=7Hz), 3.44 (1H, m), 4.25 (2H, q, J=7Hz), 6.89 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 14.43, 14.86, 39.47 ($\underline{C}CF_3$, q, J=29Hz), 59.80, 106.30, 118.84, 122.11(CF_3, q, J=280Hz), 125.23, 162.37, 165.18; *m/z* (ES^+) 268.3 (M+1).

General procedure for compounds 3a-e

A solution of **2** (2.87 g, 13.5 mmol) in formamide (20 ml) was heated up to 140˚C for 24 hours. After cooled down, saturated sodium bicarbonate solution (20 ml) was added, and the mixture was partitioned between ethyl acetate and H_2O. The organic extracts were washed with brine,

dried with $MgSO_4$ and concentrated to give a yellow solid. Silica gel chromatography using methylene chloride and methanol (99:1) as eluent gave **3** as orange solid.

6-(2,2,2-trifluoroethyl)thieno[2,3-d]pyrimidin-4(3H)-one (3b) (95.5%)

^{1}H NMR (400 MHz, DMSO-d_6): δ_H 4.05 (2H, q, J=11Hz), 7.40 (1H, s), 8.13 (1H, s), 12.56 (1H, broad s); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C 33.40 ($\underline{C}CF_3$, q, J=30Hz), 123.58, 124.07 (CF_3, q, J=277Hz), 124.61, 128.45, 146.20, 157.05, 164.54; *m/z* (ES^+) 235.2 (M+1).

6-(1,1,1-trifluoropropan-2-yl)thieno[2,3-d]pyrimidin-4(3H)-one (3d) (91%)

^{1}H NMR (400 MHz, DMSO-d_6): δ_H 1.49 (3H, d, J=7Hz), 4.28 (1H, m), 7.43 (1H, s), 8.14 (1H, s), 12.59 (2H, broad s); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C 15.24, 39.08($\underline{C}CF_3$, q, J=30Hz), 122.27, 124.80, 125.51 (CF_3, q, J=277Hz), 135.39, 146.69, 157.51, 164.40; *m/z* (ES^+) 249.5 (M+1).

General procedure for compounds 4a-e

A solution of **3** (9.6 mmol) in phosphorus oxychloride (5 ml) was refluxed for 4 hours. After cooled down, the solvent was distilled off. The residue was diluted and neutralized with saturated sodium bicarbonate solution and extracted with ethyl acetate. The organic extracts were washed with brine, dried with $MgSO_4$ and concentrated. The crude was applied to silica gel column chromatography using dichloromethane and methanol (80:2) as eluent to give **4** as orange oil.

4-chloro-6-(2,2,2-trifluoroethyl)thieno[2,3-d]pyrimidine (4b) (87%)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H 3.75 (2H, q, J=10Hz), 7.39 (1H, s), 8.87 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 35.86 ($\underline{C}CF_3$, q, J=32Hz), 121.35, 123.07 (CF_3, q, J=277Hz), 130.03, 134.28, 153.29, 154.83, 169.04; *m/z* (ES^+) 253.5 (M+1).

4-chloro-6-(1,1,1-trifluoropropan-2-yl)thieno[2,3-d]pyrimidine (4d) (89%) ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 1.69 (3H, d, J=7Hz), 3.92 (1H, m), 7.41 (1H, s), 8.85 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 15.10, 40.74 ($\underline{C}CF_3$, q, J=30Hz), 119.20, 121.46 (CF_3, q, J=280Hz), 129.51, 141.52, 152.89, 154.49, 168.26; *m/z* (ES^+) 267.3 (M+1).

General procedure for compounds MI-2-(1-5)

To a solution of **4** (2.4 mmol) and 5,5-dimethyl-2-(piperazin-1-yl)-4,5-dihydrothiazole or 2-(piperazin-1-yl)-5-(trifluoromethyl)-1,3,4-thiadiazole(2.8 mmol) in THF (20 ml), N,N-diisopropylethylamine (0.91 g, 7.1 mmol) was added. The mixture was refluxed for 6 hours. After cooled down, the mixture was partitioned between ethyl acetate and H_2O. The organic extracts were washed with brine, dried with $MgSO_4$ and concentrated to give a pale yellow solid. Silica gel column chromatography using dichloromethane and methanol (97:3) gave **MI** compounds as a pale yellow solid. Its HCl salt was obtained by adding 1 equivalent of 1N HCl solution in diethyl ether to the solution of **MI** compound in ethanol and filtrated.

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-(3-methoxypropyl)thieno[2,3-d]pyrimidine (MI-2-1)

^{1}H NMR (600 MHz, DMSO-d_6): δ_H 1.63 (6H, s), 1.91 (2H, m), 2.34 (2H, m), 3.00(2H, t, J=7Hz), 3.80 (4H, m), 3.84 (3H, s), 4.08 (4H, m), 7.42(1H, s), 8.43 (1H, s); ^{13}C NMR (150 MHz, DMSO-d_6): δ_C 23.62, 27.73, 29.28, 31.92, 43.87, 49.06, 61.31, 116.37, 118.03, 140.79, 151.91, 156.87, 167.69, 170.39; *m/z* (ES^+) 406.3 (M+1).

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-(2,2,2-trifluoroethyl)thieno[2,3-d]pyrimidine (MI-2-2)

^{1}H NMR (600 MHz, DMSO-d_6): δ_H 1.62 (6H, s), 3.83 (6H, m), 4.11 (6H, m), 7.72 (1H, s), 8.47 (1H, s), 10.40 (1H, broad s); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 28.93, 35.69 ($\underline{C}CF_3$, q, J=32Hz), 46.61, 47.77, 60.45, 73.31, 116.94, 121.82, 123.50(CF_3, q, J=277Hz), 127.50, 153.30, 158.43, 163.85, 169.94; *m/z* (ES^+) 416.2 (M+1).

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-(perfluoroethyl)thieno[2,3-d]pyrimidine (MI-2-3)

^{1}H NMR (600 MHz, DMSO-d_6): δ_H 1.63 (6H, s), 3.84 (6H, m), 4.15(4H, m), 8.23 (1H, s), 8.59 (1H, s); ^{13}C NMR (150 MHz, DMSO-d_6): δ_C ^{13}C NMR (150 MHz, DMSO-d_6): δ_C 27.66, 43.97, 45.43, 47.03, 49.67, 61.64, 76.7,115.19, 119.66, 121.21, 127.33, 155.26; *m/z* (ES^+) 452.5 (M+1).

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-(1,1,1-trifluoropropan-2-yl)thieno[2,3-d]pyrimidine (MI-2-4)

^{1}H NMR (600 MHz, DMSO-d_6): δ_H 1.55 (3H, d, J=7Hz), 1.62 (6H, s), 3.36 (2H, s), 3.82 (4H, t, J=5Hz), 4.11 (4H, t, J=5Hz), 4.36 (1H, m), 7.73 (1H, s), 8.47 (1H, s), 10.96 (1H, broad s); ^{13}C NMR (150 MHz, DMSO-d_6): δ_C 14.94, 27.00, 44.31 ($\underline{C}CF_3$, m), 57.60, 61.13, 64.89, 115.58, 121.98, 125.58 (CF_3, q, J=277Hz), 133.48, 152.79, 157.44, 168.32, 169.96; *m/z* (ES^+) 430.5 (M+1).

4-(4-(5,5-dimethyl-4,5-dihydrothiazol-2-yl)piperazin-1-yl)-6-(4,4,4-trifluorobutyl)thieno[2,3-d]pyrimidine (MI-2-5)

^{1}H NMR (600 MHz, DMSO-d_6): δ_H 1.63 (6H, s), 1.90 (2H, m), 2.35 (2H, m) 3.01 (2H, t, J=7Hz), 3.85 (6H, m), 4.09 (4H, m), 7.42 (1H, s), 8.43 (1H, s)^{13}C NMR (150 MHz, DMSO-d_6): δ_C 23.41, 27.70, 29.21, 32.4, 43.8, 45.5, 47.51, 50.42, 116.45, 118.55, 126.66 (CF_3, q, J=280Hz), 139.61, 152.02, 156.94, 167.95, 170.43; *m/z* (ES^+) 444.7 (M+1).

Supplementary Figures

Figure S1. Amino acid sequence, MLL binding data and crystal structure of menin. (A) Sequence of human menin with annotated secondary structure elements derived from the crystal structure. Fragments deleted for crystallization are shown in red. (B) Fluorescence polarization data showing binding of meninΔ10 with MLL fragments: MBM1 (MLL 4-15) – left panel and MBM2 (MLL 23-40) – right panel. (C) Overall structure of menin with bound MLL MBM1 peptide (shown in blue).

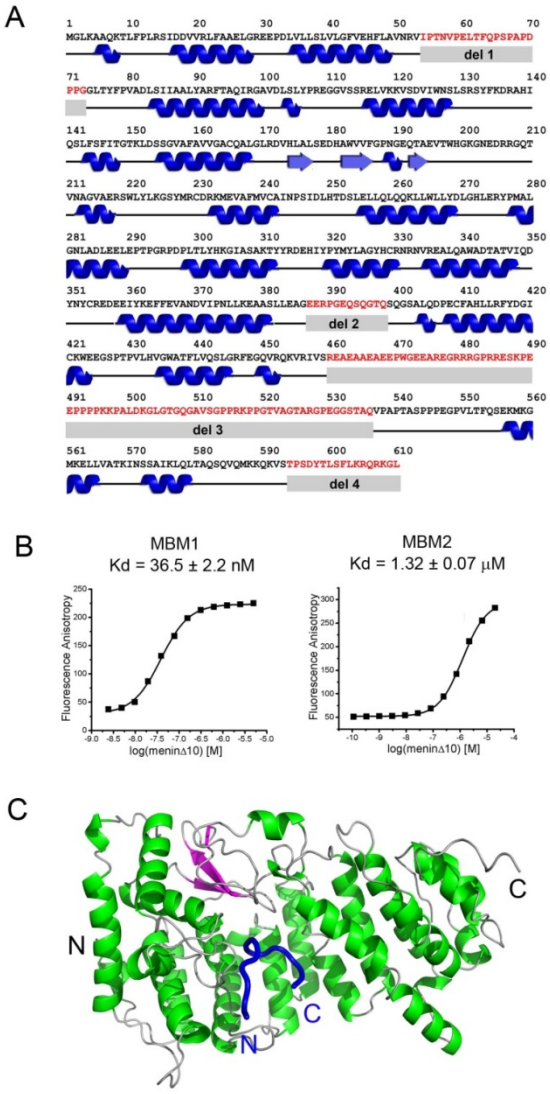


Figure S2. Menin point mutations D252K and L289K do not affect the interaction with MBM1 and menin stability. (A) Fluorescence polarization (FP) experiment showing reduced binding of low affinity MLL motif MBM2 to menin point mutants D252K and L289K. (B) FP experiment demonstrating the binding of MBM1 to wild-type menin, D252K and L289K mutants. (C) Stability of the wild-type menin and menin mutants assessed by thermal shift assays.

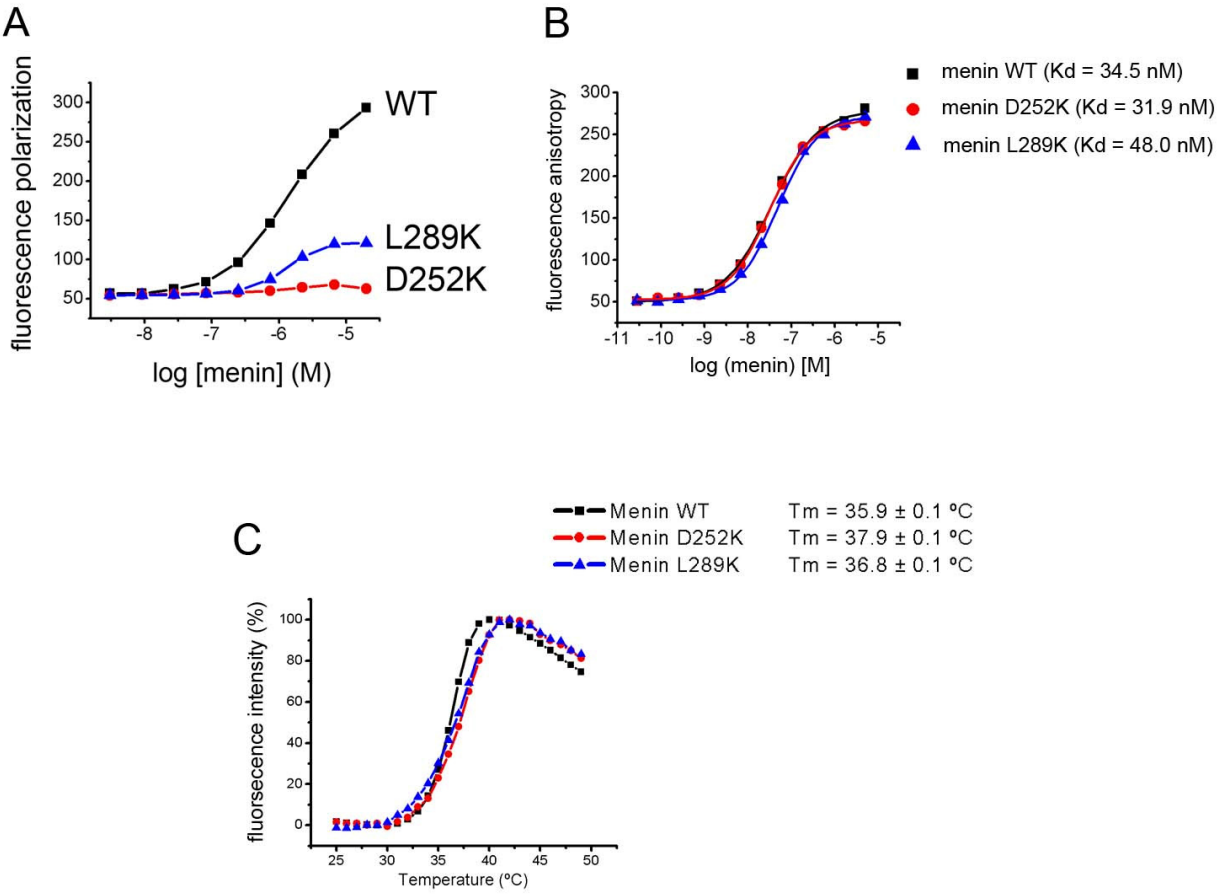


Figure S3. Colony formation assay with MI-2 and MI-2-2. Colonies for MLL-AF9 transduced BMC treated for 7 days with increasing concentrations of **MI-2** (A) and **MI-2-2** (B). Black scale bar indicates 500µm.

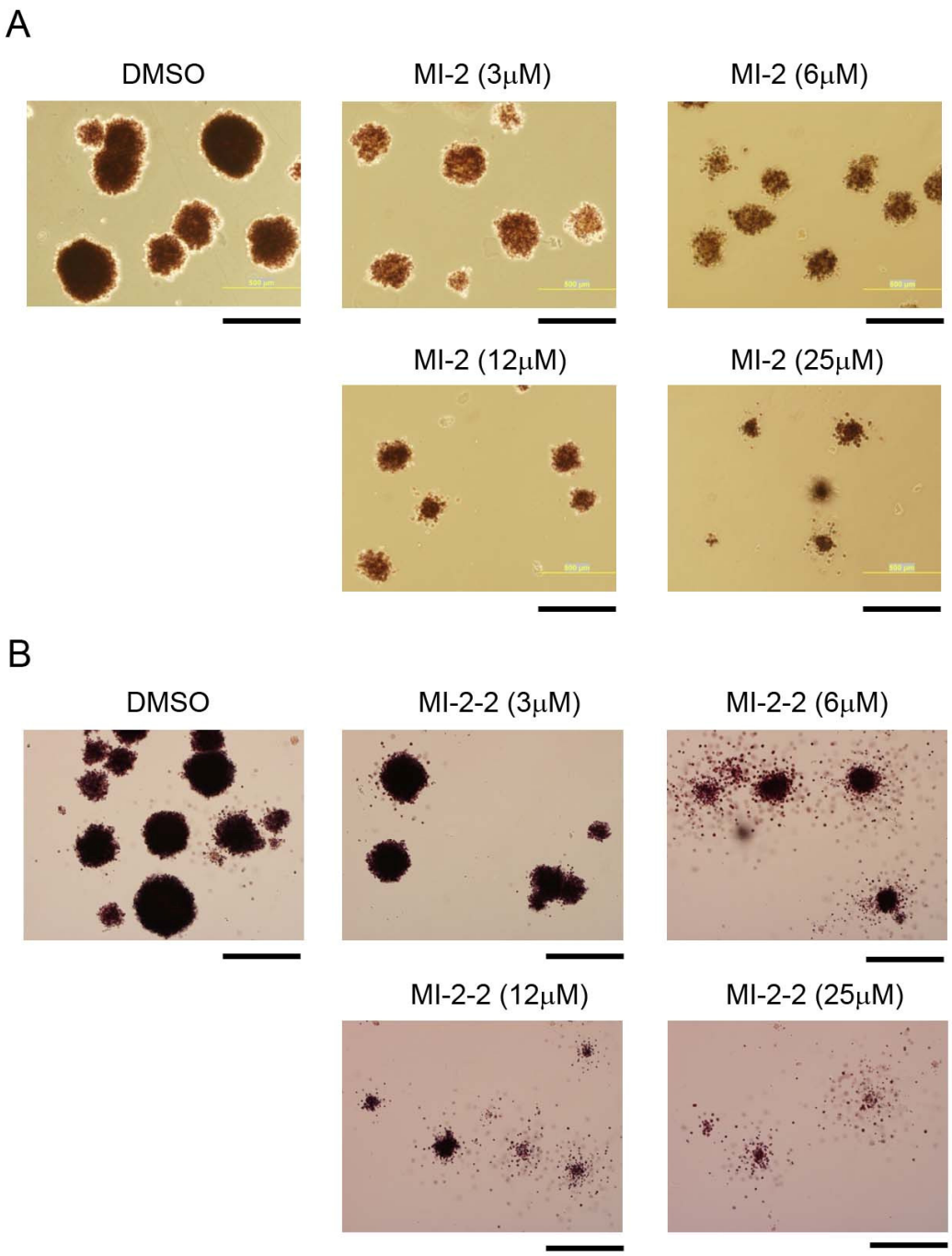


Figure S4. Treatment with MI-2-2 induces more pronounced differentiation of MLL-AF9 transformed bone marrow cells as compared to MI-2. Wright-Giemsa stained cytospins on MLL-AF9 transformed bone marrow cells after 7 days of treatment with **MI-2** (A), **MI-2-2** (B). Black scale bar indicates 50μm.

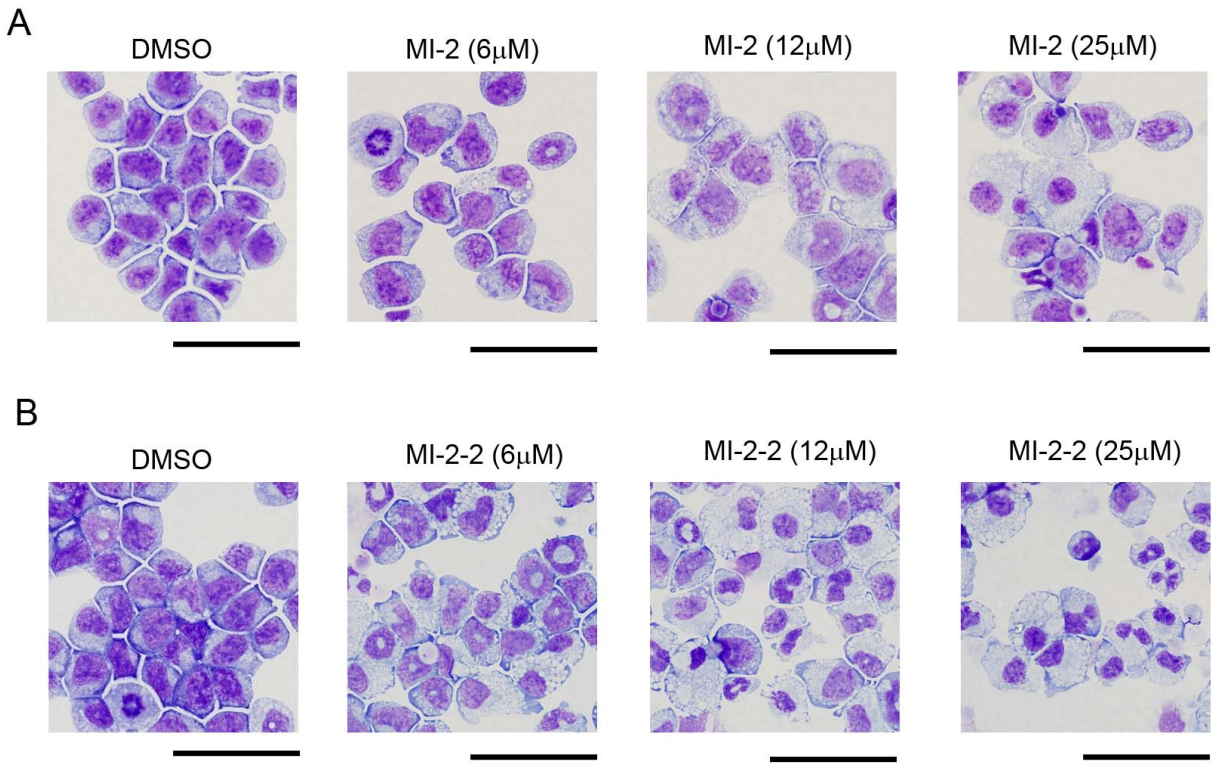


Figure S5. MTT viability assay showing weak activity for MI-2-2 in non-MLL human leukemia cells Kasumi-1 and HAL-01.

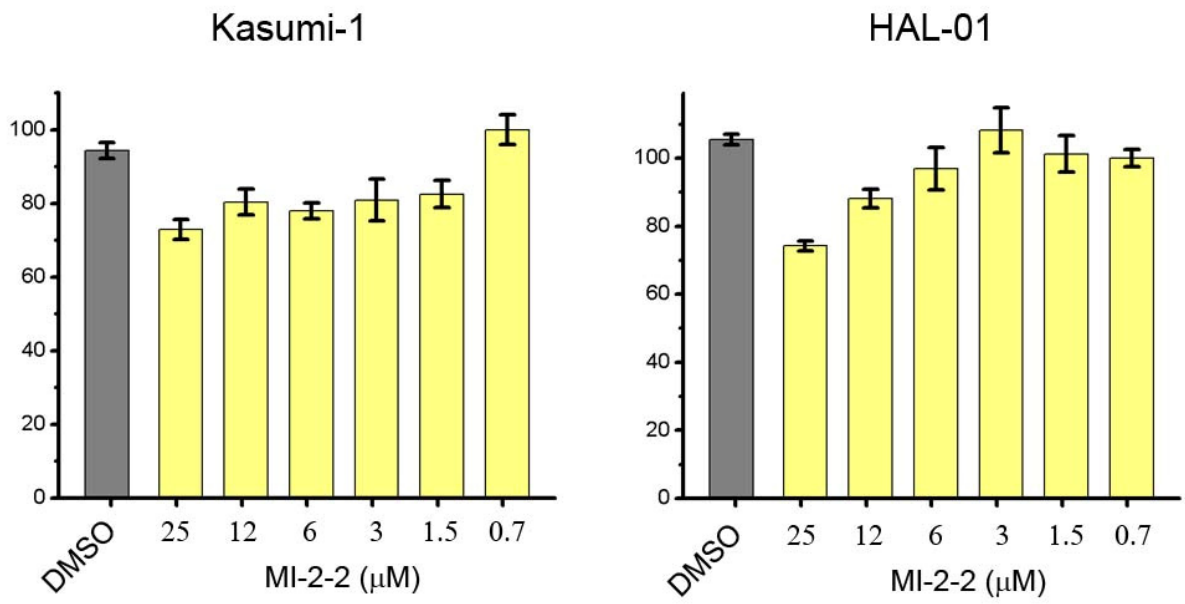


Figure S6. Human leukemia cells MV4;11 differentiate after treatment with the MI-2-2. Wright-Giemsa stained cytospins on MV4;11 cells (harboring MLL-AF4) after 10 days of treatment with **MI-2-2** and DMSO. Black scale bar indicates 50µm. The right column shows 50µm x 50µm fragments focusing on selected cells.

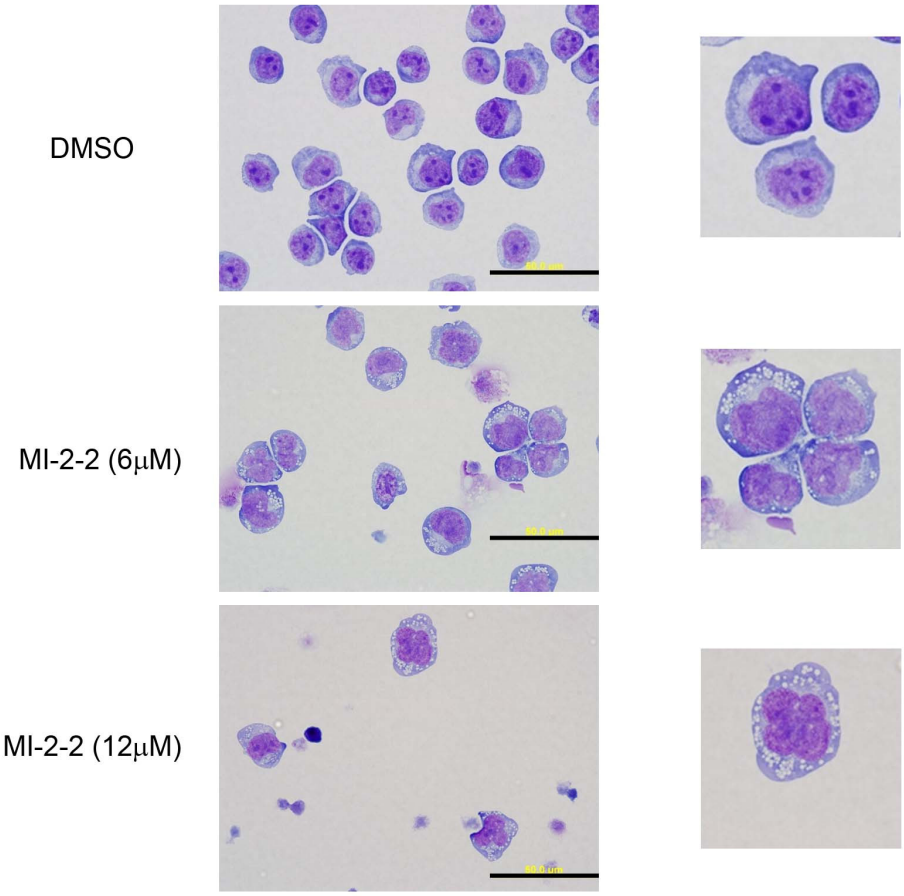


Figure S7. Second generation inhibitor, MI-2-2 has improved growth inhibition of human leukemia cell lines. Comparison of the growth inhibition of four cell lines with MLL translocations upon treatment with **MI-2** and **MI-2-2**. Experiment was carried out for 13 days.

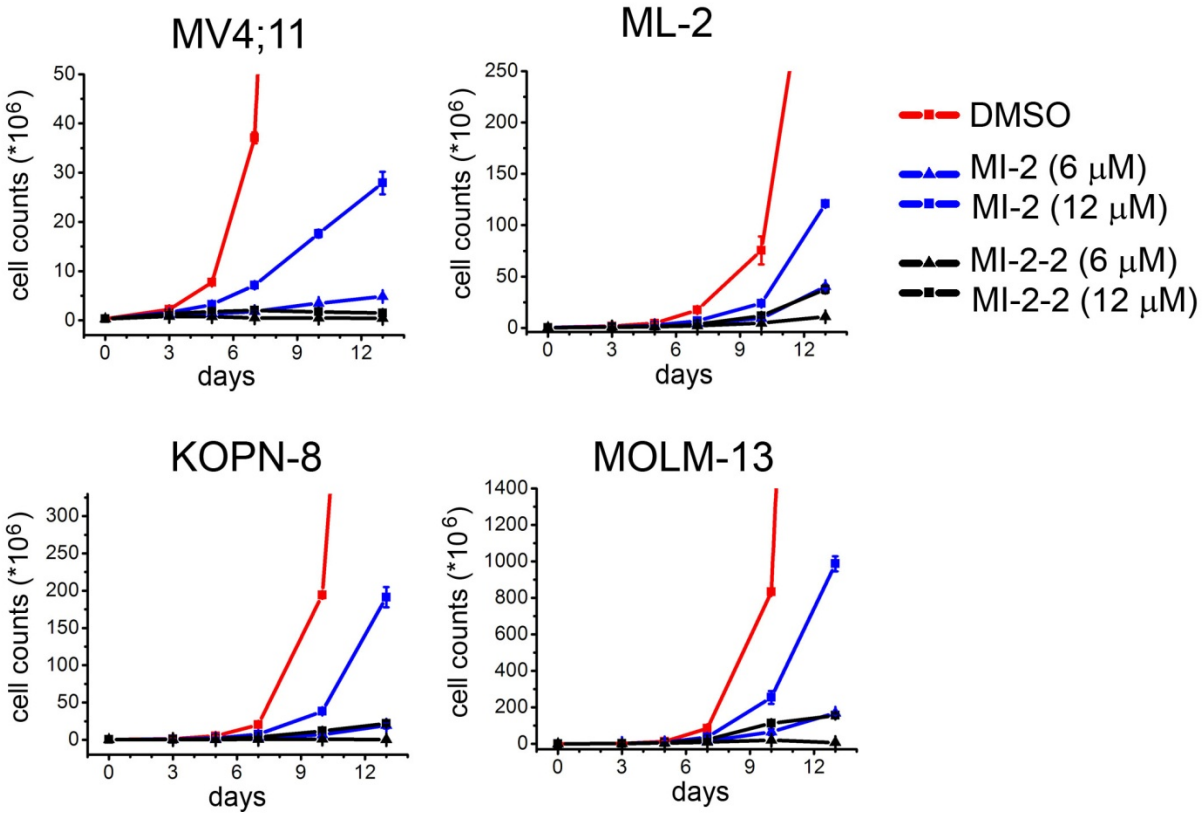
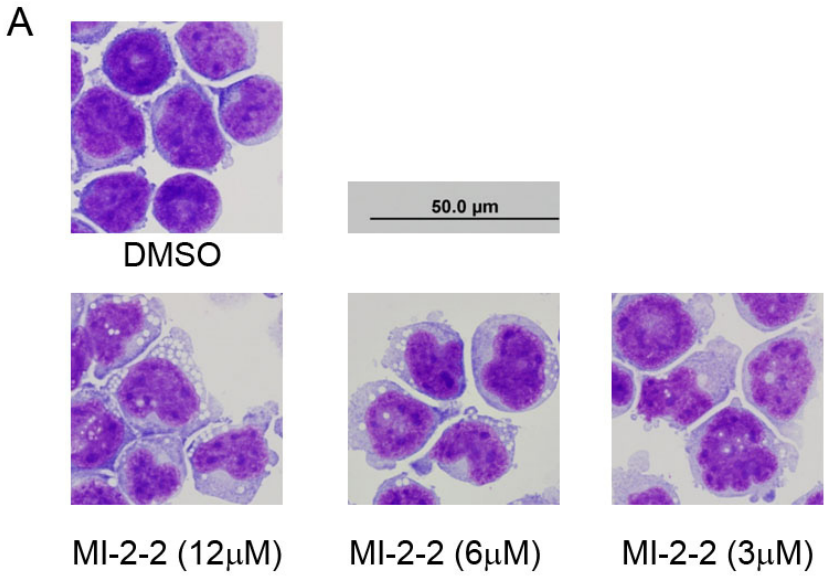


Figure S8. Differentiation of ML-2 leukemia cells after treatment with the MI-2-2. **A**. Wright-Giemsa stained cytospins for ML-2 cells (harboring MLL-AF6) after 10 days of treatment with **MI-2-2** and DMSO. Black scale bar indicates 50µm. **B**. Quantification of CD11b expression in ML-2 cells treated for 10 days with the menin-MLL inhibitors as detected by flow cytometry. Data represents the mean values for duplicates ± s.d. Experiment was performed two times.



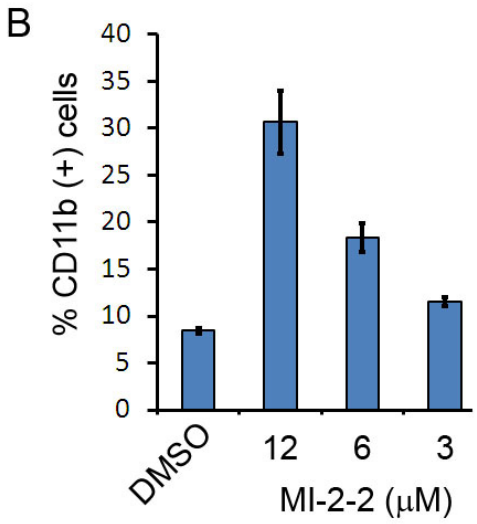
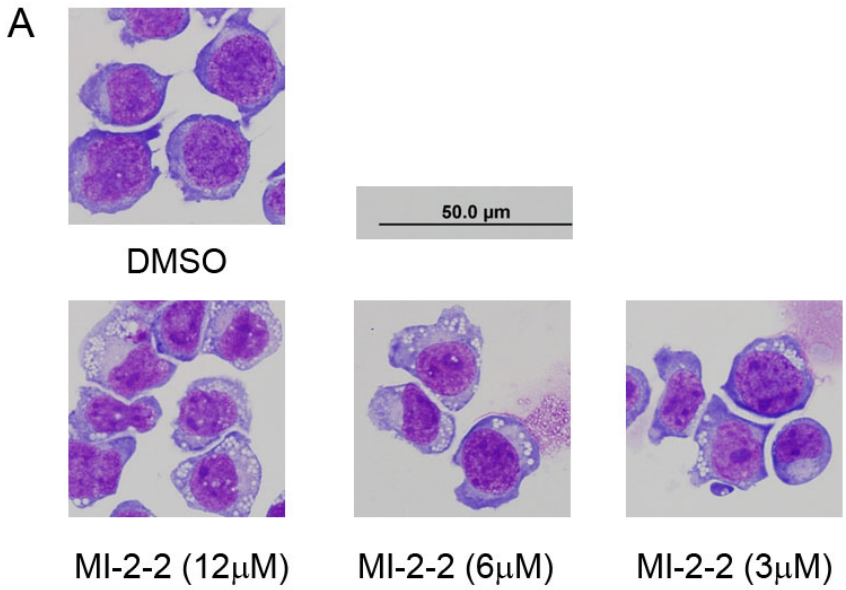


Figure S9. Differentiation of MOLM-13 leukemia cells after treatment with the MI-2-2. **A**. Wright-Giemsa stained cytospins for MOLM-13 cells (harboring MLL-AF9) after 10 days of treatment with **MI-2-2** and DMSO. Black scale bar indicates 50µm. **B**. Quantification of CD11b expression in MOLM-13 cells treated for 10 days with the menin-MLL inhibitors as detected by flow cytometry. Data represents the mean values for duplicates ± s.d. Experiment was performed two times.



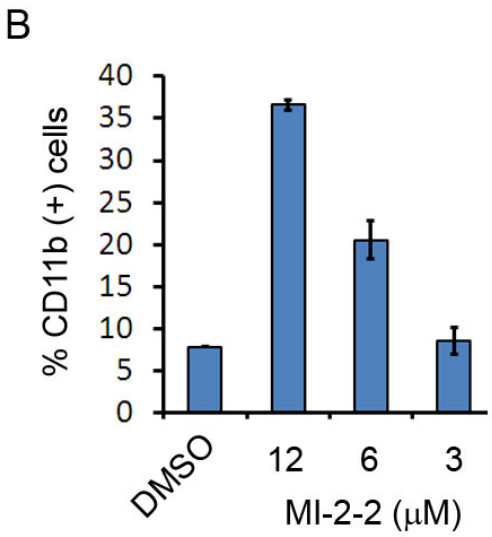


Figure S10. Differentiation of KOPN-8 leukemia cells after treatment with the MI-2-2. **A**. Wright-Giemsa stained cytospins for KOPN-8 cells (harboring MLL-ENL) after 10 days of treatment with **MI-2-2** and DMSO. Black scale bar indicates 50μm. **B**. Quantification of CD11b expression in KOPN-8 cells treated for 10 days with the menin-MLL inhibitors as detected by flow cytometry. Data represents the mean values for duplicates ± s.d. Experiment was performed two times.

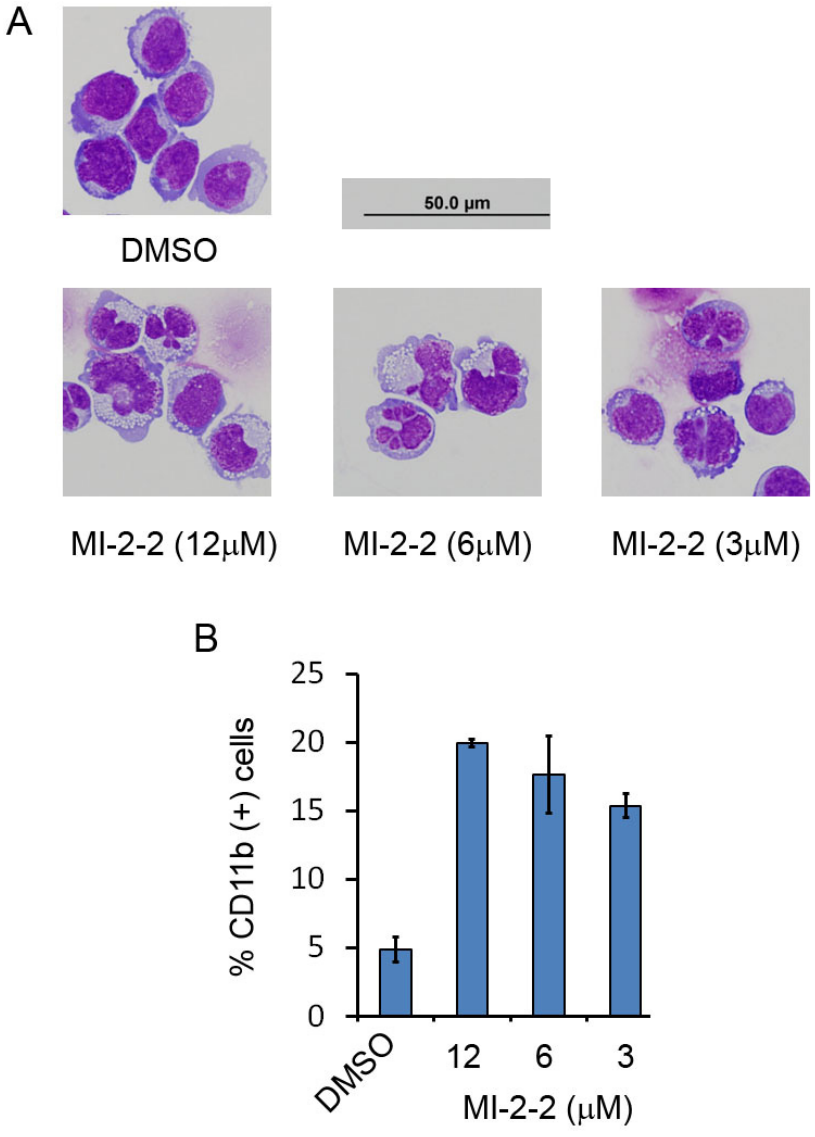
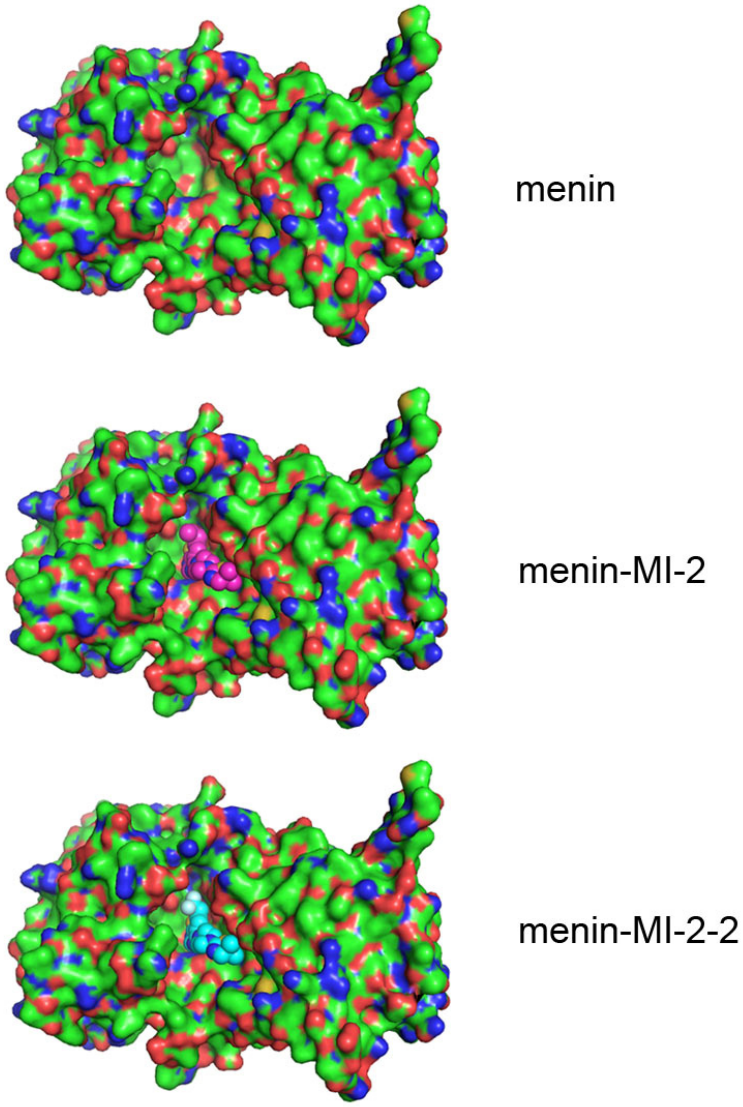


Figure S11. Surface representation of menin and menin complexes. MI-2 and **MI-2-2** are shown using space-filling representations.



Supplementary Tables

Table S1. Data collection and refinement statistics. *Numbers in parenthesis refer to the highest resolution shell.

	Menin	Menin-MBM1	Menin-**MI-2**	Menin-**MI-2-2**
PDB code	xxx	xxx	xxx	xxx
Data collection				
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions				
a, *b*, *c* (Å)	48.8, 80.2, 124.7	48.6, 80.1, 124.6	48.6, 80.1, 124.8	49.0, 80.2, 124.8
Solvent content (%)	36.6	44	40.1	36.1
Resolution (Å)	1.46 (1.49-1.46)	1.55 (1.58-1.55)	1.56 (1.59-1.56)	1.27 (1.29-1.27)
Unique reflections	85179 (3763)	70700 (3475)	60554 (3136)	129732 (6402)
R_{sym}	0.110 (0.361)	0.076 (0.583)	0.060 (0.686)	0.108 (0.646)
$I / \sigma I$	16.1 (4.5)	26.4 (2.1)	25.5 (2.0)	30.6 (2.4)
Completeness (%)	99.4 (88.1)	98.5 (97.0)	86.4 (92.1)	100.0 (99.9)
Redundancy	6.8 (5.6)	4.9 (4.5)	3.8 (3.5)	7.2 (6.2)
Refinement				
R_{work} / R_{free} (%)	14.5 / 17.6	16.0 / 18.9	17.8 / 21.4	14.9 / 18.2
No. atoms				
Protein	3831	3808	3756	3829
Water	586	479	370	621
Mean *B*-factors (Å^2)	18.12	24.5	24.29	19.57
R.m.s. deviations				
Bond lengths (Å)	0.016	0.020	0.017	0.022
Bond angles (°)	1.192	1.9	1.666	1.983
Ramachandran plot				
Most favored regions (%)	98.5	98.5	98.1	98.3
Additional allowed regions (%)	1.5	1.5	1.9	1.7