

SUPPORTING INFORMATION

The L3MBTL3 methyl-lysine reader domain functions as a dimer

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Materials and Methods

Protein expression and purification. L3MBTL3 was purified essentially as described (1-3). Briefly, cell pellets from 2 L cultures expressing His-tagged proteins were lysed with BugBuster protein extraction reagent (EMD Millipore, Darmstadt, Germany) containing 20 mM imidazole. The cell lysate was clarified by centrifugation and loaded onto a 5 mL HisTrap HP column (GE Healthcare, Piscataway, NJ) equilibrated with binding and wash buffer (50 mM sodium phosphate buffer pH 7.2, 500 mM NaCl, 30 mM imidazole) using an ÄKTA FPLC (GE Healthcare, Piscataway, NJ) at 1 mL/min. His-tagged protein was eluted using a linear gradient of elution buffer (50 mM sodium phosphate buffer pH 7.2, 500 mM NaCl, 500 mM imidazole) over 20 column volumes. Fractions containing the desired protein were confirmed by SDS-PAGE, pooled and loaded at 2 mL/min onto a HiLoad 26/60 Superdex 200 prep grade size exclusion column (GE Healthcare, Piscataway, NJ) using an ÄKTA FPLC. A constant flow of 2 mL/min size exclusion buffer (25 mM Tris·HCl pH 8.0, 250 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.02% Tween 20) was used to elute proteins. Fractions containing the desired protein were identified by SDS-PAGE, pooled and subjected to simultaneous concentration and buffer exchange using an Amicon Ultra-15 centrifugal filter unit (Millipore, Billerica, MA) and storage buffer (20 mM Tris HCl, pH 8.0, 250 mM, NaCl and 2 mM DTT for L3MBTL3 wild type and mutant proteins). Protein concentration was determined by absorbance at 280 nm using the Edelhoch method and protein purity was determined to be >95% by Coomassie.

A pET28 vector containing the coding region for residues 8-62 of CBX7 (reference sequence (NP_783640) was transformed into BL21 Rosetta DE3 pLysS cells (EMD Millipore). A 2 L culture was grown to mid log phase at 37 °C then the temperature was lowered to 18 °C and protein expression was induced by addition of 0.5 mM IPTG. Expression was allowed to continue overnight. CBX7 protein was purified essentially as described above except for the use of a buffer consisting of 25 mM Tris HCl pH 7.5, 150 mM NaCl, and 2 mM DTT for size exclusion chromatography and protein storage.

Cell lines and cell culture. Media was purchased from Gibco. HeLa cells were grown at 37°C with 5% CO₂ on 15 cm plates in Modified Eagle's Medium supplemented with 10% fetal bovine serum.

Cloning and mutagenesis. L3MBTL3 cDNA provided by the Structural Genomic Consortium and was cloned as described (2). Briefly, L3MBTL3 cDNA was PCR amplified using MGC clone corresponding to NP_001007103.1 and subsequently cloned into pACGFP N3, or (Clontech), or pCDNA3-Flag (Invitrogen) using standard cloning procedures. Site directed mutagenesis was performed with the Quick Change Mutagenesis kit (Stratagene).

L3MBTL3 co-immunoprecipitation experiments. Approximately 1 x 10⁶ HeLa cells stably expressing pACGFP N3 L3MBTL3 (wildtype full length) were seeded on 10 cm plates and then transiently transfected with 15 µg pCDNA3-Flag L3MBTL3 (wildtype 3MBT, D274A 3MBT,

D381A 3MBT, wildtype full length, D274A full length, or D381A full length) using Lipofectamine 2000 (Invitrogen) for 48 hours. When applicable, UNC1215 was added to the cells at the time of transfection to give a final concentration of 100 μ M (0.1% DMSO). Approximately 1×10^6 cells were lysed using 500 μ L of Cytobuster (Novagen) supplemented with 1X protease inhibitor cocktail (Roche no. 05056489001) and 125 U of Benzonase Nuclease (Novagen no. D00128165). Protein concentration was quantified using a Bradford assay (BioRad no. 500-0006), and then 1 mg of lysate was used for each pull-down. ANTI-FLAG M2 Affinity gel (10 μ L, Sigma Aldrich no. A2220) was used according to the manufacturer's recommendations and incubated with cell lysate at 4 °C overnight. Samples were washed three times with PBS, resuspended in 1X sample buffer (Invitrogen no. LC2676), and incubated at 95 °C for 5 minutes. Each sample was then run on a Mini-PROTEAN TGX gradient gel (BioRad no. 456-9035) followed by western blotting. Blots were blocked with 5% milk for 1 h, incubated with GFP antibody (1:10,000, Molecular Probes no. G10362) overnight at 4 °C, washed three times for 5 minutes each with PBS/0.1% Tween-20, incubated 1 h with IRDye Goat anti-Rabbit secondary antibody (1:20,000, LI-COR no. 926-32211) in the dark, and then washed three times with PBS/0.1% Tween-20. Imaging was performed on a LI-COR Odyssey.

AlphaScreen assay. Biotinylated L3MBTL3 was diluted to 400 nM in 1X assay buffer (20 mM TRIS pH 8.0, 75 mM NaCl, 2 mM DTT and 0.05% Tween-20) and transferred (4.5 μ L) into the wells of 384-well low-volume Proxiplates (PerkinElmer) by Multidrop (Thermo). To these plates 4.5 μ L of 400 nM 6His-tagged protein (WT L3MBTL3, D274A, D381A, or CBX7) in 1X assay buffer was added by Multidrop (Thermo). Next, 1 μ L of ligand (10% DMSO, UNC1215, UNC1079, or H4K20me2 peptide (residues 12-27)) was added to the plates. Ligands were transferred at 10-point, 2-fold dilutions from 1 mM in 1X assay buffer with constant concentrations of 10% DMSO. Plates were spun down at 1,200 RPM for two minutes and then incubated for 1 hour at room temperature. Next, 2 μ L of a 1:1 mixture of streptavidin-conjugate donor and nickel-chelate acceptor beads (45 μ g/mL in 1X assay buffer) were added and the plates were allowed to incubate for an additional 30 min in the dark at room temperature. After incubation, the plates were read on an EnVision multi-label reader equipped with an HTS AlphaScreen® laser (Perkin Elmer).

The IC₅₀ values reported are the average of at least 3 values \pm the standard deviation. The IC₅₀ values were calculated from replicate runs by averaging data points for each ligand concentration and plotted using 4-parameter curve fitting (GraphPad Prism 5).

LANCE TR-FRET assay. The LANCE assay was performed similarly to the AlphaScreen assay. Biotinylated L3MBTL3 and 6His-tagged protein (WT L3MBTL3, D274A, D381A, or CBX7) were diluted to 800 nM in and 1:1 mixture in 1X assay buffer (20 mM TRIS pH 8.0, 75 mM NaCl, 2 mM DTT and 0.05% Tween-20) and transferred (4.5 μ L) into the wells of 384-well low-volume Proxiplates (PerkinElmer) by Multidrop (Thermo). Next, 1 μ L of ligand (10% DMSO, UNC1215, UNC1079, or H4K20me2 peptide (residues 12-27)) was added to the plates. Ligands were transferred at 10-point, 2-fold dilutions from 1 mM in 1X assay buffer with constant

concentrations of 10% DMSO. Plates were spun down at 1,200 RPM for two minutes and then incubated for 1 hour at room temperature. Next, 4.5 μ L of a mixture of Eu-W1024-anti6XHis (2.2 nM) and Ulight-streptavidin (137.5 nM) were added and the plates were allowed to incubate for an additional 30 min in the dark at room temperature. After incubation, the plates were read on an EnVision multi-label reader (Perkin Elmer) equipped with an LANCE/DELFI A D400/630 dual mirror, UV2(TRF) 320 nm excitation filter, and two emission filters (APC 665 nm and Europium 615 nm).

The IC₅₀ values reported are the average of at least 3 values \pm the standard deviation. The IC₅₀ values were calculated using 4-parameter curve fitting (GraphPad Prism 5) from replicate runs using averaged response values for each compound concentration.

Isothermal titration calorimetry. All ITC measurements were recorded at 25 °C with an AutoITC₂₀₀ microcalorimeter (MicroCal Inc.). All protein and peptide stock samples were in the target buffer (25 mM Tris-HCl, pH 8, 150 mM NaCl, and 2 mM β -mercaptoethanol), and then diluted in the same buffer to achieve the desired concentrations: 150 μ M protein and 1 – 1.5 mM peptide. The concentration of protein stock solutions were established using the Edelhoch method, whereas peptide stock solutions were prepared gravimetrically based on molecular weight. A typical experiment included a single 0.2 μ L compound injection into a 200 μ L cell filled with protein, followed by 25 subsequent 1.5 μ L injections of compound. Injections were performed with a spacing of 180 seconds and a reference power of 8 μ cal/sec. If applicable, the heats of dilution generated were then subtracted from the protein-compound binding curves. The titration data was analyzed using Origin Software (MicroCal Inc.) by non-linear least squares, fitting the heats of binding as a function of the compound:protein ratio. The data were fit based on a one set of sites model.

Supporting Data

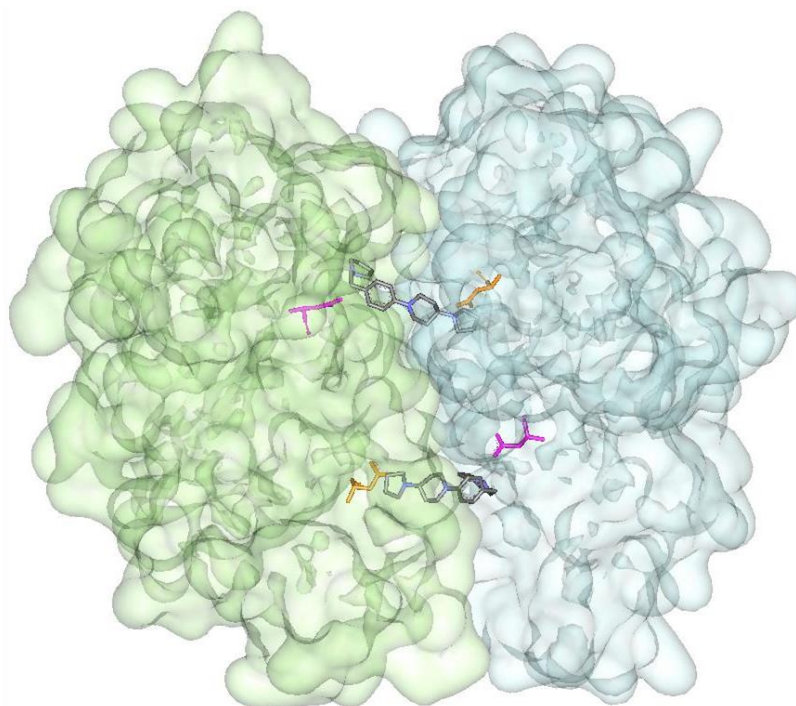
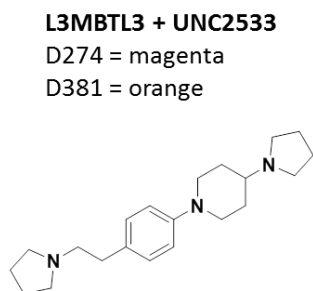


Figure S1. X-ray crystal structure of the 2:2 dimer of L3MBTL3 and UNC2533 shows a face to face arrangement wherein two copies of protein (one shown in green and one in blue) sandwich two ligand molecules (pdb 4L59). Residue D274 is highlighted in magenta and residue D381 is highlighted in orange. UNC2533 is shown in gray.

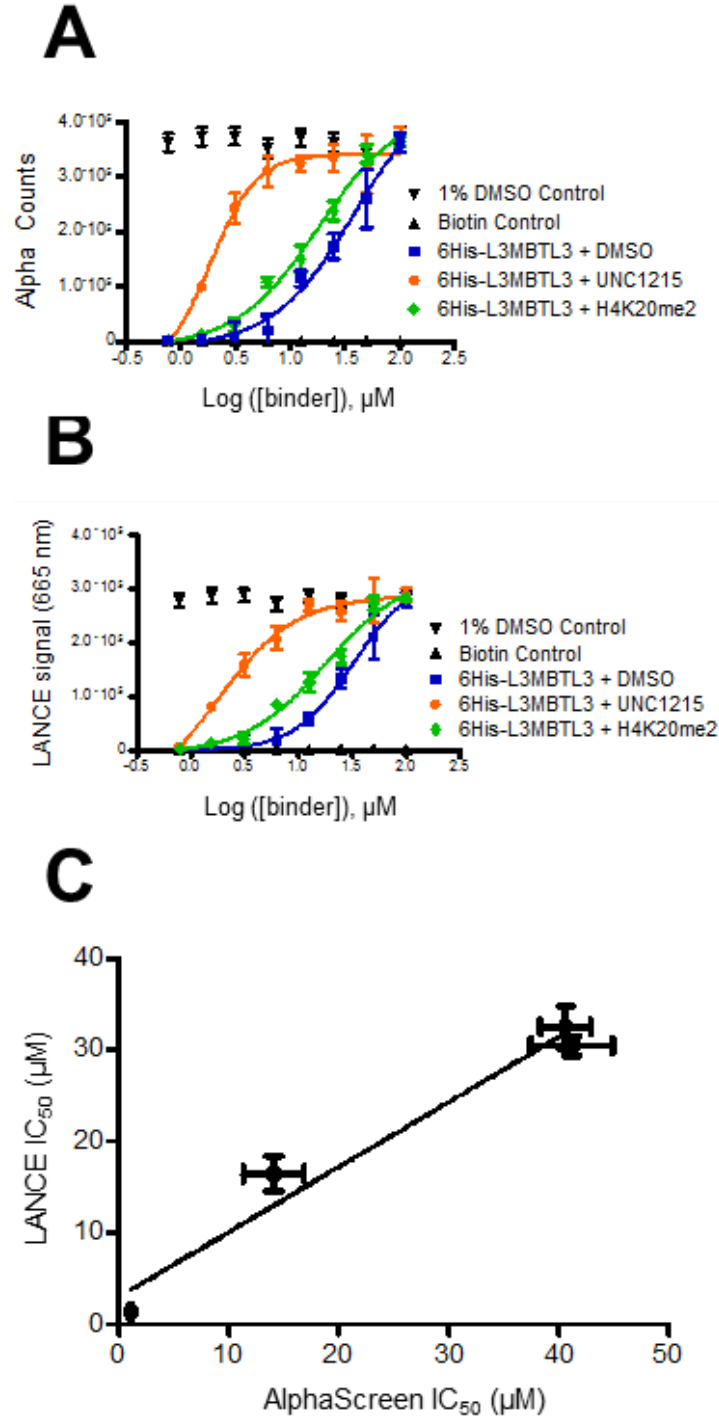


Figure S2. *In vitro* dimerization of wildtype L3MBTL3. A) AlphaScreen direct binding curves for biotinylated L3MBTL3 to 6His-L3MBTL3 in the presence of DMSO, UNC1215, and H4K20me2 peptide. B) LANCE direct binding curves for biotinylated L3MBTL3 to 6His-L3MBTL3 in the presence of DMSO, UNC1215, and H4K20me2 peptide. C) Correlation of AlphaScreen and LANCE assay results for those experiments with measurable binding. Data used for the correlation is summarized in Table 1 ($R^2 = 0.966$).

Table S1. T test results from AlphaScreen and LANCE data. P-values for UNC1215 and H4K20me2 are measured against the DMSO negative control in the biotin-L3MBTL3 and 6His-L3MBTL3 assays.

AlphaScreen IC ₅₀ (μM)			LANCE IC ₅₀ (μM)		
DMSO	UNC1215	H4K20me2	DMSO	UNC1215	H4K20me2
39.52	1.24	16.91	35.48	1.63	1.70
35.61	1.14	14.03	30.28	1.45	1.56
43.90	1.06	13.6	35.51	1.24	1.62
37.98	1.02	17.63	34.85	1.71	1.23
38.55	1.14	18.76	32.53	1.33	1.79
47.54	1.10	14.57	34.97	1.32	1.06
p-value^a	0.0000037	0.00012	p-value^a	0.00000024	0.00000031

^a P-values for UNC1215 and H4K20me2 peptide were calculated using a two-tail t test in Excel.

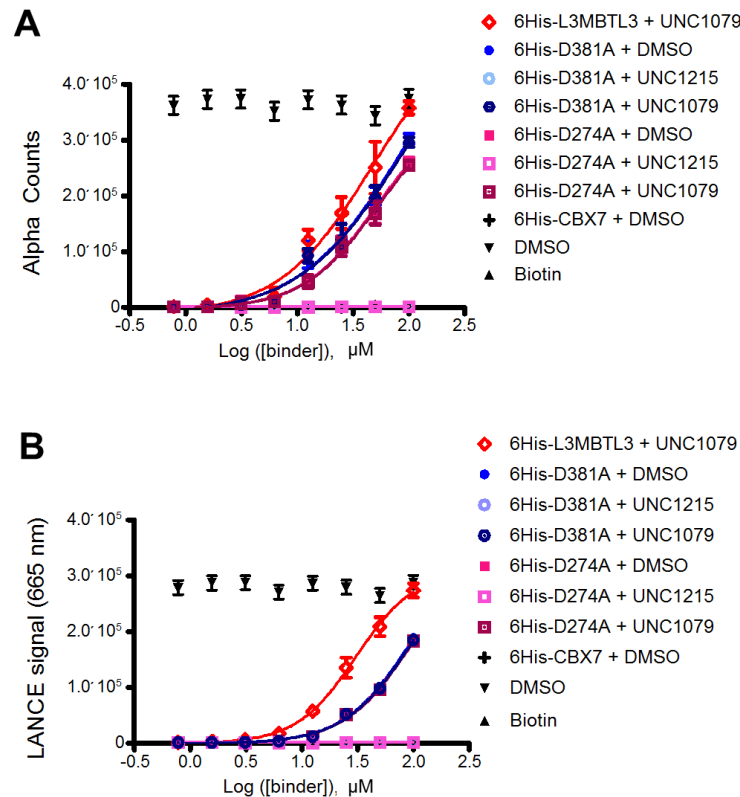


Figure S3. *In vitro* dimerization of wildtype L3MBTL3 with L3MBTL3 mutants. A) AlphaScreen direct binding curves for biotinylated wildtype L3MBTL3 and various 6His-tagged proteins (WT L3MBTL3, D381A, D274A, or CBX7) in the presence of DMSO, UNC1215, and UNC1079. B) LANCE direct binding curves for biotinylated wildtype L3MBTL3 and various 6His-tagged proteins (L3MBTL3, D381A, D274A, or CBX7) in the presence of DMSO, UNC1215, and UNC1079.

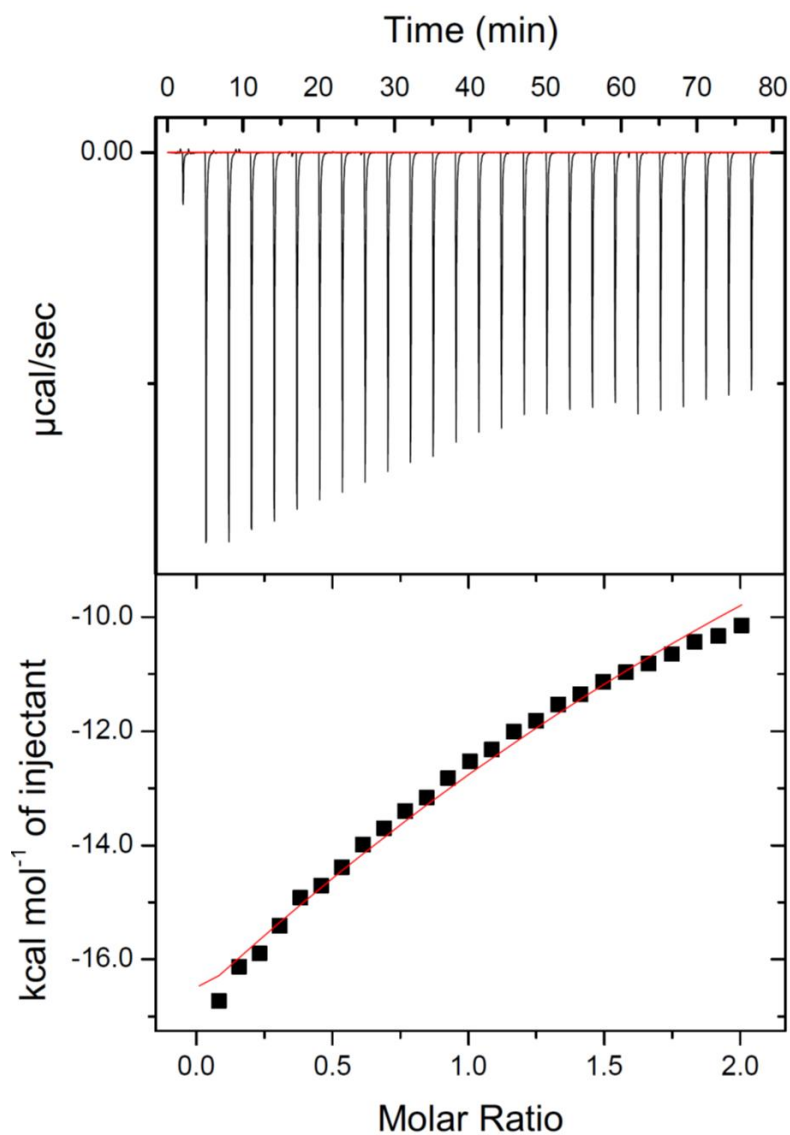


Figure S4. Isothermal titration calorimetry (ITC) of L3MBTL3 binding to H4K20me2 (residues 12 – 27). An accurate K_d value could not be calculated due to the weak affinity of the interaction and incomplete curve, but it is predicted to be $> 100 \mu\text{M}$.

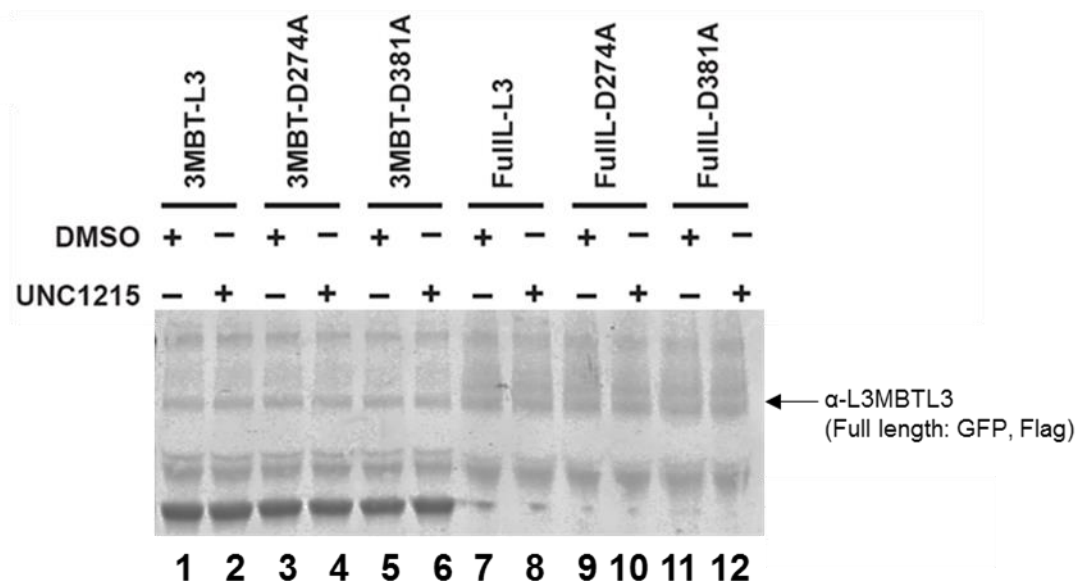


Figure S7. Western blot analysis of GFP-L3 HeLa cells transfected with Flag-tagged plasmids of L3MBTL3 (IB: L3MBTL3; Bethyl A302-852A). The non-specific band in the anti-GFP western blot in Figure 3 is not visible when input samples are blotted with an L3MBTL3-specific antibody. If this band were an isoform of L3MBTL3, it would have been visible below the full length L3MBTL3 constructs in the figure above (similarly to Figure 3). This result indicates that the extra bands visible in lanes 1, 2, 7 and 8 in Figure 3 are not isoforms of L3MBTL3.

References

1. Wigle, T.J. et al. Screening for Inhibitors of Low-Affinity Epigenetic Peptide-Protein Interactions: An AlphaScreen™-Based Assay for Antagonists of Methyl-Lysine Binding Proteins. *J Biomol Screen* **15**, 62-71 (2010).
2. James, L.I. et al. Discovery of a chemical probe for the L3MBTL3 methyllysine reader domain. *Nat Chem Biol* **9**, 184-191 (2013).
3. James, L.I. et al. Small-Molecule Ligands of Methyl-Lysine Binding Proteins: Optimization of Selectivity for L3MBTL3. *J Med Chem* **56**, 7358-71 (2013).