Supplemental Information

Discovery of a Functional Covalent Ligand Targeting an Intrinsically Disordered Cysteine Within MYC

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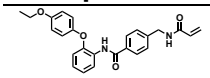
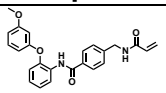
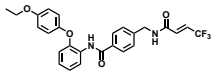
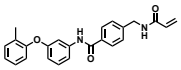
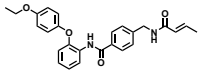
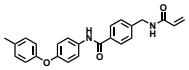
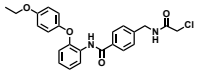
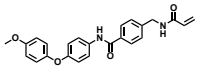
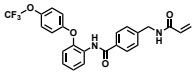
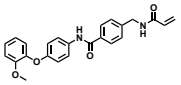
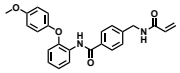
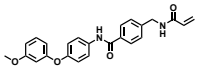
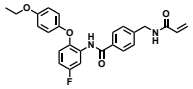
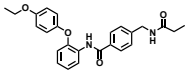
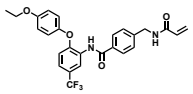
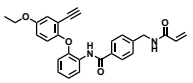
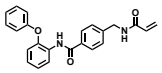
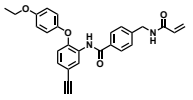
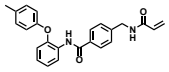
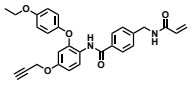
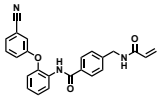
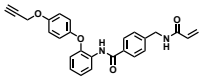
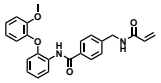
Table S4. Related to Figure 4. Structure-Activity Relationships of EN4 Analogs.

Compound	EC50 (μM)	Compound	EC50 (μM)
 EN4	12.5	 EN4-12	5.0
 EN4-1	>50	 EN4-13	>50
 EN4-2	1.7	 EN4-14	>50
 EN4-3	>50	 EN4-15	>50
 EN4-4	13.0	 EN4-16	>50
 EN4-5	2.1	 EN4-17	>50
 EN4-6	>50	 EN4-18	2.9
 EN4-7	2.2	 EN4-alkyne-1	6.3
 EN4-8	11.9	 EN4-alkyne-2	16.4
 EN4-9	10.4	 EN4-alkyne-3	7.8
 EN4-10	>50	 EN4-alkyne-4	1.4
 EN4-11	11.3		

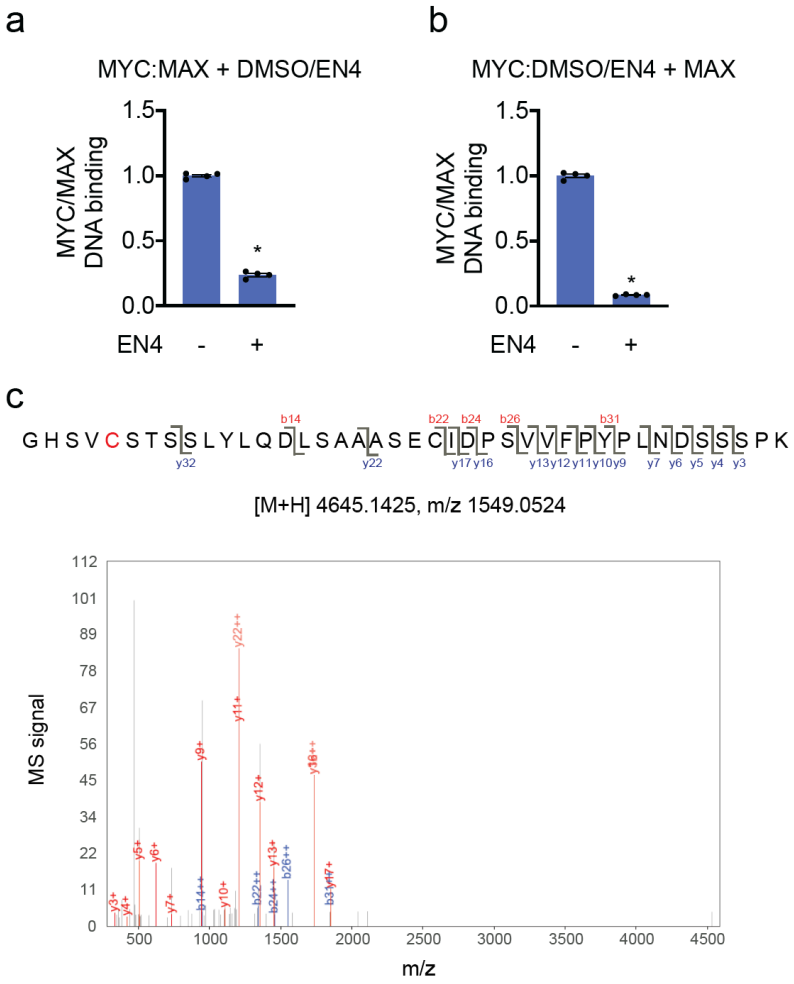


Figure S1. Related to Figure 1 and Figure 2. Characterization of EN4 inhibition of c-MYC/MAX DNA binding and interaction of EN4 with c-MYC. (a) Pure human c-MYC and MAX protein (0.2 μg each) were pre-incubated for 30 min at 37 ºC before addition of DMSO vehicle or EN4 (50 μM) for 30 min at 37 ºC prior to assessing DNA binding of the MYC/MAX complex to the E-box DNA consensus sequence. **(b)** Pure c-MYC (0.2 μg) was pre-incubated with DMSO vehicle or EN4 (50 μM) for 30 min at 37 ºC prior to adding MAX (0.2 μg) for 30 min at 37 ºC prior to assessing DNA binding of the MYC/MAX complex to the E-box DNA consensus sequence. **(c)** MS/MS analysis of EN4 adduct on MYC C171. Pure human full-length MYC was incubated with EN4 (50 μM) for 30 min. MYC was then subjected to tryptic digestion and tryptic digests were analyzed by LC-MS/MS. Shown is the EN4 adduct on C171 on a fully tryptic peptide of c-MYC. Data in **(a, b)** are shown as individual replicate values and average ± sem, n=4 biologically independent samples/group. Statistical significance was calculated with unpaired two-tailed Student's t-tests and is expressed as *p<0.05 compared to vehicle-treated controls.

Figure S2. Related to Figure 2. Sequence alignment of human JUN, c-MYC, N-MYC and L-MYC. Shown is a sequence alignment of human JUN, c-MYC, N-MYC, and L-MYC using CLUSTAL O (1.2.4). Highlighted in yellow C171 on c-MYC, which is not conserved between N-MYC and L-MYC. Highlighted in green is C99 of JUN and C117 of c-MYC.

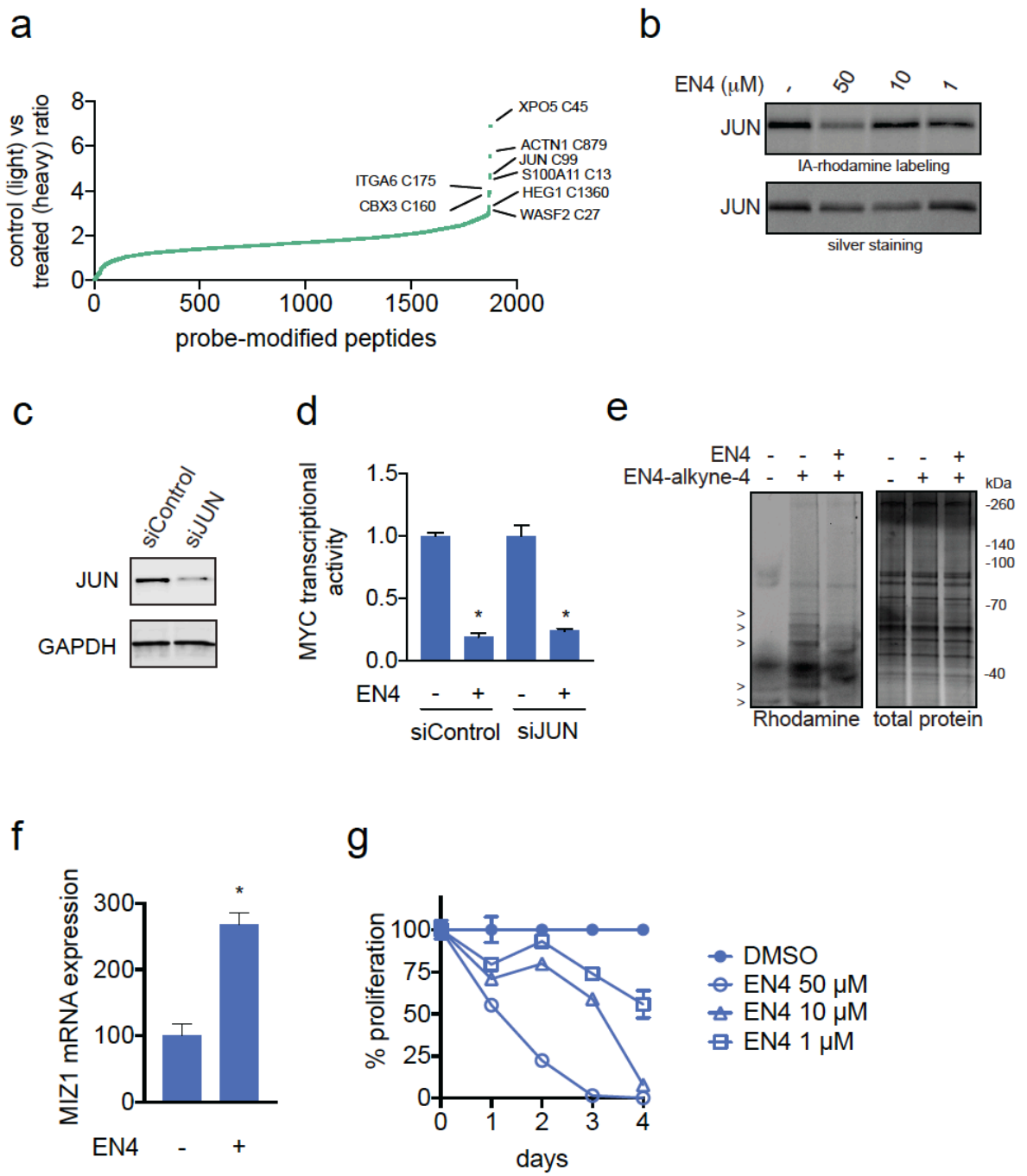


Figure S3. Related to Figures 3 and 4. Characterization of EN4 in cells. (a) isoTOP-ABPP analysis of EN4 in 231MFP breast cancer cells. 231MFP cells were treated with DMSO vehicle or EN4 (50 μM) for 4 h. Cell lysates were subsequently labeled with IA-alkyne for 1 h, followed by appendage of a TEV protease-cleavable biotin-azide linker bearing isotopically light (for control) or heavy (for EN4-treated) tags by CuAAC. Control and treated proteomes from each replicate were combined in a 1:1 ratio and subjected to avidin-enrichment, digestion by trypsin, and release of probe-modified peptides by TEV protease. Probe-modified peptides were analyzed by LC-LC-MS/MS and light to heavy ratios were quantified. Shown are those ratios with ratios >5 highlighted with the protein designation and cysteine number. Shown are average light/heavy ratios from n=3 biologically independent samples/group. **(b)** gel-based ABPP of EN4 against pure human JUN. Pure human JUN protein was pre-incubated with DMSO vehicle or EN4 for 30 min prior to labeling of protein with rhodamine-functionalized iodoacetamide (IA-rhodamine) (5 μM) for 30 min. Proteins were separated by SDS/PAGE and visualized by in-gel fluorescence and protein loading was visualized by silver staining. **(c)** JUN knockdown in HEK293T cells shown by Western blotting alongside loading control GAPDH in cells transfected with siControl or pooled siJUN oligonucleotides for 48 h. Shown is a representative gel of n=3 biological replicates/group. **(d)** MYC luciferase reporter activity in HEK293T cells transiently transfected with siControl

versus siJUN oligonucleotides treated with DMSO vehicle or EN4 (10 μM) for 24 h. Values are reported relative to vehicle-treated controls in each group. **(e)** EN4-alkyne-4 labeling of 231MFP cells. 231MFP cells were pre-treated with DMSO vehicle or EN4 (50 μM) for 2 h prior to treatment with DMSO or EN4-alkyne-4 (1 μM) for 4 h. Resulting cell lysates were subjected to CuAAC to append rhodamine-azide to EN4-alkyne-4 labeled proteins. Proteins were separated by SDS/PAGE and visualized by rhodamine fluorescence (left) or for total protein by Simple Blue staining (right). Shown are representative gels from n=3 biological replicates/group. Arrows show EN4-alkyne-4 labeled proteins that are competed by EN4 treatment. **(f)** MIZ1 mRNA expression. 231MFP breast cancer cells were treated with DMSO vehicle or EN4 (50 μM) and MIZ mRNA expression was assessed by qPCR. **(g)** 231MFP cell proliferation in cells treated with DMSO vehicle or EN4 once per day daily over 4 days, assessed by Hoechst staining. Data normalized to daily DMSO controls are shown. Data shown in **(d, f, g)** are average ± sem, n=6 for **(d),** n=3 for **(f),** and n=5-6 for **(g)** biologically independent samples/group. Statistical significance was calculated with unpaired two-tailed Student's t-tests and is expressed as *$p<0.05$ compared to vehicle-treated controls.

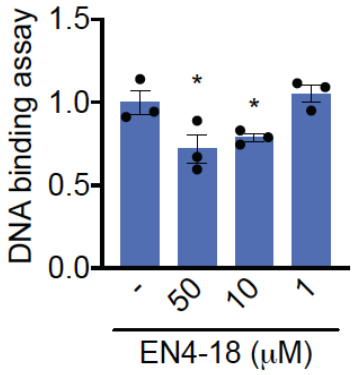


Figure S4. Related to Figure 4 and Table S4. Characterization of EN4-18 in DNA binding assays. Screening a cysteine-reactive covalent ligand library *in vitro* for compounds that would inhibit MYC/MAX binding to its E-box DNA consensus sequence. DMSO vehicle or EN4-18 were pre-incubated with pure human full-length MYC protein for 30 min before direct addition of MAX and then addition to DNA binding plates for 1 h. Data are shown as ratio relative to DMSO vehicle treated controls set to 1. Data shown are average ± sem, n=3 biologically independent samples/group. Statistical significance was calculated with unpaired two-tailed Student's t-tests is expressed as *p<0.05.