

# Supplementary Materials for

## **Trivalent PROTACs enhance protein degradation through cooperativity and avidity**

Satomi Imaide, Kristin M. Riching, Vesna Vetma, Claire Whitworth, Scott J. Hughes, Nicole Trainor, Sarah D. Mahan, Nancy Murphy, Kwok-Ho Chan, Andrea Testa, Chiara Maniaci, Marjeta Urh, Danette L. Daniels\*, Alessio Ciulli\*

\*Correspondence should be addressed to A.C. ([a.ciulli@dundee.ac.uk](mailto:a.ciulli@dundee.ac.uk)) and D.L.D. ([danette.daniels@promega.com](mailto:danette.daniels@promega.com))

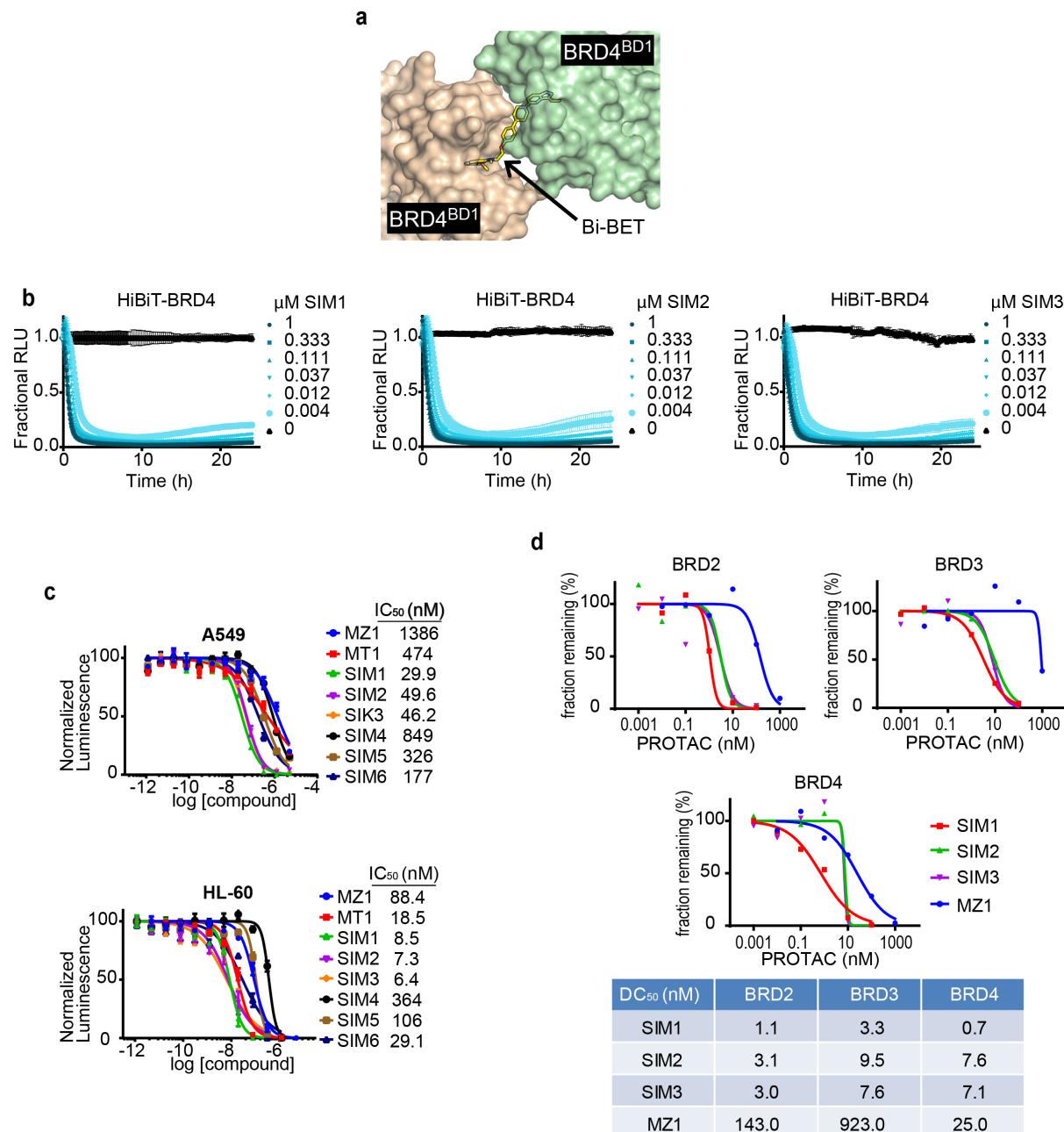
### **This PDF includes:**

Extended Data Figures and Tables  
Supplementary Note (Chemistry Methods)  
NMR spectra

### **Other Supplementary Materials for this manuscript include:**

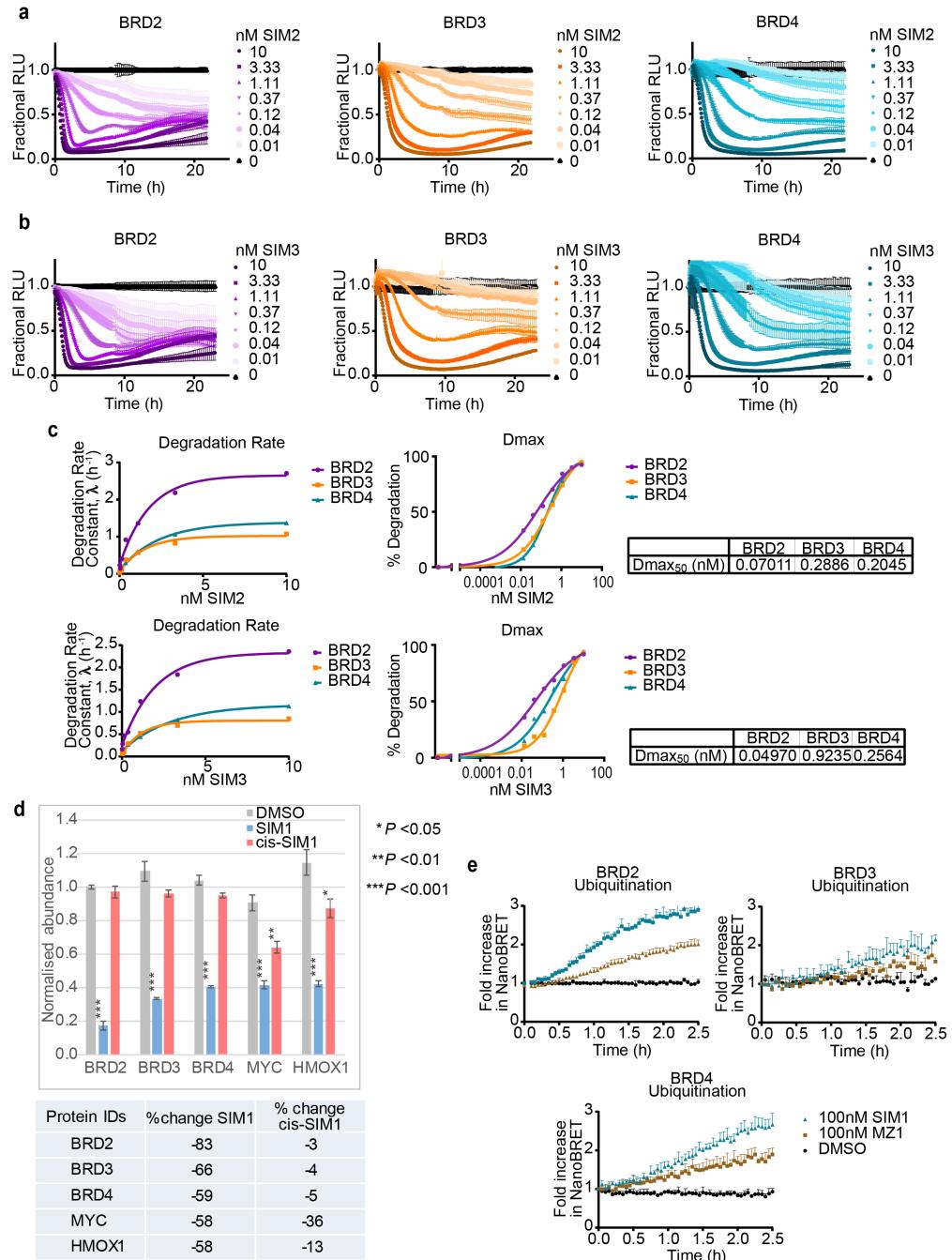
Extended Data Set (TMT labelling mass-spec proteomics)

## Extended Data Figures and Tables



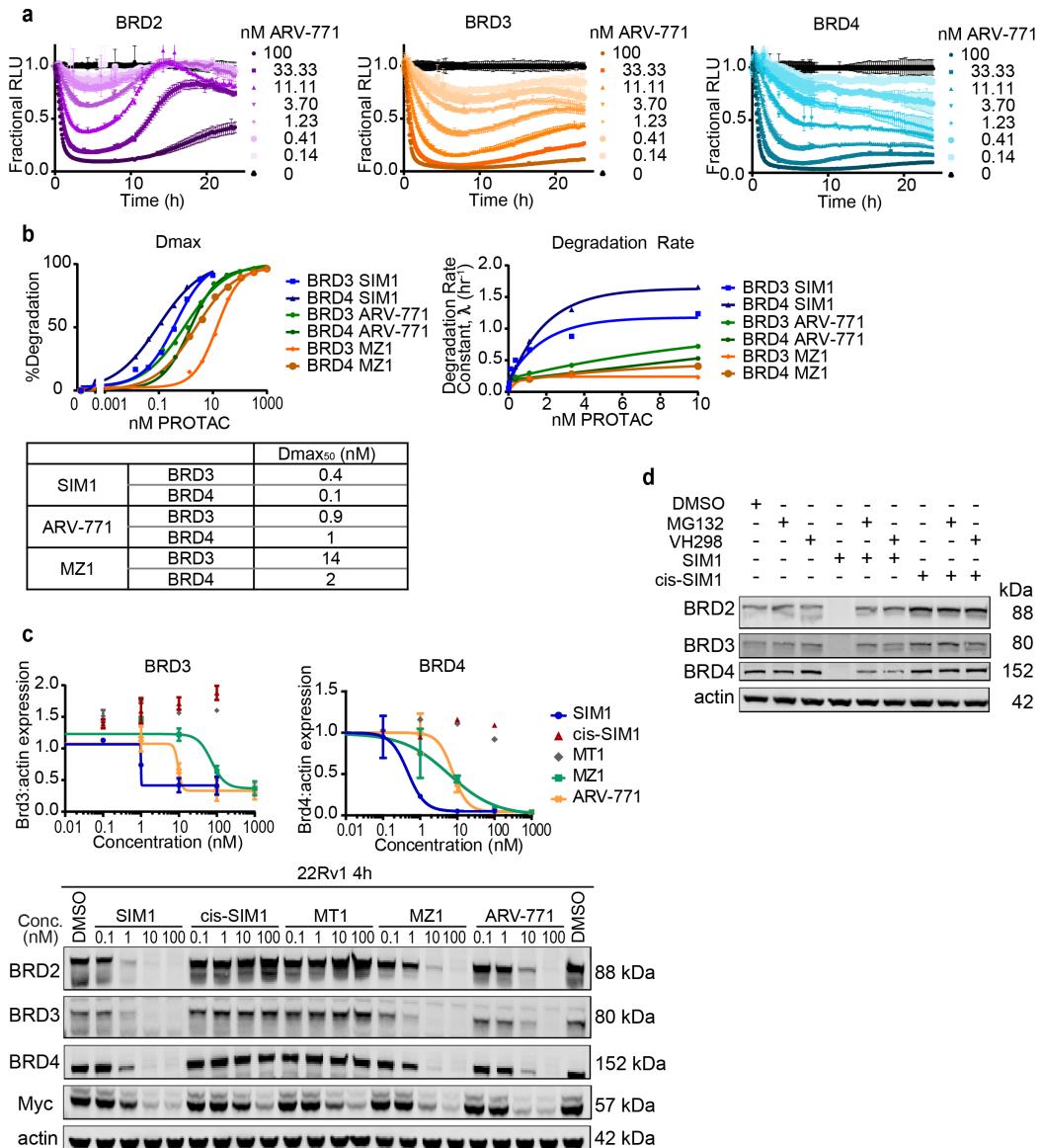
### Extended Data Fig. 1 (related to Fig. 1)

**a)** Inspection of tertiary complex crystal structures BRD4<sup>BD1</sup>:Bi-BET:BRD4<sup>BD1</sup> (PDB: 5ad3) shows the bivalent inhibitor buried inside the protein interface, suggesting derivatization would impair the binding mode. **b)** Quantitative live-cell degradation kinetics of CRISPR HiBiT- BRD4 HEK293 cells stably expressing LgBiT following treatment with serially diluted SIM1-3 in quadruplicates. Luminescence (RLU) was continuously monitored in 5-15 min intervals over a 24 h time period and fractional RLU was determined by normalization to DMSO control. **c)** Cell viability of A549 lung carcinoma cell line or HL-60 AML cell line following treatment with PROTACs or DMSO for 48 h in three replicates for each concentration point. **d)** Quantification of each BET protein levels relative to DMSO control of IB data from HEK293 cells of **Fig. 2a**.



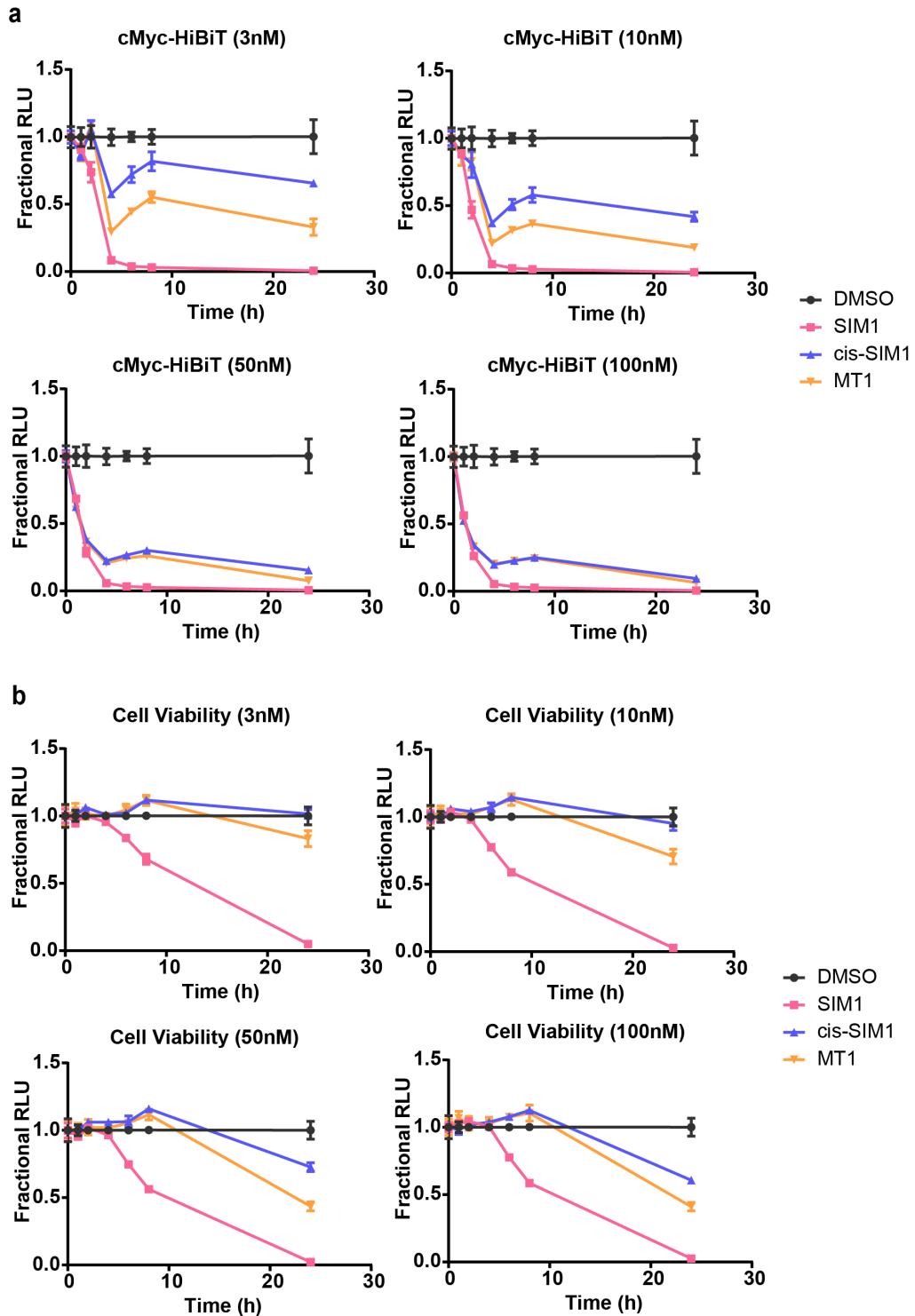
### Extended Data Fig. 2 (related to Fig. 2)

**a, b)** Quantitative live-cell degradation kinetics of CRISPR HiBiT-BRD2, BRD3, and BRD4 HEK293 cells stably expressing LgBiT following treatment with serially diluted SIM2 or SIM3 in quadruplicate. Luminescence (RLU) was continuously monitored in 5-15 min intervals over a 24 h time period and fractional RLU was determined by normalization to DMSO control. **c)** Calculation of degradation rate, degradation maximum (Dmax), and Dmax<sub>50</sub> values for SIM2 (upper set) and SIM3 (lower set) from SIM2 and SIM3 kinetic profiles shown in **Extended Data Fig. 2a** or **2b**, respectively. **d)** Quantification of representative proteins of mass spectrometry proteomics on **Fig. 2d**. **e)** NanoBRET ubiquitination kinetics of HiBiT-BET HEK293 cells expressing LgBiT and HaloTag-Ubiquitin following 100nM SIM1 or MZ1 or DMSO treatment in quadruplicate. Values are expressed as fold increase over DMSO control.



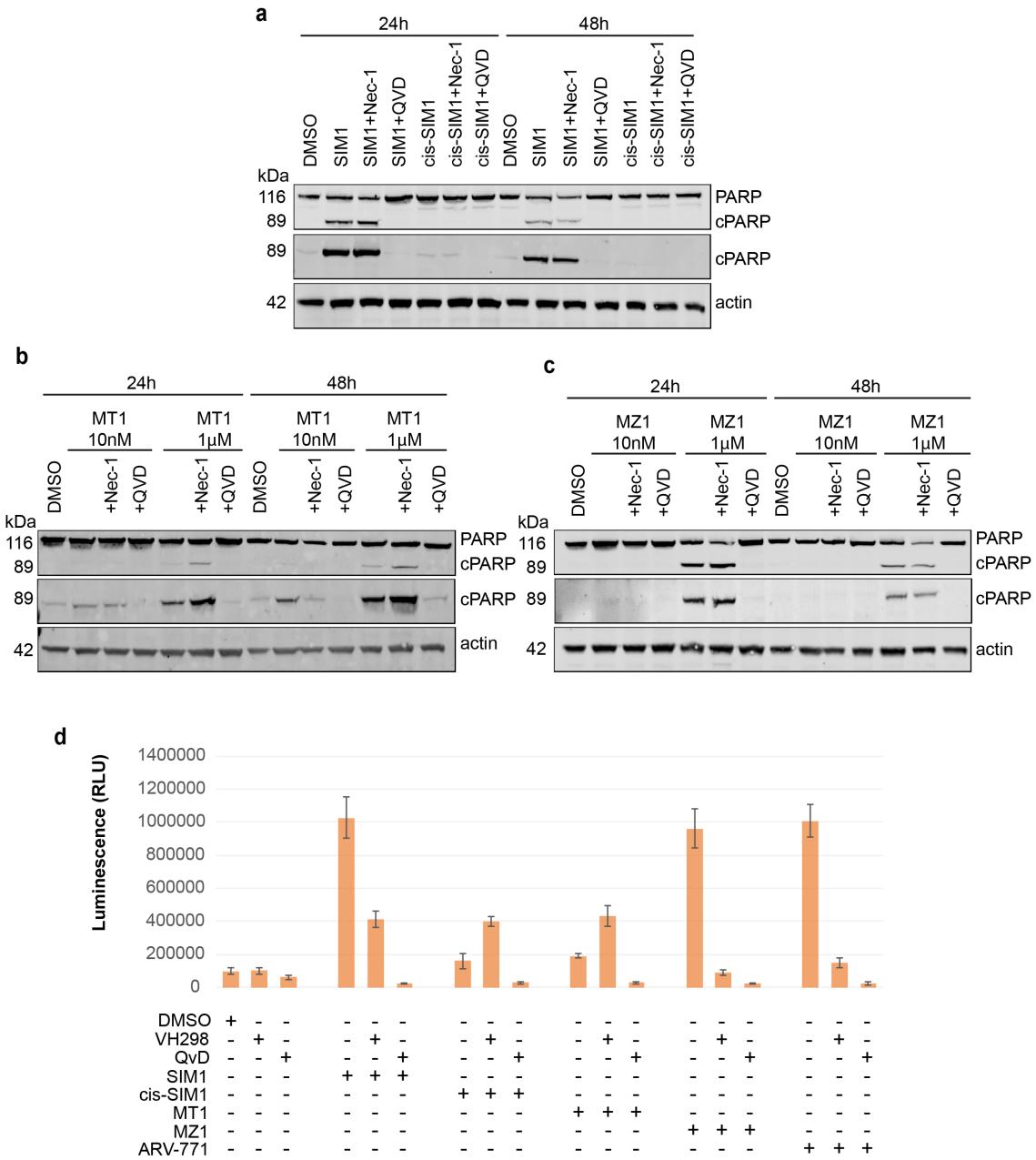
### Extended Data Fig. 3 (related to Fig. 3)

**a)** Quantitative live-cell degradation kinetics of CRISPR HiBiT-BRD2, BRD3, and BRD4 HEK293 cells following treatment with serially diluted ARV-771 in quadruplicates. Luminescence (RLU) was continuously monitored in 5-15 min intervals over a 24 h time period and fractional RLU was determined by normalization to DMSO control. **b)** Comparison of BRD3 and BRD4 degradation rates, degradation maximum (Dmax), and Dmax<sub>50</sub> values from kinetic profiles of SIM1 (Fig. 2b), ARV-771 (Extended Fig. 3a), and the previously determined MZ1 (Riching et.al. *ACS Chem. Biol.* **2018**). **c)** (upper graph) Quantified expression levels of endogenous BRD3 and BRD4 in 22Rv1 prostate cancer cell line treated with compounds for 4 h. Curves are a best fit of means from two biologically independent experiments,  $\pm$ s.e.m. (lower image) Representative images of Western blots for Fig. 3b and upper graph. Full blots can be found in Extended Data Fig. 9. **d)** Immunoblots of endogenous BRD2, BRD3 and BRD4 in MV4;11 cells treated in presence of BRD degrader or inhibitor with/without proteasome inhibitor (MG132) or VHL ligand (VH298) for 4 h. Error bars indicate the mean values  $\pm$  S.D from two biologically independent experiments. SIM1 and cis-SIM1 were used at 100nM. MG132 was used at 3 $\mu$ M. VH298 was used at 10 $\mu$ M. Full blots can be found in Extended Data Fig. 10.



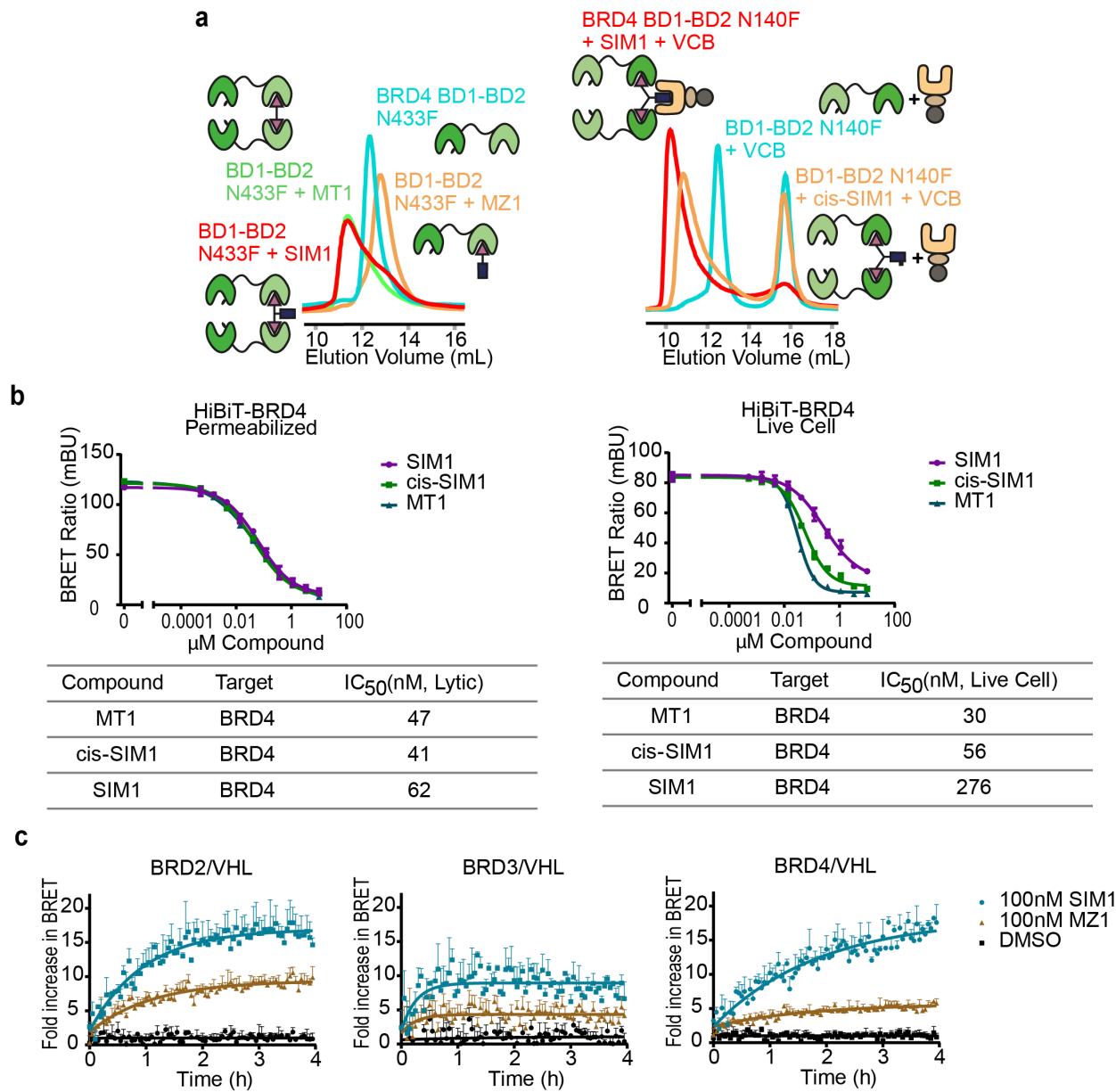
**Extended Data Fig. 4 (related to Fig. 3c)**

**a), b)** Loss in CRISPR cMyc-HiBiT protein levels (a) and correlative cell viability (b) in MV4;11 cells treated with 3nM, 10nM, 50nM and 100nM concentration of the indicated compounds in quadruplicate. Luminescence and cell viability by CellTiter-Glo were measured at various time points over 24 h and normalized to the DMSO control.



#### Extended Data Fig. 5 (related to Fig. 3d,e)

**a)** Immunoblot of PARP-cleavage in 22Rv1 cells with SIM1 or cis-SIM1 at 10nM for the indicated time point with/without the addition of caspase inhibitor (QVD-OPh, 20μM) or necroptosis inhibitor (Necrostatin-1, 20μM). **b)** Immunoblot of PARP-cleavage in 22Rv1 cells with MT1 at 10nM or 1μM for the indicated time point with/without the addition of caspase inhibitor (QVD-OPh, 20μM) or necroptosis inhibitor (Necrostatin-1, 20μM). **c)** Immunoblot of PARP-cleavage in 22Rv1 cells with MZ1 at 10nM or 1μM for the indicated time point with/without the addition of caspase inhibitor (QVD-OPh, 20μM) or necroptosis inhibitor (Necrostatin-1, 20μM). Full blots for panels (a-c) can be found in **Extended Data Fig. 8**. **d)** Caspase-Glo 3/7 assays treated in presence of BET degrader or inhibitor with/without caspase inhibitor (QVD-OPh) or VHL ligand (VH298) for 24 h in 22Rv1 cells. Error bars indicate the mean values ± S.D from three biologically independent experiments. SIM1, cis-SIM1 and MT1 were used at 100nM. MZ1 and ARV-771 were used at 1μM. VH298 was used at 10mM. QVD-OPh was used at 20μM.



**Extended Data Fig. 6 (related to Fig. 4)**

**a**) Size exclusion chromatography of complex formation after incubation of SIM1 (red, 25  $\mu$ M), MZ1 (orange, 25  $\mu$ M), MT1 (green, 25  $\mu$ M) or DMSO (cyan, 25  $\mu$ M) with 25  $\mu$ M BRD4 BD1-BD2 tandem protein (left panel: N433F mutant, right panel: N140F mutant with VCB protein). Intensity of peaks is absorbance 280nM. **b**) NanoBRET target engagement assays in quadruplicate with CRISPR HiBiT-BRD4 HEK293 cells stably expressing LgBiT performed in permeabilized and live cell formats. Cells were treated with a fluorescent BET tracer, then incubated with the indicated compounds at various concentrations to measure competitive displacement. IC<sub>50</sub> values for each compound are shown for both permeabilized and live cells. **c**) NanoBRET ternary complex kinetics of HiBiT-BET HEK293 cells expressing LgBiT and HaloTag-VHL following 100nM SIM1 or MZ1 or DMSO treatment in quadruplicates. Values are expressed as fold increase over DMSO control.

Figure 1d

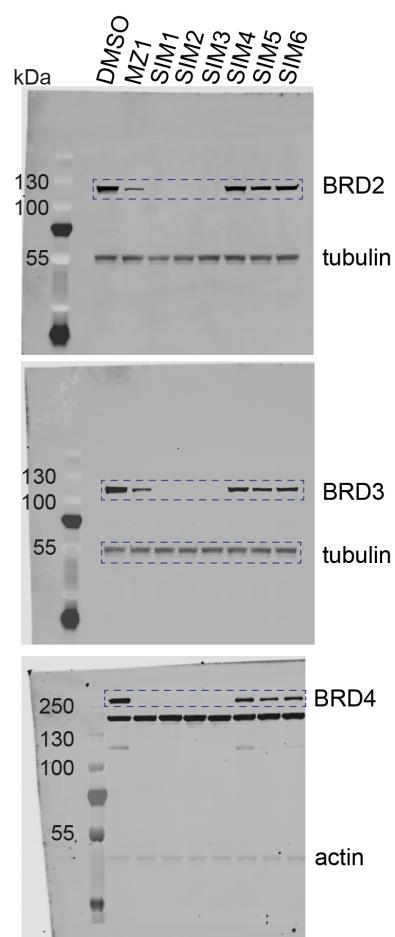
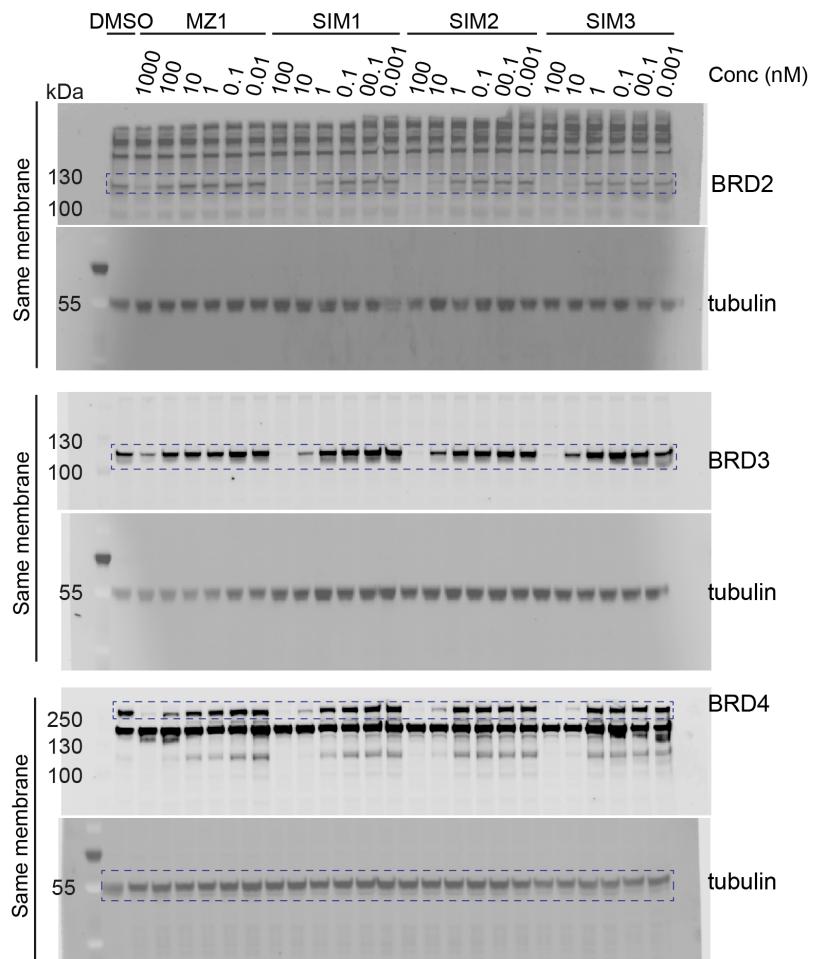
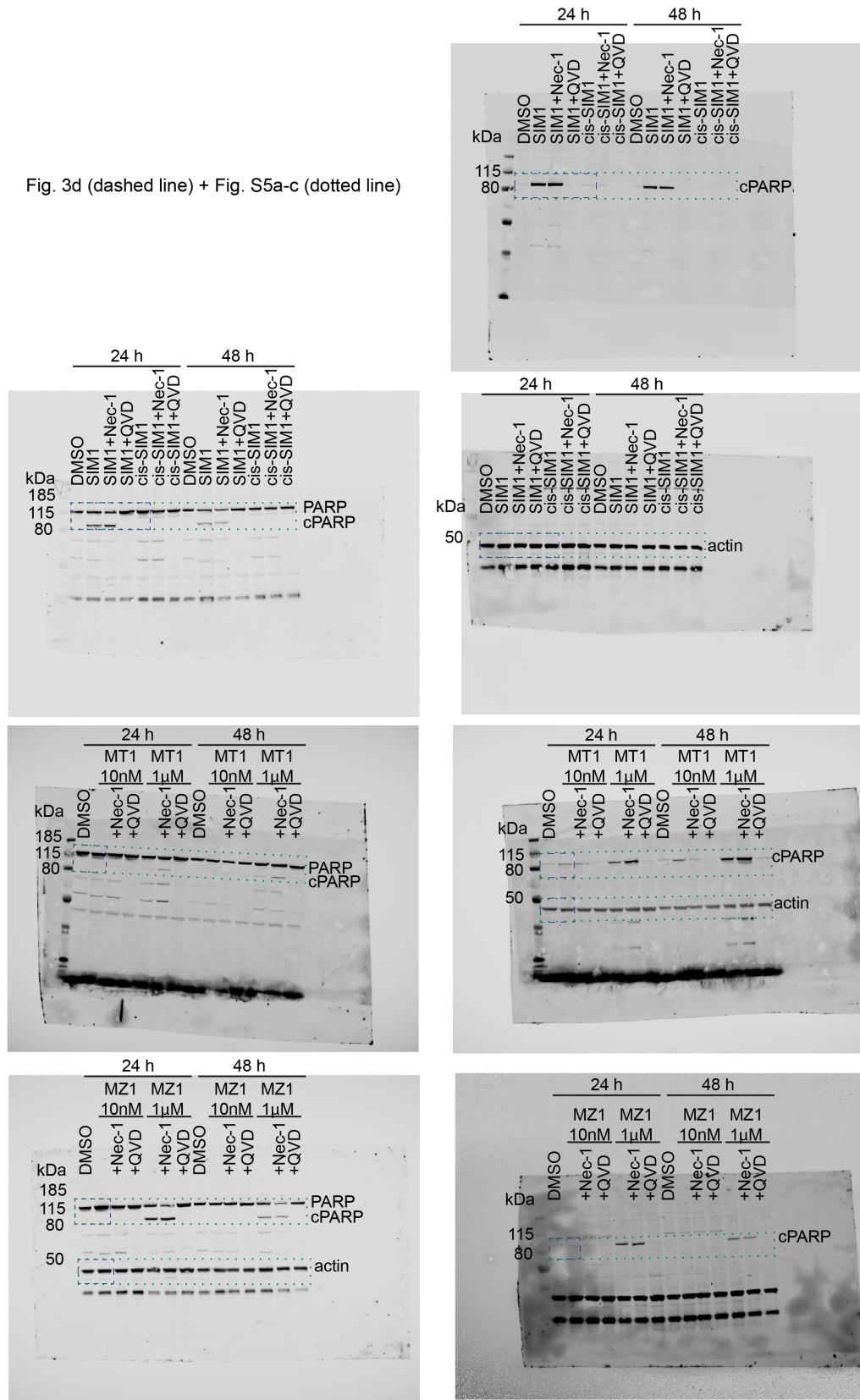


Figure 2a

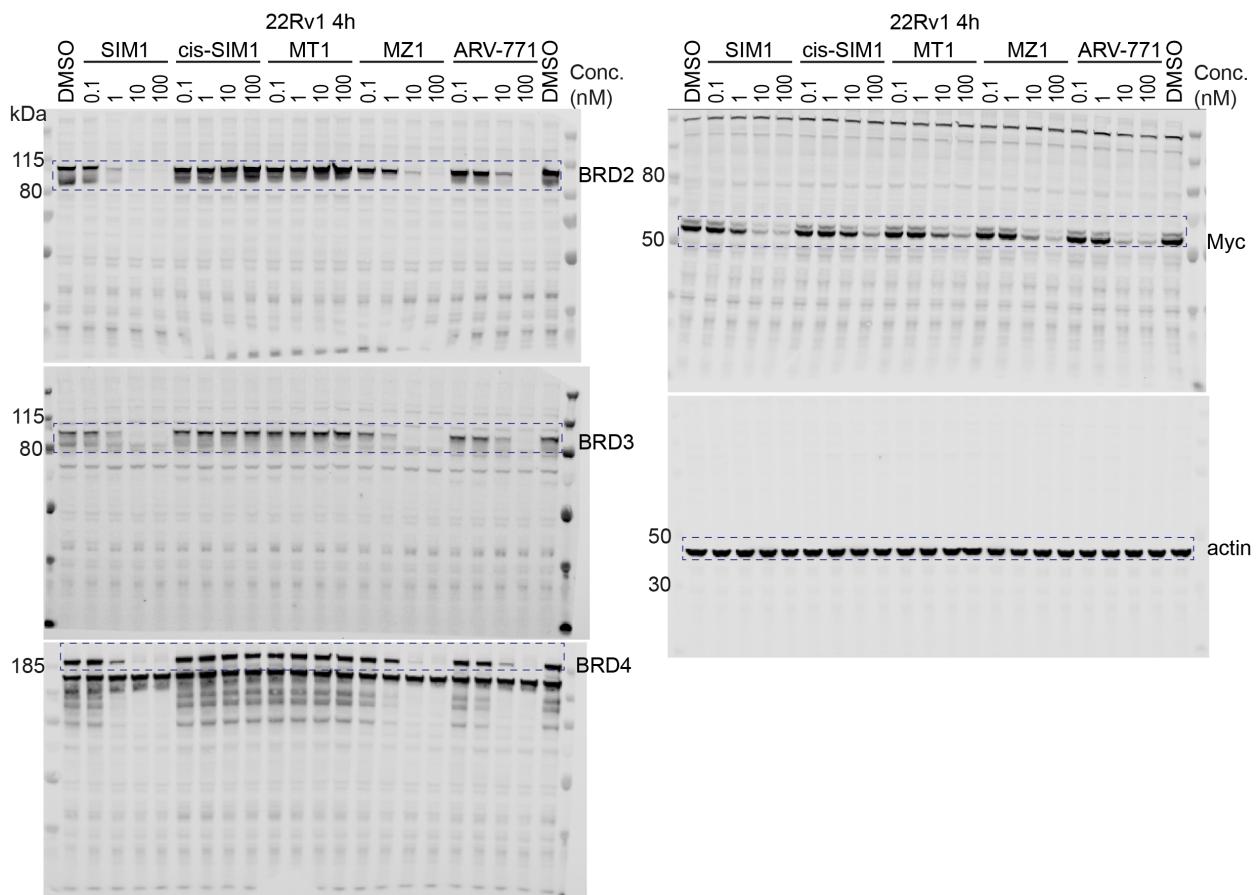


Extended Data Fig. 7. Full uncut gel images of western blot data (related to Fig. 1d and 2a)



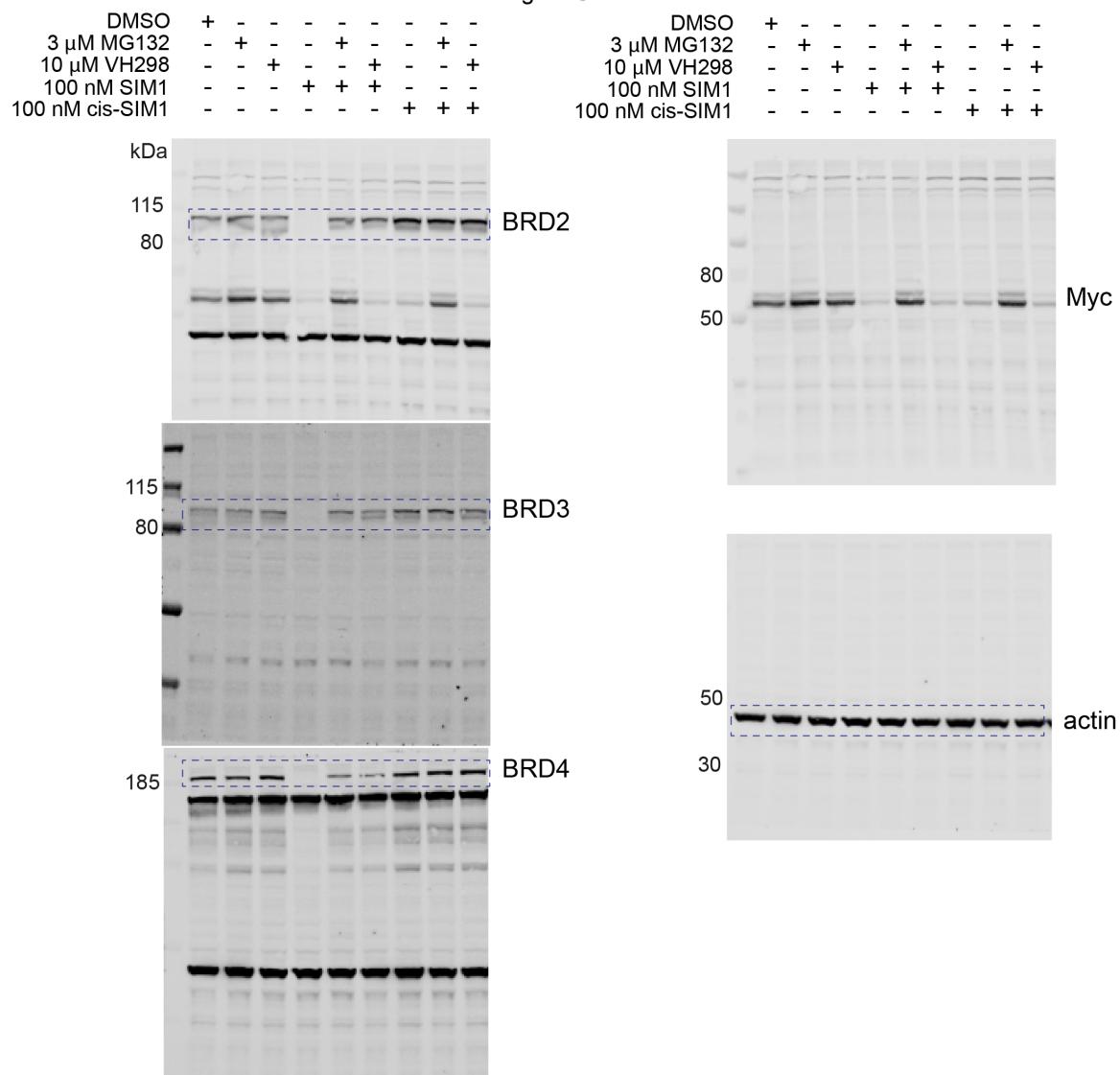
**Extended Data Fig. 8. Full uncut gel images of western blot data (related to Fig. 3d and Extended Data Fig. 5a-c)**

Figure S3c



**Extended Data Fig. 9. Full uncut gel images of western blot data (related to Extended Data Fig. 3c)**

Figure S3d



Extended Data Fig. 10. Full uncut gel images of western blot data (related to Extended Data Fig. 3d)

**Extended Data Table 1.** Experimental and fitted SPR data for SIM1 binary and ternary complex binding to immobilized VCB.

		Immobilized VCB (RU)	MW analyte (Da)	R <sub>max</sub> (1:1 complex)*	RU <sub>exp</sub>	% R <sub>max</sub> (1:1 complex)	t <sub>1/2</sub> (s)	K <sub>d</sub> (nM)
SIM1	replicate 1	1315	1619	48.6	37.3	76.7	n.d.	n.d.
SIM1 + BRD4 WT BD1-BD2 tandem	replicate 1	37.3	53611	45.66	29.8	65.3	117	57
	replicate 2	80.4	53611	98.43	65.7	66.7	141	52
	replicate 3	126	53611	154.25	96	62.2	99	50

\* Calculated based on MW of VCB protein = 43,790 Da.