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Plasticity in binding confers selectivity in ligand-induced protein degradation

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Supplementary Information

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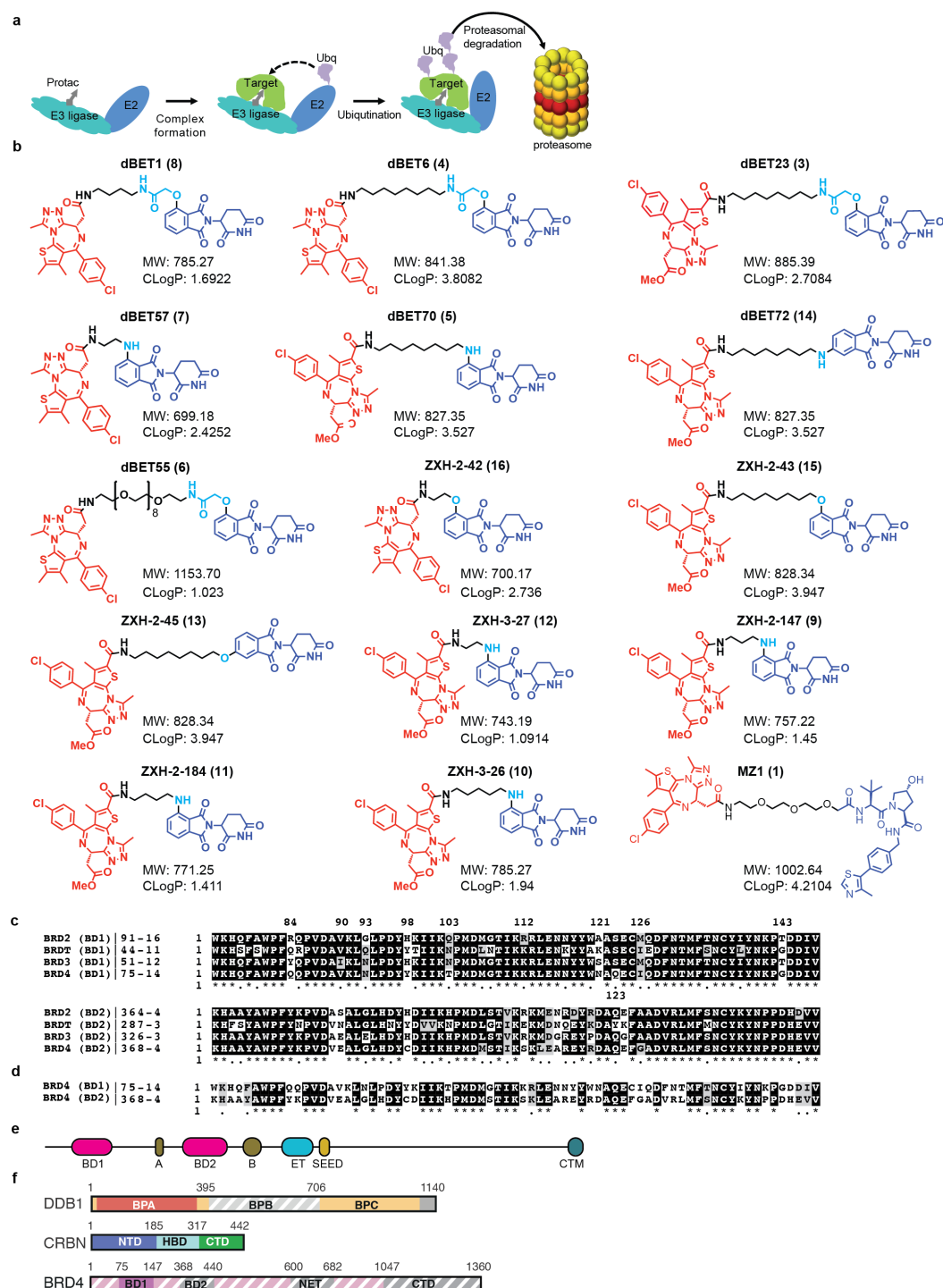
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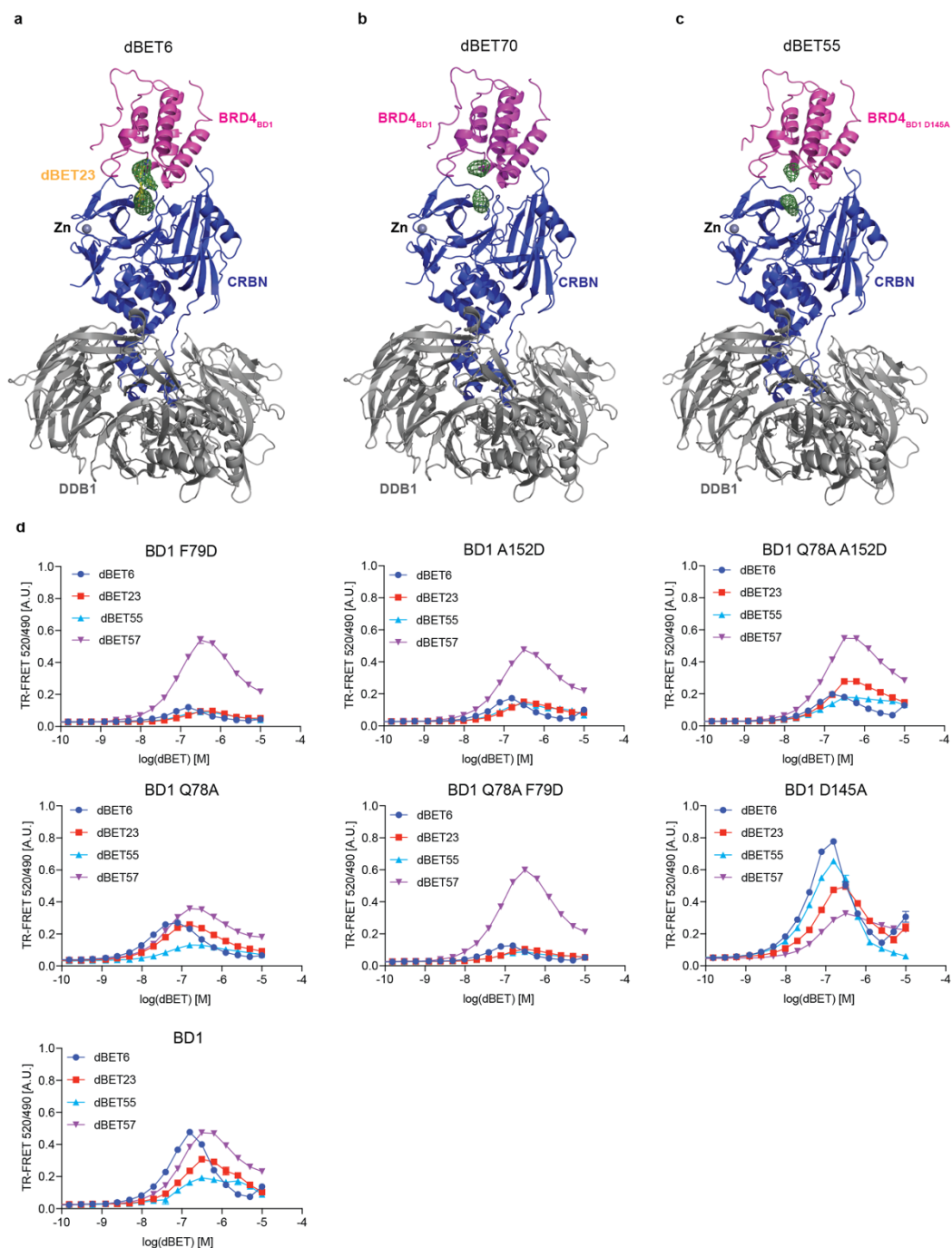


Supplementary Figure 1 | Structure of the DDB1 Δ B-CRBN-dBET23-BRD4_{BD1} complex.

(a) Schematic representation of heterobifunctional degrader (PROTAC) mediated degradation.

(b) Chemical structures, molecular weight and CLogP (calculated with ChemDraw Professional 16.0) for molecules used in this study. BET inhibitor JQ1-(S) colored in red, thalidomide moiety

colored in blue and the linker in black and cyan. **(c)** Multiple sequence alignment of BD1 and BD2 from different BET bromodomain paralogs, residue different between BRD2/3/4 are labelled, and **(d)** multiple sequence alignment of BD1 to BD2 from human BRD4. **(e)** Domain architecture of BRD4. A and B – DNA binding motifs, ET- extraterminal domain, SEED – Ser/Glu/Asp-rich region, CTM – C-terminal domain. **(f)** DDB1 Δ B, CRBN, and BRD4 domain coloring and construct boundaries referencing figure 1b. Hatched lines indicate sequences omitted from the crystallized protein construct.

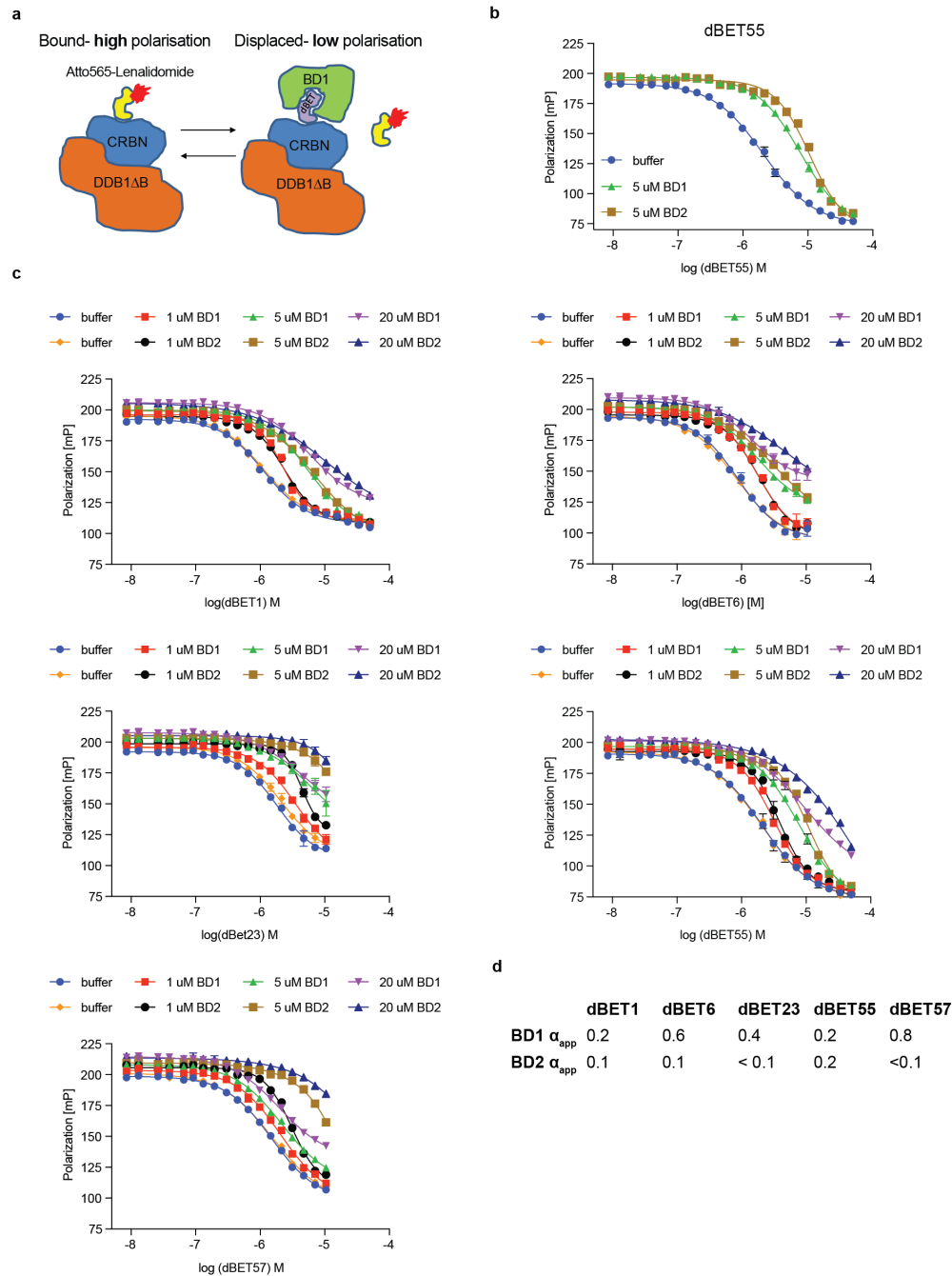


Supplementary Figure 2 | Structures of dBET6, dBET70 and dBET55 complexes.

(a) Cartoon representation of DDB1 Δ B-CRBN-dBET6-BRD4_{BD1}. The F_O-F_C map is shown as green mesh for dBET6 contoured at 4.0 σ . **(b)** Cartoon representation of DDB1 Δ B-CRBN-dBET70-BRD4_{BD1}. The F_O-F_C map is shown as green mesh for dBET70 contoured at 4.0 σ . **(c)** Cartoon representation of DDB1 Δ B-CRBN-dBET55-BRD4_{BD1/D145A}. The F_O-F_C map is shown as

green mesh contoured at 4.0σ . For **a**, **b** and **c** DDB1 is shown in grey, CRBN in blue, and BRD4_{BD1} (wild type and mutant) in magenta. **(d)** TR-FRET data underlying bar charts shown in Fig. 2a, 4a – d and Supplementary Fig. 5d – i. TR-FRET data in this figure are three independent replicates presented as means $\pm$ s.d. (n=3).

It should be noted that the structures presented here may only represent a snapshot of possible binding conformations. The weak nature of the protein-protein interactions, the non-evolved contacts and the limited constraints of poly-carbon/PEG linkers will often allow for alternative conformations. While the mutational signatures for dBET23 and dBET6 suggest that the observed structures represent a major binding mode in solution, the mutational signature for dBET70 suggests that crystal packing stabilizes the observed conformation for this ligand. While the conformation observed in the crystal structure is possible with the dBET70 ligand, it may not represent the dominant binding mode in solution.

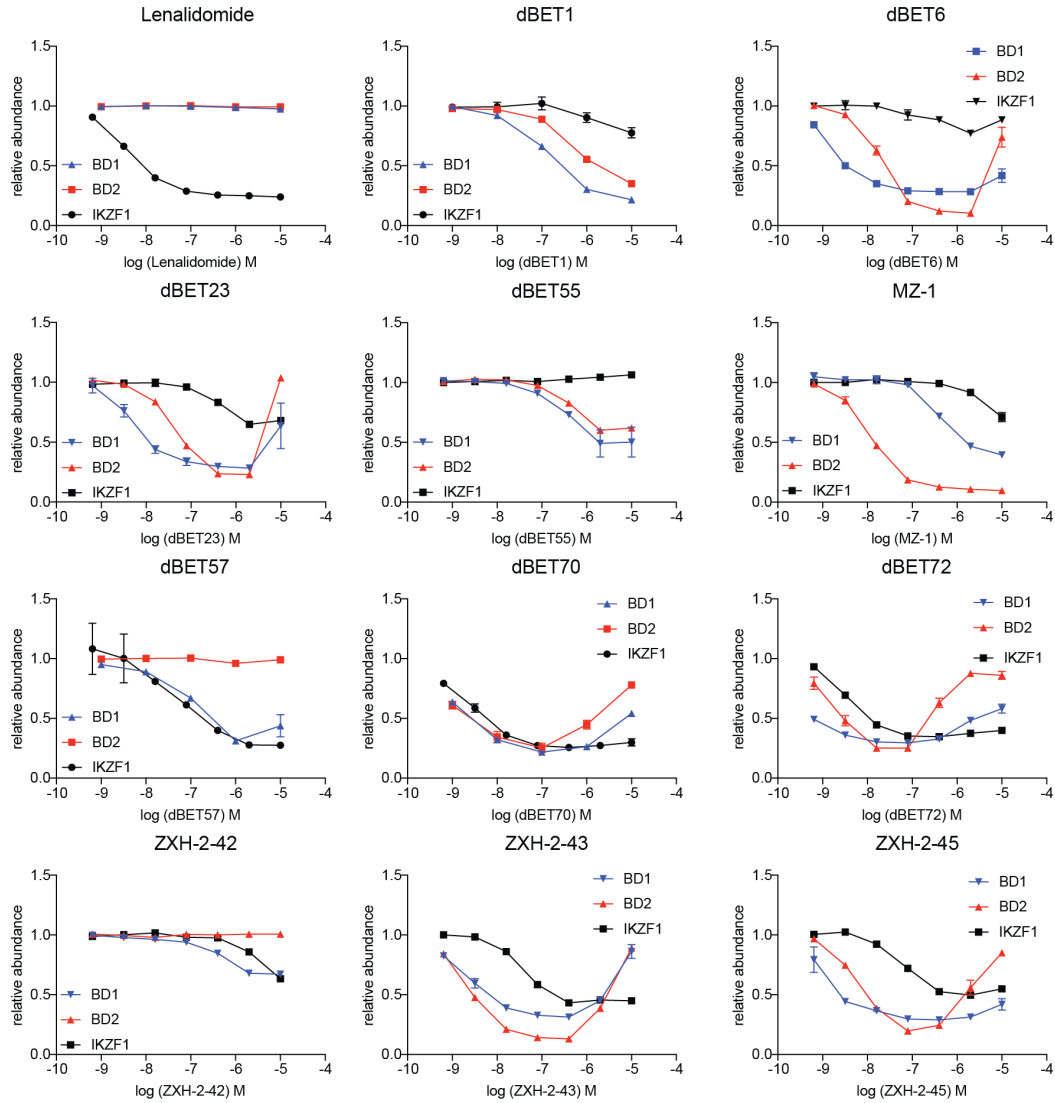


Supplementary Figure 3 | Negative cooperativity governs CRBN-dBET-BRD4 interactions.

(a) Schematic of fluorescence polarization based CRBN binding assay. Atto565-Lenalidomide fluorophore is displaced by degrader bound BRD4_{BD1/2}. **(b)** Fluorescence polarization competitive binding assay for dBET55 binding to DDB1ΔB-CRBN. Increasing concentrations of dBET55 titrated to preformed DDB1ΔB-CRBN-lenalidomide_{Atto565} complex in presence or

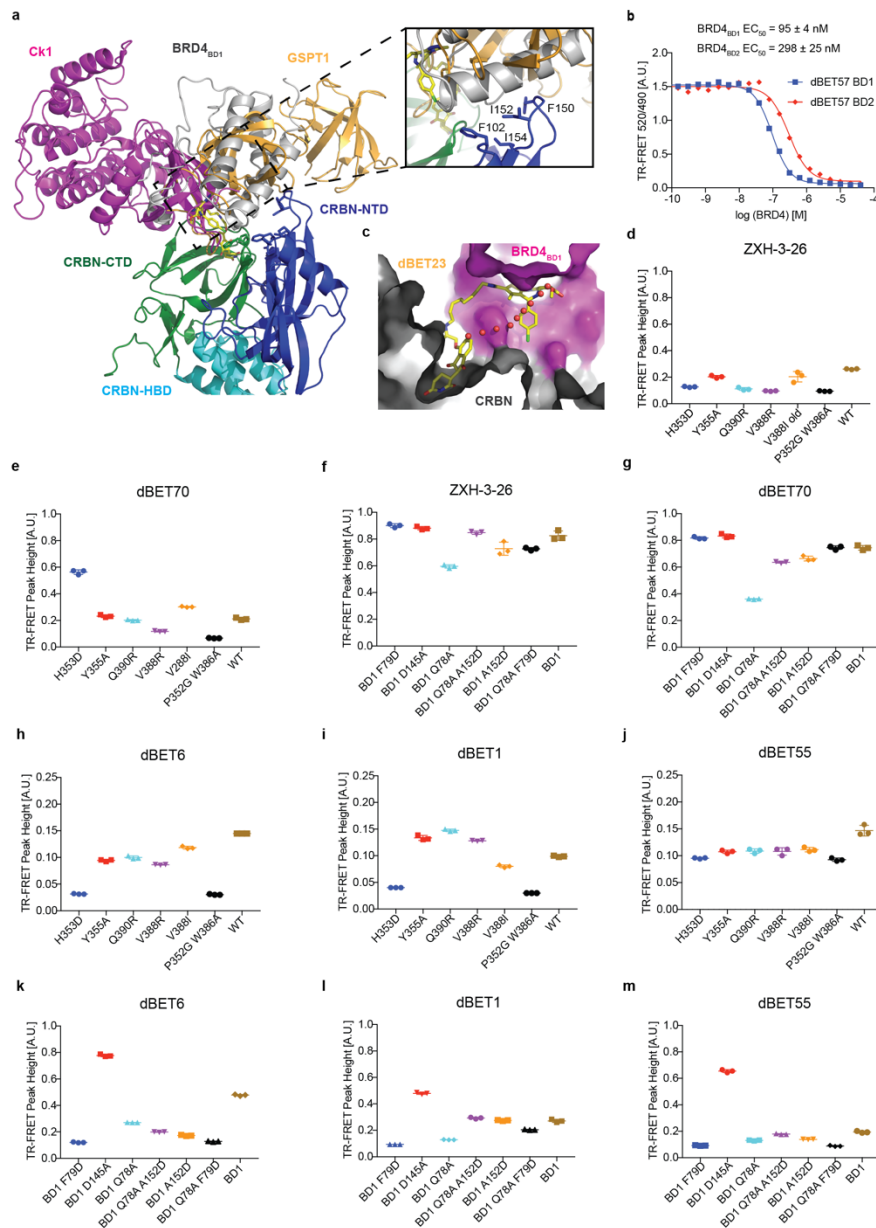
absence of BRD4_{BD1} or BRD4_{BD2}. Data is plotted as means $\pm$ s.d. from two independent replicates (n=2) for BD1 and BD2 and four independent replicates (n=4) for buffer control. **(c)** Fluorescence polarization competitive binding assay for dBET1, dBET6, dBET23, dBET55, and dBET57 binding to DDB1 Δ B-CRBN. Increasing concentrations of dBETs titrated to preformed DDB1 Δ B-CRBN-lenalidomide_{Atto565} complex in presence or absence of BRD4_{BD1} or BRD4_{BD2} at concentrations of 1 μ M, 5 μ M, and 20 μ M. The data at 5 μ M BRD4_{BD1/2} was replotted for Fig. 2c – f and panel b of this figure. Data is presented as means $\pm$ s.d. of two independent replicates (n=2). **(d)** summary of apparent cooperativity factors α_{app} .

a



Supplementary Figure 4 I | Cellular degradation of BRD4_{BD1/2}.

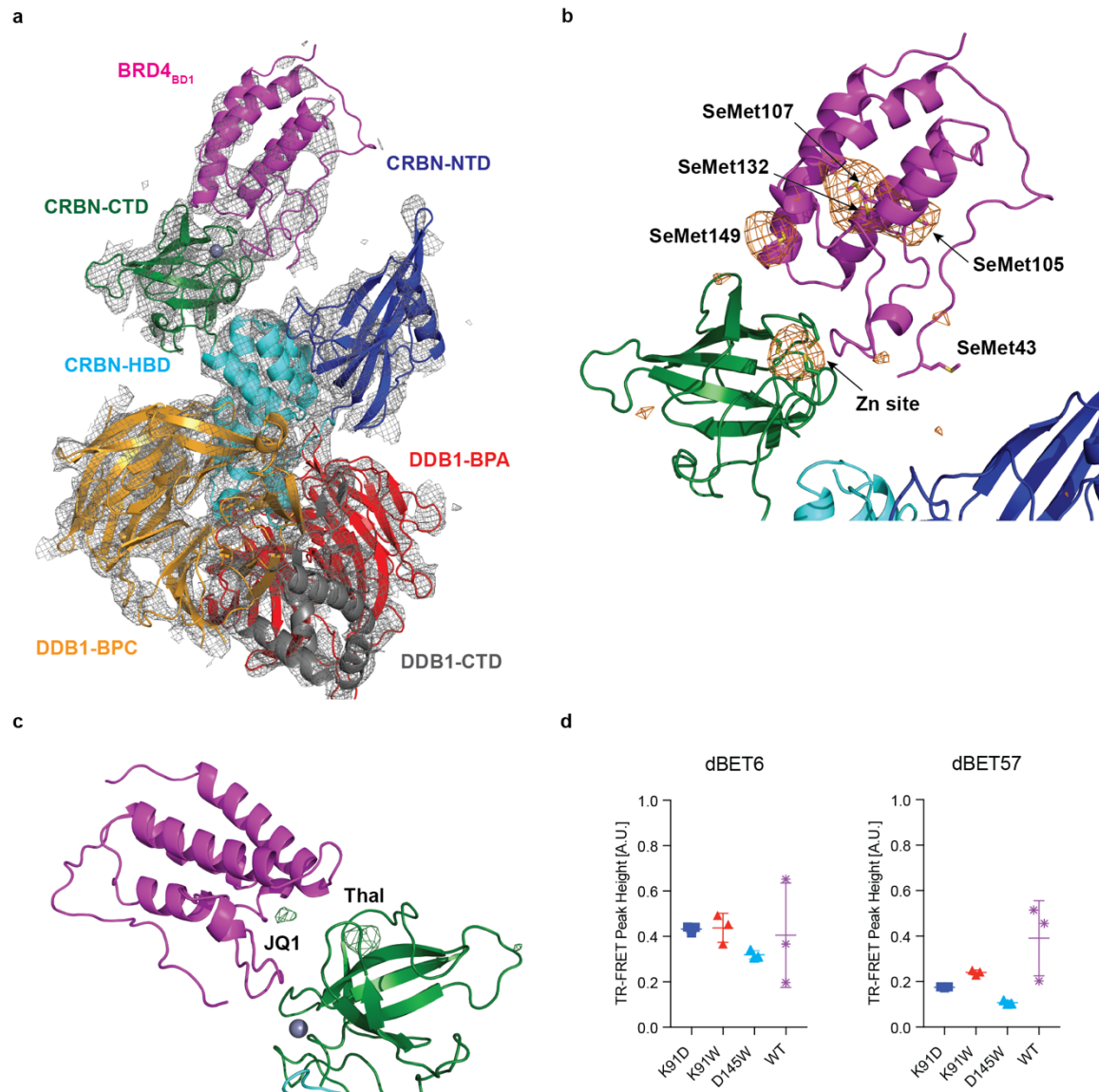
(a) Quantitative assessment of cellular degradation of a BRD4_{BD1}-EGFP/ BRD4_{BD2}-EGFP and IKZF1Δ-EGFP using flow cytometry analysis. Cells stably expressing BRD4_{BD1}-EGFP/ BRD4_{BD2}-EGFP or IKZF1Δ-EGFP with a mCherry reporter were treated with increasing concentrations of dBET1, dBET6, dBET23, dBET55, dBET57, dBET70, dBET72, MZ1, ZXH-2-42, ZXH-2-43, ZXH-2-45 and lenalidomide with the EGFP and mCherry signals quantified using flow cytometry analysis. Data is presented as means $\pm$ s.d. from three (six for lenalidomide) independent replicates.



Supplementary Figure 5 | Plasticity of CRBN-substrate interactions.

(a) CRBN utilizes different surfaces to interact with a variety with neo-substrates as illustrated by the superposition of DDB1ΔB-CRBN-dBET23-BRD4_{BD1}, DDB1ΔB-CRBN-lenalidomide-Ck1 α (pdb: 5fqd), and DDB1-CRBN-CC885-GSPT1 (pdb: 5hxb). Top right, close-up of the common hydrophobic interface between GSPT1-CRBN-NTD and BRD4_{BD1}-CRBN-NTD. **(b)** The structures of DDB1-CRBN-dBET23-BRD4_{BD1} and DDB1-CRBN-lenalidomide-CK1a suggest

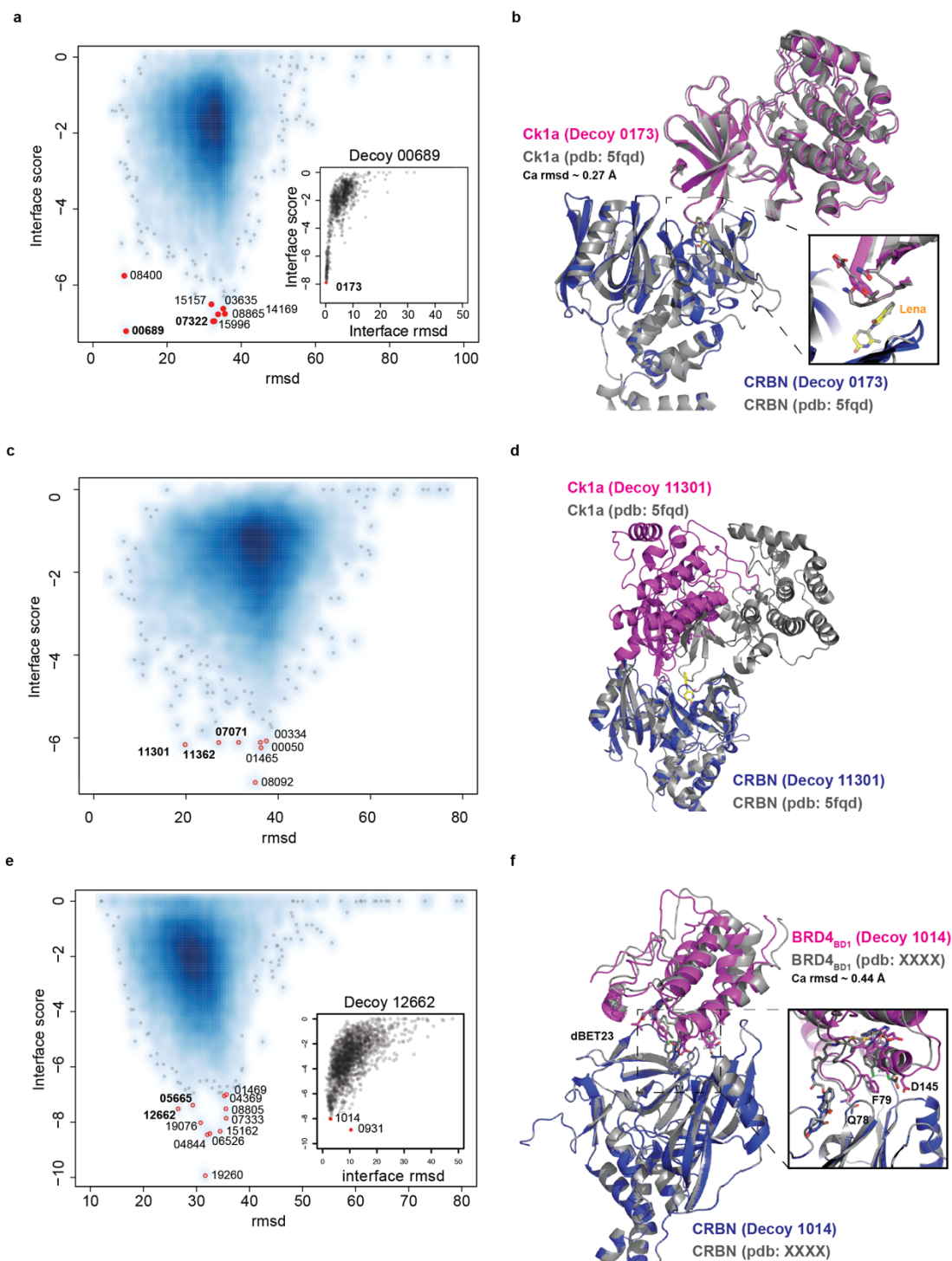
mutually exclusive binding of BRD4 with neo-substrates such as Ck1a or IKZF1/3, which is confirmed by titrating BRD4_{BD1} or BRD4_{BD2} into a preformed complex of DDB1-CRBN-dBET57-IKZF1Δ. Data is presented as mean and standard deviation of 10 technical replicates of a single experiment (n=1). **(c)** Surface representation of CRBN and BRD4_{BD1} of DDB1-CRBN-dBET23-BRD4_{BD1} crystal structure, showing dBET23 as stick representation. The hypothetical linker path from the acid position on JQ1 is shown with red spheres indicating the distance of a carbon-carbon bond and illustrating that the 2-carbon linker of dBET57 would be insufficient to bridge the gap. **(d)** TR-FRET. ZXH-3-26 degrader titrated to BRD4_{BD1}-SPYCATCHER-BODIPY and Terbium-antiHis antibody, and wild type or various mutants of His6-DDB1-His6-CRBN complex. The peak height of the dose response curve for three independent replicates was quantified and is depicted as dot-plot. TR-FRET data in this figure are independent replicates presented as means ± s.d. (n=3). **(e, h - j)** as in **d** but for dBET70, dBET6, dBET1 and dBET55, respectively. **(f)** TR-FRET. ZXH-3-26 degrader titrated to DDB1ΔB-CRBN_{SPYCATCHER-BODIPY}, Terbium-Streptavidin and wild type or mutants of BRD4_{BD1}-biotin. The peak height of the dose response curve for three independent replicates was quantified and is depicted as dot-plot. TR-FRET data in this figure are presented as means ± s.d. (n=3). **(g, k-m)** as in **f** but for dBET70, dBET6, dBET1 and dBET55, respectively.



Supplementary Figure 6 | Experimental validation of DDB1-CRBN-dBET57-BRD4_{BD1} structure.

(a) Cartoon representation of DDB1-CRBN-dBET57-BRD4_{BD1} complex with the 2F_O-F_C map contoured at 1.5 σ . Domains are colored as DDB1-BPA (red), DDB1-BPC (orange), DDB1-CTD (grey), CRBN-NTD (blue), CRBN-HBD (cyan), CRBN-CTD (green), and BRD4_{BD1} (magenta). We note that CRBN was found in a not previously observed conformation, in which the thalidomide binding CRBN-CTD domain translates and rotates away from the CRBN-HBD and

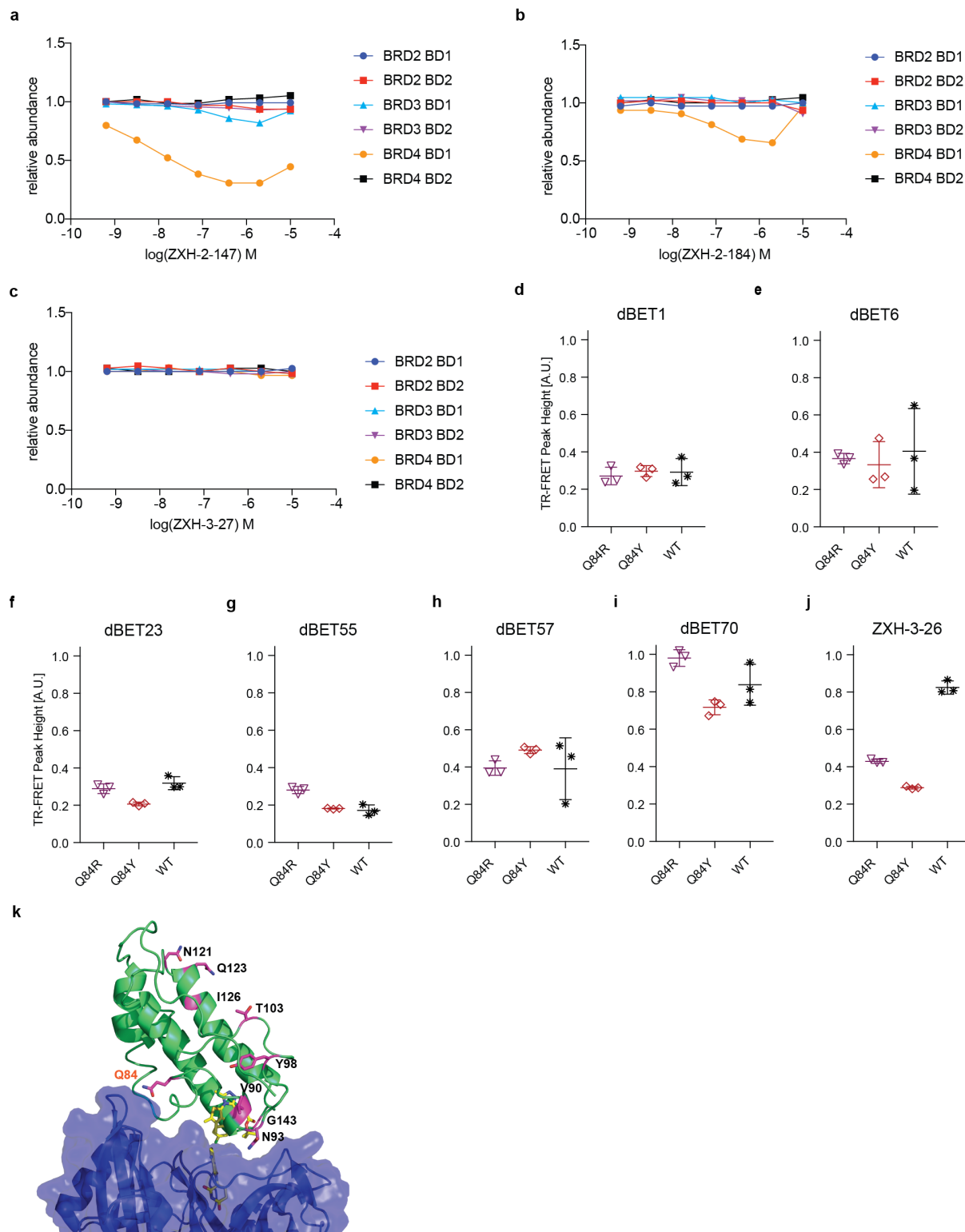
CRBN-NTD domains. This results in an open conformation that exposes large areas of CRBN that are typically buried. The high salt crystallization condition could be a driver of this structural rearrangement, and together with crystal contacts induce this conformation. We can, however, not exclude that this conformational dynamic is an intrinsic feature of CRBN to accommodate a variety of substrates and future studies are necessary to address this. Based on the compatibility of the observed BRD4_{BD1} binding conformation with the open and closed CRBN conformations, we believe that for the interpretation of our data the conformational change is negligible. **(b)** Cartoon representation of DDB1-CRBN-dBET57-SeMetBRD4_{BD1} complex. Anomalous difference map contoured at 3 σ shown in orange for data collected at the Se peak showing the position of the Se atoms and Zn. **(c)** $F_o - F_c$ map of native DDB1-CRBN-dBET57-BRD4_{BD1} contoured at 3.0 σ and shown in green, carved around the JQ1 and thalidomide sites. Positive difference density is observed for the Thalidomide (Thal) and JQ1 binding sites. **(d)** TR-FRET. dBET6 or dBET57 degrader titrated to DDB1 Δ B-CRBN_{SPYCATCHER-BODIPY}, Terbium-Streptavidin and wild type or mutants of BRD4_{BD1}-biotin. The peak height of the dose response curve for three independent replicates was quantified and is depicted as dot-plot. TR-FRET data in this figure are independent replicates presented as means $\pm$ s.d. (n=3).



Supplementary Figure 7 | Rosetta docking of CRBN-lenalidomide-Ck1 complex

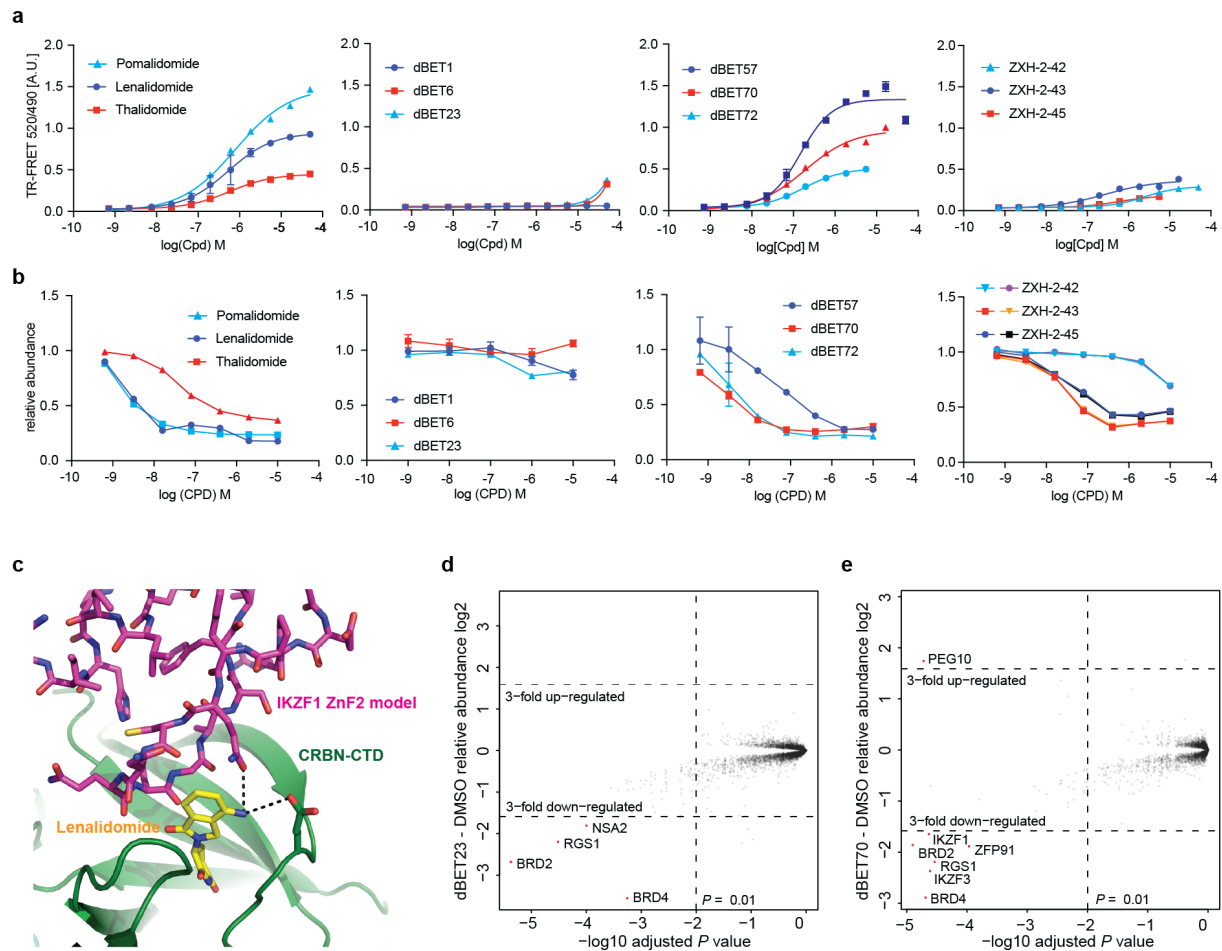
(a) Symmetric docking energy landscape for the binding of Ck1α to a CRBN-lenalidomide complex. Symmetric docking energy landscape for local perturbation docking experiments on a

lowest energy decoy 00689 is shown as insert. **(b)** Superposition of the DDB1 Δ B-CRBN-lenalidomide-Ck1 α structure (pdb: 5fqd) and the top solution, decoy 0173, from **a**. **(c)** Symmetric energy docking landscape for the binding of Ck1 α to a CRBN-lenalidomide complex. The conformer parameter file for lenalidomide was restricted to a conformer not favorable of Ck1 α binding. **(d)** Superposition of the DDB1 Δ B-CRBN-lenalidomide-Ck1 α structure (pdb: 5fqd) and the top solution from **c**. **(e)** Symmetric docking energy landscape for the binding of BRD4_{BD1} to a CRBN-lenalidomide complex. The two low energy decoys that exhibit a conformation compatible with dBET binding are indicated by bold numbers. The symmetric docking energy landscape for local perturbation docking experiments on decoy 12662 compatible with dBET mediated binding is shown as insert. **(f)** Superposition of the DDB1 Δ B-CRBN-dBET23-BRD4_{BD1} structure (pdb: 6bn7) and the top solution from local perturbation of decoy 12662.



Supplementary Figure 8 | Selective degradation of BRD4.

(a) Quantitative assessment of cellular degradation using EGFP/mCherry reporter assay. Cells stably expressing BRD4_{BD1}-EGFP (or constructs harbouring BRD2_{BD1}, BRD2_{BD2}, BRD3_{BD1}, BRD3_{BD2}, BRD4_{BD2}) and mCherry were treated with increasing concentrations of ZXH-2-147 and the EGFP and mCherry signals followed using flow cytometry analysis. **(b)** as in **a** but for ZXH-2-184. **(c)** as in **a** but for ZXH-3-27. Data in **a-c** are singlicate experiments (n=1). **(d)** TR-FRET. dBET1 degrader titrated to DDB1ΔB-CRBN^{SPYCATCHER-BODIPY}, Terbium-Streptavidin and wild type or mutants of BRD4_{BD1}-biotin. The peak height of the dose response curve for three independent replicates was quantified and is depicted as dot-plot. TR-FRET data in this figure are presented as means ± s.d. (n=3). **(e-j)** as in **d** but for dBET6, dBET23, dBET55, dBET57, dBET70 and ZXH-3-26 respectively. **(k)** Cartoon representation of docking pose from cluster 19 (see Figure 5) serving as a rationale for design of ZXH-3-26. BRD4_{BD1} shown in green and CRBN in blue. Highlighted residues of BRD4 different between BRD2/3. Residue Q84 (R in BRD2, Y in BRD3) highlighted in orange.

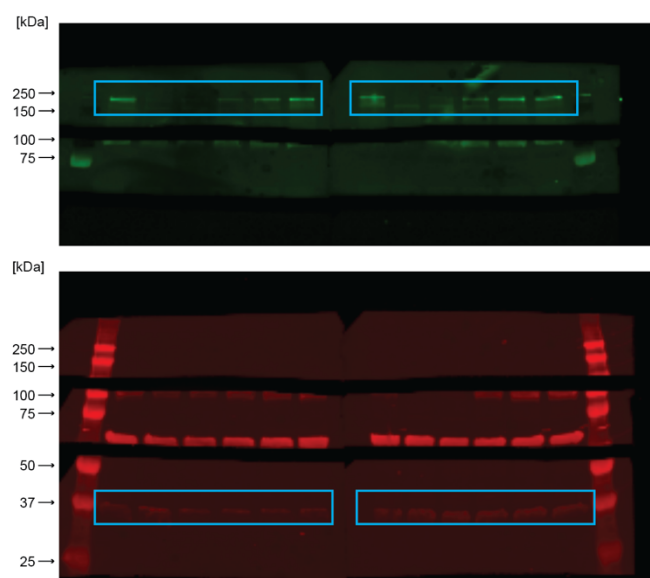


Supplementary Figure 9 | Co-degradation of IMiD neo-substrates such as IKZF1/3.

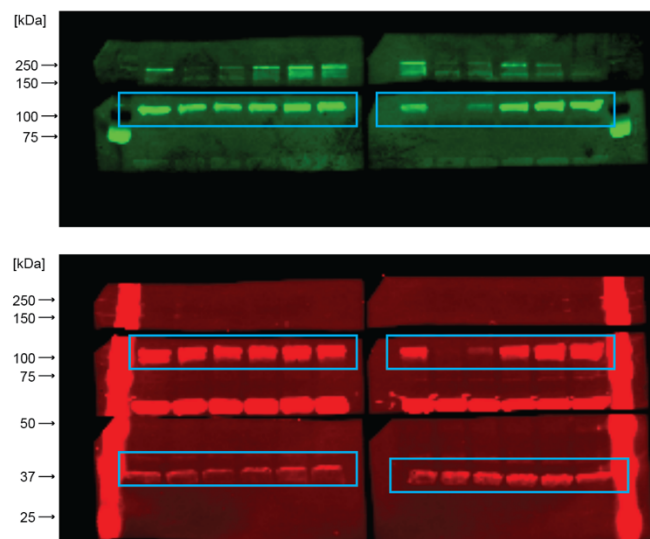
(a) TR-FRET. Titration of the indicated molecules to DDB1 Δ B-CRBN_{SPYCATCHER}-BODIPY, Terbium-streptavidin and IKZF1 Δ _{biotin}. Data in this figure are presented as means $\pm$ s.d. from three independent replicates (n=3). **(b)** Quantitative assessment of cellular degradation of a IKZF1-EGFP reporter using flow cytometry analysis. Cells stably expressing IKZF1 Δ -EGFP and mCherry were treated with increasing concentrations of the indicated molecules and the EGFP and mCherry signals followed using flow cytometry analysis. Data in this figure are presented as means $\pm$ s.d. from four cell culture replicates (n=4). **(c)** Model of a CRBN-IKZF1_{ZnF2} complex (adapted from Petzold et al., 2016) bound to lenalidomide. Potential hydrogen bonds are indicated as dashed lines. **(d)** Scatter plot depicting the fold changes in relative abundance comparing dBET23 to DMSO control treatment (MM.1s) determined using quantitative

proteomics. Negative false discovery rate adjusted *P* Values are shown on the x-axis and log₂ fold changes on the y-axis. Data shown are of three cell culture replicates measured in a single 10-plex TMT experiment. *P* Values were derived from a moderated *t*-statistic using the limma package¹ and corrected for multiple hypothesis testing (see **methods** for details). **(e)** as in **d** but for dBET70 to DMSO control. This data shows that certain degraders are capable of directly inducing binding of IKZF1/3 (and other IMiD targets) to CRBN, while others exhibit reduced or absent co-degradation of zinc-finger targets. In particular, we found that a secondary amine in the C4 position of the phthalimide (dBET57/70/72) leads to effective degradation of IKZF1/3 (and other IMiD targets), while replacing with an oxygen-ether linker (ZXH-2-42/43/45) reduces such activity, and using an oxy-acetamide linker (dBET1/6/23) fully prevents degradation of zinc finger targets. Thus, altering linker composition can be used as a strategy to tune co-degradation of IKZF1/3 and other IMiD targets.

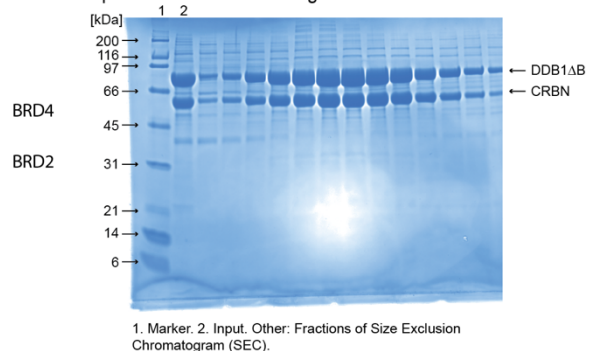
Source data to Fig. 6g



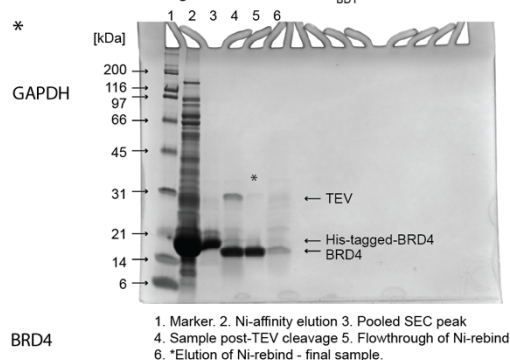
Source data to Fig. 6h



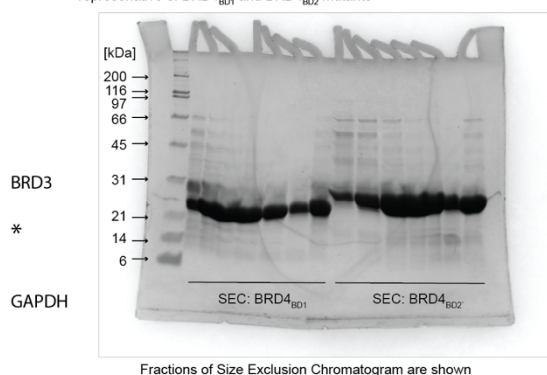
Representative SDS-PAGE gel of CRBN-DDB1ΔB



BRD3 SDS-PAGE gel of SeMet BRD4_{BD1}



SDS-PAGE gel of biotinylated BRD4_{BD1} and BRD4_{BD2} representative of BRD4_{BD1} and BRD4_{BD2} mutants



Supplementary Figure 10 | Uncropped Immunoblots.

Boxed areas correspond to image regions represented in the indicated main text and Supplementary figures. Western blots have been flipped vertically to represent increasing concentrations of Compound. SDS-PAGE gel images for representative preparations of DDB1ΔB-CRBN, SeMet-BRD4_{BD1}, biotinylated BRD4_{BD1} and biotinylated BRD4_{BD2} are shown.

Supplementary Table 1a | Data collection and refinement statistics.

	DDB1ΔB-CRBN- dBET6-BRD4 _{BD1}	DDB1ΔB-CRBN- dBET23-BRD4 _{BD1}	DDB1ΔB-CRBN-dBET55- BRD4 _{BD1} D145A
Data collection			
Space group	P 6 ₅ 2 2	P 6 ₅ 2 2	P 6 ₅ 2 2
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	115.40, 115.40, 588.14	115.57, 115.57, 596.32	115.20, 115.20, 597.14
α, β, γ (°)	90, 90, 120	90, 90, 120	90, 90, 120
Resolution (Å)	49.79 - 3.33 (3.49 - 3.33)	49.87 - 3.49 (3.68 - 3.49)	149.28 - 3.99 (4.31 - 3.99)
<i>R</i> _{merge}	0.179 (5.471)	0.128 (3.561)	0.280 (2.227)
<i>R</i> _{pim}	0.032 (0.978)	0.041 (1.173)	0.072 (0.582)
<i>CC</i> _{1/2}	1.000 (0.469)	0.999 (0.328)	0.991 (0.452)
<i>I</i> / σ <i>I</i>	16.4 (0.9)	11.4 (0.7)	7.69 (1.1)
Completeness (%)	100.0 (100.0)	99.5 (97.3)	100.0 (100.0)
Redundancy	32.2 (33.2)	11.4 (10.5)	17.0 (16.1)
Refinement			
Resolution (Å)	49.79 - 3.33 (3.45 - 3.33)	49.35 - 3.50 (3.63 - 3.50)	99.77 - 3.99 (4.13 - 3.99)
No. reflections	35251 (3287)	30453 (2671)	21193 (2038)
<i>R</i> _{work}	0.1994 (0.3605)	0.2123 (0.3551)	0.2886 (0.3757)
<i>R</i> _{free}	0.2344 (0.4380)	0.2555 (0.3848)	0.3334 (0.3912)
No. atoms	10373	10331	10291
Protein	10313	10268	10290
Ligand/ion	60	63	1
Water	0	0	0
<i>B</i> -factors	175.87	204.01	189.70
Protein	176.08	204.26	189.70
Ligand/ion	140.19	162.21	133.84
Water	-	-	-
R.m.s. deviations			
Bond lengths (Å)	0.002	0.007	0.002
Bond angles (°)	0.51	0.48	0.46

*Each dataset was collected from one crystal. *Values in parentheses are for highest-resolution shell.

Supplementary Table 1b | Data collection and refinement statistics.

	DDB1ΔB-CRBN- dBET57- SeMetBRD4 _{BD1}	DDB1ΔB-CRBN- dBET70-BRD4 _{BD1}
Data collection		
Space group	I 4 2 2	P 6 ₅ 2 2
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	313.36, 313.36, 167.37	117.60, 117.60, 597.16
α, β, γ (°)	90, 90, 90	90 90 120
Resolution (Å)	147.60 - 6.34 (7.08 - 6.34)	149.29 - 4.38 (4.90 - 4.38)
<i>R</i> _{merge}	0.165 (2.952)	0.349 (3.276)
<i>R</i> _{pim}	0.036 (0.593)	0.059 (0.548)
<i>CC</i> _{1/2}	1.000 (0.627)	1.000 (0.768)
<i>I</i> / σ <i>I</i>	15.3 (1.3)	8.5 (1.4)
Completeness (%)	98.1 (93.4)	99.7 (99.8)
Redundancy	25.4 (26.2)	36.9 (36.7)
Refinement		
Resolution (Å)	147.60-6.34 (6.57-6.34)	100.40 - 4.38 (4.54 - 4.38)
No. reflections	8964 (743)	16770 (1588)
<i>R</i> _{work}	0.3368 (0.4151)	0.2754 (0.3827)
<i>R</i> _{free}	0.3805 (0.5110)	0.3013 (0.4689)
No. atoms	10042	10314
Protein	10041	10313
Ligand/ion	1	1
Water	0	0
<i>B</i> -factors	484.99	278.70
Protein	485.00	278.71
Ligand/ion	465.40	197.08
Water	-	-
R.m.s. deviations		
Bond lengths (Å)	0.011	0.002
Bond angles (°)	1.48	0.469

*Each dataset was collected from one crystal. *Values in parentheses are for highest-resolution shell.

Supplementary Dataset 1 | Proteomics data of dBET23, dBET70 and ZXH-3-26 cellular effects.

Attached excel spreadsheet containing the raw output of statistical analysis using limma¹.

References

- 1 Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **43**, e47 (2015).