Figure 1

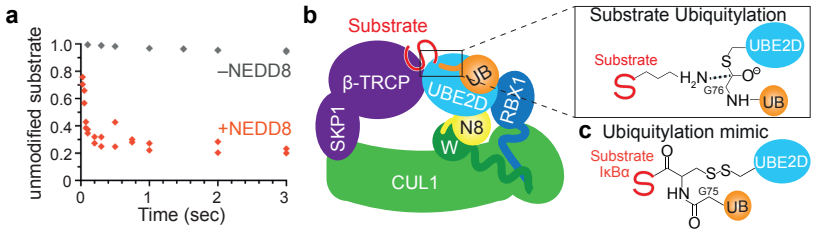

a
unmodified substrate
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
Time (sec)
–NEDD8
+NEDD8
b
Substrate
β-TRCP
SKP1
UB
UBE2D
RBX1
N8
W
CUL1
Substrate Ubiquitylation
Substrate
UBE2D
S
G76
NH
UB
c Ubiquitylation mimic
Substrate
IκBα
S
S-S
UBE2D
HN
G75
UB

Figure 2

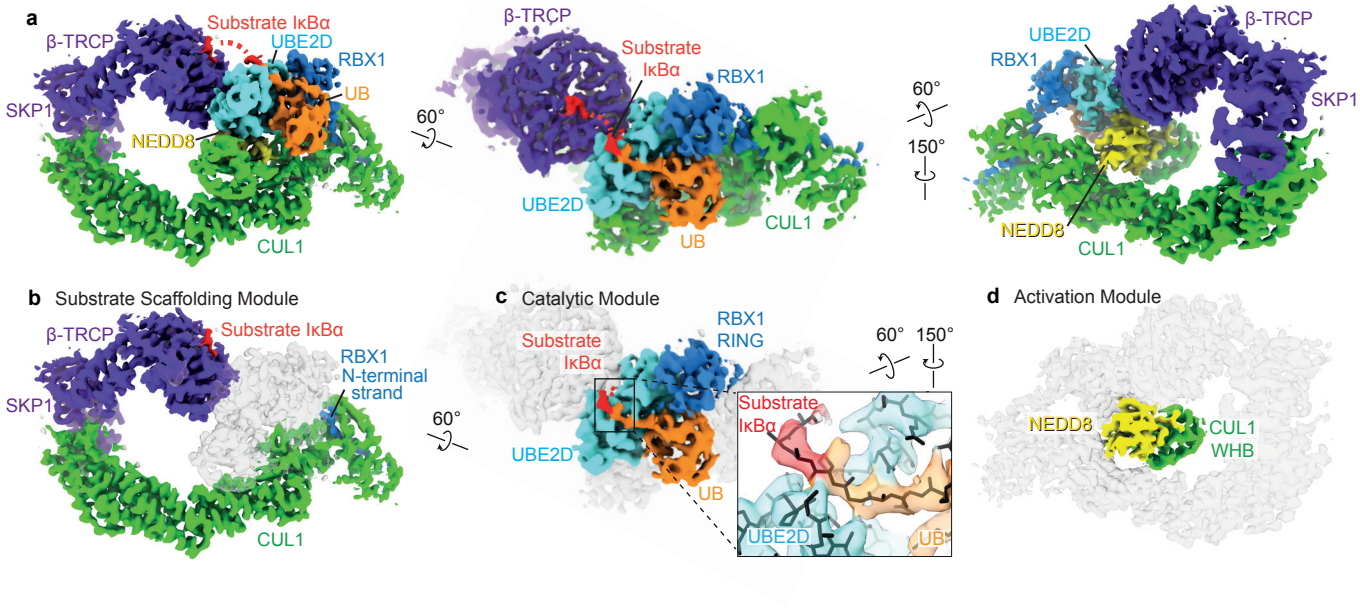

a
β-TRCP
Substrate IκBα
UBE2D
RBX1
UB
SKP1
NEDD8
CUL1
60°
β-TRCP
Substrate
IκBα
RBX1
UBE2D
UB
CUL1
60°
150°
UBE2D
RBX1
β-TRCP
SKP1
NEDD8
CUL1
b Substrate Scaffolding Module
β-TRCP
Substrate IκBα
RBX1
N-terminal
strand
SKP1
CUL1
60°
c Catalytic Module
Substrate
IκBα
RBX1
RING
UBE2D
UB
60°
150°
Substrate
IκBα
UBE2D
UB
d Activation Module
NEDD8
CUL1
WHB

Figure 3

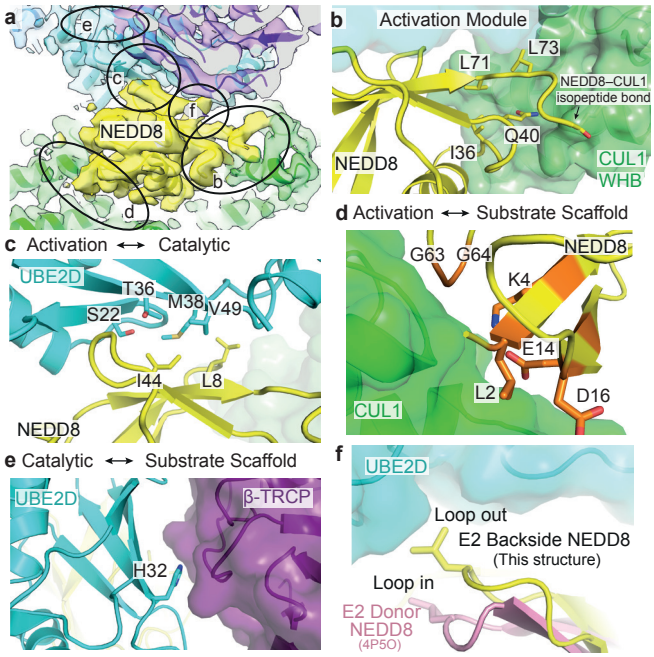
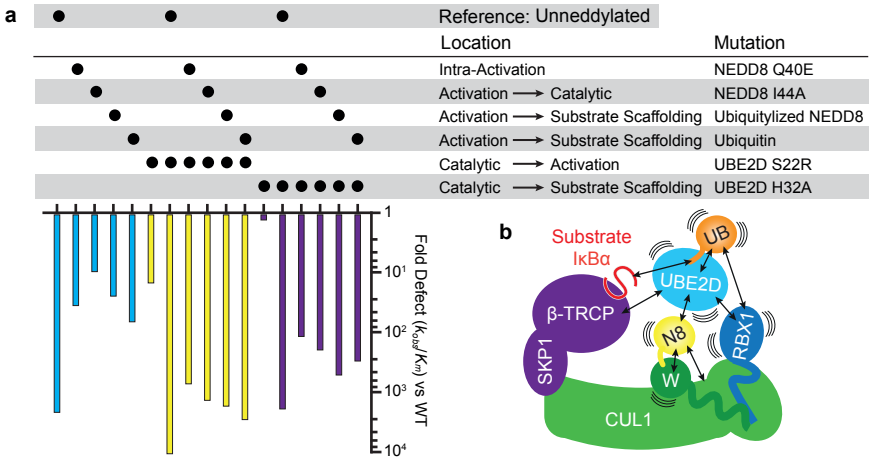
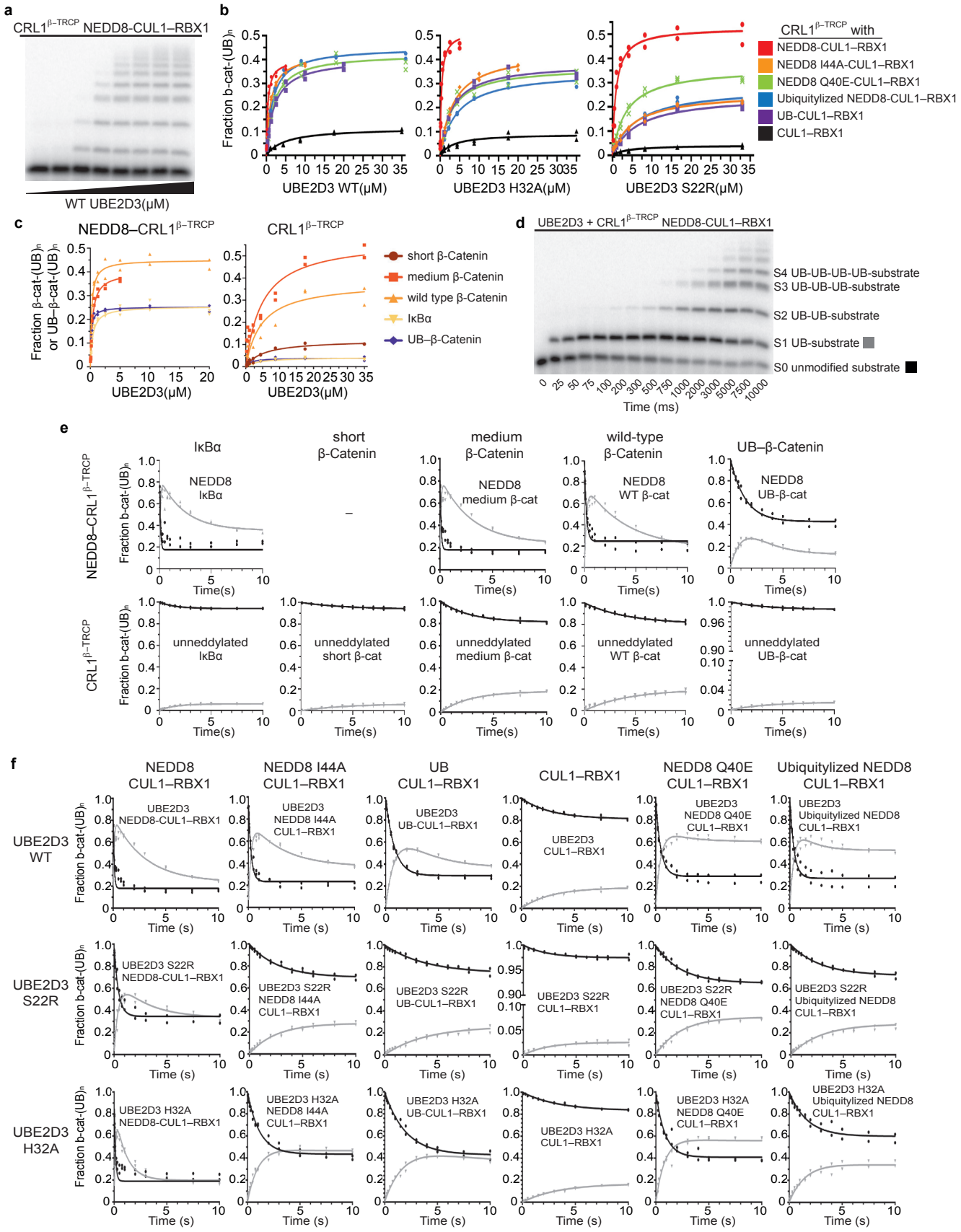

a
e
c
f
NEDD8
b
d
b Activation Module
L71
L73
NEDD8–CUL1
isopeptide bond
Q40
I36
NEDD8
CUL1
WHB
c Activation ⟷ Catalytic
UBE2D
T36
M38
V49
S22
I44
L8
NEDD8
d Activation ⟷ Substrate Scaffold
G63
G64
NEDD8
K4
E14
L2
D16
CUL1
e Catalytic ⟷ Substrate Scaffold
UBE2D
β-TRCP
H32
f
UBE2D
Loop out
E2 Backside NEDD8
(This structure)
Loop in
E2 Donor
NEDD8
(4P5O)

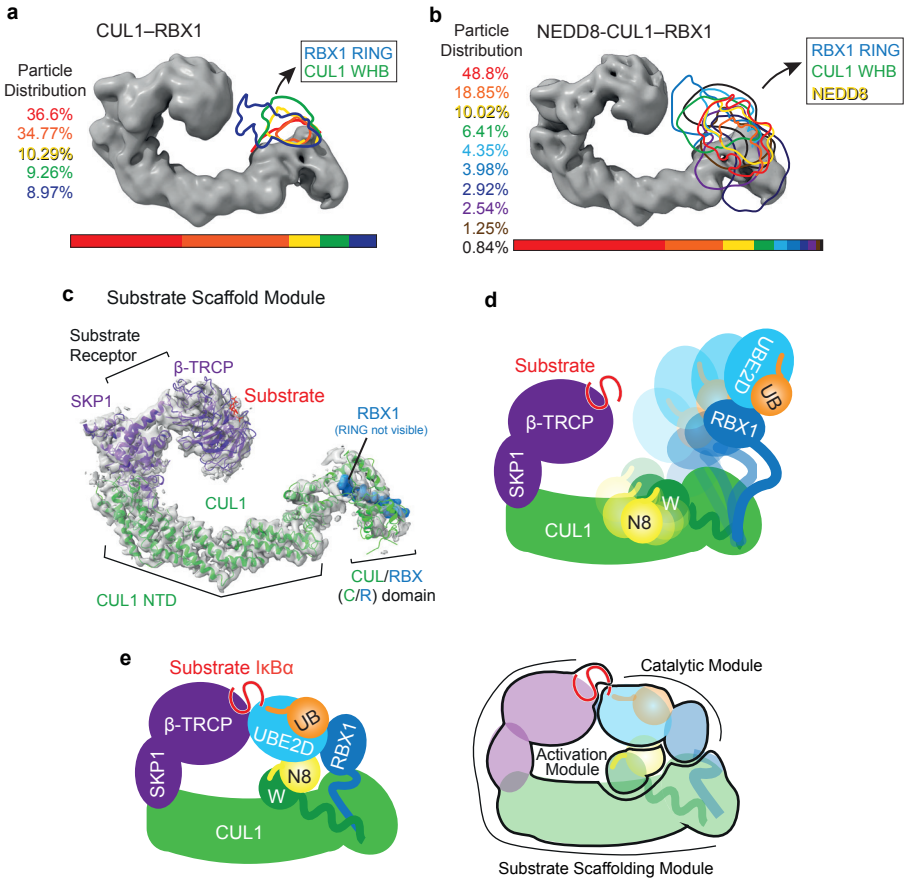
Figure 4


a
Reference: Unneddylated
Location
Mutation
Intra-Activation
NEDD8 Q40E
Activation → Catalytic
NEDD8 I44A
Activation → Substrate Scaffolding
Ubiquitylized NEDD8
Activation → Substrate Scaffolding
Ubiquitin
Catalytic → Activation
UBE2D S22R
Catalytic → Substrate Scaffolding
UBE2D H32A
1
10¹
10²
10³
10⁴
Fold Defect (kcat/Km) vs WT
b
Substrate
IκBα
UB
UBE2D
β-TRCP
SKP1
N8
W
RBX1
CUL1

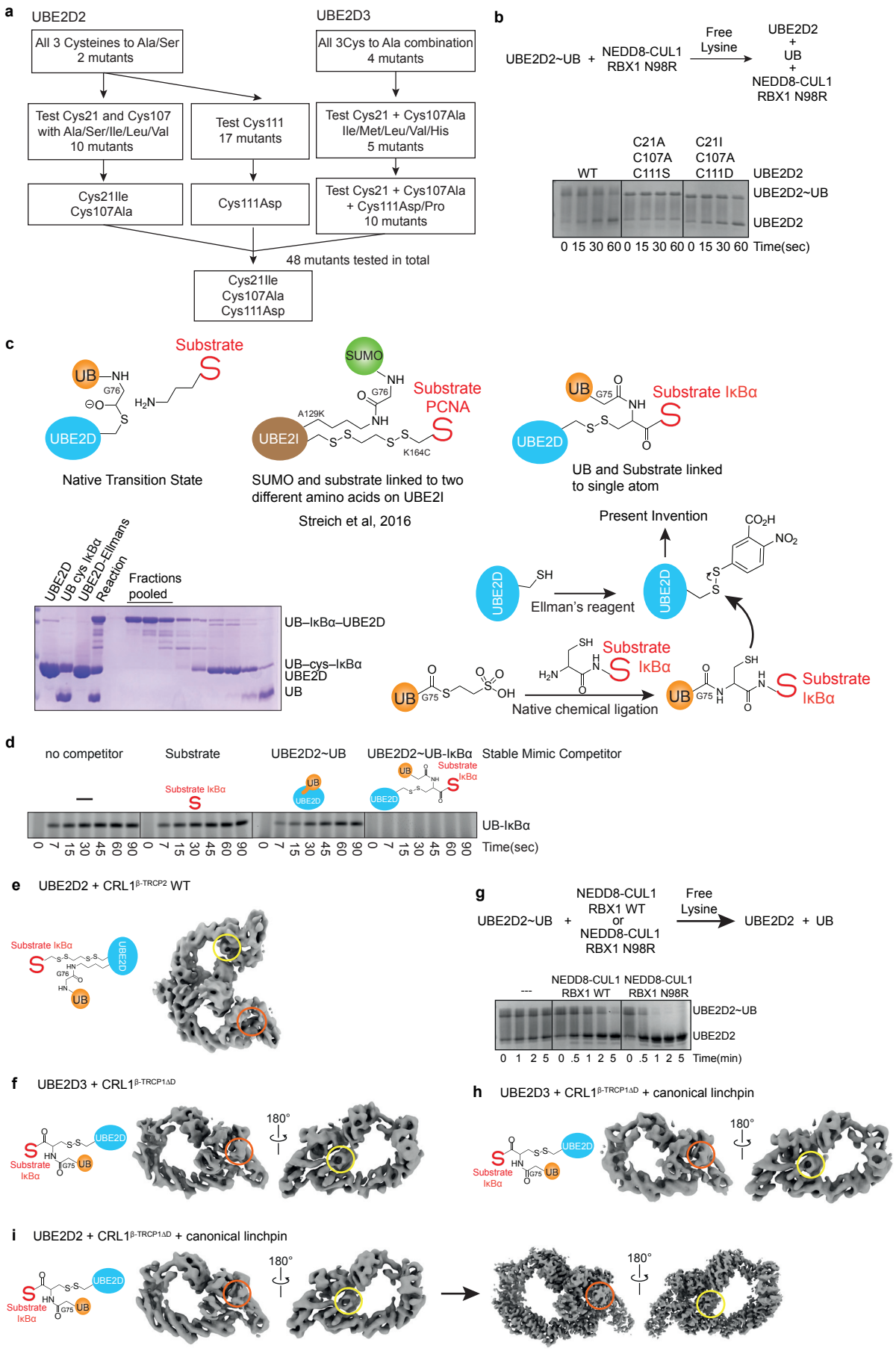
Extended Data Fig. 1



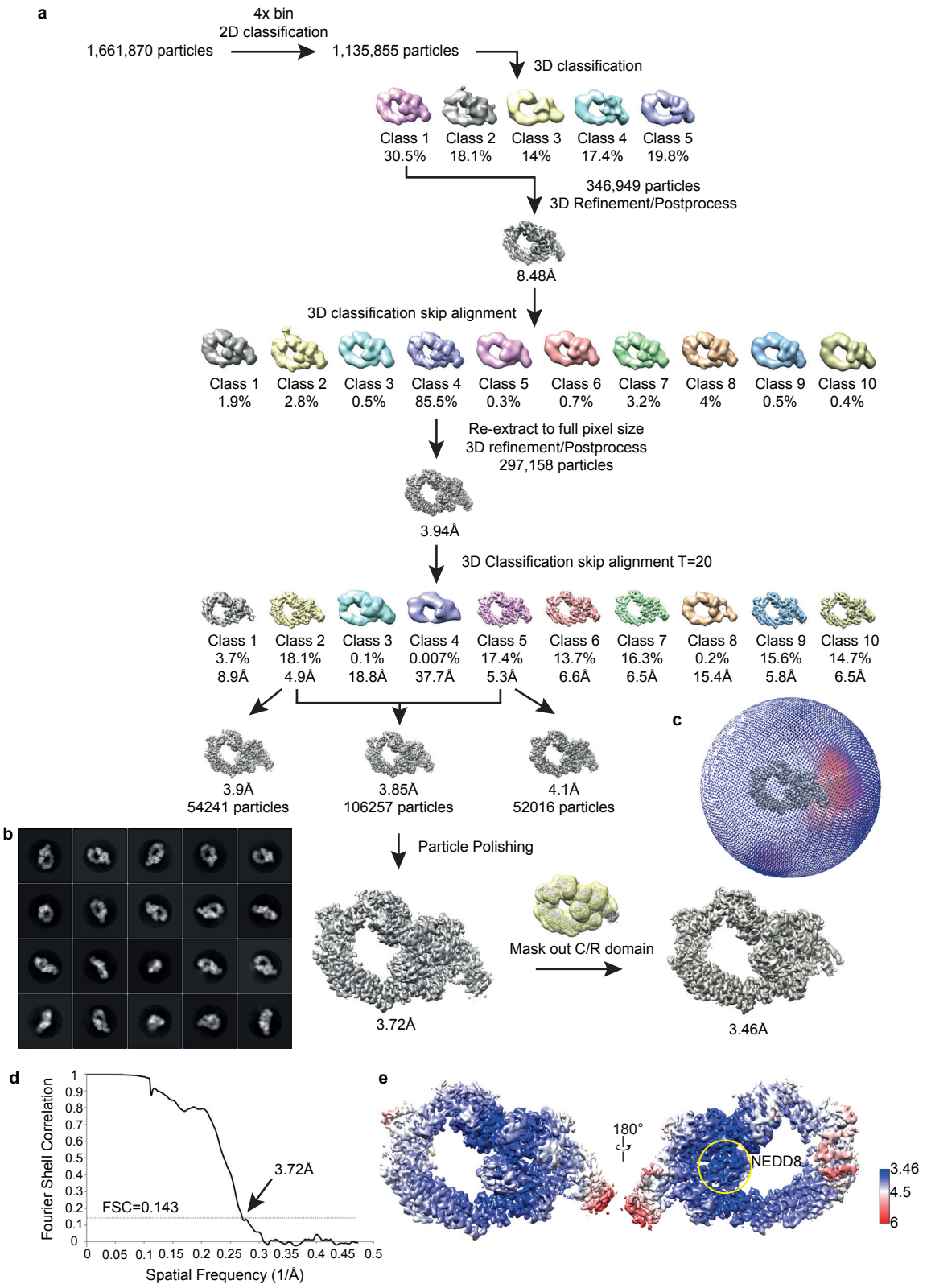
Extended Data Fig. 2



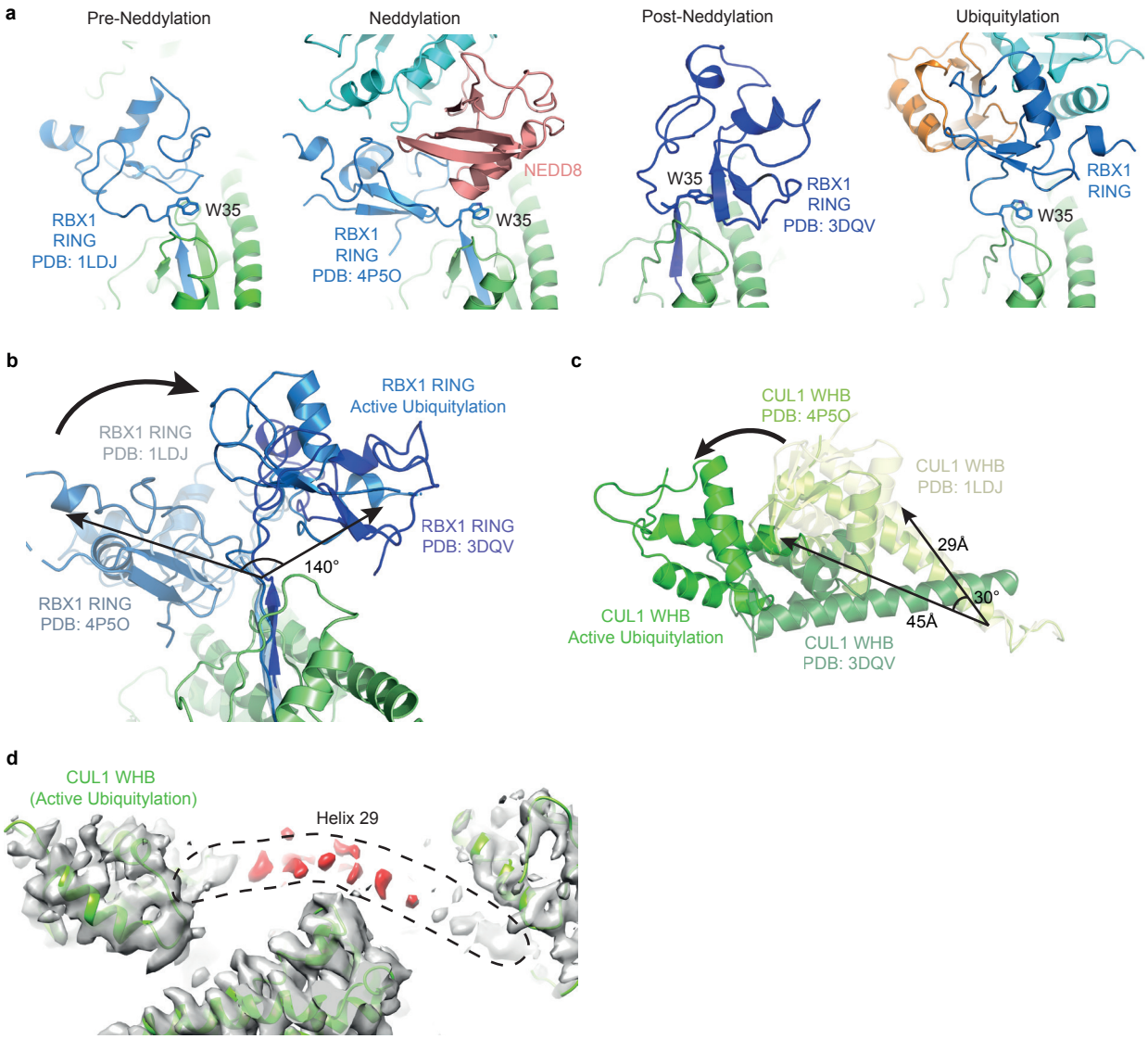
Extended Data Fig. 3



Extended Data Fig. 4

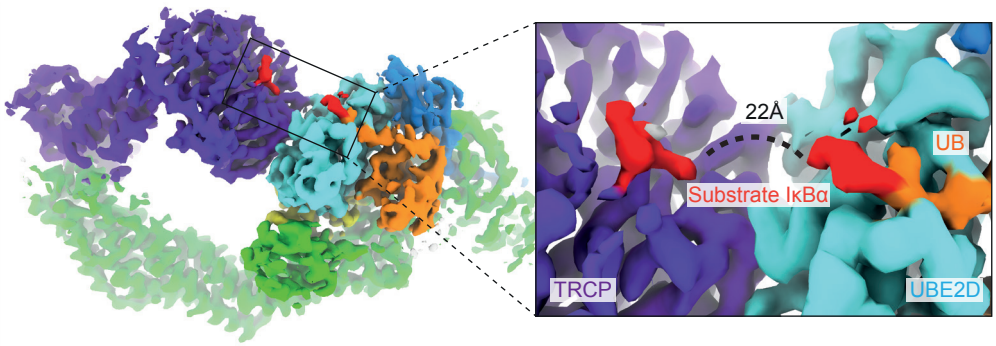


Extended Data Fig. 5



Extended Data Fig. 6

a

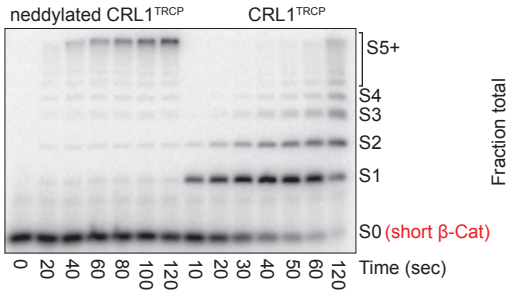


b

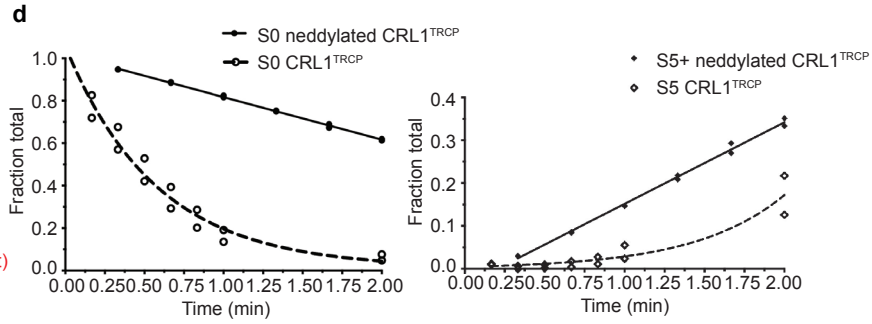
```
                                                   Substrate peptide IκBα                KERLLDDRHDSGLDSMRDEERRASY
                                                   Substrate peptide WT β-Catenin   KAAVSHWQQQSYLDSGIHSGATTAPRRASY
                                                   Substrate peptide medium β-Catenin   KAWQQQSYLDSGIHSGATTTAPRRASY
                                                   Substrate peptide short β-Catenin          KSYLDSGIHSGATTAPRRASY
NF-Kappa-B inhibitor alpha (Substrate IκBα in this study)  MEGPRDGLKKERLLDDRHDSGLDSMKDEEYEQMVKELQEI
Catenin beta-1 (Substrate β-Catenin in this study)         MEPDRKAAVSHWQQQSYLDSGIHSGATTTAPSLSGKGNPE
Cellular tumor antigen p53                                 ELNEALELKDAQAGKEPGGSRAHSSHLKSKKGQSTSRHKK
NF-Kappa-B inhibitor beta                                   MAGVACLGKAADADEWCDSGLGSLGPDAAAPGGPGLGAE
NF-Kappa-B inhibitor epsilon                               AGMSEARKGPDEAEESQYDSGIESLRSLRSLPESTSAPAS
Prolactin receptor                                         SKEHPSQGMKPTYLDPDTDSGRGSCDSPSLLSEKCEEPQA
cAMP-dependent TF ATF-4                                    YVAMIPQCIKEEDTPSDNDSGICMSPESYLGSPQHSPSTR
E3 ubiquitin-protein ligase UHRF1                          SLVLPHSTKERDSELSDTDSGCCLGQSESDKSSTHGEAAA
M-phase inducer phosphatase 1                              LEVKNNSNLQRMGSSESTDSGFCLDSPGPLDSKENLENPM
Fanconi anemia group M protein                             TECQFTNKSTSSLAGNVLDSGYNSFNDEKSVSSNLFLPFE
Programmed cell death protein 4                            EARINAKAKRRLRKNSSRDSGRGDSVSDSGSDALRSGLTV
Nuclear factor erythroid 2-related factor 2                CKAFNQNHPESTAEFNDSDSGISLNTSPSVASPEHSVESS
Growth hormone receptor                                    LLSSDHEKSHSNLGVKDGDSGRTSCCEPDILETDFNANDI
DEP domain-containing mTOR-interacting protein             KEIKIVSAVRRSSMSSCGSSGYFSSSPTLSSSPPVLCNPK
Interferon alpha/beta receptor 1                           ETNQTDEDHKKYSSQTSQDSGNYSNEDESESKTSEELQQD
Transcription factor Sp1                                   QNKKGGPGVALSVGTLPLDSGAGSEGSGTATPSALITTNM
Erythropoietin receptor                                    PELPPTPPHLKYLYLVVSDSGISTDYSSGDSQGAQGGLSD
Nuclear factor NF-Kappa-B p100 subunit                     LRGPETRDKLPSTAEVKEDSAYGSQSVEQEAEKLGPPPEP
DisKs large homolog 1                                      SQKRSLYVRALFDYDKTKDSGLPSQGLNFKFGDILHVINA
Serine/threonine-protein Kinase PLK4                       SRNSSTKSKDLGTVEDSIDSGHATISTAITASSSTSISGS
Fizzy-related protein homolog                              PEKKGLFTYSLSTKRSSPDDGNDVSPYSLSPVSNKSQKLL
Myc proto-oncogene protein                                 EEIDVVSVEKRQAPGKRSESGSPSAGGHSKPPHSPLVLKR
Heterogeneous nuclear ribonucleoprotein D0                 EGAKIDASKNEEDEGHSNSSPRHSEAATAQREEWKMFIGG
ELAV-liKe protein 1                                        DFNTNKCKGFGFVTMTNYEEAAMAIASLNGYRLGDKILQV
Protein Vpu (isolate BRU/LAI)                              LRQRKIDRLIDRLIERAEDSGNESEGEISALVEMGVEMGH

Claspin                                                    LEINDPNVISQEEADSPSDSGQGSYETIGPLSEGDSDEEIFVSKKLKNRK
Period circadian protein homolog 1                         SSIAYSLLSASSEQDNPSTSGCSSEQSARARTQKELMTALRELKLRLPPE
Bcl-2-liKe protein 11                                      TRSPLFIFMRRSSLLSRSSSGYFSFDTDRSPAPMSCDKSTQTPSPPCQAF
Period circadian protein homolog 2                         QSPDTFSLMMAKSEHNPSTSGCSSDQSSKVDTHKELIKTLKELKVHLPAD
Ubiquitin carboxyl-terminal hydrolase 37                   LSLQEFNNSFVDALGSDEDSGNEDVFDMEYTEAEAEELKRNAETGNLPHS
M-phase inducer phosphatase 2                              PLALGRFSLTPAEGDTEEDDGFVDILESDLKDDDAVPPGMESLISAPLVK
Twist-related protein 1                                        MMQDVSSSPVSPADDSLSNSEEEPDRQQPPSGKRGGRKRRSSRRSA
```

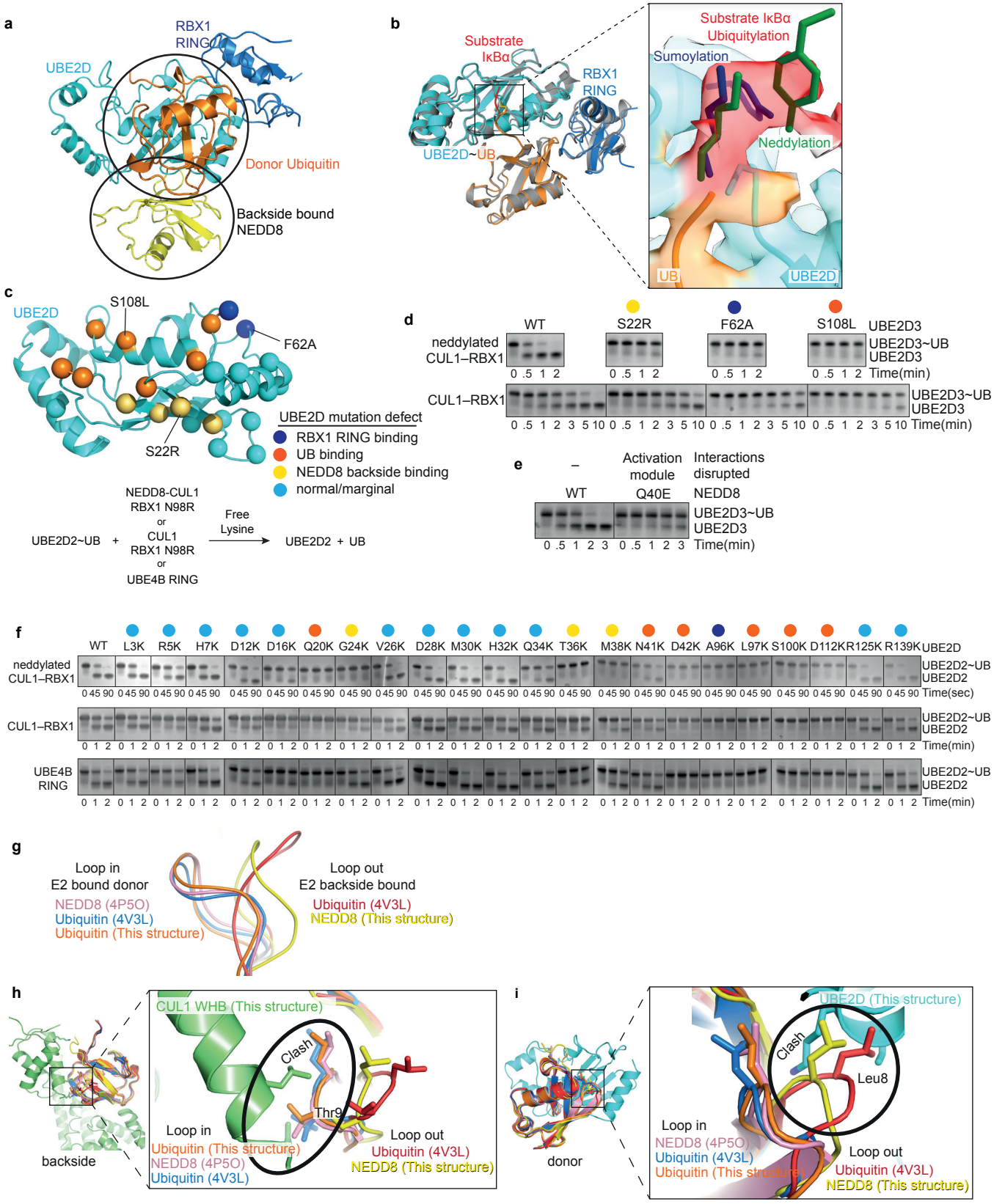
c Substrate peptide short β-Catenin



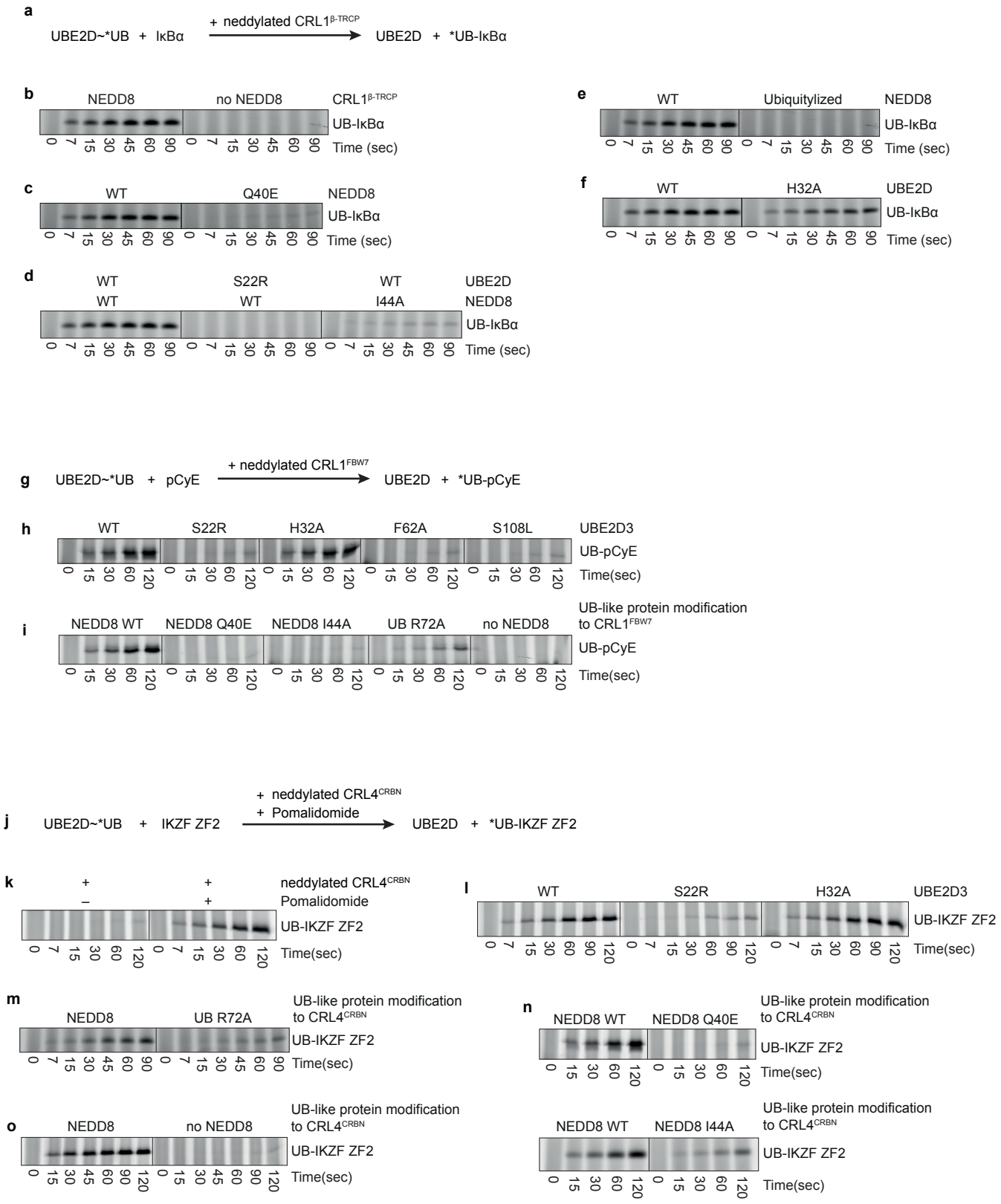
d



Extended Data Fig. 7



Extended Data Fig. 8



Extended Data Table 1 Estimates of K_m and k_{obs} for Substrate Ubiquitylation

Substrate	E2	CRL1$^{\beta\text{-TRCP}}$	K_m (10^{-9} M)	$k_{obs}^{S0\text{-}S1}$ (sec^{-1})	$k_{obs}^{S1\text{-}S2}$ (sec^{-1})	$k_{obs}^{S0\text{-}S1}/K_m$ ($M^{-1}sec^{-1}$)	Fold change
IκBα	WT UBE2D	NEDD8-CUL1–RBX1	408 ± 57	12.9 ± 1.7	0.24 ± 0.04	3.2×10^7	-
IκBα	WT UBE2D	CUL1–RBX1	4014 ± 956	0.05 ± 0.002	-	1.2×10^4	2667
WT β-cat	WT UBE2D	NEDD8-CUL1–RBX1	214 ± 30	4.8 ± 0.46	0.19 ± 0.02	2.2×10^7	-
WT β-cat	WT UBE2D	CUL1–RBX1	4638 ± 138	0.05 ± 0.005	-	1.1×10^4	2000
medium β-cat	WT UBE2D	NEDD8-CUL1–RBX1	372 ± 48	11 ± 1.00	0.24 ± 0.04	3.0×10^7	-
medium β-cat	WT UBE2D	CUL1–RBX1	4875 ± 1328	0.08 ± 0.005	-	1.6×10^4	1875
short β-cat	WT UBE2D	CUL1–RBX1	5207 ± 1051	0.02 ± 0.0004	-	3.8×10^3	
medium β-cat	WT UBE2D	NEDD8 I44A-CUL1–RBX1	1087 ± 69	3.60 ± 0.27	0.17 ± 0.03	3.3×10^6	9.1
medium β-cat	WT UBE2D	UB-CUL1–RBX1	2056 ± 170	1.0 ± 0.03	0.15 ± 0.01	4.9×10^5	61.2
medium β-cat	WT UBE2D	NEDD8 Q40E-CUL1–RBX1	1941 ± 226	1.77 ± 0.13	0.07 ± 0.01	9.1×10^5	33.0
medium β-cat	WT UBE2D	UBylized NEDD8-CUL1–RBX1	1651 ± 137	2.20 ± 0.15	0.17 ± 0.04	1.3×10^6	23.1
medium β-cat	S22R UBE2D	NEDD8-CUL1–RBX1	913 ± 135	1.89 ± 0.14	0.17 ± 0.03	2.1×10^6	14.2
medium β-cat	S22R UBE2D	NEDD8 I44A-CUL1–RBX1	5190 ± 604	0.13 ± 0.01	-	2.5×10^4	1200
medium β-cat	S22R UBE2D	UB-CUL1–RBX1	6492 ± 872	0.08 ± 0.005	-	1.2×10^4	2500
medium β-cat	S22R UBE2D	CUL1–RBX1	4007 ± 754	0.013 ± 0.002	-	3.3×10^3	9091
medium β-cat	S22R UBE2D	NEDD8 Q40E-CUL1–RBX1	3581 ± 367	0.17 ± 0.005	-	4.7×10^4	638.3
medium β-cat	S22R UBE2D	UBylized NEDD8-CUL1–RBX1	6105 ± 1030	0.12 ± 0.007	-	2.0×10^4	1500
medium β-cat	H32A UBE2D	NEDD8-CUL1–RBX1	347 ± 53	8.15 ± 1.04	0.73 ± 0.09	2.3×10^7	1.3
medium β-cat	H32A UBE2D	NEDD8 I44A-CUL1–RBX1	3431 ± 332	0.58 ± 0.04	0.12 ± 0.02	1.7×10^5	176.5
medium β-cat	H32A UBE2D	UB-CUL1–RBX1	2999 ± 301	0.34 ± 0.01	0.10 ± 0.02	1.1×10^5	272.7
medium β-cat	H32A UBE2D	CUL1–RBX1	3305 ± 1119	0.06 ± 0.003	-	1.8×10^4	1666.7
medium β-cat	H32A UBE2D	NEDD8 Q40E-CUL1–RBX1	2923 ± 459	0.81 ± 0.06	-	2.8×10^5	107.1
medium β-cat	H32A UBE2D	UBylized NEDD8-CUL1–RBX1	4303 ± 310	0.28 ± 0.02	-	6.5×10^4	461.5
UB-β-cat	WT UBE2D	NEDD8-CUL1–RBX1	148 ± 15	-	0.48 ± 0.02		
UB-β-cat	WT UBE2D	CUL1–RBX1	1503 ± 439	-	0.005 ± 0.0004		

*S0 refers to unmodified substrate, S1 to substrate modified by a single UB, and S2 to substrate modified with two UBs.
S0-S1 refers to the transition from unmodified to UB-substrate, and S1-S2 to the transition from UB-substrate to UB-UB-substrate.
Values for K_m are the best fit values derived from nonlinear regression in Prism, and value for k_{obs} are the best fit values derived from nonlinear regression in Mathematica. The measure of error is the standard error as determined by Prism and Mathematica, respectively.

Extended Data Table 2

Cryo-EM data collection, refinement and validation statistics

IκBα–UB~UBE2D crosslink (C21I C107A C111D)	UBE2D2 2x2way XL	UBE2D3 3way XL	UBE2D3 3way XL	UBE2D2 3way XL	UBE2D2 3way XL	none	none
Substrate Receptor	β-TRCP2	β-TRCP1 175-C	β-TRCP1 175-C	β-TRCP1 175-C	β-TRCP1 175-C	β-TRCP1 175-C	β-TRCP1 175-C
RBX1	WT	WT	N98R	N98R	N98R	WT	WT
NEDD8	yes	yes	yes	yes	yes	no	yes
SKP1	WT	ΔΔ	ΔΔ	ΔΔ	ΔΔ	WT	WT
	EMDB-10578	EMDB-10579	EMDB-10580	EMDB-10581	EMDB-10585 PDB 6TTU	EMDB-10582	EMDB-10583
Data collection and processing							
Microscope	Krios	Arctica	Arctica	Arctica	Krios	Glacios	Glacios
Magnification	105,000	92,000	73,000	73,000	130,000	36,000	22,000
Voltage (kV)	300	200	200	200	300	200	200
Electron exposure (e–/Å^2)	56	61.3	60.8	70	70.2	60	59
Defocus range (μm)	-1.2 ~ -3.6	-1.5 ~ -3.5	-1.5 ~ -3.5	-1.5 ~ -3.5	-1.2 ~ -3.3	-1.2 ~ -3.3	-1.2 ~ -3.3
Pixel size (Å)	1.34	1.612	1.997	1.997	1.06	1.181	1.885
Symmetry imposed	C2	C1	C1	C1	C1	C1	C1
Initial particle images (no.)	2,575,161	464,344	601,121	459,011	1,661,870	2,051,804	1,666,293
Final particle images (no.)	33,738	47,246	107,311	40,835	106,257	262,116	349,803
Map resolution (Å)	9.3	8.6	9.4	8.4	3.72	4.64	6.7
FSC threshold	(0.143)	(0.143)	(0.143)	(0.143)	(0.143)	(0.143)	(0.143)
Map resolution range (Å)	-	-	-	-	3.46 ~ 6.0	-	-
Refinement							
Initial model used (PDB code)					1LDJ 1P22 4P5O 4V3L		
Model resolution (Å)					3.7		
FSC threshold					(0.143)		
Map sharpening *B* factor (Å^2)	-578.9	-1159	-1272	-983.5	-94.2	-199	-338
Model composition							
Non-hydrogen atoms					13034		
Protein residues					1616		
Ligands					3(ZN)		
B factors (Å^2)							
Protein					91		
Ligand					224		
R.m.s. deviations							
Bond lengths (Å)					0.011		
Bond angles (°)					1.043		
Validation							
MolProbity score					2.37		
Clashscore					16.31		
Poor rotamers (%)					0.21		
Ramachandran plot							
Favored (%)					85.12		
Allowed (%)					14.88		
Disallowed (%)					0		