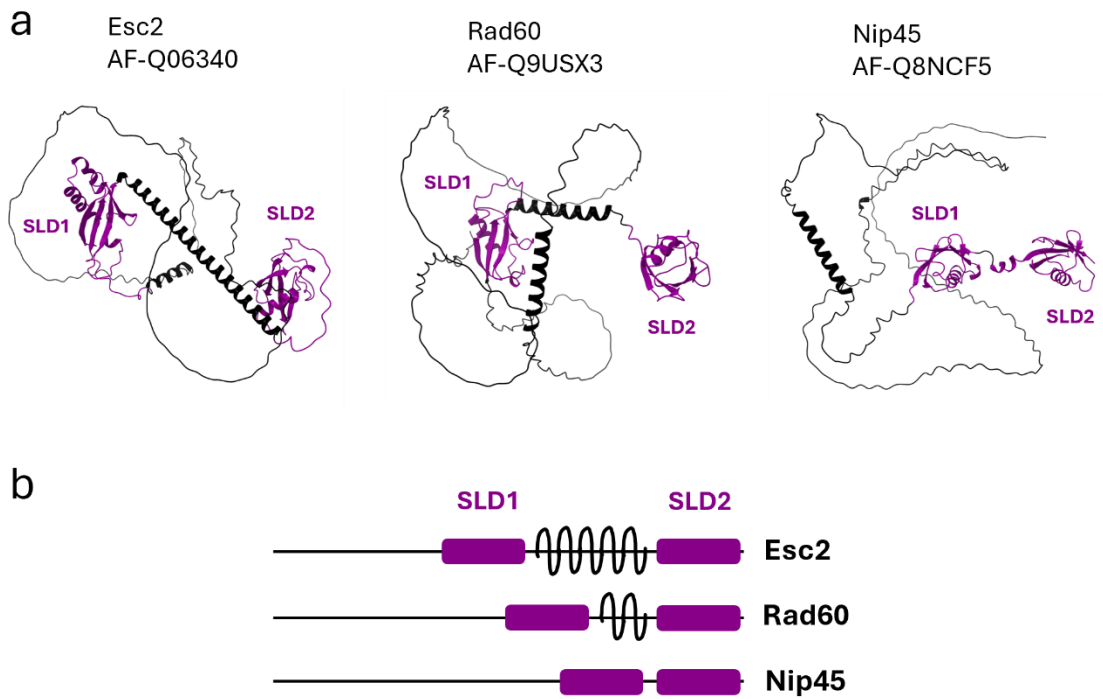


Esc2	-----MTGDSRSISEPSINLDPDNTSFSDENSDDF-FMDNSYDI	38
Nip45	MAEPVGKRGRWSGGSGAGRGGRGGWGGRRRPRAQRSPSRGTLD-VVSVDL-VTDSDEEI	58
Rad60	-----MDNLDEDDLAFFSKPIKKPPLNYAKQLIASSSDSEESELDTNKQAL	47
	. . * : . : . . : . . . :	
Esc2	DEIDHSDESNRQSVIVDSKVTVPSPKHSTLTLSDESDSAKE--QHQ--SLSRS-SSKNV	93
Nip45	LEVATARG-----AADEVEVEPEPPPGPVASRDNSNSDSEGEDRRPAGPPREP-VRRRR	111
Rad60	EHINAQK---NITHNENKSAEPLSRQSTILDADEGNQDVSD--TTPNACLNEGRHSPKS	101
	.: . : . * . . : * . : *	
Esc2	NIEDITEPKPKDKPSGRTRGRSVMKESVVE----INSSESDDLDEKFNPRSRSRSSIRS	149
Nip45	RLVLDPGEAPLPVPVYSGKVKSSLRLI-----PDDLSSLKLYPPGDEEEAE---L	157
Rad60	AISCVTQ--PVSPVYNTRAAANLRNNSINSEAAALSTTSSLLD--DDFARRLEEIDRQV--	155
	: * * : : : . * . . : ..	
	-----SLD1-----	
Esc2	ISPAGKYKRQKSSLLYTYDE-----NDDFFKELAKEAKKSTTISKESTPDQR---	196
Nip45	ADSSGLYHEGSPSPGSPWKTCLRDKDEKKKTEFLDLDNS--PLSPSPRTRKSRTHTRA	215
Rad60	----QEFEKSSSDMDVQIHT-----HKREIEEDDDNTSADVPLLKHSKSD-H---	197
	: : . : : . . : . : :	
	-----SLD1-----	
Esc2	-----KRVYNIK-FLSKLEGTINKAVQVKVLGKYEFISKILPAALDGLMKSYPKPV-	246
Nip45	LKKLSEVNKRQLDLRSCLSPKPP-----QGQEQGQGEDEVVLVEGPTLPETPRLF	265
Rad60	-----STLYHSKSEFSTNEPVISVVLQLAVIGQ-----RIPNSNISLPRDWEAPLFF	244
	. : . : : * * : : * .	
	-----SLD1-----SLD1-----	
Esc2	-----MKDIY-----KVENVTLYWNNAKLLTFMTCNSLHIP	277
Nip45	PLKIRCRADLVRPLRMSEPLQSVVDHATHLGVSPSRILLFGETELSPTATPRTLKL-	324
Rad60	KVKSNNQ--FRRVRIAYSERK-----KVDNVVLVFQNRQLWDYGTTPKGAG--	287
	.: . . : * : : * * .	
Esc2	QDFENEVSDIDVTIVSKEYEKNFEATLESKLKEEEAALLIKERQEMERKLEKKRNEQEE	337
Nip45	-----GVA-----	327
Rad60	-----MLKVDTRLVHVHAYCHSDFI-----SLKRI-----	311
	:	
	-----SLD2-----	
Esc2	EYREFESELKNVEETQEIENDTMNTKLLQEGGSLSGNSSSMEEVMRIALM-GQDNKKI	396
Nip45	-----DIIDCVVLTSSPEATET-----SQQLQLRVQGEKHKQTL	361
Rad60	--KELE-----VEKLSSVTEDSTAQTCK-----LITLLLR-SSKSEDL	346
	: : : . . : :	
	-----SLD2-----	
Esc2	YVHVRRTSPFSKIAEYYRIQKQLPQKTRVKLLFDHDELDMNECIADQDMEDEDMVDVIID	456
Nip45	EVLSRDSPLKTLMSHYEEAMGLSG-RKLSFFFDGTKLSGRELPADLGMESGDLEIIVWG-	419
Rad60	RLSIPVDFTVKDLIKRYCTEVKISFHERIRLEFEGEWLDPNQVQSTELEDEDQVSVVLD	406
	: : * : : : * : * . . : . : * . * : *	

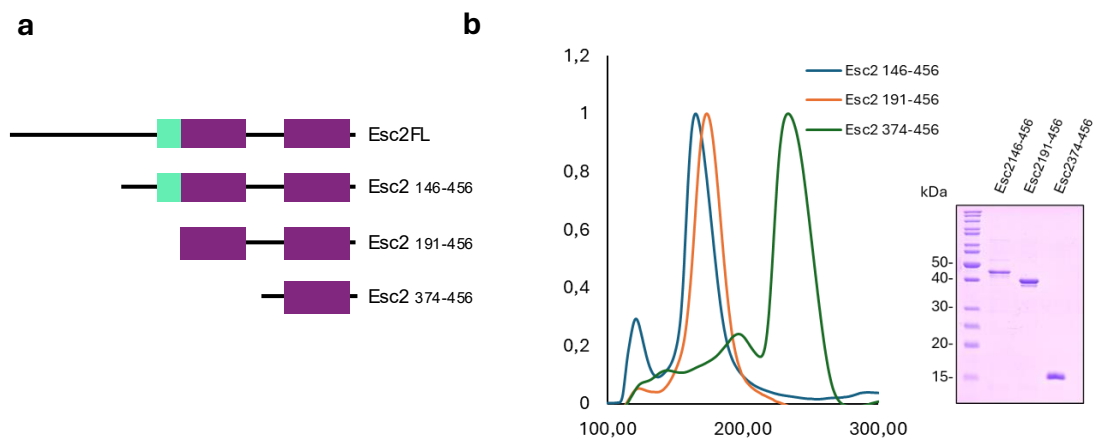
Supplementary Figure 1. Sequence alignment of RENi proteins Esc2, Nip45 and Rad60.

Asterisks, colons, periods and spaces indicate fully conserved, strongly conserved, weakly conserved, and non-conserved residues, respectively. The positions of the SLD1 and SLD2 domains are indicated above the alignment.



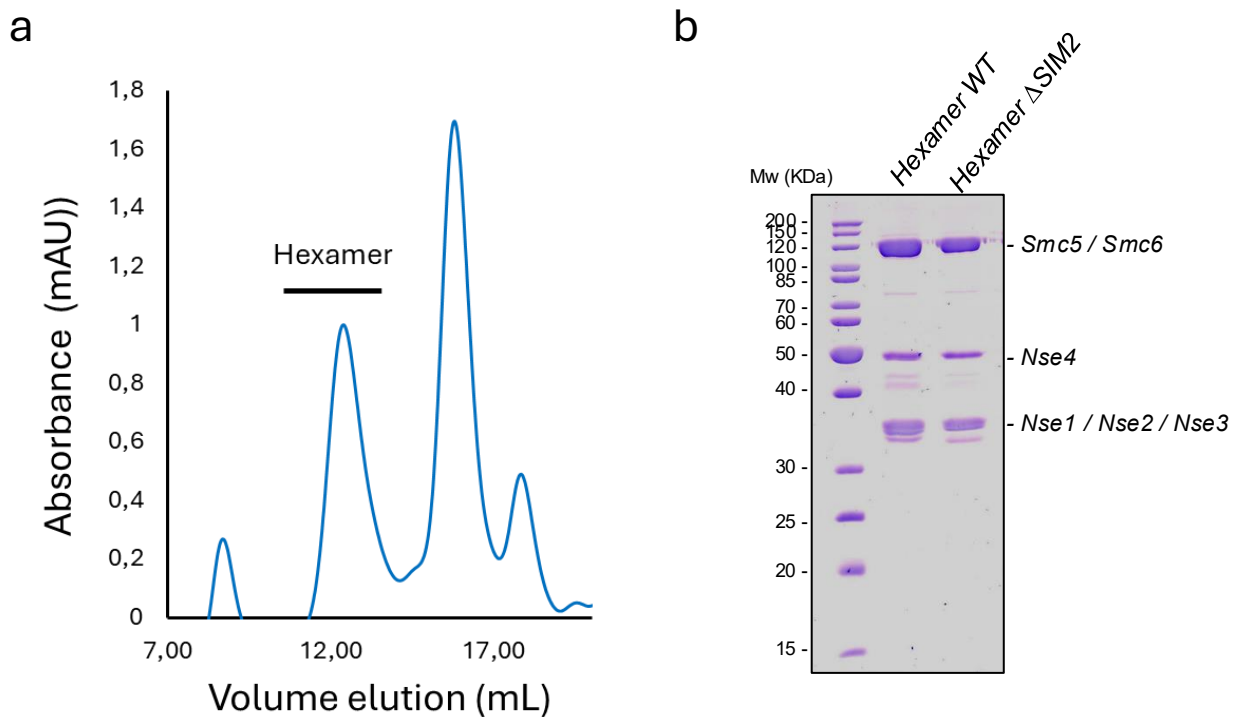
Supplementary Figure 2. Structural organization of RENi proteins.

a AlphaFold-predicted structures of the RENi proteins Esc2, Rad60, and Nip45, highlighting the two SUMO-like domains (SLD1 and SLD2) shown in purple. **b** Schematic representation of Esc2, Rad60, and Nip45. SUMO-like domains (SLD1 and SLD2) are indicated in purple, long helical regions are shown as black waves, and disordered regions are depicted as straight lines.



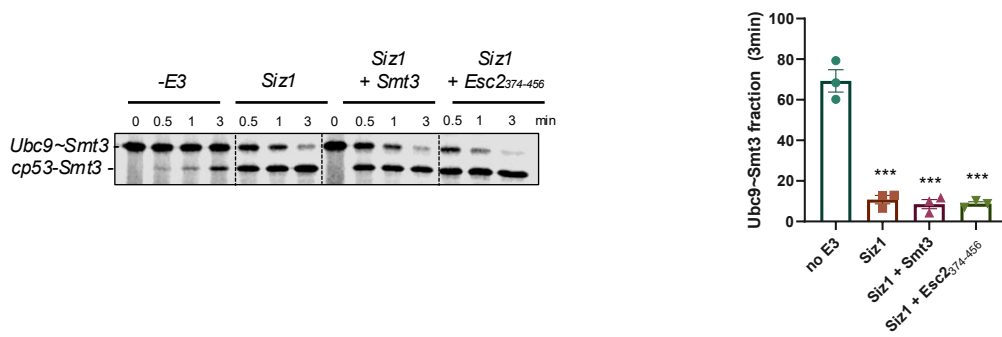
Supplementary Figure 3. Design and purification of Esc2 truncation constructs.

a Schematic representation of Esc2 and its truncation fragments. The N-terminal low-complexity region enriched in polar and charged residues is shown as thick black lines. The C-terminal globular region contains a DNA-binding domain (green) and two SUMO-like domains (SLD1 and SLD2). The three Esc2 constructs used in SUMOylation assays are indicated. **b** Purification of Esc2 truncation constructs, showing size-exclusion chromatography profiles and SDS-PAGE analysis confirming protein purity.



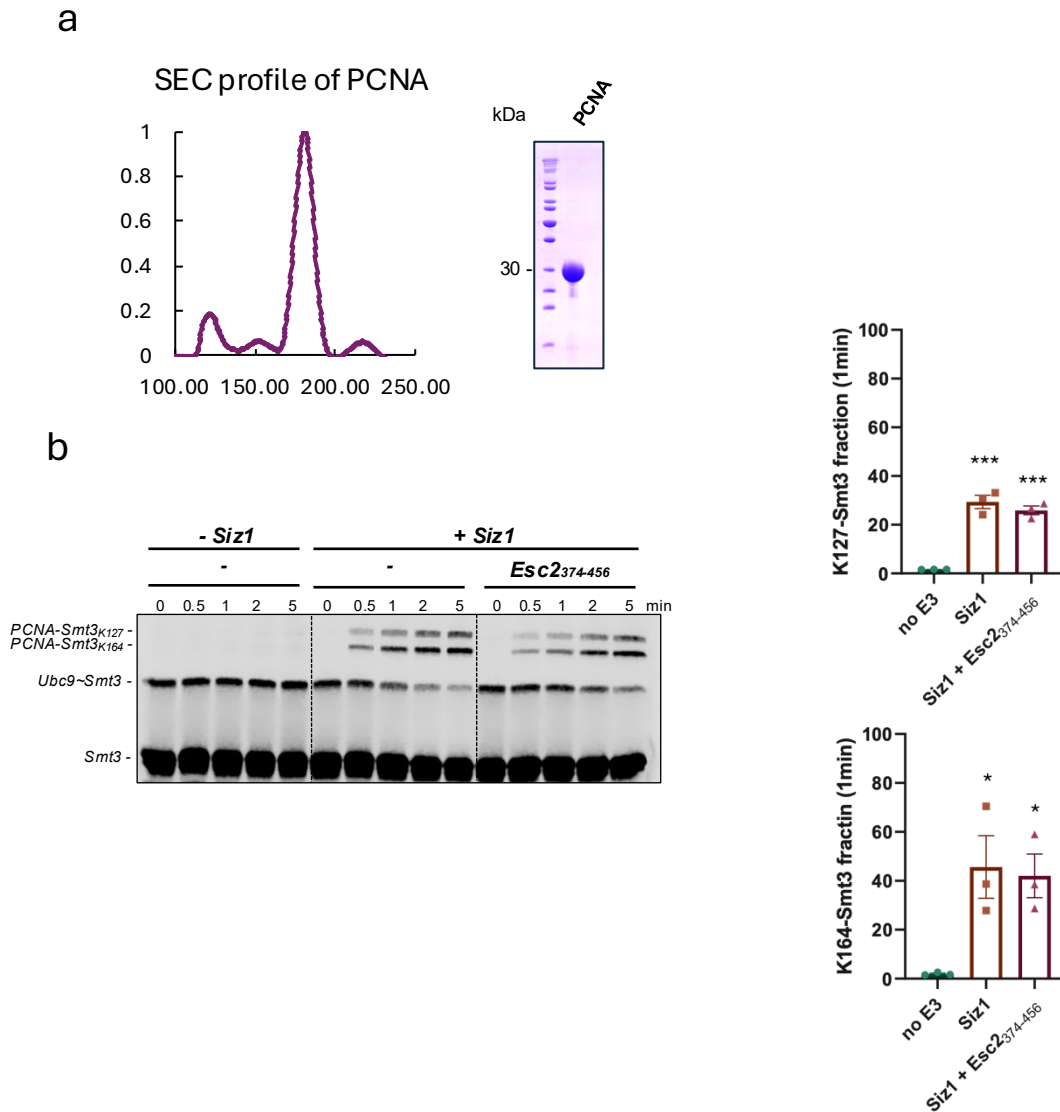
Supplementary Figure 4. Purification of the hexamer Smc5/6 complex.

a Gel filtration purification of the hexamer Smc5/6 complex using the size-exclusion chromatography column Superose 6 increase 10/300 GL (Cytiva). Fraction with the hexamer complex is indicated. **B** SDS-PAGE analysis of the wild type hexamer Smc5/6 complex (*Hexamer WT*) and the hexamer Nse2- Δ SIM2 Smc5/6 complex (*Hexamer Δ SIM2*). Source data are provided.



Supplementary Figure 5. Esc2 does not enhance Siz1-mediated SUMO conjugation of cp53.

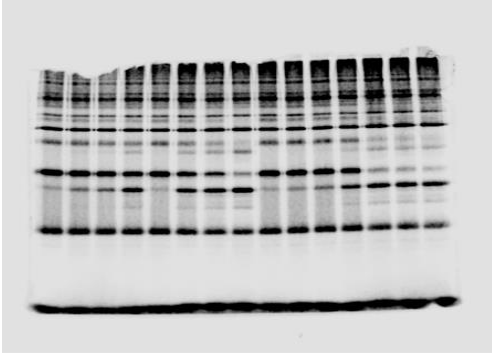
Time course of single-turnover SUMOylation reactions monitoring discharge of the *IRDye*-labeled D68R Smt3~Ubc9 thioester in the presence of cp53 and E3 Siz1 alone, with unlabeled Smt3, or with Esc2₃₇₄₋₄₉₆. Bar plots show the fraction of remaining Smt3~Ubc9 thioester after 3 min. Statistical analysis was performed as described in Methods. Exact *P* values from the left to right: 0.0006, 0.0005, 0.0004. Source data are provided.



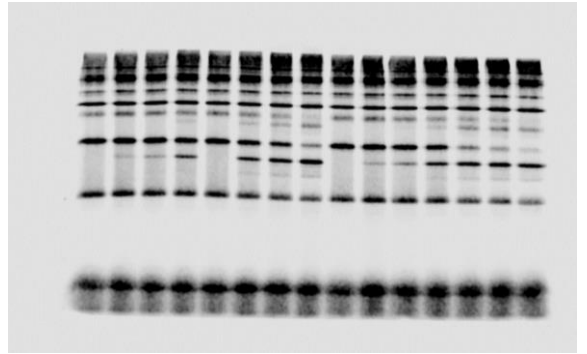
Supplementary Figure 6. Esc2 does not enhance Siz1-mediated SUMO conjugation of PCNA.

(a) Size exclusion chromatography (SEC). SEC analysis of the purified PCNA protein. SDS-PAGE gel of the purified PCNA protein. (b) Left. Time course of single-turnover SUMOylation reactions monitoring discharge of the IRDye-labeled Smt3-Ubc9 thioester to either K164 or K127 PCNA in the presence of E3 Siz1, with/without Esc2374-456. Right. Bar plots show the fraction of remaining K164 or K127 PCNA Smt3 conjugate after 1 min. Statistical analysis was performed as described in Methods. Exact P values from the left to right: 0.0005, 0.0002 (upper) and 0.0267, 0.0109 (lower). Source data are provided.

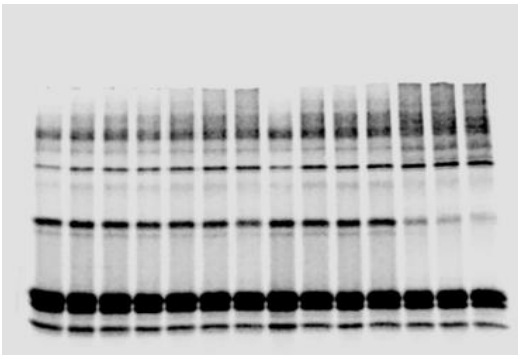
a



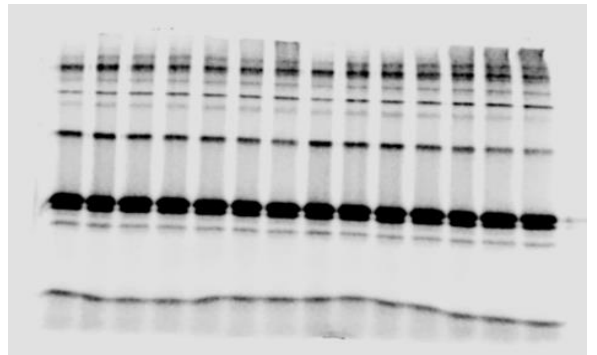
b



c



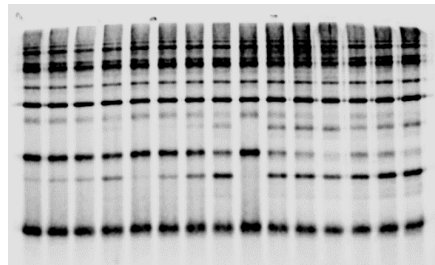
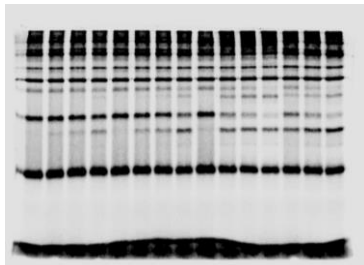
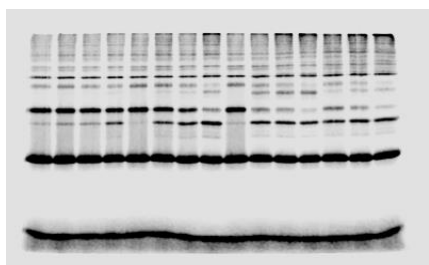
d



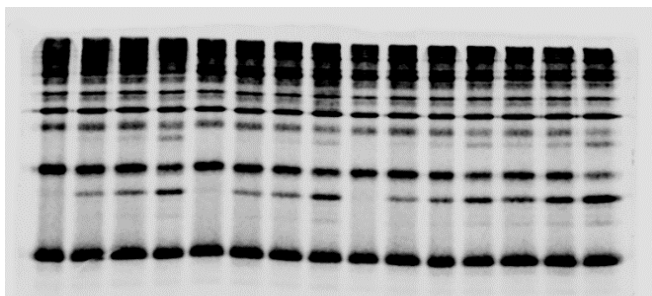
Supplementary Figure 7. Uncropped images of Figure 2.

(a)(b)(c)(d) Uncropped SDS-PAGE of corresponding panels in Figure2.

a



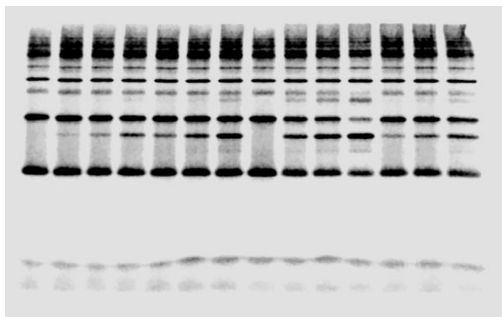
b



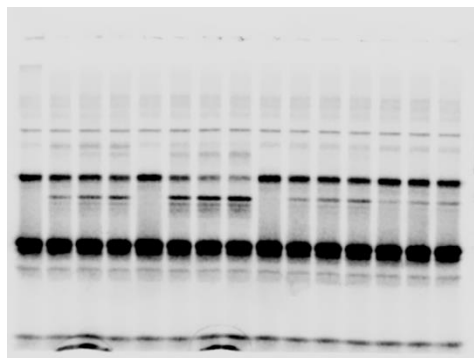
Supplementary Figure 8. Uncropped images of Figure 3.

(a)(b) Uncropped SDS-PAGE of corresponding panels in Figure 3.

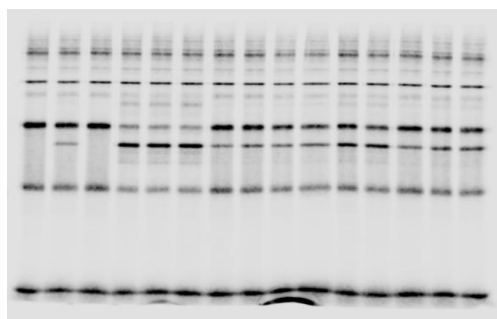
b



c



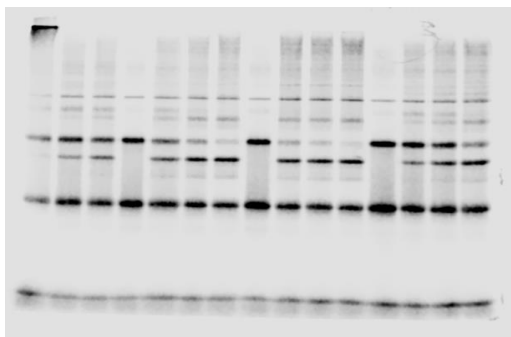
e



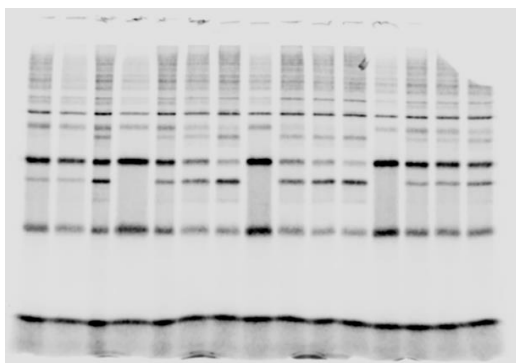
Supplementary Figure 9. Uncropped images of Figure 4.

(b)(c)(e) Uncropped SDS-PAGE of corresponding panels in Figure 4.

b



c

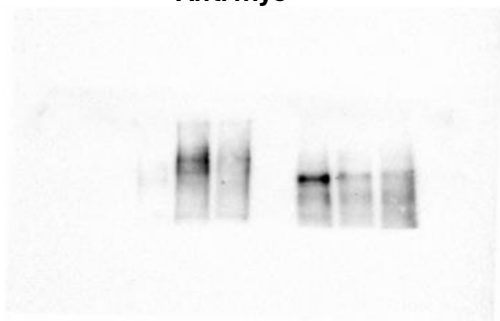


Supplementary Figure 10. Uncropped images of Figure 5.

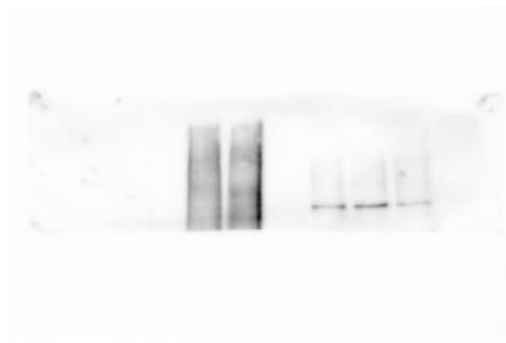
(b)(c) Uncropped SDS-PAGE of corresponding panels in Figure 5.

C

Anti myc

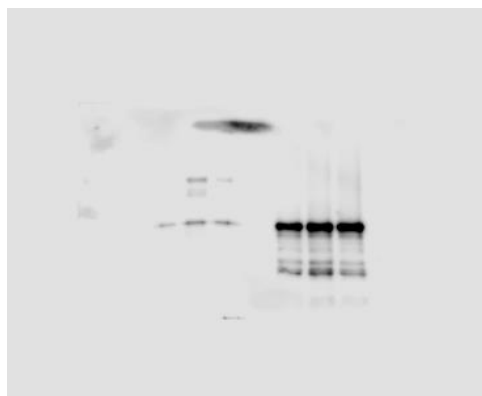


Anti Smt3

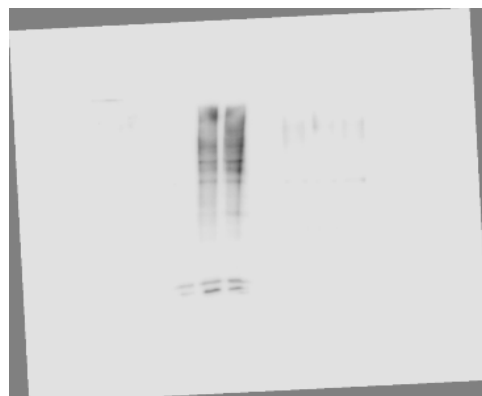


d

Anti HA



Anti Smt3



Supplementary Figure 11. Uncropped images of Figure 6.

(c)(d) Uncropped WB gels of corresponding panels in Figure 6.