

SUPPLEMENTARY FIGURES

Figure S1

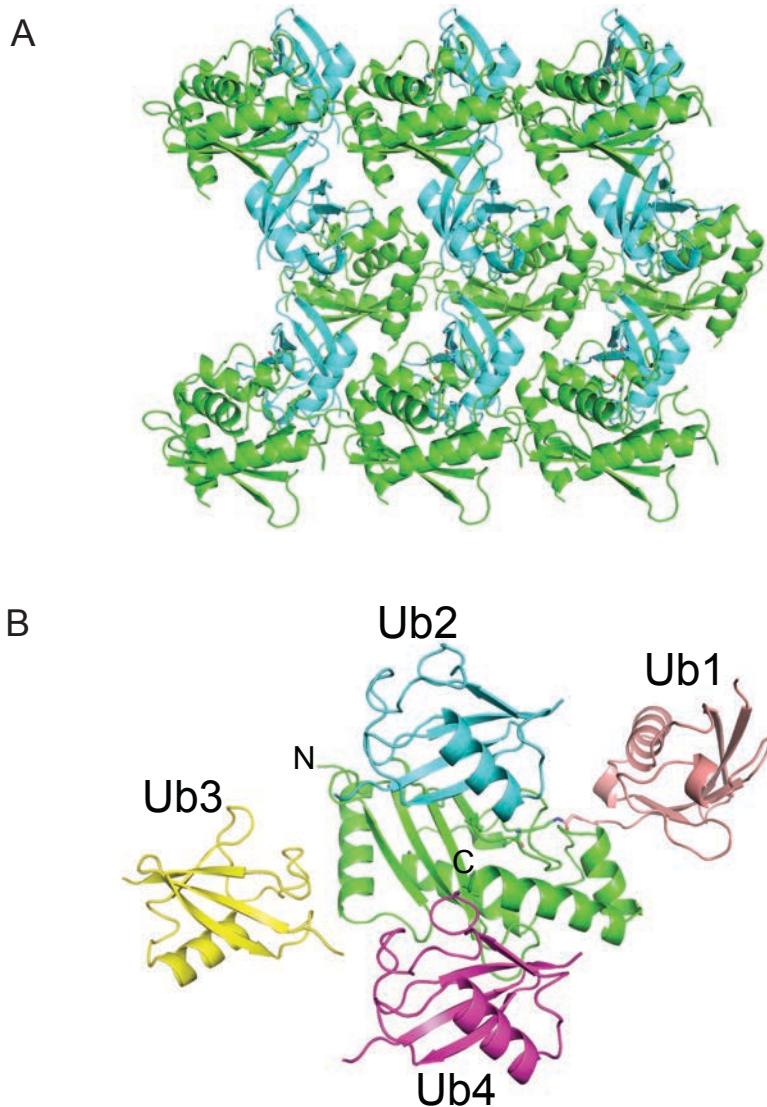


Figure S1. (A) Packing of Rad6~Ub conjugates in the crystal. Rad6 is shown in green and ubiquitin in cyan. (B) Packing interactions between Rad6 and four ubiquitin molecules in the Rad6~Ub crystal lattice. The Rad6 (green)~Ub (salmon) conjugate and three symmetry-related ubiquitin molecules that contact Rad6 in the crystal are shown as cartoon representation. Ubiquitin (Ub1) is conjugated to Rad6 via an isopeptide linkage and has a buried interface area with Rad6 of 181.5 \AA^2 . Ubiquitin (Ub2) is involved in interactions through the F4 hydrophobic patch and binds to the non-canonical backside of Rad6 with a buried interface of 446.1 \AA^2 . Ubiquitin (Ub3) interacts across the N-terminal helix of Rad6 with a buried interface of 240.8 \AA^2 . Ubiquitin (Ub4) interacts via its Ile44 surface with Rad6 with a buried interface of 331.9 \AA^2 .

Figure S2

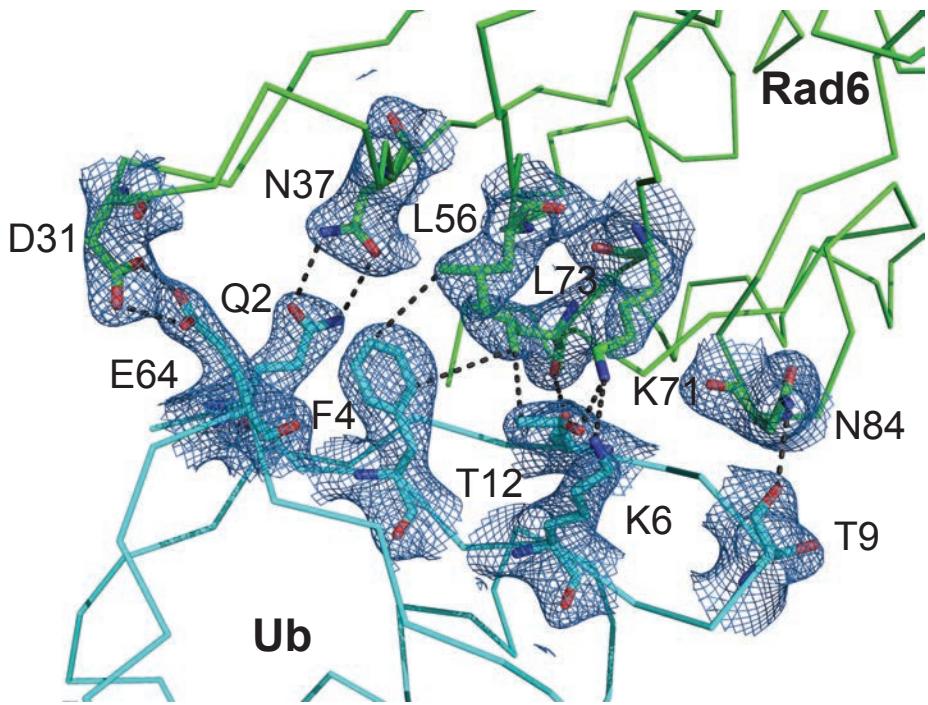


Figure S2. Omit map showing non-canonical backside interactions between Rad6 and ubiquitin in the Rad6~Ub crystal structure. The 2Fo-Fc electron density map contoured at 1.0σ .

Figure S3

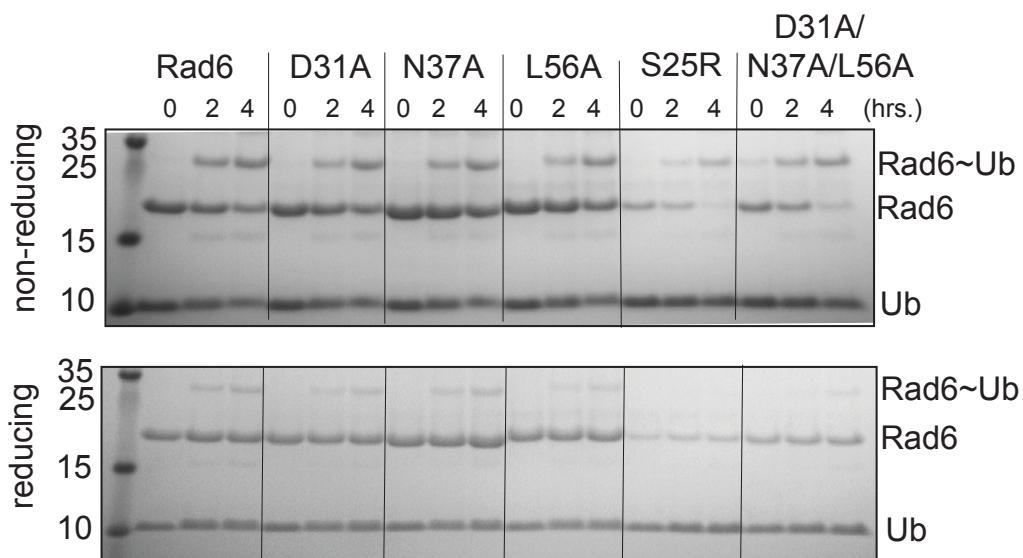


Figure S3. Charging assays for Rad6 mutants. Reactions were performed in 1.0 μ M E1, 20 μ M Rad6, 50 μ M Ub in reaction buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.5 μ M TCEP at 30°C. Thioester bond formation was monitored at the indicated time points by SDS-PAGE using sample loading buffer with or without reducing agent as indicated.

Figure S4

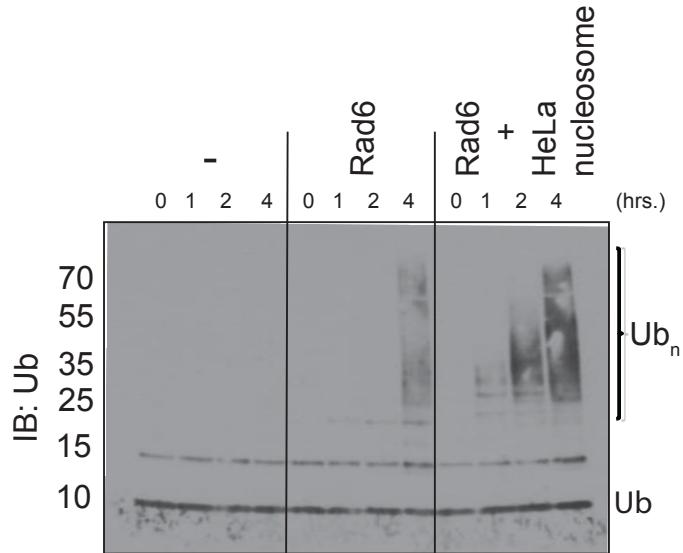


Figure S4. Rad6 ubiquitin conjugating activity in the presence and absence of HeLa nucleosomes. The ubiquitination reaction was performed at 30 °C with 2 μ M Rad6, 0.1 μ M E1, 20 μ M ubiquitin, with and without 1.25 μ M HeLa nucleosomes. Ubiquitin and ubiquitinated H2B were probed by Western blotting using anti-Ub antibody.

Figure S5

D31	N37
RAD6 <i>S.cerevisiae</i>	-----MSTPARRRLMRDFKRMKEDAPCGVSASPLD-NVMWNAVII 41
UbcB <i>Talaromyces stipitatus</i>	-----MSTSARRRLMRDFKRMQTDPPAGVSASPVD-NVMTWNAVII 41
E2 2 <i>Blastomyces dermatitidis</i>	-----MSTAARRRLMRDFKRMQTDPPAGVSASPVD-NVMTWNAVII 41
E2-17kDa <i>Botrytis cinerea</i>	-----MSTAARRRLMRDFKRMQTDPPAGVSASPVD-NVMTWNAVII 41
E2 2 <i>Trichoderma reesei</i>	-----MSTAARRRLMRDFKRMQTDPPAGVSASPVD-NVMTWNAVII 41
mus-8 <i>Neurospora tetrasperma</i>	-----MSTAARRRLMRDFKRMQTDPPAGVSASPVD-NVMTWNAVII 41
E2 2 <i>Spathaspora passalidarum</i>	-----MSTPAKRLMRDFKRMQDAPSGVSASPLD-NVMKWNAVII 41
E2-20kDa <i>Clavisporea lusitaniae</i>	-----SSLIASKRFLTPHCERNLTMSTPKRRLMRDFKRMQSDAPSGVSASPLD-NVMSWNAVII 119
E2 2 <i>Candida tenuis</i>	-----MSTPAKRLMRDFKRMQDAPSGVSASPLD-NVMTWNAVII 41
E2 7-like <i>Musa acuminata</i>	-----MAATTQASLLLQQKLRLDKMNKPVGFSAGLVDNSNFEEWAVTII 44
E2 7 iso <i>Amborella trichopoda</i>	-----MEYERATLLLQQLKEELLKNPVGFSAGLVDNDNFEEWAVTIT 43
E2 11-like <i>Phoenix dactylifera</i>	-----MASVYCGGIMANKRIKELQDLQRDPPTCSAGPAGE-DLFHWAVATIM 47
E2 D2-like <i>Chinchilla lanigera</i>	-----MALQRIQKELADLAQNFPQAFSAGPMCD-DLFWVATII 38
* : * : * : . : . : * * : * : * : * : *	
L56	
RAD6 <i>S.cerevisiae</i>	GPADTPYEDGTFRLLLEFDEEYPNPKPVHKFLSEMFPHPNVYANGIEICLDILQN----- 94
UbcB <i>Talaromyces stipitatus</i>	GPADTPFEDGTFRLMHHFEEQYPNPKPGVKFISQMFPHPNVYGTGELCLDILQN----- 94
E2 2 <i>Blastomyces dermatitidis</i>	GPADTPFEDGTFRLMHFEEQYPNPKPGVKFISQMFPHPNVYGTGELCLDILQN----- 94
E2-17kDa <i>Botrytis cinerea</i>	GPADTPFEDGTFRLMTFEEQYPNPKPAFKFISQMFPHPNVYATGELCLDILQN----- 94
E2 2 <i>Trichoderma reesei</i>	GPADTPFEDGTFRLMQFEEQYPNPKPSVKFISQMFPHPNVYATGELCLDILQN----- 94
mus-8 <i>Neurospora tetrasperma</i>	GPADTPFEDGTFRLMQFEEQYPNPKPSVKFISQMFPHPNVYATGELCLDILQN----- 94
E2 2 <i>Spathaspora passalidarum</i>	GPADTPFEDGTFRMLQFDEQYPNPKPSVKFISQMFPHPNVYASGELCLDILQN----- 94
E2-20kDa <i>Clavisporea lusitaniae</i>	GPADTPFEDGTFRMLQFDEQYPNPKPSVKFISQMFPHPNVYASGELCLDILQN----- 172
E2 2 <i>Candida tenuis</i>	GPADTPFEDGTFRMLQFDEQYPNPKPSVKFISQMFPHPNVYASGELCLDILQN----- 94
E2 7-like <i>Musa acuminata</i>	GPPDTIYEGGCNAIMSFSNSYPNSPSVRFTSEMFHNPVYSDGRVCISILHPPGDDPN 104
E2 7 iso <i>Amborella trichopoda</i>	GPPDTIYEGGCNAIMSFSNSYPNSPSVRFTSEWFHNPVYSDGRVCISILHPPGDDPN 103
E2 11-like <i>Phoenix dactylifera</i>	GAESPYAGGVFFVIMHFPPDYPKPKPVNFOTKVYHENNINSNGSICLDILKE----- 100
E2 D2-like <i>Chinchilla lanigera</i>	GPNDTPIYGQGGVFSLIAFPSSYPIFPFRVTFTTRIFHPNISKHGTICLDILGN----- 91
* : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
RAD6 <i>S.cerevisiae</i>	-----RWTPTYDVASILTSIQSLFNDPNPASPAVNEAATLFKDHDHSQYVKRVKETVEKS 148
UbcB <i>Talaromyces stipitatus</i>	-----RWSPTYDVAAILTSIQSLNNDPNTSSPANVEASNLKYDNRREYVKRVRETVEKS 148
E2 2 <i>Blastomyces dermatitidis</i>	-----RWSPTYDVAAVLTSIQSLNNDPNTGS PANVEASNLKYDNRREYTKRVRETVEKS 148
E2-17kDa <i>Botrytis cinerea</i>	-----RWSPTYDVAAVLTSIQSLNNDPNTGS PANVEASNLKYDNRREYTKRVRETVEKS 148
E2 2 <i>Trichoderma reesei</i>	-----RWSPTYDVAAVLTSIQSLNNDPNTGS PANVEASNLKYDNRKEYTKRVRETVERS 148
mus-8 <i>Neurospora tetrasperma</i>	-----RWSPTYDVAAVLTSIQSLNNDPNTGS PANVEASNLKYDNRKEYTKRVRETVERS 148
E2 2 <i>Spathaspora passalidarum</i>	-----RWSPTYDVAAVLTSIQSLNNDPNISSPANVEAANLYKDHRSQYVKRVRETVERS 148
E2-20kDa <i>Clavisporea lusitaniae</i>	-----RWSPTYDVAAVLTSIQSLNNDPNISSPANVEAANLYKDHRSQYIKRVRETVERS 226
E2 2 <i>Candida tenuis</i>	-----RWSPTYDVAAVLTSIQSLNNDPNISSPANAEAAANLYKEHRSQYIKRVRETVERS 148
E2 7-like <i>Musa acuminata</i>	YELASERWPVHTVESIVLTSIISMSSPNDPESVANEAKEWRESRDEFKKKVRIVRRS 164
E2 7 iso <i>Amborella trichopoda</i>	YELASERWPVHTVESIVLTSIISMSSPNDPESVANEAKEWRESRDEFKKKVRIVRRS 163
E2 11-like <i>Phoenix dactylifera</i>	QWSPALTIKVLLSICSLTDPPNDDPLVPEIAHIYKNQRSRYEETARAWTQKY 154
E2 D2-like <i>Chinchilla lanigera</i>	QWSPALTIKMVLLYIYNVLCDPNNPDSFHPEIANYLKNRAEYERIARRWTQKY 145
*: * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
RAD6 <i>S.cerevisiae</i>	WEDDDMDMDDDDDDDDDDDDEAD- 172
UbcB <i>Talaromyces stipitatus</i>	WED----- 151
E2 2 <i>Blastomyces dermatitidis</i>	WED----- 151
E2-17kDa <i>Botrytis cinerea</i>	WEDN----- 152
E2 2 <i>Trichoderma reesei</i>	WED----- 151
mus-8 <i>Neurospora tetrasperma</i>	WED----- 151
E2 2 <i>Spathaspora passalidarum</i>	WLEDDL--EDDEDKDE- 164
E2-20kDa <i>Clavisporea lusitaniae</i>	WDEDDD--EDGEE--EDE- 240
E2 2 <i>Candida tenuis</i>	WNDDDDDDEEDDEDEDEDDANDK 173
E2 7-like <i>Musa acuminata</i>	QEML----- 168
E2 7 iso <i>Amborella trichopoda</i>	QEAL----- 167
E2 11-like <i>Phoenix dactylifera</i>	AMG----- 157
E2 D2-like <i>Chinchilla lanigera</i>	AM----- 147

Figure S5. Multiple sequence alignment showing top twelve results of Blast search against the yeast Rad6 sequence.

Figure S6

	D31	N37
yRad6	--MSTPA--RRRLMRDFKRMKDAPPGVASPL-PDNVMVNAMIGPADTPYEDGTFRL	
hRad6b	--MSTPA--RRRLMRDFKRLQEDPPVGVSAGPS-ENNIMQWNAVIFGPEGTPFEDGTFKL	
hRad6a	-----MVWNNAVIFGPEGTPFEDGTFKL	
UBCH5C	--M---A--LKRINKELSDLARDPPAQCSAGPV-GDDMFHWQATIMGPNDSPYQGGVFLL	
UBCH5B	--M---A--LKRIHKELNDLARDPPAQCSAGPV-GDDMFHWQATIMGPNDSPYQGGVFLL	
UBC13	--M---AGLPRTIIKETQRLLAEPPVPGIKAEPD-ESNARYFVVVIAGPQDSFFEGGTFKL	
Ube2g2	GHMAGTA--LKRLMAEYKQLTLNPPEGIVAGPMNEENFEWEALIMGPEDTCFEFGVFP	
 L56		
yRad6	ILLEFDEEYPNKPPHVKFLEMFHPNVYANGEICLDILQ-----NRWTPTYDV	
hRad6b	VIEFSEEEYPNKPTVRFVLSKMFHPNVYADGSICLDILQ-----NRWSPTYDV	
hRad6a	TIEFTEEYPNKPTVRFVSKMFHPNVYADGSICLDILQ-----NRWSPTYDV	
UBCH5C	TIHFPPTDYPFKPPKVAFTRRIYHPNINNSNGSICLDILR-----SQWSPALTI	
UBCH5B	TIHFPPTDYPFKPPKVAFTRRIYHPNINNSNGSICLDILR-----SQWSPALTI	
UBC13	ELFLPEEYPMAAPKVRFMTKIHYPNVDKLGRICLDILK-----DKWSPALQI	
Ube2g2	ILSFPLDYLPLSPPKMRFTCEMFHPNIYPDGRVCISILHAPGDDPMGYESSAERWSPVQSV	
yRad6	ASILTSIQSLFNDPNPASPANVEAATLFKDHKSQYVKRVKETVEKSWEDEDDMDMDDDDDDD	
hRad6b	SSIILTSIQSLLDEPNPNSPANSQAAQLYQENKREYEKRVSAIVEQSWND-----	
hRad6a	SSIILTSIQSLLDEPNPNSPANSQAAQLYQENKREYEKRVSAIVEQSWRD-----	
UBCH5C	SKVLLSICSLLCDPNPDDPLVPEIARIYKTDRDKYNR-----ISREWTK-YAM-----	
UBCH5B	SKVLLSICSLLCDPNPDDPLVPEIARIYKTDRDKYNR-----IAREWTQK-YAM-----	
UBC13	RTVLLSIQALLSAPNPDPLANDVAEQWKTNNEAQIAE-----TARAWTRL-YAMNNI-----	
Ube2g2	EKILLSSVSMILAEPNDESGANVASKMWRDDREQFYK-----IAKQIVQK--SL-GL-----	
yRad6	DDDDDDDEAD	
hRad6b	-----	
hRad6a	-----	
UBCH5C	-----	
UBCH5B	-----	
UBC13	-----	
Ube2g2	-----	

Figure S6. Multiple sequence alignment of yeast Rad6 with human RAD6B (UBE2B), RAD6A (UBE2A), UBCH5B (UBE2E2), UBCH5C (UBE2D3) UBC13 (UBE2N) and UBE2G2.

Figure S7

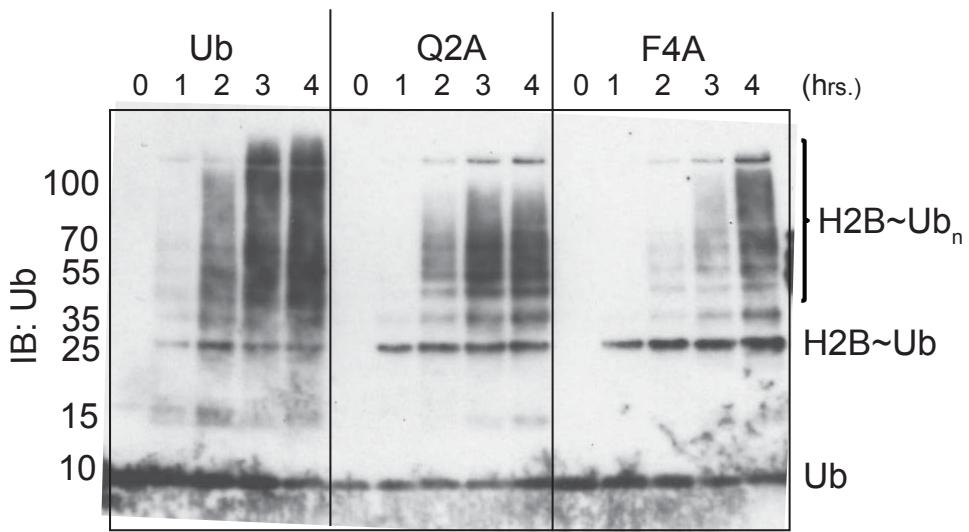


Figure S7. Histone polyubiquitination assays with wild type ubiquitin and mutants Ub^{Q2A} and Ub^{F4A} . The ubiquitination reaction was performed at 30 °C at the indicated time points with in reaction buffer containing 2 μM Rad6, 0.1 μM E1, 5 mM ATP, 5 mM MgCl_2 , 1 mM DTT, 20 μM ubiquitin, 1.25 μM HeLa nucleosomes and 50 mM Tris, pH 7.5. Ubiquitinated H2B and ubiquitin were probed by Western blotting using anti-Ub antibodies.

Figure S8

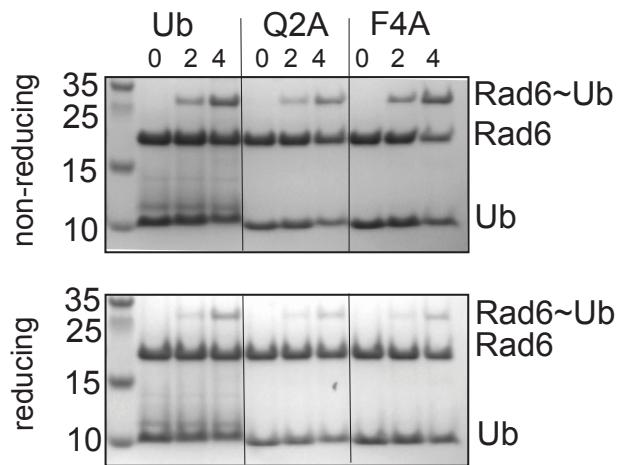


Figure S8. Charging assays for Rad6 with wild-type (Ub) and mutant ubiquitin.
Thioester bond formation between Rad6 and either wild type or mutant (Ub^{Q2A} and Ub^{F4A}) ubiquitin was monitored as described in the legend to Figure S3.

Figure S9

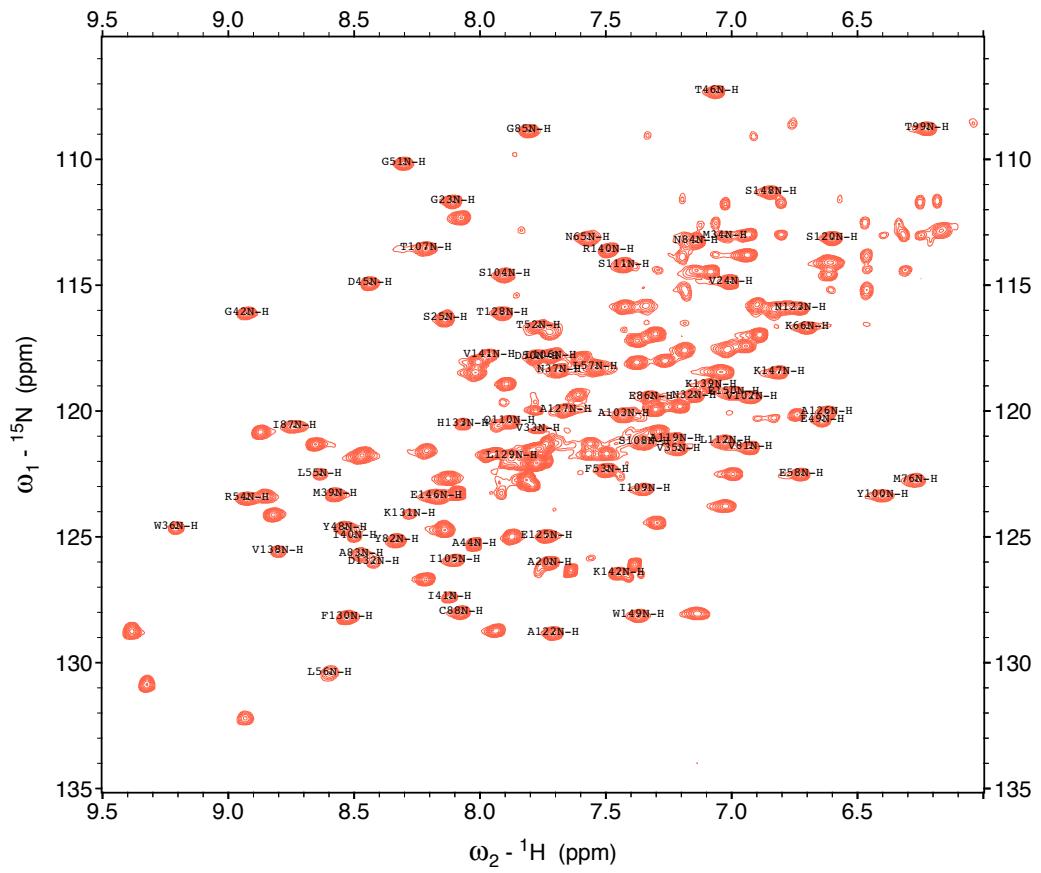


Figure S9. TROSY-HSQC spectra of 70% ^2H , ^{15}N Rad6 showing assigned residues.

Figure S10

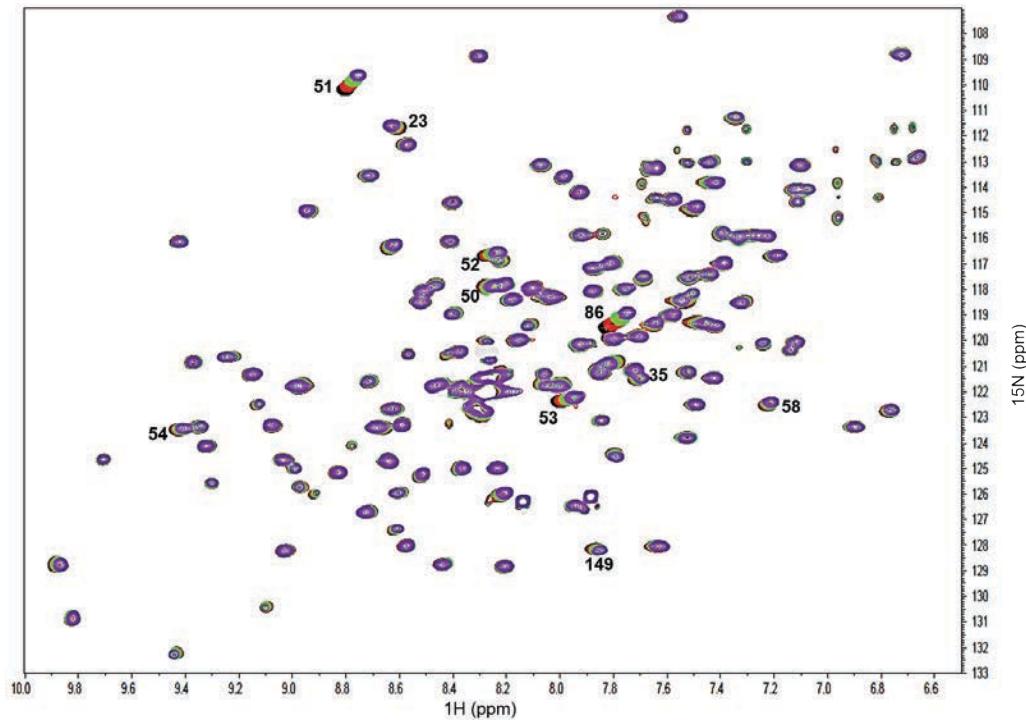


Figure S10. Interaction of Rad6 and ubiquitin as observed by TROSY-HSQC NMR.
The starting concentration of 70%-²H,¹⁵N-Rad6 was 150 μM, with ubiquitin added to a final concentration of 200 μM (black), 400 μM (red), 600 μM (green) and 800 μM (purple) for the respective titration points.

Figure S11

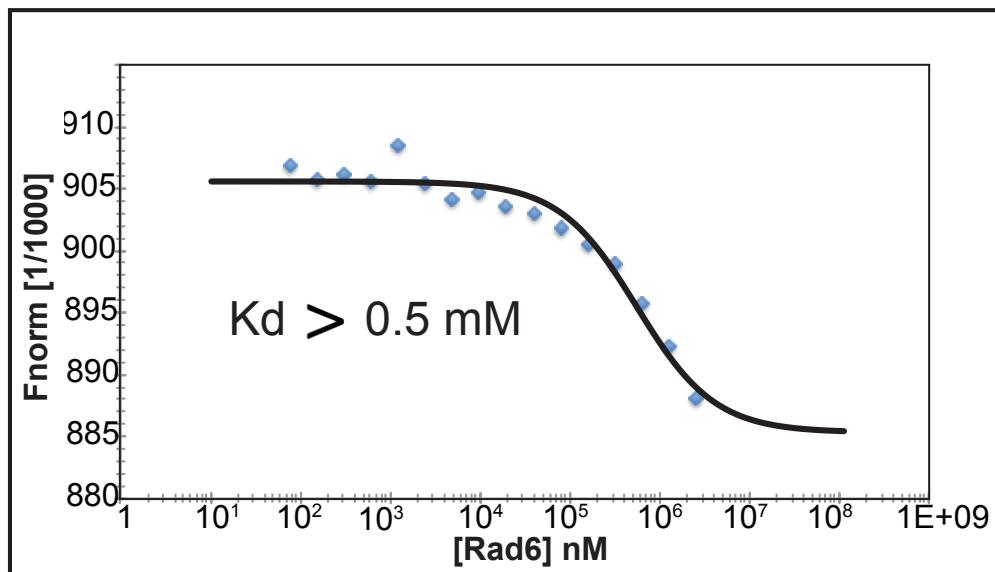


Figure S11. Interaction of Rad6 and ubiquitin monitored by microscale thermophoresis (MST). A titration series of Rad6 with protein concentrations from 2540000 – 77.0 nM was performed while the fluorescein-5 maleimide labeled ubiquitin concentration was held constant at ~150 nM. After a short incubation, the samples were loaded into standard glass capillaries and the MST analysis was performed using a Monolith NT.115 (NanoTemper). Concentrations of Rad6 on the x-axis (blue dots) are plotted in nM. The estimated dissociation constant (K_d) of 561000 ± 26800 nm determined for this interaction assuming a 1:1 binding model constitutes a lower bound, as binding could not be saturated even at the highest Rad6 concentration.

Figure S12

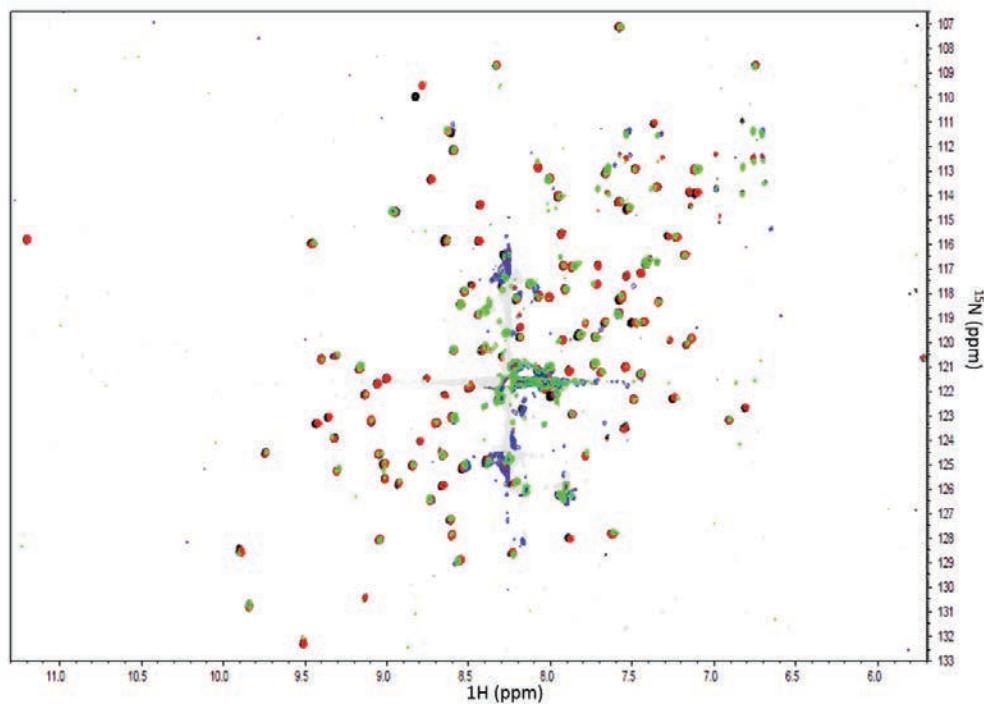


Figure S12. Oligomerization of Rad6~Ub conjugates monitored by NMR and discharging of conjugates upon addition of DTT and EDTA. Shown here are the overlaid $[^{15}\text{N}, ^1\text{H}]$ -TROSY HSQC spectra of 250 μM ^{15}N -Rad6 (black), 250 μM ^{15}N -Rad6 + 750 μM Ubiquitin (red), 250 μM ^{15}N -Rad6 + 750 μM Ubiquitin + 10 mM E1 (blue), 250 μM ^{15}N -Rad6 + 750 μM Ubiquitin + 10 mM E1 + DTT + EDTA (green).

Figure S13

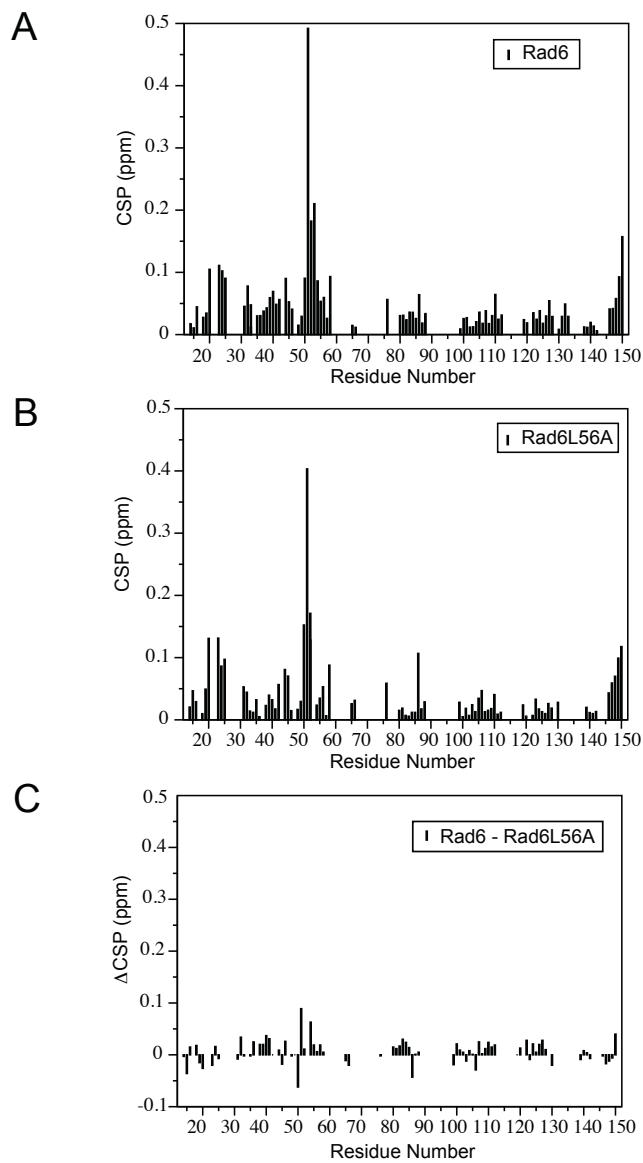


Figure S13. Monitoring of Rad6-ubiquitin interactions by chemical shift perturbation (CSP) (A) CSP data from interaction of 750 μM ubiquitin titrated into 250 μM ¹⁵N-Rad6. (B) CSP data from interaction of 750 μM ubiquitin into 250 μM ¹⁵N-Rad6^{L56A}. (C) CSP difference data of ¹⁵N-Rad6 and ¹⁵N-Rad6^{L56A} from interaction with ubiquitin.

Figure S14

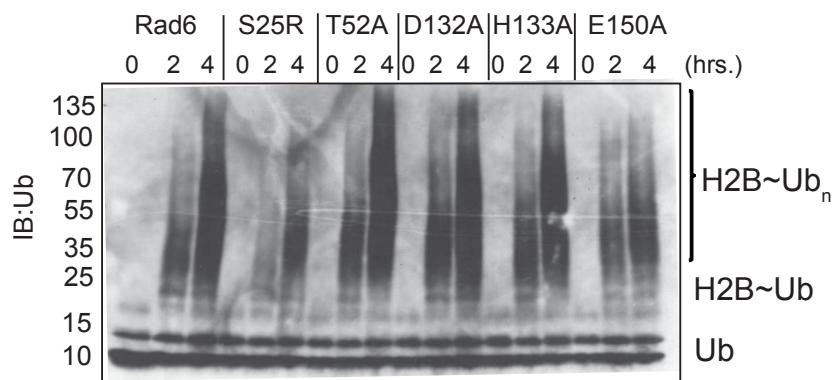


Figure S14. Histone polyubiquitination assays with Rad6 containing point substitutions at residues that show chemical shift perturbation with free ubiquitin. Activity of wild type and Rad6 mutants containing S25R, T52A, D132, H133A and E150A substitutions. The ubiquitination reaction was performed at 30 °C at the indicated time points with 2 μ M Rad6 in reaction buffer containing 0.1 μ M E1, 5 mM ATP, 5 mM MgCl₂, 1 mM DTT, 20 μ M ubiquitin, 1.25 μ M HeLa nucleosome and 50 mM Tris, pH 7.5. Ubiquitinated H2B and ubiquitin were probed by Western blotting using anti-ubiquitin antibodies.