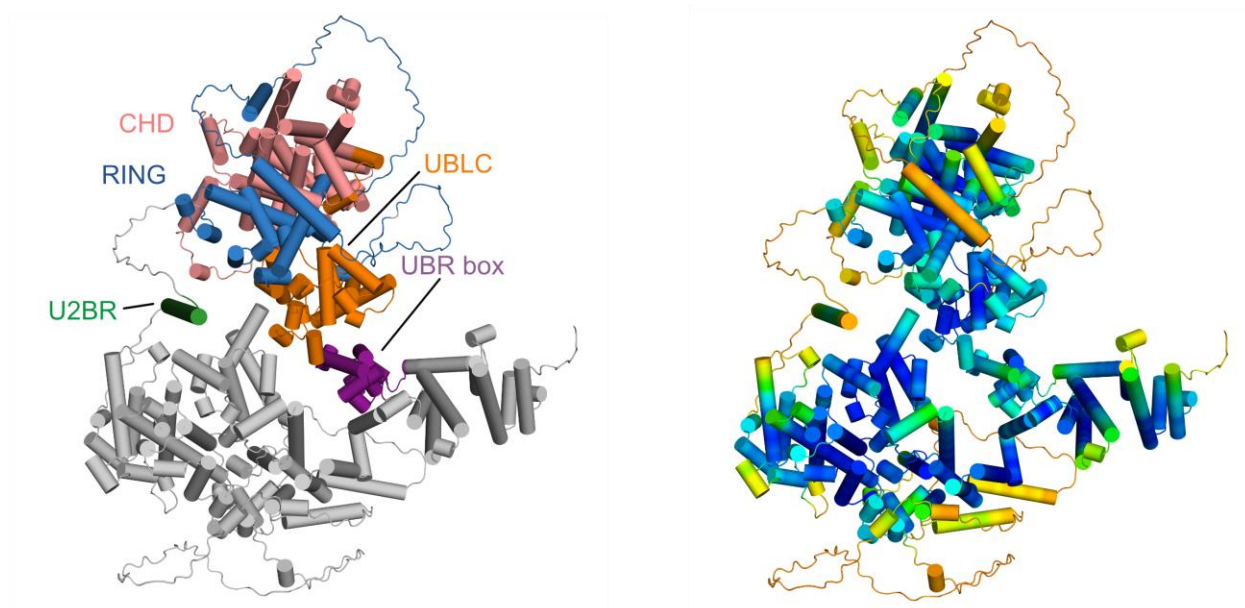


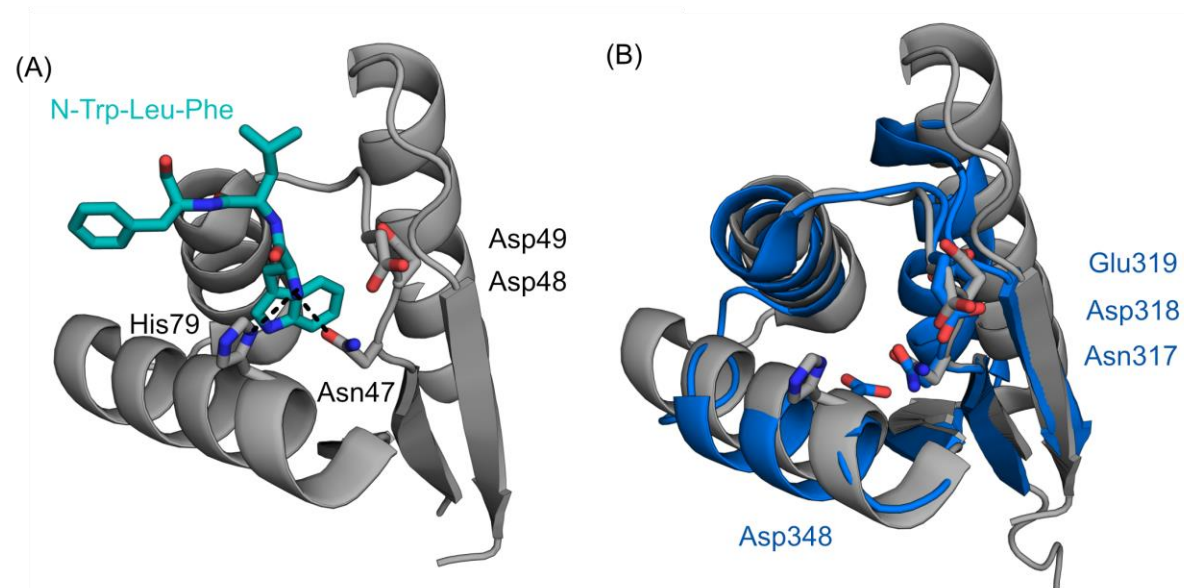
Supplementary Figure 1. Amino acid sequence alignment of *Saccharomyces* RAD6, with Human UBE2A and UBE2B, and *Arabidopsis* UBC1 and UBC2. The catalytic cysteine (Cys88) and residues that enhance Ub discharge (Asn80, Asp90 and Ser120) are indicated with red arrows. Residues are coloured green (Ile, Leu, Met, Val), orange (Gly, Pro, Ser, Thr), blue (Phe, Trp, Tyr) and red (Arg, His, Lys). The sequences and UniProt accession numbers are *Saccharomyces cerevisiae* RAD6 (P06104), *Homo sapiens* UBE2A (P49459), *H. sapiens* UBE2B (P63146), *Arabidopsis thaliana* UBC1 (P25865) and *A. thaliana* UBC2 (P42745).

UBR1	MSVADDDLGLSQG---HIRRTLRSIHNLPYFRYTRGPTERADMSRALKEFIYRYLYFVINSNGENLPTLFN--AHPKQKLSNPETVFPDSLED	89
PRT6	METNSSLFGLVSPSSHDLVIERLASVGVPPKYSRKRGLVEFVR--ANPAKISELVSAALLPTDDDKVLGLKEARERPRKSAVSPMTK---KRFRE	89
UBR1	AVDIDKITSQQTIPFYKIDESRIGDVHKHTGRNCGRKFKIGEPYLRHECGDDTCVLCHCFNPKDHNHVCVDICTEFTSGICDGDDEEAWN	184
PRT6	SMNMLQWLMFQDEP--DVSLRNLAKLNLDDQGVGGSVWGQNDIAYRRTENDPTCAICVCFQNGDHNSHDYSI---IYTGGGCDDGDDETAWK	179
UBR1	SPLHCKAEEQENDISEDPA--ATNADI---KEEDVWNSVNIALVELVLAEVFDYFIDVFNQNIIEPLPTIQKDIITIKLREMTQ--GV	261
PRT6	PDGFCSNHKGSEQIRPLSENLANSVGPILDALFTCNNKLLSAESSGKFG-----ARSDNTLVILQKMSNELTFIVVEMLLFSSMSESLLSF	267
UBR1	QGK--MYERAQFLNDL--KYENDYMGDGTAKTSPSPSPASPPLAKIDPENYTVIIN--DEYHNYSQATTALRQ--GV	334
PRT6	VSRRIISSSGLLSILLKAERFLDQDMKKLHDLFLK-----LIGDPVFKCEFAKAFVSYYPVIVSEVVKQGTDNFAKKYPLLSFTFSVQILTV	354
UBR1	PDNV--HIDLLTSRIDGEGRAMLKCSQDL--SSVLGGFFAVQTNGLSATLTSWSEYLHQETCKYIILWITHLNIPNS	409
PRT6	PTLTPFLVKEMNLLAMLGLCLSDIFVSCSGEDGLLQATKLERLCETSERVIGDLKFVMSHA-----IVSKYATHEHRELSRSLTLLT	437
UBR1	FQTTFRNMGMKTLCSYLNATECRDMTPVEKYFSNKFQKNDPPYRIDLSLADGNIPLGHHKILPESSTHLSPLINDVETPTSRYSNTRQL	504
PRT6	FAQGMNPLKRE--TGIPIDEE--NDYMHFLF--VLGHSIAVHISLLVNGTYSAA--SDEIEIENDRN--AKEEFD	501
UBR1	HILYFDNRYWKRLRKDIQNVIIPTLASSNLYKPIFCQQVVEIFNHITRSVAYMDRPGQLTAIRECVQVLETCPTNAKNIFENQSELDIVWSIID	599
PRT6	KCDGGGERYAKVGRLSHEDSVCTAIVSSSSFDSSMASEVHK--I--DPFHALLPSSAIYILIRECLKVLETCLGNDEGISKFLCKLSS	584
UBR1	FKEFCCKVEGGVLWQVOKSNLTYSYSISFKQGLYTVETLLSKVHDNPIRLPKEIISLLTLCKLFNGAWKIKRKEGEEDQNFISYLEYTT	694
PRT6	SSGRNIPESKMSWPRRDLLNVETGGS--VSSNLASSSRDPSTGLSP--LCGDIQTNLSLD--NVC	643
UBR1	SIYSIIQTAEKVSEKSDSIDSKLF--LNAIRIISFLGNRSITYKLIYDSHEVIFKFSVSHERVAFMNPLQTMLSFLIEKVSL-----	775
PRT6	GPYGVVQTDVTDADSKRVSCNSADLTKNASGLRILG--LCDWPDHYDVSSQAI SVHPLHRLLSLLIQKALRICYGESASYNGV	725
UBR1	KDAYEALEDCSDFLKISDFSLRSVVLCSQIDVGFVWRNGMSVLHQASYKKNPELGS--YSRDIHLNQLAILWERDDIPRIIYN	857
PRT6	SISHEIPHADFFSSVIGDFHPCGFSALVMEHLQIRVFCAQVIAAGMWKKNQDSALVSCWEYRSVRWSEGLELDLFLQLCC--AALAPADSYVDK	818
UBR1	ILDRWELLDWFTGEVDYQHTVYEDKISFIQQFIAFIQYILTERQYKFTFSSLKDRRMDQIKNSIIYNLYMKPLSYKLLRSVPDYLTEDTTF	952
PRT6	LLSRFGLSSYLSLNPDI--ITNEYEPVLVQEMGLGLLIQILQERRFCGLST--AESLRREIIFKLATGDFTHSQLVKSLLPRDLISK--SDELO	902
UBR1	EALKEEVSVFVEPKGLADNGVFKLKASLYAKVDPLKLLN--LENEFESSATI--IKSHLAKDKDEIAKVVLIPQVSIKQLDKD	1030
PRT6	EVLDVSVVCNPSGMNQ--GYSLQSSCWKELDLYHPRWQSRDLQSAEERFSRYCGVSALTTLQLP--WRMIYPLPKGLARIGTKCATFQIIS	983
UBR1	ALNLGAFTRTNTYFAKVY--YK--LLQVCLDMEDSTF-----LNELLHLVHGI FRDDELINGKDSI	1086
PRT6	SALYALQSGTSVKSRAPDGVLITALQLLSLSDICTQQRQNSQDCCLENSIPILELAGLEIGIAAQGTESKLSLLVLSLM--KTRMGDGRHQF	1085
UBR1	PEAYLSKPICNLLLSIANAKSDVSESIVRKADYLLEKIMMKPNELFESLIASFNGQYVNDYKDKKLROGVNLQETEKERKRLAKKHQARLLA	1181
PRT6	PEP---GSCNIISSWIGNL-----LKKFS--AIDSCMNLLQSLAPEVVGSGDFDKVMSG---STSDKKRAKAKAKERQAAIMA	1154
UBR1	KFNNQQTQKFMKEHESEFDEQDNDVDMVGE-----KVYESEDFTCALQDSSSTDFVFIIPAYHDHSPIFRPGNIFNPNFMPMWDFGYN	1264
PRT6	KMAEQSKFLSTLSSMDDDDPREFEFETSDSVMEHDSEIAVREVCSLCHDPDSKDPVSFLIFLQSKLLSF-----VDRGPPSWDQCQSEKKIS	1244
UBR1	VDGAPDLLRMNASSDSLRISSPLMLQLSDDTISESANMIESIKARLIGNGQTEKRSSDGRGKDESNMESLEIAMYQTVRNKIENMINQSLTRVDH	1274
PRT6	QPHAEANCSEKNSVGGPSTLQGRFPDIRSRQTSRRPDAGSDGFHPIDCDGVYLSGCGHVAHQSGCLERYLSLKERSGRRTVFEGAHIVDLKKKEF	1339
UBR1	ICPLCOTFSNCTPLCQTSKANTGLSL--DMFLESELSDLRLFKPFTEENYRTINSIFSLMISQCQGFDAKVR--KRANF	1397
PRT6	LCVPCRRRLANSVLPECPGDLCSVSKLQDSPRTKLRRKDALQPSLWLSEALCL--LR--SAAEVIEDGDRG--KTVPQGDGP	1510
UBR1	SHKDVSLISLVHWAN--TI--SMLEIASRLEKPYISFFR--SRQKQYKTLKNILVLCIMLFTFV--	1455
PRT6	RRKDLKSVSKMLWDFYFPKPEDKTLKRLWLPPQSIVMWDTLKYSLSMEIGTRFAKNSMLPVYCIDSLYEELKTSKGTILSVLLRVVQSSRTKNT	1605
UBR1	IGKP--SMEFE--PYQPQPDVTWNQNL--FQYIVRSALFSPVSLRQTVTEALTTFSRQFLRDLFQ	1516
PRT6	IHVQRQFVGMKHLAESICYGVSSSSSIFGSEGTGSLKNIDLWNRASDPVLAHPFSSLMWALFCLPFPFL--TCEESLLSLVHI--FHS	1694
UBR1	LSDAEQVTKLYA--KASKIGDVLKVSEQMLF--ALRTIS--DVRMEGLDSESIYDLAYTELLKSLPTIRRCVLFIKVLHVLKDSSENETLV	1603
PRT6	VSLVQTVIAYACRPSELS--ELNFGENLLNDISNALRESGGWEYFRSNNMDLS--CDI--KDTIRKYSLPFLRRCALLWLKLSKTPRKLHEESDM	1784
UBR1	INGHEVEEELEFEDTAEFVNKALKMITEKESLV--DLLTTQESIV--SHPYLENIPVEYCG--IJK--LIDLSKYLNTVYVTSQK	1679
PRT6	FDLPSDPTTNDMDFIYSPQ--SELNHVQELEKMFNIPIDIILNDELRSSTQIWLQHFQREYRVNRVRSCLCITPVVPFQLMKLPLNYQDL--	1874
UBR1	EIKLREERSQHMKNADNRDLDFKICLTCGVKVHLRA--DRHEMTKLNKNCKFKPFGAFLMPNSSEVCLHLTQPPSNIFISAPYLNSEGEVGRNAM	1771
PRT6	LQRCIKKRCVNTCTVIEEPVLCLLCGSLCSPWIWSPCCRRESGCPNMAITCGAGTGVLFLIRRTTILLQ--RFARQSPWSPYLDTFGEEDIDMI	1965
UBR1	RRGLTTTLNLKRYEHLNRLWINNEIPGYISRVMGDEFRTILS	1814
PRT6	RGRRLYLNEERYAALTYLVGSHGLD--RSSKVLGQTTIGAVLH	2006

Supplementary Figure S2A. Amino acid sequence alignment of *Saccharomyces* UBR1 (UniProt accession number P19812) and *Arabidopsis* PRT6 (UniProt accession number F4KCC2). *Saccharomyces* UBR1 domains are annotated according to the cryo-EM structure (PDB 7MEX) [51]. The zinc coordinating residues of the UBR-Box, RING finger, and UBLC domains are indicated in yellow. Red arrows indicate a putative zinc coordination site involving UBR Box (His161), WHD (Asp952) and the UBLC domain of UBR1 (His1763, Asp1775). A green box indicates a Ub binding loop in UBR1. Cyan arrows indicate UBR1 residues involved in Ub chain elongation.



Supplementary Figure S2B. AlphaFold2 model of Arabidopsis PRT6 (AlphaFold Protein Structure Database: AF_AFF4KCC2F1). Left: The predicted domain architecture of PRT6. Right: model coloured by predicted local distance difference test (pLDDT) score. Colours from high to low pLDDT; blue, cyan, green, yellow, orange.



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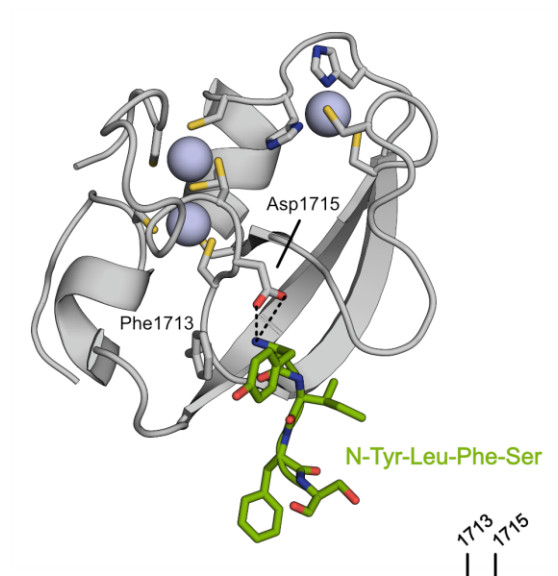
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PRT6  YYPVVISSEVVKQGTDNFAKKYPLLSTFSVQILTVPFLVKEMNLLAMLGCLSDIFVSCSGEDGLLQATKLERLCETSERVIGDLKFVMSHAI  448
UBR1  NYTVIINY-----DEYHNYSQATTALRQG--VPDN---VHI-DLLTSRIDGEGRAMLKCSQDLS-----SVLGGFFAVQTNGL  382
301F  MYKVILVN-----DDYTPMEFVIDVLQKF--FS-YDVERATQ-LMLAVHYQGKAICGV-FTAeva-----ETKV---AMVNKYARENEHPL  99
3GQ1  LYRVLILN-----DDYTPMEFVVVLERF--FN-KSREDATR-IMLHVHQNGVGVCVGY-YTYEVA-----ETKV---AQVIDSARRHQHPL  112
402X  AWKVILYN-----DDIHNFTYVTDVIVKV--VGQISKAKAHT-ITVEAHSTGQALILS-TWKSka-----EKYC---QELQ-----QNGL  176
7D34  PYRVILHN-----DNFNKREYVVQVLMKV--IPGMTVDNAVN-IMQEAHINGLAVVIV-CAQADA-----EQHC---MQLR-----GNGL  148

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Supplementary Figure S3. The UBR1 N-domain is structurally similar to ClpS proteins. **(A)** The *Caulobacter crescentus* ClpS protein bound to an N-degron (PDB 3GQ1). The α -amino group of the substrate interacts directly with His79 and Asn47. Hydrogen bonds are indicated with dashed lines. **(B)** Superposition of the N-domain of *Saccharomyces* UBR1 (blue, PDB 7MEX) and *C. crescentus* ClpS (grey, PDB 3GQ1). **(C)** Amino acid sequence alignment of PRT6 with representative ClpS domains with known three-dimensional structures. Sequences are Arabidopsis PRT6 (UniProt accession number F4KCC2), *Saccharomyces* UBR1 (PDB 7MEX), *Escherichia coli* ClpS (PDB 3O1F), *C. crescentus* ClpS (PDB 3GQ1), *Plasmodium falciparum* ClpS (PDB 4O2X), and Arabidopsis ClpS1 (PDB 7D34). Conserved binding pocket residues are indicated in red.

(A) UBR-Box



UBR4	KLCTFTITQKEFMNQHWYHCHTCKMVDGVGVCTVCAKVCHKDHEISYAKYGSFFCDCGAKED--GSC LALVK	1729
BIG	KVCTFTSSGSNFMEQHWYFCYTCDLTVSKGCCSVCAKVCHRGHRVVYSRSSRFFCDCGAGGVRGSSCQCLKP	1364

(B) DOC-1

UBR4	DFFEHNQQLTDVEFGGNDLLQVYNAQQIKHRLNSTGMYVANTKPGGFTIEISNNNSTMVM	2349
BIG	DFFERAFCITADVRLGSDAIRNGDSEGAKQSLASEDGFIESPSVPGFKISVSNPNPDIVM	2214
	**** * * * * *	
UBR4	TGMRIQIGTQAIERAPSYIEIFGRMTQLNLSRSRWFDFPFTREEALQADKKLNLFIGASV	2409
BIG	VGIRMHVGTTSASSIPSEVTIFQRSIKMDEGMRCWYDIPFTVAESLLADEDVVISVGPTT	2274
	* * * * *	
UBR4	DPAGVTMIDAVKIYGKTKEQFGWPDEPPEEFPSASVSNICPSNLNQSGTGDSDSAAPT	2469
BIG	SGTALPRIDSLEVYGRAKDEFGWKEKMDAVLDMEARV--LGHGLLL-P--GSSK-KRALA	2328
	** * * * *	
UBR4	TSGTVLERLVSSLEALESCFAVGPIIEK-	2498
BIG	QSASMEEQVIADGLKLLSIYYSVCRPRQEV	2358
	* * * *	

(C) ZZ domain

BIG	CDGCSTVPILRRRWHTVCP---DFDLCEACYEVLDA--DRLPPPHTRDHPM	2643
p62	CDGC-NGPVVGTRYKCSVCP---DYDLCSVCEGKGL-----HRG-HTK	165
PRT1	CDSCGVYPIIGDRYRCKDCKEEIGYDLCKDCYETPSKVPGRFNQQHTPDHRL	362
KCMF1	CDACLKGNFRGRRYKCLICY---DYDLCASCYESGATTT-----RHTTDHPM	52
UBR4	MVSESMVLETAENVNNGNPSPLEALLAGAEGFPMLDIPPDADDETM	2815

(D) Cysteine Rich (CR) region

UBR4	CPRCSASVPANPGVCGNCGENVYQCHKCRSINYDEKDPFLCNAC	3703
BIG	CPRCSRPTDKHGISNCHENAYQCRQCRNINYENLDSFLCNEC	3486

(E) Calmodulin binding region

UBR4	GKAPSKSELRHLYLTEKYVWRWKQFLSRRG	4113
BIG	GASQKSRTHRSDFLALKYTLRWKRRSSRTS	3904
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Supplementary Figure 4. Comparison of human UBR4 (UniProt accession number Q5T4S7) and Arabidopsis BIG (UniProt accession number Q9SRU2). BIG residues are numbered according to [103]. (A) The UBR-Box of human UBR4 (PDB 8J9R, grey cartoon) in complex with an N-degron (green sticks). Zinc coordinating residues and two residues which are essential

for substrate binding are shown as sticks. Asp1715 hydrogen bonds with the alpha-amino group of the substrate and Phe1713 is essential for both type I and II degron binding. An amino acid sequence alignment of UBR4 and BIG UBR-Box domain highlighting zinc coordinating residues (red). **(B)** An amino acid sequence alignment of the DOC-1 region of UBR4 and BIG, asterisks indicate identical residues. **(C)** An amino acid sequence alignment of the ZZ-domains from Arabidopsis BIG, human p62/sequestosome 1 (UniProt accession number Q13501), Arabidopsis PRT1 (UniProt accession number Q8LBL5) and human KCMF1 (UniProt accession number Q9P0J7). The putative zinc coordinating residues are in red. The equivalent region in human UBR4 lacks cysteines. **(D)** An amino acid sequence alignment of the UBR4 and BIG cysteine rich (CR) region, conserved cysteines are in red. **(E)** An amino acid sequence alignment of the UBR4 and BIG calmodulin binding region, asterisks indicate identical residues.