Cell, ***Volume 134***

Supplemental Data

UBXD7 Binds Multiple Ubiquitin Ligases and Implicates p97 in HIF1α Turnover

Gabriela Alexandru, Johannes Graumann, Geoffrey T. Smith, Natalie J. Kolawa, Ruihua Fang, and Raymond J. Deshaies

Table S1. Known p97 Cofactors Identified by

Mass Spectrometry in Flag-UBX Protein Immunoprecipitates

Protein Name	NPL4 69461 [a]	UFD1 38725 [a]	PLAP 87099 [a]	VCIP135 134320 [a]	p97	Flag Protein
p47	2(2) 6.0%		27(38) 42.9%	14(15) 17.6%	172(1611) 82.7%	30(164) 47.6%
UBXD8	11(209) 20.6%	7(82) 29.4%	10(15) 20.1%	11(12) 14.1%	48(3090) 57.6%	23(664) 44.3%
FAF1	22(46) 36.3%	13(15) 35.3%	2(2) 4.8%	5(5) 5.9%	84(539) 68.9%	30(205) 34.2%
UBXD7	14(33) 24.5%	4(4) 12.5%	2(2) 4.0%		77(375) 61.2%	38(224) 51.3%
SAKS1	7(30) 20.1%		26(90) 32.1%	2(2) 2.9%	68(663) 57.4%	7(26) 32.0%
UBXD6	9(16) 16.5%	4(5) 19.2%		2(2) 2.7%	55(212) 59.5%	17(94) 47.0%
UBXD5	2(3) 5.2%		6(6) 11.6%	20(25) 22.5%	124(783) 75.5%	5(16) 9.4%
UBXD1			3(3) 7.5%	2(2) 2.5%	159(1134) 84.3%	45(119) 66.9%
UBXD2					27(958) 41.1%	11(36) 34.3%
UBXD3	7(9) 14.1%	3(3) 12.2%			57(254) 64.5%	17(33) 48.9%
ASPL			10(10) 22.8%		154(1445) 78.5%	114(661) 78.8%
NPL4	49(387) 51.2%	14(22) 37.9%		2(2) 2.5%	79(414) 70.4%	49(387) 51.2%

[a] Molecular weight of the respective protein in Da.

The sequence count, the spectrum count (in parenthesis), and the corresponding percentage of sequence coverage are indicated for each interacting protein.

The results obtained for p97 and the UBX protein itself are shown as a reference.

Table S2. Ubiquitin Ligases Identified by Mass Spectrometry in Flag–(UBA-UBX) Protein Immunoprecipitates

PROTEIN NAME	IPI Identifier	MW (Da)	UBXD7		UBXD8		FAF1		SAKS1		p47	
			D	M	D	M	D	M	D	M	D	M
CRL^{Cul1} components												
Cullin 1 [a]	00014310	87388	3(3) **4.9**	6(10) **2.9**			6(8) **9.1**	5(7) **8.4**	5(7) **6.5**	2(3) **1.5**		
SKP1 [a, c]	00301364	18658	3(4) **30.7**				2(2) **10.7**	4(6) **33.6**				
RING-box protein 1, RBX1 [c]	00003386	12274		2(2) **4.1**								
F-box/WD-repeat protein 1B, FBW1B [a]	00328796	62091						2(2) **3.4**				
CRL^{Cul2} components												
Cullin 2 [a]	00014311	86983	12(16) **26.3**	23(199) **57.0**	4(12) **2.4**	8(38) **10.3**		6(6) **7.2**	3(4) **3.7**	4(11) **5.5**		2(2) **1.0**
Elongin B [a]	00410162	17911	2(2) **16.0**	5(7) **9.7**								
Elongin C [a]	00300341	12473		8(10) **20.0**	2(5) **7.0**	2(3) **5.7**						
Von Hippel-Lindau disease tumor suppressor, VHL	00027969	24153		2(2) **2.1**								
CRL^{Cul3} components												
Cullin 3 [a]	00014312	88930	13(23) **37.0**	16(30) **8.4**			15(22) **24.6**	12(12) **14.1**	3(3) **2.7**			
CUL3-interacting protein 1, KLHL12 [a]	00642182	63277	6(8) **18.1**	3(3) **1.2**					2(5) **6.4**			
BTB and Kelch domain-containing protein 2, KLHL13	00002398	73908		4(4) **1.4**								
Kelch-like protein 22, KLHL22 [b]	00156791	71667	3(3) **6.0**	8(8) **2.8**			6(6) **8.3**	8(8) **11.7**	2(2) **2.3**			

Kelch repeat and BTB domain-containing protein 7, KBTB7 [b]	00383044	77163		3(3) **1.0**			2(2) **2.6**		2(2) **2.1**			
Kelch domain-containing protein 2, KLDC2 [b]	00003793	46099	2(3) **9.3**	4(6) **3.2**								
Kelch domain-containing protein 3, KLDC3 [b]	00062558	43828		3(3) **1.7**								
Galectin-3 binding protein, LG3BP [b]	00023673	65331			2(3) **0.8**	2(3) **1.1**					3(3) **1.5**	
BTB/POZ domain-containing protein 2, BTBD2 [b]	00022826	55931							2(2) **2.9**			
CRL^{Cul4} components												
Cullin 4A	00419273	87722	5(6) **9.8**									
Cullin 4B	00643885	102756	7(9) **12.5**	10(11) **2.7**								
DNA damage-binding protein 1, DDB1 [a]	00293464	127030	11(11) **12.4**	13(13) **2.6**	2(2) **0.3**	3(4) **0.7**			2(2) **1.3**	2(2) **0.7**	2(2) **0.5**	3(3) **1.0**
DDB1- and CUL4-associated factor 1, VprBP	00329528	168936		3(4) **0.6**								
CUL4- and DDB1-associated WDR protein 2, WDR26 [a]	00414197	58604	12(19) **46.4**	9(17) **7.2**	2(4) **1.2**			5(5) **8.9**				
Bromodomain and WD-repeat domain-containing protein 2, WDR11	00412224	136685	2(2) **2.1**	5(7) **1.3**								
WD repeat protein An11 homolog, WDR68	00006754	38926			2(2) **0.9**							
Bromodomain and WD-repeat domain-containing protein 1, WDR9 [b]	00250716	257221	3(4) **2.2**									
Bromodomain and WD-repeat domain-containing protein 3, BRWD3 [b]	00167547	203596	2(2) **1.4**									
Other RING-type E3 ligases												
gp78 [a]	00423874	72996	3(3) **5.9**		2(2) **0.5**	4(6) **1.9**	2(2) **2.7**	4(6) **8.6**	3(3) **3.3**	3(6) **3.6**		3(3) **1.7**

UBR1	00217405	200209	2(2) **1.4**		3(3) **0.3**	3(3) **0.4**	2(2) **1.0**		2(2) **0.8**			
UBR2	00217407	200539	2(3) **2.1**		3(5) **0.4**	2(2) **0.2**	4(4) **2.0**	2(2) **1.0**	5(7) **2.8**	3(6) **1.3**		
Retinoblastoma-associated factor 600, UBR4 [a]	00643014	573849	12(18) **4.5**	41(49) **2.1**	3(5) **0.2**	6(6) **0.3**	9(9) **1.6**	47(56) **10.2**	18(27) **3.8**	30(74) **5.7**		2(3) **0.2**
Praja 2 [a]	00006557	78242	2(3) **5.5**		3(3) **0.7**		3(3) **3.8**		3(3) **3.1**	2(2) **1.1**		
Baculoviral IAP repeat-containing protein 2, IAP2	00013418	69900	3(3) **6.1**						2(2) **2.3**			
E3 ubiquitin-protein ligase Topors	00396077	119198		3(3) **0.6**								
RING finger protein 12, RLIM	00386464	68549							2(3) **3.5**			
RING finger protein 126, RNF126 [b]	00155562	33862				2(2) **1.4**						
HECT E3 ligases												
EDD1/UBR5 [a]	00026320	309352		2(2) **0.2**	3(3) **0.2**	9(10) **0.8**			9(10) **2.6**	9(14) **2.0**	2(2) **0.2**	
E6AP ubiquitin-protein ligase, UBE3A	00011609	100646				2(2) **0.5**				4(5) **2.2**		
E3 ubiquitin-protein ligase HUWE1	00456919	481896				6(6) **0.3**				4(7) **0.6**		
U-box E4 ligase												
Ubiquitin conjugation factor E4 B, UBE4B (Ufd2 ortholog)	00005715	146184							2(2) **1.1**	5(5) **1.5**	5(5) **1.1**	5(5) **1.4**

[a] E3 ligases/subunits identified also in p97-Myc immunoprecipitates by one spectral count or more.

[b] Putative E3 ligases/subunits that have not been yet demonstrated to possess ubiquitin-ligase activity or to be part of an E3 ligase complex. They were included in the list because their domain composition suggests they are likely to perform such function.

[c] SKP1 and RBX1 can form CRL-type of complexes with multiple cullins.

The corresponding International Protein Index (IPI) identifiers are specified and further information about the respective proteins can be found at http://www.ebi.ac.uk/IPI/IPIhelp.html. Whenever multiple isoforms were identified, the MW of the longest isoform is shown. The sequence count, the spectrum count (in parenthesis) and the abundance factor (bold) are indicated for each interacting protein. The exact sequence of the peptides identified is available upon request. D – DMSO, M – MG132.

The abundance factors (AF) were calculated by normalizing the spectrum count for the each E3 ligase to the spectrum count for p97 in the respective immunoprecipitate using the following formula:

AF = 100 x (Spectrum count E3/MW E3) / (Spectrum count p97/6 x MW p97)

The molecular weight for p97 has been amplified by six, because p97 exists as hexamers.

Table S3. Plasmids Used in this Study

RDB Number	**Plasmid Name**	Vector	ORF Source	Nucleotide Accession No.
2155	**Flag-UBXD7**	pCMV5B	Kazusa DNA Research Institute	AB018337
2143	**Flag-UBXD8**	pCMV5B	Open Biosystems	BC014001
2151	**Flag-FAF1**	pLPC	Invitrogen	BC004970
2150	**Flag-SAKS1**	pLPC	Invitrogen	BC000902
2148	**Flag-p47**	pCMV5B	G. Warren lab	AB002086
2152	**Flag-UBXD1**	pLPC	GeneCopoeia	NM_025241
2153	**Flag-UBXD2**	pLPC	GeneCopoeia	BC035594
2149	**Flag-UBXD3**	pLPC	Invitrogen	BC036417
2146	**Flag-UBXD5**	pCMV2	H. Katoh lab	BC078730
2145	**Flag-UBXD6**	pCMV2	Open Biosystems	BC020694
2144	**Flag-ASPL**	pCMV2	Open Biosystems	BC018722
2154	**Flag-NPL4**	pCMV2	G. Warren lab	BC101887
2147	**p97-Myc**	pCMV5B	G. Warren lab	BC049114

Table S4. siRNAs Used in this Study

Target gene	Dharmacon Catalog No.	Name/Sense Sequence
HIF1α [a]		CUGAUGACCAGCAACUUGAdTdT
Luciferase	P-002099-01	CAUUCUAUCCUCUAGAGGAUGdTdT
p97	M-008727-01	siGENOME SMART pool, Human VCP
UBXD7	M-023533-00	siGENOME SMART pool, Human UBXD7
UBXD8	D-010649-03	siGENOME duplex, Human UBXD8

[a] Elvidge, G.P., Glenny, L., Appelhoff, R.J., Ratcliffe, P.J., Ragoussis, J., and Gleadle, J.M. (2006). Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: the role of HIF-1alpha, HIF-2alpha, and other pathways. J Biol Chem *281*, 15215-15226.

Table S5. Primers Used for RT-PCR

Name	Sequence
HIF1α forward	5'- GAT GTA ATG CTC CCC TCA CCC AAC -3'
HIF1α reverse	5'- CAC TGG GAC TAT TAG GCT CAG GTG -3'
18S rRNA forward	5'- CGG ACA CGG ACA GGA TTG ACA GAT TG -3'
18S rRNA reverse	5'- GCA CAC GCT GAG CCA GTC AGT GTA G -3'

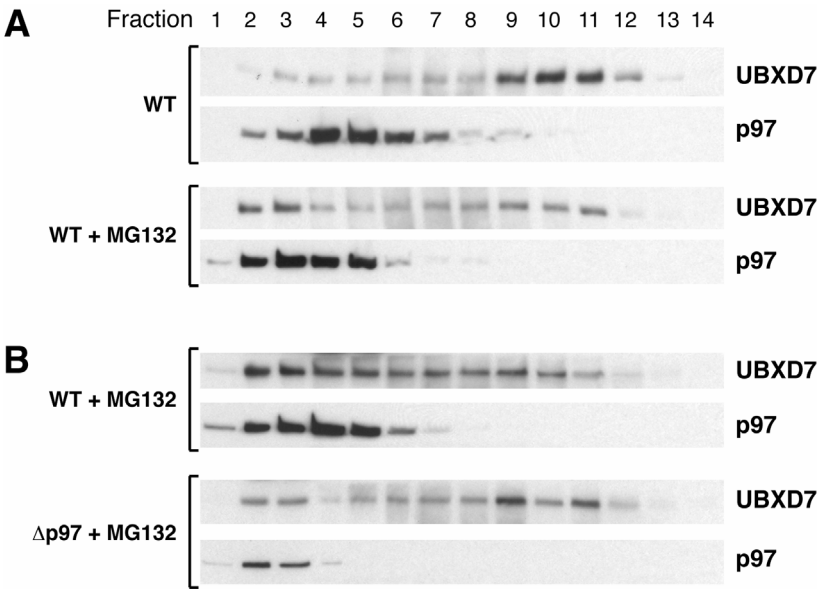

Figure S1. UBXD7 Shifts towards p97-Positive Fractions upon MG132 Treatment

HeLa cell extracts were fractionated on a Superdex 200 gel filtration column. Individual fractions were concentrated by TCA precipitation and subjected to western blotting using specific antibodies. (A) Comparison between cells treated or not with MG132.

(B) Comparison between cells treated with MG132 for 2 h, in which p97 was depleted by siRNA or not.

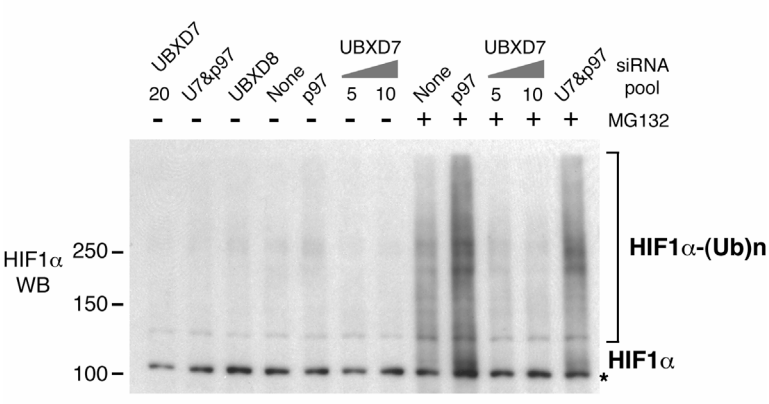

Figure S2. UBXD7 Depletion Is Partially Epistatic to p97 Depletion with Respect to Accumulation of Ubiquitinated HIF1α

The samples shown in Fig. 6A were boiled and re-analyzed by PAGE to better highlight that ubiquitinated HIF1α levels in cells depleted of both p97 and UBXD7 are intermediary between WT and p97-depleted cells.