SUPPORTING INFORMATION

Regulation of Iron Homeostasis Through Parkin-mediated Lactoferrin Ubiquitylation

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Figures

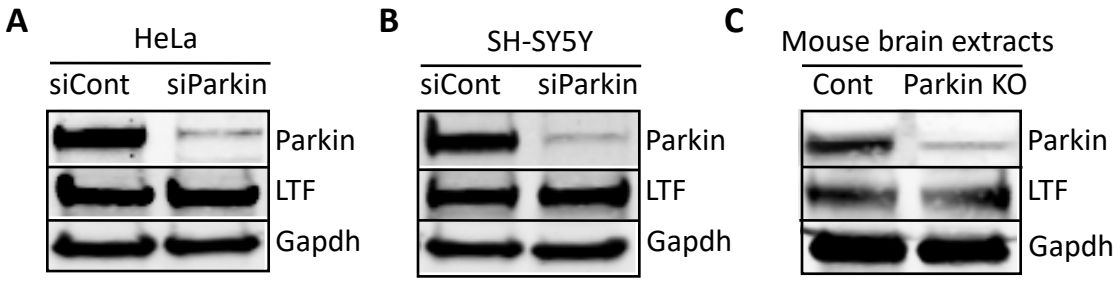

Figure S1. Depletion of Parkin does not alter LTF protein levels. (A-B) HeLa (A) or SH-SY5Y (B) cells were treated with control non-targeting siRNA (siCont) or siRNA targeting Parkin (siParkin) and extracts were immunoblotted with the indicated antibodies. Depletion of Parkin was monitored with anti-Parkin antibodies, LTF levels were monitored with anti-LTF antibodies, and anti-Gapdh antibodies were used as a control to monitor Gapdh protein levels. (C) Immunoblot analysis of control (Cont) and Parkin knock out (KO) mouse brain extracts with anti-Parkin, anti-LTF, and anti-Gapdh antibodies. (A-C) Note that LTF protein levels remain unchanged under all conditions.

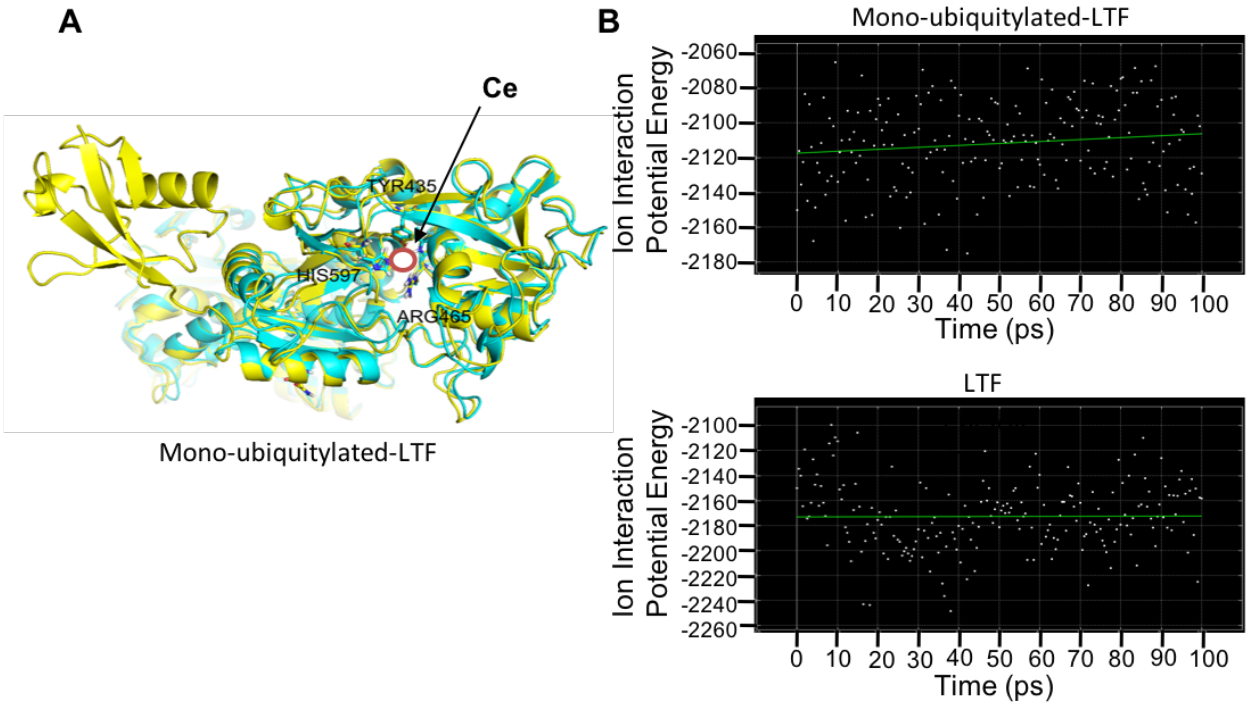

Figure S2. Molecular modeling and simulation of metal (cerium, Ce) binding to LTF and mono-ubiquitylated-LTF. (A) The mono-ubiquitylated-LTF (yellow) was modeled by conjugating the ubiquitin structure C-terminal glycine to Lysine 630 on the LTF structure (PDB: 1FCK) (corresponding to Lysine 649 on full-length LTF, see Materials and Methods). Inspection of energy minimized LTF and mono-ubiquitylated-LTF revealed a shift in the binding residues HIS597, TYR435, and ARG465 that coordinate the metal position (residue numbers correspond to residue numbers in the LTF PDB: 1FCK structure that is truncated compared to full-length LTF). (B) Molecular dynamics simulation of LTF and mono-ubiquitylated-LTF showed that the mono-ubiquitylated-LTF has an overall lower Ce binding stability than LTF with a mean initial interaction energy of ~-2120 kcal/mol compared to -2170 kcal/mol, respectively. Note that the stability of mono-ubiquitylated-LTF decreases over the 100 ps simulation with increased interaction energy while the Ce metal binding for LTF remains stable during the simulation. (x-axis: time in picoseconds, y-axis: interaction potential energy of Ce ion).

Materials and Methods

Cell culture. SH-SY5Y, HeLa, and HEK293 Flp-In T-REx LAP-tagged stable cell lines were grown in F12:DMEM 50:50 medium (GIBCO) with 10% FBS, 2 mM L-glutamine and antibiotics, in 5% CO_2 at 37° C. Cells were induced to express the indicated LAP-tagged proteins by addition of .1 μg/ml doxycycline (Sigma-Aldrich) for the indicated times.

Plasmids, mutagenesis, and generation of stable cell lines. Full length PARKIN or LTF cDNA was fused to the c-terminus of EGFP (pGLAP1 vector[1] or FLAG (pCS2-FLAG vector) as described previously[2]. The LTF single (K182A and K649A) and double (K182A/K649A) mutants were generated using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) with primers carrying the desired mutations. All mutagenesis primers were purchased from Fisher Scientific, see **Table S3** for primer sequences. The pGLAP1-PARKIN, pGLAP1-LTF-K182A, pGLAP1-LTF-K649A, and pGLAP1-LTF-K182A/K649A vectors were used to generate doxycycline inducible HeLa Flp-In T-REx stable cell lines that express the fusion proteins from a specific single loci within the genome as described previously[1, 3].

Cell extracts, immunoprecipitation and LAP purification. The indicted cell lines were harvested and cell extracts were prepared in LAP300 lysis buffer (50 mM Hepes pH 7.4, 300 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 10% glycerol) plus 0.3% NP40, 0.5 mM DTT, 10 μM MG132, 20 mM NEM and protease and phosphatase inhibitor cocktail (Thermo Scientific). Immunoprecipitations were performed as described previously[1]. Samples were resolved in a 4-20% gradient Tris gel (Bio-Rad) with TGS running buffer, transferred to PVDF membrane and

immunoblotted with indicated antibodies. Similarly LAP-tag tandem affinity purifications were performed as described previously[1]. For mouse brain extract analyses, Parkin (Park2) knock out mice Park2^{tm1Shn}/Park2^{tm1Shn} (The Jackson Laboratory, cat# TSH0059, stock #006582) or control mice (The Jackson Laboratory, cat# TSH0058, stock #000664) brains were washed once with PBS and homogenized in the presence of RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, .5% sodium deoxycholate, .1% SDS, 50mM tris, pH 8.0) with protease and phosphatase inhibitor cocktail.

Identification of Parkin associated proteins by LC-MS/MS. HEK293 LAP-tag only or LAP-Parkin inducible stable cell lines were grown in roller bottles, induced with .1μg/ml Dox, harvested and lysed in the presence of protease (Roche), phosphatase (Pierce), and proteasome inhibitors (MG132, Enzo life sciences). LAP and LAP-Parkin were purified from extracts using a tandem affinity purification protocol[1]. Eluates were resolved on a 4-12% SDS-PAG and ten gel slices corresponding to the resolved Parkin purification (and an adjacent control region) were excised and collected, washed twice with 50% acetonitrile, flash frozen, and stored until examination by mass spectrometry. Mass spectrometry-based proteomic analysis was performed at the Harvard Mass Spectrometry and Proteomics Resource Laboratory by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer as described previously[4]. The major proteins identified in the LAP-Parkin but not in the LAP-tag only purification are listed in **Table S1**.

Quantitative metal and sulfur content analysis via ICP-MS/MS. The elemental content was analyzed as described previously[5], with minor modifications to accommodate the sample

material. Briefly, HeLa cells (1×10^8) were collected by centrifugation at 1,500 *rpm* for 10 minutes. The cells were washed three times in 1 mM Na_2-EDTA (to remove cell surface–associated metals and remnants from the growth media) and briefly once in Milli-Q water. The cell pellet, after removal of water, was overlaid with 286 µl 70% nitric acid (Fisher, Optima grade, A467-500, Lot 1216040) and digested at room temperature for 24 hours and at 65 °C for 4 hours, before being diluted to a final nitric acid concentration of 2% (v/v) with Milli-Q water. Iron, zinc and sulfur contents of the cell pellets were determined by inductively coupled plasma mass spectrometry (ICP-MS/MS) on an Agilent 8800 Triple Quadrupole ICP-MS instrument, in comparison to an environmental calibration standard (Agilent 5182-4688) and a sulfur standard (Inorganic Ventures CGS1), using ^{89}Y as an internal standard (Inorganic Ventures MSY-100PPM). The levels of all analytes were determined in MS/MS mode by quantifying the most abundant isotope (^{32}S, ^{56}Fe and ^{66}Zn); while ^{66}Zn was measured directly using He in the collision/reaction cell, ^{56}Fe was directly determined using H_2 as a cell gas and ^{32}S was determined via mass-shift from 32 to 48, utilizing O_2 as a cell gas. The average of 4 technical replicate measurements was used for each individual sample or standard, the average variation in between the technical replicate measurements was 1.1% for all analytes and never exceeded 5% for any individual sample. Triplicate biological replicates were used to determine the variation in between samples, average and standard deviation between biological replicates are depicted in figures. Sulfur content was used to normalize for varying amount of cell material in between samples, since intracellular sulfur levels were unchanged in between the different samples.

Ubiquitylation reactions. Ubiquitylation reactions were carried out as described previously[6]. Briefly, GST-tagged LTF or positive control GST-tagged Tubulin or negative control GST-

tagged-GFP were incubated with or without HEK293 LAP-Parkin extracts, along with an ATP regeneration system, Ubiquitin, E1, and E2 in a buffer containing 20 mM HEPES, 5 mM NaCl, 5 mM MgCl2, DTT, MG132, protease and phosphatase inhibitor cocktail and incubated for 90 minutes at 30^{o} C. GST beads were then added for 30 minutes, washed four times with a wash buffer containing 20 mM HEPES, 100 mM NaCl, 5 mM MgCl2, 15 mM imidazole, 0.5% TritonX, BME, DTT, MG132 and a protease and phosphatase inhibitor cocktail. The beads were then boiled in 2X Laemmli sample buffer (Bio-Rad) and loaded onto a 4-20% TGX gel (Bio-Rad) followed by western transfer. The blots were subsequently probed with anti-GST and anti-Ubiquitin antibodies.

siRNA treatments. Treatment of cells with ThermoFisher control non-targeting siRNA (12935300) and siRNAs previously shown to deplete PARKIN expression[7] (1299001-HSS107594/HSS107593) was as described previously[8].

Molecular modeling. To model LTF ubiquitylation, the C-terminal glycine residue of the ubiquitin structure (PDB: 1UBI) was conjugated to lysine 630 on the LTF structure (PDB: 1FCK), which corresponded to lysine 649 on full-length LTF, using the structure joining module with C-N bond length 1.54 A and dihedral angles of 180 degrees in the UCSF Chimera program (version 1.14)[9]. After removing the solvent molecules, both the LTF and mono-ubiquitylated-LTF models were subsequently protonated and energy minimized to stabilize structure RMS gradient to less than 0.1. To estimate the binding energy of bounded ion (Ce) to LTF and mono-ubiquitylated-LTF, we performed molecular dynamics simulation using NVT ensemble[10] at 300K by monitoring the interaction potential energy between ions and the receptors. The

molecular dynamics equation was performed for 100 ps under the light-bonded constraint and solved using the The Nosé-Poincaré-Anderson (NPA) method[10]. The molecular dynamics simulation was performed using the MOE software package (version 2009)[11]. The models were visualized using the Pymol software package (version 1.8)[12].

Antibodies. See **Table S3** for a list of antibodies used for biochemical purifications, immunoprecipitations, and immunoblotting.

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Table S1. Summary of LC-MS/MS results from LAP-Parkin purifications

Swiss prot ID	Name	# of peptides	Unique	% coverage
Parkin				
O60260	Park2	270	27	46.9
Lactoferrin				
P02788	LTF	72	49	51.3
CCT Complex				
P17987	CCT-α	49	22	39.2
P78371	CCT-β	74	24	39.7
P49368	CCT-γ	49	24	35.8
P50991	CCT-δ	48	17	32.3
P02788	CCT-ε	71	25	35.1
P40227	CCT-ζ	46	19	28.5
Q99832	CCT-η	46	21	33.5
P50990	CCT-θ	82	29	48.6
Proteasome Complex				
Q15008	PSMD6	6	6	17.2
P62195	PRS8	3	3	10.1
O00231	PSD11	2	2	5.5
P43686	PRS6B	2	2	3.8
P35998	PRS7	3	2	6
Q9UNM6	PSD13	2	2	5.3
O43242	PSMD3	3	3	5.8
Q13200	PSMD2	2	2	3
p51665	PSD7	1	1	3.7
Tubulins				
P68363	TBAK	41	12	25.7
P68371	TBB2C	111	17	38.4
P07437	TBB5	10	2	6.1
Ubiquitin				
P62988	Ubiquitin	22	4	61.8
Chaperones				
P07900	HSP90a	22	12	17.4
P08238	HSP90b	4	3	4.3
P11142	HSP70/HSP7	130	29	40.2
P08107	HSP70.1	3	1	1.7
Q27965	HSP70.2	73	19	27.3
P11021	HSP70/GRP7	16	12	22.6
P38646	HSP70/GRP7	11	7	12.5
P10809	HSP60	21	14	20.2
O95816	BAG2	9	6	25.6
P31689	DNJA1	12	7	19.1
O60884	DNJA2	12	7	16
Q9Y2Z0	SUGT1	3	3	11

Table S2. LTF Ubiquitylated Peptides

Analysis 1

Query	Start	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	Peptide	Peptides	LTF isoform 1 NP_002334.2
11691	119	139	597.5474	2386.1603	2386.2175	-24	2	6	6.4	10	K.KGGSFQLNELQGLKSCHTGLR.R + GlyGly (K)	1 of 11	
													K119
11513	171	190	753.353	2257.0371	2257.0408	-1.67	1	7	0.77	1	R.FFSASCVPGADKGQFPNLCR.L + GlyGly (K)	2 of 2	
													K182
11514	171	190	753.3554	2257.0444	2257.0408	1.57	1	11	0.5	1	R.FFSASCVPGADKGQFPNLCR.L + GlyGly (K)	2 of 2	
													K182
3340	292	299	525.2677	1048.5208	1048.5301	-8.86	1	3	2.9	7	R.QAQEKFGK.D + GlyGly (K)	1 of 1	
													K296
10237	300	315	932.989	1863.9635	1863.9479	8.35	2	8	2.6	7	K.DKSPKFQLFGSPSGQK.D + GlyGly (K)	1 of 1	
													K305
11081	642	658	678.2952	2031.8637	2031.8666	-1.46	1	11	0.18	1	R.NGSDCPDKFCLFQSETK.N + GlyGly (K)	1 of 1	
													K649

Analysis 2

Query	Start	End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	Peptide	Peptides	LTF isoform 1 NP_002334.2
9491	171	190	753.3558	2257.0455	2257.0408	2.06	1	1	1	2	R.FFSASCVPGADKGQFPNLCR.L + GlyGly (K)	4 of 4	
													K182
9493	171	190	753.358	2257.0523	2257.0408	5.06	1	10	0.83	1	R.FFSASCVPGADKGQFPNLCR.L + GlyGly (K)	4 of 4	
													K182
9494	171	190	753.3588	2257.0546	2257.0408	6.11	1	13	0.54	1	R.FFSASCVPGADKGQFPNLCR.L + GlyGly (K)	4 of 4	
													K182
9496	171	190	753.36	2257.0581	2257.0408	7.65	1	9	0.49	2	R.FFSASCVPGADKGQFPNLCR.L + GlyGly (K)	4 of 4	**K182**
8077	520	535	603.2715	1806.7928	1806.7876	2.87	0	16	0.54	1	R.SNLCALCIGDEQGENK.C + GlyGly (K)	1 of 1	K535
9059	642	658	1016.944	2031.8735	2031.8666	3.39	1	18	0.061	1	R.NGSDCPDKFCLFQSETK.N + GlyGly (K)	3 of 3	**K649**
9060	642	658	678.2996	2031.8769	2031.8666	5.04	1	9	0.13	1	R.NGSDCPDKFCLFQSETK.N + GlyGly (K)	3 of 3	**K649**
9061	642	658	678.2997	2031.8774	2031.8666	5.31	1	15	0.084	1	R.NGSDCPDKFCLFQSETK.N + GlyGly (K)	3 of 3	**K649**
8110	695	709	905.9499	1809.8852	1809.8753	5.48	1	4	2.7	4	K.KCSTSPLLEACEFLR.- + GlyGly (K)	1 of 3	K695

Combined

K.KGGSFQLNELQGLKSCHTGLR.R + GlyGly (K)	1 of 11	K119
R.FFSASCVPGADKGQFPNLCR.L + GlyGly (K)	6 of 6	**K182**
R.QAQEKFGK.D + GlyGly (K)	1 of 1	K296
K.DKSPKFQLFGSPSGQK.D + GlyGly (K)	1 of 1	K305
R.SNLCALCIGDEQGENK.C + GlyGly (K)	1 of 1	K535
R.NGSDCPDKFCLFQSETK.N + GlyGly (K)	4 of 4	**K649**
K.KCSTSPLLEACEFLR.- + GlyGly (K)	1 of 3	K695

Table S3. Reagents Used

1. Primers

Name	Forward Primer	Reverse Primer	Company
LTF	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGGGAAACTTGTCTTCCTCGTCCTGC3'	5'GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACTTCCTGAGGAATTCACAGGC3'	ThermoFisher
LTF (K182A)	5'CTGTGTTCCCGGTGCAGATGCAGGACAGTTCCCCAA3'	5'TTGGGGAACTGTCCTGCATCTGCACCGGGAACACAG3'	ThermoFisher
LTF (K649A)	5'GACTGGAATAAGCAAAACGCGTCCGGGCAGTCAGATCC3'	5'GGATCTGACTGCCCGGACGCGTTTTGCTTATTCCAGTC3'	ThermoFisher
LTF (K182A-K649A)	Used K649A primers on LTF (K182A) mutant	Used K649A primers on LTF (K182A) mutant	ThermoFisher
Parkin	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGGGATAGTGTTTGTCAGGTTC3'	5'GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACACGTCGAACCAGTG3'	ThermoFisher
LCMT-1	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGGGCTTCCCTGTGCAAGAGAACTCC3'	5'GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAATAAGTTATCTCCTTCAGC3'	ThermoFisher

2. siRNA

Taget	siRNA ID	Catalog #	Company
Negative Control	N/A	12935300	ThermoFisher
PARK2	HSS107594	1299001	ThermoFisher
PARK2	HSS107593	1299001	ThermoFisher

3. Antibodies

Target	Name	Catalog #	Company
Ubquitin-K63	Ubquitin (linkage-specific K63) [EPR8590-448]	Ab179434	Abcam
Ubquitin-K48	Ubquitin (linkage-specific K48) [EP8589]	Ab140601	Abcam
LTF	Lactoferrin [2B8]	Ab10110	Abcam
Gapdh	Gapdh	GTX100118	GeneTex
Ubquitin	Mono- and polyubiquitinylated conjugates monoclonal antibody (FK2)	BML-PW8810-0100	Enzo
Parkin	Parkin	Ab15954	Abcam
Parkin	Parkin	#2132	Cell Signaling
S-Tag	S tag	GTX19321	GeneTex
GST	GST Tag (3G12B10)	66001-2-Ig	Proteintech
IgG	normal rabbit IgG	12-370	Sigma Aldrich
IgG	normal mouse IgG	sc-2025	Santa Cruz

4. Proteins

Name	Details	Catalog #	Company
LTF	GST-Lactoferrin, Human	H00004057-P01-2ug	Novus
Tubulin	GST-Tubulin, Human	SRP5148-20UG	Sigma
Ubc7	His-UBE2G1 (UBC7), Human	009-001-U35S	Rockland
E1	His-E1, Human	BML-UW9410-0050	Enzo
Ubquitin	His-Ubiquitin, Human	BML-UW8610-0001	Enzo
GFP	GST-GFP	Torres Lab	Torres Lab

5. Affinity Resins

Name	Details	Catalog #	Company
Protein A Beads	Affi-Prep Protein A Media	156-0005	BIO-RAD
Protein G Beads	Pierce™ Protein G Magnetic Beads	88847	ThermoFisher
GST Beads	Pierce™ Glutathione Magnetic Agarose Beads	78601	ThermoFisher
S Beads	S-protein Agarose	69704-4	Millipore