Cell

Supplemental Information

PARIS (ZNF746) Represses PGC-1α Contributing to Neurodegeneration in Parkinson's Disease

Joo-Ho Shin, Han Seok Ko, Hochul Kang, Yunjong Lee, Yun-Il Lee, Olga Pletinkova, Juan C. Troconso, Valina L. Dawson and Ted M. Dawson

Supplemental Figure Legends

Figure S1, related to Figure 1

Characterization of PARIS

(A) Multiple sequence Alignment of human, mouse and rat PARIS reveals highly conserved amino acid sequence among the different species. The KRAB domain (blue bar) and zinc finger domains (black bars) are indicated.

(B) Northern blot analysis of *PARIS* gene expression in different tissues. Relative levels of PARIS normalized to *GAPDH* control is indicated in bottom panel.

(C) Regional analysis of PARIS protein expression in various mouse tissues. Relative levels of PARIS normalized to β-actin control is indicated in bottom panel. Repeated three times with similar results.

(D) Representative confocal images with (a) anti-parkin antibody alone, (b) anti-PARIS antibody alone, or (c) anti-parkin along with anti-PARIS antibodies reveal that there is no channel crosstalk

and that endogenous PARIS and parkin are co-localized mostly in the cytoplasm of SH-SY5Y dopaminergic-like cells. Parkin (green); PARIS (red); Nucleus (DAPI, blue), n = 4.

(E) Immunoblot analysis using a polyclonal PARIS antibody in SH-SY5Y cells transduced with lentiviral shRNA-dsRed or shRNA-PARIS. The polyclonal PARIS antibody used in these studies is specific for PARIS since it recognizes a single band on immunoblot from mouse brain (see Figure S1E). β-actin was used as a loading control, *n* = 3.

Figure S2, related to Figure 2

Protein interaction mapping between parkin and PARIS

(A) Co-immunoprecipitation between parkin and PARIS in mouse brain using antibodies to parkin, PARIS. Mouse IgG was used as a control.

(B) Full-length WT PARIS and the F2 fragment of PARIS interact with parkin.

(C) PARIS interacts with parkin's RING1 and RING2 domains. Immunoprecipitated MYC-parkin deletions bind FLAG-PARIS except for the IBR domain (lane 8 on left bottom panel).

(D) Immunoprecipitated FLAG-PARIS interacts with WT and the C-terminal RING domains of parkin, but not the N-terminal UBL-SH domain (bottom right panel).

Figure S3, related to Figure 3

Parkin ubiquitinates PARIS *in vitro*.

(A) *In vitro* ubiquitination reactions with GST-PARIS, E1, E2s UbcH2 (2), UbcH3 (3), UbcH5a (5a), UbcH5b (5b), UbcH5c (5c), UbcH6 (6), UbcH7 (7), UbcH8 (8), and His-tagged parkin were performed at pH = 7.5 showing that parkin ubiquitinates PARIS in the presence of various E2 enzyme including UbcH5c, *n* = 3.

(B) *In vitro* ubiquitination reactions with His-tagged parkin, E1, E2s UbcH2 (2), UbcH3 (3), UbcH5a (5a), UbcH5b (5b), UbcH5c (5c), UbcH6 (6), UbcH7 (7), UbcH8 (8), in the absence of GST-PARIS

were performed at pH = 7.5 showing that the ubiquitin signal (Panel A) was derived from PARIS and not parkin, *n* = 3.

(C) *In vitro* ubiquitination reactions with E1, E2s UbcH2 (2), UbcH3 (3), UbcH5a (5a), UbcH5b (5b), UbcH5c (5c), UbcH6 (6), UbcH7 (7), UbcH8 (8), in the absence of GST-PARIS and His-tagged parkin were performed at pH = 7.5 showing that there is no ubiquitin signal in the absence of parkin and PARIS, *n* = 3.

(D) CHIP, which acts as a E4 for parkin (Imai et al., 2002), enhances the ubiquitination of PARIS by parkin, but it has no affect in the absence of parkin, *n* = 3.

(E) OTU1, a K48-linkage specific deubiquitinating enzyme eliminates the ubiquitination of PARIS by parkin and CHIP indicating that parkin ubiquitinates PARIS via K48 linkages. K48 ubiquitin linkages were confirmed by immunoreactivity with a K48-specific anti-ubiquitin antibody (Apu2) and no immunoreactivity with a K63-specific anti-ubiquitin antibody (Apu3), *n* = 3.

(F) Parkin mediated ubiquitination of FLAG-PARIS in SH-SY5Y cells is via K48 linkages as co-expression of OTU1 eliminates the ubiquitination, *n* = 3.

Figure S4, related to Figure 5

PARIS is a transcriptional repressor.

(A) GAL4-luciferase assay demonstrates that PARIS decreases promoter activity, which is recovered by co-expression of WT parkin, but not Q311X mutant. Relative luciferase activity compared to *Renilla* luciferase control is indicated in the histogram (*n = 3*). Immunoblot analysis confirms the expression of FLAG-PARIS, MYC-Parkin, and MYC-Q311X parkin. β-Actin was used as a loading control. (bottom of panel). *$p < 0.05$, **$p < 0.01$ in a ANOVA test followed by Student-Newman-Keuls post-hoc analysis. Schematic representation of the promoter construct is indicated at the top of the panel.

(B) PARIS represses the mouse *PGC-1α* promoter luciferase reporter activity. Co-transfection of SH-SY5Y cells with PARIS and the mouse PGC-1α promoter (-2533 to +78), results in decreased activity of PGC-1 α promoter. Introduction of WT parkin rescues PARIS suppression of the mouse *PGC-1α* promoter. Relative luciferase activity compared to *Renilla* luciferase control is indicated in histogram (*n = 4*). Immunoblot analysis confirms the expression of FLAG-PARIS, MYC-Parkin and β-actin was used as a loading control (bottom of panel). Data are expressed as mean ± S.E.M. *$p < 0.05$ in a ANOVA test followed by Student-Newman-Keuls post-hoc analysis. Schematic representation of the promoter construct is indicated at the top of the panel.

(C) Schematic representation of the PGC-1α WT, IRS1-M, IRS2-M, IRS3-M and IRS123-M promoter reporter constructs harboring the T→G point mutation on the IRS elements is indicated at the top of the panel. The basal promoter activity of PGC-1α WT, IRS1-M, IRS2-M, IRS3-M and IRS123-M promoter reporter constructs were monitored in the presence and absence of PARIS. Relative luciferase activity compared to *Renilla* luciferase control is indicated in histogram (*n = 3*). Data are expressed as mean ± S.E.M. *$p < 0.05$ in a ANOVA test followed by Student-Newman-Keuls post-hoc analysis. Immunoblot analysis confirms the expression of FLAG-PARIS. β-actin was used as a loading control.

(D) Schematic representation of PARIS zinc finger mutants (see Table S3).

(E) The basal promoter activity of the human PGC-1α promoter reporter construct in SH-SY5Y cells was monitored in the presence and absence of WT PARIS and the 8 zinc finger PARIS mutants depicted in panel A. Relative luciferase activity compared to *Renilla* luciferase control is indicated in the histogram, *n = 3*. Immunoblot analysis confirms the expression of GFP-PARIS, and PARIS zinc finger mutants. β-actin was used as a loading control (Top Panel). Data are expressed as mean ± S.E.M. *$p < 0.05$ in a ANOVA test followed by Student-Newman-Keuls post-hoc analysis.

(F) EMSA reveals that GST-PARIS binds to ^{32}P-labeled WT (WT-^{32}P) IRS oligonucleotides (IRS1-WT, IRS2-WT, IRS3-WT) of the human *PGC-1 α* promoter, whereas the PARIS C571A mutant has substantially reduced binding (*n* = 3). The arrow indicates the specific PARIS-shifted probe. NS, nonspecific; FS, fragmented PARIS-shifted probe; FP, free probe.

(G) Schematic representation of the promoter construct of human *PEPCK*.

(H) Schematic representation of the promoter construct of human *G6Pase*.

(I) PARIS does not repress the IRS-containing genes, *PEPCK* and *G6Pase*. Co-transfection of SH-SY5Y cells with PARIS and the rat *PEPCK* (rPEPCK) promoter-luciferase reporter (-2100 to +68), results in increased promoter activity of *rPEPCK*. While overexpression of PARIS does not alter mouse *G6Pase* (mG6Pase) promoter (-484 to +66) activity. Relative luciferase activity compared to *Renilla* luciferase control is indicated in histogram, *n* = 3. Immunoblot analysis confirms the expression of FLAG-PARIS and β-actin was used as a loading control (top of panel). Data are expressed as mean ± S.E.M. ***$p < 0.001$ in a unpaired two-tailed Student's *t*-test.

(J) PARIS occupies the endogenous *PEPCK* and *G6Pase* promoter. ChIP assay monitoring the occupancy of the endogenous *PEPCK* and *G6Pase* promoter by PARIS in SH-SY5Y, *n* = 3.

(K) PARIS occupies the endogenous *PEPCK* and *G6Pase* promoter. ChIP assay shows increased endogenous binding of PARIS in human PD and aged-matched control striatum to the IRS region of the human *PGC-1α* promoter and occupies the *PEPCK* and *G6Pase* promoter (CTL *n* = 3; PD *n* = 4; see Table S5 for details on human brain samples).

Figure S5, related to Figure 6

Identification of PGC-1α and NRF-1 as *in vivo* targets of accumulated PARIS in PD brain

(A) Real-time qRT-PCR of qRT-PCR of IRS (PEPCK-like motif)-containing genes and PGC-1α dependent genes reveals that PGC-1α and NRF-1 are reduced in PD striatum compared to age-matched controls (*n = 4* per group). Relative mRNA levels of PGC-1α, parkin, PARIS and PGC-1α

dependent genes normalized to GAPDH are indicated. See Table S4 for qRT-PCR primers and Table S5 for details on human brain samples. No mRNA for APOC3 and TAT was detected with 2 different pairs of primers.

(B) Immunoblot analysis shows that PGC-1α and NRF-1 protein levels are reduced in PD striatum compared to age-matched controls, *n = 4* per group. Parkin redistributes to the insoluble fraction consistent with its inactivation in PD.

(C) Quantitation of the immunoblots in panel B normalized to β-actin, *n = 4* per group; see Table S5 for details on human brain samples).

(D) a-f, Regression analysis of the quantified level of PARIS, PGC-1α, and NRF-1 shows there is a strong negative correlation between the protein levels of PARIS and PGC-1α (R^2=0.5195, $p < 0.05$) and NRF-1 (R^2=0.8015, $p < 0.01$) in the striatum and between PARIS and PGC-1α (R^2=0.6955, $p < 0.05$) and NRF-1 (R^2=0.5979, $p < 0.05$) in the SN and a positive correlation between PGC-1α and NRF-1 in the striatum (R^2=0.6827, $p<0.05$) and in the SN (R^2=0.6488, $p<0.05$), *n = 4* per group. See Table S5 for details on human brain samples.

(E) Relative mRNA levels as determined by real-time qRT-PCR of IRS (PEPCK-like motif)-containing genes, PGC-1α, PARIS, parkin and PGC-1α dependent genes normalized to GAPDH in the SN of the lentiviral-mediated conditional parkin knockout model, *n* = 4 per group. See Table S4 for qRT-PCR primers.

(F) Representative photomicrographs of laser capture microdissection (LCM) of dopaminergic neurons from conditional parkin KO mice. Upper panels are immunofluorescent images of TH and either GFP (left panel) or GFPCre (right panel) in conditional parkin KO mice before LCM. Lower panels are after LCM

(G) Robust reduction of PGC-1α mRNA in microdissected parkin KO dopaminergic neurons. PARIS level was assessed to monitor RNA quality (negative control). Values were normalized to GAPDH, *n* = 3 per group. ***$p < 0.001$ in a unpaired two-tailed Student's *t*-test.

(H) PGC-1α and NRF-1 levels are not altered in the ventral midbrain (VM) of 18-24 month old germline parkin exon 7 KO mice compared to age-matched WT controls mice where is PARIS is modestly upregulated, WT, *n = 4*; KO, *n = 5*.

(I) Quantitation of the immunoblots in panel H normalized to β-actin, WT, *n = 4*; KO, *n = 5*. All data are expressed as mean ± S.E.M. Asterisk indicates statistical significance (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) in a unpaired two-tailed Student's *t*-test.

Figure S6, related to Figure 7

Lack of Degeneration of GABAergic Neurons

(A) Representative SN sections stained with α-GAD 65/67, Robust viral expression was evaluated by GFP immunofluoresence in SNpr (top panel). The GABAergic neuronal marker shows no difference between AAV1-GFP and AAV1-PARIS between noninjected side and injected side, *n* = 6 per group. (B) Equivalent protein levels of GAD 65/67 were confirmed by immunoblot analysis, n=5 per group. (C) Quantitation of the immunoblots in panel C normalized to β-actin, *n = 5*. Data are expressed as mean ± S.E.M. Statistical significance was evaluated ANOVA with the Student-Newman-Keuls post hoc test.

Supplementary Tables:

Table S1, related to Figure 4

	Final Diagnosis	Age	PMD
Control	Control	61	15
	Control	67	6
	Control	68	10
	Control	49	15
AR-PD	PD	62	15
	PD	65	4
	PD	52	18
	PD	68	10

AR-PD cingulate cortex used for immunoblot in Figure 4A, B. Abbreviations: PD, Parkinson's disease; PMD, post-mortem delay

Table S2, related to Figures 4 and 5

	Final Diagnosis	Age	Sex	Race	PMD
Control	Control	87	F	W	7
	Control	89	M	W	8.5
	Control	71	M	W	16
	Control	79	M	W	16
PD	PD w/dementia	76	M	W	17
	PD w/dementia, neurodegeneration, occipital infarct	83	M	W	5
	PD, multiple infarcts/contusions	80	F	W	6
	PD, Neocortical	71	M	W	8
	PD w/dementia	73	M	W	6.5

Human postmortem tissues used for immunoblot in Figure 4C, D and ChIP in Figure 5 F, G.
Abbreviations: PD, Parkinson's disease; W, white; B, black; PMD, post-mortem delay.

Table S3, related to Figures 5, S4

Mutation	Target genes		Primers (5'-3')
	PARIS	F	ACC TGC GCC ACG GCT GGG AAG AGC TTC
M1	C458A	R	GAA GCT CTT CCC AGC CGT GGC GCA GGT
	PARIS	F	AGC CTG AGC GCG GCC CAG CGC AGC TGT
M2	H471A	R	ACA GCT GCG CTG GGC CGC GCT CAG GCT
	PARIS	F	GAG TGC GGC CGT GCC TTC ACG CGC CCC
M3	C518A	R	GGG GCG CGT GAA GGC ACG GCC GCA CTC
	PARIS	F	CAC CTC ATC CGC GCT CGC ATG CTG CAC
M4	H528A	R	GTG CAG CAT GCG AGC GCG GAT GAG GTG
	PARIS	F	TTC CCC TGC ACC GAG GCT GAG AAG CGC TTC
M5	C543A	R	GAA GCG CTT CTC AGC CTC GGT GCA GGG GAA
	PARIS	F	CAC TAC CGA ACG GCC ACG GGC GTG CGG
M6	H560A	R	CCG CAC GCC CGT GGC CGT TCG GTA GTG
	PARIS	F	ACC TGC ACC GTC GCC GGC AAA AGC TTC
M7	C571A	R	GAA GCT TTT GCC GGC GAC GGT GCA GGT
	PARIS	F	CAC CTC CGC AAG GCC CAG CGC AAC CAT
M8	H584A	R	ATG GTT GCG CTG GGC CTT GCG GAG GTG

List of primers used for site-directed mutagenesis for PARIS used in Figure 5H-J and Figure S4D-F. Abbreviations: F, forward; R, reverse.

Table S4, related to Figures 6 and S5

Target genes		Mouse primers (5'-3')	Human primers (5'-3')
PGC-1α	F	AGCCGTGACCACTGACAACGAG	TCCTCACAGAGACACTAGACA
	R	GCTGCATGGTTCTGAGTGCTAAG	CTGGTGCCAGTAAGAGCTTCT
PEPCK	F	CTGCATAACGGTCTGGACTTC	CAAGACGGTTATCGTCACCCA
	R	CAGCAACTGCCCGTACTCC	GAACCTGGCATTGAACGCTT
G6Pase	F	CGACTCGCTATCTCCAAGTGA	GTGTCCGTGATCGCAGACC
	R	GTTGAACCAGTCTCCGACCA	GACGAGGTTGAGCCAGTCTC
IGFBP-1	F	ATCAGCCCATCCTGTGGAAC	GAGCACGGAGATAACTGAGGA
	R	TGCAGCTAATCTCTCTAGCACTT	GCCTTCGAGCCATCATAGGTA
APOC3	F	TACAGGGCTACATGGAACAAGC	CTCCCTTCTCAGCTTCATGC *or* TGCAGGGTTACATGAAGCACG
	R	CAGGGATCTGAAGTGATTGTCC	GTCTGACCTCAGGGTCCAAA *or* CTCCAGTAGTCTTTCAGGGAACT
TAT	F	TGCTGGATGTTCGCGTCAATA	TGTGTCCCCATCTTAGCTGAT *or* TACAGACCCTGAAGTTACCCAG
	R	CGGCTTCACCTTCATGTTGTC	AATGGTACAGGGTCCCAAAATG *or* TAAGAAGCAATCTCCTCCCGA
SOD1	F	CCAGTGCAGGACCTCATTTT	AGGGCATCATCAATTTCGAGC
	R	TTGTTTCTCATGGACCACCA	GCCCACCGTGTTTTCTGGA
SOD2	F	CCGAGGAGAAGTACCACGAG	TTGGCCAAGGGAGATGTTAC
	R	GCTTGATAGCCTCCAGCAAC	AGTCACGTTTGATGGCTTCC
GPX1	F	CCGTGCAATCAGTTCGGACA	GCACCCTCTCTTCGCCTTC
	R	TCACTTCGCACTTCTCAAACAAT	TCAGGCTCGATGTCAATGGTC
CAT	F	AGCGACCAGATGAAGCAGTG	CGCAGAAAGCTGATGTCCTGA
	R	TCCGCTCTCTGTCAAAGTGTG	TCATGTGTGACCTCAAAGTAGC
NRF1	F	GTTGGTACAGGGGCAACAGT	CTTACAAGGTGGGGGACAGA
	R	TCGTCTGGATGGTCATTTCA	GGTGACTGCGCTGTCTGATA
TFAM	F	CCAAAAAGACCTCGTTCAGC	CCGAGGTGGTTTTCATCTGT
	R	CTTCAGCCATCTGCTCTTCC	TCCGCCTATAAGCATCTTG
UCP2	F	ACTTTCCCTCTGGATACCGC	GCATCGGCCTGTATGATTCT
	R	ACGGAGGCAAAGCTCATCTG	TTGGTATCTCCGACCACCTC
UCP3	F	CTGCACCGCCAGATGAGTTT	AGCCTCACTACCCGGATTTT
	R	ATCATGGCTTGAAATCGGACC	CGTCCATAGTCCCGCTGTAT
ANT1	F	GTCTCTGTCCAGGGCATCAT	ATGGTCTGGGCGACTGTATC
	R	ACGACGAACAGTCTCAAACG	TCAAAGGGGTAGGACACCAG
ATP5B	F	GAGGGATTACCACCCATCCT	GCACGGAAAATACAGCGTTT
	R	CATGATTCTGCCCAAGGTCT	GCCAGCTTATCAGCTTTTGC
CYTC	F	CCAAATCTCCACGGTCTGTTC	GGTGATGTTGAGAAAAGGCAAG
	R	ATCAGGGTATCCTCTCCCCAG	GTTCTTATTGGCGGCTGTGT
COX II	F	ACGAAATCAACAACCCCGTA	TTCATGATCACGCCCTCATA
	R	GGCAGAACGACTCGGTTATC	TAAAGGATGCGTAGGGATGG
COX IV	F	ACCAAGCGAATGCTGGACAT	CCGCGCTCGTTATCATGTG
	R	GGCGGAGAAGCCCTGAA	CGTTCTTTTCGTAGTCCCACTTG
GAPDH	F	AAACCCATCACCATCTTCCAG	AAACCCATCACCATCTTCCAG
	R	AGGGGCCATCCACAGTCTTCT	AGGGGCCATCCACAGTCTTCT
Parkin	F	TGGAAAGCTCCGAGTTCAGT	CAGCAGTATGGTGCAGCGGA
	R	CCTTGTCTGAGGTTGGGTGT	TCAAATACGGCACTGCACTC

PARIS	F	AGTTGGACTCTGGAGCAGGA	GCTGGAATTTCCGGTGTAAACC
	R	GCTGCTGTGTTGAGCTTCAG	GGGGTCCAAGATGGCCTCT

Primers used for real-time qRT-PCR in Figure 6A and Figure S5A, E. Abbreviations: PGC-1α, peroxisome proliferators-activated receptor γ coactivator-1α; PEPCK, phosphoenolpyruvate carboxylase; G6Pase, glucose-6-phosphatase; IGFBP-1, insulin-like growth factor binding protein 1; APOC3, apolipoprotein C-III; TAT, tyrosine aminotransferase; SOD, superoxide dismutase-1; GPX1, Glutathione Peroxidase 1; CAT, Catalase; NRF1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A; UCP, uncoupling protein; ANT1, adenine nucleotide translocator; ATP5B, ATP synthase, H+ transporting, mitochondrial F1 complex; CYTC, cytochrome c; COX, cytochrome C oxidase; F, forward primer; R, reverse primer(5'-3'); N/A, not applicable

Table S5, related to Figures 5, 6, S4, S5

	Final Diagnosis	Age	Sex	Race	PMD	Tissue
Control	Control	62	M	W	14	Str
	Control	59	M	W	12	Str
	Control	79	M	W	16	Str, SN
	Control	53	M	W	N/A	Str
	Control	73	F	W	9	SN
	Control	85	M	B	6	SN
PD	PD, CVD	85	F	W	9	Str, SN
	PD, W/D	60	M	W	16	Str
	PD, W/D, ND, OI	83	M	W	5	Str, SN
	PD, W/D	84	F	W	11	Str, SN
	PD, W/D	71	M	W	24	SN

Human postmortem tissues used for immunoblot and real time qRT-PCR analysis in Figure 6A-C and Figure S5A-D and ChIP analysis in Figure S4K. Abbreviations: CVD, cerebrovascular disease; W/D, with dementia; ND, neurodegeneration; OI, occipital infarction; W, white; B, black; PMD, post-mortem delay; N/A, not available; Str, striatum; SN, substantial nigra.

Supplemental Experimental Procedures

Antibodies

To generate a peptide antigen of PARIS, a peptide containing amino acids 572 to 590 [GKSFIRKDHLRKHQRNHAA] was generated from the C-terminal region and cross-linked to keyhole limpet hemocyanin. The conjugated peptide was used to immunize a New Zealand white rabbit (JH 786-789) (Cocalico Biologicals). Antisera were purified by affinity chromatography using the same peptide immobilized on SulfoLink gel matrix (Pierce,), according to manufacturer's protocol. The quality of antibody against PARIS is shown in Figure 1B and S1.

Database Searching

Full-length PARIS sequence of human (Accession: Q6NUN9), mouse (Accession: XP_909399) and rat (Accession: XP_231752) were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and entered as a query for multiple alignment search (ClustalW, http://www.ebi.ac.uk/Tools/clustalw2/). Jalview (http://www.jalview.org/) was used for color-coding editing of multiple alignments. Phylogenetic comparison was performed in ClustalW.

Northern blot analysis. A multiple human tissue Northern blot (Clontech) was hybridized using the PARIS cDNA probe (Y2H clone) labeled with DIG-DNA Labeling Mixture (Roche Diagnostics). Hybridization and washing were performed according to manufacturer's instructions, and the PARIS mRNA was detected using a DIG Luminescent Detection Kit (Roche Diagnostics). Levels of PARIS mRNA were normalized to GAPDH.

Plasmid Constructions

Full-length, deletion mutants, Q311X, R42P, R275W, G430D and C431F parkin were cloned into pRK5-MYC vector, and full-length HA-ubiquitin was cloned into pRK5-HA vector as described

previously (Chung et al., 2001; Zhang et al., 2000). Full-length parkin and truncations encoding amino acids 1-198 (UBL-SH) and 220-465 (R1-IBR-R2) were cloned into pRK5-HA vector between *SalI* and *NotI* sites. Full-length PARIS cDNA (IMAGE: 30347892; Open Biosystems) was cloned into the mammalian expression vector pCMV-Tag2A (Stratagene) between *EcoRI* and *XhoI* sites. PARIS cDNA sequences encoding amino acids 1-322, 322-644, and 1-164 were cloned into pCMV- Tag2A vector between *EcoRI* and *XhoI* sites to generate PARIS truncations. For the ZNF398 vector, its cDNA was amplified with *EcoRI* or *XhoI* site-flanked primers and inserted into pCMV-Tag2A vector. The sequences were confirmed by automated DNA sequencing.

Site-directed mutagenesis

The expression plasmid, pEGFP-PARIS mutants was generated using a QuikChange site-directed mutagenesis kit (Stratagene). The sequences were confirmed by automated DNA sequencing. Primers used are listed on Table S3.

Purification of GST-PARIS recombinant proteins

Full-length PARIS (a.a. 1-644) and PARIS zinc finger domain (ZFD) (a.a. 453-589) cDNA were PCR-amplified from pCMV-Tag2A-PARIS plasmid and cloned into pGEX-6P-1 vector (GE Healthcare). The sequences were confirmed by automated DNA sequencing. The plasmids were transformed to BL21 pLys, which were then grown in the presence of 0.1 mM IPTG for 4 h at 30°C. Cells were lysed by sonication in a TNE buffer (10 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1mM EDTA) containing 0.1% Triton X-100 and protease inhibitors and finally centrifuged at 14,000 rpm for 30 min at 4°C. After centrifugation, the supernatant was recovered, and the GST-PARIS and GST-PARIS-ZFD were purified with glutathione Sepharose 4B (GE Healthcare). The GST protein was also prepared as a control. The purity and quantity of GST-PARIS and GST-PARIS-ZFD were analyzed by SDS-PAGE with a well-defined BSA concentration standard.

In vitro interaction assay

For *in vitro* protein-protein interaction assays, 0.2 μg of GST or GST–PARIS were incubated for 1 h at 4°C with 20 μl of glutathione-sepharose beads, respectively. After washing, GST or GST-PARIS conjugated beads were resuspended in 100 μl binding buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS) including the protease inhibitor cocktail (Roche), and incubated for 2 hrs at 4°C with the His-Parkin (Boston Biochem). After extensive washing, retained proteins were eluted by boiling in SDS protein loading buffer and analyzed by immunoblotting using anti-GST and anti-PARIS antibodies.

In vitro ubiquitination assay

GST-PARIS, E1 (50 nM) and different E2s (UbcHs) (50 nM) were incubated with His-tagged parkin (100 nM) in presence or absence of CHIP at 37 °C in reaction buffer containing 50 mM Tris-Cl, pH 7.5, 2.5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP. For reducing conditions, samples were treated with SDS sample buffer and the boiled supernatants were separated by 8−16% gradient SDS−PAGE. Both polymerized ubiquitin chains and ubiquitinated proteins were detected by immunoblot with anti-ubiquitin (DAKO), anti-ubiquitin, K48-specific (Apu2, Millipore), anti-ubiquitin, K63-specific (Apu3, Millipore), anti-ubiquitin, K63-specific (HWA4C4, Millipore), and anti-PARIS antibody. Recombinant E1, UbcHs and ubiquitin were purchased from Calbiochem. GST-PARIS was purified from *Escherichia coli* strain, BL21 pLys (Stratagene).

Co-immunoprecipitation. For co-immunoprecipitation from cell cultures, SH-SY5Y cells were transfected with 2 μg of each plasmid, unless otherwise indicated. After 48 h, cells were washed with cold PBS and harvested in immunoprecipitation buffer (1% Triton X-100, 2 μg/ml aprotinin,

and 100 µg/ml PMSF in PBS). The lysate was then rotated at 4°C for 1 h, followed by centrifugation at 14,000 rpm for 20 min. The supernatants were then combined with 50 µl of protein G Sepharose (Amersham Biosciences) preincubated with antibodies against FLAG or MYC (Sigma; Roche, Indianapolis, IN), followed by rotating at 4°C for 2 h. The protein G Sepharose was pelleted and washed three times using immunoprecipitation buffer or buffer with additional 500 mM NaCl, followed by three washes with PBS. The precipitates were resolved on SDS-PAGE gel and subjected to immunoblot analysis. Immunoblot signals were visualized with chemiluminescence (Pierce, Rockford, IL). For co-immunoprecipitation of endogenous proteins from mouse brain, adult human brain was homogenized in lysis buffer [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10 mM Na-β-glycerophosphate, Phosphatase Inhibitor Cocktail I and II (Sigma), and Complete Protease Inhibitor Mixture (Roche)], using a Diax 900 homogenizer (Heidolph). The tissue homogenate was incubated on ice for 30 min and mixed twice for complete lysis. The samples were then centrifuged at 52,000 rpm at 4°C for 20 min. The supernatant was used for immunoprecipitation with one of the following antibodies: mouse or rabbit IgG (mIgG or rIgG), anti-PARIS, or anti-parkin. Immunoprecipitates were separated by SDS-PAGE and subjected to immunoblot analysis with an anti-PARIS or anti-parkin antibody. Immunoblot signals were visualized with chemiluminescence. For mapping of the binding region between parkin and PARIS, MYC- or HA-tagged parkin deletion constructs were transfected with full-length FLAG-PARIS or FLAG-tagged PARIS deletion fragments were co-transfected with full-length parkin. Transfections and co-immunoprecipitation was performed as described above.

Cellular ubiquitination assay. For the ubiquitination assay, SH-SY5Y cells were transiently transfected with 2 µg of pRK5-Myc-tagged parkin or Myc-tagged parkin (C431F, G430D, R275W, Q311X), pCMV-FLAG-PARIS, and 2 µg of pMT123-HA-ubiquitin plasmids for 48 h. Total cell lysates were prepared by harvesting the cells after washing with PBS, followed by solubilizing the

pellets in 200 µl of 2% SDS, followed by sonication. The lysates were then rotated at 4°C for 1 h, diluted to 1 ml with PBS, and then boiled and sonicated. The samples were used as input and for immunoprecipitation. Immunoprecipitation was performed with an antibody against FLAG. The precipitates were subjected to immunoblotting with anti-HA or anti-FLAG antibodies.

Immunocytochemistry. About 5 x 10^4 SH-SY5Y cells or rat cortical neurons were seeded onto polylysine-coated sterile glass cover slips in a 24-well culture plate. After attachment, cells were washed once with PBS and fixed in 3% paraformaldehyde (w/v) for 20 min. The fixed cells were washed three times with PBS before permeabilization in 0.2% (v/v) Triton X-100 in PBS for 5 min. Blocking was then carried out with 5% goat serum in PBS for 1 h. This was followed by incubation in primary antibodies for 1 h at 25°C and secondary antibodies for another hour at 25°C. Immunofluorescent images were acquired on a Carl Zeiss confocal microscope. For immunohistochemistry with mouse brain, animals were perfused with PBS followed by 4% paraformaldehyde. Brains were post-fixed with 4% paraformaldehyde, cryoprotected in 30% sucrose. Sagittal or coronal sections were cut throughout the whole brain and sections were reacted with rabbit polyclonal anti-PARIS and visualized with biotinylated goat anti-rabbit IgG, followed by streptavidin-conjugated horseradish peroxidase (HRP) (Vectastain ABC kit, Vector Laboratories). Positive immunostaining was visualized with 3,3′-diaminobenzidine (DAB, Sigma) after reaction with hydrogen peroxide (DAB kit, Vector Laboratories). Stained sections were mounted onto slides and analysed by Stereo Investigator software (MicroBrightfield).

Preparation of tissues for immunoblot. The tissues including nine different organs, eight different brain regions from C57/BL6 mouse, human brain, and mouse brain were homogenized in lysis buffer [10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10 mM Na-β-glycerophosphate, Phosphate Inhibitor Cocktail I and II (Sigma), and Complete Protease Inhibitor

Mixture (Roche)], using a Diax 900 homogenizer. After homogenization, samples were rotated at 4°C for 30 min for complete lysis, then the homogenate was centrifuged at 52,000 rpm for 20 min, and the resulting fractions were collected. Protein levels were quantified using the BCA kit (Pierce) with BSA standards and analyzed by immunoblot. Immunoblotting was performed with an antibody of interest and was performed with chemiluminescence (Pierce). The densitometric analyses of the bands were performed using ImageJ (NIH, http://rsb.info.nih.gov/ij/). Data are expressed as mean ± S.E.M. The results were evaluated for statistical significance by applying the unpaired two-tailed Student's *t-test* or Student-Newman-Keuls. Differences were considered significant when $p < 0.05$.

Luciferase Assay. SH-SY5Y cells were transiently transfected with pCMV-empty vector or pCMV-FLAG-PARIS with either wild type or Q311X parkin. In addition, each well was co-transfected with pGL3-Basic, pGL3-PGC-1α promoter-Luciferase, -pGL3-PGC-1α promoter deletion mutant (a gift from Akyoshi Fukamizu, University of Tsukuba, Japan) (Daitoku et al., 2003), pGL3 Hygro-rat PEPCK, pGL3 MOD-mouse G6Pase (kindly provided by Richard O'Brien, Vanderbilt University, USA) (Boustead et al., 2003) for firefly Luciferase assay and 0.1 μg pRL-TK vector (Promega) for *Renilla* luciferase control. Cells were harvested 48 h post-transfection and lysates were assayed sequentially for firefly and *Renilla* luciferases, using the Dual-Luciferase Reporter Assay System (Promega) with a Monolight 3010 luminometer (Analytical luminescence Lab), according to the manufacturer's instructions. Firefly luciferase readings were normalized to *Renilla* readings.

CAST (Cyclic Amplification and Selection of Targets)

We followed a previous published protocol with modification (Voz et al., 2000). Briefly oligonucleotides containing random 26 nucleotides (CAST26 – CTGTCGGAATTCGCTGACGT-(N)26-CGTCTTATCGGATCCTACGT) were used for generation of random double-strand

oligomers for the first round of CAST, 400 pmol of CAST26 were applied into 100 µl of PCR buffer containing 1 µmol of CAST-C (ACGTAGGATCCGATAAGACG), 200 µM dNTP, and 10 units of Taq (Invitrogen) and incubated as follows: 5 min at 94°C, 20 min at 65°C, and 20 min at 72°C. Fifty µl of random double-strand oligomers were subjected to pull-down with GST-ZF-PARIS (322-644 a.a.) bound to Glutathione Sepharose beads in mixture of 50 µg of BSA and 50 µg of poly polydeoscyinosinic-deoscycytidylic acid (Sigma) in 500 µl of binding buffer containing 10 mM Tris (pH 7.5), 200 mM NaCl, 10% glycerol, 50 mM $ZnCl_2$, 1 mM $MgCl_2$, and 1 mM DTT. The oligonucleotides were extracted from the beads by applying 100 µl of distilled H_2O, followed by phenol extraction and ethanol precipitation. An elute was used for the subsequent PCR in the presence of 200 pmol of each primer CAST-N (CTGTCGGAATTCGCTGACG) and CAST-C with 25 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. After four rounds of selection were done, an additional three rounds of selection were performed by EMSA. An eluate from 4th round CAST was amplified with 1 µl of [α-^{32}P]-dCTP (GE Healthcare), incubated with GST-ZF-PARIS, loaded on PAGE. DNA was extracted from the shifted band on EMSA and subsequently used for a second round of selection performed as described above. Following a total of seven selections, oligomers were cloned into the pGEM-T Easy vector according to the manufacturer's protocol (Promega). Twenty-four independent clones were sequenced and 19 clones were identifiable. Among 19 clones, 3 clones were duplicated and the final 16 clones were aligned with MACAW software (NCBI, http://iubio.bio.indiana.edu/soft/molbio/ncbi/old/macaw/).

EMSA

GST and GST-PARIS, GST-C571A-PARIS were prepared as described above. The different probes for the WT-IRS and Mutant-IRS with mutations in the consensus sequence (underline) were synthesized as followed:

IRS1-WT: ^{986}AGTGTGTTGGTATTTTTCCCTCAGTTC^{960},

IRS1-MT: ^{986}AGTGTGTTGGTATTGTTCCCTCAGTTC^{960},

IRS2-WT:^{596}ACATACAGGCTATTTTGTTGATTAAAC^{570},

IRS2-MT:^{596}ACATACAGGCTATTGTGTTGATTAAAC^{570},

IRS3-WT:^{364}GCCACTTGCTTGTTTTGGAAGGAAAAT^{338},

IRS3-MT:^{364}GCCACTTGCTTGTTGTGGAAGGAAAAT^{338}.

The complementary probes were annealed in buffer consisting of 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), and 1 mM EDTA, subsequently end-labeled with [γ-^{32}P]ATP (GE Healthcare) in present of T4 polynucleotide kinase (Promega), and finally purified with the QIAquick Nucleotide removal kit (Qiagen). Probe-protein binding reactions were performed for 10 min at room temperature in 25 µl of binding buffer consisting of 10 mM Tris (pH 7.9), 4% glycerol, 100 mM KCl, 50 mM $ZnCl_2$, 1 mM DTT, 1 mg polydeoxyadenylic acid-polythymidylic acid (Sigma), and 10 µg of BSA. Probe-protein complexes were analyzed on 5% nondenaturing polyacrylamide gels and electrophoresis was carried out at 4°C.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was carried out according to manufacturer's instruction (Millipore) with modification. Briefly, powderized brain (mouse and human) was suspended in 1% formaldehyde in PBS for 20 min at room temperature and SH-SY5Y cells were fixed with 1% formaldehyde for 10 min at 37°C. Glycerol quenched samples were lysed in 1 ml of SDS buffer containing protease inhibitors. The lysates were incubated for 10 min on ice and sonicated to shear DNA. The samples were centrifuged at 10,000 x g at 4°C for 10 min and supernatant was taken. Pre-cleared samples were incubated with either PARIS or rabbit IgG (rIgG)-agarose bead followed by a number of washes. Elutes were subjected to reverse cross-linking and DNA was recovered by phenol-chloroform-ethanol purification. PCR was performed using template DNA and the following primers:

hPGC-1α promoter (forward, 5'-ACATACAGGCTATTTTGTTGATTAAAC-3'; reverse, 5'-ATTTTCCTTCCAAAACAAGCAAGTGGC-3'),

hG6Pase promoter (forward, 5'- GTAGACTCTGTCCTGTGTCTCTGGCCTG-3'; reverse, 5'-GGTCAACCCAGCCCTGATCTTTGGACTC-3'),

hPEPCK promoter (forward, 5'- GACTGTGACCTTTGACTATGGGGTGACATC-3'; reverse, 5'-CTGGATCACGGCCAGGGTCAGTTATGC-3')

hGAPDH promoter (forward, 5'-TACTAGCGGTTTTACGGGCG-3';

reverse, 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'),

mPGC-1α promoter (forward, 5'-CAAAGCTGGCTTCAGTCACA-3';

reverse, 5'-TTGCTGCACAAACTCCTGAC-3'),

and *mGAPDH* promoter (forward 5'-TGGGTGGAGTGTCCTTTATCC-3';

reverse 5'-TATGCCCGAGGACAATAAGG-3').

Real-time Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen), and cDNA was synthesized from total RNA (1.5 μg) using a First-strand cDNA synthesis kit (Invitrogen). Aliquots of cDNA were used as templates for real-time qRT-PCR procedure. Relative quantities of mRNA expression were analyzed using real-time PCR (Applied Biosystems ABI Prism 7700 Sequence Detection System, Applied Biosystems). The SYBR greenER reagent (Invitrogen) was used according to the manufacturer's instruction. For microdissected specimens, RNA was extracted with proteinase K/acid phenol method (Khodosevich et al., 2007). To eliminate DNA, dissolved RNA was treated with DNase I (RNase free, Stratagene) for 15 min at 37˚C and purified by RNeasy kit (Qiagen).

RNA was directly used for qRT-PCR according to the manufacturer's instruction (QuantiTect SYBR Green RT-PCR kit, Qiagen). The primer sequences are listed in Table S4.

AAV1-plasmid construction and generation of AAV1 virus

cDNAs for PARIS and parkin were sub-cloned into an AAV1 expression plasmid (AAV/CBA-WPRE-bGHpA) under the control of a CBA (chicken beta-actin) promoter and containing WPRE (woodchuck hepatitis virus post-transcriptional-regulatory element), and bovine growth hormone polyadenylation signal flanked by AAV2 inverted terminal repeats (ITRs). dGFP (destabilized GFP) was cloned into the same AAV expression vector backbone and was used as control vector. High-titer AAV virus generation and purification were performed as described in detail elsewhere (During et al., 2003).

Lentiviral shRNA constructs

MISSION short hairpin RNA (shRNA) plasmids encoding small interfering RNAs (siRNAs) targeting parkin or PARIS were purchased from Sigma (St Louis, Mo). TRCN0000000285 and TRCN0000000283 vectors successfully knockdown human parkin. Three plasmids (TRCN0000156627 TRCN0000157534 and TRCN0000157931) were effective in knocking down PARIS expression. As a control, shRNA-dsRed co-expressing GFP and short hairpin sequence (AGTTCCAGTACGGCTCCAA) under the control of the EF1α and human U6 promoter was used. For knockdown human parkin or PARIS in SH-SY5Y cells, two lentiviral vectors were combined and the TRCN0000157931 lentiviral vector was used to knockdown mouse PARIS *in vivo*.

Stereological assessment

For stereotaxic injection of AAV1 overexpressing GFP, PARIS, or parkin and lentivirus overexpressing PGC-1α, GFP or GFPCre, experimental procedures were followed according to the guidelines of Laboratory Animal Manual of the National Institute of Health Guide to the Care and Use of Animals, which were approved by the Johns Hopkins Medical Institute Animal Care

Committee. Six-week-old male C57BL mice (Charles River Laboratories, Inc) or 6~ 8 week old parkin$^{flx/flx}$ mice were anesthetized with pentobarbital (60 mg/kg). An injection cannula (26.5 gauge) was applied stereotaxically into the substantia nigra (anteroposterior, -3.0 mm from bregma; mediolateral, 1.2 mm; dorsoventral, 4.3 mm). The infusion was performed at a rate of 0.2 µl/min and wound healing and recovery were monitored after the injection was done. Four weeks, 3 months and 10 months after injection, animals were anesthetized and perfused with PBS followed by 4% paraformaldehyde. Brains were post-fixed with 4% paraformaldehyde, cryoprotected in 30% sucrose, and processed for immunohistochemistry. Forty-µm coronal sections were cut throughout the brain including substantia nigra and every 4th section was utilized for analysis. For tyrosine hydroxylase (TH) or glutamate decarboxylase 65/67 (GAD 65/67), sections were reacted with a 1:1000 dilution of rabbit polyclonal anti-TH (Novus) or anti-GAD65/67 (Chemicon) and visualized with biotinylated goat anti-rabbit IgG, followed by streptavidin-conjugated horseradish peroxidase (HRP) (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Positive immunostaining was visualized with 3,3′-diaminobenzidine (DAB, Sigma) after reaction with hydrogen peroxide (DAB kit, Vector Laboratories). Stained sections were mounted onto slides and counterstained with thionin for Nissl substance. Total numbers of TH-, and Nissl-stained neurons in substantia nigra pars compacta were counted using the Optical Fractionator probe of Stereo Investigator software (MicroBrightfield, Williston, VT). For Nissl counting, a cell was defined as a bright blue-stained neuronal perikarya with a nucleolus. Nissl positive counts were restricted to Nissl+/TH+ neurons along with large Nissl+ neurons with dopaminergic-like morphology, that contain little or no TH immunostaining,

Laser Capture Microdissection (LCM)

Approximately 6 week old parkin$^{flx/flx}$ mice injected with either lentiviral GFP (*n* = 3) or lentiviral GFPCre (*n* = 3) were transcardially perfused by autoclaved 1 x PBS for 3 min (10 ml/min), 2%

paraformaldehyde (resolved in autoclaved PBS) for 5 min (10 ml/min), and 20% sucrose for 5 min (10 ml/min). The brains were rapidly removed and frozen on dry ice. In order to preserve fluorescence and RNA integrity, a RNase inhibitor and autoclaved PBS were used during all staining procedures. Fifteen micron-thick coronal sections of the midbrain on superfrost glass slide were incubated with blocking solution for 30 min and rinsed with 1 x PBS followed by incubation with rabbit anti-TH (1:50) and mouse anti-GFP (1:50) for 3-4 h. Rinsed sections were incubated with Cy3-conjugated anti-rabbit (1:25) and Cy2-conjugated anti-mouse (1:25) for 1 h. Sections were rinsed with 1 x PBS three times and were washed once again with DEPC-treated water. Double, TH and GFP, positive neurons were obtained by LCM (P.A.L.M., Microlaser Technologies). Microdissected cells were directly used for RNA extraction.

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