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Regulation of Iron Homeostasis Through Parkin-mediated Lactoferrin Ubiquitylation

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Abstract

Somatic mutations that perturb Parkin ubiquitin ligase activity and the misregulation of iron homeostasis have both been linked to Parkinson's disease. Lactotransferrin (LTF) is a member of the transferrin iron binding proteins that regulate iron homeostasis and increased levels of LTF and its receptor have been observed in neurodegenerative disorders like Parkinson's disease. Here, we report that Parkin binds to LTF and ubiquitylates LTF to influence iron homeostasis. Parkin-dependent ubiquitylation of LTF occurred most often on lysines (K) 182 and 649. Substitution of K182 or K649 with alanine (K182A or K649A) led to a decrease in LTF ubiquitylation and substitution at both sites led to a major decrease in LTF ubiquitylation. Importantly, Parkin-mediated ubiquitylation of LTF was critical for regulating intracellular iron levels as overexpression of LTF ubiquitylation site point mutants (K649A or K182A/K649A) led to an increase in intracellular iron levels measured by ICP-MS/MS. Consistently, RNAi-mediated depletion of Parkin led to an increase in intracellular iron levels in contrast to overexpression of Parkin that led to a decrease in intracellular iron levels. Together, these results indicate that Parkin binds to and ubiquitylates LTF to regulate intracellular iron levels. These results expand our

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Author Contributions

A.A.G. and J.Z.T. contributed to the design, execution and analysis of experiments. M.A., H.D., and W.J.D., contributed to the experimentation. S.S. performed the ICP-MS/MS analyses. E.F.V. and Y-C.L. performed LTF structure modeling. W.C., J.C., L.W.G., and J.P.W. performed mass spectrometry characterization of LTF ubiquitylation. A.A.G. and J.Z.T. wrote the final version of the manuscript.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Tables, figures, and experimental methods (PDF)

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understanding of the cellular processes that are perturbed when Parkin activity is disrupted and more broadly the mechanisms that contribute to Parkinson's disease.

Parkinson's disease (PD) is a debilitating neurodegenerative disease whose incidence has increased over the last decade and it presents a major public health epidemic[1]. The *PARK2* gene that encodes the Parkin E3 ubiquitin ligase is found mutated in familial forms of PD[2]. Two pathological hallmarks of PD are the aggregation of α-synuclein in Lewy bodies and the accumulation of iron[3]. Lactotransferrin (lactoferrin, LTF) is a member of the transferrin iron binding proteins that transport iron and regulate intracellular iron levels[4-6]. X-ray structures of LTF show that is displays a bilobal architecture with one iron binding site within each lobe[7, 8]. Increased levels of LTF and its receptor have been reported within nigral neurons in PD patients and in other neurodegenerative disorders like Alzheimer's disease[4, 9-11]. Iron homeostasis is important for maintaining normal physiology of neuronal cell populations and iron accumulation leads to neurotoxicity[12]. Due to this dysregulation of iron homeostasis during PD progression, iron chelators have been proposed as a potential therapeutic strategy[13, 14]. Although many models exist for how iron-mediated cell death occurs in PD, most agree that an excess of reactive iron (Fe^{2+}) leads to the generation of reactive oxygen species that induces oxidative stress and promotes neuronal cell death through toxic reactions that include mitochondrial dysfunction, lipid peroxidation, and protein misfolding[13, 14]. Interestingly, transferrin (Tf) containing endosomes have been shown to contact the mitochondria and may function to deliver iron directly to the mitochondria[15], which could be a mechanism contributing to mitochondrial dysfunction. However, it remains unclear whether other members of the transferrin family, like LTF, function in a similar manner. Although the dysregulation of Parkin and LTF had been previously linked to the accumulation of iron in populations of neuronal cells that undergo cell death in PD, a direct connection between the two had not been established.

To better understand the role of Parkin dysfunction in PD, we sought to identify novel Parkin ubiquitylation substrates. First, we established a HEK293 doxycycline-inducible localization and affinity purification (LAP= EGFP-TEV-S-Peptide)-tagged Parkin stable cell line and utilized it to express and tandem affinity purify LAP-Parkin[16, 17]. Eluates were analyzed by mass spectrometry to identify Parkin associating proteins (Figure 1A). This analysis identified Parkin (270 peptides) and Lactotransferrin (LTF, 72 peptides) as the most abundant proteins (Figure 1B and Table S1). Additionally, tubulin isoforms and proteasome subunits, known Parkin-interacting proteins[18-23], were also identified along with subunits of the CCT/TRiC complex (Chaperonin containing T-complex/TCP-1 ring complex), which is critical for tubulin folding and for blocking the fibrillation of α-synuclein that is a pathological hallmark of PD (Figure 1B and Table S1)[24-26]. Due to the importance of LTF in iron homeostasis and its misregulation in PD[4], we sought to further validate the Parkin-LTF interaction. Reciprocal coimmunoprecipitation experiments from SH-SY5Y neuronal cells and HeLa cells with anti-Parkin and anti-LTF antibodies showed that LTF co-immunoprecipitated with Parkin and Parkin co-immunoprecipitated with LTF (Figure 1C-F). Similarly, *in vitro* protein binding reactions with GST-Parkin and FLAG-LTF showed that LTF co-immunoprecipitated with Parkin (Figure 1G). Together these data indicated that LTF was associating with Parkin.

To understand the significance of the Parkin-LTF association, we asked if LTF was ubiquitylated and whether its ubiquitylation was Parkin-dependent. LAP-LTF was immunoprecipitated from control siRNA (siCont) or Parkin siRNA (siParkin) treated cells and its ubiquitylation was monitored by immunoblot analysis with anti-ubiquitin antibodies. LAP-LTF was ubiquitylated in the siCont sample and this ubiquitylation was substantially decreased upon Parkin depletion with siParkin (Figure 2A). Next, we asked if LTF was a Parkin substrate using an *in vitro* reconstituted ubiquitylation assay[27]. GST-LTF, GST-Tubulin (positive control) or GST-GFP (negative control) were incubated with an ATP-regeneration system, ubiquitin, E1 ubiquitin-activating enzyme, E2 ubiquitin conjugating enzyme, and wildtype (WT) or LAP-Parkin overexpressing HEK293 cell extracts. Parkin substrate ubiquitylation was then monitored by immunoprecipitating the GST-tagged proteins and performing an immunoblot analysis with anti-ubiquitin and anti-GST antibodies. We observed ubiquitylation of GST-LTF and GST-Tubulin (a known substrate of Parkin[18]) as a ladder of increasing molecular weight bands (Figure 2B). Next, we analyzed LTF ubiquitylation reactions by mass spectrometry and determined that LTF was ubiquitylated at 7 different lysine (K) residues with K182 and K649 being the most frequently modified sites (Table S2). Mapping of the ubiquitylation sites onto the human LTF crystal structure (PDB 1FCK) showed that all sites were on exposed loops (Figure 2C).

Next, we analyzed the contribution of the most frequently modified lysines (K182 or K649) to the overall ubiquitylation of LTF by substituting them with alanines and assessing LTF ubiquitylation. LAP-LTF-WT or LTF single (K182A or K649A) or double (K182A/K649A) ubiquitylation site point mutants were expressed in HeLa cells, immunoprecipitated, and their ubiquitylation status was monitored using anti-K48 and anti-K63 ubiquitin linkage specific antibodies. Single K182A or K649A point mutants showed a reduction in K63-linked ubiquitylation of LTF and ubiquitylation of the LTF K182A/K649A double point mutant was highly impaired compared to the WT control (Figure 2D). Together these data indicated that LTF was a Parkin substrate and that K182A and K649A were the most frequently ubiquitylated sites and accounted for the majority of LTF ubiquitylated species. Interestingly, LTF protein levels remained unchanged in HeLa or SH-SY5Y cells treated with Parkin siRNA or in Parkin knock out mice brains (Figure S1), indicating that Parkin did not regulate the levels of LTF.

Next, we sought to determine if ubiquitylation at K182 or K649 could influence the ability of LTF to regulate intracellular iron levels. Extracts from control HeLa cells or HeLa cells overexpressing LTF wild type, K182A single point mutant, K649A single point mutant, or K182A/K649A double point mutant were analyzed for intracellular sulfur (S), iron (Fe) and zinc (Zn) levels using Inductively Coupled Plasma Mass Spectrometry (ICP-MS/MS). For all ICP-MS/MS analyses, Fe and Zn levels were normalized to total S levels. Overexpression of the K649A variant alone or the K182A/K649A double point mutant resulted in a similar significant increase in intracellular Fe compared to the control, while the Zn levels did not significantly change (Figure 3A,B). We hypothesized that if Parkin was ubiquitylating LTF on K649A to regulate iron levels, then modulation of Parkin levels would also affect intracellular iron levels. To test this, we performed RNAi-mediated depletion of Parkin levels and again analyzed the extracts for total S, Fe and Zn. Decreasing Parkin levels led to a significant increase in Fe content compared to the control, while the Zn levels did not

significantly change. (Figure 4A,B). In contrast, extracts from cells that were overexpressing Parkin showed a significant decrease in total Fe levels compared to the control, while the Zn levels did not significantly change (Figure 4C,D). Together these data demonstrated that Parkin abundance directly or the substitution of the Parkin-dependent LTF ubiquitylation site (K649A) affects intracellular iron levels, consistent with a role for Parkin in regulating iron levels through LTF ubiquitylation. To begin to understand how Parkin-mediated ubiquitylation of LTF on K649 was affecting LTF metal binding, we performed molecular dynamics simulations to compare the interaction potential energy in the LTF structure to the mono-ubiquitylated-LTF structure. LTF structural studies have shown that the structure of LTF remains essentially the same irrespective of whether iron (Fe), cerium (Ce), or copper (Cu) is bound[28-31]. For this analysis, we used the PDB: 1FCK LTF structure that is bound to Ce[30]. First, the mono-ubiquitylated-LTF was modeled by conjugating the ubiquitin structure C-terminal glycine to K649 of human lactoferrin (see Materials and Methods) (Figure S2A). The molecular dynamics simulation showed that the mono-ubiquitylated-LTF had an overall lower Ce binding stability than LTF with a mean initial interaction energy of ~−2120 kcal/mol compared to −2170 kcal/mol. Furthermore, the stability of mono-ubiquitylated-LTF decreased over the 100 ps simulation with increased interaction energy while the Ce metal binding for LTF remained stable during the simulation (Figure S2B). Inspection of energy minimized LTF and mono-ubiquitylated-LTF structures revealed a shift in residues HIS597, TYR435, and ARG465 that coordinate metal binding[29]. This *in silico* molecular analysis indicated that LTF ubiquitylation at K649 led to structural changes that destabilize metal coordination in the binding site and therefore could modulate metal (including iron) binding activity.

In summary, we have discovered a previously undescribed link between Parkin and LTF that influences iron homeostasis. We identified lactoferrin (LTF, lactotransferrin) as a Parkin interacting protein and validated this interaction through reciprocal co-immunoprecipitations from cells and in binding reactions *in vitro*. Increased levels of LTF and its receptor have been reported within nigral neurons in PD patients and in other neurodegenerative disorders[4, 9] Iron homeostasis is important for maintaining normal physiology of neuronal cell populations and iron accumulation leads to neurotoxicity[12]. Due to the importance of LTF in iron homeostasis and its misregulation in PD, we sought to further define the significance of the Parkin-LTF interaction. We determined that LTF was ubiquitylated in a Parkin-dependent manner in cells and *in vitro* through K63 linkages. Moreover, we mapped the sites of 7 lysines on LTF lysine that were ubiquitylated, with K182 and K649 being the most abundantly modified. Substitution of K182 or K649 with an alanine (K182A or K649A) led to a decrease in LTF ubiquitylation and the double point mutant led to a major decrease in LTF ubiquitylation. Importantly, Parkin-mediated ubiquitylation of LTF was critical for LTF's ability to modulate iron levels as overexpression of LTF ubiquitylation site point mutants K649A or K182A/K649A led to an increase in intracellular iron levels measured by ICP-MS/MS. Consistently, RNAi-mediated depletion of Parkin also led to an increase in intracellular iron levels in contrast to overexpression of Parkin that led to a decrease in intracellular iron levels. Together, our data suggest that Parkin binds to and ubiquitylates LTF to influence intracellular iron levels. We propose that Parkin ubiquitylation of LTF at K649 perturbs LTF's ability to accumulate intracellular iron levels

and that depletion of Parkin, or substitution of K649 on LTF, allows LTF to accumulate intracellular iron levels. The ability of Parkin to influence iron levels through LTF ubiquitylation may have direct implications to the increased iron levels that are observed in the nigral cells of PD patients. These results expand our understanding of the cellular processes that are perturbed when Parkin activity is disrupted and more broadly the mechanisms that contribute to PD. LTF is a member of the transferrin iron binding proteins that transport iron and regulate intracellular iron levels[4, 5] It is currently unclear as to whether other isoforms of LTF like deltalactoferrin[32] or other transferrin iron binding proteins are regulated through ubiquitylation and whether their ubiquitylation is Parkin-mediated. This study should help stimulate additional work related to Parkin-mediated iron regulation in PD and other neurological disorders. We note that the regulation of iron levels through Parkin-mediated ubiquitylation of LTF that we observe *in vitro* cannot solely account for the dysregulation in iron levels seen in PD patients and that other mechanisms are likely to exist. For example, the protein levels of divalent metal transporter 1 (DMT1), which transports metals including iron into the cell from the extracellular environment, have been shown to be regulated through Parkin-mediated ubiquitylation and proteasome-dependent degradation and elevated levels of DMT1 have been observed in PD patients[4, 33, 34]. Furthermore, it's important to note that LTF can also influence the levels of other proteins with roles in iron homeostasis[35]. For example, studies analyzing the effect of bovine lactoferrin (bLf) on the inflammation response showed that the addition of exogenous bLf could inhibit iron overload by reducing the production of the pro-inflammatory cytokines IL-6 and IL-1β and regulating iron metabolism through the upregulation of the iron exporter ferroportin (Fpn) and the transferrin receptor 1 (TfR1) and down-regulation of ferritin (Ftn)[35, 36]. Thus, whether the changes in intracellular iron levels that we observe upon LTF ubiquitylation are directly related to LTF function or to the function of downstream iron regulating proteins that are modulated by LTF remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

PD	Parkinson's Disease
PARK2	Parkin
LTF	Lactotransferrin

LAP	localization and affinity purification
ICP-MS/MS	inductively coupled plasma mass spectrometry

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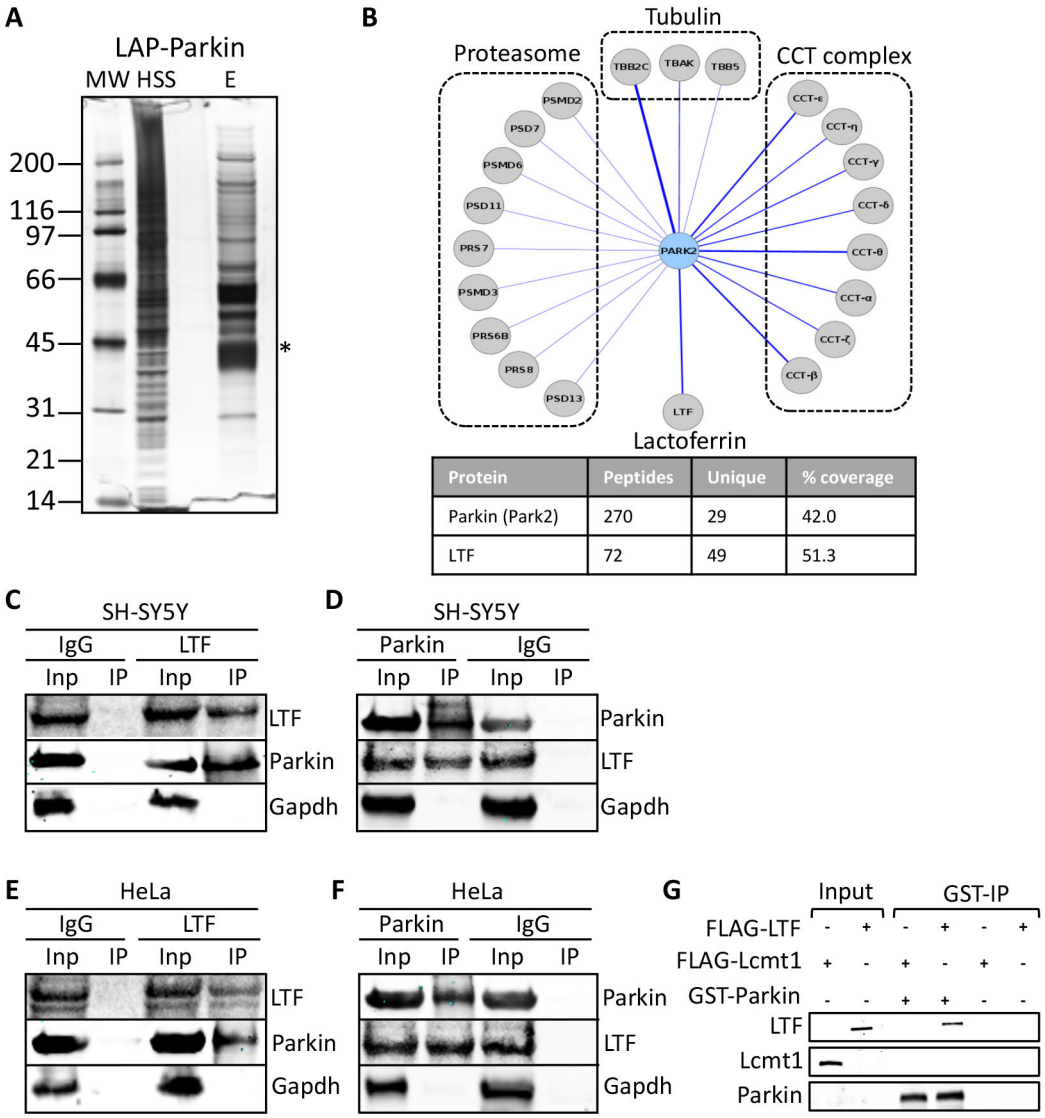

Protein	Peptides	Unique	% coverage
Parkin (Park2)	270	29	42.0
LTF	72	49	51.3

Figure 1.

LTF co-purifies and associates with Parkin. (A) LAP-Parkin tandem affinity purification. MW= molecular weight marker, HSS= high spin supernatant, E= final eluates. Asterisk denotes the Parkin protein band. (B) Cytoscape visualization map of Parkin associated proteins, identified by mass spectrometry, showing the major classes of co-purifying proteins. Bottom panel highlights the identification of lactoferrin (LTF) as a Parkin co-purifying protein. The protein name, number of peptides identified, number of unique peptides, and the percent protein coverage are indicated. See Table S1 for a complete list of Parkin co-purifying proteins identified by mass spectrometry. (C-F) SH-SY5Y or HeLa cell extracts were used to perform reciprocal co-immunoprecipitation (Co-IP) experiments using anti-lactoferrin (LTF), anti-Parkin and control IgG antibodies. Note that endogenous Parkin IPs with endogenous LTF (C,E) and endogenous LTF IPs with endogenous Parkin (D,F). Inp indicates input and IP indicates immunoprecipitation. (G) *In vitro* binding assays performed in the presence or absence of *in vitro* transcribed/translated FLAG-LTF, FLAG-Lcmt1, or

GST-Parkin. GST-Parkin was immunoprecipitated (GST-IP) and eluates were analyzed by immunoblotting with the indicated antibodies. Note that FLAG-LTF IPs with GST-Parkin, whereas control FLAG-Lcmt1 does not.

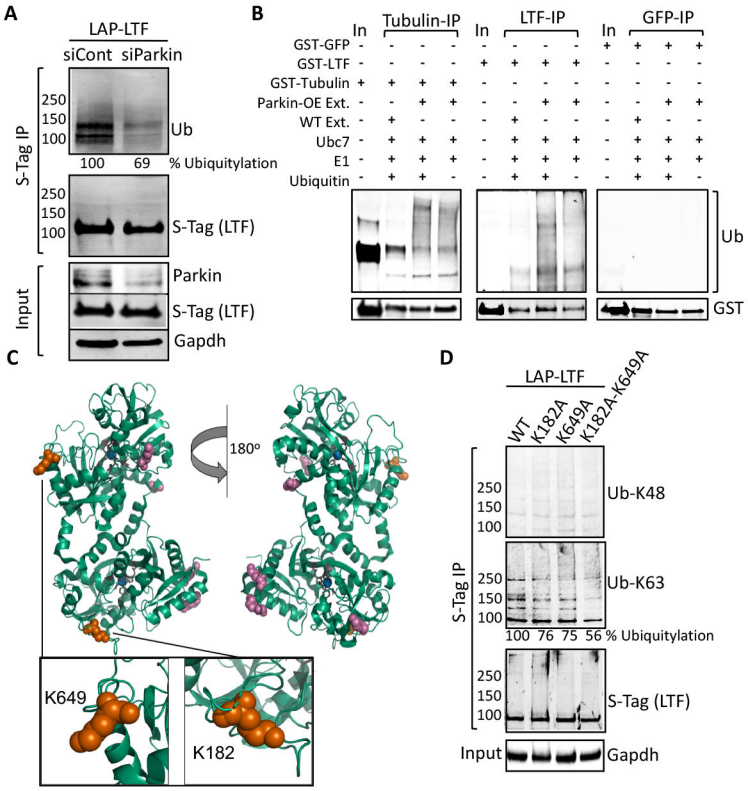

Figure 2.

LTF is a substrate of Parkin. (A) Immunoblot analysis of LAP-LTF immunoprecipitated from HeLa cells treated with control non-targeting siRNA (siCont) or siRNA targeting Parkin (siParkin). Immunoprecipitation of LAP-LTF (S-Tag-IP) was monitored with anti-S-Tag antibodies. LAP-LTF ubiquitylation was monitored with anti-ubiquitin antibodies (Ub). Depletion of Parkin was monitored with anti-Parkin antibodies and anti-Gapdh antibodies were used as a control to monitor Gapdh protein levels. IP indicates immunoprecipitation. (B) *In vitro* ubiquitylation assays with or without recombinant GST-LTF, GST-Tubulin (positive control) or GST-GFP (negative control); an ATP-regeneration system; ubiquitin; E1 ubiquitin-activating enzyme; E2 ubiquitin conjugating enzyme (Ubc7) and wildtype (WT) or LAP-Parkin overexpressing (OE) HEK293 cell extracts (Ext). GST-tagged proteins were immunoprecipitated and their ubiquitylation was monitored with anti-ubiquitin antibodies (Ub). IP indicates immunoprecipitation. (C) LTF ubiquitylation reactions were analyzed by mass spectrometry and the most abundant Parkin-mediated LTF ubiquitylation sites, K182 and K649, were mapped onto the human LTF structure PDB 1FCK (represented as orange spheres). Five additional ubiquitylation sites are represented in magenta spheres. Fe bound to LTF is represented in blue spheres. For a complete list of identified LTF ubiquitylation sites see Table S2. (D) LAP-LTF-WT or LTF single (K182A or K649A) or double (K182A/K649A) ubiquitylation site point mutants were expressed in HeLa cells, immunoprecipitated (S-Tag IP) and their ubiquitylation was monitored using anti-K48 and anti-K63 ubiquitin linkage specific antibodies. IP indicates immunoprecipitation.

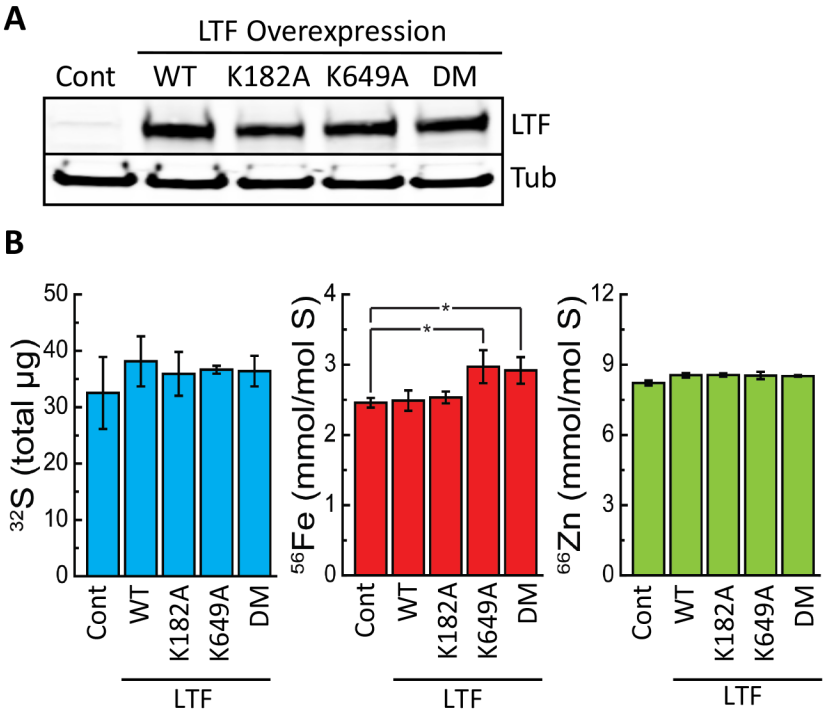

Figure 3.

Overexpression of LTF ubiquitylation site mutants influences intracellular iron levels. (A) Extracts from control HeLa cells (Cont) or HeLa cells overexpressing LAP-LTF wild type (WT), lysine 182 to alanine (K182A) point mutant, lysine 649 to alanine (K649A) point mutant, or the double point mutant (K182A/K649A, indicated by DM) were analyzed for sulfur (^{32}S), iron (^{56}Fe), and zinc (^{66}Zn). (B) The total ^{32}S levels (in µg) were used to quantify^{56}Fe and ^{66}Zn levels (in mmol/mol S). Bar graphs show mean ± standard deviation from 3 replicate samples. A *t*-test was used to calculate p values ($\alpha < 5\%$) in the indicated comparisons.

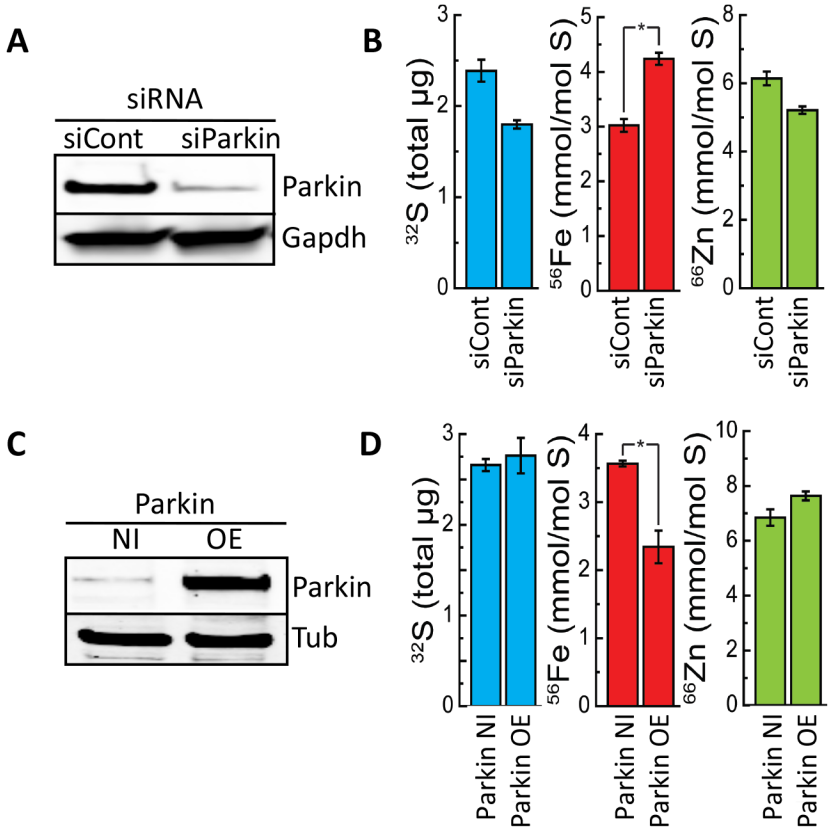

Figure 4.

Parkin levels influence intracellular iron levels. (A,B) Extracts from HeLa cells transfected with control non-targeting siRNA (siCont) or Parkin siRNA (siParkin) were analyzed for total sulfur (^{32}S), iron (^{56}Fe), and zinc (^{66}Zn). (C,D) Extracts from the HEK293 LAP-Parkin inducible cell line that was not induced (Parkin NI) or induced to overexpress LAP-Parkin (Parkin OE) were analyzed for total sulfur (^{32}S), iron (^{56}Fe), and zinc (^{66}Zn). NI indicates not induced and OE indicates overexpression. (B,D) The total ^{32}S levels (in µg) were used to quantify ^{56}Fe and ^{66}Zn levels (in mmol/mol S). Bar graphs show mean ± standard deviation from 3 replicate samples. A *t*-test was used to calculate p values ($\alpha < 5\%$) in the indicated comparisons.