Lysine 63-linked polyubiquitination is dispensable for Parkin-mediated mitophagy*

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*Running title: Role of K63-linked polyubiquitination in mitophagy by Parkin

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Background: K63-linked ubiquitination in mitochondria occurs in PINK1/Parkin-mediated mitophagy, and its important roles have been proposed. Results: The suppression of K63-linked ubiquitination did not modulate PINK1/Parkin-mediated mitophagy and Drosophila mitochondrial phenotypes. Conclusion: K63-linked ubiquitination is dispensable for PINK1-Parkin pathway. Significance: This is the first to report the biological significance of K63-linked ubiquitination in PINK1-Parkin pathway in vitro and in vivo.

ABSTRACT

PINK1/Parkin-mediated mitophagy is thought to ensure mitochondrial quality control in neurons as well as other cells. Upon the loss of mitochondrial membrane potential (ΔΨm)³, lysine 63 (K63)-linked polyubiquitin chains accumulate on the mitochondrial outer membrane in a Parkin-dependent manner. However, the physiological significance of K63-linked polyubiquitination during mitophagy is not fully understood. Here, we report that the suppression of K63-linked polyubiquitination through the removal of Ubc13 activity essentially affects neither PINK1 activation nor the degradation of depolarized mitochondria. Moreover, the inactivation of Ubc13 did not modulate the mitochondrial phenotypes of PINK1 knockdown Drosophila. Our data indicate that the formation of K63-linked polyubiquitin chains on depolarized mitochondria is not a key factor for the PINK1-Parkin pathway as was once thought.

Mutations of the Parkin and PINK1 genes cause selective degeneration of the midbrain dopaminergic neurons in autosomal recessive juvenile PD (1,2). The Parkin and PINK1 genes encode a ubiquitin-ligase (E3) and a serine/threonine protein kinase, respectively (3-7). Loss of the Parkin and PINK1 genes in Drosophila leads to the degeneration of the mitochondria in tissues with high-energy demands, such as the muscles and sperm, and genetic analysis has demonstrated that PINK1 is an upstream regulator of Parkin, suggesting an important role of Parkin and PINK1 in mitochondrial maintenance in the midbrain dopaminergic neurons that are affected in PD (8-10). A series of cell biological studies have provided strong evidence that Parkin cooperates with PINK1 to induce mitochondrial autophagy or mitophagy when the mitochondria are damaged (11-16). The reduction of ΔΨm leads...
Role of K63-linked polyubiquitination in mitophagy by Parkin

to the accumulation and activation of PINK1 in
the mitochondria (12,17), which leads to the
phosphorylation of a latent form of Parkin,
priming its E3 activation (17,18). PINK1 also
phosphorylates ubiquitin (19-21), which in turn
fully activates Parkin E3 activity, leading to
Parkin translocation from the cytosol to the
mitochondria and the subsequent ubiquitination
of mitochondrial proteins (14,15). Ubiquitin
modification on the mitochondria induces the
LC3-mediated autophagic elimination of the
damaged mitochondria, a process known as
mitophagy (11). The ubiquitination of
mitochondrial proteins mainly produces
K63-linked polyubiquitin and only a small
portion of K48 linkages (22,23). The
K63-linked polyubiquitin chain is proposed to
activate PINK1 (24) and the mitochondrial
translocation of Parkin (25). We examined the
impact of K63-linked polyubiquitination on
PINK1/Parkin-mediated mitophagy in cells and
mitochondrial maintenance in
Drosophila
and report that K63-linked polyubiquitination is
dispensable for PINK1 activation, mitochondrial
clearance and
Drosophila mitochondrial homeostasis.

EXPERIMENTAL PROCEDURES
Antibodies, Reagents, Plasmids and Cell Lines –
The following antibodies were used in the
western blot analysis: anti-PINK1 (1 : 1,000
dilution; Novus, BC100-494), anti-Mfn1 (1 : 1,000
dilution; Abnova, clone 3C9), anti-Ubc13
(1 : 1,000 dilution; Life technologies, clone
4E11), anti-polyubiquitin (1 : 1,000 dilution;
MBL, clone FK2), anti-K63-linked polyubiquitin
(1 : 1,000 dilution; Cell Signaling Technology, clone
D7A11), anti-K48-linked polyubiquitin
(1 : 1,000 dilution; Cell Signaling Technology, clone
D9D5), anti-Tom20 (1 : 1,000 dilution; Santa Cruz
Biotechnology, FL-145), anti-HA (1 : 1,000
dilution; Roche, clone 3F10),
anti-FLAG-HRP (1 : 2,000 dilution; Sigma-Aldrich, clone
M2), anti-Actin (1 : 10,000 dilution; Millipore, MAB1501),
anti-Hsp60 (1 : 10,000 dilution; BD Biosciences,
clone 24/Hsp60), anti-NDUFS3 (1 : 10,000
dilution; Abcam, 17D95), anti-
Drosophila
Hsp60 (1 : 1,000 dilution; Cell Signaling, D307)
and anti-
Drosophila Mitofusin (dMfn) (1 : 2,000
dilution; made in-house). The following
antibodies were used for immunocytochemistry
analysis: anti-polyubiquitin (1 : 250 dilution;
MBL, clone FK2), anti-K63-linked polyubiquitin
(1 : 50 dilution; Millipore, clone
Apu3) and anti-Tom20 (1 : 1,000 dilution; Santa
Cruz Biotechnology, FL-145). Mouse
embryonic fibroblasts (MEFs) harboring
wild-type or homozygousloxP-flanked
Ubc13 alleles (26) were stably transfected with Cre
recombinase controlled by Tet-On systems.
Ubc13 genes were floxed out following
Cre-mediated excision by treatment with 1
µg/ml doxycycline (Dox) for 72 hr to generate
Ubc13−/− MEFS. Wild-type Ubc13 MEFS were
also treated with Dox as a control. The plasmids
encoding GFP-Parkin, HA-Parkin and
PINK1-FLAG have been previously described
(15,27). MEFs and HeLa cells were retrovirally
transfected with pMXs-puro harboring
PINK1-FLAG, HA-Parkin and GFP-Parkin, and
the infected cells were selected with 1
µg/ml
puromycin. The mitochondrial uncoupler
carbonyl cyanide m-chlorophenyl hydrazine
(CCCP) and the ubiquitin-activating enzyme
(E1)-specific inhibitor UBEI-41 were purchased
from Sigma-Aldrich. The mitochondrial
uncoupler valinomycin and TUBE1-agarose
were obtained from Wako and LifeSensors,
respectively.

Immunocytochemical and Biochemical Analyses –
Cells plated on 3.5-mm glass-bottom dishes
(MatTek) were fixed with 4% paraformaldehyde
in PBS and permeabilized with 50 µg/ml
digitonin in PBS. The cells were stained with
anti-Tom20 or anti-ubiquitin antibodies. The
cells were imaged using laser-scanning
microscope systems (LSM510 META, Carl
Zeiss). Phos-tag western blotting was performed
as previously described (18).

Drosophila Genetics – Fly experiments were
performed as described (28). The w
1118
(w−)
line was used as a wild-type genetic background.
The
Ubc13 RNAi
line was obtained from the Vienna
Drosophila
RNAi Center and was
characterized in (29). Other fly stocks used in
this study have been previously described (8).

RESULTS AND DISCUSSION
Because Ubc13 is an E2 enzyme crucial for
generating K63-linked chains (30), we tested
PINK1/Parkin-mediated mitophagy in Ubc13
mutant cells to estimate the effects of K63-linked polyubiquitin chain formation. In the Ubc13 mutant cells harboring the loxp-flanked Ubc13 gene, Ubc13 can be inactivated by Dox-induced flox-out. We inactivated Ubc13 by Dox treatment and induced the mitochondrial translocation of GFP-Parkin and the accumulation of ubiquitin chains using CCCP. The mitochondrial translocation of GFP-Parkin occurred with similar efficiency (Fig. 1, A and B). In contrast, the accumulation of total ubiquitin (Fig. 1C and E) as well as K63-linked polyubiquitin (Fig. 1D and E) in the mitochondria was dramatically reduced in the absence of Ubc13 activity. Accumulation of K48-linked polyubiquitin in the mitochondrial fractions was similar between Ubc13+/+ and Ubc13−/− MEFs expressing GFP-Parkin (Fig. 1E).

Polyubiquitination induces the degradation of mitochondria outer membrane proteins through the proteasome and recruits LC3-mediated autophagy machinery (22). To test whether autophagy is altered in Ubc13−/− MEFs, we examined the levels of Mfn1, a known substrate of Parkin E3; a mitochondrial outer membrane protein, Tom20; and a matrix protein, Hsp60. The time-dependent degradation of Mfn1, Tom20 and Hsp60 in Ubc13−/− MEFs was comparable to that in Ubc13+/+ MEFs (Fig. 2A). When Parkin is activated upon CCCP treatment, Parkin is subjected to autodegradation by the proteasome (18). The degradation efficiency of HA-tagged Parkin was similar between Ubc13+/+ and Ubc13−/− MEFs, suggesting that the formation of K63-linked polyubiquitin affects neither the activation of Parkin nor the autophagic clearance of mitochondria.

It has been proposed that K63-linked ubiquitination of PINK1 by TRAF6 is required for the mitochondrial accumulation of PINK1 and mitochondrial translocation of Parkin upon a reduction of ΔΨm (24). PINK1 stabilization on the mitochondrial outer membrane stimulates its dimerization and is closely correlated with its autophosphorylation at S228 and S402 in an intermolecular fashion (31), through which PINK1 kinase activity is thought to be activated (32). We estimated the extent of PINK1 accumulation and PINK1 autophosphorylation by conventional western blot and phos-tag western blot analyses, respectively (Fig. 2B). However, there was no evidence that PINK1 accumulation and autophosphorylation were altered in the absence of Ubc13, suggesting that the formation of the K63-linked polyubiquitin chain is not a key factor in PINK1 regulation in mitophagy.

Because MEFs are derived from a heterogeneous population of cells, the response to PINK1/Parkin-mediated mitophagy might differ among different batches of cells. To exclude this possibility, we used the same batch of Ubc13 mutant cells, which were treated with or without Dox. PINK1/Parkin-mediated mitophagy was induced by CCCP treatment for up to 24 hr. We again confirmed that the efficiency of the degradation of HA-Parkin, Mfn1 and Tom20 is comparable between Dox-treated and non-treated cells (Fig. 2C). It has been reported that Parkin is also involved in xenophagy for Mycobacterium tuberculosis, in which the co-localization of a K63-linked ubiquitin chain with phagosomes containing M. tuberculosis was observed (33). Because the formation of K63-linked ubiquitination, the subsequent accumulation of the ubiquitin-adaptors and the autophagy machinery are Parkin-dependent, K63-linked ubiquitination likely mediates the recruitment of autophagy-related proteins, as proposed in mitophagy. K63-linked ubiquitination is also observed in Salmonella xenophagy (34). However, the recruitment of the autophagy machinery occurred with the same efficiency in Salmonella xenophagy (35). The results describing both mitophagy and xenophagy suggest that the autophagy machinery can recognize other polyubiquitin linkages in addition to K63 or that K63-linkage is not involved in this step. Although K63-linked ubiquitination is not essentially required for mitochondrial translocation of Parkin, the inhibition of all of ubiquitination reactions by an E1-specific inhibitor completely suppresses Parkin translocation, suggesting that ubiquitination is part of the regulation in Parkin translocation (Fig. 2D).

The formation of K63-linked polyubiquitination by Ubc13 and Uev1a is involved in the TNF signaling in both mammals...
(36) and Drosophila (29). Knockdown of Bendless (Ben), an ortholog of Ubc13, suppresses TNF signaling in Drosophila, suggesting that the formation of K63-linked polyubiquitination is inhibited (29). Muscular mitochondria in the thorax, in which Ben was inactivated, showed a normal gross morphology, implying that K63-linked ubiquitination is dispensable for mitochondrial maintenance under steady-state conditions (Fig. 3A). In contrast, PINK1 activity is essential for maintaining mitochondrial homeostasis because inactivation of PINK1 largely leads to mitochondrial degeneration, as previously described (Fig. 3A) (8). The mitochondrial degeneration by PINK1 inactivation was no longer modulated by the suppression of Ben activity, even in old flies (Fig. 3A). Consistent with the histochemical analysis, levels of a mitochondrial outer membrane protein Mitofusin, which is a ubiquitination substrate of Parkin, as well as the mitochondrial complex I subunit NDUFS3 were not altered by Ben inactivation (Fig. 3B,C). In addition, the absence of Ben did not affect mitochondrial ATP production (Fig. 3D).

In conclusion, this study revealed that K63-linked ubiquitination is dispensable for the PINK1-Parkin pathway. Although K63-linked ubiquitination by Parkin has been suggested to be important for the suppression of protein toxicity by Parkin, further investigations will be required to determine whether specific roles of K63-linked ubiquitination in the PINK1-Parkin pathway exist (37,38).

NOTE. After submission of this manuscript, two studies using siRNA against Ubc13 reported that Ubc13 has a role for the autophagy process of Parkin-mediated mitophagy (39) and Parkin translocation (40). As we also observed some delay in mitophagy in our initial study using siRNA, we feel that certain sequences of siRNA affect mitophagy.
REFERENCES


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FOOTNOTES
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3The abbreviations used are: ΔΨm, mitochondrial membrane potential; K63, lysine 63; PD, Parkinson's disease; MEF, mouse embryonic fibroblast; E1, ubiquitin-activating enzyme; Mfn1, Mitofusin1; Dox, doxycycline; CCCP, carbonyl cyanide m-chlorophenyl hydrazine; siRNA, small interfering RNA; shRNA, short hairpin RNA; dMfn, Drosophila Mitofusin; CBB, Coomassie Brilliant Blue.
FIGURE LEGENDS

FIGURE 1. The loss of Ubc13 activity impairs the accumulation of K63-linked ubiquitin chains during Parkin-mediated mitophagy. A, MEFs retrovirally introduced with GFP-Parkin were treated with Dox to remove Ubc13 genes and then treated with 30 μM CCCP for 6 hr. Parkin and mitochondria were visualized with GFP fluorescence (green) and anti-Tom20 (red), respectively. B, the mitochondrial translocation efficiency of Parkin treated as in A was graphed. The values represent the means ± SE of the percentages of cells exhibiting mitochondrial recruitment in three independent experiments. The translocation efficiency was similar in Ubc13+/+ and Ubc13−/− (3 hr, P < 0.8024; 6 hr, P < 0.1309 by Student’s t-test). C, ubiquitin accumulation was detected with anti-polyubiquitin (red) in cells treated as in A. D, accumulation of a K63-linked ubiquitin chain was detected with anti-K63-linkage specific ubiquitin antibody (red) in cells treated as in A. Scale bars = 10 μm. E, accumulation of K63-linked polyubiquitin (K63-Ub) but not of K48-linked polyubiquitin (K48-Ub) was reduced in the absence of Ubc13 activity. Crude mitochondrial fractions from MEFs expressing GFP-Parkin (1 x 10⁶) treated with (+) or without (−) 30 μM valinomycin for 6 hr were prepared. Polyubiquitin purified with TUBE1-agarose in the mitochondrial fractions were detected by western blot. Poly-Ub; polyubiquitin. All experiments were repeated at least three times in A-D and two times in E and representative results were shown.

FIGURE 2. Suppression of K63-linked ubiquitin chain formation does not affect PINK1 activation or mitochondrial clearance. A, MEFs expressing HA-Parkin were treated with 30 μM CCCP for up to 24 hr and were subjected to western blot analysis. Mfn1 and Tom20 were used as markers of mitochondrial outer membrane proteins. Hsp60 was used as a marker of mitochondrial matrix proteins. Actin was used as a loading control. B, MEFs expressing PINK1-FLAG were treated with 30 μM CCCP as in A. The autophosphorylation of PINK1 and accumulation of PINK1 were estimated by phos-tag western blot with anti-PINK1 (Phos-tag WB) and conventional western blot with anti-FLAG (WB). C, MEFs harboring loxP-flanked Ubc13 were treated with or without Dox for 72 hr and were further treated with CCCP for the indicated time periods. The degradation of Parkin, Mfn1 and Tom20 were analyzed by western blot analysis. D, HeLa cells stably expressing GFP-Parkin were pretreated with 60 μM UBE1-41 (E1 inhibitor) or DMSO solvent for 1 hr and were further treated with or without 20 μM CCCP for 3 hr. GFP-Parkin and mitochondria were visualized with GFP signal (green) and anti-Tom20 (red), respectively. Scale bars = 10 μm. All experiments were repeated at least three times in A-C and two times in D.

FIGURE 3. Inhibition of Ubc13 does not modulate the mitochondrial phenotypes caused by PINK1 inactivation. A, fluorescent images of the indirect flight muscle in 7- and 30-day-old adult flies expressing the indicated shRNAs are shown. To visualize the mitochondria, the mitoGFP (green) transgene was co-expressed and the muscle tissue was counterstained with phalloidin (magenta). Representative images from three independent samples in each genotype are shown. Experiments were repeated two times. Scale bar = 10 μm in the fluorescent images. B, the protein levels of Drosophila Mitofusin (dMfn), complex I subunit NDUFS3 and Hsp60 from the thoraxes of 7-day-old adult flies were analyzed by western blot. CBB staining around the dMfn migration position confirms that approximately equivalent amounts of protein were loaded. C, the band intensities of dMfn and NDUFS3 were normalized to each CBB signal. The values (arbitrary units) represent the mean ± SE from 4-5 independent samples as in B. Although dMfn and NDUFS3 levels showed increasing and decreasing tendencies, respectively, with PINK1 inactivation as reported (28), there were no statistical differences between any combinations. N.S., not significant. n = 4-5. D, ATP contents of thorax muscle tissues of 7-day-old adult flies were measured. ATP contents were normalized against the protein levels. The values represent the mean ± SE from five independent samples. * p < 0.05, ** p < 0.01 by Tukey-Kramer test.
Fly genotypes used in A-D are as follows: UAS-mitoGFP/UAS-LacZ RNAi; MHC-GAL4/+ (LacZ RNAi), UAS-mitoGFP/UAS-Ben RNAi; MHC-GAL4/+ (Ben RNAi), UAS-mitoGFP/UAS-LacZ RNAi; MHC-GAL4, UAS-PINK1 RNAi/+ (PINK1 RNAi, LacZ RNAi), UAS-mitoGFP/UAS-Ben RNAi; MHC-GAL4, UAS-PINK1 RNAi/+ (PINK1 RNAi, Ben RNAi).
Figure 1

Role of K63-linked polyubiquitination in mitophagy by Parkin

A. GFP-Parkin, Tom20, Overlay

B. % of cells with Parkin translocation

C. GFP-Parkin, Ubiquitin, Overlay

D. GFP-Parkin, K63-Ubiquitin, Overlay

E. Valinomycin: – + – +

Ubc13+/+ Ubc13-/–

250 150 100 75 kDa

poly-Ub

K63-Ub

K48-Ub
Figure 2

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Figure 3

A

LacZ RNAi
Ben RNAi
PINK1 RNAi
LacZ RNAi
PINK1 RNAi
Ben RNAi

Day7
Day30

B

+ PINK1 RNAi

LacZ RNAi
Ben RNAi
LacZ RNAi
Ben RNAi

C

Relative dMfn levels
Relative NDUFS3 levels

LacZ RNAi
Ben RNAi
LacZ RNAi
Ben RNAi
LacZ RNAi
Ben RNAi

+ PINK1 RNAi

D

ATP/Protein (nmol/mg)

LacZ RNAi
Ben RNAi
LacZ RNAi
Ben RNAi

+ PINK1 RNAi

N.S.
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