Figures and figure supplements

MUL1 acts in parallel to the PINK1/parkin pathway in regulating mitofusin and compensates for loss of PINK1/parkin

Jina Yun, et al.
Figure 1. Overexpression of MUL1, but not MUL1 LD, suppresses PINK1/parkin mutant phenotypes. (A) Protein domain organization of Drosophila MUL1. TM1, TM2, and RNF represent transmembrane domains 1 and 2, and the RING Finger domain, respectively. The position of the mutation in the ligase dead (LD) version of MUL1 is marked with a red asterisk. (B) Sequence alignment of MUL1 in various species in the highly conserved RNF domain. A highly conserved histidine residue (marked as red) was mutated to alanine in MUL1 LD, ablating ligase activity. (C–C″) Dopaminergic neurons stained with an anti-TH antibody in red and mitochondria labeled with mitoGFP in Figure 1. Continued on next page.
green. Neurons in the PPL1 cluster are shown. While mitochondria in wild-type dopaminergic neurons are dispersed (C), mitochondria in PINK1 mutant dopaminergic neurons are clumped (C’, white arrow heads). This phenotype is suppressed by MUL1 overexpression driven by TH-Gal4 (C”). Scale bars: 10 µm. (D–E” and J–M”) Confocal images of the IFM from thoraces double labeled with mitoGFP and phalloidin (red) (D–D”, J–J”, L–L”), or double labeled with mitoGFP and TUNEL (red) with lower magnification (E–E”, K–K”, M–M”). Scale bars: 5 µm. MUL1 overexpression is driven by Mef2-Gal4. In wild-type (D), mitochondria have a regular size and shape, and are localized in between myofibrils. In PINK1 mutants (D”), mitochondrial size becomes irregular, and the GFP signal is reduced. Large mitochondrial clumps also appear. PINK1 mutant muscle is TUNEL-positive (E’). (F–F”) Tousidine blue staining of muscle. Compared with the wild-type (F), PINK1 mutant muscle shows vacuolation indicating muscle degeneration (F’). These PINK1 mutant phenotypes (D’, E’, F”) are almost completely suppressed by MUL1 overexpression (D”, E”, F”). (Ga–Ga”, Gb–Gb”) EM images of mitochondria in muscle. (Gb–Gb”) Single mitochondrion (outlined with dashed lines) from white boxes in Ga–Ga”. Scale bars: 1 µm (Ga–G’a) 0.5 µm (Gb–G”b). In wild-type (Ga and Gb), mitochondria have compact and organized cristae whereas mitochondria from PINK1 mutants (Ga’, Gb’) are swollen with fragmented cristae, and this is rescued by MUL1 overexpression (Ga”, Gb”). (H) Images of thoraces. Arrows point to thoracic indentations due to muscle degeneration. Compared with WT, PINK1 mutants have thoracic indentation due to muscle degeneration. Compared with WT, PINK1 mutants have thoracic indentation due to muscle degeneration. MUL1 overexpression, but not MUL1 LD overexpression, suppresses PINK1 mutant thoracic indentation. (I) qPCR analysis shows that MUL1 and MUL1 LD mRNA are expressed at similar levels in muscles. The data are shown as the mean ± SEM from three experiments (RNA from ten 5-day-old fly thoraces for each genotype). The statistical analysis was done using One-way ANOVA with Tukey’ multiple comparisons test. ns: not statistically significant. MUL1 LD overexpression in the PINK1 mutant background does not suppress the formation of mitochondrial clumps (J”) or TUNEL-positivity (K”). (L–M”) Overexpression of MUL1, but not MUL1 LD, suppresses parkin mutant phenotypes.

DOI: 10.7554/eLife.01958.003

Figure 1—figure supplement 1. MUL1, but not its ligase-dead version (MUL1 LD), is able to self-ubiquitinate in vitro.

DOI: 10.7554/eLife.01958.004
Figure 2. MUL1 regulates mitochondrial morphology. (A) A schematic depicting the Drosophila MUL1 genomic region (cytological location 64A4). MUL1 coding and untranslated regions (dark and open rectangles, respectively) are depicted. The P element, MUL1\textsuperscript{EY}, inserted in the 5' UTR, is shown as an inverted triangle. The deleted region in the MUL1\textsuperscript{A6} allele is indicated by parentheses. (B) RT PCR shows that flies carrying the MUL1\textsuperscript{EY} allele have detectable but reduced levels of MUL1 transcripts. However, no MUL1 transcript is detected in flies homozygous for the MUL1 deletion, MUL1\textsuperscript{A6}. (C) qPCR shows that MUL1\textsuperscript{EY} allele has approximately a 60% reduction of MUL1 transcript compared to the wild-type (WT). No MUL1 transcript is detected in flies homozygous for MUL1\textsuperscript{A6}. (D) MUL1 RNAi line reverses the suppression of PINK1 mutant mitochondrial phenotypes due to MUL1 overexpression. (E) Muscle fibers stained with mitoGFP in green and actin in red. Compared with the WT, flies homozygous for the MUL1 deletion or expressing MUL1 RNAi show slightly elongated mitochondria. In contrast, when MUL1 is overexpressed using the Mef2-Gal4 driver, mitochondria are significantly smaller. (F) Salivary glands, with cell boundaries labeled with rhodamine phalloidin in red, and mitoGFP in green. In WT, mitochondria are tubular and evenly distributed. In contrast, in cells expressing MUL1 RNAi (driven by OK6-Gal4) mitochondria are fewer in number and found in clumps. In contrast, MUL1 overexpression (also driven by OK6-Gal4) results in fragmented mitochondria and irregular cell boundaries. (G) Quantification of mitochondrial number and size in salivary glands (mean ± SEM, n > 6 larvae for each genotype). * Significantly different from wild-type, p<0.05 (One-way ANOVA with Tukey’s multiple comparisons test). DOI: 10.7554/eLife.01958.005
Figure 3. MUL1 physically binds to Mfn, and promotes ubiquitination-mediated Mfn degradation. (A and B) Western blots and quantifications of Drp1 and Mfn levels in vivo. Analysis of lysates from thoraces show that MUL1 overexpression reduces Mfn levels (B) but not Drp1 levels (A). The data are shown as the mean ± SEM from three experiments (each experiment was done with lysate from 8 thoraces for each genotype). The statistical analysis was done using One-way ANOVA with Tukey’s multiple comparisons test. ns: not statistically significant. ** Significantly different, p<0.01. (C) Western blots of Mfn levels in S2 cells either not treated or treated with control, PINK1, parkin or MUL1 RNAi. Quantification of relative Mfn levels shows that there is an increase in Mfn levels in cells treated with RNAi to PINK1, parkin, or MUL1 (mean ± SEM. ** Significantly different from cells not treated with RNAi, p<0.01, One-way ANOVA with Tukey’s multiple comparisons test). (D) Co-immunoprecipitation using lysates from S2 cells transfected with the indicated constructs. The INPUT represents 2% of total lysate to monitor protein expression (top panel). MUL1-GFP is co-immunoprecipitated with Mfn-myc using both anti-GFP and anti-Myc antibodies. Mfn-myc also co-immunoprecipitates with HA-Parkin, which serves as a positive control. The interaction between Mfn-Myc and MUL1-GFP was specific, as confirmed by separate immunoprecipitation control experiments (Figure 3—figure supplement 1). (E) Mfn ubiquitination levels in S2 cells. S2 cells are treated with dsRNA designed to silence various genes and transfected with Mfn-Flag. Immunoprecipitation was performed with anti-Flag antibody, and Western blots were probed with anti-Ubiquitin antibody and an anti-Flag antibody. Relative ubiquitination levels compared to control are shown below (mean ± SEM). ** Significantly different from control, p<0.01 (One-way ANOVA with Tukey’s multiple comparisons test). (F) In PINK1 mutant thoraces, where Mfn levels are increased, MUL1 overexpression (driven by Mef2-Gal4) reduces the increased Mfn levels. Relative Mfn levels compared to control are shown below (mean ± SEM). ** Significantly different, p<0.01 (One-way ANOVA with Tukey’s multiple comparisons test).

DOI: 10.7554/eLife.01958.006
Figure 3—figure supplement 1. MUL1 co-immunoprecipitates with Mfn in S2 cells.
DOI: 10.7554/eLife.01958.007
Figure 4. Generation and expression of the IFM-GAL driver, mfn overexpression, but not loss of drp1, induces PINK1/parkin-mutant like pathology. (A–J) Different developmental stages of flies expressing GFP under Mef2-Gal4 (A–E) or IFM-Gal4 (F–J). (A) Third instar larvae show GFP expression in whole body muscles. (B) At the early pupal stage, GFP is expressed in a similar pattern as in larvae. However, the GFP expression pattern becomes more specific at the late pupal stage (C), in which the strongest GFP signal is seen in the thorax, and a weaker signal is observed in the head and abdomen (arrows). (D) In an adult fly, dorsal view shows GFP signal in the thorax, upper abdomen and legs. (E) GFP is also expressed in adult head and legs, marked with arrows. (F) Flies expressing GFP under IFM-Gal4 show no GFP expression in third instar larvae, or in early pupae (G). (H) GFP is strongly expressed only in the thorax at the late pupal stage, but not in other areas (arrows). (I) In the adult fly, GFP signal is highly concentrated in the thorax. No GFP expression in abdomen and legs is observed, arrows. (J) In contrast to GFP expression under Mef2-Gal4, IFM-Gal4 does not express in adult head or legs, as indicated with arrows. (K–P, T–Y) Confocal images of muscle double labeled with mitoGFP (green) and phalloidin (red) (K–M, T–V), or those labeled with mitoGFP and TUNEL (red) at lower magnification (N–P, W–Y), respectively. (Qa–Sb) EM images of mitochondria in muscle. Single mitochondrion from the black-boxed area in Qa, Ra, Sa is shown in Qb, Rb, Sb. Scale bars: 1 µm (Qa, Ra, Sa) and 0.5 µm (Qb, Rb, Sb). Compared with wild-type (K and N), parkin null mutant (L and O) shows overall reduced levels of mitoGFP signal, large mitochondrial clumps, and muscle cell death. Similar phenotypes are observed with mfn overexpression (M and P), and these phenotypes are suppressed by MUL1 overexpression (T and W). As a control, parkin overexpression also suppresses phenotypes due to mfn overexpression (U and X). Importantly, drp1 null (drp1+/drp1−) mutant muscle does not have any mitochondrial clumping or TUNEL-positivity seen in loss of parkin function or mfn overexpression (V and Y). mfn overexpression is driven by IFM-Gal4. Scale bars: 5 µm.

DOI: 10.7554/eLife.01958.008
Figure 5. MUL1 acts in parallel to the PINK1/parkin pathway. (A–H) Images of thoraces of various mutants. Arrows point to thoracic indentations due to muscle degeneration. PINK1 MUL1 and parkin MUL1 double mutants have more severe thoracic indentation compared to either mutant alone. Remarkably, the severe thoracic indentation phenotype in parkin MUL1 double mutants is almost completely suppressed when mfn is also knocked down. (I–P) Mitochondria are labeled using an anti-ATP synthase antibody in the IFM. While PINK1, parkin, and MUL1 mutant show slightly elongated mitochondrial morphology, PINK1 MUL1 and parkin MUL1 double mutants exhibit highly elongated and interconnected mitochondria. These phenotypes can be suppressed by mfn knockdown. Instead of using mitoGFP, we utilized anti-ATPase antibodies that allow better visualization of the enhancement phenotypes seen with double mutants. (Q) Relative ATP levels in whole flies of various mutants (mean ±SEM from three experiments, five 5-day-old flies for each genotype). ** and *** significantly different from wild-type, p<0.01 and p<0.001, respectively (One-way ANOVA with Tukey’s multiple comparisons test). # Significantly different from parkin mutant and MUL1 mutant, both p<0.01 (Two-way ANOVA with Tukey’s multiple comparisons test). (R) In vivo ubiquitination assay of Mfn. S2 cells were treated with the indicated RNAi, transfected with Flag-Mfn, and treated with proteasome inhibitor MG132. Immunoprecipitations were performed using anti-Flag antibody, and western blots were probed with antibodies against anti-Ubiquitin antibody (P4D1) or anti-Flag antibody. Relative ubiquitination levels compared to control are shown in the lower panel (mean ±SEM). ** and *** Significantly different from control, p<0.01 and p<0.001, respectively (One-way ANOVA with Tukey’s multiple comparisons test). # Significantly different from MUL1 RNAi #1 and parkin RNAi, both p<0.01. & Significantly different from MUL1 RNAi #2 and parkin RNAi, p<0.001 and p<0.01, respectively (Two-way ANOVA with Tukey’s multiple comparisons test). (S) Western Figure 5. Continued on next page
Figure 5. Continued

blot analysis of Mfn levels in vivo and quantification (mean ± SEM from three experiments, eight third instar larvae for each genotype). * and ** significantly different from wild-type, p<0.05 and p<0.01, respectively (One-way ANOVA with Tukey’s multiple comparisons test). # Significantly different from parkin mutant and MUL1 mutant, both p<0.01. & Significantly different from PINK1 mutant and MUL1 mutant, both p<0.01 (Two-way ANOVA with Tukey’s multiple comparisons test).

DOI: 10.7554/eLife.01958.009

Figure 5—figure supplement 1. MUL1 acts in a parallel pathway to the PINK1/parkin pathway.
DOI: 10.7554/eLife.01958.010
Figure 6. MUL1’s function in mitochondrial morphology and Mfn levels is conserved in human cells. (A–D”) HeLa cells transfected with GFP-MUL1 (A–B”) or GFP-MUL1 LD (C–D”) are marked with asterisks, while cells not transfected serve as internal controls. Mitochondria are labeled with mitotracker in red (B and D). (B’ and B”, D’ and D”) Higher magnification images of mitochondria within white boxes in B and D. Cells expressing GFP-MUL1 have clustered mitochondria in the perinuclear region (B). Mitochondria are also small and fragmented (B”), as compared to cells not expressing GFP-MUL1 (B”). Importantly, GFP-MUL1 LD does not result in localization of mitochondria to the perinuclear region (D) or in mitochondrial fragmentation (D”). (E) Western blot analysis of Mfn1 and Mfn2 levels after CHX treatment. HeLa cells expressing scrambled shRNA or MUL1 shRNA are treated with CHX. Mfn1 and 2 levels at each time point are normalized with Actin. The relative portion of remaining Mfn1 and 2 as compared to time point 0 was calculated and plotted (E).
treatment are more stable than those in cells expressing scrambled shRNA. (F) Expression of transfected GFP-MUL1 and GFP-MUL1 LD in HeLa cells, as detected using anti-GFP antibody. (G) Western blot analysis of endogenous MUL1 levels in HeLa cells stably expressing scrambled shRNA and MUL1 shRNA. MUL1 shRNA expressing cells have reduced levels of endogenous MUL1. (H) Human MUL1 sequence and deletion in MUL1 knockout (MUL1−/−) HeLa cells, generated using the CRISPR/Cas 9 system. Sequences targeting MUL1 are highlighted in blue. Red letters indicate start codon. Red dashes represent deleted bases. Deleted eight base pairs include the start codon of MUL1. (I) Western blot analysis of Mfn1 and Mfn2 levels in wild-type and MUL1−/− HeLa cells treated with CHX for the indicated time. Remaining Mfn1 and Mfn2 levels at each time point were plotted below. (J) Western blot showing no MUL1 expression in MUL1−/− HeLa. Arrowhead points to MUL1 protein. Asterisk indicates a non-specific band.

DOI: 10.7554/eLife.01958.011
Figure 7. Neither MUL1 knockout nor overexpression affects Parkin-mediated mitophagy. (A–C) HeLa cells (control, MUL1 knockout or PINK1 knockout) were transfected with YFP-Parkin, treated with either DMSO or antimycin A, and immunostained with an anti-Tom20 antibody which labels mitochondria. (A) HeLa cells treated with DMSO as a control. (B) Following treatment with antimycin A for 3 hrs, Parkin is recruited to mitochondria, as shown by co-localization of Parkin and the mitochondrial marker. In MUL1 null cells, Parkin recruitment to mitochondria is not affected, whereas in PINK1 null cells (positive control), Parkin recruitment to mitochondria is abolished. (C) After 24 hrs of antimycin A treatment, Parkin returns to the cytosol and the mitochondrial signal disappears. In MUL1 null cells, mitochondrial disappearance occurs similarly as with WT, whereas in PINK1 null cells (positive control), mitochondria are not eliminated. (D–F) Quantification of cells with Parkin recruited to mitochondria after 3 hrs of antimycin A treatment (D) and with few or no mitochondria after 24 hrs of antimycin A treatment (E) and after 16 and 20 hrs of antimycin A treatment (F). The data are shown as the mean ± SEM from three experiments (n ≥ 100 for each genotype). *** Significantly different from wild-type, p<0.001. ns: not statistically significant (One-way ANOVA with Tukey’s multiple comparisons test). While Parkin translocation and mitochondrial disappearance are significantly blocked in PINK1 knockout cells, there is no significant difference between HeLa cells and MUL1 knockout cells in these processes. (G) HeLa cells stably expressing YFP-Parkin and mito-RFP are transfected with Flag-MUL1, treated with DMSO or antimycin A, and immunostained with anti-Flag antibody. 3-hour antimycin A treatment causes Parkin localization to mitochondria in cells with or without MUL1 expression. (H) Quantification of cells with Parkin recruited to mitochondria after 1.5 or 3 hrs Antimycin A treatment. Both 1.5 and 3 hrs of antimycin A treatments results in similar levels of Parkin localization to mitochondria.
recruitment to mitochondria. The data are shown as the mean ± SEM from three experiments (n ≥ 100 for each genotype). ns: not statistically significant (One-way ANOVA with Tukey’s multiple comparisons test).

DOI: 10.7554/eLife.01958.012

Figure 7—figure supplement 1. MUL1 knockdown does not affect Parkin-mediated mitophagy.

DOI: 10.7554/eLife.01958.013
Figure 8. Loss of both MUL1 and parkin aggravates mitochondrial damage and induces degeneration-like phenotypes in mouse cortical neurons. (A) MUL1 targets mitochondria in the cell bodies and axons of mouse primary cortical neurons. Neuronal mitochondria were labeled by DsRed-Mito or stained with an antibody against mitochondrial marker, TOM20 or Cytochrome C (Figure 8—figure supplement 1). (B) Levels of endogenous MUL1 in neurons transfected with scrambled or MUL1 shRNA. Note that partial suppression of endogenous MUL1 may reflect relative low transfection rate (20%) in the neuronal culture. (C–F') Mitochondria in live cortical neurons were co-labeled by expressing CFP-mito, which targets all mitochondria, and by loading fluorescent dye TMRE, which stains healthy mitochondria dependent upon membrane potential ($\Delta\psi_m$). Loading TMRE also labels mitochondria in glia in the culture. The edges of neuron cell bodies are marked with white solid lines, and the nuclei are outlined with white dashed lines. In contrast to other neurons, parkin knockout neurons with MUL1 knockdown show reduced TMRE intensity (F and F'), indicating decreased $\Delta\psi_m$. Scale bars: 10 µm. (G) Quantification of relative TMRE intensity. TMRE intensity measured from each group of neurons was normalized to WT neurons transfected with scrambled shRNA. The data are shown as the means ± SEM from three experiments. (n ≥ 12 for each group). *** Significantly different from wild-type neurons transfected with scrambled shRNA, p<0.001. ns: not statistically significant (One-way ANOVA with Tukey’s multiple comparison test). Figure 8. Continued on next page
comparisons test). # Significantly different from wild-type neurons transfected with MUL1 shRNA and parkin KO neurons transfected with scrambled shRNA, p<0.001 and p<0.01, respectively (Two-way ANOVA with Tukey’s multiple comparisons test). (H–M) MUL1 knockdown in parkin KO neurons results in enhanced fragmentation of neurites. Representative wild-type (H and I) and parkin KO (J–K″) cortical neurons transfected with scrambled or MUL1 shRNA and labeled with GFP (confirming transfection of shRNA and labeling axons and dendrites). (K–K″) Higher magnification of a white box in K showing the soma and proximal dendrites labeled with an anti-MAP2 antibody (red). Arrows point to the GFP- and MAP2-labeled dendrites, and arrowheads indicate GFP-labeled but MAP2-negative fragmented axons. Scale bars: 20 µm. (L and M) Quantitative analysis showing enhanced process fragmentation (L) and dendritic retraction (M). The data are shown as the means ± SEM from three experiments (process fragmentation phenotype: n ≥ 115 for each genotype, dendritic retraction: n ≥ 127 for each phenotype). *, **, and *** Significantly different from wild-type neurons transfected with scrambled shRNA, p<0.05, p<0.01, and p<0.001, respectively. ns: not statistically significant (One-way ANOVA with Tukey’s multiple comparisons test). # Significantly different from wild-type neurons transfected with MUL1 shRNA and parkin KO neurons transfected with scrambled shRNA, both p<0.001. & Significantly different from wild-type neurons transfected with MUL1 shRNA and parkin KO neurons transfected with scrambled shRNA, both p<0.01 (Two-way ANOVA with Tukey’s multiple comparisons test).
DOI: 10.7554/eLife.01958.014

Figure 8. Continued

Figure 8—figure supplement 1. MUL1 localizes to mitochondria in mouse cortical neurons.
DOI: 10.7554/eLife.01958.015
**Figure 8—figure supplement 2.** MUL1 knockdown increases Mfn2 levels in mouse cortical neurons.

DOI: 10.7554/eLife.01958.016
Figure 9. Models for how MUL1 interacts with PINK1/parkin. (A) Schematic depictions of how MUL1, PINK1, Parkin, and Mfn interact in the mitochondria. In mammalian cells, upon mitochondrial damage (CCCP or antimycin A treatment), PINK1 is stabilized onto the mitochondrial OM of damaged mitochondria, with its kinase domain facing the cytosol (Zhou et al., 2008). PINK1 recruits Parkin onto the OM, either through direct phosphorylation or indirect interaction with other proteins (not depicted here) (Jin and Youle, 2012). Parkin then ubiquitinates multiple substrates on the OM, including Mfn. MUL1, a mitochondrial OM-anchored ligase with its RNF domain facing the cytosol, also mediates ubiquitination of Mitofusin. (B) The PINK1/parkin pathway and MUL1 act in parallel to regulate mfn, and maintain mitochondrial function and tissue health. Reducing either PINK1/parkin or MUL1 leads to increased levels of Mfn. Significant elevation of Mfn leads to mitochondrial dysfunction and tissue damage, similar to what is observed in PINK1/parkin mutants. Loss of both PINK1/parkin and MUL1 leads to significantly higher Mfn levels, associated with severe mitochondrial dysfunction and tissue damage. OM: mitochondrial outer membrane; IM: mitochondrial inner membrane.

DOI: 10.7554/eLife.01958.017