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MitoFLUX-EM reveals damaged mitochondria are captured by pre-existing mitolysosomes during PINK1-dependent mitophagy in the heart

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Abstract

Damaged mitochondria are cleared by PINK1-Parkin mitophagy, but the pathway is incompletely understood *in vivo*. Here, we developed MitoFLUX-EM, combining electron microscopy with nanoscale ^{15}N isotope tracing to resolve the temporal sequence of mitophagy intermediates *in vivo*, and applied it to a cardiomyopathy mouse model (CHCHD10 S59L) with high PINK1-dependent mitophagy. Unexpectedly, we found that damaged mitochondria are directly captured by pre-existing mitolysosomes, resulting in multisegmented mitolysosomes that sequestered as many as 33 mitochondria at distinct stages of ultrastructural degradation. Autophagosomal-like membranes arising from the mitolysosomes preferentially engaged the intact outer membrane of damaged mitochondria and neither protein removal nor rupture of the outer membrane was required for mitophagy, contrary to prior models. Pink1 loss disrupted this pathway, causing accumulation of aggregates within mitochondria and reduced survival. These findings reveal that PINK1-dependent mitophagy proceeds through a novel form of microautophagy to safeguard cardiac mitochondrial quality *in vivo* and establish MitoFLUX-EM as a platform for resolving organelle turnover.

Introduction

Mitochondria produce ATP through oxidative reactions that can damage them. With age, these damaged mitochondria accumulate in post-mitotic cells and are believed to contribute to degenerative disorders such as Parkinson's disease and cardiomyopathy ¹. One pathway to limit this damage is PINK1-Parkin mitophagy, which degrades damaged mitochondria through a form of selective autophagy ("self-eating") ²⁻⁴. The pathway is formed by two proteins, the kinase PINK1 and the E3 ubiquitin ligase Parkin, that are mutated in the most common forms of recessive Parkinson's disease. These proteins are highly expressed in the central nervous system as well as striated muscles, including the heart.

The PINK1-Parkin pathway has been extensively characterized using rapidly dividing cancer cells lines overexpressing Parkin ⁵. This system allows for the robust and synchronous activation of mitophagy, making it a useful, if artificial, system to study PINK1-Parkin mitophagy. In this system, PINK1 initially senses mitochondrial damage ⁶⁻⁹, and activates the E3 ubiquitin ligase Parkin to mark the surface of the damaged mitochondria with ubiquitin ⁵. Ubiquitination of the outer mitochondrial membrane (OMM) by Parkin can cause its rupture through the removal and degradation of OMM proteins by VCP/P97 and the ubiquitin-proteasome system (UPS), respectively ¹⁰⁻¹³. Whether OMM rupture is required for mitophagy, however, has been unclear. On the one hand, it has been proposed that blocking OMM protein removal and OMM rupture (through proteasome or VCP inhibition) inhibits mitophagy ¹¹⁻¹⁴. This model was extended by the Levine lab to propose that OMM rupture is required for mitophagy because it exposes the inner mitochondrial membrane protein PHB2, which, in turn, serves as an essential LC3 receptor in PINK1-Parkin mitophagy ¹⁵. On the other hand, ubiquitin-binding autophagy adaptors, such as OPTN and NDP52, have been shown to be necessary and sufficient to promote mitophagy downstream of Parkin OMM ubiquitination ^{16,17}. Why these would require OMM rupture to facilitate mitophagy is not clear. Additionally, an early

report found autophagosomal membranes preferentially bind the intact OMM rather than the exposed IMM in cultured cells during PINK1-Parkin mitophagy¹⁰. What role OMM rupture plays during PINK1-Parkin mitophagy under physiologic conditions *in vivo* is similarly unresolved.

Once ubiquitinated, mitochondria in cultured cells are captured by a cup-shaped vesicle called an autophagophore (also known as, an isolation membrane) that expands along the mitochondrial surface to form a double-membraned vesicle called an autophagosome⁴. The autophagophore grows via bulk lipid transfer from adjacent ER (via ATG2) and ATG9 vesicles from the Golgi apparatus—but the autophagophore and the resulting autophagosome are isolated from other membrane-bound organelles. The autophagosome subsequently fuses with a lysosome to form an autolysosome, in which the captured mitochondrion and inner autophagosomal membrane are degraded by acid hydrolases. During mitophagy, these intermediates are often referred to as mitophagophores, mitophagosomes, and mitolysosomes (MLs), respectively, to indicate that mitochondria are the selective cargo undergoing autophagy.

The autophagosome/mitophagosome intermediate defines this canonical PINK1-Parkin mitophagy pathway as a form of macroautophagy^{4,10}. This contrasts with microautophagy, in which the autophagic cargo is directly captured by the lysosome, without passing through an isolated autophagosome¹⁸. In mammalian systems, microautophagy is believed to mediate the turnover of small cargos such as individual proteins or small fragments of organelles such as mitochondrial derived vesicles (MDVs). The term micromitophagy has been coined to describe the latter process in which the OMM derived MDVs (60 – 150 nm in diameter) bud off and are captured into late endosomes or primary lysosomes^{19–22}. Recently, it was shown that vesicles derived from the inner mitochondrial membrane can also be delivered to primary lysosomes through a microautophagy pathway²³. Additionally, mitochondria have been reported captured into Rab5-positive endosomes and then delivered to lysosomes²⁴. The microautophagy of whole organelles, by contrast, is

thought to be rare in mammals and has only been observed in the lysosome-related organelles of specialized phagocytic cells²⁵. Macroautophagy is believed to be the primary mode for autophagy of intact mitochondria in mammals *in vivo*, although detailed ultrastructural examination is lacking.

Examination of PINK1-Parkin mitophagy *in vivo* is more challenging than in cell culture systems, because it requires a stressor that predictably damages mitochondria and a method to measure mitophagy deep within the affected tissue. The latter has been helped immensely by the development of mitophagy flux reporters, such as mito-QC and mt-Keima^{26–28}, but these are limited by light resolution, which makes mitophagy intermediates difficult to define. Transmission electron microscopy (TEM) is the gold-standard for assessing mitophagy intermediates², as the 2 – 4 nm lateral resolution (in heavy metal-stained sections) can resolve membrane structures, particularly in serial section. However, TEM lacks a temporal component to understand the sequence and tempo of mitophagy *in vivo*.

Here, we address these limitations by developing an approach called MitoFlux-EM that combines TEM of mitophagy intermediates with ¹⁵N flux measurements by NanoSIMS.^{29–32} We use Mito-FLUX EM in a mitochondrial cardiomyopathy model (*Chchd10*^{S59L/+}; hereafter, C10 SL), known to have frequent mitophagy events secondary to mitochondrial protein misfolding^{33–35}. Surprisingly, we find that damaged mitochondria are captured into pre-existing MLs by a PINK1-mediated microautophagy pathway that is distinct from that used in cultured cells. Additionally, we find that OMM rupture likely inhibits rather than promotes mitophagy in the heart and further demonstrate that OMM protein removal is not required for the capture of damaged mitochondria by PINK1-Parkin mitophagy. Finally, we demonstrate that PINK1-Parkin mitophagy is required for the clearance of aggregate-laden mitochondria and for survival in this cardiomyopathy model.

Results

PINK1-Parkin pathway captures damaged mitochondria through a novel microautophagy pathway

Mitophagy in C10 SL hearts is observed in the early symptomatic phase (125 days)³³, suggesting it is an early response to mitochondrial damage from C10 protein misfolding. Altered heart tissue levels of PINK1 and Parkin imply their involvement in this pathway³³; however, a causal relationship has not yet been established.

We first tested whether mitophagy in C10 SL hearts depends on the general autophagy gene *Atg7*, by crossing C10 SL to a line with muscle-specific KO of *Atg7* (using the Ckmm-Cre driver)^{36,37}. ATG7 is required for attachment of LC3 to the expanding autophagophore membrane, which can be monitored as a gel shift from a cytosolic (LC3-I) to a membrane bound (LC3-II) form, following LC3 lipidation. As expected, LC3-II formation was largely blocked in *Atg7* KO hearts, and the constitutive autophagy substrate, p62, increased (Extended Data Fig. 1A). To measure mitophagy flux, mice were injected with adeno associated viral (AAV) particles expressing tandem acid-sensitive (GFP) and acid-resistant (mCherry) fluorescent proteins targeted to the mitochondrial matrix (MTS(Su9)-GFP-mCherry). This follows a similar principle as the mito-QC reporter: if the mitochondrial reporter protein is delivered to the acidic lumen of a lysosome, the GFP is quenched and degraded whereas the mCherry remains fluorescent, giving MLs a high mCherry/GFP signal²⁸. Mice were sacrificed three weeks after injection and delivery of mitochondria to LAMP1-positive lysosomes was tracked by the appearance of co-localized mCherry+/GFP- puncta. Mitophagic flux was increased in the hearts of C10 SL mice and was substantially reduced but not eliminated when combined with *Atg7* KO (Figure 1A and B and Extended Data Fig. 1B). This is consistent with prior observations that the LC3 conjugation system facilitates but is not strictly required for mitophagy in the heart^{36,38}.

We next tested whether PINK1-Parkin mitophagy pathway is activated in C10 SL mice by measuring levels of the specific product of PINK1 phosphorylation, pS65-ubiquitin, using a sensitive ELISA³⁹. Consistent with PINK1-Parkin activation, pS65-Ub was highly elevated in C10 SL hearts and required PINK1 expression (Figure 1C and D). To assess whether Pink1 is required for mitophagy, we examined tissue from the mouse hearts by TEM. Pink1 KO strongly reduced (but did not eliminate) the characteristic mitophagosomal structures (MLs and apparent mitophagosomes) in the tissue (Figure 1E and F). This demonstrates that Pink1 strongly promotes mitophagy in the C10 SL hearts.

Having established that there is robust activation of PINK1 mitophagy in the heart, we next characterized the mitophagy intermediates that had formed *in vivo* by light microscopy and serial section electron microscopy. Close observation of the fluorescent mitophagy reporter revealed that MLs in the heart have a complex structure (Figure 1G). Several red puncta, presumably partially degraded mitochondria, co-localized with large structures that were immunopositive for the endolysosomal marker LAMP1. The high mCherry (acid-resistant) and low GFP (acid-sensitive) signal from the tandem reporter strongly suggests these mitochondrial fragments were in an acidic compartment, as expected for a ML.

To investigate further, we performed serial section TEM of samples from C10 SL mice (Figure 1H and Extended Data Fig. 2A and B). This confirmed the complex structure of MLs: they were separated into multiple compartments by several layers of electron-dense membranes; each compartment typically containing a single mitochondrial remnant. The sequestered mitochondria within a single ML often exhibited different grades of ultrastructural degradation, ranging from fully intact inner and outer mitochondrial membranes with normal appearing cristae (grade 1) to swelling of the cristae but intact inner and outer membranes (grade 2) to loss of all membranes

except for an occasional detected cristae (grade 3) to a completely granular appearance with no detected cristae (grade 4).

These structures observed *in vivo* were notably different than what had been reported in cultured cells¹⁰. This prompted us to directly compare PINK1-mitophagy in the C10 SL hearts with PINK1-mitophagy in HeLa cells expressing exogenous mCh-Parkin (HeLa^{mCh-Parkin}), following CCCP treatment for 2 hrs (Figure 1I - L). As expected, Parkin activation in this condition lead to a substantial increase in large YFP-LC3 positive autophagophores and autophagosomes, quantified by an automated detection algorithm (Figure 1I and J and Extended Data Fig. 1C). By TEM, MLs identified in HeLa cells had a single boundary membrane and an electron dense matrix, suggesting recent fusion between an autophagosome and a primary lysosome. In some rare cases, the electron dense inner autophagosomal membrane was visible around the mitochondrion within the lysosome (Extended Data Fig. 2E). These were distinguished from MLs in the heart by having a single limiting membrane, the absence of segmentation by limiting membranes into multiple compartments, and the residual appearance of the primary lysosome (Figure 1L and Extended Data Fig. 2C).

We next compared the early intermediates in the two mitophagy pathways by serial section EM (Figure 1H and L and Extended Data Fig. 2A - C). In HeLa cells, isolated autophagophores and autophagosomes, which had distinct electron dense double membranes, were easily identified (Figure 1L and Extended Data Fig. 2C). As expected, their autophagosomal membranes were non-contiguous with other organelles, but they formed contact sites with the mitochondria being sequestered and the ER, which is a source of phospholipid for membrane expansion. In the heart, mitochondria in the process of being sequestered could also be readily identified in serial sections (Figure 1H and Extended Data Fig. 2A and B). These were contacted by electron dense autophagosomal-like (APL) double membranes, which had a similar ultrastructural appearance to

autophagosomal membranes in cultured cells. Surprisingly, however, these APL membranes were not isolated from other organelles but instead were directly contiguous with the membranes of the ML. The APL membranes appeared to arise as folds from the surface of the segmented MLs that then expanded in close apposition to the mitochondrial surface. The multi-compartmented structure of the MLs further suggested damaged mitochondria are captured sequentially. Together, this suggests that PINK1-Parkin mitophagy proceeds through a protrusion-type microautophagy pathway in the heart, in contrast to the canonical macro-autophagy pathway observed in cultured cells.

Developing MitoFLUX-EM to track the in vivo lifecycle of mitochondria following damage

The ultrastructural appearance of mitophagy intermediates in the C10 SL heart suggests a staged progression of mitophagy *in vivo*, in which mitochondria are initially captured by APL membranes that extend from a pre-existing ML. However, EM alone lacks temporal information to order events along the mitophagy pathway.

To address this challenge, we developed a new approach for analyzing mitophagy *in vivo* called MitoFLUX-EM (Figure 2A). MitoFLUX-EM combines the ultrastructural resolution of TEM with temporal information provided by nanoscale measurements of ^{15}N flux. ^{15}N flux primarily reflects bulk protein turnover, as ~97.5% of heart nitrogen is in protein. This allows the temporal ordering of intermediates by their relative protein ages. To achieve direct correlation, ultrathin sections on a formvar slot grid are first imaged by TEM. The formvar is then transferred with the section to a Silicon chip for NanoSIMS 50L analysis, which provides information on protein turnover via $^{15}\text{N}/^{14}\text{N}$ ratio measurements (detected as $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N}$) as well as histological information from the cellular

distribution of common elements like phosphorus (^{31}P) and sulfur (^{32}S). MitoFLUX-EM can also be performed on serial sections, enabling, for the first time, volumetric correlated TEM-NanoSIMS.

We applied this technique to C10 WT and SL mice. They were pulsed for 2 weeks through food pellets in which the sole nitrogen source was ^{15}N (^{15}N , 98%). This period is roughly equivalent to the half-life of cardiac mitochondria in mice (estimated to be 17.2 days⁴⁰). For a set of animals, the ^{15}N label was chased for 1, 2, or 4 weeks by switching back to standard ^{14}N chow. Mitochondria and lysosomes (as well as the contents of individual ML compartments) were systematically segmented from aligned TEM and NanoSIMS images. The submicron registration of the NanoSIMS and EM images allowed confident ^{15}N flux measurement of 5,575 organelles (including unsequestered mitochondria, lysosomes, and mitochondria sequestered in MLs) in the 2D dataset and 77 organelles in the 3D dataset. Additionally, m-line measurements were sampled from several sarcomeres in each cell to compare turnover of the myofibrillar proteins to mitochondria and lysosomes. ^{15}N fractional incorporation was determined from the median pixel value within each organelle, making measurements robust to edge pixels and facilitating comparison between organelles of different sizes. The median relative Poisson counting error of 3.18% across organelles the dataset (Extended Data Fig. 3A). Organelles generally showed uniform ^{15}N fractional incorporation as reflected in the relatively low coefficient of variation among pixels within the ROI (median = 4.17%) (Extended Data Fig. 3B). Measurements were also robust to the contour of the ROI, as shown by the strong correlation between the initial ROIs and ROIs after eroding the edge by 1 pixel (Extended Data Fig. 3C and D).

C10 SL protein misfolding induces increased protein turnover in the heart in vivo

We first compared protein turnover in the C10 WT vs. SL cardiomyocytes. At steady-state, organelles with faster protein turnover will incorporate more ^{15}N during the pulse, while those with slower protein turnover will incorporate less. Consistently, myofibrils, known to have faster protein turnover, had a higher fractional ^{15}N incorporation (i.e., $^{15}\text{N}/(^{14}\text{N}+^{15}\text{N})$) than adjacent mitochondria, known to have slower protein turnover, in both genotypes ⁴¹. Unexpectedly, however, the protein turnover rate of both sarcomeres and mitochondria was substantially higher in the C10 SL cardiomyocytes, evidenced by greater ^{15}N fractional incorporation (Figure 2B - F). This was surprising as we and others have previously shown that the integrated stress response (ISR) is strongly activated by the mitochondrial stress in the C10 SL hearts, through the OMA1-DELE1 pathway ^{35,42}. At least in cultured cells exposed to acute stress, the ISR strongly slows protein turnover ⁴³, although protein synthesis may resume if the stress is prolonged ⁴⁴. To confirm protein turnover was elevated in C10 SL hearts with an orthogonal approach, groups of four mice of each genotype were fed heavy lysine for 2 weeks, and protein lysates from the heart were analyzed by LC-MS/MS based proteomics (Figure 2G and H). As in the NanoSIMS analysis, heavy amino acid incorporation was similar among individuals of the same genotype but was substantially higher in C10 SL vs. WT mice, consistent with faster protein turnover. Together this suggests that C10 SL protein misfolding induces a state of increased protein turnover in cardiomyocytes, even in the setting of chronic ISR activation.

PINK1-Parkin activation is not inhibited by chronic co-activation of the integrated stress responses in vivo

We next considered how co-activation of the OMA1-DELE1 ISR may shape the PINK1-Parkin response. Studies in cultured cells have highlighted that new PINK1 synthesis and import arrest is

required for PINK1-Parkin activation ⁶, and that this may be decreased if the DELE1-ISR is co-activated ^{45,46}. To test this relationship *in vivo*, we analyzed C10 SL heart samples with or without *Dele1* (*Dele1*^{+/-} vs. *Dele1*^{-/-}). These samples were generated for a previous publication, in which *Dele1* KO was found to mildly decrease mitophagy intermediates quantified from electron micrographs ³⁵. We first confirmed that the ISR is activated in these samples by measuring proteins that are known transcriptional targets of the ISR by proteomics (Extended Data Fig. 4A - C). We then measured PINK1-Parkin activity in the samples by monitoring levels of the PINK1 product pS65-Ub. We reasoned that if the DELE1-ISR is limiting for PINK1 activity, then PINK1 activity should be higher in the C10 SL; *Dele1*^{-/-} samples. Contrary to this prediction, PINK1 activity was not significantly different (and in fact trended lower) in C10 SL; *Dele1*^{-/-} hearts (Extended Data Fig. 4D), demonstrating that the co-activated DELE1 ISR is not limiting for PINK1-Parkin activation *in vivo*. Notably, the reduced pS65-Ub correlated with the reduced number of mitophagy intermediates observed previously in C10 SL; *Dele1*^{-/-} samples ³⁵. We also tested previously published samples from a second model of mitochondrial cardiomyopathy due to CHCHD10 protein misfolding (C10 G58R with or without *Dele1*) and observed a similar correlation between the number of mitophagy intermediates (measured by TEM in our prior publication ³⁵) and pS65-Ub levels across the samples. In this case, both mitophagy and pS65-Ub were increased in the absence of *Dele1* (Extended Data Fig. 4E). These data demonstrate a correlation between mitophagy intermediates and PINK1-Parkin activation. Additionally, they demonstrate that the DELE1-ISR does not consistently suppress PINK1 activation in the heart *in vivo*.

Characterization of lysosomes in the heart by MitoFlux-EM

We next focused on systematically evaluating each intermediate within the mitophagy pathway, starting with lysosomes. As expected, primary lysosomes (PLs) and MLs could be seen in most fields from C10 SL mice but were infrequent or absent in fields from WT mice (Figure 3A and B). Primary lysosomes are reported to have at least two distinct morphological appearances by TEM: a single membrane bound structure with an electron-dense lumen containing granules of heterogenous size (hereafter, PL-speckled), and a structure with prominent membrane whorls but limited luminal space (hereafter, PL-whorled)⁴⁷. These two morphological types were frequent in the C10 SL hearts and had distinct elemental compositions (Figure 3C - F). PL-speckled were sulfur-rich (Figure 3C and E), possibly due to their storage of the sulfur-containing amino acids cysteine and cystine in addition to their higher protein concentration⁴⁸. By contrast, PL-whorled were phosphorus-rich, likely due to their high membrane content and lower protein concentration. Notably, PL-speckled had markedly higher ¹⁵N fractional incorporation than PL-whorled, suggesting a ¹⁵N flux (Figure 3F). This is consistent with the PL-speckled lysosomes being the primary source of hydrolytic enzymes, a group of fast turnover proteins relative to mitochondrial proteins, as measured by LC-MS/MS (Figure 2H).

Lysosomes with speckled and whorled morphologies could be found in isolation, but, surprisingly, could also be found as components of complex MLs and mitophagophore-like structures (Figure 3D). When the whorl-type lysosomes were found connected to MLs their membranes were typically continuous with the ML membrane that, in turn, formed distinct compartments to contain mitochondria in the process of degradation. Consistently, the membranous component of the ML was compositionally similar to PL-whorled and similarly had a low ¹⁵N fractional incorporation. Together these findings suggest that the two morphologically and elementally distinct primary lysosomes have distinct functions in the heart. Those with a speckled morphology and recently incorporated nitrogen are likely the source of hydrolytic enzymes. By

contrast, those with a whorled morphology and slower nitrogen turnover may be a source of membrane to initiate or expand MLs or, alternatively, may represent redundant membrane generated in the expansion of the ML membranes.

APL membranes have higher affinity for the OMM than the IMM of damaged mitochondria

Next, we focused on the ultrastructure of mitochondria undergoing mitophagy *in vivo*. Studies in cultured cells have proposed that OMM protein removal and OMM rupture is required for PINK1-Parkin mitophagy^{11,13–15}, but the relationship between OMM rupture and mitophagy *in vivo* is largely unknown.

We first considered mitochondria in the process of being captured by an APL membrane (which we referred to as mitophagophores) (Figure 4A - C). As noted above, the APL membranes were distinguished by their high electrodensity, which exceeded that of neighboring mitochondrial and ER membranes (Figure 4A). In roughly half of cases, these were connected to MLs in the profiles (similar to what was observed by serial sections above), while in the other half they were apparently isolated from MLs in the section (Extended Data Fig. 5A). For all cases in which the membrane was cleanly cross-sectioned, the APL membrane closely tracked the intact OMM, with a median distance of 15.3 nm between the two membranes (Figure 4C). In the vast majority of cases, the OMM was intact (Figure 4B). We next examined mitochondria within MLs that had cristae with normal (grade 1) or mild (grade 2) deformation, suggesting recent capture (Figure 4B). Consistent with the analysis of mitophagophores, all but one had a mostly intact OMM. Together this suggests that the APL membrane can bind and expand along the OMM surface during PINK1-Parkin mitophagy *in vivo*.

Mitochondria with complete OMM rupture (hereafter, “mitoplasts”) were also frequently observed in the sample, as reported previously³⁵. Notably, these were never found within mitophagosomes or MLs (Figure 4B and E). Rarely, we observed partial OMM rupture in a mitochondrion that was partially wrapped by an APL membrane. In these cases, contact between the APL membrane and the mitochondrion was observed exclusively where the OMM was intact. To further assess this rare population of partially wrapped and ruptured mitochondria, we examined these events among images and samples from C10 SL; *Dele1*^{+/-} hearts that were originally generated for a previous publication³⁵ (Figure 4D and E and Extended Data Fig. 5B). Notably, in each case the APL membrane extended along the intact OMM up to the point of rupture but did not directly contact the exposed IMM (Figure 4D and E and Extended Data Fig. 5B). This suggests that the APL membranes have a substantially higher affinity for the OMM than the IMM during PINK1-Parkin mitophagy in the heart. These intermediates may be resolved by fission of the ruptured portion, as in many cases the mitochondrion was constricted near the rim of the APL membrane with extrusion of the ruptured portion of the mitochondrion (Figure 4E). Completion of fission in this model would allow capture of a portion of the damaged mitochondrion but would produce a mitoplast as a byproduct.

Our observation that the APL preferentially binds the OMM would predict that OMM proteins are the predominate *in vivo* targets of Parkin activated by PINK1. To test this prediction, we performed diGly ubiquitin proteomics on heart samples from C10 SL mice with one or no copies of PINK1. Consistent with this prediction, PINK1-dependent ubiquitinated peptides were predominately derived from OMM localized proteins (Figure 4F and G and Extended Data Fig. 5C). These included well-defined Parkin substrates identified in cultured cells (including VDAC1-3, MFN1, and C1SD1) as well as some targets that are specific mitochondria in striated muscle (e.g., CPT1B). Interestingly, ubiquitinated IMS and IMM proteins were also identified as increased in C10

SL; Pink1^{+/-} mice vs C10 WT; Pink1^{+/-} hearts, but did not show the same PINK1 dependence. Notably, this subset of ubiquitinated IMS/IMM proteins also had reduced total protein abundance in C10 SL vs. C10 WT hearts, in contrast to OMM proteins, suggesting that they are selectively degraded independently of the PINK1-Parkin pathway (Extended Data Fig. 6A). Together this demonstrates that PINK1-Parkin predominately ubiquitinates OMM proteins on damaged mitochondria and validates several PINK1-Parkin pathway targets for the first time *in vivo*.

We next examined the temporal dynamics of mitoplasts by tracking their relative ¹⁵N incorporation in the pulse-chase dataset. While mitophagophores and partially ruptured mitochondria had a similar protein age as intact mitochondria within the tissue, the protein age of mitoplasts was highly variable (Figure 4H - J). As a group, mitoplasts contained significantly older protein than intact mitochondria (Figure 4K). Additionally, among the group were extreme population outliers (Figure 4K and L). This included one with a median incorporation of 0.142 in its center – a level similar to the average intact mitochondrion following a 2-week chase – and less than all mitochondria in the pulsed dataset (Figure 4J).

Consistently, mitoplasts observed in the 2-week chase dataset also showed a highly variable median incorporation but had higher ¹⁵N incorporation relative to the intact mitochondrial population (Figure 4L and M). Together this demonstrates that loss of the OMM slows the rate of protein turnover within mitochondria. This is plausibly due to block of mitochondrial import required for the biogenesis of ~99% of mitochondrial proteins⁴⁹. As these mitoplasts are not efficiently removed by mitophagy, we speculate their lifecycle may end with spontaneous rupture of the IMM.

OMM protein removal is not required for capture of damaged mitochondria in autophagosomes

Our observations *in vivo* were surprising given some prior reports in cultured cells, which suggest that PINK1-Parkin mitophagy requires both the removal of OMM proteins and/or the consequent OMM rupture^{10–13,15,50}.

This prompted us to evaluate the relationships among OMM protein removal, autophagic capture of mitochondria, and OMM rupture at an early time point (2 hrs) following Parkin activation. For this purpose, we developed a stable HeLa cell line that allows the simultaneous assessment of OMM protein removal (by tracking endogenously tagged MFN2-Halo) and capture by autophagosomes (labelled by YFP-LC3) following recruitment of exogenously expressed mCherry-Parkin (Figure 5A - D and Extended Data Fig. 4C - F). As expected, MFN2-Halo was degraded to ~50% following Parkin activation (Figure 5A, B, and D). YFP-LC3 positive puncta formed around mitochondria with low MFN2-Halo expression, as expected (Figure 5D and Extended Data Fig. 6B - E).

Next, we tested whether OMM protein removal is required for autophagic capture of mitochondria. Notably, blocking the removal of MFN2-Halo with VCP inhibitor (VCPi) NMS-873 increased rather than decreased the number of mitophagosomes, demonstrating that OMM protein removal is not required for capture of mitochondria by autophagy (Figure 5D and Extended Data Fig. 6D). The modest increase in the number of autophagic structures with VCPi is consistent with prior reports⁵¹. To investigate events downstream of autophagic capture of mitochondria, we monitored the appearance of mitophagosomes using the mitophagy flux reporter mt-Keima²⁶ (Figure 5E). VCPi slightly decreased the appearance of MLs, consistent with a known downstream requirement for VCP in the autophagy-lysosomal pathway⁵¹, although the difference did not reach significance. Under these conditions, a VCP-dependent decrease in MFN2-Halo could also be detected by flow cytometry (Figure 5E). Together these findings demonstrate that OMM protein removal by the VCP-

UPS pathway is not required for the capture of mitochondria by autophagosomes during PINK1-Parkin mitophagy, contrary to conclusions of prior reports^{11–13,50}.

We next evaluated the ultrastructure of autophagosomal structures formed in the HeLa^{mCh-Parkin} cells treated with CCCP for 2 hrs, using correlated light-electron microscopy (Figure 5F - H). YFP-LC3 rings and crescents directly correlated with the expected ultrastructural appearance of mitophagophores and mitophagosomes, respectively (Figure 5F and G). Notably, the double membranes of the autophagosomal structures were generally electrodense in comparison neighboring mitochondrial and ER membranes, similar to what we observed for the APL membranes in C10 SL mouse hearts (Figures 1, 4A, and 5H, and Extended Fig. 2). Among the 80 autophagy events imaged across two samples (38 mitophagophores and 42 mitophagosomes), 78 had a fully intact OMM (Figure 5I). The two events with clear OMM rupture were partially wrapped, and the mitophagophore membrane contacted only the intact OMM. This is consistent with an early prior report that found autophagosomal membranes preferentially bind the OMM during PINK1-Parkin mitophagy in cultured cells¹⁰, but is at odds with other models for PINK1-Parkin mitophagy that require OMM rupture for autophagosomal engagement¹⁵. Together, these findings are consistent with our observations *in vivo*: PINK1-Parkin mitophagy is preferentially initiated around mitochondria with intact OMMs. Additionally, our findings demonstrate that VCP can remove MFN2-Halo from the OMM without causing OMM rupture.

Tracking mitophagy progression by MitoFlux-EM

We next considered the degradation of mitochondria along the autophagy-lysosomal pathway by MitoFLUX-EM. As in the serial sections described above, mitochondria with different ultrastructural grades of degradation (1 – 4) could be identified within compartments bounded by the electrodense

membranes of the ML (Figure 6A - C). The number of membranes surrounding the contents increased with progression of grades, from the double membrane of the initial mitophagophore to several layers of double membranes around some grade 3 and 4 contents (Figure 6A). Additionally, as the mitochondria remnants progressed to more advanced grades of degradation, their area decreased (Figure 6C). The relative concentrations of nitrogen and sulfur per pixel also decreased while the relative concentration of phosphorus per pixel increased. This change in elemental composition likely reflects a progressive decrease in protein concentration and increase in phospholipid concentration within a contracting degradative compartment.

We next examined the protein age of mitochondria at each stage of mitophagy. The complex ML structures in C10 SL hearts have previously been interpreted as stalled mitophagy intermediates³³. If this were true, the aging sequestered mitochondria should be enriched with the “older” ¹⁴N due to little metabolic exchange with ¹⁵N during the pulse. In contrast to this prediction, sequestered mitochondria in the initial stages of mitophagy (up to grade 2) had a similar protein age as unsequestered mitochondria (Figure 6B and D), distinguishing them from the older mitoplasts and the younger PL-speckled observed in the same samples (Figure 4M and 6E, respectively). This suggests that mitochondria pass through the initial stages of mitophagy quickly relative to a mitochondrial half-life within the ML. Consistently, late-stage mitophagy intermediates (3 and 4) were more abundant in the tissue than early-stage mitophagy intermediates (MP, stage 1, stage 2) (Extended Data Fig. 7A), a steady-state that would be explained by the later stages having a longer duration.

Consistent with their older age, median ¹⁵N incorporation of late-stage ML contents (3 and 4) after the pulse was variable but significantly less than that of unsequestered mitochondria and early-stage ML contents (Figure 6B). Although lower than unsequestered mitochondria after the pulse, the median value of late state ML contents was still substantially higher than that of

unsequestered mitochondria after a 1-week chase (Figure 6D). Assuming all ^{15}N is mitochondrial-derived, this would imply progression to these late stages takes less than 1 week on average. This assumption is complicated, however, by potential mixing of mitochondrial contents with lysosomal contents after mitochondrial membrane breakdown. Some of these lysosomal contents have lower ^{15}N incorporation (e.g., membranes of the ML) but others have higher ^{15}N incorporation (e.g., hydrolytic enzymes), as noted above. Indeed, a subset of stage 3 compartments had a higher median incorporation than early-stage intermediates (dashed box in Figure 6B) and appeared as “hot spots” within the ML (Extended Data Fig. 7B and C, yellow arrows), suggesting admixture of younger lysosomal and older mitochondrial contents. Together these findings demonstrate that ultrastructural degradation of mitochondria within the ML correlates with their median protein age and a change in their elemental composition. These findings additionally suggest that transit through the initial stages of mitophagy is swift relative to the protein half-life of mitochondria in the heart. This is contrary to a prior interpretation based on ultrastructure assessment alone, which posited mitophagy intermediates in C10 SL hearts are the result of stalled mitophagic flux³³.

Damaged mitochondria are captured by pre-existing MLs in the heart

As noted above, most mitochondria undergoing degradation were part of a weblike ML that had multiple compartments separated by the electron-dense ML membranes (Figure 6F and G). This suggested that new mitochondria captured by APLs were sequestered sequentially into a ML. Consistent with this interpretation we found that mitochondrial remnants in separate compartments within single MLs had distinct protein ages (Figure 6H). Together, this suggests that individual mitochondria are incorporated into the ML through sequential mitophagy events and that degradation occurs asynchronously in each compartment.

The MitoFLUX-EM analysis up to this point was limited to 2D profiles of ML, which can be complicated by connections between apparently separate objects in the unexamined z-plane, as was apparent in the serial sections above (Figure 1H and Extended Data Fig. 2A and B). To address this limitation, we developed a new procedure for volumetric correlative TEM- NanoSIMS analysis, using serial sections (Extended Data Figure 8). Two MLs and surrounding mitochondria that were imaged and segmented from 22 serial sections ($z = \sim 70$ nm) (Figure 7A - F). As the lateral resolution of NanoSIMS was ~ 70 nm, this allowed NanoSIMS measurement of 3D organelles with an approximately isometric voxel. ML1, containing nine compartments, was captured at high EM resolution (0.9 nm/px), whereas ML2, containing 33 compartments, was captured at a lower EM resolution (3 nm/px) (Figure 7A). Each compartment of the two MLs contained a single mitochondrion. As in the 2D sections, intact mitochondria (grade 1 or 2) inside and outside the ML had a similar median ^{15}N incorporation, whereas degraded mitochondria (grade 3 or 4) within the ML had a decreased median ^{15}N incorporation (Figure 7B and Extended Data Fig. 9A - C). Likewise, each ML contained mitochondria at different ultrastructural stages of degradation and different levels of median ^{15}N fractional incorporation.

The volume also clearly showed the relationship between ML1 and surrounding organelles. In serial sections, the ML membranes were contiguous with a PL-whorled structure, from which the membranes appeared to arise (Figure 7C and D). There was also a close relationship between the ML and ER, which may be source of phospholipids, and the Golgi apparatus, which delivers lysosomal hydrolases to lysosomes through clathrin-coated vesicles (Figure 7E). Similar relationships were seen between ML2 and neighboring ER and Golgi but interpretation was limited by the lower EM resolution.

The ultrastructure of mitochondria in each compartment of ML1 was almost always uniform throughout the stack (either grade 1 - 2 or grade 3 - 4), consistent with our observations in the 2D

profiles. There was one notable exception, however: a mitochondrion, in the process of microautophagy, had a region inside the ML that was partially degraded (grade 3; blue dotted outline in 7C and D) and a portion still outside the ML with intact cristae (grade 1; white dotted outline in 7C and D). This event differed from those in serial sections described above in that the mitochondrion was captured by enfolding of the ML membrane around the mitochondrion rather than protrusion of APL membranes. Notably, the ^{15}N incorporation differed between the regions within and without the ML. The enclosed portion had a ^{15}N hot spot co-localizing with the region of ultrastructural degradation (arrowhead in 7D), suggesting newer protein in this area than the rest of mitochondrion. This hot spot could be followed in serial sections that were independently measured by NanoSIMS. Similar hot spots were noted in neighboring compartments, where they also co-localized with areas that had the ultrastructural appearance of degradation (Figure 7D - F). Between the enclosed and exterior portions, the mitochondrion was constricted by the edge of the ML and a bundle of ER- and T-tubules (Figure 7C and E), suggesting the two portions may be in the process of division by fission. This would allow the portion inside the ML to be captured, while releasing an unsequestered mitochondrion into the sarcoplasm. Together, our findings demonstrate that damaged mitochondria are incorporated into pre-existing MLs.

PINK1-dependent mitophagy is critical for survival and limits accumulation of intramitochondrial protein aggregates

Next, we tested whether this microautophagy damage response is protective or harmful, by assessing the dependence of survival on ATG7, PINK1, and Parkin expression. Consistent with the response being strongly protective, C10 SL; *Pink1*^{-/-} mice died substantially earlier than their C10 SL; *Pink1*^{+/-} littermates (median survival 139 vs. 371 days) (Figure 8A). C10 SL mice with *Prkn* KO

from the heart had a similar phenotype (median survival 140 days), demonstrating that this response is Parkin-dependent (Extended Data Fig. 10A). Finally, this early lethality was phenocopied by *Atg7* KO in muscle (median survival of 179 days) (Figure 8B), demonstrating that it is most likely due to the promotion of mitophagy by PINK1-Parkin and not separate PINK1-Parkin function. Together this suggests that PINK1-Parkin mitophagy is strongly protective in the heart. In the absence of PINK1-Parkin mitophagy, mice die shortly after the start of the early symptomatic phase, when mitochondrial failure is first observed^{34,42,52}.

To assess the effect of PINK1 absence in the C10 SL hearts, we re-examined the TEM images for changes in the ultrastructure of mitochondria that could not be captured by PINK1-dependent mitophagy. Strikingly, we observed a distinctive population of mitochondria with grain-like protein aggregates in the IMS (Figure 8C - E). These events were extremely rare in the presence of PINK1 (Figure 8C and Extended Data Fig. 10B and C). These aggregates accumulated both within an expanded cristae lumen, as well as an expanded IMS between the OMM and boundary IMM (Figure 8E). Serial sections showed that the aggregates were usually contained within intact mitochondria but occasionally were present in MDVs bounded by a single membrane (Figure 8E, bottom). These aggregate-laden MDVs were observed in the TEM fields that had the highest burden of aggregate-containing mitochondria and plausibly formed by the budding of IMS aggregates adjacent to the OMM. On review of images in the pulsed MitoFLUX-EM dataset from C10 SL; *Pink1*^{+/+} animals, a single aggregate-laden mitochondrion was found (Figure 8F and G). This mitochondrion, which was not captured in the initial segmentation of mitochondria in the sample, had a lower ¹⁵N fractional incorporation (0.058) than all mitochondria in the dataset, consistent with its being a very old structure. Indeed, its ¹⁵N fractional incorporation was below that of the extracellular space (0.096), suggesting that it failed to incorporate any new protein during the 2-week pulse. Together,

these findings suggest that PINK1-Parkin mitophagy protects the hearts of C10 SL mice by selectively clearing mitochondria with increased aggregate burden.

Discussion

Although the PINK1-Parkin pathway is well characterized in cultured cells, the mechanisms mediating mitophagy *in vivo* are less well defined. Here, we identify an unexpected PINK1-Parkin micro-autophagy pathway used by cardiomyocytes *in vivo* that degrades intact mitochondria within autolysosomes/mitolysosomes (Figure 8H).

In this microautophagy pathway, damaged mitochondria are sequentially captured into pre-existing MLs, in which multiple mitochondria are degraded in separate compartments asynchronously. In many cases, capture was mediated through APL membrane protrusions that were contiguous with the ML membranes. These APL membranes have a similar electrodense appearance to the membranes of both the cardiac ML and autophagosomes in cultured cells. Efficient capture of mitochondria into MLs depended on ATG7, suggesting that the LC3-conjugation system may be involved in stabilizing these membranes and/or contact sites with the damaged mitochondrion, although future work will be needed to investigate this further. This mechanism superficially resembles that used by some species of yeast to capture peroxisomes into the vacuole, which also depends on the LC3-conjugation system for efficient micro-autophagy of large organelles^{53,54}. In addition to protrusion, the ML membrane was also observed to capture damaged mitochondria by an enfolding mechanism, in which a large portion of a mitochondrion was enveloped into the ML to form a new compartment.

We envision microautophagy by either mechanism, protrusion or enfolding, may be initiated by a contact site between the damaged mitochondrion and the membrane of the ML (Figure 8H).

This would be followed by either expansion of an ML membrane fold or enfolding of the ML membrane around the mitochondrion, depending on the local geometry. Adaptors containing both ubiquitin-binding and LIR domains, such as Optineurin and NDP52, may help establish the initial contact sites between the ubiquitinated mitochondrion and LC3 on a pre-existing ML membrane and then guide expansion of the membrane along the OMM until it is fully captured by the ML. These MLs may originate with an initial macroautophagy event, which subsequently grows through the sequential capture of damaged mitochondria by microautophagy. These active, mitophagosomal structures may be augmented by fusion with primary lysosomes, which would explain the presence of primary lysosomes as apparent components of these MLs. This microautophagy mechanism may be more efficient for supporting high and sustained mitophagic flux in long-lived cells, in comparison to successive cycles of mitophagosome formation, lysosomal fusion, and lysosomal reformation required to sustain macroautophagy. Additionally, this microautophagy pathway may be particularly adaptive in spatially constrained cells, such as cardiomyocytes. Notably, this microautophagy pathway is distinct from what has been called micromitophagy previously to specifically describe the degradation of mitochondrial derived vesicles (60 – 150 nm in diameter) in late endosomes/multivesicular bodies or lysosomes^{19–22,55}. We suggest that the definition of micromitophagy should be broadened to encompass selective microautophagy of mitochondria in all forms and the term mitolysosome-mediated micromitophagy (MiMM) be used for the specific process described here.

Although the focus of this work has been on mitophagy, it is possible that analogous microautophagy mechanisms are used for other forms of selective autophagy under conditions of high autophagy flux and/or severe spatial constraint. For instance, complex autophagy-related structures called inclusion bodies are frequently reported in skeletal muscle under proteostatic stress, including in a sporadic condition known as inclusion body myositis⁵⁶. Additionally, complex

autophagosomal structures are frequently observed in neurons in Alzheimer's disease and were recently reported within neuronal Lewy body-like protein aggregates in a cellular model of Parkinson's disease^{57,58}, both likely in response to pathogenic protein misfolding. These have been traditionally viewed as stalled autophagy intermediates arising from blocks in autophagy-lysosomal flux, similar to how mitophagy intermediates were previously interpreted in the C10 SL model³³. The present work suggests an alternative possibility: namely, that these large, complex structures may be active degradative compartments that have adapted to chronic autophagy flux. The MitoFLUX-EM method introduced here may help resolve these questions by determining whether ultrastructurally-defined intermediates are metabolically active (e.g., able to exchange ¹⁵N) or inert.

Surprisingly, we found that most mitochondria captured by PINK1-Parkin mitophagy under physiological conditions *in vivo* had an intact OMM and that areas of OMM rupture were not engaged by autophagosomal membranes. Consistently, we found that the PINK1-Parkin pathway preferentially ubiquitinates OMM proteins *in vivo*. This prompted us to investigate links among OMM protein removal following Parkin ubiquitination, OMM rupture, and capture by an autophagosome, using a new reporter cell line that allowed simultaneous assessment of OMM protein removal and capture of mitochondria by autophagosomes. We established that OMM protein removal was not required for autophagosomal capture and further demonstrated that most of the mitochondria that are captured by autophagosomes in cultured cells have an intact OMM. This is consistent with an early report from the Mizushima lab that reached the same conclusion¹⁰. They observed in cultured cells that the autophagosomal membrane binds the OMM but not the exposed IMM following rupture, similar to our *in vivo* findings in the mouse heart. They also observed that OMM rupture is rare after 6 hrs but common by 12 hours, following PINK1-Parkin activation in mouse embryonic fibroblasts¹⁰. This likely explains why we did not observe substantial OMM rupture 2 hrs after of

PINK1-Parkin activation, despite robust mitophagosome and mitolysosomal formation. OMM rupture may occur only after prolonged VCP and UPS activity on the mitochondrial surface.

These findings are seemingly incompatible with an often cited model proposed by the Levine lab ¹⁵. This model posits that OMM rupture is required in PINK1-Parkin mitophagy to expose PHB2 for LC3 binding. Also seemingly incompatible with the Levine model, we recently found that knockdown of PHB2 increases both PINK1-Parkin dependent and independent mitophagy, measured with the mt-Keima reporter ⁵⁹. Instead, these findings are consistent with the ubiquitin-binding adaptor model. In this model, OMM proteins heavily ubiquitinated by Parkin attract ubiquitin-binding autophagy adaptors like OPTN and NDP52, which initiate mitophagosome formation. These findings also suggest a reinterpretation of prior findings from several labs, which showed that VCP-UPS is required for both OMM protein removal and the lysosomal degradation of all cellular mitochondria following sustained uncoupling (for ~24 hrs) ¹⁰⁻¹³. These observations are likely true but unrelated, as we find that blocking OMM protein removal by VCP inhibition does not prevent the formation of mitophagosomes. Mitochondrial clearance is likely slowed by VCP-UPS inhibition at a step after mitophagosome formation, as demonstrated for starvation induced autophagy ⁵¹, and not because MFN2 or another OMM protein needs to be removed by the VCP-UPS pathway for the formation of a mitophagosome, as has been previously assumed ¹⁰⁻¹³.

Our optimized MitoFlux-EM workflow allowed for the first volumetric dataset of directly correlated TEM and ¹⁵N flux, building on recent advances in correlative EM-NanoSIMS techniques ^{29,60-64}. In addition to giving volumetric information, this technique allows independent verification of NanoSIMS measurements by allowing repeated measurements across physically separated sections. This greatly increases the confidence in these measurements for rare events, such as hot spots of increased ¹⁵N fractional incorporation within MLs observed here. We anticipate this method may also be useful for studying other complex processes resulting from disrupted

proteostasis *in vivo*, including the relationship between organelles and protein aggregates in models of neurodegeneration and alterations in lysosomal flux in lysosomal storage disorders.

Resource availability

Lead contact

Further information and requests for reagents will be fulfilled by Lead Contact Derek Narendra (derek.narendra@nih.gov).

Materials availability

All constructs or cell lines generated in this study are available from the lead contact upon request and completion of a Material Transfer Agreement.

Data code and availability:

- Custom code mentioned throughout the methods is available through the public repository: <https://github.com/NarendraLab/MitoFLUX-EM/>. ChatGPT-5 was used in the generation of custom code.

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Author contributions

J.D.P. and D.P.N. led the project. J.D.P., X.H., C.G., M.W., D.N.D., H.L., T.M.D., Y.L., and D.P.N. designed and performed experiments. W.S. supported the project by providing supervision, suggestions, and reagents. J.D.P. and D.P.N. wrote an initial draft of the manuscript. All authors edited the manuscript.

Declaration of interests

Mayo Clinic and W.S. hold a patent related to PRKN activators (Small Molecule Activators of Parkin Enzyme Function, US patent, 11401255B2; August 02, 2022). All other authors declare they have no competing interests

Statistical analysis

All statistics were calculated in Graphpad Prism 10 or Python. Python code was implemented in Jupyter notebooks and used NumPy and pandas for numerical computation and data handling, SciPy for statistical testing, and statsmodels where applicable for statistical modeling and multiple-comparison procedures. Scikit-learn was used for analyses requiring machine-learning and clustering routines. Specific statistical tests used in each comparison are noted in Figure Legends.

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Methods

Mouse models. Mice were maintained on a 12-hour light/12-hour dark cycle, with food and water provided *ad libitum* unless otherwise stated. Generation of CHCHD10 S59L mice was described previously⁶⁷. *Atg7^{fl/fl}* mice were a generous gift from Masaaki Komatsu³⁷. *Prkn^{fl/fl}* mice were a generous gift from Michael Jurczak⁶⁸. *Ckmm*-Cre (JAX stock #006475) and *Pink1^{-/-}* mice (JAX stock #017946) were obtained from Jackson laboratory and their generation was described previously^{69,70}. All animal studies were approved by the Animal Care Use Committee at the NINDS intramural research program (ASP1395). Both sexes were used in all studies.

Immunoblotting and ELISA testing. Immunoblotting and densitometric measurements were performed as described previously⁶⁷. In brief, mouse frozen hearts were lysed in RIPA buffer (Cell Signaling Technology, #9806) with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, #5872). Tissue samples were cut into small pieces on ice, followed by sonication. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Thermo Scientific, #23225). Approximately 30 µg of total protein from each sample was separated on Criterion TGX Precast Gels (4–15%; Bio-Rad, #5671084) and subsequently transferred onto nitrocellulose membranes (Bio-Rad, #1704272). The following commercially available primary antibodies were used for immunoblotting: p62 (Abcam, #ab56416), LC3 (Cell Signaling Technology, #2775), HSP90 (Proteintech, #13171-1-AP), CHCHD2 (Proteintech, #19424-1-AP), CHCHD10 (Sigma-Aldrich, #HPA003440), OPA1 (BD Biosciences, #612606), MTHFD2 (Proteintech, #12270-1-AP), and PYCR1 (Proteintech, #13108-1-AP).

To measure pS65-ub, an MSD-ELISA assay that was previously validated and developed was used³⁹. Briefly, 1 µg/ml of pS65-Ub antibody (Cell Signaling Technology #62802) in sodium carbonate buffer (200 mM Na₂CO₃, pH 9.7) was used to coat the bottom of 96 well ELISA plates

overnight at 4°C. Mouse heart lysates were prepared as for immunoblotting and proteomics experiments. After overnight capture antibody incubation, 30 µg protein lysate was incubated on the plate for 2 hours at room temperature with gentle agitation. The plate was washed 3 times with 1xTBST and an antibody against total ubiquitin (ThermoFisher #14-6078-37) was used at 1 µg/mL for 2 hours at room temperature, followed by 3 washes in 1xTBST. A sulfo-tag coupled anti-mouse secondary detection antibody (#R32AC-1) was incubated for 1 hour at room temperature, again followed by 3 washes with 1xTBST. Measurements were performed in technical duplicate and the values were averaged. Values from Pink1 KO heart samples run in parallel were used for background subtraction. Measurements were obtained using the Meso QuickPlex SQ120 (Meso Scale Diagnostics, Rockville, MD, USA) in MSD Read Gold buffer.

Confocal analysis of MTS(Su9)-GFP-mCherry puncta in mouse heart tissue. Mice 17 – 26 weeks old were injected by tail vein with AAV9 MTS(Su9)-GFP-mCherry (1.5×10^{12} vector genomes / mouse) suspended in PBS (without divalent cations) with 0.001% Pluronic F68. Mice were sacrificed by perfusion three weeks after AAV injection. A block of midventricular heart tissue was sliced using a mouse heart slicer (Zivic instruments). The block of heart tissue was post-fixed in 4% PFA. Tissues were then sectioned at 35 µm using the Compressstome VF-510-0Z (Precisionary). Free floating sections were obtained and washed with PBS twice for 5 minutes each and in 0.1% Triton X-100 in PBS (PBST) twice for 10 minutes each. Sections were then blocked in 0.4% PBST with 10% Normal donkey serum (NDS) for 2 hours at room temperature. Tissues were placed with primary antibodies overnight in 0.3% PBST with 3% NDS. The next day, tissues were washed with 0.1% PBST 4 times for 10 minutes each and then incubated with secondaries in 0.1% PBST for 1-2 hours at room temperature. Tissues were washed with 0.1% PBST twice for 10 minutes each and with PBS 2x for 5 minutes each at room temperature. Counter staining was done with DAPI for 10 minutes. All tissues

were mounted on slides and coverslipped with prolong diamond mounting media and sealed with nail polish. Sections were imaged using an Olympus (Evident) FV3000 microscope, 60X confocal fields were semi-automatically acquired by choosing a central field near the middle of the specimen and then acquiring a 16-field mosaic of the surrounding area. Individual fields were analyzed in FIJI, using the mQC counter macro with the following settings: green=1 red=2 radius=2 ratio=1 red_0=1⁶⁵. A polygon tool was used to select the area of the field with predominately cardiomyocytes, while excluding visible blood vessels, presumed macrophages, and auto-fluorescent RBCs, which may be falsely identified as mitolysosomes within the cardiomyocytes. Fields were excluded if less than 50% of the area contained cardiomyocytes. The output ratio and selection images from the mQC counter macro were monitored to ensure that MLs were being correctly called. If a sample repeatedly resulted in poor segmentation using the settings above, it was excluded from the analysis. This semi-automated analysis was not performed blind to genotype.

Confocal and flow cytometry analysis of mitophagy in HeLa cells. HeLa cells were endogenously tagged with MFN2-Halo and mCherry-Parkin as described previously⁵⁹. The line additionally expressed dCas9-KRAB BFP. The line was further modified to stably express exogenous YFP-LC3 by lentiviral transduction using the plasmid pLVX-LC3-YFP (addgene: #99571), a kind gift from Edward Campbell. Cells were maintained in High Glucose DMEM (Gibco) with 10% FBS and Pen/Strep. The endogenous MFN2-HaloTag was labelled using the Halo ligand conjugated to JF646 (Promega) according to manufacturer's instructions. Cells were fixed with 4% PFA in 1X PBS prior to imaging. Imaging was performed using an Olympus (Evident) FV3000 microscope with a 60X objective.

Automated quantification of puncta from the confocal images was performed by first segmenting cells using CellPose3 followed by a custom python script modified from the PunctaFinder python code ⁶⁶ (Multiscale_puncta_finder.ipynb). The main modifications to PunctaFinder were mild Gaussian smoothing for detection and to perform hierarchical analysis at several spatial scales with an explicit cross-scale de-duplication procedure. These modifications were made as the size of the puncta in this mitophagy context were highly variable. The YFP-LC3 image was first Gaussian-filtered with a standard deviation of 0.6 pixels for the purposes of candidate punctum estimation. Every eligible pixel within each cell segmentation was evaluated using a square candidate window with a size of 5, 7, and 9 pixels with a 2 pixel wide local background border. A candidate was provisionally accepted if the mean YFP-LC3 intensity in the candidate window if three conditions were met: (1) its intensity was at least 1.5 fold greater than the local background border, (2) its intensity was at least 2 fold greater than the average YFP-LC3 intensity of the whole cell, and (3) the CV within the square candidate window was less than or equal to 0.60. The boundary of the candidate was then estimated using the PunctaFinder derived variable-size segmentation procedure. As some small and larger puncta were missed in initial versions of the script, and so an additional layer was added to attempt detections at 10 pixels and 3 pixels. The 10 pixel pass only contributed puncta if there segmented equivalent diameter was at least 8 pixels. The 3 pixel pass only contributed puncta if its segmented equivalent diameter was in the range of 2.0 – 4.5 pixels. For the 3-pixel detection window the CV was relaxed to less than or equal to 0.75 owing to its small size. Candidates identified at different scales were considered duplicate detections when their seed positions were within 2 pixels or the segmented masks had an overlap coefficient of at least 0.5. If two puncta were duplicates in the 5/7/9 pixel multiscale detection procedure, the one with the highest normalized detection score was preferentially retained, with whole-cell intensity ratio, agreement between segmented diameter and detection

scale, and detection scale used sequentially as tie-breakers. The normalized detection score was calculated as follows:

$$S(normalized) = \min(\frac{R_{local}}{1.5}, \frac{R_{cell}}{2.00}, \frac{0.60}{CV})$$

where:

R_{local} = mean candidate intensity / mean surrounding intensity

R_{cell} = mean candidate intensity / mean whole-cell intensity

CV = standard deviation of candidate intensity / mean candidate intensity

During the de-duplication procedure, the 5-, 7-, or 9-pixel detection windows had precedence over the 10-pixel rescue detection which in turn had precedence over the 3-pixel rescue detection. So, a punctum detected with the 10- or 3-pixel windows could contribute an additional punctum only if it did not duplicate a higher-priority detection.

Flow cytometry was performed as described previously using a CellStream (Cytex Biosciences) flow cytometer⁵⁹. Analysis on MFN2-Halo degradation was performed using the cell line described above. Mitophagy analysis was performed using a HeLa cell line that stably expresses mt-Keima and YFP-Parkin, in addition to dCas9-Zim3 BFP and was generated as described previously⁵⁹.

SILAM analysis of mouse heart tissues. Groups of 4 mice per genotype were fed Mouse Express® L-Lysine (¹³C₆, 99%) mouse feed ad libitum for 2 weeks. Mice were sacrificed for cardiac perfusion with PBS and heart tissue was flash frozen. For each sample 5 – 12 mg of heart tissue was used. The

heart tissue was cut into small pieces in three volumes of 50 mM Tris HCL pH7.4 buffer with proteinase inhibitor and phosphatase inhibitor on ice. The sample was sonicated and protein concentration was normalized to 200 mg / 90 μ L. The sample was then fractionated into soluble and insoluble fractions, by adding Triton X-100 to a final concentration of 1%. The sample was mixed well and then left to sit on ice for 30 minutes. The sample was then cleared by centrifugation at max speed (21,130 g) for 10 minutes at 4°C using a tabletop centrifuge. The 40 μ L of supernatant was transferred to a clean tube and 10 μ L of 10% SDS was added to give a final concentration of 2% SDS. This was saved as the soluble fraction. The pellet from the prior centrifugation step was solubilized by resuspending in 60 μ L of 2% SDS 50 mM Tris HCL pH7.4 with proteinase inhibitors and phosphatase inhibitors. The sample was incubated at room temperature for about 25 minutes, mixed well, and then 30 μ L was transferred to a clean tube. This was saved as the insoluble fraction. The fractions were subsequently alkylated with iodoacetamide and digested to peptides with trypsin. Samples were run on a Vanquish Neo couple with Orbitrap Ascend Mass Spectrometer using a DDA LC/MS/MS data acquisition mode. The databased search was performed using Proteome Discoverer 3.2 searching against the Sprout Mouse databased. The soluble and insoluble groups were analyzed separately.

Subsequent analysis was performed from the protein group ion intensities calculated by Proteome Discoverer using a custom python script (C10WTvsSL_SILAM_analysis.ipynb). For each protein and sample the fractional incorporation of the heavy label was calculated from the ion intensities as (heavy / (light + heavy)). Within the soluble and insoluble fractions, the replicate values were summarized using the geometric mean and were expressed as the log2 of C10 SL/C10 WT. Protein wise comparisons were made using a two-sided unpaired two sample t-test using the library `scipy.stats.ttest_ind`. Multiple comparisons was controlled for using the Benjamini-Hockberg procedure with a false discovery rate of 5%. Mitochondrial proteins were identified using the

MitoCarta3.0 database ⁷¹. Lysosome and myofibril gene sets were derived from the UniProt controlled-vocabulary terms SL-0158 and SL-0312, respectively, for the organism identifier 10090 and the proteome identifier UP000000589. Lysosomal hydrolases were defined as the subset of SL-0158 (lysosome) proteins carrying an Enzyme Commission classification of EC class 3 but excluding EC 3.6 (to remove ATPases, GTPases, etc.). Macroautophagy associated proteins were defined as those with the text “Macroautophagy” in the Reactome Pathways field. For protein-level and replicate level analysis the following statistical approaches were used. For the protein-level analysis protein-level medians were compared between C10 WT and SL using a two-sided Mann-Whitney U test, requiring at least three observations per genotype. Multiple comparisons were corrected using the Benjamini-Hochberg procedure and adjusted p-values were reported. For replicate level analysis the median fraction incorporation for all proteins in a given group (e.g., mitochondrial proteins) was calculated for each replicate. WT and SL replicates medians were then compared using a two-sided Mann Whitney U test when at least two replicates per genotype were available. The p values were adjusted across panels using the Benjamini-Hochberg procedure.

Proteomic analysis of C10 SL mouse heart tissue with and without one copy of endogenous

Dele1* or *PINK1. Heart samples were lysed in RIPA buffer with protease and phosphatase inhibitors added. Samples were sonicated and cleared by tabletop centrifugation at the top speed (21,130 g). Proteins were alkylated with iodoacetamide and digested with trypsin. Label free quantification data was acquired in DIA acquisition mode using a Vanquish Neo coupled with Orbitrap Ascend Mass Spectrometer. The database search and protein group ion intensity calculations were performed in Spectronaut 20.3, with the database search performed against the Sprout Mouse database. Protein-level abundance values were then imported into a custom python workflow. All positive, finite abundance measurements were log2 transformed. Proteins were retained for

analysis if all replicates were available for at least one genotype. When an experimental group had no observed values, missing values were imputed randomly from the lower end of the dataset distribution defined as follows: $X_{\text{imp}} \sim N(\mu - 1.8\sigma, (0.3\sigma)^2)$ where μ and σ are the mean and standard deviation of observed log₂ intensities in that sample, similar to imputation scheme performed in the software package Perseus (<https://maxquant.net/perseus/>)⁷². This was done to allow quantification of proteins that are detectable under one experimental condition but not others. Protein-wise comparisons were made using two-sided Welch two-sample *t*-tests and required at least two replicates per experimental group after imputation. These nominal p-values were adjusted using the Benjamini-Hockberg procedure. Proteins were classified as significant in the volcano plots if their adjusted p-value was less than 0.05 and their absolute log₂ fold change was at least 1. Proteins were annotated as mitochondrial, lysosomal, or autophagy related as noted above. Dele1-dependent genes were defined as described previously, using the core set of genes that were upregulated in a Dele1-dependent manner across three mitochondrial cardiomyopathy models³⁵.

For ubiquitin proteomics of the mouse heart, three mouse samples per group (C10 WT; Pink1^{+/-}, C10 SL; Pink1^{+/-}, C10 SL; Pink1^{-/-}) were used. For some samples hearts from two animals were combined to give sufficient tissue (~160 mg) for protein extraction. Tissue was lysed in 2 mL 8M urea lysis buffer following the protocol of the PTMScan® Ubiquitin Remnant Motif (K-epsilon-GG) Kit protocol) (Cell Science Technology, Cat#5562S) with phosphatase inhibitor added. The tissue was cut into small pieces, sonicated, and centrifuged as above to clear insoluble material. The proteins were digested with trypsin and then enriched for the diGly containing peptides following the CST PTMScan protocol. Mass spectrometry analysis was performed as above. Data was acquired using DDA mode with analysis was performed using Proteome Discoverer 3.2 (Thermo), using the Sprout mouse database as the reference. Downstream analysis was performed

using a custom python code after filtering for mitochondrial proteins (based on their inclusion in MitoCarta 3.0).

Transmission Electron Microscopy. Mice were deeply anaesthetized with isofluorane and then transcardially perfused with freshly prepared fixative solution containing 2% paraformaldehyde, 2% glutaraldehyde, and 2 mM calcium chloride in 0.1M sodium cacodylate buffer (CB) (Electron Microscopy Sciences (EMS), 15710, 16120 and 11653, respectively). After fixation, hearts were removed and immersed in the same fixative. Using a heart slicer matrix (Zivic Instruments, HSMS001-1), a 1 mm-thick coronal slice was cut from the middle of the heart, and the left side of the left ventricle was cut into sub-millimeter pieces. Tissues were stored in the same fixative at 4°C overnight or until processing for EM.

After aldehyde fixation, tissue was rinsed with CB and then postfixed and stained with a reduced osmium solution containing 1% osmium (EMS, 19150) and 1% potassium ferrocyanide (Sigma, P3289) in CB for one hour on ice. Reduced osmium was used for membrane fixation, which maintains the physiologic apposition of the inner and outer autophagosomal membranes⁷³. After rinsing with CB, tissue was rinsed with 50 mM sodium acetate buffer, pH 5.2 (EMS, 21120) and then incubated overnight in 1% uranyl acetate (EMS, 22400) in acetate buffer at 4°C. Tissue was then rinsed with acetate buffer and dehydrated through a series of 5-minute ethanol rinses (50%, 70%, 95%, 100% ethanol x 2) and then infiltrated with EMBED812 epoxy resin (EMS, 14120) (at least one hour steps in 50%, 75%, 95%, and then 100% resin x 2) and then embedded in a flat mold with the muscle fibers oriented parallel to the tip of the embedding mold to obtain longitudinal sections through cardiomyocytes. After 48 hours in an embedding oven at 65°C, polymerized samples were trimmed and sectioned to a thickness of 70 nm (EM UC7 ultramicrotome, Leica Biosystems).

Ultrathin sections were picked up on slot grids with a thick-formvar coating (EMS, FF-2010-CU-TH) that were glow discharged immediately before use (Ted Pella, Pelco EasiGlo). Sections were viewed using a JEOL 1400Flash transmission electron microscope (JEOL USA Inc.) operated at 120KV. Images were acquired using a 29 Mpix CMOS detector (Advanced Microscopy Techniques, Biosprint29).

The 70 nm sections on the Formvar support were then transferred to clean 5 mm silicon chips for direct correlation NanoSIMS analysis. Briefly, to transfer a section onto a silicon chip, a ~25 μ l drop of filtered ultrapure water was placed on the section-side of the slot grid, which was then placed on a silicon chip and dried on a slide warmer at 60°C. The grid was subsequently removed using double-sided tape positioned at the grid periphery and lifted away, leaving behind the Formvar layer and thin sections firmly adhered to the chip.

NanoSIMS analysis

The NanoSIMS analysis was performed as previously described previously⁷⁴. Briefly, detectors were set to the mass channels ^{16}O , $^{12}\text{C}_2^1\text{H}$, $^{12}\text{C}^{14}\text{N}$, $^{12}\text{C}^{15}\text{N}$, ^{31}P , and ^{32}S for all analyzed fields. Fields of view were $\leq 20 \times 20 \mu\text{m}$ and were imaged at a resolution of 256×256 pixels. On average, approximately 30 image planes were recorded per field to ensure that the error on counting statistics remained below 5% in most cases. For most samples, 70 nm sections were used for directly correlated EM and NanoSIMS. However, for one sample (sample 2) analyzed early in the project, an alternative procedure was used: adjacent thick (~400 nm) and thin (~70 nm) sections were imaged by TEM with direct correlation performed on the thick section.

Multimodal registration of 2D TEM and NanoSIMS data to generate the 2D MitoFLUX-EM

dataset. Preprocessing of NanoSIMS images involved aligning and summing the depth planes to increase ion count statistics for each field. To sum and align the depth planes, a custom macro in (FIJI Is Just) ImageJ was used, which included the following set of commands:

```
run("Re-order Hyperstack ...", "channels=[Frames (t)] slices=[Channels (c)] frames=[Slices (z)]");

run("Linear Stack Alignment with SIFT MultiChannel", "registration_channel=3
initial_gaussian_blur=1.60 steps_per_scale_octave=3 minimum_image_size=64
maximum_image_size=1024 feature_descriptor_size=4 feature_descriptor_orientation_bins=8
closest/next_closest_ratio=0.92 maximal_alignment_error=25 inlier_ratio=0.05
expected_transformation=Rigid interpolate show_info show_transformation_matrix");

run("Z Project...", "projection=[Sum Slices]");

run("Hyperstack to Stack");
```

This produced a TIFF stack that contained 6 or 7 channels of summed mass counts. A python script that performs this function was also developed and has been made available as part of the Jupyter notebook: [Align_3D_Stack_NanoSIMS_End_to_End_Python_Pipeline.ipynb](#).

To register the TEM image to the corresponding NanoSIMS TIFF stack, the summed $^{12}\text{C}^{14}\text{N}$ image was first up-sampled to match the pixel dimensions of the TEM image in FIJI with the `resize` command, using an exact integer multiple of the EM pixel size. For registration, a composite of one or more overlapping 5K TEM images was used. The resized $^{12}\text{C}^{14}\text{N}$ NanoSIMS image was then used as the registration target, using the TrakEM2 plugin in FIJI. Both rigid and non-linear transformation were used to achieve submicron registration over the $\sim 300\ \mu\text{m}^2$ fields. The registered TEM image was then used to segment organelles using the python library Napari to generate separate labels

TIFFs for each organelle class. Lysosomes and their mitochondrial contents were manually segmented. Mitochondria were segmented using the MitoNet deep-learning model implemented in the Napari plug-in Empanda⁷⁵, as described previously³⁵, and then manually corrected in Napari. Segmentations for most samples were performed on the TEM images in manner that was blind to information from NanoSIMS. This procedure was modified for sample 2, however, for which the correlation between TEM and NanoSIMS was less precise owing to direct correlation on the thick section. In this case, the boundaries of organelles were segmented using the thick, thin, and $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N}$ ratio image as references. All segmentations were reviewed by 2 – 3 of the study investigators, who reached a consensus.

Using the label TIFFs and summed NanoSIMS TIFF stacks as input, a custom python script was generated to extract NanoSIMS counting statistics for each organelle with a label segmented from the correlated EM (Measure_2D_NanoSIMS.ipynb). This script conservatively downsampled the label TIFFs to match the original 256 px X 256 px resolution of the NanoSIMS stacks and then extracted both raw counts and counts for each pixel normalized to the average of the field for each channel in the summed NanoSIMS TIFF stack. Pixels in the down-sampled labels were retained only if they were completely contained within the high-resolution contour of the original label. In addition to these strict labels, eroded labels were also generated to remove the edge pixels. For the elements ^{32}S and ^{31}P , ion counts per pixel normalized to the field average were generally used to control for field-to-field variability. For calculating $^{12}\text{C}^{15}\text{N}$ fractional incorporation the raw $^{12}\text{C}^{15}\text{N}$ and $^{12}\text{C}^{14}\text{N}$ ion counts were used with the formula: $^{12}\text{C}^{15}\text{N} / (^{12}\text{C}^{14}\text{N} + ^{12}\text{C}^{15}\text{N})$. The pixel-level median ^{15}N fractional incorporation value was the main measurement used for each organelle to minimize edge effects and to have similar counting statistics across organelles of varying sizes. Numpy arrays corresponding to each segmented organelle were stored as .npz files. In parallel, area measurements were performed for each organelle based on the segmentation from the EM image.

To limit mitochondria to compositionally typical examples in the sample, mitochondria that were outliers in terms of the 32S, 31P, or total nitrogen ($^{12}\text{C}^{14}\text{N} + ^{12}\text{C}^{15}\text{N}$) were filtered out using Tukey's interquartile range (IQR) method which flags data points lying 1.5 X above or below the IQR. This was needed as the C10 SL sample contained a few very large, swollen mitochondria that were not typical of the cellular population and to remove potential segmentation errors.

Each mitochondrion sequestered within a mitolysosome was additionally annotated for its grade of ultrastructural degradation, based on its appearance in 5K EM micrographs. Scoring was performed by two raters who were blind to the NanoSIMS data, using the predefined scoring criteria:

Grade 1 – Apparently undigested. All cristae clear and distinguishable, similar to mitochondria outside mitolysosome.

Grade 2 – Partially digested. Some cristae with normal morphology still visible but clear disruption of morphology

Grade 3 – Moderately digested. No normal cristae, but few remnants that could be cristae, can be very granular.

Grade 4 – Severely digested. No cristae recognizable, very granular.

To obtain sarcomere measurements, the positions of M-lines on the $^{12}\text{C}^{14}\text{N}$ image of the NanoSIMS stacks were recorded in FIJI using the multi-point tool. A custom python script (Measure_2D_NanoSIMS_sarcomere_mline_ROIs.ipynb) was used to define a 3 px by 3 px square ROI centered on each recorded M-line coordinate and to extract NanoSIMS measurements from these ROIs. The resulting sarcomere measurements were added as additional rows to the overall dataset generated from the manually segmented organelles described above. All measured objects

appear in the dataset named “MitoFLUXEM_2D_dataset_cleaned_annotations.csv”. Custom python code (Graph_2D_data.ipynb) was used to analyze and graph the data from this dataset. One analysis cell additionally accesses segmented data, stored as .npz files.

Multimodal registration of TEM and NanoSIMS serial sections to generate the 3D MitoFLUX-EM

dataset. For volumetric multimodal registration, the same imaging and initial NanoSIMS pre-processing procedures were followed as above for 2D. Briefly, an area that could be identified within the 22 serial sections (on a single formvar-coated single slot grid) was imaged first by TEM and then by NanoSIMS. The NanoSIMS image for each sections were aligned using the FIJI function SIFT MultiChannel on the $^{12}\text{C}^{14}\text{N}$ channel. A python version of this pipeline (Align_3D_Stack_NanoSIMS_End_to_End_Python_Pipeline.ipynb) is available (see code availability section). A ratio stack was then generated in FIJI by dividing the $^{12}\text{C}^{15}\text{N}$ channel by the $^{12}\text{C}^{14}\text{N}$ channel. The individual stacks were then realigned using SIFT. The EM images were manually aligned to the corresponding NanoSIMS ratio images by rigid transformations using the TrakEM2 FIJI plugin. Organelles were then manually segmented from the TEM image stacks using the procedure described in the 2D section.

To quantify volumetric ^{15}N fractional incorporation in mitolyosomes and surrounding unsequestered mitochondria, the following workflow was used. First, a 50% eroded version of the label TIFF stack was generated for each organelle class, using a custom python script (Erode_from_edges_CPU.ipynb). Eroded TIFF stacks allowed measurements that exclude the edge voxels in the volume, which are more likely to contain isotopic information from neighboring structures. The aligned ratio NanoSIMS stack, label TIFF stacks, and eroded label TIFF stacks were then down-sampled in parallel to the resolution of the acquired NanoSIMS dataset, using a custom

python script (Downsample_Volumetric_NanoSIMS_and_labels_stacks.ipynb). The script uses the block size of pixels of equal value to determine the scaling factor, trims partial pixel blocks from the edges of the cropped stacks, and down-samples the pixel blocks by the estimated scaling factor to retain the modal value in each detected block of pixels. As a results, each pixel block of the up-sampled NanoSIMS ratio image in the EM space becomes a single pixel in the down-sampled image. The label images were down-sampled in parallel, using the same trim, block size, and modal value retention logic. The size of pixel blocks and partial boxes on the edge of the image were determined by manual inspection of the ratio images in FIJI. This procedure retained the original measurements of NanoSIMS ratio stack prior to resampling while preserving alignment to the labels. Volumetric measurements for each labelled organelle were then extracted from the down-sampled NanoSIMS ratio image obtained using a custom script (Measure_Volumetric_NanoSIMS_stack.ipynb).

^{15}N fractional incorporation was calculated from the $^{15}\text{N}/^{14}\text{N}$ ratio data as follows: $\text{ratio} / (\text{ratio} + 1)$. For mitochondria sequestered in mitolyosomes, the grade of ultrastructural degradation was scored by two raters from the EM stacks using the same criteria as described above for the 2D images, with the following modification: separate ratings for a mitochondrion were given for each section in which the mitochondrion appeared, and the modal rating among sections was assigned as the overall rating for the mitochondrion in the stack. Data from the two mitolyosomes and surrounding mitochondria were combined into the dataset of volumetric measurements and graphed using a custom python script (Graph_3D_data.ipynb). The custom HSI Hue color map was generated by exporting the corresponding look up table from FIJI (LUT.csv), which is available at: <https://github.com/NarendraLab/MitoFLUX-EM/>.

To visualize volumetric ^{15}N fractional incorporation in mitolyosomes and surrounding unsequestered mitochondria, the following workflow was developed. The label TIFF stacks for each

organelle class generated as described above, were rescaled from their native x, y, z dimensions (where z was assumed to be 70 nm, based on the estimated section thickness) to an isometric space in which x, y, and z were all 10 nm, using a custom python script (Resample_anisotropic_mask_GPU.ipynb – a CPU-only version has also been made available). This involved down-scaling in x and y and up-scaling in z. Up-scaling in z was achieved through smooth interpolation between the sections. The isometric TIFF stacks were then visualized in ChimeraX. In the visualizations the unsequestered mitochondria and mitochondria sequestered in mitolysosomes, were colored for median ¹⁵N fractional incorporation based on the values calculated and look up table described above.

Correlative Light-Electron Microscopy

HeLa LC3-GFP, dCas9-KRAB BFP, MFN2-Halo were plated on gridded glass bottom dishes (Mattek, P35G-1.5-14-C-GRD). To induce mitophagy and capture early phases of mitochondrial engagement with LC3-GFP labeled APLs, cells were treated with CCCP for 2 hours in the incubator, and then fixed with 4% paraformaldehyde (EMS, 15710) in 0.1M Sorensen's phosphate buffer (PB) (EMS, 11601-10) for 12 minutes. Cells were rinsed twice with PB, and then confocal imaging was performed in PB (Olympus, FLUOVIEW FV3000). Z-stacks of cells with distinct LC3-GFP labeled crescent and ring structures were obtained with a PlanApo N 60x1.42 oil objective. Lower magnification phase contrast images were collected to record the location of the cells relative to the grid pattern in the coverslip.

After confocal imaging, cells were rinsed once with 0.1M sodium cacodylate buffer (CB) (EMS, 11653), and then fixed with 2% paraformaldehyde (EMS, 15710), 2% glutaraldehyde (EMS,

16120), and 2 mM calcium chloride (Quality Biological, Inc., 351-130-721) for at least 30 minutes, then stored at 4°C in fixative until processing for EM.

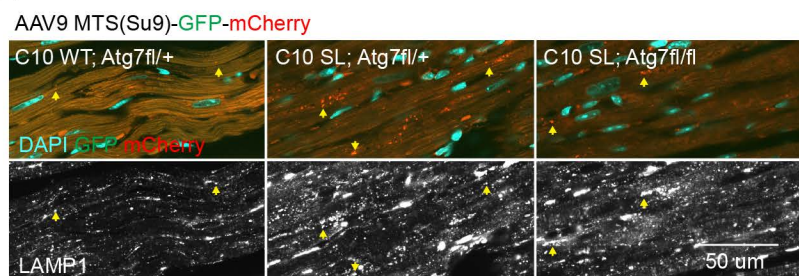
Cells were postfixed and stained in reduced osmium solution containing 0.5% osmium tetroxide (EMS, 19150) and 0.5% potassium ferrocyanide (Sigma Aldrich, P3289) in CB for 45 minutes, then processed as described above for heart tissue until the 50% ethanol dehydration step at which point the gridded coverslip was detached from the Mattek dish as follows: The Mattek dish containing 50% ethanol was placed in the bottom of a 60 mm glass Petri dish to which about 2 mL of acetone (Sigma Aldrich, 270725) was added such that the acetone contacted the bottom of the dish and advanced up the side of the Mattek dish about half way, to allow the acetone to dissolve the adhesive attaching the glass to the plastic dish. The cover was put on the glass Petri dish and after about two hours the Mattek dish was inverted, and while working under a dissecting microscope (Zeiss, Stemi508), the gridded glass coverslip was removed from the bottom of the Mattek dish with a pair of forceps and quickly placed in a new 35 mm plastic Petri dish containing 50% ethanol. Dehydration and infiltration were performed and then samples were embedded by laying the coverslip cell side up on glass slide and then resin filled gelatine capsules were inverted over the glass surface (EMS, 70110). The assembly was placed in a 65°C embedding oven for 48 hours to polymerize.

To separate the polymerized resin-containing gelatin capsules (and cells) from the glass coverslip, the back of the glass slide was warmed with a flame until pulling on the gelatin capsules caused the resin to pop off cleanly from the glass coverslip and glass slide. When cleanly separated, the grid pattern etched on the coverslip surface is visible on the resin block face. The block was trimmed to the coordinates recorded by confocal microscopy and 100 nm-thick serial sections were cut and collected on formvar-coated slot grids (FF-2010-CU-TH). Cells imaged by confocal microscopy were re-located in the TEM thin sections. Structures that aligned with LC3-

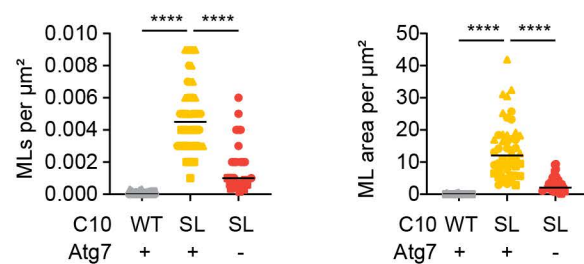
GFP labeled objects were imaged across serials sections. Serial sections were aligned using the TrakEM plugin of Fiji ⁷⁶.

Figure 1

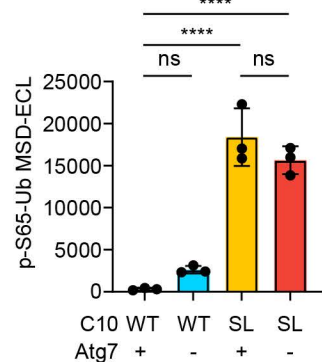
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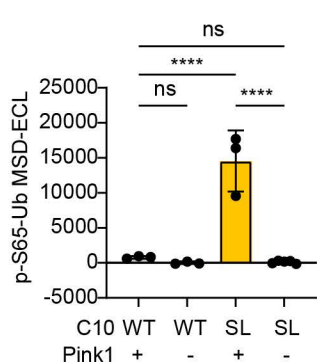
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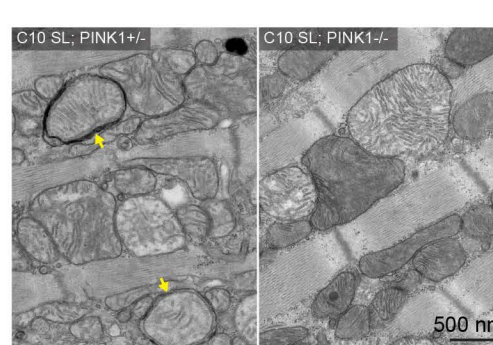
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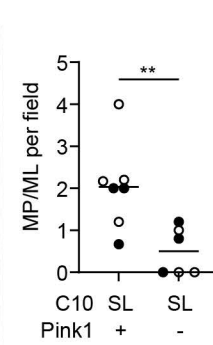
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E

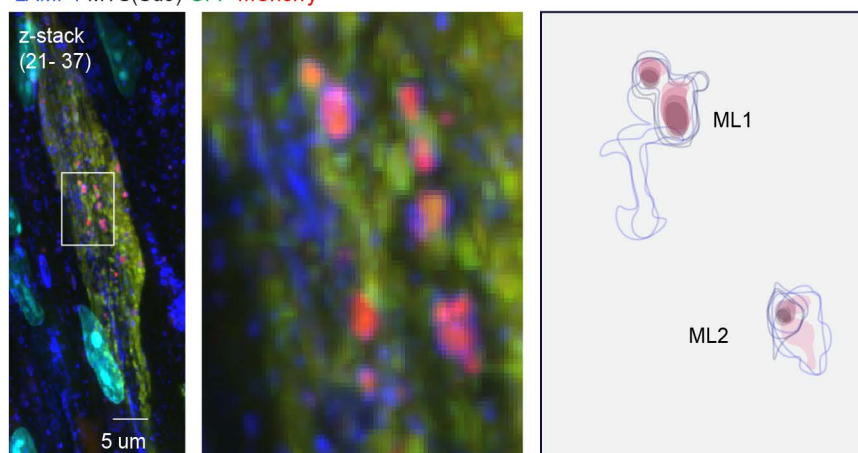


F

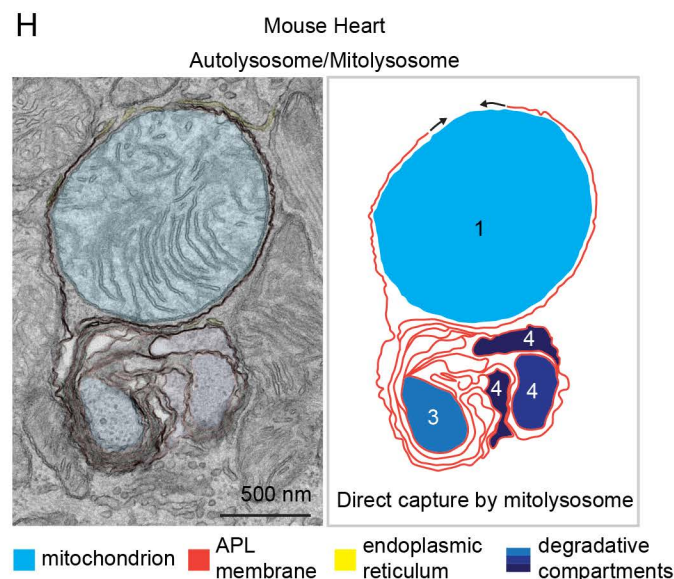


G

LAMP1 MTS(Su9)-GFP-mCherry Mouse Heart

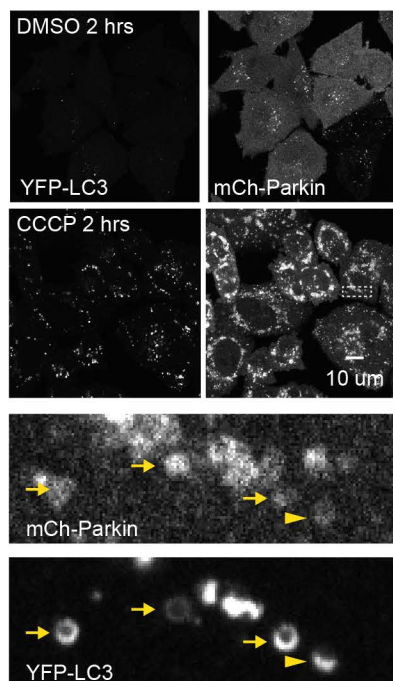


H

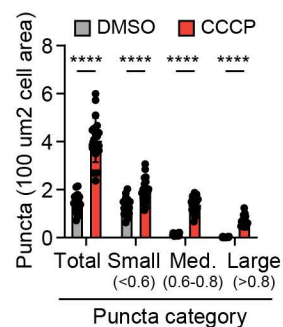


I

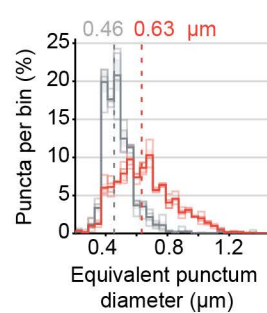
HeLa Cells



J



K



L

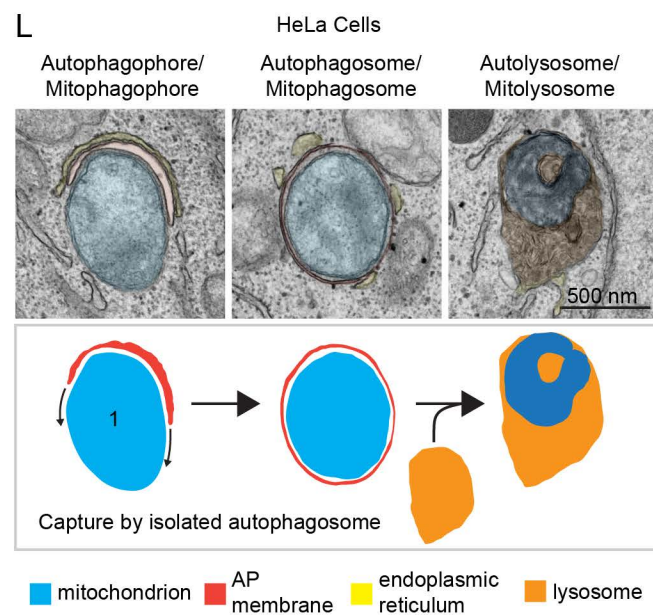
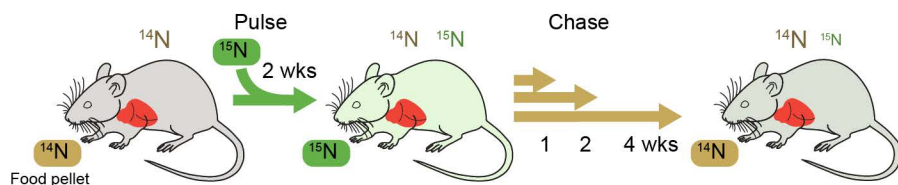


Figure 1. PINK1 mediates the capture of damaged mitochondria through protrusion of autophagophosomal-like (APL) membranes from mitolysosomes. (A) Confocal images of heart sections from C10 WT or SL mice with or without one functional copy of the canonical autophagy gene *Atg7*. Mice were systemically injected with the mitophagy reporter, AAV9 MTS(Su9)-GFP-mCherry, three weeks prior to sacrifice. Heart sections were additionally labelled for lysosomes using antibodies against Lamp1 and for nuclei with DAPI. Acidic mitolysosomes (MLs) appear as red puncta in the merged image. These often co-localized with the lysosomal marker (yellow arrows). (B) Confocal fields shown in (A) were quantified for the number and total area of MLs, using the mQC counter macro in FIJI ⁶⁵. N = 3 mice of each genotype. 9 - 17 fields were measured per sample. Datapoints represent the measurements of each field. Statistical testing was performed using Welch ANOVA testing followed by a Dunnett's T3 multiple comparisons test. (C and D) Graphs show levels of the specific PINK1 product, pS65-ubiquitin, in heart lysates measured with a sensitive ELISA in mice with or without one copy of *Atg7* (C) and with or without one copy of *Pink1* (D). N = 3 mice per genotype. Each datapoint represents one mouse. Statistical testing was performed using One-Way ANOVA followed by Holm-Šidák's multiple comparisons test. (E) TEM images of C10 SL hearts with or without one functional copy of *Pink1*. C10 SL; *Pink1*^{+/-} hearts had frequent mitophagosomes (MPs) and MLs (yellow arrows) that were rarely seen in C10 SL; *Pink1*^{-/-} hearts. (F) Quantification of mitophagic events in electron micrographs like those shown in (E). Two mice were analyzed per genotype. Datapoints reflect the average number of events per field in each heart area examined. Analysis was performed by a rater blind to genotype. Statistical testing was performed using the Mann Whitney U test. Scalebar, 500 nm. (G) A maximum intensity projection (left) from a confocal stack of a C10 SL; *Atg7*^{fl/+} mouse heart, as described in (A and B) and tracing of mCherry+/GFP+ structures and Lamp1+ structures from the z-stack (right). These images are part of the same dataset analyzed in Figure 1A and B. (H) EM image of a mitolysosome with membrane

protrusions extending around a partially enclosed mitochondrion from the heart of a C10 SL mouse. Serial sections of this mitolysosome and others from the same tissue dataset were analyzed (Figure 5 and Extended Figure 2). Numbers on the traced image indicate the grade of ultrastructural degradation of each captured mitochondrion in the mitolysosome; Grade 1: normal; Grade 2: mild; Grade 3: severe with cristae remnants; Grade 4: severe with only granular material. Scale bar, 500 nm. (I) Confocal images of HeLa cells endogenously tagged with MFN2-Halo (not shown) and exogenously expressing mCherry-Parkin and YFP-LC3 treated with CCCP for 2 hrs. (J) Quantification of YFP-LC3 positive puncta from images as in (I) by automated detection according to equivalent punctum diameter in μm . Parkin activation increases total YFP-LC3 puncta and especially larger puncta. (K) Histogram of same data in (J) showing the size distribution of YFP-LC3 puncta detected in the respective conditions. (L) TEM from HeLa cells treated as in (I) showing structures in the progression of mitophagy from isolated autophagophores to autophagosomes to autolysosomes. Tracings of each mitophagy intermediate are shown below. Scale bar, 500 nm.

Figure 2

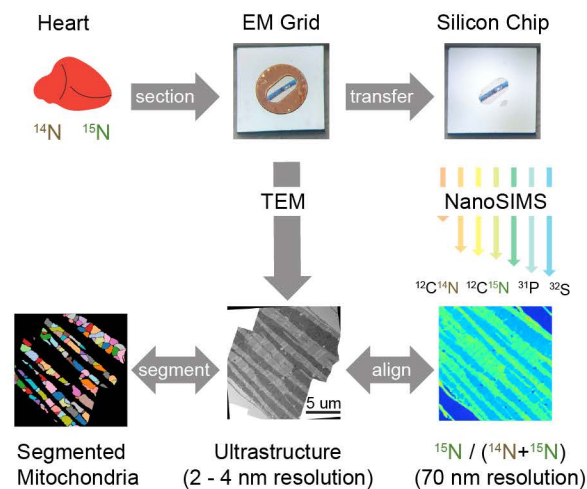
A

In vivo metabolic labeling of protein with ^{15}N 

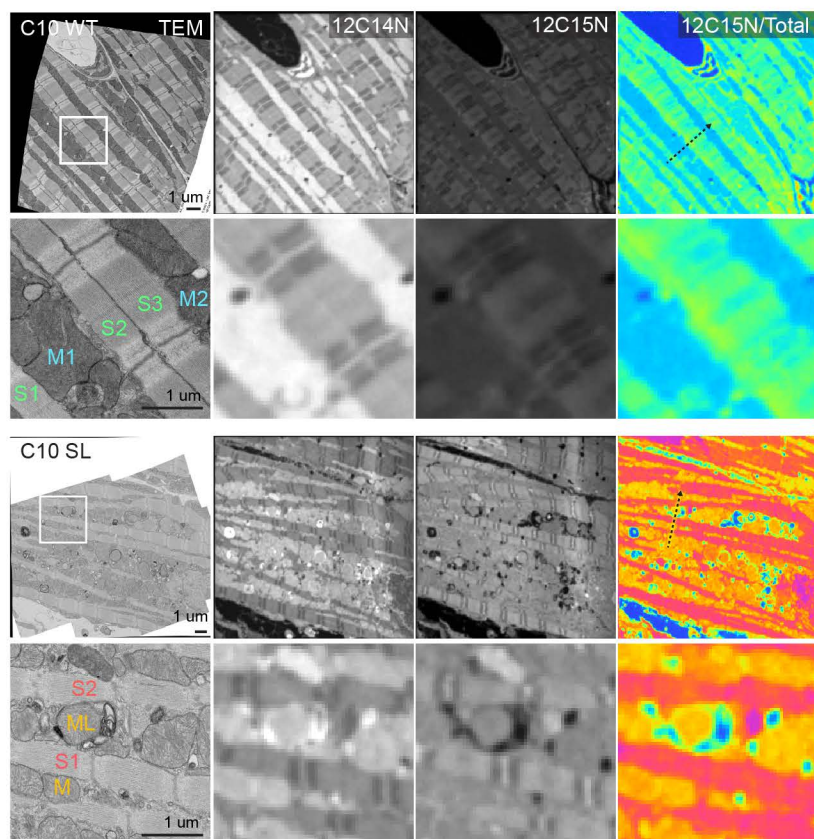
MitoFLUX-EM Dataset

2D					Pulse - Chase 1 wk				
Groups	Mice	Fields	Cells	Organelles	Groups	Mice	Fields	Cells	Organelles
C10 WT	2	10	14	1319	C10 SL	1	8	16	1377
C10 SL	2	10	19	1405					
3D					Pulse - Chase 2 wk				
Groups	Mice	Fields	Sect.	Organelles	Groups	Mice	Fields	Cells	Organelles
C10 SL	1	1	24	77	C10 SL	1	5	8	763
					Pulse - Chase 4 wk				
Groups	Mice	Fields	Cells	Organelles	Groups	Mice	Fields	Cells	Organelles
C10 SL	1	5	9	881					

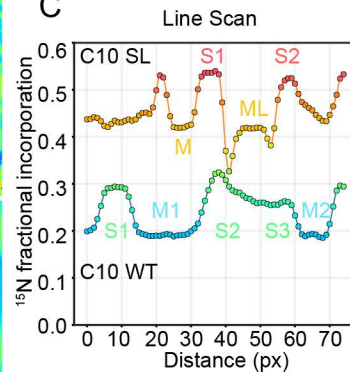
MitoFLUX-EM Analysis



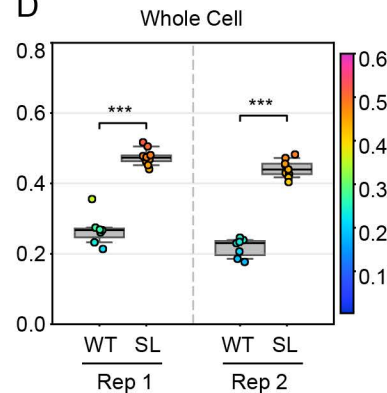
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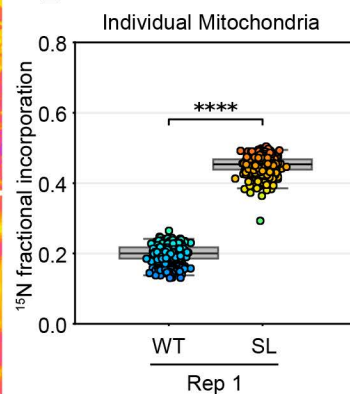
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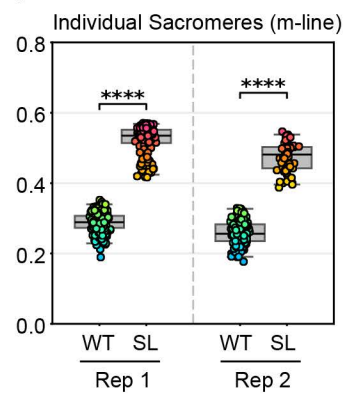
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E

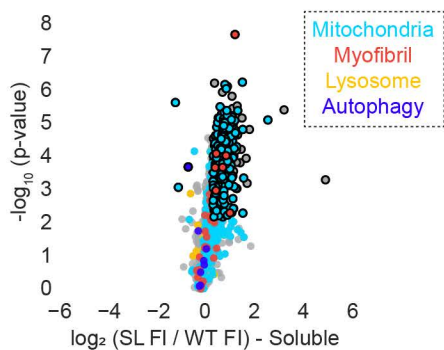


F



G

Heavy lysine incorporation into heart proteome



H

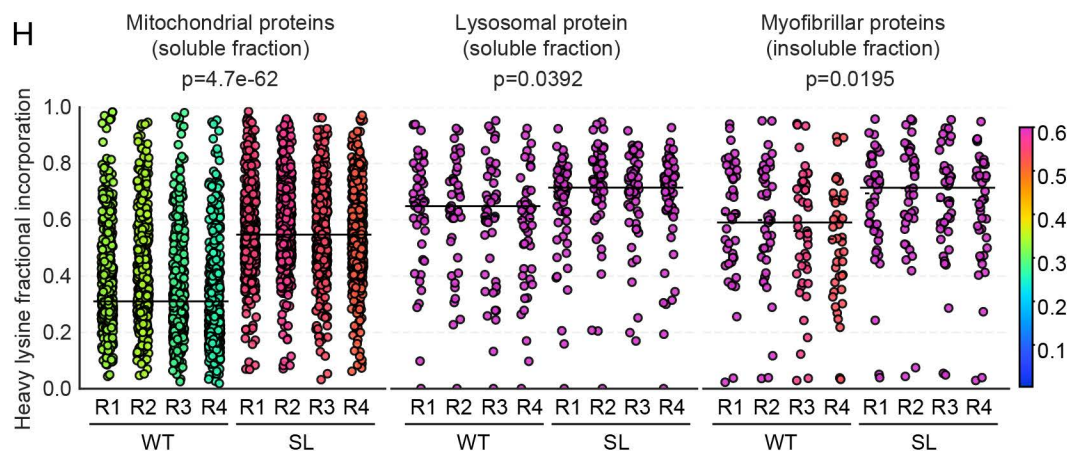


Figure 2. MitoFLUX-EM and pulsed SILAM reveal elevated global protein turnover in the hearts of C10 SL mice. (A) Schematic demonstrates the workflow of MitoFLUX-EM and dataset generated from C10 WT and SL mice. Mice were pulsed with ^{15}N -labelled food for 2 weeks followed by chase for 0, 1, 2, or 4 weeks. The heart samples were fixed and prepared for conventional thin section EM. After imaging by EM, each section or ribbon of serial sections (for volumetric MitoFLUX-EM) was transferred along with its formvar support to a silicon wafer for correlated NanoSIMS analysis. Scale bar, 5 μm . (B) Aligned TEM and correlated NanoSIMS images of $^{12}\text{C}^{14}\text{N}$ counts (reflecting tissue nitrogen before pulse), $^{12}\text{C}^{15}\text{N}$ counts (reflecting tissue nitrogen incorporated after the pulse), and fractional incorporation of ^{15}N ($^{12}\text{C}^{15}\text{N}/(^{12}\text{C}^{14}\text{N}+^{12}\text{C}^{15}\text{N})$). Mitochondria (M), sarcomeres (S), and a mitolysosome (ML) show distinct levels of ^{15}N fractional incorporation. Additionally, incorporation is globally increased in C10 SL vs. WT samples. Boxes indicate regions of magnification, while black dotted arrows show the trajectory for profile plots in (C). Eroded ROIs were used for ^{15}N measurements. Scale bars, 1 μm . (C) Profile plots from C10 WT and SL samples in (B) demonstrate that ^{15}N fractional incorporation is increased globally in SL samples, and that there is a similar relative increase in ^{15}N incorporation in sarcomeres (S) vs. mitochondria (M), reflecting faster protein turnover in sarcomeres vs. mitochondria. (D) Quantification of whole cell ^{15}N incorporation data from C10 WT and SL mice acquired by NanoSIMS (same dataset as used in B and C). Rep1 and Rep2 represent pairs of age-matched C10 WT and SL mice. Datapoints represent measurements from individual cardiomyocytes. Eroded ROIs were used for ^{15}N measurements. (E and F) Quantification from same dataset as (D) showing ^{15}N fractional incorporation measurements from individual segmented mitochondria (E) or 3 px X 3 px square ROIs centered on the m-line of sarcomeres (F) from the indicated images. Each datapoint represents a mitochondrion or sarcomere measurement in (E) and (F), respectively. Statistical testing for panels (D – F) was performed using the Mann U Whitney test, p-values were adjusted for the number of tests

performed in the respective panels using the Holm procedure. (G) Volcano plot comparing heavy arginine fractional incorporation in soluble proteins isolated from C10 WT vs. SL hearts following a 2-week pulse by LC-MS/MS. (H) Heavy arginine fractional incorporation measurements from same experiment described in (G) grouped by organelle localization. C10 SL hearts show increased fractional incorporation in this pulsed SILAM experiment. Heart samples from four mice (R1 – R4) for each genotype were tested for (G and H). Statistical testing was performed using two-sided Mann–Whitney U tests, with P values adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) procedure.

Figure 3

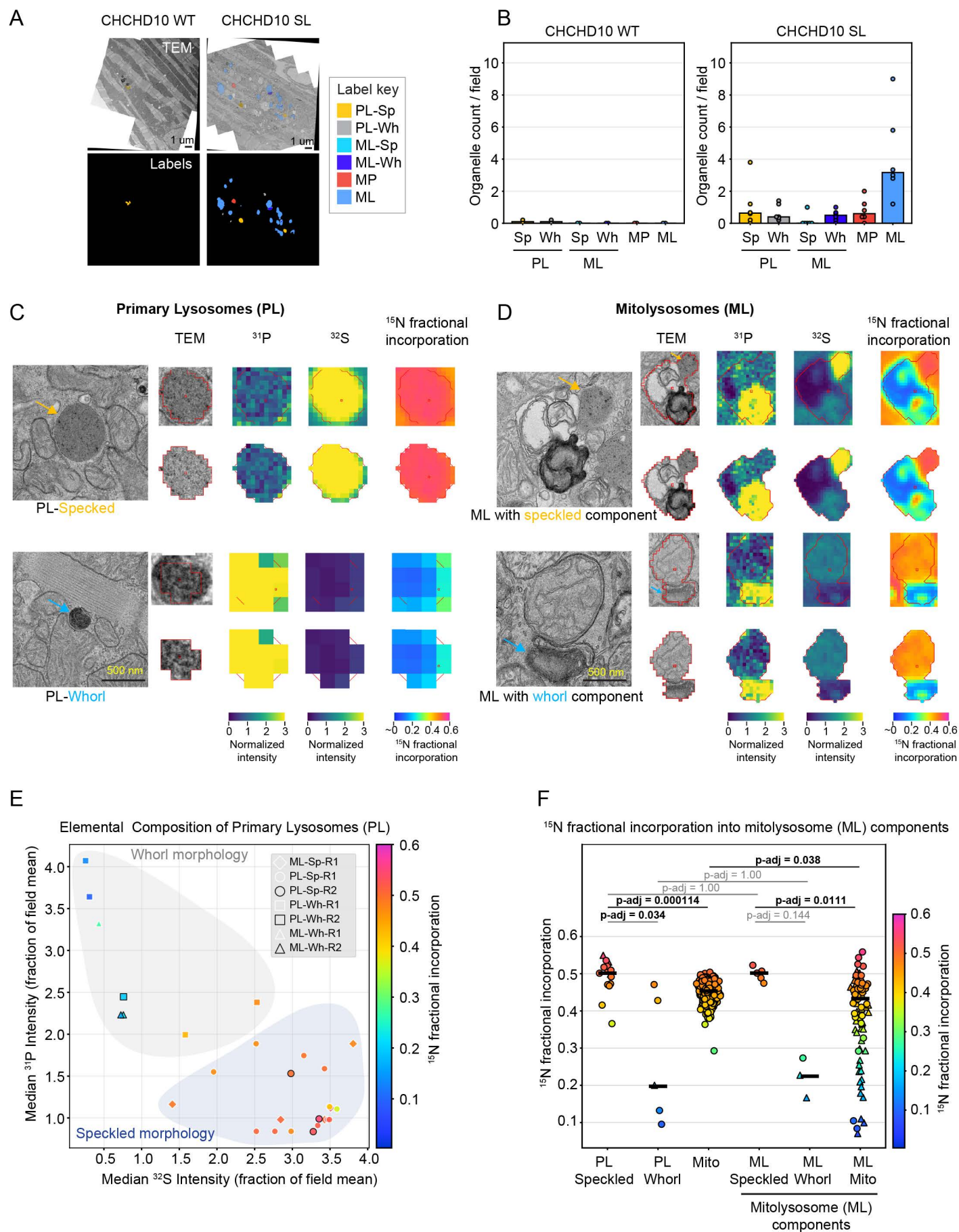


Figure 3. Abundance and composition of primary lysosomes in C10 SL mouse hearts. (A)

NanoSIMS-aligned TEM and labels for identified primary lysosomes with speckled (PL-Sp) or whorled (PL-Wh) morphologies within a representative field from C10 WT and SL mice. When these structures were touching or contiguous with mitolysosomes (ML), these were labelled ML-Sp and ML-Wh, respectively. Mitophagophores (MP) were additionally labelled. Scale bars, 1 μ m. (B) Quantification of fields such as those shown in (A). N = 4 mice for C10 SL and 2 mice for C10 WT. At least 4 EM fields were quantified per mouse. (C and D) Representative correlated EM-NanoSIMS images of morphologically defined lysosomes observed isolated (C) or touching or contiguous with MLs (D). Speckled vs. whorled lysosomes have distinct ^{32}S and ^{31}P elemental signatures and differential ^{15}N fractional incorporation reflecting differential nitrogen flux. Eroded ROIs were used for measurements. Scale bars, 500 nm. (E) Scatterplot demonstrating clustering of morphologically distinct lysosomes by elemental composition (^{32}S vs. ^{31}P) whether found isolated or as part of a ML. Datapoints represent a single organelle measured from N = 2 C10 SL mice (R1 and R2). Eroded ROIs were used for measurements. (F) Comparison of ^{15}N fractional incorporation in morphologically distinct lysosomes to unsequestered mitochondria and mitochondria within MLs. Statistical testing was performed using the non-parametric Kruskal-Wallis test followed by post-hoc Mann-Whitney U tests with Holm p-value correction. Eroded ROIs were used for measurements.

Figure 4

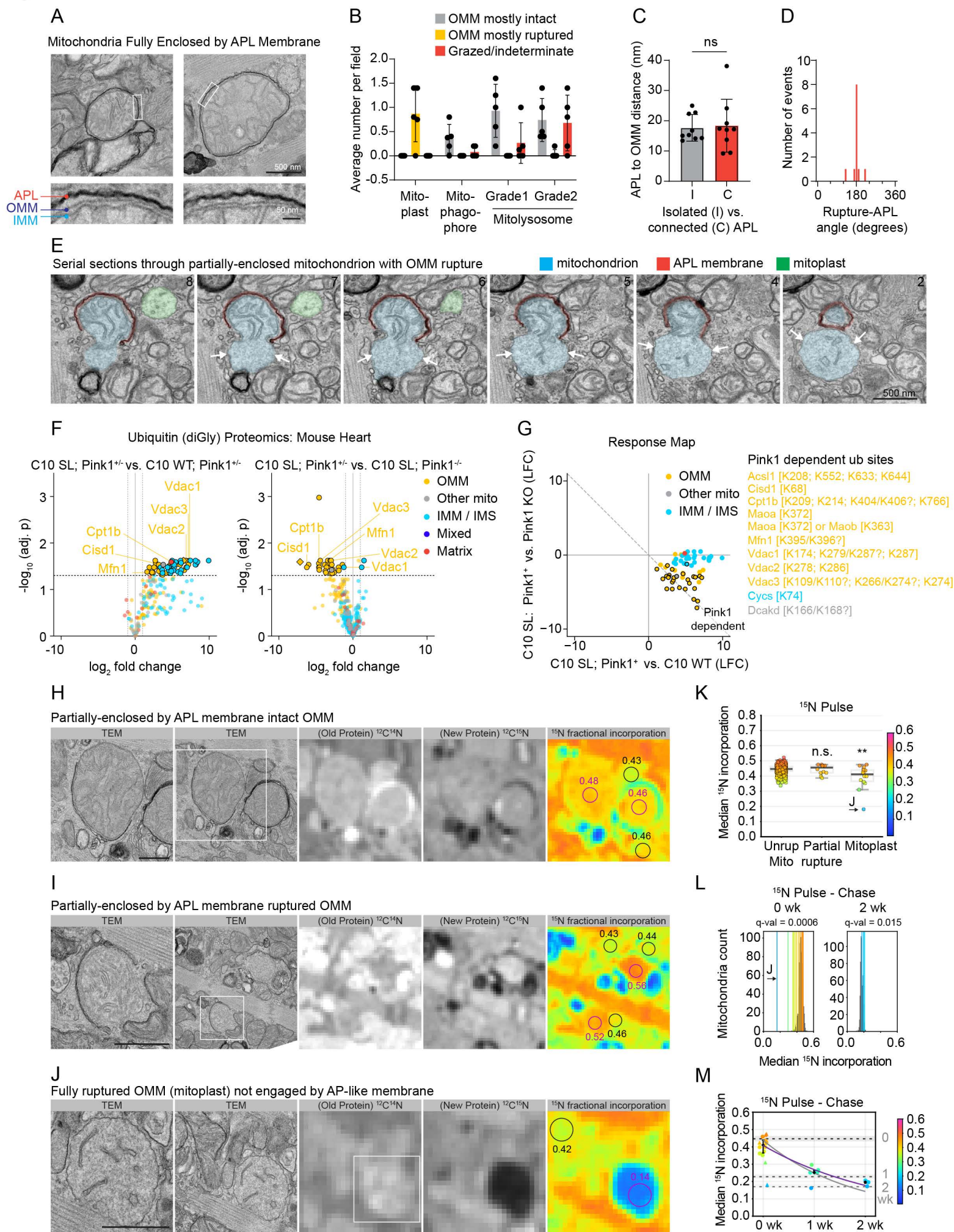


Figure 4. Analysis of outer mitochondrial membrane (OMM) rupture of unsequestered mitochondria and mitochondria undergoing mitophagy in C10 SL hearts. (A) Representative electron micrographs of mitochondria undergoing mitophagy showing that the contact site between the mitochondrion and autophagophosomal-like (APL) membrane is made at an unruptured portion of the OMM. Scale bars, 500 nm; scale bars on insets, 50 nm. (B) Graph depicting frequency of events with OMM per field among unsequestered mitochondria and mitochondria undergoing mitophagy. N = 4 C10 SL mice. At least 4 EM fields were quantified per mouse. (C) Distance between the APL and OMM was measured for events with incomplete capture of mitochondria by the APL, some of which were apparently isolated (I) from MLs and others of which were connected (C) to MLs. Three measurements taken near each edge and center of the APL-mitochondria contact site were averaged. Each datapoint corresponds to the mean of three measurements for a single event. Statistical testing for (B and C) was performed using the non-parametric Kruskal-Wallis test followed by post-hoc Mann-Whitney U tests with Holm p-value correction. (D) Histogram of events from C10 SL; *Dele1*^{+/-} mice with partial OMM rupture and incomplete capture by the APL, showing the relationship between the APL and the OMM rupture. Lines were drawn between the midpoint of the APL and the centroid and the OMM rupture and the centroid, and the angle between the lines was measured (all events measured are shown in Extended Data Fig. 4). The median value was ~180° indicating the APL and OMM rupture are on opposite sides of the mitochondrion. The 12 events were from N = 2 mice. The samples were generated and used in a previous publication³⁵. (E) Serial sections through an event with partial OMM rupture (white arrows) and incomplete capture of a mitochondrion (shaded blue) by the APL (shaded red) from the heart of a C10 SL; *Dele1*^{+/-} mouse as in (D). A neighboring mitoplast is shaded green. The serial section number is indicated in the upper right of each image; serial section 3 was damaged and is not shown. Scale bar, 500 nm. (F and G) PINK1-dependent mitochondrial ubiquitination sites in the mouse heart were determined by

diGly-based ubiquitin proteomics of filtered mitochondrial proteins. Mitochondrial peptides that were significantly increased in the C10 SL; Pink1+/- vs. C10 WT comparison and significantly decreased in the C10 SL; Pink1-/- vs. C10 SL; Pink1-/- comparison were considered *Pink1*-dependent. These were predominately sites on OMM localized proteins. (H – J) Representative correlated NanoSIMS and EM of mitochondria with (I and J) and without OMM rupture (H), undergoing mitophagy (H and I) vs. unsequestered (J). Mitochondria with incomplete capture by the APL have a similar protein age (based on ^{15}N fractional incorporation) as surrounding unsequestered mitochondria, whether (I) or not (H) they are partially ruptured. By contrast, mitochondria lacking an OMM (J) have much older protein age than surrounding mitochondria. Circles indicate locations of ROIs (diameter = 5 pixels) for the median ^{15}N fractional incorporation measurements shown on images to compare neighboring organelles. Non-eroded ROIs were used for measurements to include the maximum number of events. Scale bars, 500 nm. (K) Graph comparing median ^{15}N incorporation for unruptured, partially ruptured, and fully ruptured (mitoplasts) mitochondria following the 2-week ^{15}N pulse. Black arrow is the event shown in (J). (L) Histograms showing the relationship between each mitoplast (colored lines) in the dataset relative to the distribution of unruptured mitochondria (gray histogram) in the dataset after the 2-week pulse (left) and following a 2-week (right) chase. Black arrow is the event shown in (J). Non-eroded ROIs were used for measurements to include the maximum number of events. (M) Graph shows trendlines for mitoplasts (black) vs. unruptured mitochondria (gray) across the chase timepoints with individual colored datapoints for each mitoplast. Dataset is the same as used in graphs in (K and L). Reference horizontal dotted lines and gray bars indicate the median and the 95% confidence interval of unruptured mitochondria. Mitoplasts have lower ^{15}N fractional incorporation before the pulse but higher after the chase, consistent with slower protein turnover. Non-eroded ROIs were used for measurements to include the maximum number of events.

Figure 5

HeLa: mCh-Parkin; GFP-LC3; MFN2-Halo

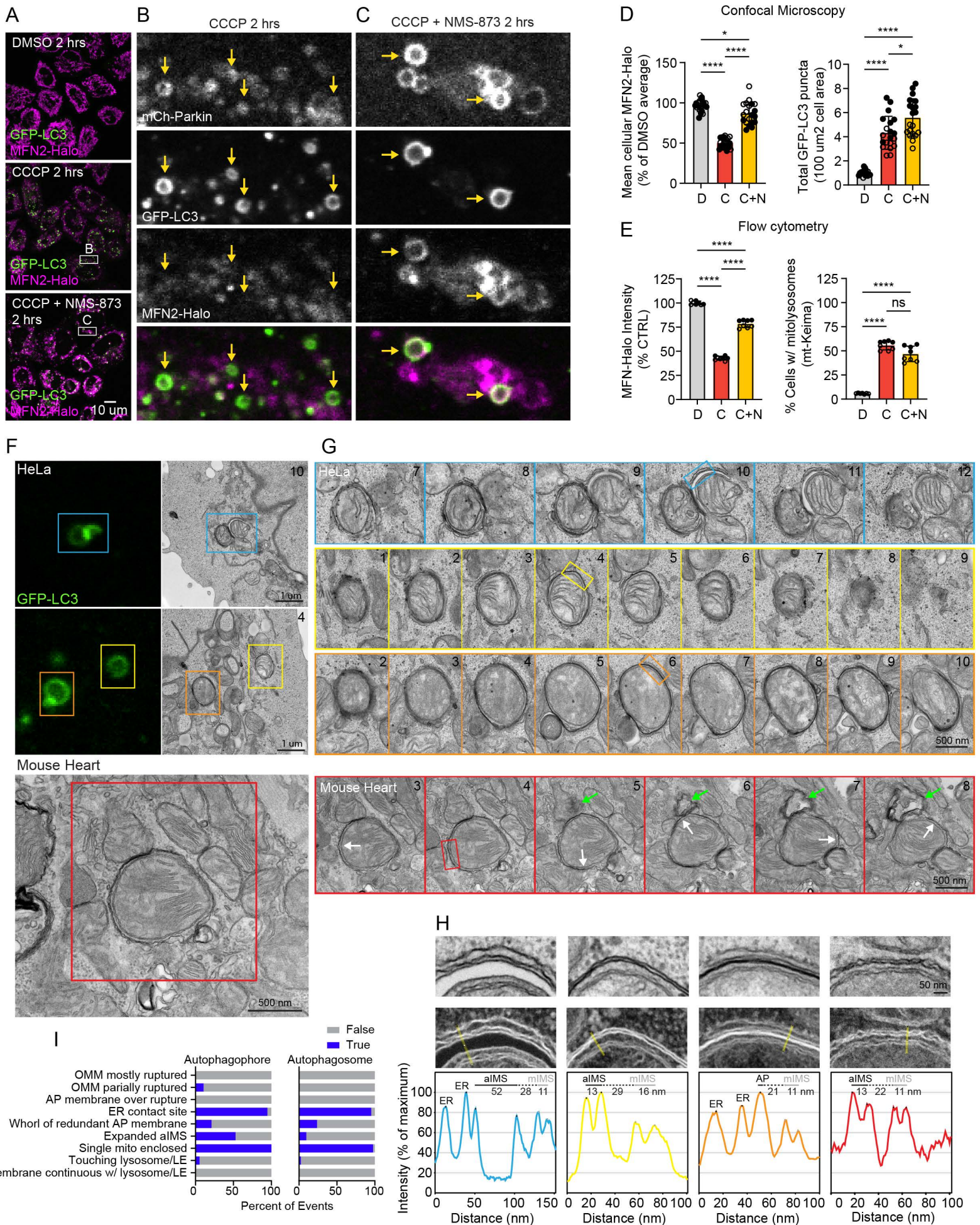


Figure 5. Removal of outer mitochondrial membrane (OMM) proteins and OMM rupture are not required for capture of mitochondria in autophagosomes. (A - D) Autophagosomes imaged by confocal microscopy in HeLa cells stably expressing YFP-LC3, mCh-Parkin, and endogenously tagged MFN2. Mitophagy was activated with CCCP treatment for 2 hrs with or without block of OMM protein removal by VCP inhibitor NMS-873 (pre-treated for 30 min prior to addition of CCCP for 2hrs). White boxes in (A) show the areas magnified in (B) and (C). Yellow arrows in (B and C) point to autophagosomes, which have low MFN2-Halo levels due to Parkin degradation in CCCP treated (B) but partially retained MFN2-Halo in cells with NMS-873 co-treatment (C). (D) Graphs quantify MFN2-Halo and YFP-LC3 puncta measured from confocal images. Puncta were detected automatically from confocal images using a custom script based on PunctaFinder⁶⁶. Datapoints represent confocal fields, which were measured from N = 4 total replicates separated over two occasions. (E) Graphs represent flow cytometry data of MFN2-Halo intensity (left) and mitophagosome accumulation (right). The left graph used the same line as in (A - D), while the right graph used a HeLa line stably expressing YFP-Parkin and mt-Keima. Datapoints represent 8 total replicates per condition separated over two occasions. Statistical testing in (D and E) was performed using Welch ANOVA testing followed by a Dunnett's T3 multiple comparisons test. (F - H) Mitophagy events from the HeLa YFP-LC3 line used in (A - D) were imaged by correlative light-EM microscopy and serial sectioning following 2 hr CCCP treatment and compared to mitophagy events in the C10 SL mouse heart. Boxes in (F) indicate the areas magnified in (G) imaged across serial sections, and boxes in (G) show areas magnified in (H). White arrows in (G) show areas in mitochondria where intact OMM and IMM are visible. Green arrow indicates a mitolysosome connected to engulfed mitochondrion. The serial section number is indicated in the upper right of each image. Scale bars in F of HeLa cells, 1 μ m. Scale bars in F of mouse heart, 500 nm. Scale bars in G, 500 nm. (H) shows images and line profiles through the autophagosomal and adjacent

mitochondrial membranes, comparing the autophagy intermembrane space (aIMS), mitochondrial intermembrane space (mIMS), and contact site distances. In addition to the magnified image, an inverted image, which was used for profile measurements, is shown together with the location of the linescan. Scale bar, 50 nm. (I) Quantification of mitophagy events from the HeLa YFP-LC3 line used in (A – D) treated with CCCP for 2 hrs. A total of 38 mitophagophore and 42 mitophagosome events were counted from 2 replicates, performed on the same occasion. One replicate also was used for the correlative images shown in (F – H).

Figure 6

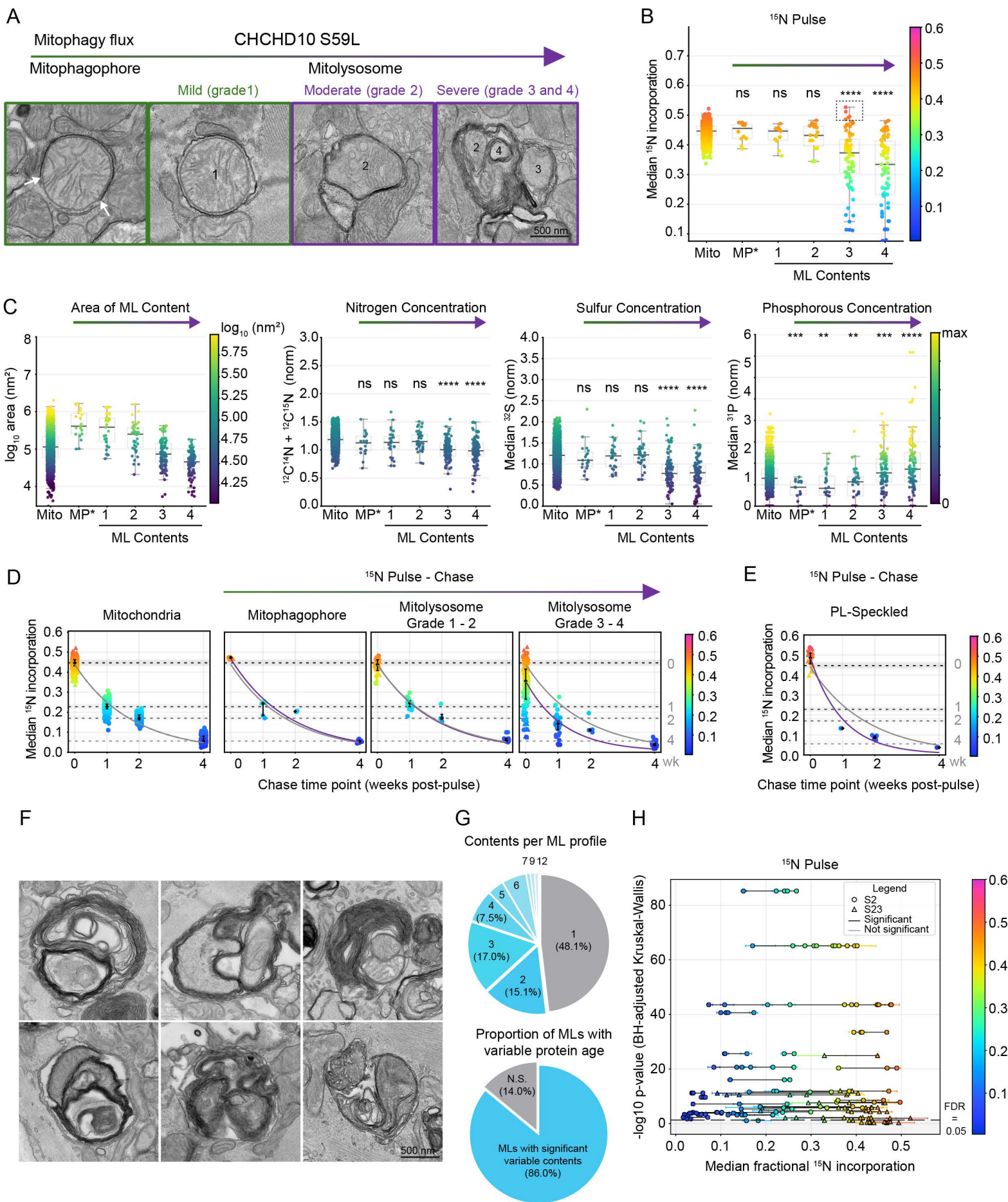


Figure 6. MitoFLUX-EM demonstrates mitochondria are actively and asynchronously degraded in multicompartmented MLs. (A) Representative electron micrographs showing mitochondria with successive grades of ultrastructural degradation (1 – 4), within mitophagic structures of increasing complexity in C10 SL hearts. Mitophagophores in the graphs include all events with incomplete APL membrane wrapping, without regard to whether they are apparently isolated from a ML or not. Scale bar, 500 nm. (B) ^{15}N fractional incorporation of unsequestered mitochondria, mitophagosomes, and sequestered mitochondria grouped by grade of ultrastructural degradation. Dotted box indicates a subpopulation of grade 3 ML contents with higher median ^{15}N fractional incorporation than low grade contents. Non-eroded ROIs were used for measurements to include the maximum number of events. (C) Additional measurements of events in (B) including area, total nitrogen ($^{12}\text{C}^{14}\text{N} + ^{12}\text{C}^{15}\text{N}$), sulfur (^{32}S), and phosphorous (^{31}P). Statistical testing for (B and C) was performed using the non-parametric Kruskal-Wallis test followed by post-hoc Mann-Whitney U tests with Holm p-value correction (each group was compared to unsequestered mitochondria for pairwise comparisons). One datapoint was higher than the ^{31}P plotting area and was excluded. Non-eroded ROIs were used for measurements to include the maximum number of events. (D and E) Pulse-chase trends for the events in (B) or, for comparison, primary lysosomes with a speckled morphology (PL-Speckled) (E). Purple line shows the trend for the organelle analyzed in comparison to gray trendline for unsequestered mitochondria. PL-Speckled have faster turnover than mitochondria as expected, whereas the ^{15}N turnover of early sequestered mitochondria is similar to unsequestered mitochondria. (F) Representative electron micrographs of multi-compartmented MLs, showing sequestered mitochondria with different grades of ultrastructure degradation. The two MLs on the left occurred in the same field. Scale bar, 500 nm. (G) Charts represent the number of ML compartments detected in cross-section (top) and the proportion of multi-compartmented MLs with significantly different ^{15}N fractional incorporation among compartments, based on testing

described in (H). (H) Chart shows median incorporation for contents for each multi-compartmented ML in the pulse dataset. Datapoints show the median incorporation of each content while whiskers show the interquartile range. Contents belonging to a ML are connected by a black line. Above the gray area and dotted line shows the cutoff for significance. For statistical analysis, the distribution of measurements for each contents of the same ML were compared to each other using Kruskal-Wallis test, with correction of multiple comparisons performed using the Benjamini-Hockberg false discovery rate procedure. Non-eroded ROIs were used for measurements to include the maximum number of events.

Figure 7

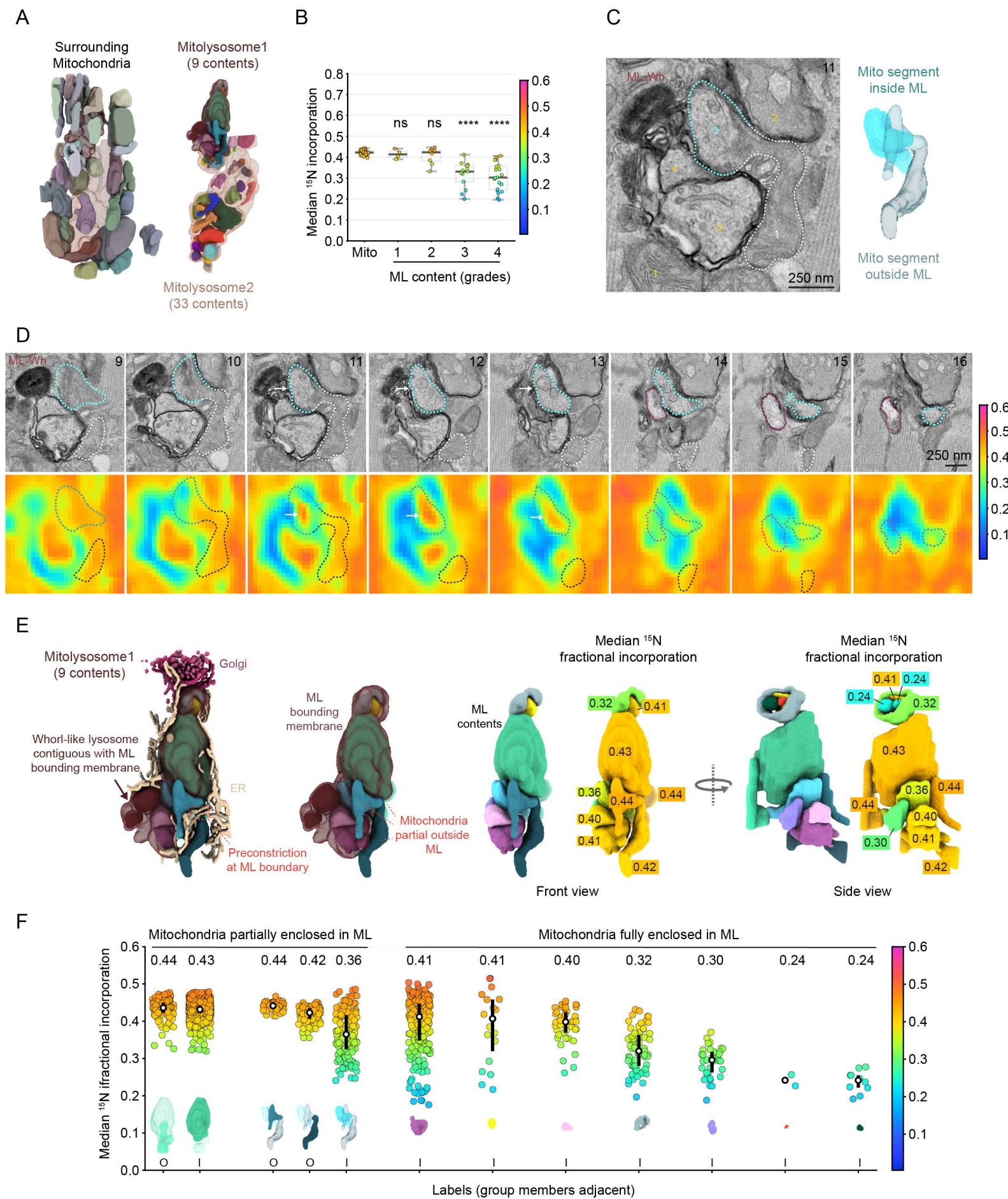


Figure 7. Volumetric MitoFLUX-EM reveals sequestered mitochondria of varying protein age in separate compartments of a mitolysosome. (A) Volumetric reconstructions are shown for two adjacent mitolysosomes (right) and surrounding mitochondria that were segmented from 22 aligned TEM sections. (B) Median incorporation for each of the sequestered and unsequestered mitochondria was measured from the segmented volume for each object. Volumetric ROIs were eroded by 50% prior to measurement to minimize contribution of edge voxels. Statistical testing was performed using the non-parametric Kruskal-Wallis test followed by post-hoc Mann-Whitney U tests with Holm p-value correction. Eroded ROIs were used to minimize edge pixels. (C) Representative TEM section (left) and volumetric reconstruction (right), showing a mitochondrion in the process of microautophagy by an enfolding mechanism, which is partially outside (blue dotted line) and partially inside the ML (white dotted line). The neck of the mitochondrion is constricted by ER (e) and a t-tubule (t). A ML-Whorl component of the ML has electrodense membranes that are apparently contiguous with those that form the compartments of the ML. Numbers indicate grading of the ultrastructure; the portion of the mitochondrion inside the ML has substantially degraded ultrastructure (grade 3) compared to the portion outside (grade 1). Scale bar, 250 nm. (D) Representative serial sections of aligned TEM and NanoSIMS images through Mitolysosome 1, including the section shown in (C). A hot spot of elevated ^{15}N fractional incorporation (arrow) co-localizes with a region of degradation in the sequestered portion of a mitochondrion undergoing microautophagy. Serial section number is indicated in the upper right corner of each image. The full series of sections is shown in Extended Figure 6. Scale bar, 250 nm. (E) Volumetric reconstruction of Mitolysosome 1 shown in relationship to surrounding organelles. It is additionally recolored according to the median ^{15}N fractional incorporation of each mitochondrion sequestered within the ML, highlighting their variable protein ages. (F) Graph shows individual ^{15}N fractional incorporation values for voxels corresponding to mitochondria sequestered within Mitolysosome 1. Portions of

two partially sequestered mitochondria that are inside (I) or outside (O) the ML are graphed separately. Eroded ROIs were used to minimize edge pixels.

Figure 8

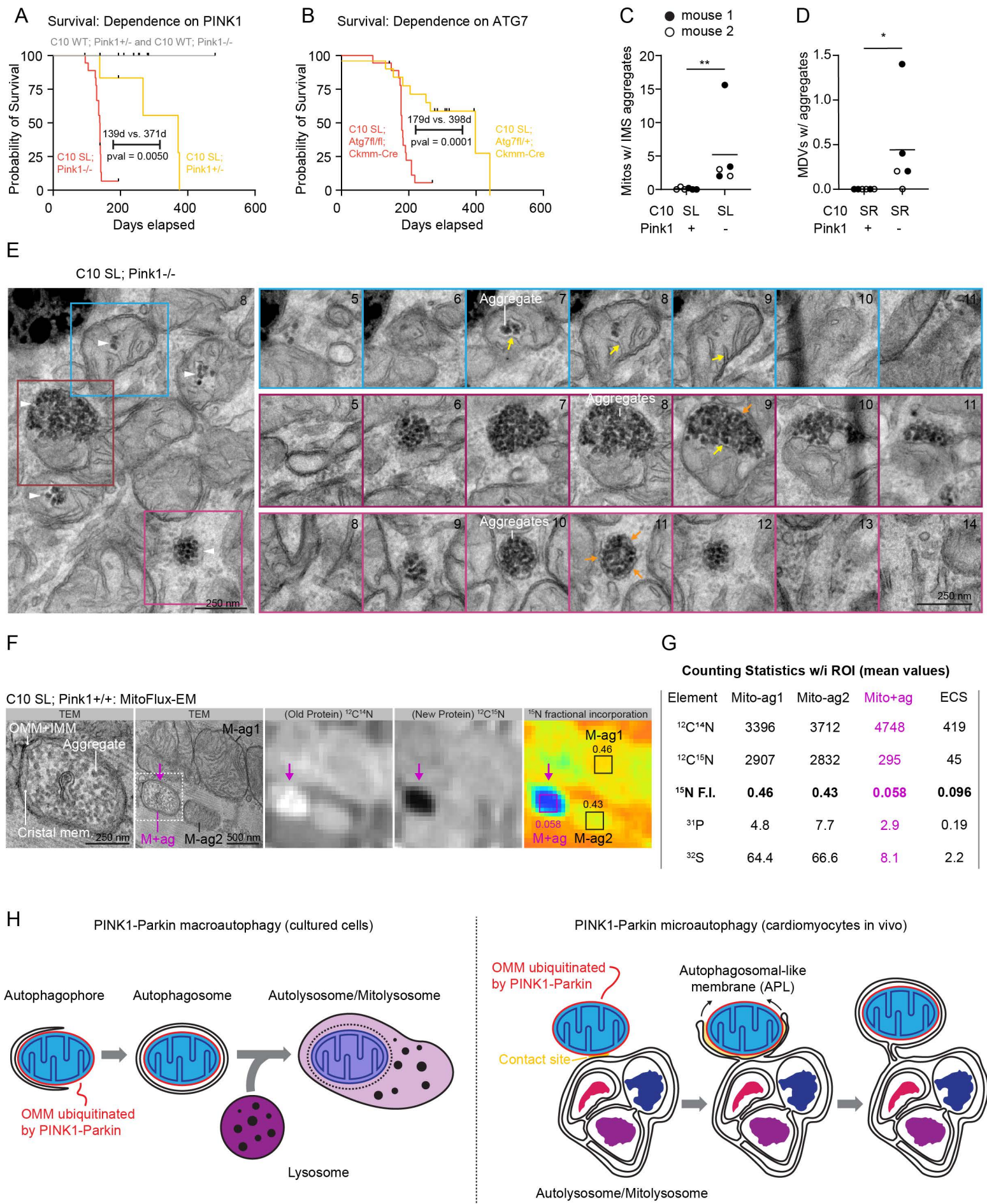
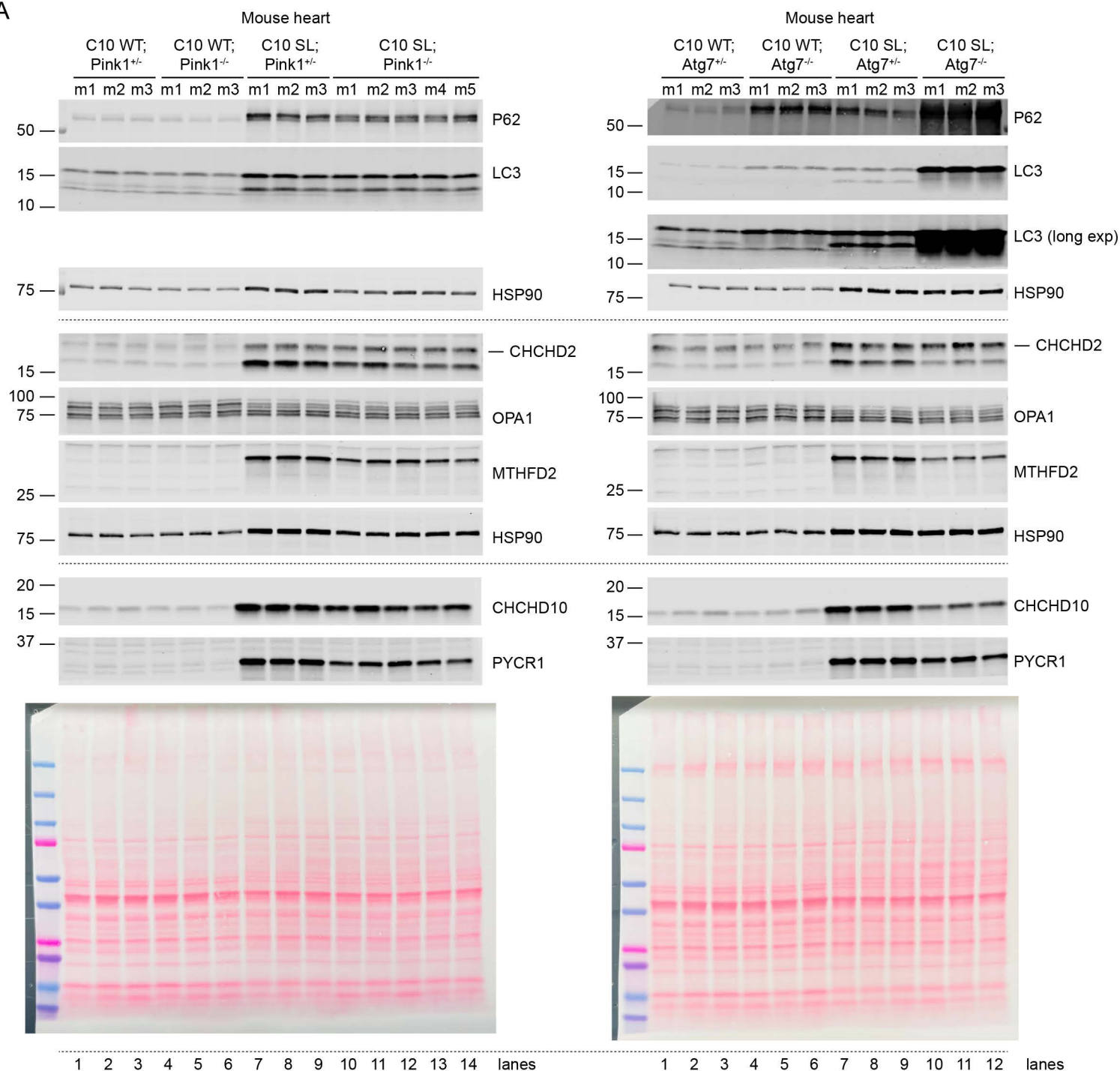


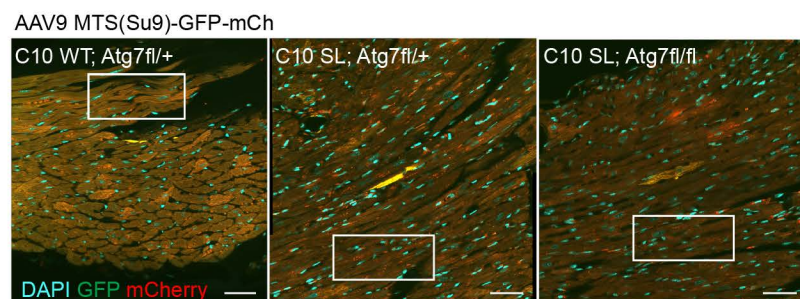
Figure 8. PINK1-mitophagy prevents accumulation of mitochondrial protein aggregates and is required for survival in C10 SL mice. (A) Survival of C10 SL mice with or without one functional copy of *Pink1*. Tick marks represent age of animals still alive at the time of census. (B) Survival of C10 SL mice with or without one functional copy of *Atg7*. Tick marks represent age of animals still alive at the time of census. Statistical testing in (A and B) was performed using the log-rank (Mantel-Cox) test. (C and D) Quantification of IMS aggregates (C) and mitochondrial derived vesicles containing IMS aggregates (D) in electron micrographs. Two mice were analyzed per genotype. Datapoints reflect the average number of events per field in each heart area examined. Analysis was performed by a rater blind to genotype. Statistical testing was performed using the Mann Whitney U test. This is graphed from the same dataset that was used for the graph that appears in (Figure 1E and F). (E) Serial TEM images C10 SL; *Pink1*^{-/-} hearts obtained from one of the samples used in (C) Mitochondrial intermembrane space protein (IMS) aggregates were frequent (white arrowheads). Yellow arrows indicate IMM, and orange arrowheads indicate OMM, showing the aggregates are confined to the intermembrane space Serial section number is indicated in the upper right corner of images. Scale bar, 250 nm. (F) A mitochondrion with IMS aggregates identified in the 2-week pulse MitoFlux-EM dataset. ¹⁵N fractional incorporation into the mitochondrion with IMS aggregates (M+ag) is compared to two adjacent mitochondria without aggregates (M-ag1 and M-ag2). Scale bar, 250 nm. (G) Table shows the mean counts for the 9-pixel ROIs shown in (F) in comparison to a measurement taken from the extracellular space (ECS) within the same field. (H) Model compares the canonical PINK1-Parkin macroautophagy pathway in cultured cells to the novel PINK1-Parkin microautophagy pathway described here, in which damaged mitochondria marked by the PINK1-Parkin pathway are directly captured by pre-existing mitolysosomes through the protrusion of autophagosomal-like membranes.

Extended Data Figure 1

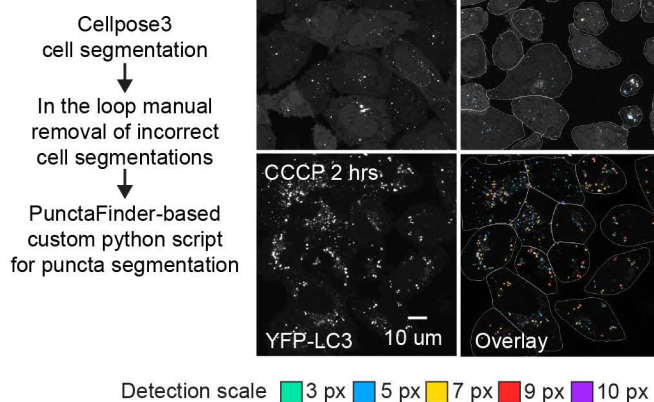
A



B

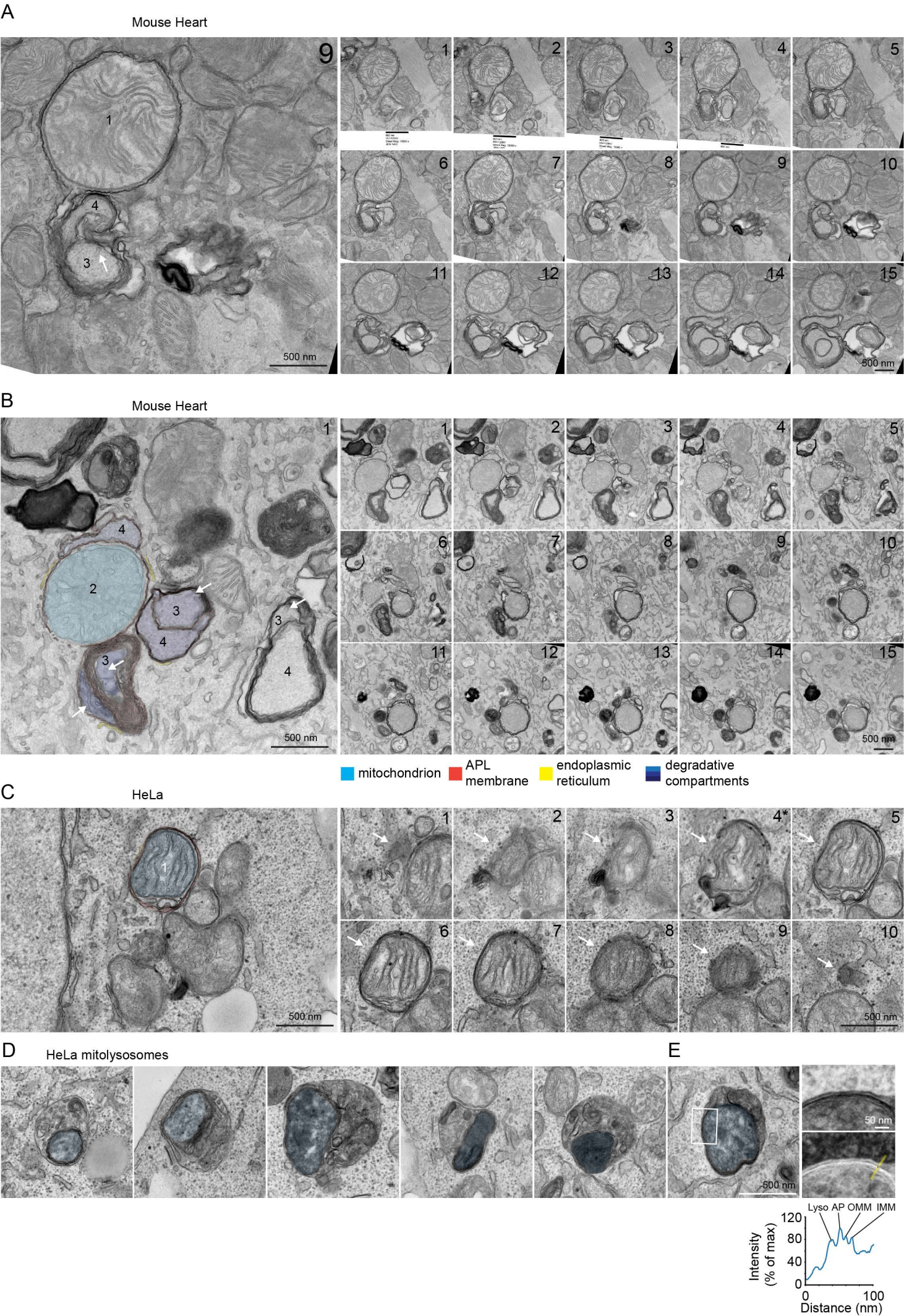


C



Extended Data Figure 1. LC3-II and P62 are elevated in C10 SL hearts and LC3-II conjugation is blocked by ATG7 KO. (A) Immunoblot of C10 SL hearts lysates with Pink1 KO (left) or *Atg7* KO (right). CHCHD10 and CHCHD2 accumulate in hearts of C10 SL mice. LC3-I and LC3-II are elevated in C10 SL, but LC3-II is blocked by *Atg7* KO. OPA1 is cleaved by OMA1 to a similar extent in C10 SL cells but expression of integrated stress responsive genes MTHFD2 and PYCR1 genes are reduced in the absence of PINK1 or ATG7. CHCHD2 and CHCHD10 levels are also moderately reduced in PINK1 and *Atg7* KO heart lysates. (B) Full size confocal image which also appears in (Figure 1A) shows the location of the magnified fields in (Figure 1A). (C) Pipeline for quantification of YFP-LC3 puncta. Cells were first identified using Cellpose3. Subsequently puncta were identified across different sized detection scales, using a custom python script based on PunctaFinder⁶⁶. Images show YFP-LC3 signal for cells after treatment with CCCP or DMSO for 2 hrs together with the puncta automatically identified with the PunctaFinder-based script.

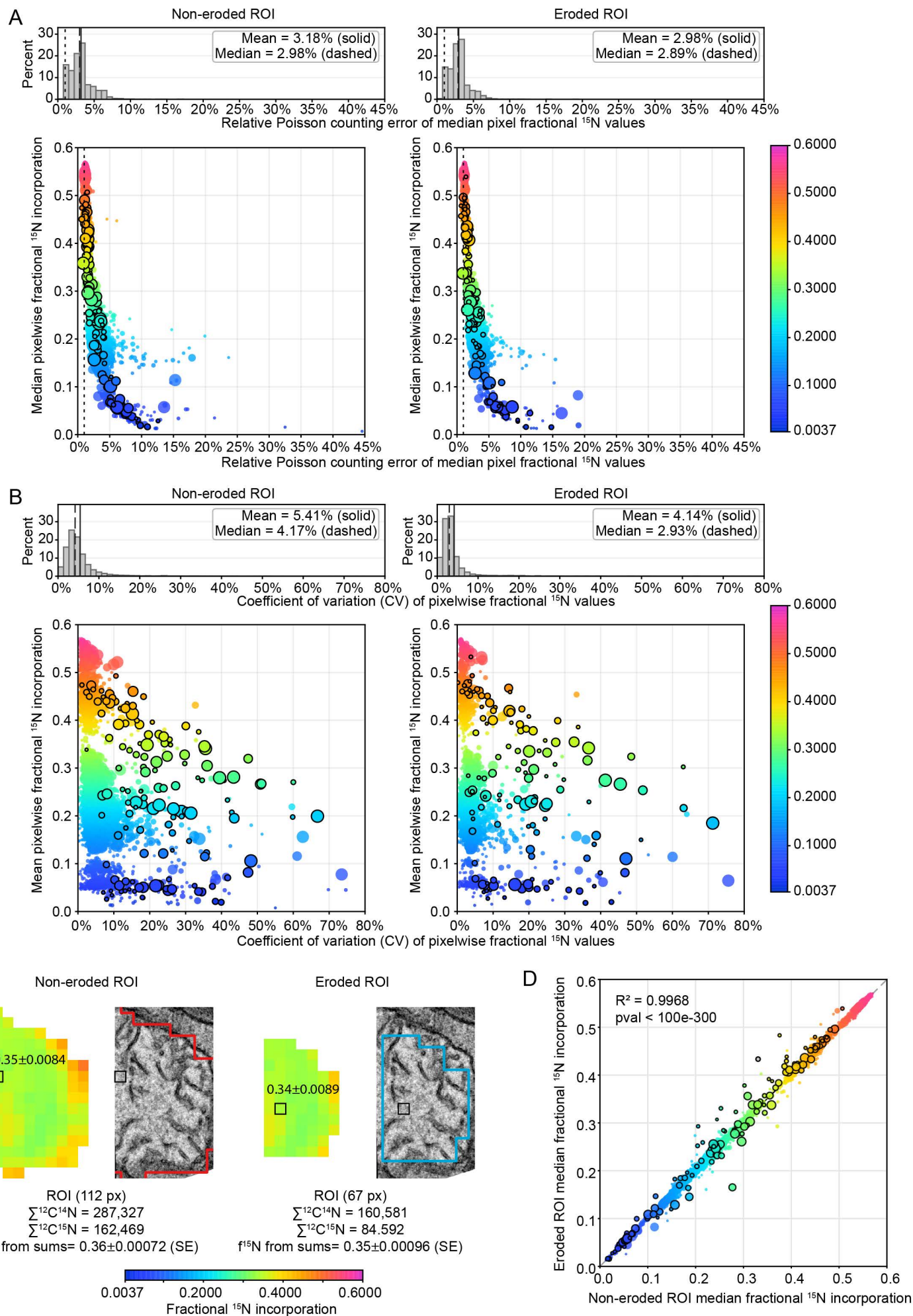
Extended Data Figure 2



Extended Data Figure 2. Serial section EM of mitophagy in heart vs. cultured HeLa cells. (A and B)

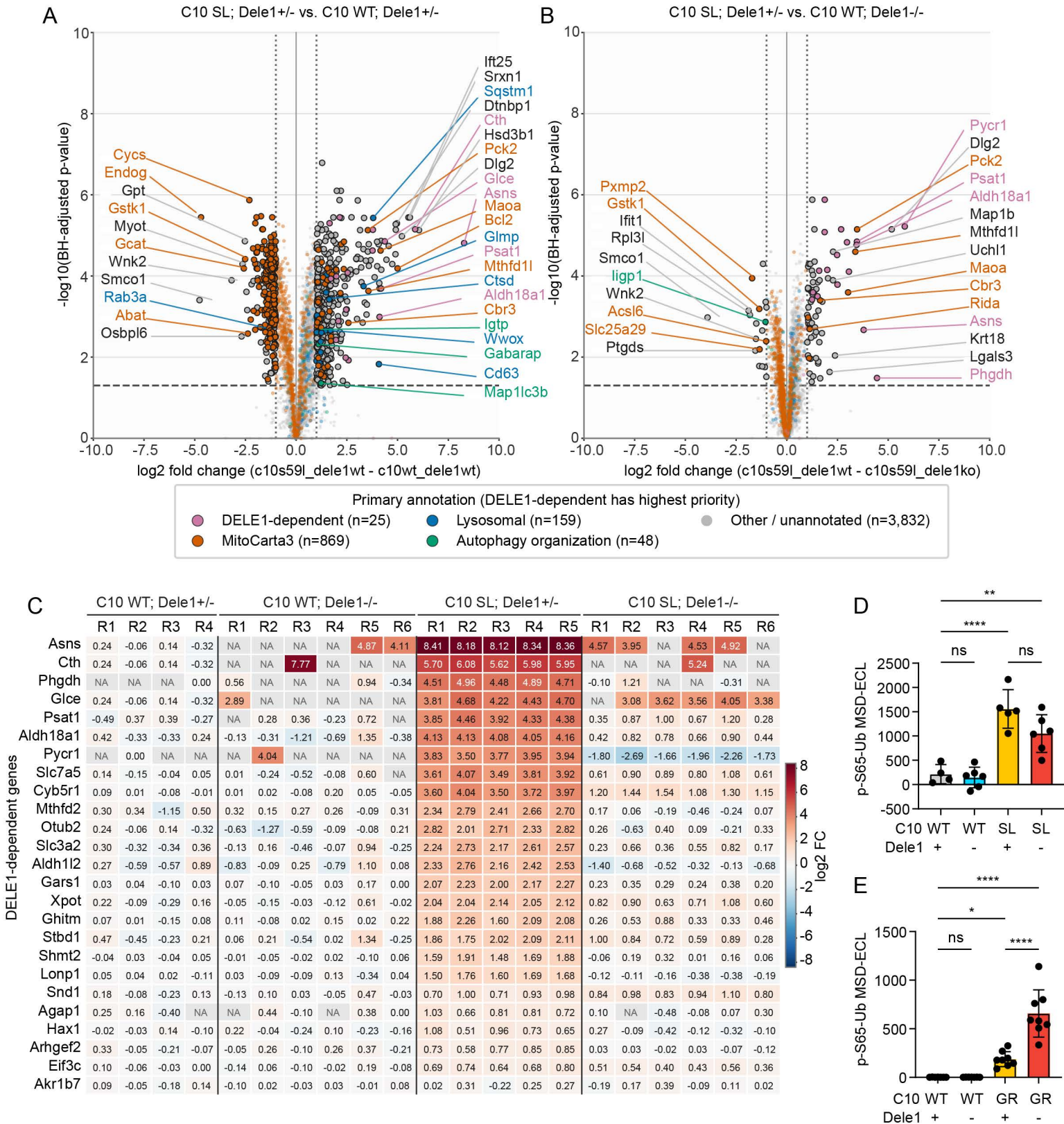
Serial sections through mitochondria in the process of being captured by APL membranes in C10 SL mouse hearts. In each case, these were contiguous with the membranes of complex MLs that contained multiple compartments, each of which contained mitochondrial remnants at different stages of ultrastructural degradation. The grade of ultrastructural degradation of the engulfed mitochondria is indicated; Grade 1: normal; Grade 2: mild; Grade 3: severe with cristae remnants; Grade 4: severe with only granular material. Scale bars, 500 nm. (C) EM serial sections show that in HeLa cells, autophagosomes are not connected to lysosomes. Serial section numbers are indicated in the upper right of each image. The asterisk on section 4 indicates this section was imaged at a lower magnification than the other sections (1.8nm/px v. 0.9 nm/px). Scale bar, 500 nm. (D and E) TEM images of mitolysosomes in HeLa cells. Putative engulfed mitochondria are shaded dark blue and the ultrastructural degradation grade is indicated as above. In some cases (E), the limiting membrane of the lysosome as well as an apparent electrodense autophagosomal membrane, IMM and OMM could be resolved. The insert from the boxed area is enlarged to show layers of membrane at the edge of the mitolysosome. In addition to the magnified image, an inverted image, which was used for profile measurements, is shown together with the location of the linescan through the area showing intensity peaks at each membrane. Scale bar for mitolysosome images, 500 nm; scalebar in linescan insert, 50 nm.

Extended Figure 3



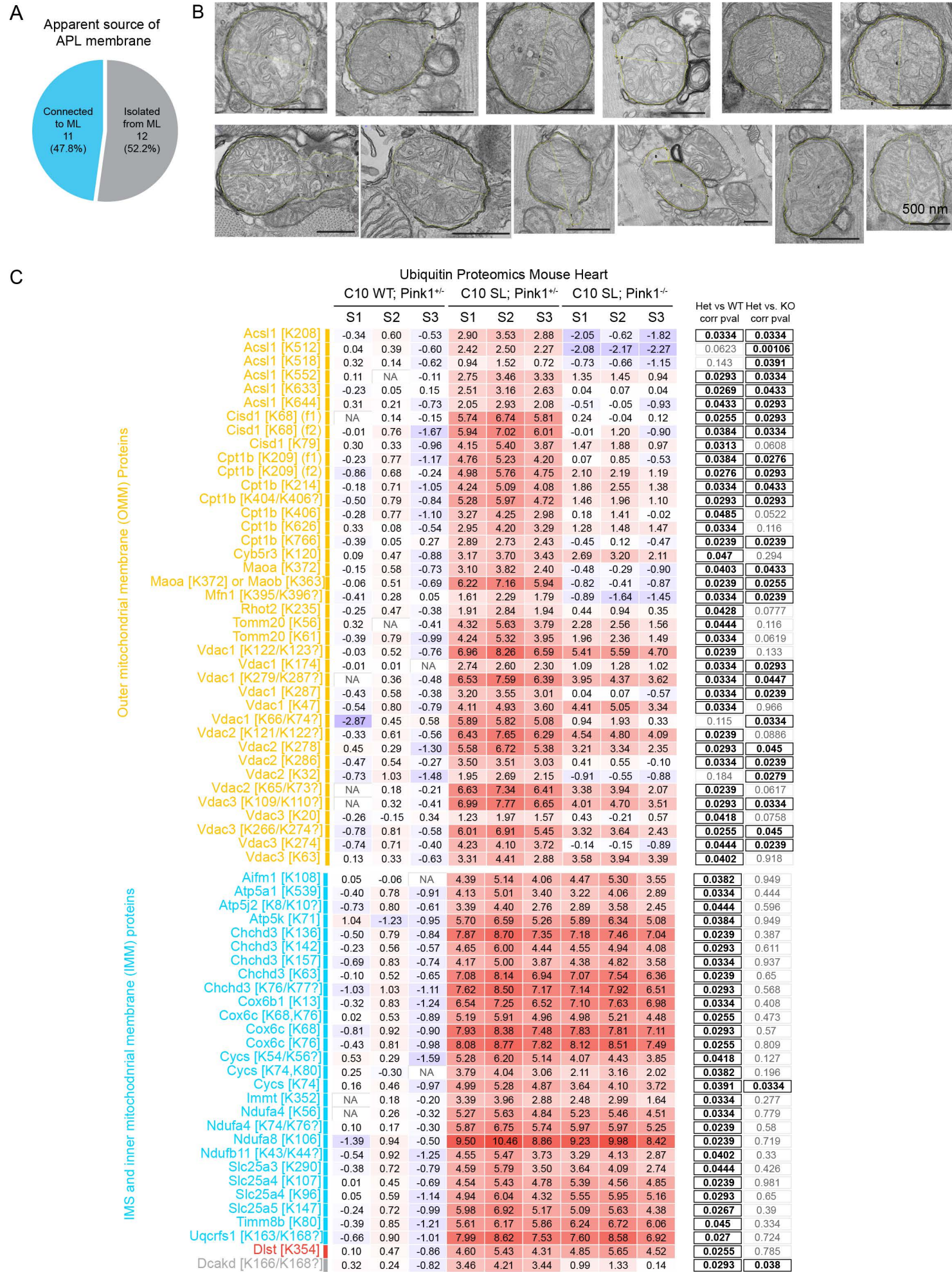
Extended Data Figure 3. Validation and sensitivity analyses of MitoFlux-EM measurements from segmented organelles in 2D dataset. (A) Graphs show the relative poisson counting error for the ^{15}N fractional incorporation value for the median pixel within each organelle ROI for both the non-eroded and eroded ROIs. (B) Graphs show the coefficient of variation of pixel-wise ^{15}N fractional incorporation values within each non-eroded and eroded organelle in the 2D dataset. (C) Example shows a mitochondrion with pixel-wise measurements within the non-eroded and eroded ROI. The ^{15}N fractional incorporation values are similar whether derived from the summed ^{14}N and ^{15}N counts or the median pixel-wise ^{15}N fractional incorporation value. They are also similar whether the non-eroded or eroded ROI is used. (D) Graph demonstrates that ^{15}N fractional incorporation values derived from non-eroded or eroded ROIs are tightly correlated.

Extended Figure 4



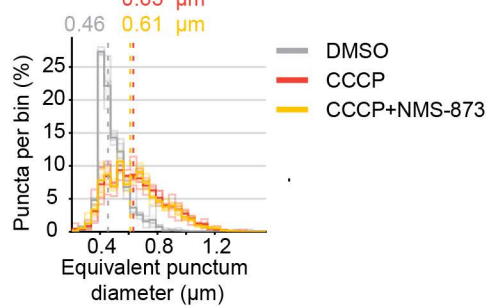
Extended Data Figure 4. Dele1-dependent mt-ISR is not required for PINK1 activation *in vivo*. (A and B) Volcano plots comparing proteome of C10 SL heart lysates with or without one copy of *Dele1*, graphed from label free quantification proteomics data. Genes previously identified as Dele1-dependent based on transcriptomics in mouse hearts are colored pink³⁵. These were increased by C10 SL when Dele1 was present (A) but not when it was absent (B). 4 – 5 samples were analyzed per group. These heart samples were used in a previous publication³⁵. (C) Heat map of same data shown in (A and B) highlighting the Dele1-dependent gene subset. (D) The specific PINK1 substrate pS65-Ub was measured by ELISA from the same heart lysates analyzed by proteomics in (A - C). (E) pS65-Ub was measured in heart lysates from C10 GR mice with or without one copy of *Dele1*. These heart samples were used in a previous publication³⁵.

Extended Figure 5



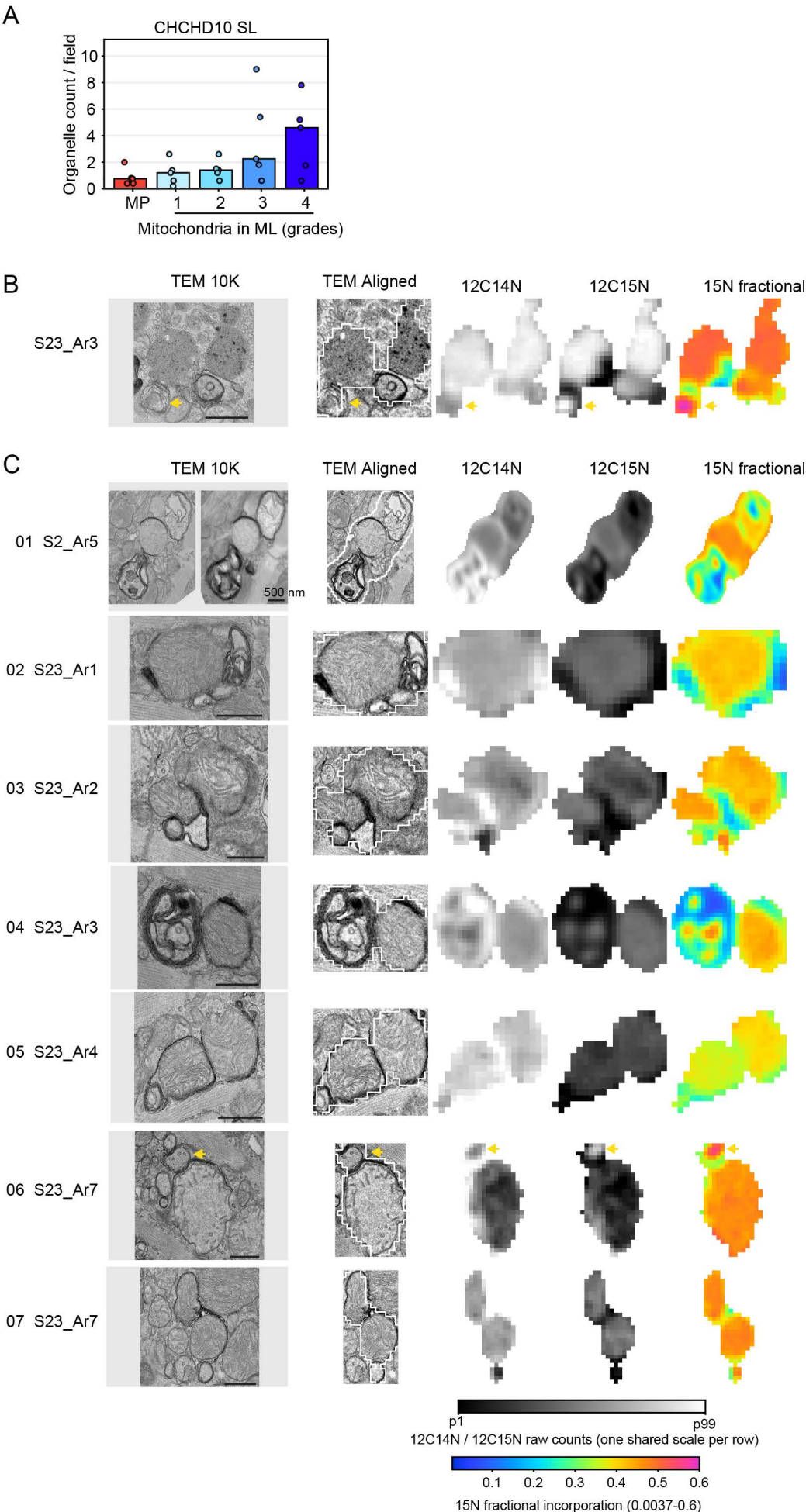
Extended Data Figure 5. Autophagosomal-like membranes preferentially bind regions with intact OMM on partially ruptured mitochondria and OMM ubiquitination sites are preferentially dependent on PINK1 expression. (A) Chart shows the proportion of events in which the autophagophore-like membrane contacting a partially wrapped mitochondrion was connected to an existing mitolysosome in the MitoFLUX-EM 2D dataset. (B) Electron micrographs of partially ruptured mitochondria measured for data shown in (Figure 4D). Overlaid are the traced ruptured OMM, APL membrane, and centroid. Lines connecting the midpoints of the OMM rupture to the centroid and the APL membrane to the centroid are shown. Angles between these lines are reported in (Figure 4D). Scale bars, 500 nm. (C) Heat map shows $\log_2$ fold changes relative to the control group (C10 WT; Pink1^{+/-}) for all differentially regulated diGly peptides detected in the ubiquitin proteomics experiments that also appears in (Figure 4F and G).

A



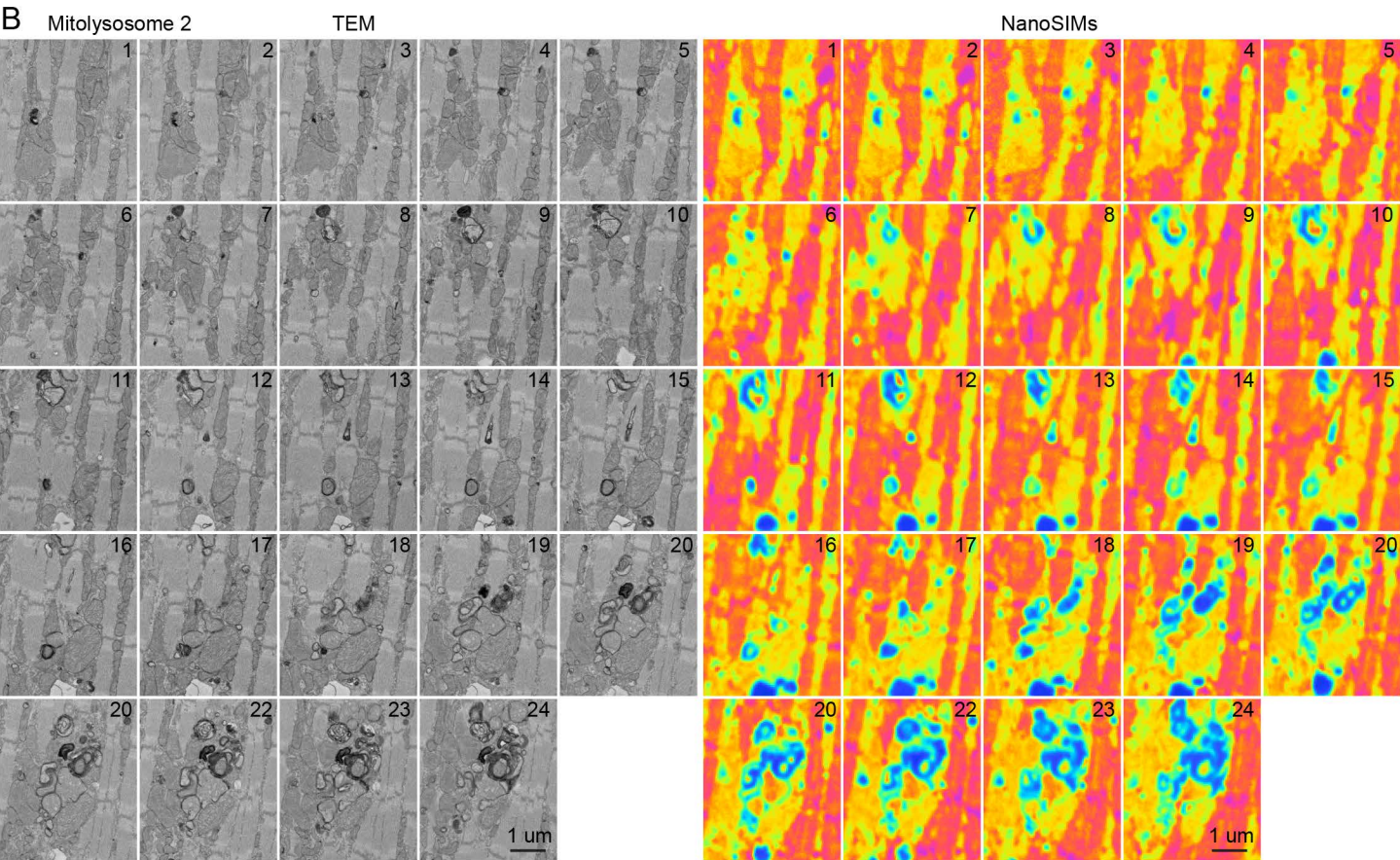
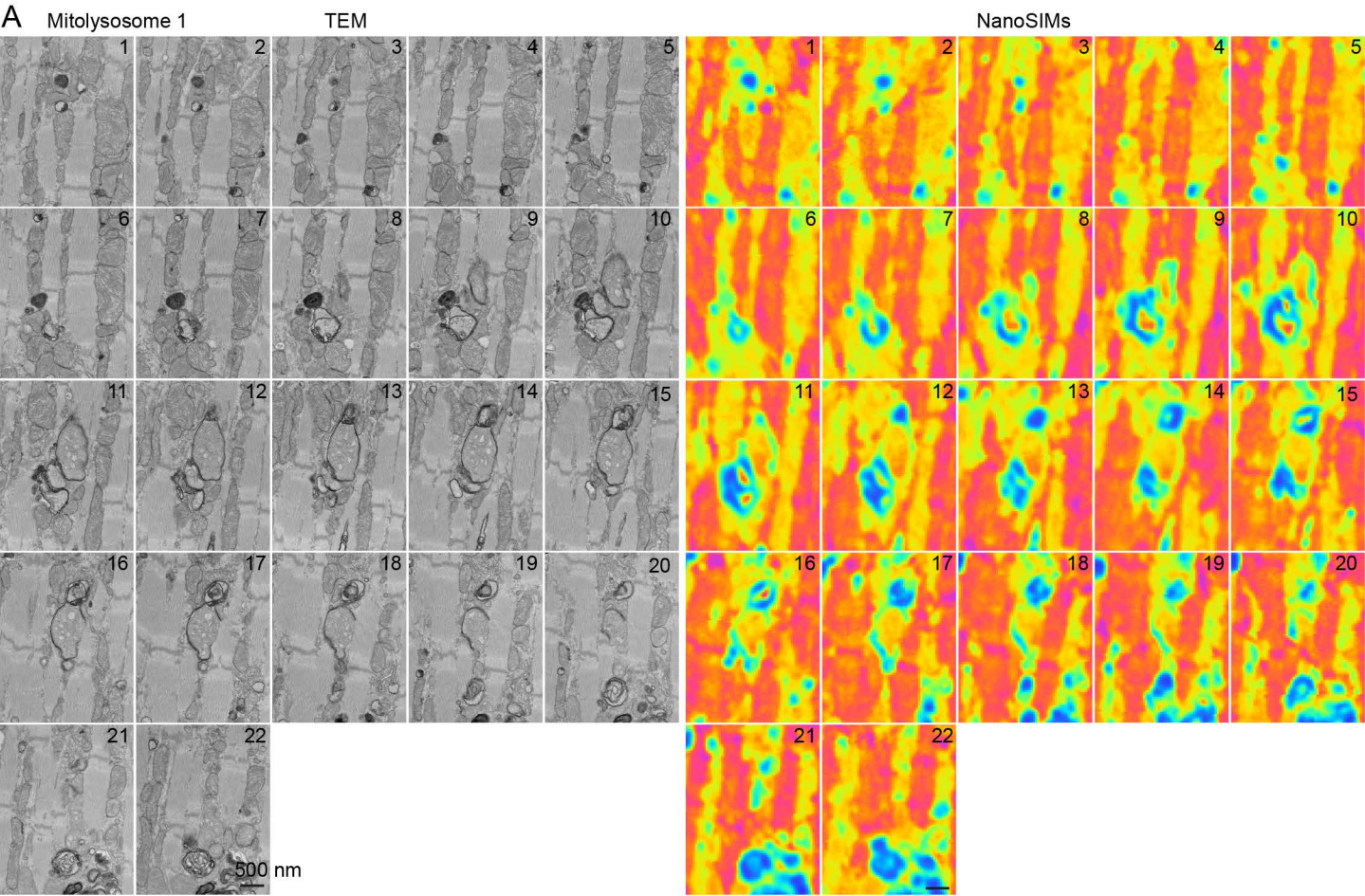
Extended Data Figure 6. Autophagosomal-like membranes preferentially bind regions with intact OMM on partially ruptured mitochondria and OMM ubiquitination sites are preferentially dependent on PINK1 expression. (A) Comparison of total protein abundance for differentially ubiquitinated proteins that localize the OMM vs. those that do not localize to the OMM, as defined by the experiment shown in (Figure 4F and G). Total protein abundance from soluble and insoluble fractions were derived from the same experiment shown in (Figure 2G and H) by summing the light and heavy peptide ion intensities for each peptide. (B) Confocal images show a parallel experiment as shown in (Figure 5A-D), in which the Halo ligand was omitted and the cells were immunostained for the mitochondrial marker cytochrome c after fixation. This demonstrates that the YFP-LC3 puncta generally form around mitochondria. This co-localization experiment was N = 1. (C) Parkin recruitment was measured as the percent of cell area with high mC-Parkin intensity. For each replicate, the mC-Parkin intensity was determined as the average threshold for all CCCP treated cells in the replicate calculated using the Otsu method. Parkin recruitment was increased with CCCP (C) as expected compared to vehicle (D) and was not affected by VCP inhibition with 20 μ M NMS-873 (C+N). (D) Large YFP-LC3 puncta (≥ 7 pixels in equivalent diameter) were increased with CCCP treatment and were further increased with CCCP + VCP inhibitor co-treatment. These measurements are from the same samples shown in (Figure 5A-D). (E) Histogram shows the average equivalent diameter of YFP-LC3 puncta for the samples shown in (Figure 5A-D).

Extended Figure 7



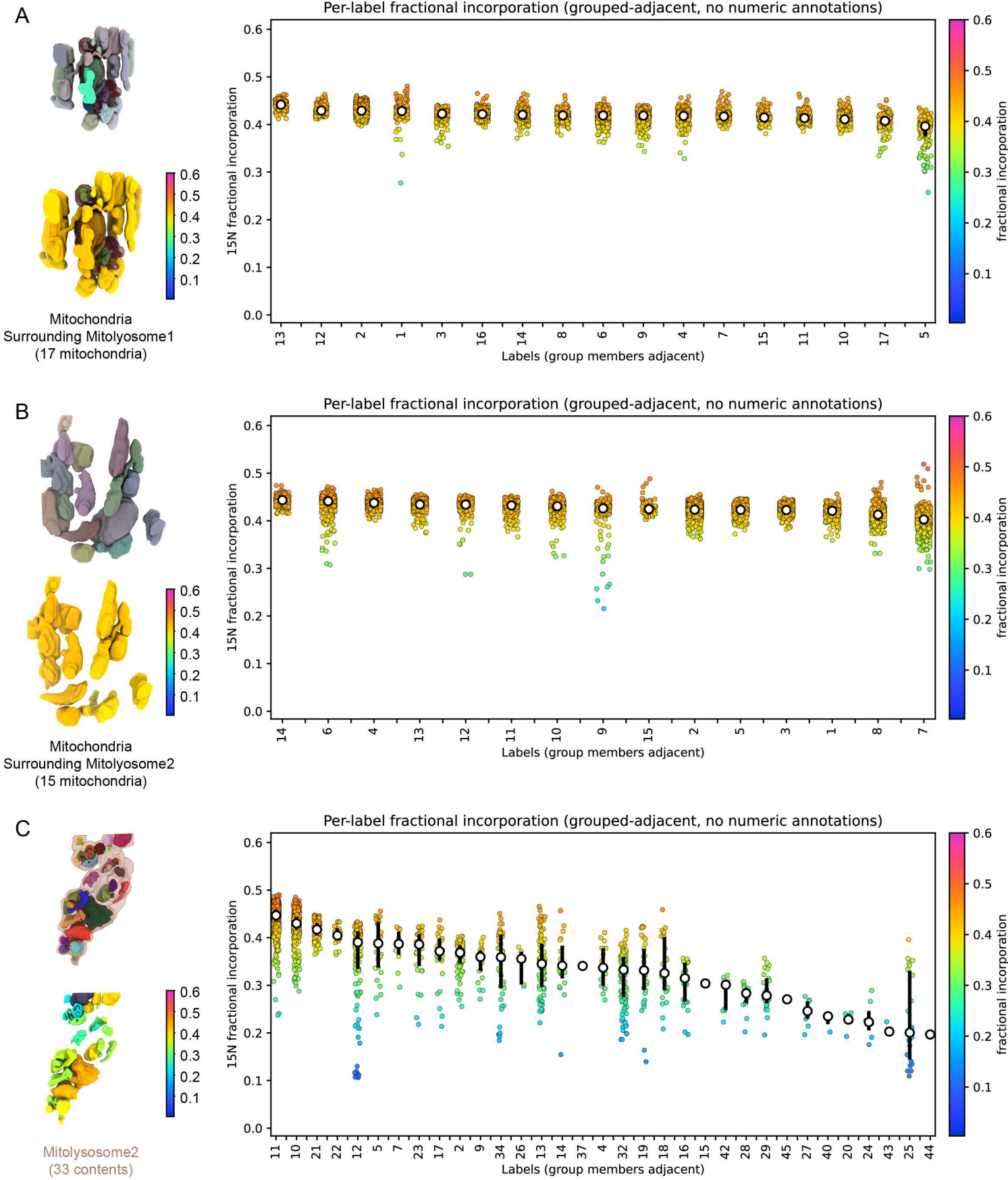
Extended Data Figure 7. Autophagosomal-like membranes are often connected to pre-existing mitolysosomes in C10 SL hearts. (A) Bar graph shows the frequency of sequestered mitochondria in the MitoFLUX-EM 2D dataset by grade of ultrastructural degradation. Partially wrapped events, mitophagophores (MP), are also shown. Datapoints correspond to heart samples from 4 C10 SL mice. (B) Correlated TEM and NanoSIMS of a mitolysosome containing a “hotspot” of ^{15}N fractional incorporation (yellow arrow) that co-localizes with a grade 3 mitolysosome-sequestered mitochondrion. Another hotspot is seen in an example in (C) also indicated by yellow arrow. The median value of these events is higher than that of “earlier” mitophagy events (grade 1 or 2 sequestered mitochondria or mitophagosomes), suggesting the introduction of newer protein, perhaps, from lysosomes. (C) Correlated TEM and NanoSIMS of all partially wrapped events from the 2D pulse dataset in which the autophagosomal-like membrane is connected to the ML in the profile.

Extended Data Figure 8



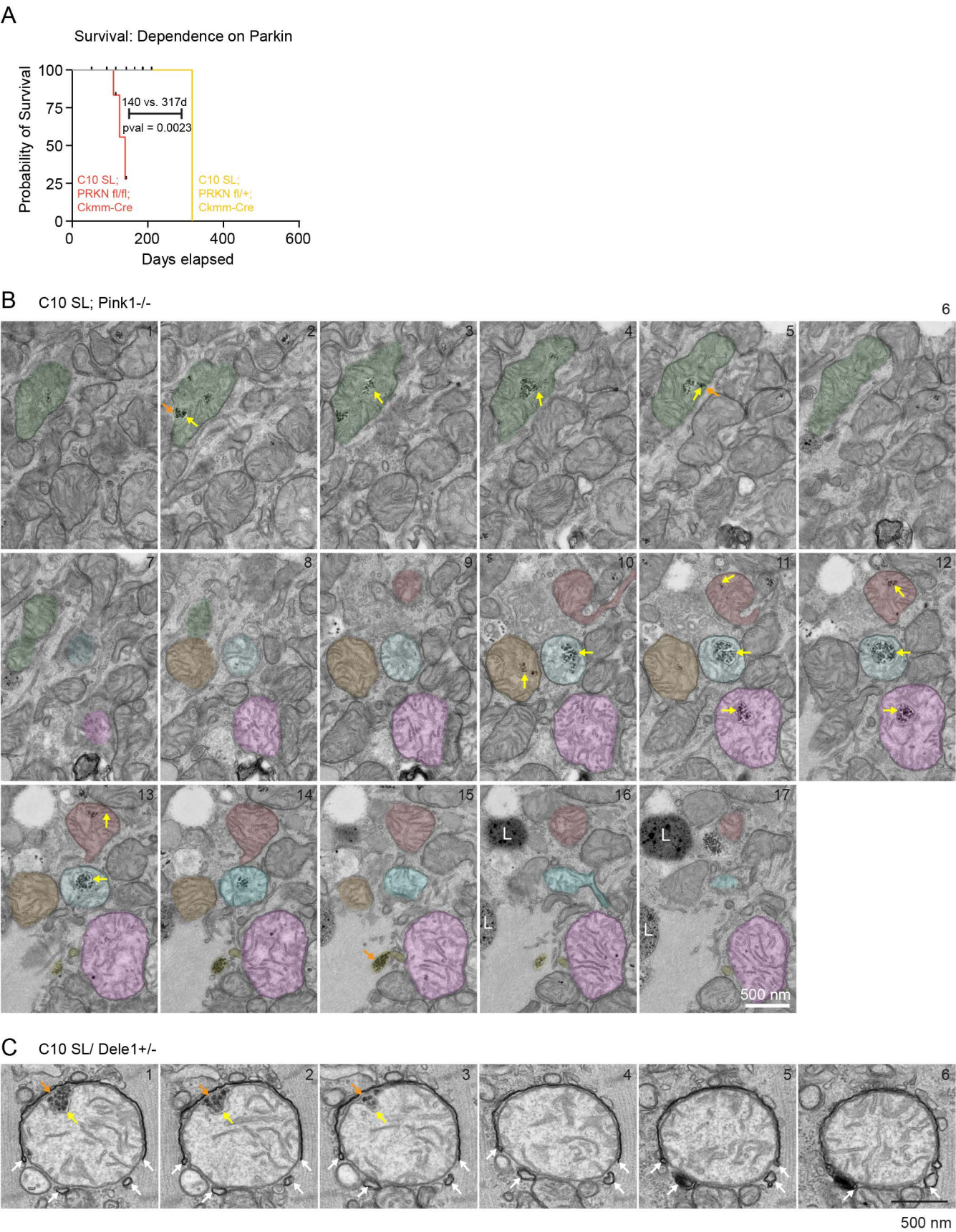
Extended Data Figure 8. Serial sections for volumetric MitoFLUX-EM analysis. (A) TEM serial sections acquired at 0.9 nm/px resolution of mitolysosome 1 (left) that were aligned to corresponding NanoSIMS serial section images (shown are the $^{15}\text{N}/^{14}\text{N}$ ratio images, right) to extract ^{15}N fractional incorporation data from the 3D volume. Scale bar, 500 nm. (B) TEM serial sections acquired at 3 nm/px resolution of mitolysosome 2 (left) that were aligned to NanoSIMS serial section images and used to extract ^{15}N fractional incorporation values from the 3D volume. Scale bar, 1 μm .

Extended Figure 9



Extended Data Figure 9. Volumetric MitoFLUX-EM reveals sequestered mitochondria of varying protein age in separate compartments of a mitolysosome in contrast to surrounding unsequestered mitochondria. (A - C) Graphs shows individual ^{15}N fractional incorporation values for voxels corresponding to mitochondria surrounding Mitolysosome 1 (A), mitochondria surrounding Mitolysosome 2 (B), and mitochondria sequestered within separate compartments of Mitolysosome 2 (C). To the left of each graph are volumetric reconstructions of the mitochondria represented in the respective graph.

Extended Figure 10



Extended Data Figure 10. C10 SL mice depend on Parkin for survival. (A) Survival of C10 SL mice with or without one functional copy of *Parkin* in their striated muscle due to muscle specific expression of Cre (Ckmm-Cre). Tick marks represent age of animals still alive at the time of census. (B) Serial sections from C10 SL; *Pink1* KO mouse showing color-shaded mitochondria containing aggregates within the IMS, bound by inner membranes (yellow arrows) and outer membranes (orange arrows). Serial section numbers are shown in the upper right corner of images. Lysosomes, L. Scale bar, 500 nm. (C) Serial TEM images of C10; *Dele1*^{+/-} heart shows rare example of mitochondrion with aggregates in the presence of PINK1. This mitochondrion is in the process of being sequestered by APL membranes (white arrows). Serial section numbers are shown in the upper right corner. Scale bar, 500 nm. The samples used in (C) are the same as was used in a previous publication ³⁵.