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**Broad activation of the ubiquitin–proteasome system by Parkin is critical for mitophagy**

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**Objectives**

Parkin, an E3 ubiquitin ligase, is a Parkinson’s disease related protein that functions to ensure mitochondrial integrity. Parkin migrates from the cytosol onto dysfunctional mitochondria and promotes mitochondrial degradation by mitophagy, a mitochondrial specific form of autophagy. Mitochondria are targeted for degradation when Parkin attaches a chain of the protein ubiquitin to the outer membrane. In flies with a Parkin mutation, the failure to degrade malfunctioning mitochondria led to their accumulation. Disease alleles in the Parkin gene have an impaired enzymatic function. Parkinson’s disease has been characterized by toxic accumumulation intracellular protein aggregates called Lewy bodies which were previously associated with Parkin activation of the ubiquitin proteosome system (UPS). Lewy bodies being the result of UPS malfunction. More recently studies have focused on Parkin’s role in activation of the autophagic pathway, specifically mitophagy.

Chan et al.’s objectives included monitoring the changes in a mitochondrial proteome in response to Parkin and to determine the proximal function of Parkin by clarifying its association to both the UPS and the autophagic pathway. The two pathways, the UPS which relies on K48-linked polyubiquitination (Lysine 48) and the autophagic pathway which is K63-linked (Lysine 63) lead to separate fates in the cell. The UPS, when activated by Parkin, results in mitochondrial outer membrane protein degradation. The K63-linked polyubiquitinization on the other hand recruits the machinery necessary for autophagy like the ubiquitin binding adapters HDAC6 and p62/SQSTM1.

**Approach and Results**

In order to illicit a Parkin-mediated response, carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used to depolarize the mitochondrial outer membrane. Using the stable isotope labeling by amino acids in cell culture (SILAC) analysis, a mass spectrometry approach, Parkin- expressing HeLa S3 cells treated with CCCP for 2 hours were analyzed. The results showed the most significantly altered protein levels in comparison with untreated cells. Out of 2979 different protein groups a total of 1013 mitochondrial proteins were characterized with a significance of < 0.01. Parkin expression was 13 fold that of non CCCP treated HeLa S3 cells. Autophagy-related proteins like NBR1, p62/SQSTM1, LC3 and GABARAPL2 levels were found to be elevated. The increased expression of proteins like V-type proton ATPase which are components of lysosomes and endosomes also indicates autophagy related protein increase. Proteins related to the UPS were also found to be increased (ubiquitin 9 fold). 26S proteosome’s subunit 20S and 19S proteins were shown to be increased. Many of the mitochondrial outer membrane proteins like Tom70 were shown to be significantly decreased also. This study focused on the outer membrane proteins, this being the site of interaction with Parkin. Out of the proteins quantified only 5% were from the mitochondrial outer membrane they totaled 20% of the proteins with reduced amounts. Mfn1 and Mfn2 mitochondrial fusion proteins and Miro1 and Miro2 mitochondrial transport proteins were also reduced. The mitochondrial fission factor DRP1 is a protein linked to mitochondrial fragmentation that was significantly increased. This indicates that Parkin prevents defective mitochondria from fusing to neighboring mitochondria.

Immunoblotting was used to elucidate Parkin’s role in mitochondrial outer membrane proteolysis. Cloned Parkin-expressing HeLa S3 cells were treated with 20 µm of CCCP or the vehicle and compared to parent HeLa S3 cells that do not express Parkin. CCCP was shown to cause degradation of Mfn1, Mfn2 and Tom70 within 1 hour in the Parkin-expressing cells while parent cells and non-CCCP treated cells showed no change. Several other proteins were shown to be degraded in a Parkin-dependent manner although at a slower rate. VDAC1 (solute transport), BAK (apoptosis), Fis1 (mitochondrial fission) and Tom 20(protein import) were all shown to be examples. This is noteworthy as none of the inter-membrane or matrix proteins were shown to be affected including cytochrome c and Hsp60. When incubated with the proteasome inhibitors MG132or epoxomicin, treatment with CCCP did not cause the proteolysis of any of the outer membrane proteins analyzed. This indicates Parkin function’s to mediate the degradation of depolarized mitochondrial outer membrane proteins is dependent on the UPS. The author has indicated similar results when valinomycin was used rather than CCCP to depolarize mitochondria. Similarly, treatment with the complex I inhibitor rotenone inhibited Parkin recruitment to depolarized mitochondria. This indicates that Parkin is probably activated exclusively by mitochondrial depolarization.

In a second immunoassay mouse embryonic fibroblasts (MEFs) were treated with either the DMSO vehicle or CCCP. Normal MEFs were compared with MEFs containing a knockout gene for Atg3. Atg3 is similar to E2 enzyme and is essential for At8/LC3’s conjugation to phosphatidylethanolamine. This conjugation is essential for autophagosome activation. The autophagosome pathway was defective in the mutant MEFs while as in the other trials Parkin-mediated mitochondrial outer membrane degradation proceeded in response to CCCP. Epoxomicin was also able to prevent this degradation as in HeLa S3 cells. This is indicative of Parkin-mediated outer membrane proteolysis being a result of the UPS and independent of autophagic activity.

The SILAC analysis revealed a 9-fold increase in K48 ubiquitin and a 28-fold increase in K63 ubiquitin after CCCP treatment. In a third assay immunoblot analysis was used to confirm these results using K48 and K63 specific antibodies. In non Parkin-expressing HeLa S3 cells, CCCP treatment led to no accumulation of K48 or K63 protein on mitochondria. On the contrary, in Parkin-expressing HeLa S3 cells CCCP treatment led to the accumulation of both polyubiquitin proteins on mitochondria. This was both Parkin and CCCP dependent and is representative of Parkin’s function of recruitment to depolarized mitochodria.

Because the SILAC data also showed an accumulation of 26S proteasome subunits, both the alpha and beta subunits were stained by anti-proteasome staining in separate assays. Alpha was stained with PSMA2 and Beta with PSMB5. Prior to CCCP treatment the subunits protein was diffused throughout the cells cytosol. Upon 4 hours of CCCP treatment there was distinct proteasome enrichment on mitochondria, again indicating that upon depolarization the UPS is activated in a Parkin-dependent manner.

To elucidate the temporal relationship between the proteolysis of mitochondrial outer membrane proteins and mitophagy, the proteins Tom20 (outer membrane protein) and Hsp60 (matrix protein) were compared via staining at both 4 and 24 hours. Two groups of HeLa S3 were compared as before, one receiving the DMSO vehicle and the other receiving CCCP. In the DMSO group mitochondria remained diffused throughout the cell cytosol during the entire assay. In the CCCP treatment group however after 4 hours mitochondria were divided into two distinct populations. One population formed aggregate clusters at the nuclear periphery. These mitochondria remained positive for both Tom20 and HSP60. The other group was mitochondria negative for Tom2o that were dispersed in the cell periphery. After 24 hours the CCCP treatment approximately 20% of these had complete loss of Tom20. Greater than 60% of these were still positive for Hsp60. Similarly, Parkin-expressing MEFs with the Atg3 knockout treated with CCCP did not undergo mitophagy but did show loss of Tom20. These findings indicate that loss of Tom20 is correlated with Parkin-mediated activation of the UPS and mitochondrial outer membrane proteolysis and is separate from the autophagic pathway.

In the dispersed population of mitochondria in the previous experiment LC3B, an autophagosome marker, was found to be associated at a rate of approximately 90% while absent from the aggregated mitochondria. In the next experiment proteasome inhibitor MG132 was used to prevent loss of Tom20 after 4hours of CCCP treatment. Parkin recruitment was unaffected. Without MG132 90% of CCCP treated cells showed 30 or more Tom20 negative dispersed mitochondria. In MG132 treated cells more than 90% had no dispersed Tom20 negative mitochondria. The same results were found with epoxomicin. In order to further exemplify the division between the UPS and mitophagy, cells and were subjected to a 100 minute CCCP pulse and then allowed to recover for 12 hours and 24 hours. At 12 hours 90% of cells had dispersed and fragmented mitochondria and at 24 hours approximately 30% of cells had complete mitochondrial loss. Pretreatment with MG132 inhibited mitochondria loss at both time frames. This indicates that Parkin-mediated autophagy is proteasome dependent.

Parkin expressing dopaminergic neuroblastoma cells SHSY-5Y also showed Tom20 negative dispersal while retaining Hsp60 after CCCP treatment. These cells also retained Tom20 when pretreated with epoxomicin.

In summary the authors have demonstrated that Parkin activation is triggered by mitochondrial depolarization. Parkin-mediated outer membrane proteolysis is a result of the UPS and independent of autophagic activity. Upon depolarization the UPS is activated in a Parkin-dependent manner. Loss of Tom20 is correlated with Parkin-mediated activation of the UPS and mitochondrial outer membrane proteolysis and is separate from the autophagic pathway and Parkin-mediated autophagy is proteasome dependent.

**Future Research**

Future research on mitophagy will require a matrix protein marker for accurate assessment. In the past Tom 20 has been used to indicate mitophagy but in light of the aforementioned properties of Parkin, a matrix protein would be a better indicator. Secondly, clarify the role of mitofusins in the autophagic pathway. Mitofusins have been referenced as sites of polyubiquitinization and degradation in a Parkin-related manner. These are blocked by proteosome inhibitors and the question is presented, are mitofusions critical for Parkin-mediated proteolysis? It has however, been shown that mitochondrial proteome remodeling is necessary for mitophagy. Does mitochondrial proteolysis by Parkin remove a negative inhibitor of mitophagy and if so what is it? Does Parkin mediated activation of UPS have other functions for mitochondria besides autophagy? Also why Atg-null cells were not tested for polyubiquitin proteins K48 and K63 activity? Lastly, the actual signal that causes mitochondria to disperse or aggregate was never elucidated it was only correlated with depolarization and loss of Tom20.