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**Molecular chaperone-mediated rescue of mitophagy by a Parkin RING1 domain mutant**

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**Objectives/Introduction:** Parkinson’s disease is one of the most common degenerative diseases, and one characteristic of Parkinson’s disease is the accumulation of damaged mitochondria by inhibiting the ubiquitin-protein ligase, Parkin which cells use to attempt to degrade the mitochondria by a process known as mitophagy, or autophagy to be exact in this instance. However, as is often the case, Parkin is not the only factor that can be influenced by Parkinson’s mutations; PINK1 is a protein kinase which has been shown to phosphorylate Parkin and mediate it to localize to damaged mitochondria. This study focuses on Parkin function. Contributing to Parkin function includes three specific domains along the Parkin sequence; one ubituin (Ubl) domain and two RING domains. The RING domains are located on the C-terminus, and the ubiquitin domain is oriented toward the N-terminus. The RING1 domain of Parkin is specifically in question, and subsequent experiments show that it is the RING domain, and not the ubiquitin domain which governs most of the function of Parkin to localize to damaged mitochondria,and in fact this explains why mutations in the RING domains would cause improper function of Parkin because the sequences within RING domains have major structural influences over protein folding. This localization of Parkin to the damaged mitochondria and subsequent perinuclear accumulations of the mitochondria can be induced by introducing certain uncoupling agents into the cell culture, because Parkin is stimulated to localize to damaged mitochondria which have had depolarization events occurring along the mitochondrial membrane. Mutations in the RING1 domain significantly reduce this function. This study aims to find a way in which the mutant form of Parkin (FLAG-Parkin (C289G)), with mutations in the RING1 domain, can regain the wild type function within the cellsby the assistance of particular chaperone proteins. Chaperones participate in proper folding of newly synthesized proteins and aggregation prevention of inclusion bodies; formed from mutated Parkin accumulations. The co-chaperone, DnaJ/Hsp40-like HSJ1, will be used to try and regain function of mutated Parkin, and it has one N-terminus J domain region and two C-terminus ubiquitin interacting motifs which are believed to play a role in proper binding to Parkin and thus facilitating chaperone activity. However, mutations in the HSJ1 ubiquitin domains still show some retained function, while mutations in J domains significantly abolish the activity of the chaperone.

**Experimental Approach and Results:** All of the experimental tests involved in this study, in general, involved the use of fluorescence microscopy, using staining techniques with MitoDsRed mitochondrial marker and also green fluorescent protein Hsp70. Transfection of specific strains involved were transfected into SK-N-SH neuroblastoma cells to analyze gene expression. Some of the players involved in these experiments are FLAG-Parkin(WT), FLAG-Parkin(R42P), FLAG-Parkin(C289G), HSJ1a(WT), HSJ1a(H31Q), and HSJ1a(∆UIM). First, immunocytochemistry techniques analyzing the Parkin wild type and mutated forms at rest were employed to show relative aggregation activity of the proteins while under no induced environmental stresses. Results showed that a very small minority of Parkin wild type cells had incidence of Parkin aggregation and protein misfolding (5% of cells), and Parkin(R42P), with mutations in the ubiquitin domains showed similar activity to wild type cells with only a slightly higher rate of aggregation occurrence at 22% of cells. However, it was shown that the Parkin(C289G) strain, with mutations in the RING1 domain, was extremely aggregation prone under no environmental stress (86% of cells). Next, in order to prove that the aggregations observed in the cytoplasm of cells containing Parkin(C289G) were due to protein aggregations GFP-Hsp70 along with Parkin(C289G) were transfected in SK-N-SH cells and after viewing the cells under a fluorescence microscope it was confirmed that the aggregates were of protein nature because the majority of aggregate inclusion bodies did test positive for Hsp70. Then, SK-N-SH cells were transfected with MitoDsRed mitochondrial marker and one of the three Parkin strains, C289G, R42P, or WT, and then these cells were treated with an uncoupling agent, carbonyl cyanide m-chlorophenylhydrozone (CCCP), to cause a depolarization in mitochondrial membranes and to subsequently analyze Parkin function when induced to perform its cellular activities. Analyses were done by quantifying overlap of mitochondrial marker with the Parkin strains, because this would show if Parkin is associating with mitochondria during uncoupler treatment. Before treatment of these cells, however, it was shown experimentally that without the uncoupling agent none of the Parkin strains associated with the marker. After four hours of treatment with CCCP Parkin(WT) expressed cells showed the induction of mitochondria to accumulate in perinuclear clusters where there was significant overlap of marker with Parkin(WT) in 91% of cells. The Ubl mutant, FLAG-Parkin(R42P) was also shown to associate with mitochondria by having similar overlapping patterns as the wild type strain in 100% of cells. Parkin(C289G), however, was shown to not associate with mitochondria and only 2% of cells with this mutant form showed any accumulation of mitochondria in perinuclear structures; Parkin(C289G) remained in distinct cytoplasmic inclusions. These results showed that the RING1 domain governs the proper folding necessary to stimulate localization of Parkin to damaged mitochondria because the C289G strain with RING1 mutations failed to localize to damaged mitochondria. Then, to show that the RING1 domain mutant ParkinC289G fails to induce autophagy on its own, SK-N-SH cells were transfected with either the wild type Parkin or C289G with mutations in the RING1 domain and these cells were also loaded with an autophagy marker GFP-LC3; these cells were then treated with the CCCP uncoupling agent. It was shown that cells containing Parkin(WT) had significant overlap of GFP-LC3 with mitochondrial marker and Parkin(WT) marker, while the RING1 domain mutant cells showed no overlap of GFP-LC3 with mitochondria, and this suggests that RING1 mutants do not induce autophagy. Next, to demonstrate that the chaperone HSJ1a does reduce Parkin(C289G) inclusion formation SK-N-SH cells were cotransfected with Parkin(C289G) and also either myc-HSJ1a, HSJ1a(H31Q)(with mutations in the N-terminus J domain), or HSJ1a(∆UIM)(with mutations in the C-terminus ubiquitin domain), to test which domain within HSJ1a, if any, rescues the function of Parkin(C289G). Co-expression of HSJ1a(WT) with Parkin(C289G) showed a significant reduction in the amount of Parkin aggregates leading to inclusion bodies, from 81% of cells down to 17% inclusion cells. HSJ1a(∆UIM) had a similar effect on the reduction of inclusion body formation of Parkin(C289G) as the wild type HSJ1a (from 81% to 27%), which would suggest that the J domain retains the function of being able to reduce inclusion bodies because in HSJ1a(∆UIM) the ubiquitin domain is mutated. As expected, mutations in the J domain abolished HSJ1a’s ability to reduce inclusion body formation, and this confirms that the J domain of HSJ1a regulates Parkin aggregation**.** Finally, after it was also shown that HSJ1a and similarly HSJ1a(∆UIM) restored relocalization of Parkin to damaged mitochondria, in order to test if the HSJ1a chaperone can save Parkin autophagy induction, SK-N-SH cells were transfected with Parkin(C289G), HSJ1a(WT), Hsp60 red fluorescent protein marker (for Parkin), and autophagy marker GFP-LC3. In cells expressing just Parkin(WT) there was significant overlap between GFP-LC3 and Hsp60 markers (68%). In cells expressing only Parkin(C289G) there was no overlap of the Hsp60 and GFP-LC3 markers which indicates that autophagy had not been induced in those cells. However, cells expressing Parkin(C289G) with HSJ1a experienced increased levels of overlap between the markers, from 0% to 16%! This data strongly suggests that the HSJ1a chaperone can actually restore at least some of the original function of Parkin RING1 domain mutants.

**Conclusion:** The very important take home message from this study is that it has been successfully demonstrated that the HSJ1a chaperone can actually, to some degree, save several of the functions of a Parkin ubiquitin-protein ligase. HSJ1a has been shown to not only reduce the number of inclusion bodies formed by aberrant Parkin protein folding, but can also allow Parkin(C289G) mutants with mutations associated with the RING1 domain to regain the functions of relocalization to damaged mitochondria and also the function of induced autophagy after depolarization by uncoupling agents. Also, it was shown that the J domain region of the chaperone HSJ1a most likely plays the pivotal role of retaining the function of HSJ1a on Parkin. It is important to realize how this data can help shed light into the treatment and prevention of Parkinson’s disease.

**Future Research Ideas:** One particular aspect of this study that interested me, and that I thought needed additional study, was the fact that although in most cases the HSJ1a variant that had a mutation in the ubiquitin domain (HSJ1a(∆UIM)) showed similar expression as the wild type HSJ1a, it did not exactly match expression. In every case, when analyzing which strains of HSJ1a restore functions of Parkin(C289G) the HSJ1a(∆UIM) strain restored function in an intermediate amount compared to the wild type strains and strains with mutations in the J domain. Wild type domains with both J and Ubl domains intact restored function almost completely, and HSJ1a(H31Q) with no functional J domain but a functional Ubl domain showed almost no recovery of functions. However, the HSJ1a(∆UIM) strain with no Ubl domain but a functional J domain showed regain of function to a greater extent than HSJ1a(H31Q) and to a lesser extent than HSJ1a(∆UIM). I think that this establishes a possible function for the ubiquitin domain to act as an enhancer to the J domain, possibly allowing a conformational change in the protein at the J domain to allow a tighter bind of the substrate. Studies could be done in which the ubiquitin domain is knocked out and replaced with a foreign, nonmutated, domain to see if cells expressing this mutant strain recover less function than the wild type strain of HSJ1a. Or an inversion of the ubiquitin domain could be processed, so as to keep the same sequences in the molecule but to test if this abolishes some enhancer function of the normal orientation of the ubiquitin region.