GFP Tagged Mitochondrial IMG1 and BSCI Proteins Disrupt Normal Huntingtin Inclusion Body Formation in *Saccharomyces cerevisiae*

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Introduction

Neurodegenerative diseases

Many neurodegenerative diseases have similar pathogenesis and are characterized by aggregates of unfolded proteins and these protein aggregates take many different forms depending on the disease (1, 2). Alzhziemser disease shows extracellular structured amyloid beta plaques while amyotrophic lateral sclerosis (ALS) is characterized by aggregates of superoxide dismutase (SOD) in motor neurons (3,4). The proteins associated with each disease are expressed widely in the brain and other tissues, yet each is toxic in a different, highly specific group of neurons and produces a distinct pathology (2, 3, 5). Huntington's disease (HD) is one of many neurodegenerative diseases with an autosomal dominant manner of inheritance. The prevalence of HD is 10.6–13.7 individuals per 100,000 (6).

Huntington's Pathology

The hallmark symptom of Huntington's disease is Huntington's chorea, involuntary hyperkinetic movements and others include impaired judgement and memory, slurred speech and personality changes which become more severe as the disease progresses (7, 8, 9). HD patients typically die within 17 years of diagnosis from various complications such as accidents, aspiration and dysphagia (10, 11). The majority of cell death occurs in medium-size spiny GABAergic neurons in striatum and pyramidal neurons in cerebral cortex resulting in these cognitive deficits (12, 13). Signs of pathology initially appear in the caudate nucleus and putamen and as the disease progresses, it spreads to the cerebral cortex including frontal, parietal, and temporal regions, although these changes are initially less obvious than those found in the striatum which makes this disease difficult to diagnose. Huntington's disease is a result of

the expansion of glutamine repeats in the Huntingtin protein (Htt) found on the fourth chromosome (14). Deletion of Htt in mice results in embryonic lethality, suggesting a critical, yet unidentified role of Htt during normal development (10, 15, 16). A typical number of glutamine repeats ranges from 10 to 36, however when the amount of glutamine repeats exceeds 40, it is considered mutant huntingtin (mHtt) and eventually develops into Huntington's disease (17, 18, 19). mHtt fractures in multiple places due to this elongates glutamine repeat chain (polyQ tract) The cleavage releases an N-terminal fragment containing the poly-Q tract, which has a tendency to unfold (20, 21). These unfolded proteins forms intracellular aggregates known as inclusion bodies (IBs) in individuals affected by Huntington's disease (22).

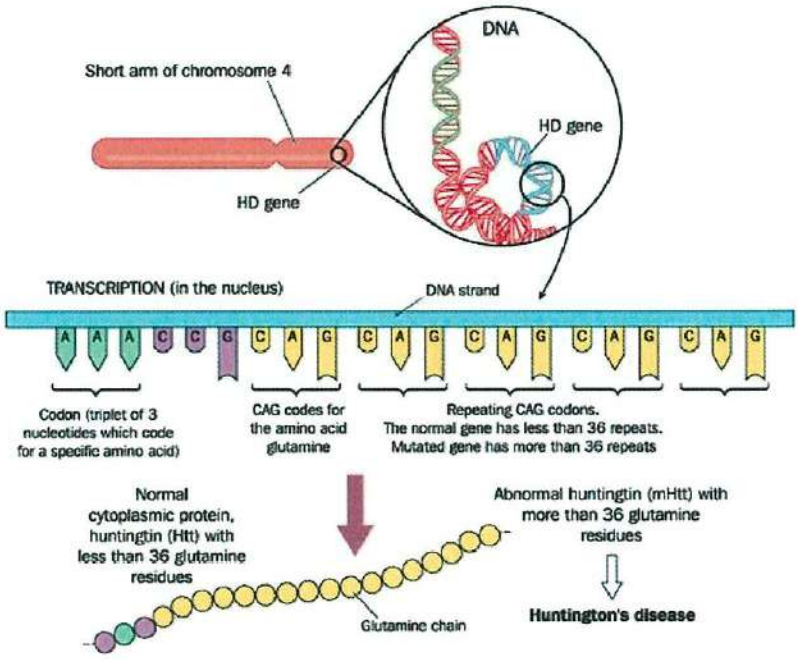

Figure 1. Shows the nucleotide sequence for Huntingtin protein and the CAG repeats which code for the amino acid glutamine. An abnormal number (>40) of CAG repeats creates a long chain of glutamines, also referred to as a polyQ tract. The long polyQ tract leads the unfolding of the huntingtin protein and forms unfolded aggregates in the cells with mHtt.

Source: "Huntington's Disease - Genetics Home Reference - NIH." U.S. The National Library of Medicine, National Institutes of Health, ghr.nlm.nih.gov/condition/huntington-disease#inheritance. (46)

Heat Shock Proteins

Heat shock proteins (HSPs) are chaperone proteins that function to refold unfolded protein and are a protein quality control mechanism to promote protein homeostasis by interacting with different types of folding intermediates and off-pathway folding products (23-26). Additionally, it has been shown that HSPs are what drive the aggregation of mHtt in cells and play a major role in structuring the IB (26). These HSPs are present in the IB demonstrating that the IB is made up of more than just unfolded mHtt (5, 13, 27, 28).

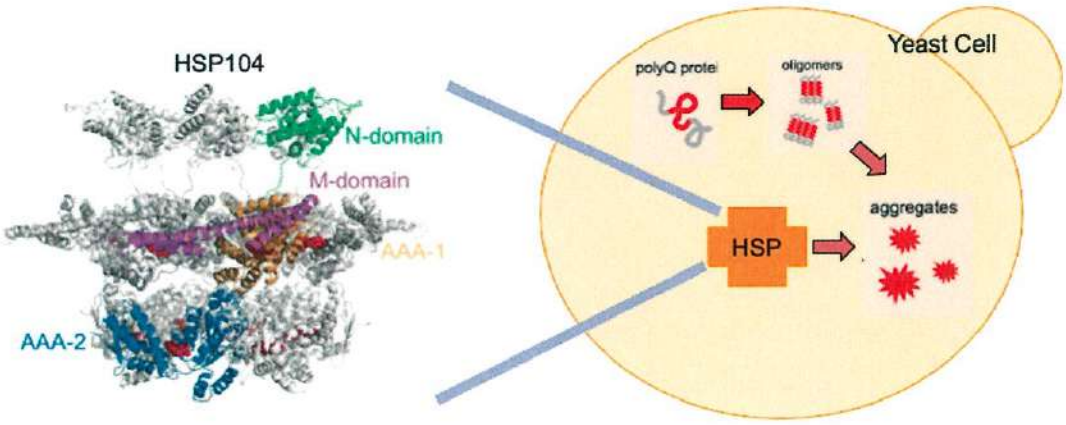

Figure 2. Model of Yeast cell recruitment of HSP in response to unfolded mHtt (shown as polyQ protein) and the refolding and reorganization of mHtt into aggregates and IBs inside the yeast cell. First, it seems that, but is not definite, the protein fractures along the N-terminus and then layers itself to form oligomers. In other neurodegenerative diseases, the unfolded protein may stay in this form but in HD, the recrution of HSPs attempts to refold the proteins and creates IBs (large aggregates) of these oligomers. Pictured on the left is the ribbon diagram of HSP104, a heat shock protein known to colocalize with mHtt IBs. It shows the specific binding sites of HSP104 and it is unknown which site it uses to interact with unfolded Huntingtin.
Source: Student generated image

Since HSPs function in prevention of the earliest aberrant protein interactions, therapeutic approaches modulating HSP or other chaperone protein expression and function can be promising for treatment of Huntington's diseases. In *Saccharomyces cerevisiae*, HSP104 has been found to catalyze the formation and disaggregation of these IBs, demonstrating that it plays a role in regulating mHtt IBs (29, 30). It has been proven that after introducing mHtt tagged with GFP into a *hsp104Δ* strain, there were no IBs formed, supporting the previously made hypothesis (30, 31). Additionally, HSP104 has been found to colocalize with mHtt inclusions as well as small particles of unfolded mHtt in cells (31, 32, 33).

Protein Aggregates

To study these aggregates, we used a budding yeast model system, *Saccharomyces cerevisiae*. These eukaryotic cells act as models for human cells and enable us to study the growth of these aggregates (5, 33, 34, 35).

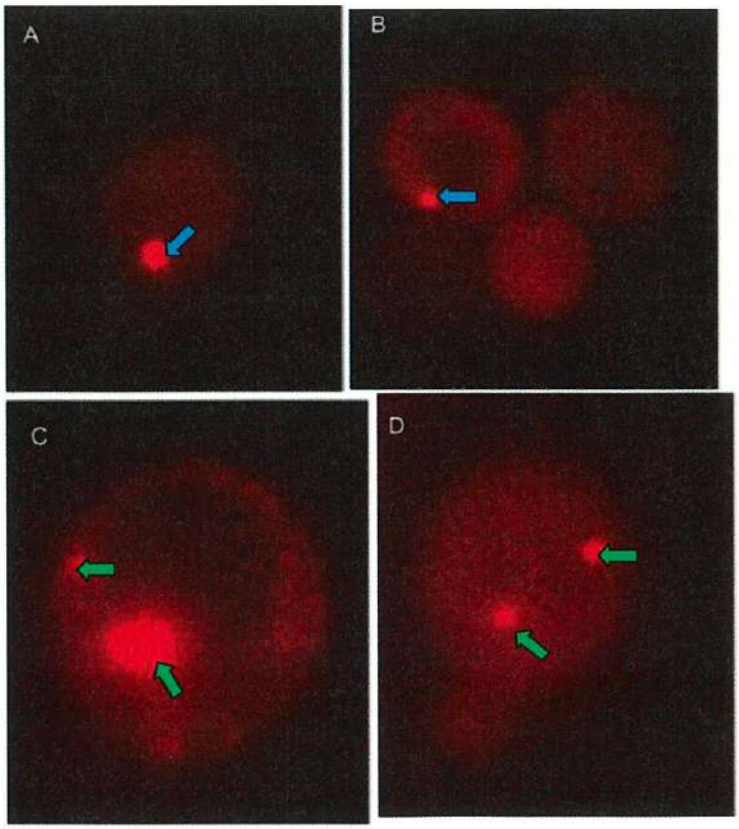

Figure 4. Images of IBs tagged with mCherry in *S. cerevisiae* viewed under a fluorescence microscope. The blue arrows point to what is considered a "normal" or typical IB with smooth edges, independent of other organelles and have uniform intensity shown all throughout in images A and B. The green arrows in images C and D show a cells with multiple IB and although this is relatively uncommon, it is still an abnormal result and is cause for further investigation.
Source: Student generated image

The issue of unfolding proteins is very old and goes back to the first eukaryotic cells and the basic processes to deal with the unfolding has not changed much since then. However, there are drawbacks to using this model such as the lack of multicellularity and inflammatory processes as seen in mammals at all levels. Highly specialized functions of neuronal cells cannot be fully studied in yeast cells, although basic features (e.g., endo- and exocytosis as rudimentary functions of vesicle transport and neurotransmitter release) may be present (28). Overall, they are a good model for the ongoing scan as it can properly form IB in the same manner as humans which is what we are studying.

We studied these cells in log phase during cell growth in which it is characteristic of about 20-40% of budding cells to have one intracellular IB which is visible by fluorescence microscopy. Normal IBs are ovoid shaped with smooth edges, independent of other organelles and have uniform intensity shown all throughout. *S. cerevisiae* contains many of the same proteins as humans, especially heat shock proteins which is important in this study.

Multiple Proteins Present in mHtt IB

In addition to HSP104, other proteins have been found in mHtt inclusion bodies leading us to look in other directions other than just chaperone proteins. Rnq1, a prion protein which plays a role in misfolding peptides, has been found to be present in mHtt IBs as well (36, 37, 38). The study of yeast prions has resulted in significant contributions to explaining fundamental mechanisms of protein misfolding and the study of Rnq1 enables us to ameliorate our understanding of mHtt unfolding and misfolding (39, 40). Similar to *hsp104Δ* strain, *Rnq1Δ* strains expressing mHtt tagged with GFP showed no large aggregates or even small particles of mHtt, again showing how this protein is essential for inclusion formation (41). It has been

determined that Rnq1 determines the structural properties of mHtt inclusions and are crucial for inclusion formation along with HSP104.

Purpose of the Project

Our study focuses mostly finding and identifying other proteins, HSPs as well as others also present in the inclusion or affect inclusion formation in hopes to find what is regulating the formation of mHtt inclusions. The presence of HSP104 and Rnq1 suggest that other proteins are present in the inclusion and play a role in keeping the IBs together as they are not membrane bound (33). We compiled a collection of about four hundred proteins that could possibly be found in the inclusion based on predictions made by other papers as well as proteins that are commonly known to work with or recruited by HSP104 and/or Rnq1. These proteins were then tagged with GFP in cells that contained mHTT tagged with mCherry to look for abnormalities in the inclusions and inclusion frequency as well as colocalization. Using this method, we were able to identify other proteins of interest that could possibly play a role in mHtt IB formation and regulation.

Materials and Methods

LiAc-Mediated Yeast Transformation

Yeast cells with a previously performed genomic integration of gfp (Yeast GFP Clone Collection, Thermofisher Scientific) were grown in YPD at 30 °C overnight in 5mL falcon tubes. Each strain in the clone collection has a different gene fusion, the parental strain was haploid *S. cerevisiae* strain BY4741. The next day, the cells are diluted and ensure that they are in mid-log phase (OD 0.1-0.3 at 600nm). 1.5 mL of the cell suspension was added to an eppendorf tube and spun for 5 minutes at 5000rpm in a tabletop centrifuge to pellet the cells. The supernatant was

decanded and resuspended in 1.0 mL of .1 M LiAc. Pellet cells again in a tabletop centrifuge for 5 minutes at 5000rpm and remove residual LiAc. The cells were resuspended in 240 μL of Polyethylene glycol (PEG) using a vortex and 36 μL of 1.0 M LiAc and 25 μL of SS-DNA were added to the solution. Then, 1 μL of the plasmid containing mHtt-mCherry and the Lew 2 gene was added and the solution was vortexed 3 times for 2-3 seconds as not to break the SS-DNA. This was then incubated at 30 °C for 30 minutes in a water bath. Next, the cells were heat shocked in a water bath at 42 °C for 45 minutes. This solution was then added to a microfuge at 5000rpm for 2 minutes and all the transformation mix was removed with a micropipette. The pellet was resuspended in 100 μL of water and spread on a warmed plate using a glass spreader with selectable media that is devoid of Lew 2 to ensure that only cells that were transformed and have the plasmid survive. Finally, parafilm was added to edge of plates to prevent them from drying out and incubate upside down for 2-4 days at 30 °C. After the cells grow in the plates, one colony was selected and added to YPD medium and inoculated in a falcon tube. If the size differences of the colonies on one plate were relatively large, then two colonies were chosen, one small and one large. These tubes were incubated overnight at 30 °C and were used the next morning for slide preparation.

Fluorescent Microscopy Slide Preparation

First, the parental strain was OD to ensure that the cells are in log phase and are growing exponentially (OD 0.1-0.3 at 600nm). If they were not in mid log phase, they were either left to grow for 3 more hours or were diluted with YPD. The cells were then spun in a microcentrifuge for 2 min 30 sec at 5000rpm. Decant majority of supernatant and resuspend pellet in the small amount of remaining liquid. Less than 2 μL of the liquid is added to the slide and covered with a

coverslip. Lastly, oil is added to the lenses to direct more light the objective and observe a clearer image and the slide is placed upside down to view the image.

Fluorescent Microscopy Settings and Data Collection

We used a Nikon Eclipse Ti-S inverted epifluorescence microscope, equipped with a SOLA Light Engine, QImaging Monochrome Digital Camera, FITC and RFP filter sets (Chroma Technologies), CFI Plan Apo Lambda 100x/N.A. 1.45 oil immersion objective, and NIS-Elements Advanced Research software. The exposure time was set for 100ms, hardware gain of 1.8 and a step of 0.3 microns with a range of 4.5 microns. These settings were kept constant for every strain we imaged. For each strain, about 5-7 images were taken to image at least 40 cells per strain to get an accurate representation. When analysing these cells, we recorded number of total cells, percent of cells with IBs, number of "normal/typical" IBs and number of abnormal IBs determined by size, shape and intensity of IBs as well as if there were multiple IBs per cell. Additionally, the localization of gfp was noted and checked to see if it was where it is predicted to be (ex. Membrane proteins are in the membrane and not in the vacuole) and checked for colocalization with the mCherry-mHtt IB. Everyday, the parent strain BY4741 was checked first before observing the other strains to establish a normal control. The strains viewed that day were compared to the parental strain (*S. cerevisiae* with only the plasmid mCherry-mHtt and no gfp) from that day to account for any disparities.

Results and Discussion

BCS1 Tagging Induces Oxidative Stress

When genes are tagged with gfp, it alters the protein that results from the gene and as a result, the protein may end up abnormal. For this reason, any cells tagged with gfp that has abnormal IBS and/or multiple IBs indicates that the tagged gene has an effect on IB formation. For the parental strain BY4741 during log phase, it is characteristic of 20-40% of cells to have one intracellular IB. We compared the percentage of IBs in the multiple parental strains we studied to those of other genes we looked at.

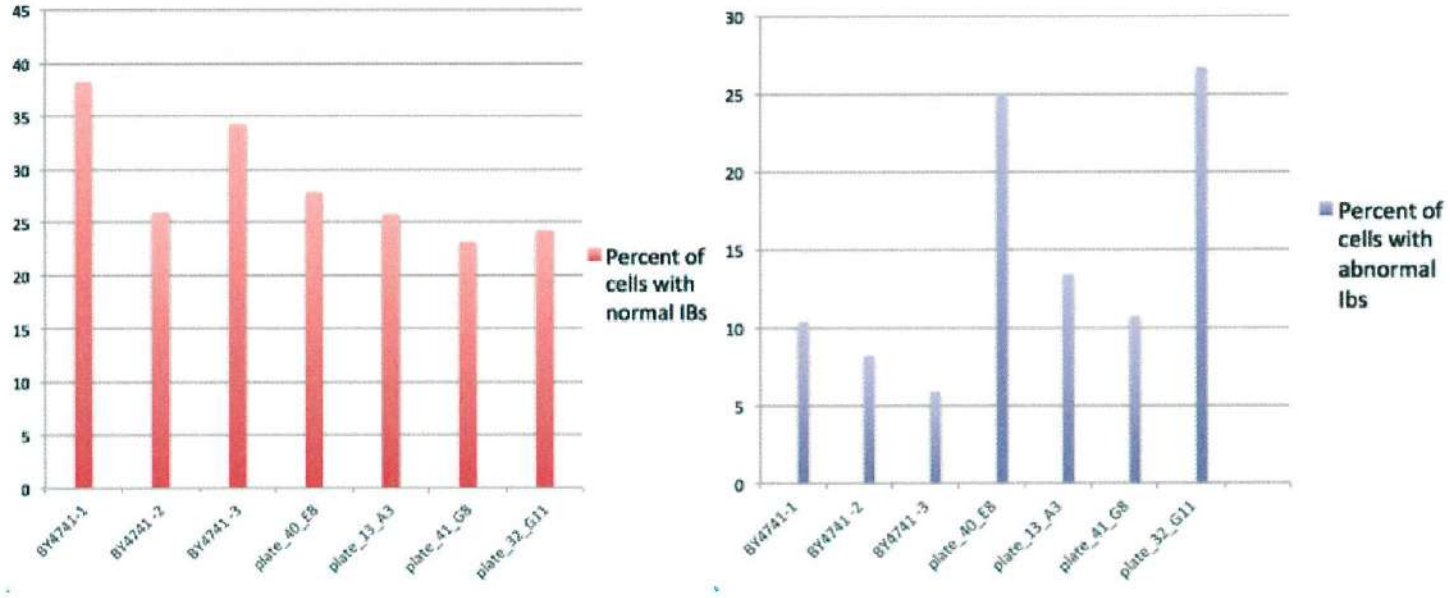

Figure 5. Chart A shows the percent of cells with normals IBs of three imagging sessions of the parental strain and three other strains according to their plate and well numbers which have corresponding gene number to tagged gfps. Chart B shows the percent of cells with abnormal IBs of both the parental strain and other strains according to their plate and well numbers which have corresponding gene number to tagged gfps. The average of the parental strain is 20-40% of cells have normal IBs and theoretically there should be no abnormal IBs since there are no proteins interacting with the IB but these changes can we accounted to the age of the cell, mutations in the parental strain itself or of the cells not being in log phase. These are three of fifty tagged proteins that were observed in this study.

One protein that is extremely interesting is BCS1 which is found in Plate 40 E8 and is shown in Figure 5 and Figure 6. Although it has an average number of normal IBs, a large percent of its cells contain abnormal IBs. Abnormal IBs is a vague description and can come in many forms and for BCS1, this comes in the form of one very large aggregate as well as multiple smaller aggregates in 25% of cells. We can hypothesize that this is due to the tagging and disrupting of the protein which impacts the ability of mHtt to aggregate and form IBs. BCS1 protein plays a major role in the mitochondrial functions and translocates the C-terminal domain of Rip1p to an intermediate step of the Complex III assembly (46). It mediates the export of Rip1p across the mitochondrial inner membrane, a process that is very important to respiration and the Complex III assembly(47). Mutations or disrupts to this protein have been known to cause oxidative stress, a sign of pathogenesis for multiple diseases (48). Oxidative stress can result in and is usually accompanied by unfolding of proteins and can be used to explain why the tagging of BCS1 has an impact on the IB formation and aggregation of unfolded protein in yeast cells (49). BCS1 shows no colocalization to the IB but rather is seen in the mitochondria where it is predicted to be, which indicates that it is not found in the IB. But the large IB along with smaller aggregates in one cell suggests that there is more unfolded protein in the cell than average. Additionally, this pattern is evident in many cells from this strain and all the cells with abnormal IBs are similar in terms of large IBs and small aggregates which demonstrates that it is a characteristic of disrupted BCS1 function.

This sizeable amount of unfolded mHtt in the cells can be accredited to the oxidative stress caused by the tagging of BCS1 with gfp and the disruption of the Complex III assembly that we hypothesize may accompany it. BCS1 was the only protein that is a part of the Complex

III assembly which was studied, however other mitochondrial proteins were observed and they appeared to have no impact on IB formation. The Complex III process is essential for cell function but the cell itself was not damaged by the tagging of BCS1 which leads us to presume that changing that specific protein leads to IB formation. This protein and other mitochondrial proteins were included in the clone collection due to the increasing studies showing a correlation between Complex I protein mutations (mitochondrial diseases) and neurodegeneration (50,51). These correlations have been shown with Parkinson's, Alzheimers and other neurodegenerative disease.

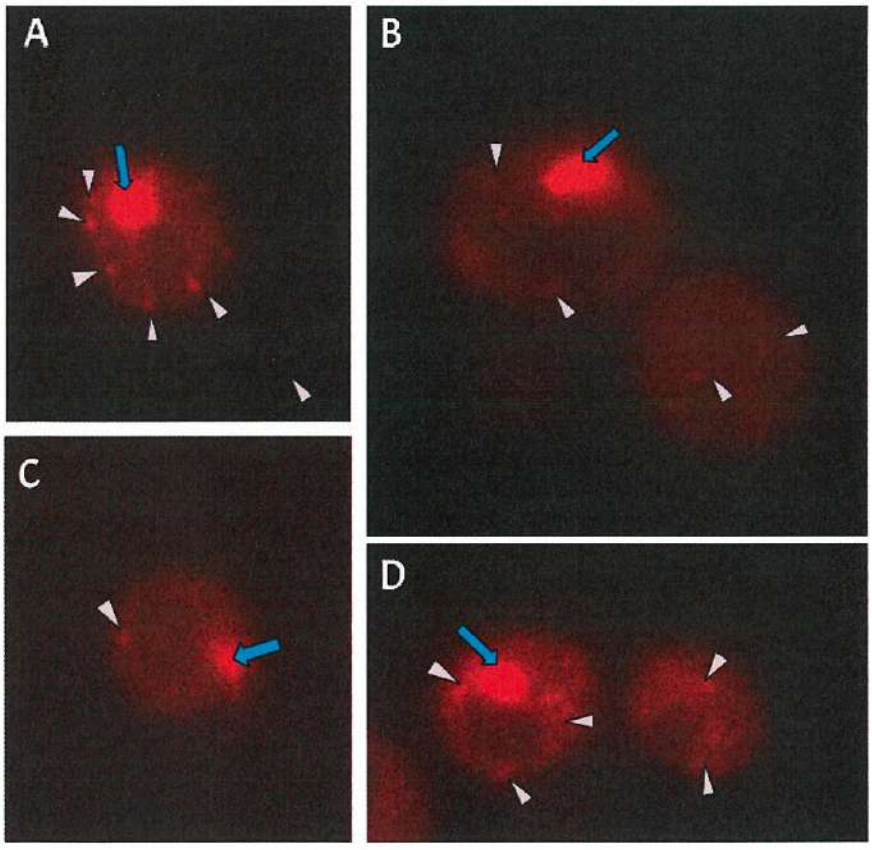

Figure 6. Images A, B, C and D are from Plate 40 E8 and have BCS1 tagged with gfp and show examples of the large IBs and small aggregates that is characteristic of BCS1 tagged with gfp and is present in 25% of cells. The long blue arrows point to the large IBs shown to be almost the size of the nucleus and the white arrowheads point to the smaller aggregates that are not as lighter but still have a high intensity of fluorescence indicating that it includes mHtt.

IMG1 Strain with Similar Aggregate Pattern

The pattern of abnormally large IBs accompanied by multiple smaller aggregates in the same cell, which was seen in the BCS1 strain, is very unique that when another strain with a similar pattern was found, we immediately took great interest in it.

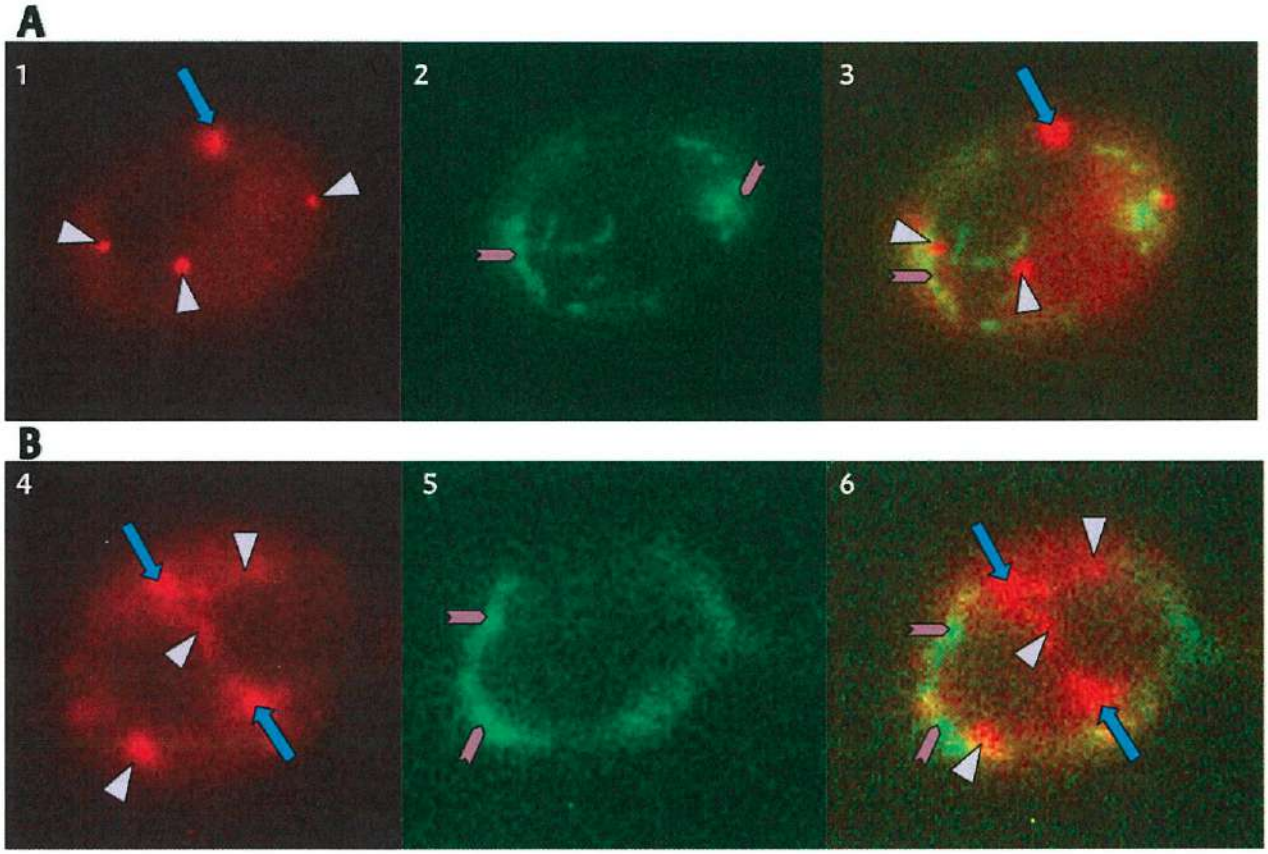

Figure 7. All the images above are IMG1 tagged with gfp. Images 1, 2 and 3 are all the same cell identified as shown in different channels and Images 4, 5 and 6 are the same cell identified as B shown in different channels. 1 and 4 show the red channel in which mHtt is seen. The long blue arrows point to the large IBs shown to be almost the size of the nucleus and the white arrowheads point to the smaller aggregates that are not as lighter but still have a high intensity of fluorescence indicating that it includes mHtt. 2 and 5 show the green channel where the gfp is shown which is IMG1 in this strain. The dark purple banners point to the mitochondria which is evident in green. 3 and 6 are combined channels showing both the mHtt IBs and IMG1 in the mitochondria in red and green respectively.

The protein tagged with gfp in this strain was identified to be IMG1 (Figure 7), which is found in Plate 32 G11 shown in Figure 5 as having similar statistics to BCS1 with a large number of abnormal IBs. IMG1is a mitochondrial protein just like BCS1, and although it was not a part of the Complex III process like BCS1, IMG1 is an important protein and is required for respiration and mitochondrial function. IMG1 is responsible for the synthesis of mitochondrial genome-encoded proteins, including at least some of the essential transmembrane subunits of the mitochondrial respiratory chain. This demonstrates its necessity to the body and how tagging it and disrupting its function can result in abnormal mHtt IBs. The abnormal shape of the IB suggests that it is not regulated and is growing out of control which is another sign of abnormal aggregation in these cells. It is hypothesized to follow the same procedure as BSCI in terms of how the tagging of a mitochondrial protein plays a role in IB formation. The abnormally large amount of unfolded mHtt in the cells can be accredited to the oxidative stress caused by the tagging of IMG1 as altering the respiration chain changes all aspects of the cell since respiration is such an important process. This specific pattern that has shown up in mitochondrial proteins and is enough of a correlation to look into more mitochondrial proteins in the future and observe to see if they present with the same types of IBs. Moreover, this result is interesting because there is no colocalization observed and IMG1 is in the mitochondria as expected, which is seen in figure 7, but these proteins, both IMG1 and BSCI, have known functions in the mitochondria have an effect on the unfolding of a developmental protein that takes place in the cytoplasm of the cells.

Mitochondrial Proteins and Neurodegeneration

The connection between mitochondrial proteins and neurological disease is new but has many studies to show the correlation that they both progress simultaneously. The heme metabolism plays a central role in mitochondrial functions, and several evidences indicate that alterations of the heme metabolism are associated with neurodegenerative disorders. Moreover, heme can also promote oxidative stress, thus performing as a double-face molecule with both positive and negative properties (42). Both heme deficiency and excess are fatal to cells and result in neurological deficits, signalling that mitochondrial functions play an important role in survival of neuronal cells. This information is helpful when studying the pathogenesis of neurodegenerative disease, but gives little information about how these proteins affect IB formation when there are no neural cells to observe. However, this gives an explanation for why mitochondrial proteins influence mHtt IBs, a theory that previously had no reasonable justification.

Conclusion

The presence of abnormal IB as a result of tagged gfp indicates that the tagged protein may potentially play a role in IB formation. Since tagging proteins sometimes results in disruption of the proteins, the change in IBs from the parental strain is likely accredited to the tagging and disruption of the specific protein. Yeast cells were used for this study because of a fact known as "homology", or the similarity of genes between eukaryotic cells. The genes being studied are the same in yeast as they are in humans, or they have a very similar form and create similar proteins to those in humans which makes yeast a viable option for basic cellular processes. The research has identified two main proteins that have significantly changed the

types of IBs present in the yeast cells. IMG1 and BSCI, both mitochondrial proteins, have had a drastic impact on the aggregation in cells with mHtt. For the parental strain BY4741, it is characteristic of cells to have one intracellular IB while in log phase. However, both IMG1 and BSCI strains produced cells with abnormally large IBs accompanied by multiple smaller aggregates in the same cell while in log phase. This is a very unique pattern and has only been seen so abundantly in these two proteins, which were the only two mitochondrial proteins observed in this section of the study. However, there are more mitochondrial proteins in the clone collection and they will be compared to these two proteins in terms of function and their effect on mHtt unfolding and IB formation.

There has been an established correlation between mitochondrial degeneration and other neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease. This connection makes it less surprising and more understandable as to why two mitochondrial proteins had a greater impact on IB formation than other proteins in the clone collection including chaperone proteins that interact with HSP104 and Rnq1, which are two proteins that are known to be necessary for IB formation. BSCI is an intermediary protein that is very important in the Complex III assembly and IMG1 is a protein responsible for the synthesis of mitochondrial genome-encoded proteins, including at least some of the essential transmembrane subunits of the mitochondrial respiratory chain. Both IMG1 and BSCI are proteins that are vital to respiration and mitochondrial function and tagging them disrupts their functionality, resulting in mitochondrial dysfunction. Mitochondrial dysfunction has been described as an early pathological mechanism delineating the selective neurodegeneration that occurs in Huntington's disease (43). Based on previously published papers and the correlations between mitochondrial

proteins and IB formation found in this study, new avenues of study can come about and scientists can find different ways to alter the pathogenesis of Huntington's disease.

Future Research

This study is part of an ongoing scan meaning that there are more proteins to be observed in the manner and analysed to check for colocalization with mHtt IBs or abnormal IBs in the presence of those tagged proteins. These proteins are chosen from a clone collection which includes chaperone proteins, scaffolding proteins and other proteins mentioned in published literature that have been predicted to play a role in IB formation. After identifying other proteins that impact IB formation when tagged, which are IMG1 and BSCI, the next steps would be to create a deletion strain without the specific protein and see what impact it has on IB formation. This same procedure was done with HSP104 and Rnq1 and the absence of IB in the hsp104Δ strain and the Rnq1Δ strains support the hypothesis that they are necessary for IB formation (29, 31, 32). If the protein in question is deleted and the strain produced very little or no IBs, it demonstrates that the protein is important for IB formation however this can also go the other way and the presence of normal IBs can prove that the protein in question has no direct relationship to IB formation. Additionally, proteins that interact with IMG1 and BSCI and perform similar operations should be added to the clone collection and should be studied as they could also influence IB formation.

Applications of Study

This study is the beginning of understanding the factors of IB formation and these discoveries can help create treatment options in the future to downregulate the unfolding of the Huntingtin protein and contain the IBs and prevent them from becoming toxic to the cell. One of

the big questions in the field is why Huntingtin is expressed in all the cells in the body and IBs are present in all the cells in the body, but the majority of cell death occurs in medium-size spiny GABAergic neurons in striatum and pyramidal neurons in cerebral cortex. It is known that the cell type-specific characteristics, such as axon length and differences in synapses, contribute to the vulnerability of specific neuronal populations to mutant huntingtin expression (44, 45). However it has been postulated that differences in biochemical content may also contribute to the differential vulnerability (10, 45). Understanding which proteins are present in IBs can aid in determining why these IBs become toxic to striatal neurons. Determining which mitochondrial defects affect which parts of the brain can also play a role in differentiating between all these neurodegenerative diseases. Ultimately, the more information present about the progression of HD and the process of creating IBs in eukaryotic cells, the easier it is to generate a treatment for this disease.

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