Building an *in vivo* model of Tau aggregation in *Saccharomyces cerevisiae*

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BUILDING AN *IN VIVO* MODEL OF TAU AGGREGATION IN *SACCHAROMYCES CEREVISIAE*

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A Project Submitted in Partial Fulfillment

of the Requirements for

Biology Departmental Honors

in the Department of Biology

The School of Arts and Sciences

Rhode Island College

April 2019

Approved:

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ABSTRACT

Protein aggregates, which can result in cellular toxicity, are found in the brains of people afflicted with neurodegenerative diseases. One of the proteins found to aggregate is Tau, a protein that stabilizes microtubules in neuronal cells. Based on its amino acid sequence, Tau is likely to be N-terminally acetylated by the complex NatA, which is responsible for the acetylation of a majority of proteins with this post-translational modification. Mutations in the NatA complex result in a variety of pleiotropic detrimental phenotypes, showing that acetylation is a crucial modification for many proteins. Without the presence of NatA – and therefore without acetylation – Tau may be more prone to aggregation and toxicity. By expressing Tau in yeast strains with and without the presence of NatA, the possible effects of acetylation or lack thereof can be determined by examining protein expression and aggregation within the cells by SDS-PAGE and SDD-AGE. Initial results indicate that Tau aggregates within these yeast cells although the nature and effect of the aggregates is still being determined. This project has allowed for the creation of a system by which Tau aggregates can be examined *in vivo.*

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Neurodegenerative Diseases

Neurodegenerative diseases are disorders which affect the vertebrate nervous system; they have been known to cause cognitive, behavioral, and motor function which progresses to eventual incapacitation[1]. Many symptoms of neurodegenerative diseases (ND) overlap with psychiatric symptoms, particularly in the earlier phases of ND; this can lead to problems with misdiagnoses and ineffectual treatments[2]. Proper diagnosis and treatment of ND is critical, especially as the numbers of people living with ND increase; it is expected that by 2050 there will be 18.5 million people living with ND[2]. It is therefore crucial to further investigate the underlying mechanisms that are responsible for disease onset and progression in the hopes of finding potential therapies and targets.

Neurodegeneration occurs in many forms, and although symptoms often overlap, there are many distinct disorders. The most well-known neurodegenerative disorder is Alzheimer's disease, although that only accounts for about 50-70% of dementia cases; the remaining cases of dementia are generally attributed to vascular dementia, frontotemporal lobar degeneration, corticobasal degeneration, progressive supranuclear palsy, and dementia with Lewy bodies[3]. In addition, there are other neurodegenerative disorders that are not typically associated with dementia but rather with other symptoms, such as progressive non-fluent aphasia, amyotrophic lateral sclerosis, Huntington disease, and Parkinson's disease[2,4,5]. Certain disorders, such as corticobasal degeneration and progressive supranuclear palsy, can result in early motor problems, while others such as Alzheimer's disease and some subtypes of frontotemporal lobar degeneration present only with personality and behavioral changes at the onset[3]. As previously mentioned, this can frequently result in misdiagnoses, especially in younger patients with early-onset conditions[2,3].

In many cases of neurodegeneration, misfolded proteins aggregate within the central nervous system and spread to new regions, increasing clinical symptoms as the damage spreads[1]. Synapses between neurons may falter, and communication between neural pathways is affected[1]. The different protein aggregates may be localized to certain regions of the brain, which may account for variation in symptoms between disorders[1]. Multiple proteins have been linked to aggregation correlating with neurodegenerative disease, including β-amyloid, TDP-43, α-synuclein, and tau.

Many disorders can be categorized as synucleinopathies or tauopathies[5]. These characterizations are based off of the accumulation of α-synuclein or Tau aggregates in the nervous system, respectively, although co-localization of these proteins has been noted[6]. α-synuclein is a 140-amino acid protein typically found in the axon terminals of neurons; it plays a role in synaptic function[6,7]. The structure can be divided into three main regions: the N-terminal region, the hydrophobic region, which likely forms a beta sheet, and the acidic C-terminal region[8]. α-synuclein has been found localized in the nucleus and cytosol as well as associated with the membrane[9]. Synucleinopathies, which can be thought of as a form of protein-misfolding disorders, typically contain α-synuclein in intracellular inclusions known as Lewy bodies[7,9]. Lewy bodies are characteristic in Parkinson's disease, one of the most well-known synucleinopathies, as well as dementia with Lewy bodies, the second most common form of dementia[7]. Lewy bodies can be found in many regions of the central nervous system, including the *substantia nigra*, *locus ceruleus*, *nucleus basalis*, hypothalamus, cerebral cortex, and cranial nerve motor nuclei[11].

α-synuclein is encoded by the gene SNCA, and mutations within this gene have been linked with Parkinson's disease, likely due to amino acid substitutions or conformational changes within α-synuclein resulting from the mutation[11]. These gene mutations are not common and only account for about 1% of cases, but it is also possible that variants within this gene increase the risk of Parkinson's even if they do not directly cause it[11]. SNCA gene triplication and duplication has also been seen in cases of familial Parkinson's; it has been

suggested that overexpression of the gene may be a factor in these patients[11]. The potential for toxicity due to overexpression was shown in a yeast model as well; increasing the levels of α-synuclein twofold resulted in cytoplasmic inclusions and cell death[11]. It has also been suggested that different variants of the SNCA promoter may be associated with increased susceptibility to Parkinson's[11].

Microtubule-Associated Protein Tau

Like α-synuclein, Tau is found in neuronal cells; it is a microtubule-associated protein which promotes microtubule polymerization and stability of polymerized microtubules[12]. Six different forms of Tau exist in the adult human brain, ranging from 352 to 441 amino acids and produced via alternative splicing[12]. The isoforms differ based on the presence or absence of 29- or 58-amino acid sequences (N) near the N-terminus of the protein, and whether or not they have three or four tandem repeats (R) near the C-terminus[12]. The full-length form of the protein is 2N/4R, but 0N/3R, 0N/4R, 1N/3R, 1N/4R, and 2N/3R are all expressed within the central nervous system in adults as well, although only the shortest 3R isoform of Tau is seen in fetal neurons[12,13]. 4R Tau has been shown to promote microtubule assembly around three times more than 3R Tau[14]. In addition, granule cells and pyramidal cells within the human hippocampus have differing amounts of 3R and 4R Tau expression; differing degrees of microtubule polymerization and assembly may be unique within varying cell types, suggesting a reason for the differing isoforms[14].

It has been previously suggested that some mutations resulting in frontotemporal dementia with Parkinsonism cause increased levels of 4R Tau compared with 3R Tau[13]. Both 3R and 4R Tau are capable of promoting microtubule polymerization; however, 4R Tau is capable of significantly stabilizing microtubules to a greater extent, suppressing shortening rates and reducing the length shortened overall[13]. An increase in the levels of 4R Tau may potentially shift microtubule dynamics within the cell; a specific ratio of 4R to 3R may be needed in order to keep the microtubule dynamics within the narrow range necessary for functionality and prevent polymerization from occurring too quickly or slowly[13]. Changes in these microtubule dynamics

within the cell may affect the cell's capability to function. Proper microtubule function is necessary for maintenance of cellular morphology and intracellular transport, both of which are crucial for neuronal cell health[13].

In addition, mutations in the Tau protein can reduce its binding affinity for microtubules; $^{G}272^{V}$, $^{P}301^{L}$, $^{V}336^{M}$, and $^{R}401^{W}$ mutants of Tau all showed reduced affinity for microtubule binding and reduced capability to facilitate microtubule polymerization[15]. Impairing the proper function of Tau, even if the ratio of 4R to 3R remains the same, could also negatively affect the neuronal cells.

The hallmark of neurodegenerative diseases classified as tauopathies is the formation of Tau aggregates known as neurofibrillary tangles[16]. In many cases, the Tau found in these aggregates is hyperphosphorylated[16]. In normal adult neuronal cells, the phosphorylation of Tau is highly regulated by a variety of protein kinases and phosphatases[16]. The phosphorylation of Tau plays a role in Tau-microtubule interactions and is suggested to serve as a negative regulator, decreasing the ability of Tau to bind to the microtubule[16,17]. In addition, phosphorylation may affect the ability of Tau to bind to regulatory proteins[16]. The Tau found in fetal neurons is also hyperphosphorylated[15,16]. It is possible that in tauopathies, neuronal cells respond to some stressor by attempting to return to this initial proliferative phase, at least partially[16].

Hyperphosphorylation is strongly implicated in Tau aggregation; one theory suggests that Tau aggregates via its microtubule-binding repeats, but the basic regions near these repeats, and the acidic C-region segment, inhibit Tau-Tau interaction[18]. Phosphorylation of these regions neutralize these domains and allow interaction[18]. Aggregation of Tau proceeds by monomeric Tau proteins associating with each other and forming dimers; a conformational change then results in larger oligomers[19]. Multiple studies suggest that Tau oligomers may be more cytotoxic than larger aggregates, which appear as filaments in which a portion of each Tau protein adopts a beta-sheet conformation; the beta-sheets stack in parallel[19,20]. In the earlier stages of aggregation, Tau oligomers are not ubiquitinated, but ubiquitination - a process which

allows for degradation of potentially toxic protein species - occurs during the transition from oligomers to filamentous aggregates; it is possible that an abundance of hyperphosphorylated Tau exceeds the proteasome's capacity, and the formation of aggregates occurs as a way to shift the Tau into a more stable form[19]. In addition, it has been theorized that certain post-translationally modified forms of Tau - such as hyperphosphorylated Tau - are directly toxic even before their formation into aggregates, and the formation of aggregates is an attempt to sequester cytotoxic species of protein[20].

In Alzheimer's disease patients, the Tau present in neurofibrillary tangles is often truncated at the C-terminus, suggesting another post-translational modification that may be relevant to propensity to aggregation, at least in this specific disease[21]. This truncation may be linked to exposure to amyloid precursor protein (APP, cleaved to amyloid-beta, Aβ), which suggests a potential link between their pathologies; unlike frontotemporal dementia with Parkinsonism, in which only Tau aggregates are present, Alzheimer's disease includes both neurofibrillary tangles of Tau protein and extracellular amyloid plaques composed of Aβ[21]. Although previously thought of as independent pathologies, amyloid plaques and neurofibrillary tangles have been linked by recent studies showing that exposure to Aβ increases neurofibrillary tangles in a murine model[21]. As it has been also suggested that amyloid plaques occur prior to Tau pathology, it is possible that the presence of Aβ plaques result in changes within the cell that alter the modification state and conformation of Tau, leading to aggregation[22]. This also implies that Aβ aggregation may be the trigger for Alzheimer's disease, while Tau aggregation may occur as a downstream effect[22].

It is also possible that interactions between α-synuclein and Tau may play a role in the formation of aggregates. Co-occurrence of α-synuclein and Tau inclusions has been previously noted in human ND patients[23]. Normal α-synuclein is capable of self-polymerization, while this is less likely to occur with Tau; α-synuclein can potentially act as a cofactor to initiate Tau fibrillization[23]. Tau fibrillization in the presence of α-synuclein likely results from polymers of α-synuclein that act as chaperones to produce a conformational change[23]. In addition, rates of α-

synuclein polymerization were shown to increase significantly in the presence of Tau, suggesting that Tau is capable of promoting α-synuclein fibrillization as well[23].

Mechanisms already correlated with Tau aggregation include the previously-discussed post-translational modifications (PTMs): phosphorylation and truncation. As any modification to a protein has the capability to affect its structure - and therefore its function - it is reasonable to assume that other modifications may also play a role in the propensity of Tau to aggregate within the cells. One such modification, which has not been widely studied in this regard, is N-terminal acetylation.

N-Terminal Acetylation

Discovered over fifty years ago, N-terminal acetylation - the addition of an acetyl group to the N-terminus of a protein, thus changing the positive charge present on the N-terminus to a neutral charge - is one of the most common protein modifications in eukaryotic organisms, occurring in 85% of proteins in humans and 50% of proteins in yeast[24]. The difference in these numbers suggests that acetylation patterns differ between species although the fundamental process remains the same, and indeed, this was shown to be true. For instance, about half of human proteins with methionine-lysine termini are acetylated, but yeast proteins with this terminus are not acetylated[25].

N-terminal acetylation is performed by one of six different N-terminal acetyltransferase (NAT) complexes - NatA through NatF - and the sequence of amino acids present in the protein's N-terminus determines which NAT is responsible for acetylation of that protein[24]. For a majority of proteins, it is only the first and second amino acid of the sequence that are responsible for affecting acetylation[25]. Serine and alanine termini are most likely to be acetylated; methionine, glycine, and threonine are the next most likely, and 95% of acetylated proteins have one of these amino acids at their end terminus[26].

NatA is the most active NAT complex, responsible for acetylating a majority of proteins - specifically those with Ser-, Ala-, Thr-, Gly-, Cys-, and Val- termini exposed by cleavage of the initial methionine[25]. In contrast, NatB and NatC acetylate proteins with initial methionine

residues; NatB acetylates proteins with methionine followed by asparagine, aspartate, or glutamate while NatC acetylates those with methionine followed by isoleucine, leucine, phenylalanine, or tryptophan[27]. NatD is specialized for acetylating the N-terminus of histone proteins, and NatE acetylates proteins that have leucine at position 2 and proline at position 4[29]. NatF is responsible for acetylating proteins with initial methionine residues, specifically those followed by lysine, valine, alanine, or a second methionine[28].

Given that cleavage of the initial methionine residue is a common post-translational modification in eukaryotic proteins, it follows that the NAT complex - NatA - responsible for acetylating proteins with this cleaved methionine is therefore responsible for acetylating the most protein. NatA is composed of three subunits; in yeast, the main subunits are Nat1 and Ard1[24]. Ard1 is the catalytic subunit, while Nat1 is responsible for anchoring Ard1 to the ribosome, allowing co-translational modification[25]. Both of these subunits are required for proper function of NatA, and the over-expression of both genes is necessary for overproduction of the NatA complex[29]. Over-expression of only one subunit does not result in any significant increase in acetyltransferase activity, but when both are over-expressed, activity can increase up to 20-fold[30]. In the same way, null mutants of Nat1, null mutants of Ard1, and double null mutants (Nat1/Ard1) in *Saccharomyces cerevisiae* all show very similar phenotypes, showcasing how both subunits are necessary for the proper function of the final protein[30].

Since NAT complexes are responsible for acetylating proteins with a variety of functions, lacking one or more NAT complexes leads to defects in a variety of cellular processes and pathways which can be completely unrelated to one another[30]. In *Saccharomyces cerevisiae,* loss of N-terminal acetylation, caused by knockout of Nat1 and/or Ard1, results in several phenotypes including slow growth, inability to sporulate, and failure to enter G_0 when necessary[30]. Decreased mating, increased sensitivity to heat shock, and increased sensitivity to sodium chloride were also observed in mutant *Saccharomyces cerevisiae* with reduced N-terminal acetylation[28]. Despite these defects, these strains remain viable, suggesting that N-

terminal acetylation, although clearly important to the function of many proteins and the overall health of the cell, may not be completely necessary in all normally-acetylated proteins[29].

In humans, however, disturbances to N-terminal acetylation are more prominent and more likely to result in lethality. Ogden syndrome, an X-linked disorder, correlates to a mutation in the gene which encodes the catalytic subunit of the NatA complex[31]. Symptoms include cranial abnormalities, hypotonia, and cardiac problems, which eventually lead to death during infancy[32]. Deregulation of NatA expression is also linked to the development of tumors[32]. The human homolog of the *Saccharomyces cerevisiae* Nat1 unit, NATH, is found over-expressed in papillary thyroid carcinomas and is found in dividing tissues, suggesting that it might play a role in a cell's ability to proliferate[33]. Increased protein synthesis would be necessary in rapidly-growing tumor cells, which would then result in the acetylation of more proteins overall.

The severity of effects in humans is likely due to the increased amount of proteins that are N-terminally acetylated in humans as opposed to *Saccharomyces cerevisiae,* as well as the increased complexity of the processes involved in a multicellular organism like *Homo sapiens* compared with a single-celled organism such as *Saccharomyces cerevisiae.*

The higher amount of proteins acetylated in humans rather than yeast could partially be due to a larger amount of NatB substrates; 19% of acetylated human proteins are acetylated by NatB, while only 9.5% of acetylated yeast proteins utilize NatB[25]. In addition, a larger amount of amino acid sequences in humans can result in N-terminal acetylation - for instance, human proteins with the N-terminal sequence Met-Lys are acetylated while yeast-proteins with this N-terminus are not[25]. The increase in acetylation in multicellular eukaryotes such as humans is also likely due to the presence of NatF, which is not present in the single-celled eukaryotes such as *S. cerevisiae*[28]. Divergence of both the NAT complexes themselves and their human protein targets could be responsible for the increased percentage of N-terminally acetylated proteins in humans[25].

Although it is clear that the pleiotropic phenotypes seen with a lack of acetylation is likely caused by the wide variety of proteins which receive this modification, and disruption to this

modification results in the disruption of various other cellular pathways, it is not currently known what the true function of acetylation is. It is apparently an irreversible process[34]. N-terminal acetylation occurs when the nascent polypeptide chain is ~50 amino acids long, and can be considered a co-translational modification[24]. As eukaryotic proteins fold co-translationally, it is reasonable to assume that N-terminal acetylation may play a role in protein folding as well[24].

One study shows evidence that N-terminal acetylation can be responsible for the creation of degradation signals in proteins with this modification[34]. Initially this seems impossible, since so many proteins, especially in higher eukaryotes, are acetylated, and many of them last a reasonable length of time before being degraded. However, other processes could also play a role. N-terminal acetylation may form degradation signals, but if the protein associates with chaperone proteins, forms a complex, or folds in a way that blocks the N-terminus from associating with ubiquitin ligase or any other enzymes responsible for degradation, it may block the degradation signal from being recognized[34]. On the other hand, if the protein is slow to properly fold or form a complex, the N-terminal degradation signal may remain open to the cellular environment and become recognized by ubiquitin ligase or others, resulting in quicker degradation; this suggests that N-terminal acetylation may potentially play a role in regulation and quality control[34].

As expected from this potential role as a degradation signal, N-terminal acetylation does not protect against degradation by the proteasome[35]. It is possible that protease resistance, folding, and thermal stability may be impaired in proteins without acetylation, but there is little evidence for these direct effects[35]. It was shown in alpha-synuclein that N-terminal acetylation increases stabilization and helical propensity as well as decreasing rates of aggregation, likely due to secondary structure changes that resulted from the change from an N-terminal positive charge in the non-acetylated form to a neutral charge[36]. Acetylation increases helicity in proteins, and this effect on the protein's structure will cause varying effects depending on the particular protein and its function[37].

It has also been shown that N-terminal acetylation is far more common in cytosolic proteins - those that remain in the cytoplasm - than those which are sent to the endoplasmic reticulum for secretion out of the cell[27]. Acetylation appears to inhibit protein targeting to the ER; most secretory proteins are unmodified, and the presence of acetylation decreases the chances of a protein being translocated and secreted out of the cell[27]. It is possible, therefore, that the presence of acetylation is a marker that results in a protein staying within the cytoplasm instead of being moved to the ER[27].

It has also been shown that lack of acetylation can be beneficial for certain proteins; in human hemoglobin, for instance, the acetylated form results in a reduced affinity of hemoglobin to oxygen[38]. It has also been suggested that some proteins are only acetylated because their amino acid sequences are favorable to the NAT complex, and the protein does not actually require acetylation for its proper function[38]. This could occur because evolution results in certain sequences being conserved for other reasons - for instance, a serine at position 2 of the amino acid sequence promotes phosphorylation and initial methionine cleavage as well as N-terminal acetylation, but it is entirely possible that the protein only requires one of these for its function[38].

Acetylation depends upon acetyl-CoA levels, since it is an essential cofactor in the process of N-terminal acetylation; this provides a clear link between cellular metabolism and acetylation[39]. Inhibiting acetyl-CoA production in cells has resulted in lower levels of N-terminal acetylation as well as lower levels of apoptotic sensitivity[39]. It is therefore clear that acetylation is a widespread process that affects many aspects of life within the cell, and its exact functionality likely cannot be narrowed down to just one factor.

In the same way, even the NAT complexes themselves may have multiple functions. New evidence suggests that NAT complexes are also capable of acting as propionyltransferases, transferring a propionyl group from propionyl-CoA to the N-terminus of a protein[40]. Propionylation occurs at a lower rate than acetylation, which could be at least partially explained by the lower concentration of propionyl-CoA in cells compared to that of acetyl-CoA[40].

It is a naturally occurring process; NAT complexes are capable of propionylating proteins both *in vitro* and *in vivo*[40]. Currently, the modifications of acetylation and propionylation are not linked, but this shows how the NAT complexes may have a role in multiple cellular pathways.

Prevention of acetylation obviously occurs, as previously discussed, when one or more of the NAT complexes is not present or otherwise nonfunctional, but this is not the only way to block acetylation. Presence of a proline at the first or second position of a polypeptide chain prevents acetylation; this is known as the (X)PX rule[38]. Proteins with a proline at the second position remain non-acetylated even when the primary amino acid is serine or alanine, two amino acids which generally result in N-terminal acetylation by NatA[38]. Knowing this (X)PX rule allows for manipulation of amino acid sequences to prevent a protein's acetylation, allowing it to be studied for potential changes in stability or any other functions. There is tentative experimental evidence for proline residues being acetylated in yeast, although this has not been proven; if it is true, it could potentially be another difference in acetylation between multicellular and unicellular eukaryotes[38].

***Saccharomyces cerevisiae* as a Model System**

S. cerevisiae is a valuable tool as a model organism. Although a unicellular eukaryote, it is still capable of carrying out many of the basic cellular processes - such as acetylation - which are important to life; unlike prokaryotes, it is capable of many post-translational modifications[41]. Practically speaking, yeast are straightforward to work with, inexpensive, and have a short generation time[42]. Yeast are consistently used to produce foreign proteins, which allows this organism to be the model system for many different fields of research[41].

Yeast are used as a model to study neurodegenerative diseases[42]. Although these diseases themselves may only be present in some multicellular organisms, the processes which occur in affected organisms can also be observed in a unicellular system. Studying tauopathies, for instance, is entirely capable in *S. cerevisiae;* expression of Tau in yeast does not result in a negative phenotype[42]. This provides a perfect basis for studies of Tau; if the protein alone does not result in a notable stress response of any kind, experimental

manipulations can more easily be performed. One such manipulation which has been previously studied is the co-expression of Tau and α-synuclein; as co-localization has been noted in clinical samples, the association continues to be investigated[42].

α-synuclein itself has caused growth delays when expressed in *S. cerevisiae;* when it was co-expressed with Tau, the growth delays seen were comparable to that of when α-synuclein was expressed alone, with no additive effect resulting from the inclusion of Tau[42]. As previously stated, expression of Tau on its own did not result in arrested growth[42]. The presence of α-synuclein affected solubility of Tau, resulting in a higher fraction of insoluble (aggregated) Tau protein than what was seen in cells with Tau alone[42]. α-synuclein was also found to increase the levels of phosphorylated Tau[42]. It has been previously suggested that α-synuclein forms a complex of sorts with GSK3β (the kinase which mediates phosphorylation) and Tau which results in increased phosphorylation; this finding suggests that it is capable of forming a similar complex with a yeast orthologue of GSK3β[42,43]. These results do show that Tau is phosphorylated and is capable of aggregating within *S. cerevisiae* although no negative phenotypes were directly associated with increased levels of Tau phosphorylation and aggregation; this result could potentially just be explained by the difference between yeast cells and human neurons[42].

Tau phosphorylation or hyperphosphorylation is not only observed in the presence of α-synuclein; human protein Tau expressed in yeast on its own, without any other foreign proteins, is capable of aggregating and being phosphorylated[44]. In *S. cerevisiae,* this process is controlled by yeast kinases; two suggested to be particularly important are Mds1 and Pho85, which are the orthologues of the human kinases GSK3β and cdk5, respectively[44]. Tau expressed in yeast strains lacking Pho85 were shown to have increased amounts of phosphorylation and aggregation, suggesting that Pho85 - and therefore possibly cdk5 in neuronal cells - acts as a negative regulator of these processes[44]. Absence of Mds1, on the other hand, resulted in decreased amounts of hyperphosphorylated Tau[44]. Both 3R and 4R isoforms of Tau were used in the study, and although both were phosphorylated, there was a

marked difference in that 3R was not seen to undergo extensive aggregation while 4R was[44]. Overall the finding that the 4R isoforms aggregate more readily than the 3R isoforms implies that the extra microtubule-binding domain may potentially be relevant in the formation of aggregates[44]. That being said, binding of Tau to yeast microtubules has not been previously noted, suggesting that it may be the structure of this domain, rather than its function, which is particularly important in aggregate formation[44]. Because Tau is not currently known to bind to *S. cerevisiae* microtubules, studies regarding microtubule binding must be done outside the cell; that being said, the Tau protein used in said studies can be expressed and purified from yeast cells, reducing the need to use the more expensive, complicated, and time-consuming mammalian cell cultures. Tau-4R purified from yeast retains its ability to bind to microtubules and has been noted to do so *in vitro*[45].

Phosphorylation was decreased in the absence of mds1, and ability to bind increased; likewise, phosphorylation increased in the absence of the negative regulator Pho85 and ability to bind to microtubules decreased[45]. Hyperphosphorylated Tau isolated from neuronal cells of Alzheimer's disease patients also was shown not to have normal interactions with microtubules, confirming this finding[45]. A mutant isoform of Tau (P301L) was also used in this study, and it was shown to aggregate on the microtubules themselves[45]. Mutations in the gene encoding for Tau can lead to a variety of mutant Tau forms, which may contribute to disease by being more prone to aggregation[45]. The hyperphosphorylated form disturbed the overall structure and organization of the microtubules but did not appear to form large clusters on them; it was assumed that this form resulted in smaller aggregates[45].

Another factor potentially affecting the behavior of Tau protein is oxidative stress, in which reactive oxygen species or free radicals are formed within the cell[46]. It has been previously suggested that oxidative stress may play a role in tauopathies, playing a role in the formation of neurofibrillary tangles in conjunction with hyperphosphorylation[46]. Treatment of yeast cells with ferrous sulfate resulted in increased levels of insoluble Tau in wild-type cells[46]. Both mutant Tau forms and the wild-type 2N-4R isoform consistently showed these increased

levels of aggregation; however, it was noted that the mutant forms showed significantly higher levels of aggregation than that of the the 2N-4R isoform, suggesting that the mutants are more susceptible to aggregation induced via oxidative stress[46]. This experiment was also performed on cells lacking the Pho85 kinase, although the effect was not as noteworthy as that of the wild-type cells[46]. Analysis by Western blot suggested that Tau - or at least the 2N-4R isoform - could actually be dephosphorylated under oxidative stress conditions[46]. Overall, this suggests that oxidative stress acts in parallel to phosphorylation to cause aggregation[46]. Iron ions have also been found in Lewy bodies, and addition of iron ions to yeast cells expressing alpha-synuclein showed increased aggregation, suggesting that oxidative stress may play a role in many different forms of neurodegenerative disease, and this can also be easily replicated in this yeast model[47].

It is clear, therefore, that multiple factors likely correlate with increased aggregation. Phosphorylation has been previously linked with acetylation; however, the effects of other post-translational modifications have not been extensively examined. N-terminal acetylation is the most common protein modification[24], yet it has not been considered as a potential factor which could affect aggregation. *S. cerevisiae* is capable of N-terminally acetylating proteins, and has previously been characterized as a useful model system for protein expression *in vivo*[41]. This project has involved expressing Tau in *S. cerevisiae* with the goal of creating a system with which to examine the effects of acetylation on Tau *in vivo.*

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MATERIALS AND METHODS

Yeast growth

Unless otherwise stated, all cultures were grown in YPD media at 30°C, and a roller drum or shaking platform was used to allow for aeration. Heat shock treatments were performed by growing the yeast at 37°C for at least four hours.

Yeast strains

74D-694 (psi- WT) cells were used as a parent strain and served as the wild-type control for all experiments, as well as providing the wild-type background for integrant strains. 74D-694 cells with the the nat3 subunit of NatB knocked out served as the parent strain for all ΔNatB integrants mentioned. 64D-694 cells with the mak3 subunit of NatC knocked out served as the parent strain for all ΔNatC integrants mentioned. ΔNatA strains had both the Ard1 and Nat1 subunits of ΔNatA knocked out.

Plasmids

pYX212-Tau (2N4R) with +URA selection marker

pRS304-GPD (Tau-GFP) with +TRP selection marker

pRS304-GPD (Tau-A2P) with +TRP selection marker

pRS304-GPD (Tau-A2P-GFP) with +TRP selection marker

Integration of plasmids into yeast genome

Each plasmid (Tau, Tau-A2P, Tau-GFP, Tau A2P-GFP) was linearized by restriction digest with the enzyme Bsu36I prior to integration. 10mL of cells were spun down and washed before resuspension in 50 uL of 100 mM LiAc prior to a 15-minute 30°C incubation period. The cell suspension was then added to the solution of linearized plasmid and 300 uL of PEG-LiAc was added prior to another incubation period of 30 minutes at 30°C. Heat shock at 42°C for 20 minutes then followed. Cells were pelleted and resuspended in sterile water before plating on

selective media (SD-URA for Tau integrants, SD-TRP for Tau-A2P, Tau-GFP, or Tau A2P-GFP integrants).

SDS-PAGE

2.5 ODs of yeast cells were lysed via NaOH lysis and resulting lysates were resuspended in 50 uL of SDS sample buffer (2.25 mM BME, 1.3 mM SDS, 10 mM glycerol, 0.07% bromophenol blue, 12% 1 M Tris-HCl 6.8). 8% polyacrylamide gels allowed for resolving of proteins, which were then transferred onto a PVDF membrane and visualized via standard Western blot procedure using anti-Tau and anti-GPD antibodies.

SDD-AGE

40 ODs of cells were spun down and washed, keeping everything on ice. Standard glass bead lysis was used to lyse the cells. Samples were then added to SDD-AGE sample buffer (24% 1M Tris-HCl 6.8, 15% glycerol, 3 mM SDS, 3 mM bromophenol blue) and incubated at 30°C for 5 minutes. Lysates were loaded onto a 1.25% agarose gel containing 0.0002% SDS in a Tris-glycine buffer. 1x LRB was used as a running buffer. Gels were resolved for roughly 40 minutes at 160V and transferred onto a PVDF membrane at 20V for 3 hours. Visualization was done using standard Western blot procedure with anti-Tau antibody.

Microscopy

A wet mount of 10 uL of exponentially growing culture was prepared, and excess light exposure on the slide was minimized by keeping the slide in an enclosed container until visualization. Slides were visualized via fluorescence microscopy at 40x magnification.

Oxidative stress treatment

100 mL cultures were treated with 1M $FeSO_4$ for two hours before centrifugation and lysis in order to cause oxidative stress.

RESULTS

Expression of the Microtubule-Associated Protein Tau in *Saccharomyces cerevisiae*

As Tau is a protein found in mammalian neurons, our model system of *S. cerevisiae* are not capable of endogenously producing this protein of interest[1]. Standard LiAc/PEG yeast transformation protocol is useful but has to be consistently repeated since future generations of cells do not retain the plasmid. As a result, the transformation protocol was adapted to include the linearization of the Tau plasmid via restriction enzyme (Fig. 1) to allow for insertion directly into the yeast genome to ensure that the production of Tau remained constant from one generation to the next.

Although *S. cerevisiae* do not endogenously produce Tau, they are capable of post-translational modifications such as N-terminal acetylation, which is performed by one of several N-acetyltransferase (NAT) complexes[2]. Based on its amino acid sequence, Tau is predicted to be acetylated by NatA. In order to determine the effect of acetylation or lack thereof, four strains were chosen for integration with the Tau plasmid: wild-type, ΔNatA, ΔNatB, and ΔNatC. The Tau plasmid also contained a marker for growth on selective media (in this case, +URA) which ensured that only cells which successfully took up the plasmid would grow on SD-URA plates. As each integration resulted in multiple colonies, several colonies were selected for screening in order to determine the most efficient production of Tau in each strain. The colonies were restruck on a selective plate and lysed in order to examine expression of Tau, which was visualized via Western blot (Figure 2A, 2B). These Western blots were used to determine the best integrant colony which was done by examining the blot for the most effective protein expression (that is, the darkest band), and this process was repeated for all four strains.

Following this process, the selected integrant colonies were frozen down as new strains of yeast for use in future experiments. Expression of Tau was again confirmed by Western blot of the four new strains and their parent strains, which lack Tau (Figure 3). In order to examine whether any growth differences were qualitatively observed, a spot plate of the parent strains and the Tau strains was performed (Figure 4A). The presence of Tau did not appear to inhibit

growth; conversely, in some cases, the strains with Tau seemed to have increased growth compared with the wild-type or ΔNat knockout parent strain.

Production of Tau-A2P Strain

Although the ΔNatA+Tau strain created is assumed to have unacetylated Tau, it is also true that this strain has a large amount of other proteins which are typically acetylated. Yeast strains lacking NAT complexes typically show phenotypic deficits in temperature resistance, growth, mating, and other variables[3,4]. Because of this, it could potentially be difficult to determine whether any differences seen between the WT+Tau and ΔNatA+Tau strain are solely due to the acetylation status of Tau, as opposed to a downstream effect based on the complete loss of the NatA complex. To account for this, a version of Tau containing a substitution at the second position (alanine to proline) was also utilized. A proline at the second position blocks acetylation[5], so by integrating this version of the Tau plasmid into wild-type yeast, we could have unacetylated Tau in a completely healthy background. The same protocol for integration into the yeast genome was followed; the selective marker here was +TRP so the successful integrants would grow on SD-TRP media. Integrant colonies were screened by selection of the colony with the best protein expression, analyzed via Western blot (Figure 5A). The Tau-A2P plasmid was also integrated into ΔNatA cells (Figure 5B). Expression of Tau was again confirmed for all of the Tau-producing strains via Western blot (Figure 5C). Growth differences were examined via spot test (Figure 4B). There did not appear to be any differences between the growth of strains producing Tau and the wild-type strain (Figure 4B).

Visualization of Tau Aggregation in *S.cerevisiae* by SDD-AGE

Although SDS-PAGE is a very useful tool for determining whether or not a protein is expressed, this method is not particularly conducive to examining aggregation; aggregates do not typically survive the denaturing conditions, and are also typically too large for the pores of a polyacrylamide gel. In order to visualize Tau aggregates in *S. cerevisiae,* we utilized SDD-AGE (Semi-Denaturing Detergent Agarose Gel Electrophoresis) which has previously been utilized to examine other forms of aggregates. SDD-AGE has previously been used for visualization of

Sup35 prion aggregates in yeast, but has never before been used to visualize Tau aggregates. In order to determine whether or not this method would be sufficient for visualization of Tau aggregates, Sup35-HA prion aggregates were utilized as a control. As seen in Figure 6, the Sup35-HA aggregates were higher on the gel than the Tau aggregates; this indicates the Tau aggregates are smaller than Sup35 aggregates, but a quantitative comparison is not possible due to the current lack of a proper standard or ladder for this method.

As this method requires a large OD of cells, we next wanted to determine whether or not it was feasible to use frozen pellets or if this method always had to be started from fresh pellets (Figure 7). WT+Tau cells were flash-frozen via liquid nitrogen and placed in a -80°C cooler. Another sample of ΔNatC+Tau cells were placed in the freezer without flash-freezing. As comparison, a fresh sample of WT+Tau cells was taken, and then all three samples were lysed and run on the gel. The lanes of both frozen samples had no visible signal on the Western blot, suggesting that some aspect of the freeze-thaw cycle disrupts aggregation, and therefore freezing down pellets is not adaptable to this method. In the future, it may be useful to determine whether or not the total proteome is disrupted by the freeze-thaw cycle, or whether this may be a Tau-specific effect.

Next, multiple strains of Tau-producing yeast were examined via this method to determine Tau aggregation between strains with and without acetylation (Figure 8, Figure 9). Although the strains of different aggregates are not identical, they are consistently similar in size. Small differences may be noted, such as a slight shift up in Tau-A2P (Figure 9) but without a ladder there is no way to quantify this kind of minor shift.

In addition to directly examining the differences between strains, we were also aiming to examine the potential differences in aggregates when exposed to cellular stress. In an attempt to examine this, a sample of WT+Tau yeast was treated for two hours with 1M $FeSO_4$, which results in oxidative stress for the cells[6]. This sample and a control sample of WT+Tau yeast which had not received the oxidative stress treatment were examined via SDD-AGE (Figure 10). A slight shift upward may be apparent in the 2-hr treatment lanes, but as before with the

different strains, small shifts are difficult to quantify or fully claim without a standard. Future experiments will involve examining the different strains when under some form of cellular stress to see whether there is a noticeable difference between the aggregates of different strains when exposed to the same stressor.

Examination of Cellular Stress by Microscopy

Microscopy is often used as a tool to visually examine differences between cells. In particular, green fluorescent protein (GFP) can be a valuable tool for examining localization and therefore aggregation, since GFP is easily visible in cells with the aid of a fluorescent microscope. In order to determine whether differences between localization and/or amount of GFP-tagged protein can be visualized using this method, strains containing GFP fused to heat shock protein 104 were examined. Two different strains - one [*PSI+*] and one [*psi-*] - were examined with and without the presence of a heat shock treatment in order to qualitatively determine if a difference was visible in the amount and localization of Hsp104-GFP (Figure 11). In both cases the heat shock treatment resulted in brighter patches of Hsp104-GFP, although it is important to note that this was qualitative and no quantification or cell-counting was performed to determine significance.

Following this determination, the psi- strain containing Hsp104-GFP was transformed with either the empty vector or the Tau plasmid and again examined under the microscope to examine whether or not the presence of Tau within the cells appeared to be a version of cellular stress analogous to heat shock which would result in increased levels of this chaperone protein (Figure 12). Qualitatively, it did appear that for at least some of the cells, the presence of Tau was a stressor which increased in increased levels of chaperone proteins, which were seen as brighter green cells.

Production and Examination of Strains Containing Tau-GFP

Since examining cellular stress by chaperone expression does not directly examine the effects of Tau aggregates, new strains were created which had a Tau-GFP construct. Once again, the plasmid was linearized and integrated directly into the yeast genome, and several

integrant colonies were selected for screening from each strain (Figure 13A). The Tau-GFP plasmid was integrated into wild-type, ΔNatA, and ΔNatC cells. In addition, a construct containing A2P-Tau-GFP was also integrated into WT and ΔNatA yeast for the same purpose as the creation of the initial Tau-A2P strain; any differences seen could then be assumed to be the result of the acetylation status of Tau rather than downstream effects of lacking the NatA complex completely. Expression of Tau for all of the GFP strains was again confirmed via Western blot (Figure 13B). Growth differences were visualized via spot test (Figure 14). Expression of Tau-GFP was confirmed via fluorescence microscopy (Figure 15).

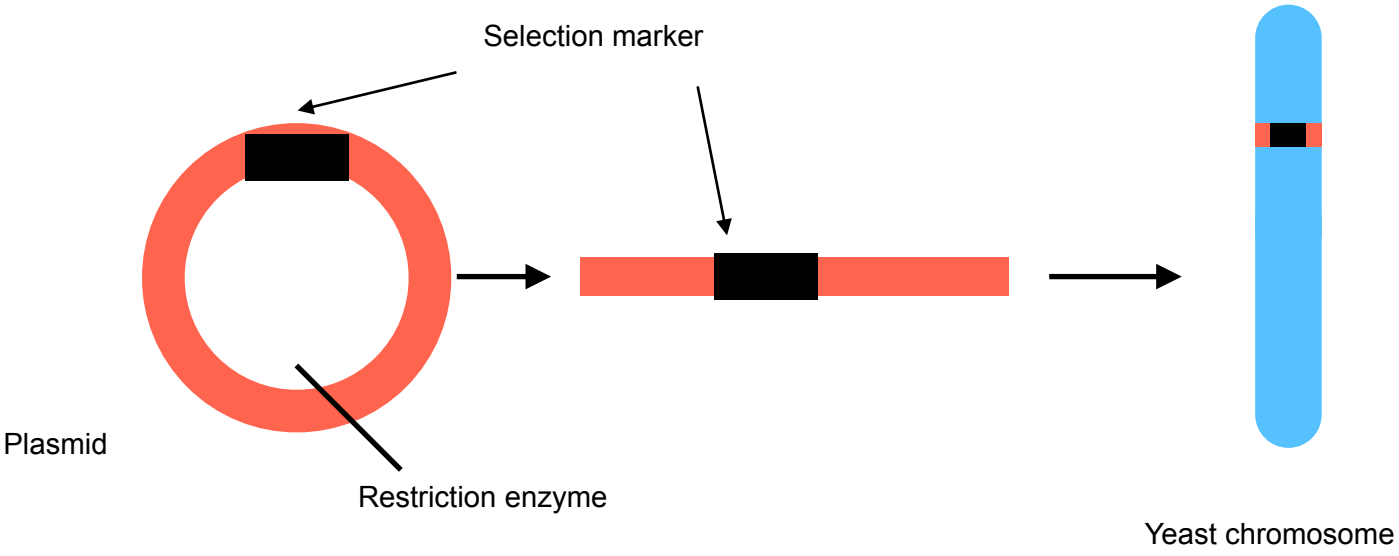


Figure 1: Schematic explaining the integration of genetic material into the *Saccharomyces cerevisiae* genome. LiAc transformation of yeast cells was adapted to include the linearization of the plasmid of interest in order to insert into the chromosome.

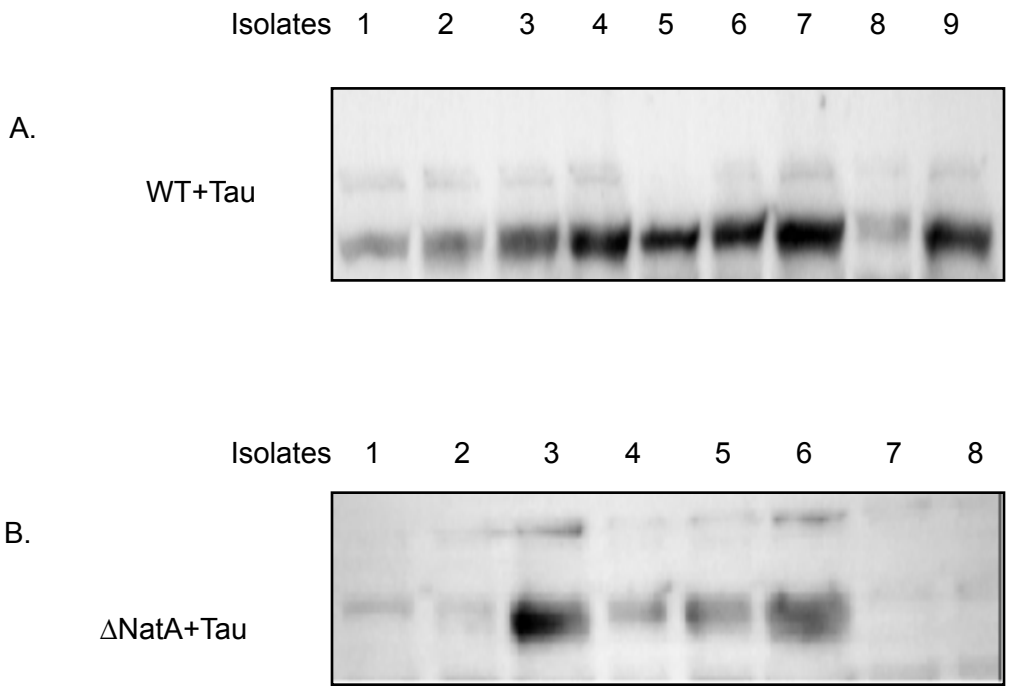


Figure 2: Examples of isolate screening Western blots. Following integration, colonies which successfully grew on selective media were isolated and restruck on a second plate. Each of those colonies was then lysed, and the lysates ran on SDS-PAGE. A: Expression of Tau in wild-type yeast integrated with the linearized Tau plasmid. B: Expression of Tau in ΔNatA yeast integrated with the linearized Tau plasmid.

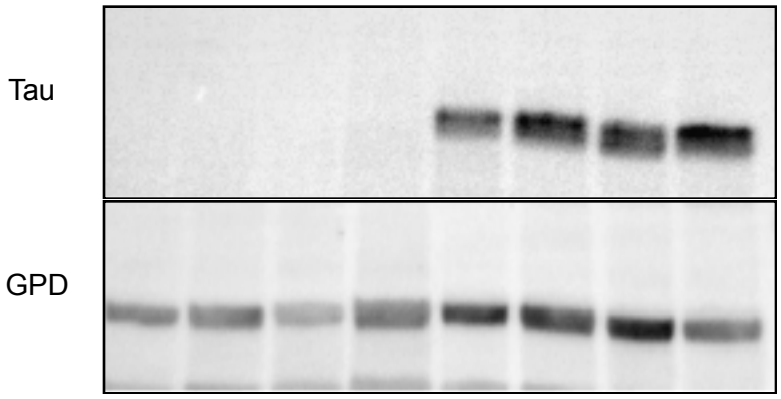
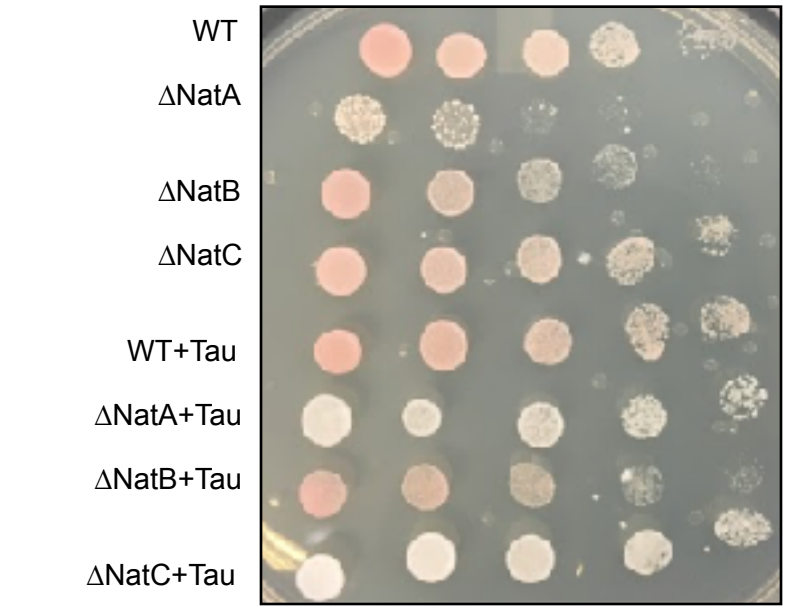
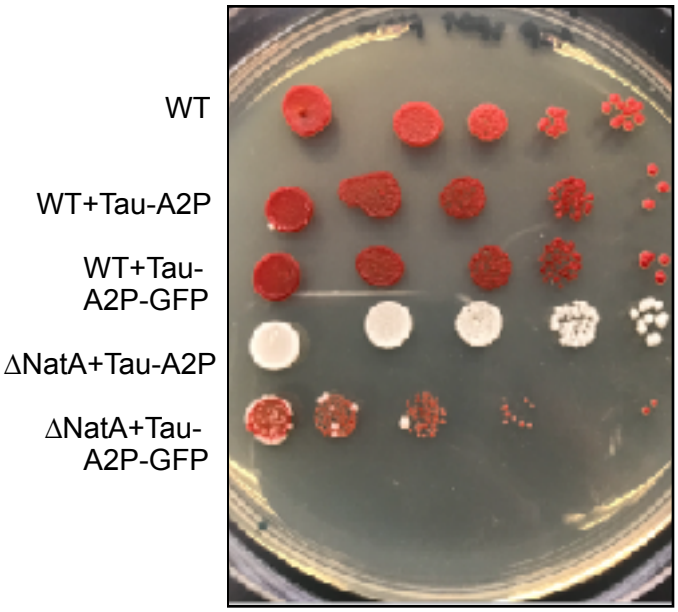


Figure 3: Initial Tau-producing strains and parent strains. Tau expression visualized via Western blot with anti-Tau antibody and anti-GPD load control.



A.



B.

Figure 4: Spot test showing parent strains and mutant strains. A: Parent strains and initial Tau-producing strains. B: Strains containing Tau-A2P. Spot tests generated by diluting a saturated overnight culture by a factor of 1:10 for each successive spot.

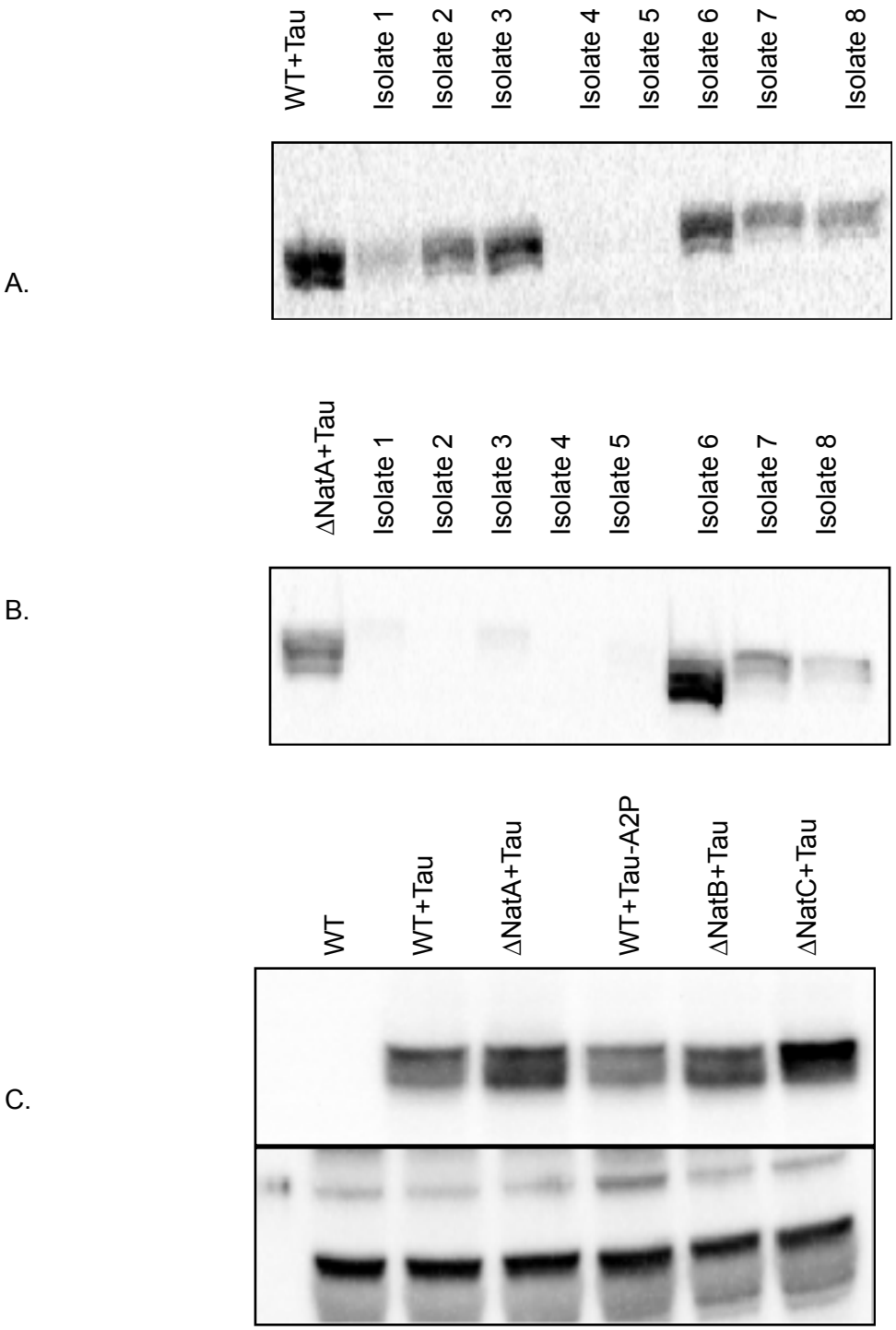


Figure 5: Expression of Tau-A2P. A: Expression of Tau in integrant colonies of WT+Tau-A2P, compared to the previously-created WT+Tau strain, visualized via Western blot with anti-Tau antibody. B: Expression of Tau in integrant colonies of ΔNatA+Tau-A2P, visualized via Western blot with anti-Tau antibody. C. Western blot of Tau-producing strains with GPD load control.

Figure 6: SDD-AGE of Tau aggregates andSup35-HA prion aggregates. Sup35-HA prion aggregates were run as a control, since Tau has never been visualized by this method previously. Anti-Tau antibody was used as the primary, and anti-HA was added to the secondary antibody solution.

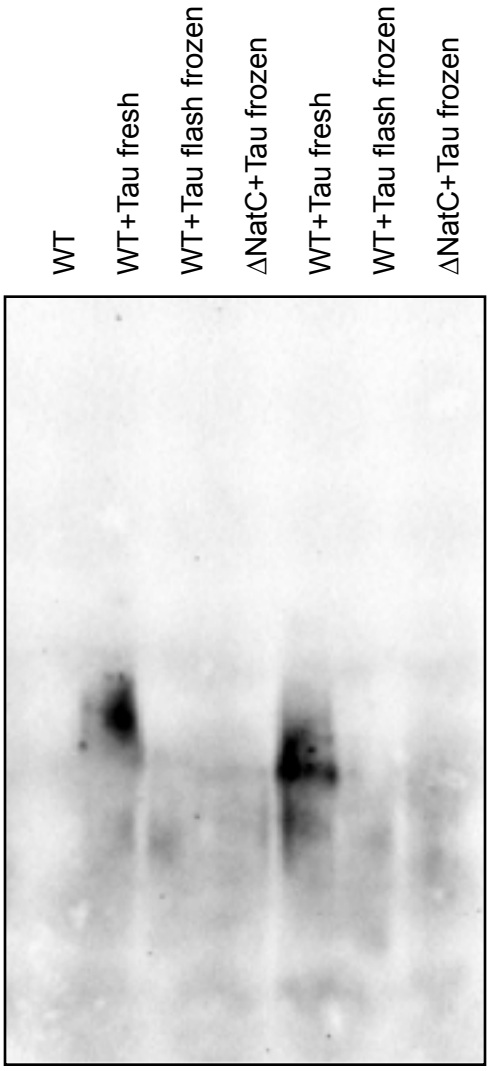


Figure 7: SDD-AGE of fresh and frozen pellets. Visualized via Western blot using an anti-Tau antibody.

Figure 8: SDD-AGE comparing the Tau aggregates of three Tau-producing strains.
Visualized via Western blot using an anti-Tau antibody.

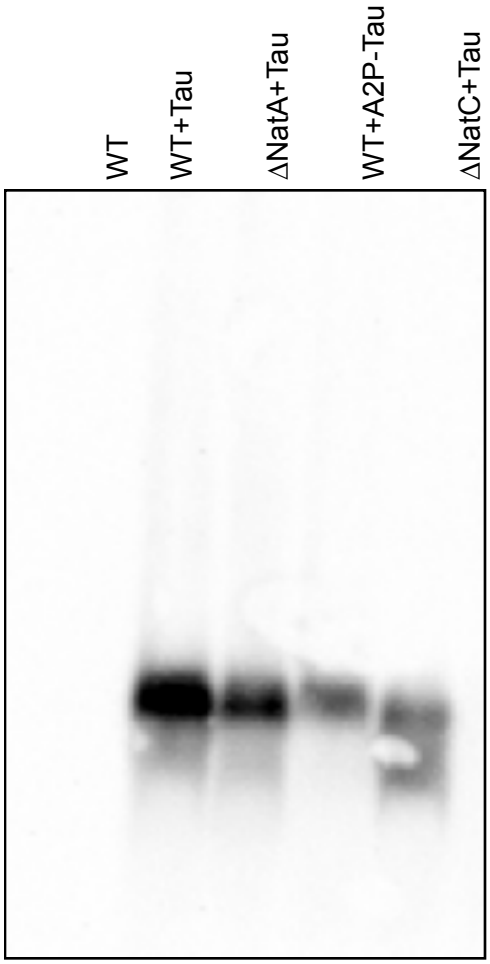


Figure 9: SDD-AGE showing WT Tau compared with ΔNatA, A2P, and ΔNatC. Visualized via Western blot using an anti-Tau antibody.

Figure 10: Effects of oxidative stress on WT+Tau yeast. Effects of oxidative stress and/or heat stress will be examined in the different strains. Visualized via Western blot, using an anti-Tau antibody.

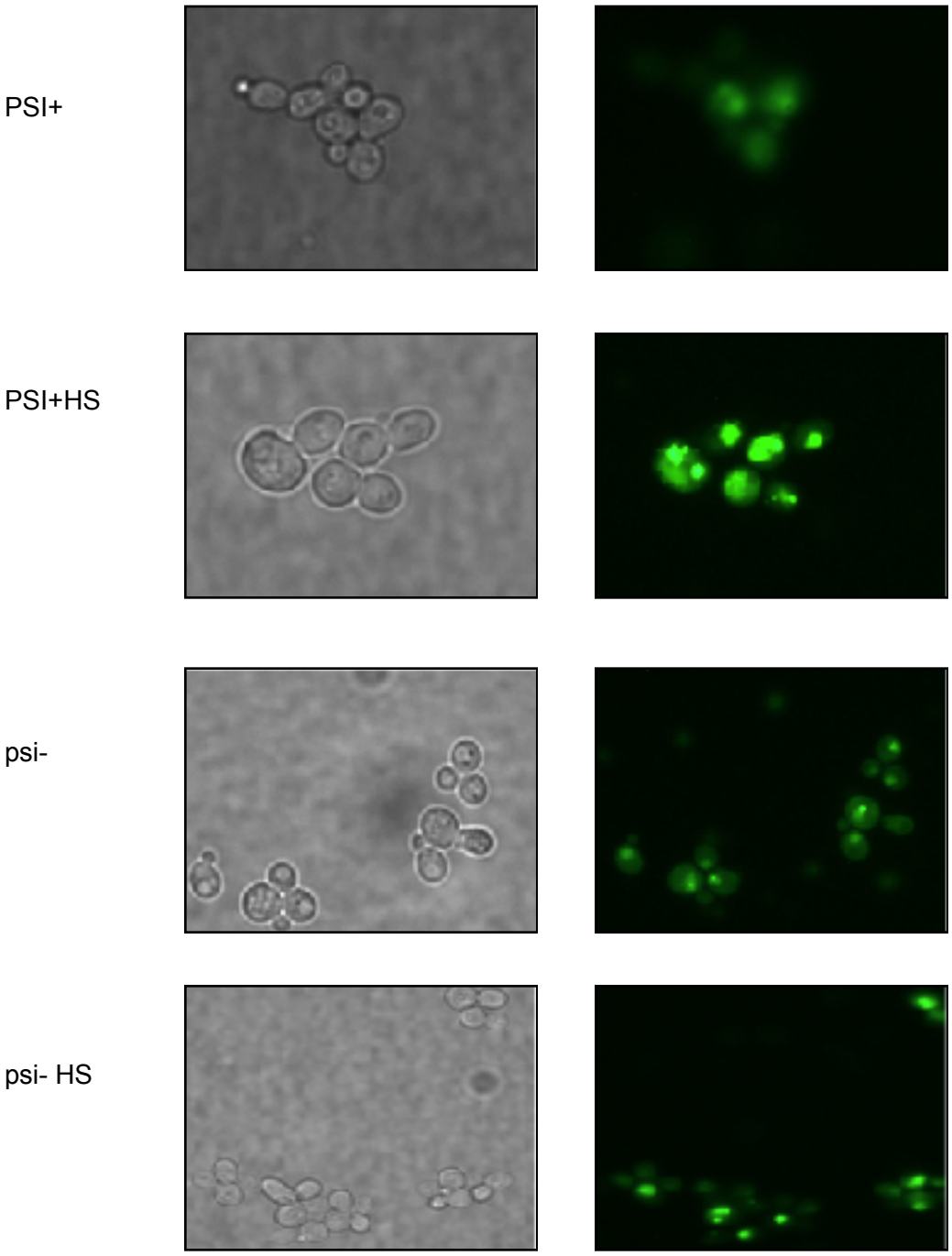


Figure 11: Qualitative microscopy of Hsp104-GFP cells. Microscopy, with the aid of GFP-tagging proteins, can be another method to examine cellular stress. Cells with GFP-tagged heat shock protein 104 (Hsp104) were heat shocked to qualitatively examine differences in GFP expression and determine whether this is an optimal method to potentially examine cell stress.

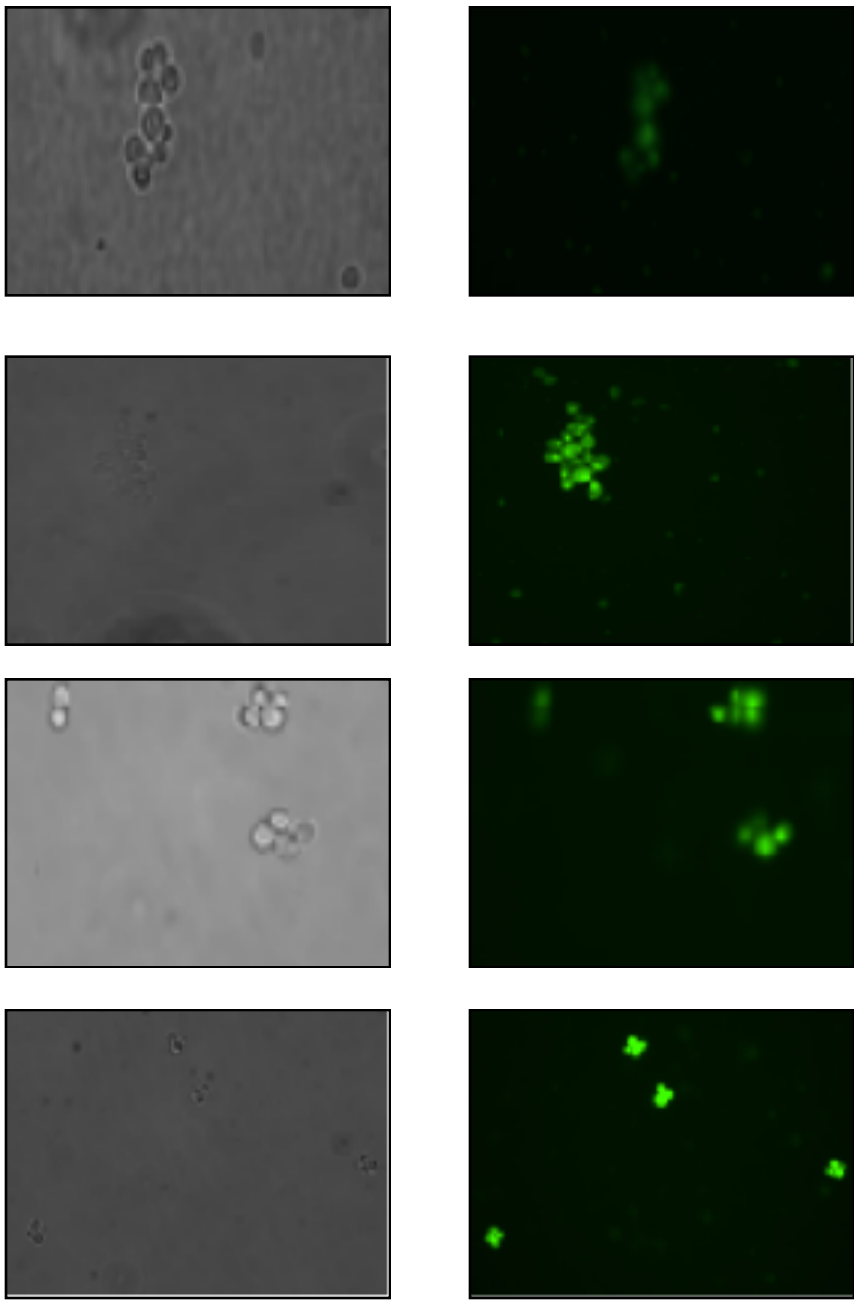


Figure 12: Qualitative microscopy of psi- Hsp104-GFP cells either transformed with the Tau plasmid or an empty vector.

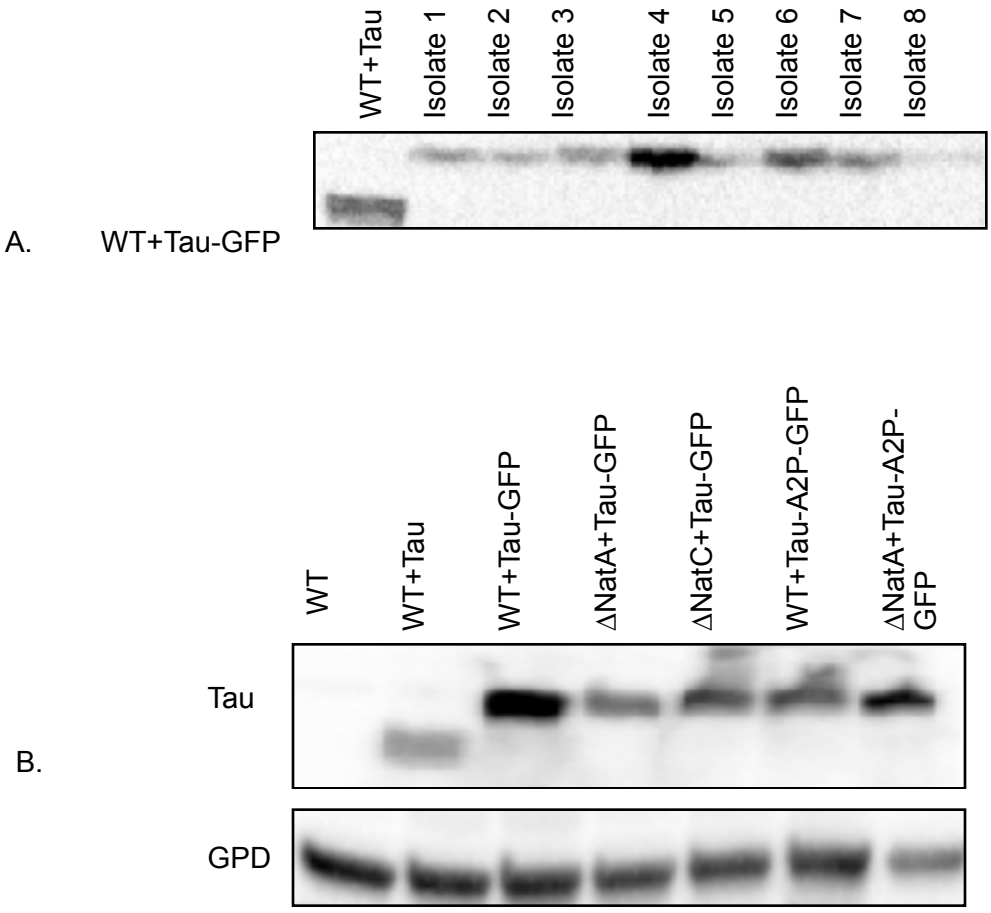


Figure 13: Tau expression of Tau-GFP strains. A: Sample isolate Western blot showing the expression of Tau-GFP in the WT+Tau-GFP integrant colonies compared to WT+Tau. B: Expression of Tau-GFP in the final strains, visualized via Western blot.

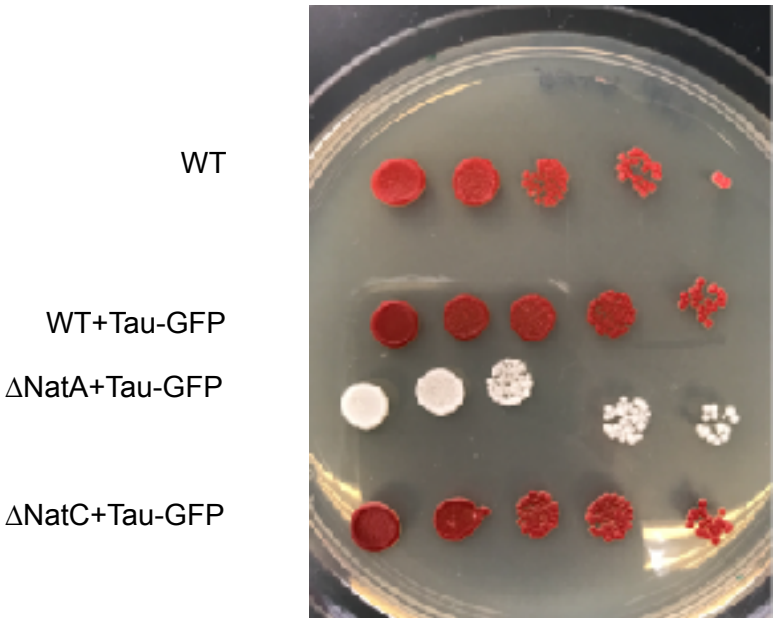


Figure 14: Spot test of GFP strains. Saturated overnight cultures were diluted 1:10 for each successive spot.

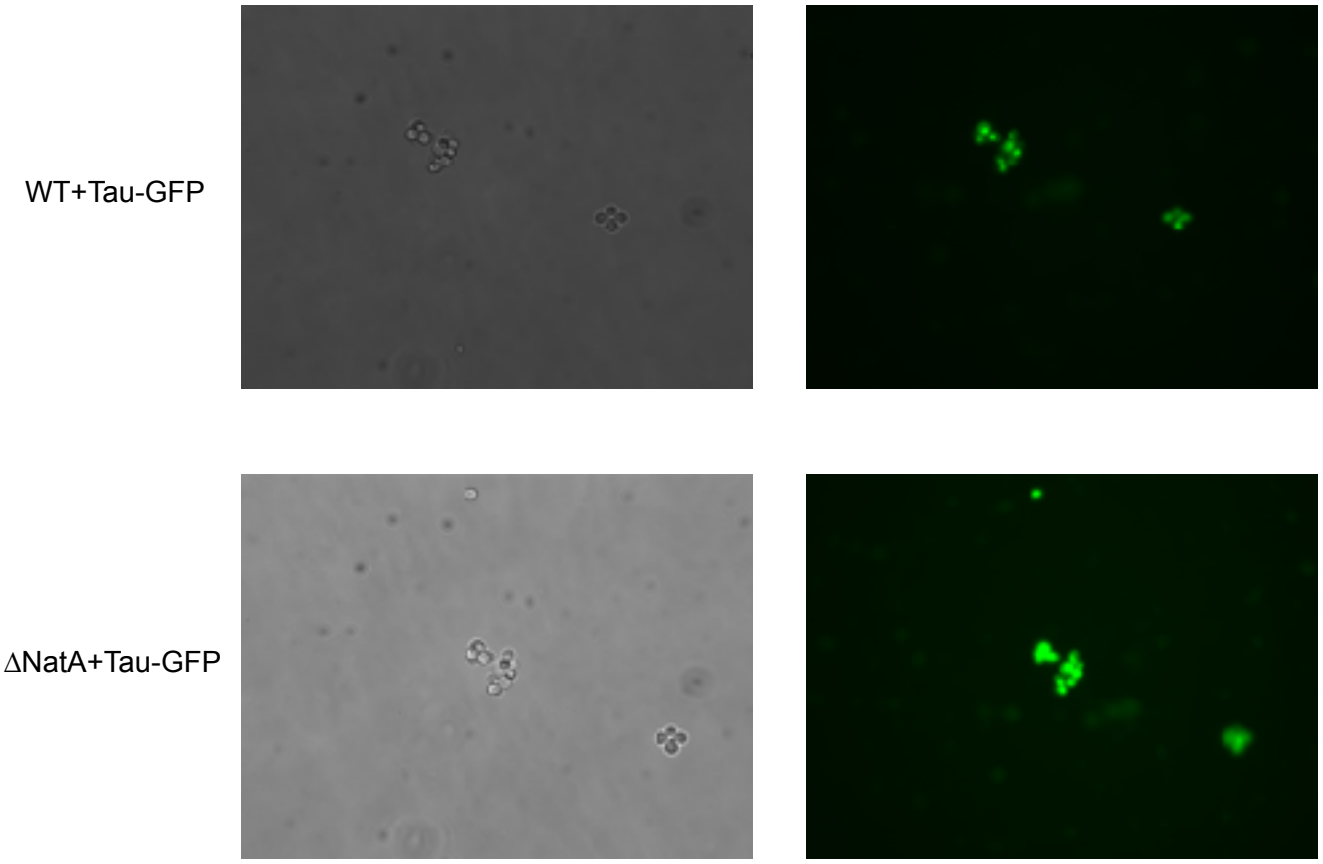


Figure 15: Representative sample images of Tau-GFP strains under the microscope.

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DISCUSSION

Although N-terminal acetylation is a very common post-translational modification, its effects on the protein Tau have not been properly examined. The amino acid sequence of Tau suggests that it is a suitable candidate for acetylation by the NatA complex; an alanine residue exposed by methionine cleavage, as seen in Tau, is a common target for NatA[1,2]. In addition, acetylation of Tau's N-terminus has been observed in both Tau from Alzheimer's patients and normal Tau[2]. It is reasonable to predict that if this modification is indeed present, then it likely has some effect on the protein. N-terminal acetylation changes the positive N-terminus to a neutral charge by the addition of an acetyl group[3]. Any change in charge could potentially affect the propensity of a protein to interact with other molecules within the cell; it could also affect the way in which the protein folds. It is important to examine all factors which may play a role in Tau's structure and function; changes in any of these pathways could contribute to aggregation and tauopathy. Other modifications such has hyperphosphorylation have previously been correlated with increased aggregation; although these studies do not discuss acetylation directly, they do show how changes to the protein's structure can affect stability and function.

S. cerevisiae provide a useful model organism for protein expression; their eukaryotic structure provides at least a basal similarity to human cells[4]. They endogenously contain the NAT complexes responsible for acetylation as well as the molecular machinery to perform other post-translational modifications[4]. *S. cerevisiae* have been previously utilized as a model to study neurodegenerative disease, and expression of Tau in yeast is efficient and does not cause negative phenotypes[5]. Based on amino acid sequence, Tau should be acetylated in wild-type yeast. Expressing Tau in *S. cerevisiae* allows for experiments *in vivo* which can examine changes to acetylation status. By examining the difference in results between the strain containing Tau-A2P and the strain which lacks NatA entirely, for instance, it can be determined whether the potential effects seen are due to the effect of Tau specifically lacking

acetylation or due to an overall reduction of acetylated proteins within the cell and disruption of other cellular pathways that may result in downstream effects on Tau.

The formation of Tau aggregates is heavily implicated in neurodegenerative disease[6]. Any method which can characterize or examine Tau aggregates could provide insight into factors affecting aggregation as well as allow for characterization of these aggregates. This project showcases the first occurrence of SDD-AGE being utilized to examine Tau aggregation. SDD-AGE has been previously utilized to examine other protein aggregates, such as Sup35 prion aggregates from *S. cerevisiae,* but it has not been previously used as a method to examine Tau. Our results show that this method is a viable option for visualizing Tau aggregates. Although no notable differences have been seen in aggregate sizes between the strains, future work will attempt to examine the effect of different treatments and how the strains respond. It could be possible that under normal conditions, unacetylated and acetylated Tau aggregate at similar rates, but with the addition of a further stressor, changes in aggregation dynamics may be visible. Increased aggregation has been noted under various experimental conditions, including mutations in the Tau protein[7], oxidative stress[7], and the presence of α-synuclein[5]. This suggests that aggregation is affected by multiple factors which may work in parallel with one another, and disease conditions may not be directly caused by any one on its own. It is therefore crucial to examine the interplay of different conditions. Heat and oxidative stress are typically utilized in yeast models to examine cell stress, and these will likely be the first conditions examined further. Preliminary work was done with an oxidative stress treatment (Figure 10); these results are not fully conclusive but may indicate that oxidative stress does slightly increase aggregate size in WT+Tau yeast, suggesting that this treatment may be a viable method for affecting aggregation rate/amount. Further directions will include utilizing some form of oxidative stress treatment like this on all of the different yeast strains as well as testing heat stress to determine whether that could also differentially affect aggregate size between strains.

These differences in aggregate size will also be examined via microscopy. Preliminary data suggests that fluorescence microscopy is a viable tool for examining localization of GFP-tagged proteins in *S. cerevisiae* cells; although none of the data collected has been quantified, this again suggests that this method could be utilized in the future to examine differences between strains. The same sort of conditions which will likely be examined via SDD-AGE - that is, heat stress and oxidative stress - will also be used to treat cells prior to microscopy in order to potentially visualize the differences in aggregation within the cells themselves. Ideally, the results gained from microscopy and the results gained from SDD-AGE could together provide a more complete picture of Tau aggregation within the strains. Fluorescence microscopy could also provide insight into localization of aggregates within cells, particularly if it differs among conditions.

So far, this project has established that *S. cerevisiae* is a useful model for examining Tau aggregation. Tau has been constitutively been expressed in multiple strains with and without acetylation, and although precise effects of acetylation have yet to be conclusively determined, it is safe to say that this system provides a valuable basis for further experiments regarding acetylation's effects. We have shown that Tau aggregates within yeast strains and these aggregates can be visualized by way of SDD-AGE, and GFP-tagged Tau aggregates can be visualized via fluorescence microscopy. The results shown here have laid the groundwork, allowing for future experiments which aim to determine the effect of acetylation or lack thereof on aggregation of Tau. As any modification to a protein can cause structural changes, which can therefore impact its interactions with other proteins, it is important to examine all of these modifications to Tau in the hopes of finding ways to potentially reduce aggregation.

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