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YEAST AS A MODEL ORGANISM TO STUDY DISEASES OF PROTEIN MISFOLDING

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ABSTRACT

Several neurodegenerative disorders, such as Parkinson's and Huntington's disease, are associated with protein misfolding, aggregation, and accumulation. Generally, these diseases strike in midlife either directly or indirectly leading to death. While the clinical and pathological hallmarks of these diseases have been known for a few decades, it was not until recently that the first molecular players were identified. Several of those players still remain unidentified. Therefore, the molecular mechanisms that lead to the selective degeneration of specific neuronal populations are largely unknown.

Cell and animal models have been used to investigate both the normal and pathological functions of some of the proteins already identified, such as α-synuclein in Parkinson's disease, and huntingtin in Huntington's disease, but slow progress has complicated the development of therapeutics for these diseases.

We developed a novel cellular model for synucleinopathies based on the expression of human α-synuclein in the yeast *Saccharomyces cerevisiae* to investigate the molecular mechanisms linking α-synuclein to those diseases. We also explored yeast models for Huntington's disease based on the expression of the exon 1 of the huntingtin protein. Our studies revealed that a simple doubling in the level of expression of α-synuclein in the cell, similarly to what is observed in certain families with Parkinson's disease, is a critical determinant of its localization and cellular effects. Analyses of the biological consequences of α-synuclein expression in yeast showed the protein is associated with lipid metabolism and intracellular trafficking, results that we confirmed in a genetic screen performed with the yeast gene deletion collection. A similar screen with

huntingtin identified protein folding and degradation as being the major pathways involved in modifying huntingtin toxicity in yeast.

Drug screens performed with several models for different neurodegenerative diseases showed no overlapping hits, suggesting neurodegeneration follows different pathways in different diseases.

Finally, our studies established a connection between α-synuclein and oxidative stress, which may help to explain the intimate connection between oxidative stress and mitochondrial dysfunction and Parkinson's disease.

Taken together, our studies establish yeast as a powerful system for learning about the basic molecular mechanisms underlying neurodegenerative diseases, especially Parkinson's disease. Thus, yeast models emerge as privileged systems for the initial studies that may enable the development of novel therapeutic approaches.

RESUMO

Várias doenças neurodegenerativas, como as doenças de Parkinson e Huntington, estão associadas com alterações conformacionais em proteínas, agregação e acumulação. Geralmente estas doencas surgem a partir da meia-idade, levando à morte, directa ou indirectamente. Apesar de as características clínicas e patológicas destas doenças ja serem conhecidas há algumas décadas, so recentemente foram identificados alguns dos intervenientes moleculares, e outros ainda estão por identificar. Assim, os mecanismos moleculares que levam à degeneração selectiva de populações neuronais específicas ainda são largamente desconhecidos. Modelos celulares e animais têm sido utilizados para investigar tanto a função normal como patológica de algumas das proteinas já identificadas, como a α-synuclein ou huntingtin, nas doenças de Parkinson ou Huntington, respectivamente, mas o progresso tem sido lento, complicando o desenvolvimento de terapêuticas para estas doenças.
Nesse sentido, desenvolvemos um novo modelo celular para synucleinopatias, baseado na expressão em levedura de α-synuclein humana, para investigarmos os mecanismos moleculares que relacionam a α-synuclein com essas doenças. Tambem explorámos modelos de levedura para a doença de Huntington, baseados na expressão do exão 1 da proteina huntingtin. Os nossos estudos revelaram que uma simples duplicação no nível de expressão de α-synuclein na célula, à semelhança do observado em famílias com doença de Parkinson, é um factor crítico na determinação da sua localização e distribuição e efeitos celulares. Análises das consequências biológicas da expressão da α-synuclein em levedura mostraram que a proteina está associada ao metabolismo de lípidos e transporte intracelular, resultados estes confirmados num screening genético efectuado com a colecção de knock outs em levedura. Um screening

semelhante com a proteína huntingtin identificou o folding e a degradação de proteínas como as principais vias involvidas na modulação da toxicidade da huntingtin em levedura.

Screenings de drogas realizados com vários modelos para diversas doenças neurodegenerativas não revelaram resultados em comum, sugerindo que a neurodegeneração segue vias diferentes nas diversas doenças.

Finalmente, os nossos estudos estabeleceram uma relação entre α-synuclein e stress oxidativo, o que pode ajudar a explicar a relação entre o stress oxidativo e disfunção mitocondrial e a doença de Parkinson.

De uma forma global, os nossos estudos estabeleceram a levedura como um sistema poderoso para se aprender sobre os mecanismos moleculares básicos que causam as doenças neurodegenerativas, especialmente a doença de Parkinson. Assim, modelos em levedura constituem sistemas privilegiados para o desenvolvimento inicial de novas intervenções terapêuticas.

RÉSUMÉ

Plusieurs maladies neurodégénératives, comme le sont celles de Parkinson et Huntington, sont associées a des altérations "conformationnelles" en protéines, agrégation et accumulation. En général, ces maladies surgissent à partir de "l'âge mûr" et conduisent au décès directement ou indirectement. Bien que les caractéristiques cliniques et pathologiques de ces maladies soient connues depuis déjà quelques décennies, certains intervenants moléculaires ont été que récemment identifiés, et d'autres ne l'ont toujours pas été.

Ainsi, les mécanismes moléculaires qui conduisent à la degeneration sélective de populations neuronales sont encore amplement inconnus.

Les modèles cellulaires et animales sont encore utilisées pour la recherche concernant aussi bien le fonctionnement moléculaire comme pathologique de certaines protéines déjà identifiées, comme l'α-synucleine ou huntingtin, pour les maladies de Parkinson ou Huntington respectivement, mais les progrès sont lents, retardant ainsi le développement thérapeutique pour ces maladies.

Nous avons développé un nouveau modèle cellulaire pour synucleinopathies, basée sur l'expression en levure de l'α-synucleine humaine, pour nous permettre la recherche des mécanismes moléculaires qui fait la relation entre l' α-synucleine humaine et ces maladies. Nous avons aussi exploré des modèles de levure pour la maladie de Huntington, basée sur l'expression de « l'exon » 1 de la protéine hutingtin.

Nos études ont révélé qu'une simple duplication au niveau de l'expression de α-synucleine dans la cellule, comme cela arrive dans les familles avec la maladie de Parkinson, est un facteur critique dans la détermination de sa localisation et distribution des effets cellulaires.

L'analyse des conséquences biologiques de l'expression de l'α-synucleine en levure a démontré que la protéine est associée au métabolisme de lipides et transport intracellulaire, ces résultats pouvant être confirmés au moyen du screening génétique effectué avec la collection des knock out en levure.

Un screening ressemblant avec la protéine huntingtin a identifié le folding et la dégradation de protéines comme les principales voies présentes dans la toxicité de huntingtin en levure.

Screenings de drogues réalisés avec divers modèles pour diverses maladies neurodégénératives n'ont pas révélé des résultats communs, suggérant que la neurodègèneration suive des différentes voies dans les diverses maladies.

Enfin, nos études ont établies qu'il existe une relation entre l'α-synucleine et le stress oxydatif, ce qui peut aider a expliquer la relation entre le stress oxydatif et la dysfonction mitochondrial et la maladie de Parkinson.

D'une manière générale, nos études ont établi la levure comme un système puissant, nous permettant d'apprendre à propos mécanismes moléculaires basiques qui provoquent les maladies neurodégénératives, en particulier la maladie de Parkinson.

Ainsi, des modèles en levure constituent des systèmes privilégies pour le développement initial de nouvelles interventions thérapeutiques.

OVERVIEW

The molecular basis of most neurodegenerative disorders is presently unclear despite the significant advances in recent years. The mapping and identification of genes associated with these devastating disorders enabled the development of multiple study models.

The convenient handling and easy manipulation have made the yeast *Saccharomyces cerevisiae* one of the most valuable model systems in which to dissect fundamental biological processes. The remarkable conservation of gene function between yeast and higher eukaryotes, including humans, affords the possibility of identifying and characterizing conserved genes and pathways, which hold promise for the development of novel avenues for therapeutic intervention.

This thesis describes the development of a novel yeast model for studying Parkinson's disease and also molecular, genetic, and chemical-genetics approaches towards the elucidation of the basic mechanisms underlying neurodegeneration associated with protein misfolding and aggregation.

ORGANIZATION OF THE THESIS

This thesis is organized into six chapters. The first chapter reviews and introduces the main concepts and the current level of understanding of the diseases that ere studied in the chapters that follow (Parkinson's and Huntington's disease). The second chapter presents the yeast model of Parkinson's disease based on the heterologous expression of a-synuclein. In the third chapter, genetic screens with the Parkinson's disease and Huntington's disease models are presented. The fourth chapter constitutes a chemical-genetic approach towards the identification of drugs with potential to treat neurodegenerative diseases. The fifth chapter addresses the molecular mechanisms affected by increased expression of a-synuclein. Finally, general conclusions and discussion are presented in chapter six.

ABBREVIATIONS

3D	Three dimensional
αSyn	Alpha-synuclein
Aβ	Amyloid β-peptide
AD	Alzheimer's disease
Amp^r	Ampicilin resistant
APP	β-Amyloid precursor protein
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
cDNA	Complementary DNA
CFP	Cyan fluorescent protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
Da	Dalton
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxiribonucleic acid
dNTP	Deoxinucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
EGFP	Enhanced green fluorescent protein
EM	Electron microscopy
ERAD	ER associated protein degradation
EtBr	Ethidium bromide
FAP	Familial amyloid polyneuropathy

FLIM	Fluorescence lifetime imaging microscopy
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
Gal	Galactose
GFP	Green fluorescent protein
HD	Huntington's disease
HRP	Horseradish peroxidase
Hsp	Heat shock protein
Ig	Immunoglobulin
kb	kilo base pair
kDa	kilo Dalton
LB	Lewy body
LiAc	Lithium acetate
MCS	Multiple cloning site
mRNA	Messenger RNA
NaOAc	Sodium acetate
NEM	N-ethylmaleimide
NMR	Nuclear magnetic resonance
N-terminal	Amino terminal
OD	Optical density
O/N	Overnight
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PD	Parkinson's disease
PEG	Polyethylene glycol
Pfu	*Pyrococcus furiosus*

PMSF	Phenylmethylsulphonyl fluoride
PrP	Prion protein
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	rotations per minute
RT	Room temperature
SD	Synthetic defined minimal medium
SD-X	Synthetic defined minimal medium lacking nutrient X
SDS	Sodium dodecyl sulphate
SGD	*Saccharomyces* genome database
SL	Synthetic lethal
Taq	*Thermus acquaticus*
TCA	1,1,1-trichloroacetic acid
TE	Tris/EDTA
ThT	Thioflavine T
T_m	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
TSE	Transmissible spongiform encephalopathy
UPP	Ubiquitin-proteasome pathway
WT	Wild type
WWW	World wide web
YFP	Yellow fluorescent protein
YNB	Yeast nitrogen base
YPD	Yeast extract/peptone/dextrose media

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1. GENERAL INTRODUCTION

1.1 Protein Folding and Misfolding

Structurally, a nascent polypeptide is a very unstable entity that is present in environments that are not always favorable for adopting the proper fold and can often be destabilizing (Barral, Broadley et al. 2004; Dobson 2004). Among the various forces involved in the folding of a polypeptide chain – Van der Waals force, electrostatic force, hydrogen bonding, and hydrophobic force – there is strong and increasing evidence that the hydrophobic force is, generally, the dominant force in determining the overall folded structure (Dobson 2004).

Protein folding is a complex process in which a variety of factors play a specific role. The classic work of Christian Anfinsen in the 1950s on the enzyme ribonuclease revealed the relationship between the primary amino acid sequence of a protein and its conformation. Anfinsen showed that the information encoded in the primary sequence of ribonulcease was sufficient for correct refolding after denaturation, i.e., the formation of the native protein fold from the unfolded state is a spontaneous process determined by the global free energy minimum (Anfinsen 1973). It was later found that this was not the case for every protein although the primary structure is an important factor in the folding process.

More than thirty years ago, Levinthal (Levinthal 1969) pointed out that proteins can not fold by randomly checking all possible conformations of their unfolded states, because that process would take longer than the age of the Universe. This is known as the Levinthal paradox.

Traditionally, the native state of a protein was seen as the most thermodynamically stable conformation of the polypeptide chain under physiological conditions.

A more recent view of folding *in vitro*, however, says that the pathway from an unfolded polypeptide chain to the folded native state is a stochastic process that occurs on a rather flat energy landscape (Cohen and Kelly 2003; Daggett and Fersht 2003; Dobson 2003; Dobson 2003). Like any stochastic process, there is a finite possibility for polypeptides to misfold and adopt non-native states where they might be at least transiently stable (kinetic traps). Proteins could be trapped in non-native states in various ways, for example, as partially folded intermediates or as a result of interactions with other molecules or proteins with the same or different sequences.

Protein folding *in vivo* is, naturally, a quite different story. In a living cell, folding conditions have been optimized for millions of years of natural selection and evolution. This explains the ability of proteins to fold under conditions that could appear to be counterproductive for efficient folding because of the high temperature, the extremely crowded milieu reaching extreme concentrations of ~100-400 mg/ml (Figure 1.1), and the large number of 'non-native' proteins present (Ellis 2001; Minton 2001; Hall and Minton 2003; Dobson 2004). Given the circumstances, it is quite an accomplishment for 'healthy' cells to avoid the accumulation of aggregated proteins.

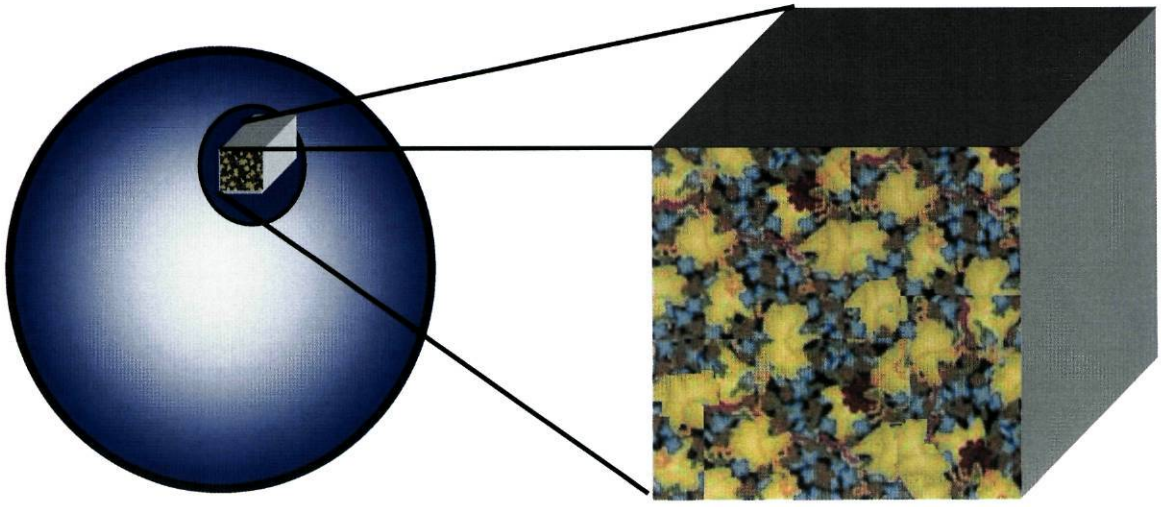


Figure 1.1. The crowded environment inside a living cell. Schematic representation of the interior of a cell, showing the ribosomes and nascent polypeptide chains. The concentration of proteins in this environment reaches an amazing ~100-400 mg/ml (Adapted from (Minton 2001)).

The intrinsic ability of polypeptide structures to fold to specific structures has been exploited, regulated, and controlled in biological systems, through evolution. Many conformational states, other than native globular structures, have been exploited, including unfolded or partially folded states. For example, unfolded or partially unfolded states are known to be important in events such as protein trafficking to specific cellular compartments, translocation across membranes, and the degradation of proteins that are damaged or no longer needed (Dobson 2003). Some proteins are also 'intrinsically unfolded' (random coil), lacking unique globular structures even under physiological conditions, at least in the absence of interacting partners.

To overcome the highly crowded, severely destabilizing environment in the cell, complex and sophisticated systems have evolved for regulating and controlling folding processes. These include molecular chaperones, folding catalysts, and quality control systems that monitor the folding process and ensure that

incorrectly folded proteins are rapidly targeted for refolding, reactivation or even destruction, to avoid any potential harmful effects in the cell. This synthesis of proteins followed by their degradation implies that cells 'waste' a lot of energy to produce proteins that may never become functional and are rapidly degraded (Walter and Buchner 2002). Recent studies confirm that a significant percentage of newly synthesized proteins (10-50%) are targeted for degradation by the ubiquitin proteasome system (Wickner, Maurizi et al. 1999; Schubert, Anton et al. 2000).

When misfolded proteins are no longer salvageable, escape the cellular quality control systems or establish undesired interactions with other biological molecules, cytotoxicity and disease phenotypes may develop. The origin of the misfolding may be genetic, involving mutations but it may also be sporadic for reasons that are not fully understood but that relate to the inability of the cells to deal with the misfolded proteins in appropriate ways.

1.2 Cellular Quality Control Mechanisms

Since protein misfolding is as old as life itself (ever since proteins exist), a large number of quality control mechanisms have evolved to cope with it. These cellular quality control mechanisms are highly conserved. All proteins face similar problems in living cells, as mentioned earlier, so it is not surprising that the general quality control mechanisms are highly conserved, especially among all eukaryotes, from yeast to man.

The next section will next introduce two major classes of quality control mechanisms – molecular chaperones and the ubiquitin proteasome pathway.

1.2.1 Molecular Chaperones

Molecular chaperones are proteins with the ability to assist the proper folding of other proteins, promote correct interactions within themselves and between different polypeptides, and prevent improper and unproductive interactions, including aggregation (Walter and Buchner 2002; Barral, Broadley et al. 2004).

It is now clear, based on investigations of transient and equilibrium intermediates *in vitro*, that partially folded intermediates, as found with newly synthesized proteins in the cell, are particularly prone to aggregate, possibly via specific intermolecular interactions between hydrophobic surfaces of structural subunits (Hartl and Hayer-Hartl 2002). Generally, chaperones transiently shield the non-native structures of proteins and assist with their proper folding, without contributing conformational information to the folding process. Chaperones typically work through cycles of substrate binding and release, regulated by various co-factors and ATPase activity. Molecular chaperones are found in all compartments of a cell where folding and other conformational adjustments occur.

Several processes, besides protein synthesis, can generate unfolded proteins. At high temperatures or in the presence of certain chemicals, proteins can become labile and may unfold. The loss of the native structure leads to a loss of function and may lead to the accumulation of protein aggregates. Cells respond to this threat by activating the heat shock response, or stress response, producing increased amounts of specific protective proteins (Ellis 1987; Lindquist and Craig 1988; Lindquist 1992; Parsell and Lindquist 1993).

Chaperones are involved in most, if not all, cellular processes, including signal transduction, vesicular trafficking, protein targeting and degradation (Hartl 1996).

According to their molecular weight (and also amino acid sequence), molecular chaperones are divided into several classes. Cells usually express multiple

members of the same chaperone class. The yeast *Saccharomyces cerevisiae*, for example, produces 14 different versions of the chaperone Hsp70 (Craig et al., 1999). The sequence homology between chaperones of the same class is usually high and they are structurally and functionally related, whereas there is hardly any homology between chaperones from different classes. Nonetheless, most molecular chaperones share common functional features.

The main property of any molecular chaperone is its ability to bind unfolded or partially folded polypeptides. This association, which is rather promiscuous, efficiently suppresses protein aggregation. Most native proteins and late folding intermediates are no longer substrates for molecular chaperones, as they do not expose hydrophobic patches. Chaperones are also capable of inducing conformational changes in their target proteins. Some have the ability to unfold protein substrates and are, therefore, connected to the cellular protein degradation systems. Others act upon protein aggregates, to reactivate misfolded proteins. But unfolding may also be important during the folding pathways by avoiding the trapping in states of energy minima and offering a new chance to achieve its native structure. Another important aspect of chaperone action is their ability to release bound polypeptides in a controlled fashion through a conformational switch powered by ATP hydrolysis, in most cases.

1.2.2 Ubiquitin-Proteasome Pathway

The ubiquitin-proteasome pathway (UPP) was recognized as the major system for degradation of short lived proteins in eukaryotes, in 1984, by the Varshavsky laboratory. The UPP constitutes an important component of the cellular protein quality control mechanisms that impacts on virtually all aspects of cell biology. Misfolded and damaged proteins are directed to the UPP for degradation to avoid

accumulation and the potentially adverse effects. The proteasome is also important for the regulation of protein lifetime for functional purposes – mitotic cyclins, for example, are proteins involved in the cell cycle that are degraded by the proteasome in order to enable the cell to proceed in the cell cycle (Glotzer, Murray et al. 1991; Hershko, Ganoth et al. 1991). Most proteasome degradation is ubiquitin-dependent, i.e., proteins destined for degradation are conjugated to a poly-ubiquitin chain that is then recognized by the proteasome (Glickman and Ciechanover 2002). Ubiquitination, the attachment of ubiquitin (a highly conserved protein of 76 amino acids, ~ 8.6 kDa) to a polypeptide chain, is an ordered process in which an ubiquitin-activating enzyme (E1) activates and transfers ubiquitin to an ubiquitin-conjugating enzyme (E2), which then acts in concert with a ubiquitin ligase (E3) to transfer ubiquitin to a lysine residue on the target substrate, through an isopeptide bond to ubiquitin's C terminus (Ciechanover, Heller et al. 1980; Hershko, Ciechanover et al. 1980). Substrate recognition by the 26S proteasome complex requires at least four ubiquitin moieties (Weissman 2001). The proteasome complex consists of a 20S core that contains the proteolytic activities and typically a 19S cap that cleaves ubiquitin moieties from the substrate, unfolds the polypeptide and feeds it through the narrow channel to the proteolytic chamber of the 20S core (Braun, Glickman et al. 1999; Verma, Aravind et al. 2002; Yao and Cohen 2002), where three distinct peptidase activities [chymotryptic-like (Tyr or Phe at position 1), tryptic-like (Arg or Lys at position 1) and peptidylglutamyl peptide hydrolyzing (Glu at position 1)] cleave the substrate into short peptides.

The role of ubiquitination in the cell goes beyond the role in protein degradation. Ubiquitination is also a post-translational modification that regulates protein localization and activity, much like phosphorylation. This ubiquitination can occur as mono-ubiquitination or poly-ubiquitination with non-standard lysine linkages (Hicke 2001).

Thus, problems with the UPP will affect the protein homeostasis in the cell. Mutations in genes involved in the UPP have been linked to several neurodegenerative diseases associated with protein misfolding. This will be discussed in more detail in the following sections.

1.3 Protein Misfolding Diseases

Protein misfolding diseases are a group of diseases that arise due to or associated with the misfolding of one or more proteins. They are believed to result from the failure of proteins to reach their active state or from the accumulation of abnormally folded proteins. Proteins, as the main effectors in the cell, play underpinning roles in all biological processes. It is, therefore, not surprising that the number of these diseases is always expanding, as new proteins are identified and their functions understood. From the classic examples of sickle cell anemia to cancer and a variety of neurodegenerative diseases, this group of disorders promises to continue to entertain the minds of investigators for generations to come.

Neurodegenerative diseases are characterized by a slow, progressive, and inexorable loss of specific neuronal cell populations and are associated with protein aggregates. Some can occur either sporadically or by the inheritance of specific gene mutations, as in Parkinson's disease, Alzheimer's disease, amyotropic lateral sclerosis or even the prion diseases. Other diseases, such as Huntington's disease, seem to be inherited exclusively due to genetic factors.

A common feature of these diseases, besides protein aggregation, is the extensive evidence of oxidative stress, an imbalance between the production and clearance of reactive oxygen species (ROS) by cellular antioxidant systems,

which might be responsible for the dysfunction or death of neuronal cells that contributes to disease pathogenesis.

Another commonality to many neurodegenerative diseases is that they give rise to the deposition of proteins in the form of 'amyloid fibrils' (broadly defined) and plaques (Tan and Pepys 1994; Thomas, Qu et al. 1995; Koo, Lansbury et al. 1999; Dobson 2001). Such deposits can form in the brain, liver, spleen or in skeletal tissue, depending on the disease. Each amyloid disease involves the aggregation of a specific protein, together with a range of other components, including other proteins and carbohydrates. There is no apparent connection between the soluble form of each of the 20 or so proteins involved in amyloidoses. Interestingly, the aggregated forms of these proteins share many common characteristics: amyloids show green birefringence upon binding of the dye Congo red and show other specific optical properties upon binding of other dyes; the fibrillar structures have very similar morphologies and sizes; they show a characteristic cross-beta X-ray diffraction pattern, revealing that the core structure is composed of β-sheets having strands running perpendicular to the axes of the fiber (Sunde and Blake 1997) (Figure 1.2). The aggregated forms of the proteins are also thermally stable, and SDS- and protease-resistant. By definition, amyloid is an extracellular type of protein deposit. The term is also applied, in a broader sense, to other proteins that share the aforementioned biophysical properties even though they accumulate inside the cell.

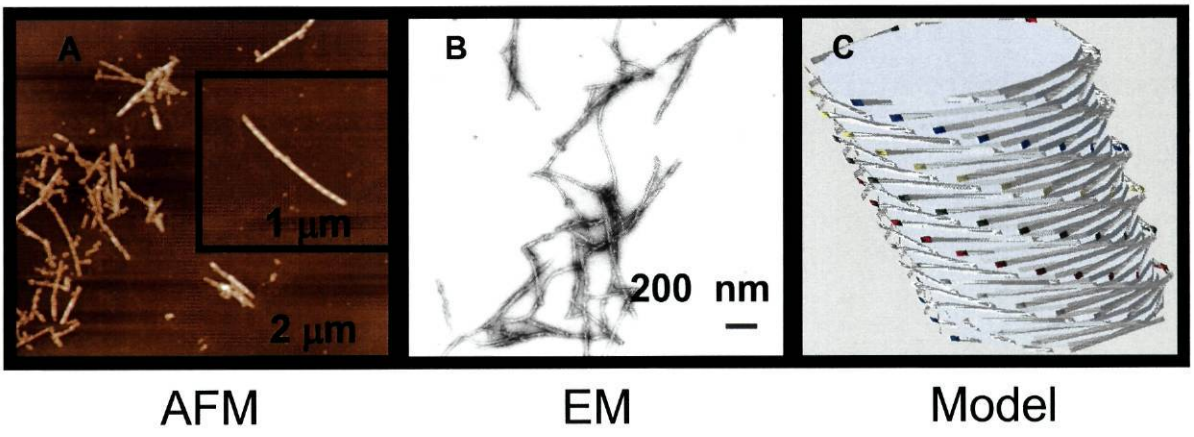


Figure 1.2. Different views of amyloid fibers. AFM **(A)** and EM **(B)** are two common methods for visualizing amyloid fibers (αSyn fibers are shown). In the fiber, the beta-sheets are perpendicular to the axis of the fiber, as depicted in the model presented **(C)**.

The behavior of many amyloidogenic proteins *in vitro* shows that the self-assembly process does not require other components. Different models have been put forward to explain the self-assembly process, but it typically follows a nucleated conformational conversion (Figure 1.3) (Serio, Cashikar et al. 2000). If the end product of the assembly reaction (the amyloid fibers) is added to soluble protein, the assembly reaction follows a much faster mechanism (seeded reaction) than if the soluble protein is just incubated over time, without seeds (unseeded reaction). These kinetics are typical for many amyloids.

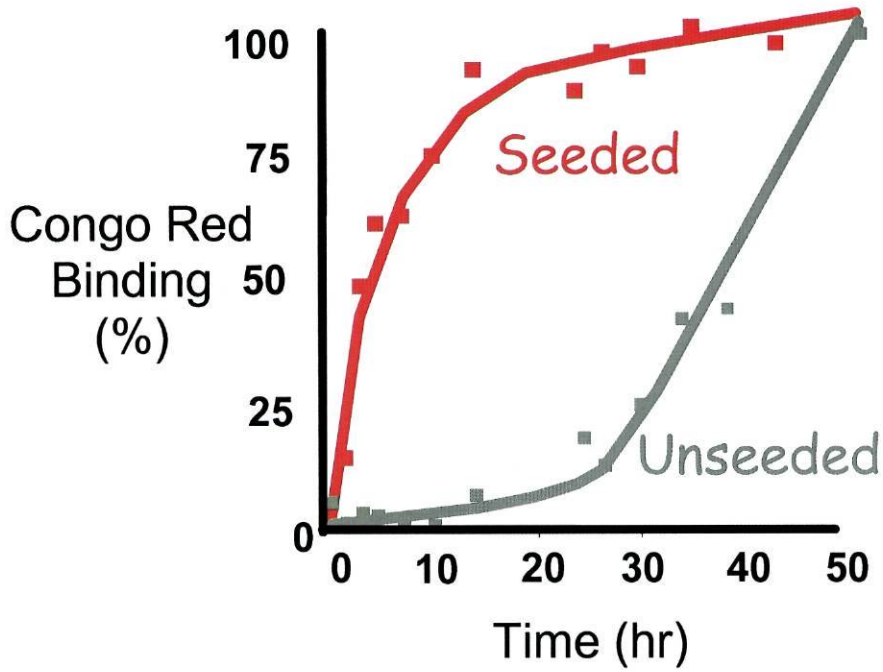


Figure 1.3. Many amyloids follow a nucleation-dependent polymerization. A seeded reaction in which pre-formed amyloid fibers (seeds) are added to soluble protein proceeds without the typical lag phase observed when seeds are not added.

Recent studies have suggested that the ability of polypeptide chains to form amyloid structures is not a unique property of those proteins that deposit in the amyloid diseases. It can be considered a generic feature of polypeptide chains (Chiti, Webster et al. 1999; Dobson 1999; Fandrich, Fletcher et al. 2001). Although the ability to form amyloid fibrils appears generic, the propensity to do so varies dramatically depending on the primary structure of the protein. This is intuitive if we think of the different chemical properties of the 20 naturally occurring amino acids.

Despite the strong connection between protein misfolding and aggregation and disease, the manner by which it results in disease is not understood. In some cases, it seems that the deposition of protein aggregates may physically disrupt

the functioning of specific organs. In other cases, it seems that the lack of functional protein, due to its recruitment into the aggregates, results in the failure of crucial cellular processes (Thomas, Qu et al. 1995). However, for some neurodegenerative diseases it appears that the symptoms result from the destruction of cells by a "gain of toxic function" that results from the aggregation process (oligomers, protofibrils, fibrils or other intermediates) or by a combination of both this gain of toxic function and a loss of normal function of the protein (Caughey and Lansbury 2003). Nonetheless, inclusion bodies are not normal structures and their existence argues for underlying problems in protein homeostasis.

The quality control systems discussed earlier (molecular chaperones and UPP) are key factors that affect the formation of inclusions. The accumulation of misfolded proteins, and mutations in several UPP components that cause neurodegeneration (Table 1.1) provide evidence for its involvement in protein misfolding diseases (Berke and Paulson 2003).

Table 1.1. Genetic evidence for UPP involvement in neurodegenerative disease.

Disease	Protein	UPP component
Parkinson's disease	Parkin	E3 ligase
	UCH-L1	Deubiquitinating enzyme
Alzheimer's disease	Ub^{+1}	Ubiquitin

(Adapted from Berke and Paulson, 2003)

1.3.1 Parkinson's Disease

Parkinson's disease (PD) is one of the most common progressive, neurodegenerative disorders that affects about 2% of people over 65 years old and 4-5% of people over 85 (between one and one-and-a-half million Americans). It was James Parkinson, an English physician, who first described the disease, in 1817 (in the days of the industrial revolution) (Parkinson, 1817).

PD is characterized by loss of dopaminergic neurons in the substantia nigra (Figure 1.4) and is accompanied by muscle rigidity, bradykinesia, resting tremor and postural instability. Although the underlying causes for neuronal cell loss are unknown, the symptoms of PD can be effectively, albeit transiently, treated by replacement of dopamine (via L-DOPA) or by dopamine agonists (2000).

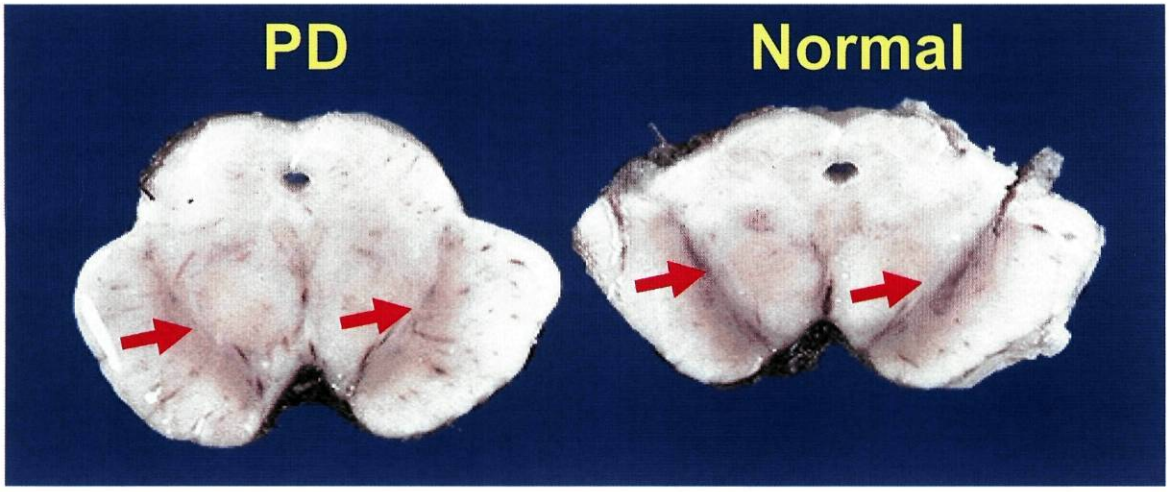


Figure 1.4. Loss of dopaminergic neurons in the substantia nigra in PD. In a PD brain the darker area (red arrows) is significantly smaller than in the brains of age matched controls.

A variety of autonomic symptoms are also known to be constant clinical features of PD. Were there any patients who suffered from PD before the 19th century? PD, as well as other neurodegenerative diseases, is mostly a disease of the elderly, so it is not surprising that only when life expectancy started increasing did it become more common. The industrial revolution represents an important landmark in terms of improving the quality of life. Nonetheless, it is interesting to note that some believe that Leonardo da Vinci, who lived in the 15th and 16th centuries, had already described the abnormalities that appear in PD patients (Calne, Dubini et al. 1989).

The neuropathological hallmark of idiopathic PD is the presence of concentric hyaline cytoplasmic inclusions called Lewy bodies (LB) (Figure 1.5), first described by Frederich H. Lewy in 1912. LBs are spherical inclusions of 5-25 μm in diameter, seen as a dense eosinophilic core with a pale surrounding halo in the cytoplasm of affected neurons. LBs were first seen in the substantia innominata and the dorsal vagal nucleus in PD (Lewy, 1912).

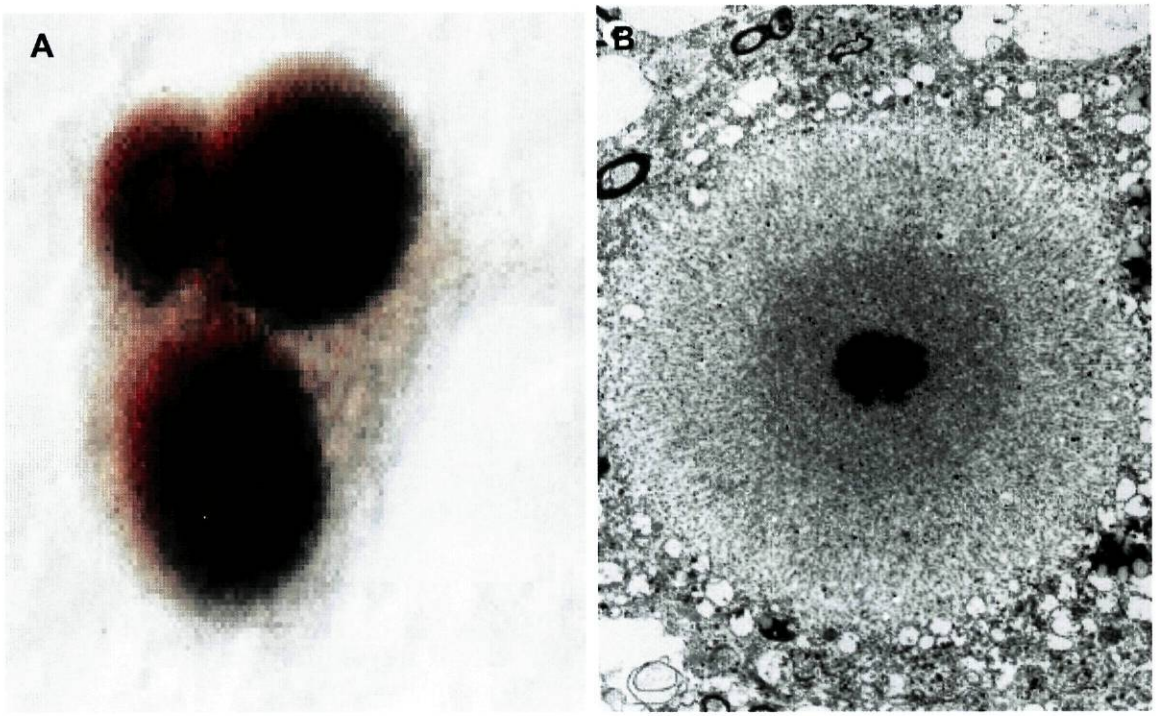


Figure 1.5. Classical Lewy bodies (LBs) in the substantia nigra. **A**, immunohistochemistry with antibodies for α-synuclein. **B**, electron microscopy showing fibrillar α-synuclein in a LB. Adapted from (Forno, DeLanney et al. 1996; Spillantini, Schmidt et al. 1997).

It is now known that LBs can be seen in pigmented neurons of the substantia nigra in almost every case of PD (Jellinger 1987). However, LBs have also been observed in the brains of asymptomatic individuals (Nussbaum and Polymeropoulos 1997). It is not clear whether so-called incidental LB disease is actually preclinical PD or is a case where rapid fibril formation precluded accumulation of other toxic intermediates, such as protofibrils.

LBs were identified in many regions of the brain, not only in familial and sporadic PD but also in dementia with LB (DLB), a neurodegenerative disorder that is distinguishable from PD. All LBs were shown to contain the protein α-synuclein

(Spillantini, Schmidt et al. 1997; Wakabayashi, Matsumoto et al. 1997; Baba, Nakajo et al. 1998; Irizarry, Growdon et al. 1998; Takeda, Mallory et al. 1998; Goedert 2001). LBs are often encountered in other neurodegenerative disorders such as familial Alzheimer's disease, Down syndrome and Hallervorden-Spatz disease. All these LBs have been reported to contain α-synuclein (Arawaka, Saito et al. 1998; Lippa, Fujiwara et al. 1998; Wakabayashi, Yoshimoto et al. 1999).

1.3.1.1 Genetics of PD

A genetic component in PD was thought to be unlikely until recently, because of the long preclinical phase that makes a family history difficult to discern. A high percentage of the nigral neurons (50-60%) can be lost with no obvious clinical consequence. Moreover, initial studies of twins showed equally low rates of concordance in monozygotic and dizygotic twins (Tanner, Ottman et al. 1999). Only when improved positron-emission tomography (PET) imaging methods were available to monitor the number of dopaminergic neurons in the substantia nigra it became clear that the concordance among monozygotic twins was significant, even in presymptomatic PD.
Familial PD with specific genetic defects may account for fewer than 10% of all cases of PD (Gasser 2001); however, the identification of these rare genetic defects and the functions of those genes has provided tremendous insight into the pathogenesis of PD. This knowledge is essential for developing new avenues for therapeutic intervention.

Several genes have now been linked to PD, and a number of other genetic linkages have been identified that may cause PD (Table 1.2).

Table 1.2. Genes associated with Parkinson's disease.

Locus	Chromosome location	Gene	Mode of Inheritance	Lewy Bodies
PARK1	4q21.3	α-synuclein	AD	Yes
PARK2	6q25.2-27	*Parkin*	AR	No (except one case)
PARK3	2p13	unknown	AD	Yes
PARK4	4p15	α-synuclein	AD	Yes
PARK5	4p14	*UCH-L1*	AD	ND
PARK6	1p35-p36	unknown	AR	ND
PARK7	1p36	*DJ-1*	AR	ND
PARK8	12p11.2-q13.1	unknown	AD	No
PARK9	1p36	unknown	AR	?
PARK10	1p32	unknown	Late-onset susceptibility gene	?

AD, autosomal dominant; AR, autosomal recessive; ND, not determined

1.3.1.1.1 PARK1

The first gene to be linked to PD (PARK1) was the gene encoding α-synuclein (αSyn). In 1997, a mutation in the αSyn gene was identified in an Italian family and in three unrelated families of Greek origin with autosomal dominant inheritance for PD (Polymeropoulos, Lavedan et al. 1997). This represented a major breakthrough in the research of PD pathogenesis. Due to the central role

of αSyn in PD and other neurodegenerative disorders, this gene will be discussed in greater detail in a separate section (1.3.1.2).

1.3.1.1.2 PARK2

PARK2 was the second PD gene to be discovered. It constitutes an important locus for autosomal recessive PD and isolated early-onset cases (Lucking, Durr et al. 2000). The gene was designated *parkin* and encodes a protein of 465 amino acids, and has some similarity to ubiquitin at its amino-terminus and a RING-finger motif at the carboxy-terminus (Kitada, Asakawa et al. 1998). The spectrum of parkin mutations now includes at least 60 different mutations, including exonic deletions, insertions, and several missense mutations (Periquet, Lucking et al. 2001).

The modular architecture of parkin led to insight into its function by comparison to several other proteins with similar structures (Dawson 2000). Parkin was shown to be an E2-dependent E3 ubiquitin-protein ligase (Shimura, Hattori et al. 2000; Zhang, Gao et al. 2000; Chung, Dawson et al. 2001). It appears to use both UbcH7 and UbcH8 as its E2 ligases and it also utilizes the ER-associated E2s Ubc6 and Ubc7 (Chung, Dawson et al. 2001).

Mutations in parkin impair the binding to E2s, suggesting that disruption of the E3 ligase activity is probably the cause of autosomal recessive PD. It is, therefore, of great importance to identify the substrates of parkin. Conceivably, dysfunction of the proteasomal processing of one or more of parkin's substrates may lead to dopaminergic dysfunction. Several potential substrates for parkin have already been identified. Synphilin-1, a protein of unknown function identified as an αSyn-interacting protein (Engelender, Kaminsky et al. 1999), was recently found to be a substrate for parkin-targeted ubiquitination (Chung, Zhang et al. 2001). Synphilin-1 is also present in LBs, and co-expression of synphilin-1 with αSyn in

cultured cells results in the formation of LB-like aggregates, containing both proteins, that become ubiquitinated in the presence of parkin (Chung, Zhang et al. 2001). The observation that patients with parkin mutations do not have LBs led to the speculation that pathogenic mechanisms caused by mutations in the parkin gene are different from those that occur in sporadic PD and in PD due to mutations in αSyn (Dawson 2000). On the other hand, the interactions of synphilin-1 with both αSyn and parkin suggest a common link between different causes of PD. This also establishes a connection between αSyn and parkin. The sequestration of parkin in inclusions may contribute to its loss of function, meaning that LB-associated proteins would not be ubiquitinated and the formation of LBs would be impaired (Dawson 2000; Chung, Dawson et al. 2001). This is important because LBs may actually represent a protective mechanism neurons developed to avoid the accumulation of toxic intermediate species throughout their cytoplasms. Interestingly, a patient with a R275W mutation in one parkin allele and a 40 bp deletion on exon 3 in the other allele revealed the presence of LB pathology in regions typically affected in PD (Farrer, Chan et al. 2001). The R275W mutation reduces the catalytic activity of parkin but it still retains substantial enzymatic activity (Chung, Zhang et al. 2001). This mutation appears to be the exception that proves the rule. It also indicates that parkin is required for the formation of LB pathology, which may, at least in part, contribute to neuronal cell death by sequestering parkin, which is important for the degradation of specific proteins (Chung, Dawson et al. 2001).

Other parkin substrates have been identified, including CDCrel-1, a synaptic vesicle-associated protein that belongs to a family of septin GTPases (Zhang, Gao et al. 2000). CDCrel-1 has been suggested to regulate synaptic vesicle release in the nervous system (Beites, Xie et al. 1999), but it is not yet known whether it is involved in the release of dopamine (DA). If this is true, it may suggest another link between parkin, its substrates, and the parkinsonian state.

An O-glycosylated form of αSyn (aSp22) was ubiquitinated, *in vitro*, by normal but not by mutant parkin (Shimura, Schlossmacher et al. 2001). Unglycosylated αSyn is the major species in the brain and does not appear to be a parkin substrate (Chung, Zhang et al. 2001; Shimura, Schlossmacher et al. 2001). Thus, the real relevance of parkin mutations in the ubiquitination of aSp22 is unclear.

A putative G-protein-coupled protein receptor, the parkin-associated endothelial-like receptor (Pael-R), was found to be another parkin substrate (Imai, Soda et al. 2001). Overexpression of Pael-R causes cell death through the induction of the unfolded protein response (UPR). Pael-R accumulates in an insoluble form in autosomal recessive juvenile parkinsonism (AR-JP). Co-expression of parkin (but not the truncated form) protects against Pael-R-induced cell death (Imai, Soda et al. 2000).

1.3.1.1.3 PARK5

A mutation in the gene (PARK5) encoding for ubiquitin carboxy-terminal hydrolase L1 (*UCH-L1*) in two family members of a small German kindred with autosomal dominant PD has also been described (Briggs, Mortier et al. 1998). However, the penetrance of this mutation may not be 100%, because neither of their parents had been diagnosed with PD. Also, this mutation has not been identified in any other individuals. Nevertheless, recent functional studies suggest it may be causally related to PD (Liu, Fallon et al. 2002).

1.3.1.1.4 PARK7

Mutations in the gene (PARK7) encoding DJ-1 in isolated communities in the Netherlands have been shown to cosegregate with PD (Bonifati, Rizzu et al. 2003). Because the function of DJ-1 is largely unknown, it is difficult to appreciate how it may cause PD. Nevertheless, DJ-1 was identified as a hydroperoxide-responsive protein that becomes more acidic following oxidative stress, suggesting it may function as an antioxidant protein (Mitsumoto and Nakagawa 2001). Even though the function of DJ-1 is not known, the knowledge of its crystal structure constitutes an important piece of information that should enable a deeper understanding into its connection to PD (Honbou, Suzuki et al. 2003; Lee, Kim et al. 2003; Wilson, Collins et al. 2003; Wilson, St Amour et al. 2004).

Several other loci have been linked to PD cases, both autosomal dominant and recessive. Until the genes involved are identified it is not possible to close the loop and link all the multiple genes and the sporadic cases of PD into a common pathogenic biochemical pathway.

What is clear is that protein mishandling and oxidative stress are central in PD pathogenesis.

1.3.1.2 α-Synuclein

The discovery that mutations in the presynaptic protein αSyn are linked to PD was a major leap forward in PD research. However, wildtype (WT) αSyn is also deeply connected to several neurodegenerative diseases, including PD. These diseases are characterized by the presence of Lewy bodies and Lewy neurites in the brain, inclusions in which fibrillar αSyn is the major component.

1.3.1.2.1 αSyn Structure and Interacting Partners

αSyn belongs to a family of three different genes, αSyn, βSyn and γSyn, which have been described in vertebrates, and seem to be highly conserved among distantly related vertebrate species (Maroteaux, Campanelli et al. 1988; Jakes, Spillantini et al. 1994; George, Jin et al. 1995). The name 'synuclein' was chosen because the protein was found in the *syn*apse and in the *nucl*ear envelope (Maroteaux, Campanelli et al. 1988). αSyn was not found in the nucleus in several subsequent studies, so it appears to be a presynaptic protein. The structure of αSyn is still unknown, but under physiological conditions it seems to be mostly unstructured (random coil) (Weinreb, Zhen et al. 1996; Kim 1997). All synuclein proteins consist of a highly conserved amino-terminal domain that appears to adopt an α-helical conformation under certain conditions (George, Jin et al. 1995). αSyn has a middle hydrophobic domain (residues 61-95) also referred to as NAC (non Aβ component) and a C-terminal region rich in prolines and the acidic residues glutamate and aspartate (Figure 1.6). The α-helical N-terminal domain has an 11-amino acid repeat with a highly conserved motif (KTKEGV). This domain is thought to adopt amphipathic a-helical structure similar to that of apolipoproteins of the class A_2. The N-terminal region also harbors one of two characteristic signatures for fatty acid binding proteins (FABP) (Sharon, Goldberg et al. 2001) and is important for lipid binding (Perrin, Woods et al. 2000).

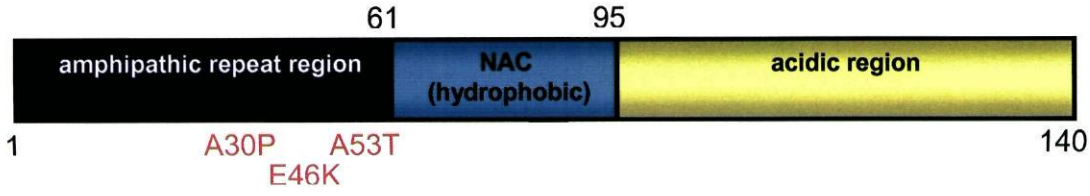


Figure 1.6. Schematic representation of the α-synuclein protein. Three regions can be defined in αSyn: the N-terminal region with amphipathic repeats; a central hydrophobic region called NAC; and a C-terminal acidic region.

αSyn seems to bind exclusively to acidic phospholipids, especially phosphatidic acid, and to vesicles with small diameters. This is likely to target the protein to specific populations of membranes or vesicles (Davidson, Jonas et al. 1998). The N-terminal domain is also likely to be responsible for the protein's ability to disrupt lipid bilayers (Jo, McLaurin et al. 2000). The conservation of the α-helical domain among the synuclein proteins suggests that all three synucleins may be involved in similar biological processes that involve lipid binding.

The central region of αSyn (NAC) comprises the highly amyloidogenic part of the protein. This amyloidogenic part is responsible for properties that distinguish αSyn from the other members of the synuclein family: (i) the ability to undergo a conformational change from random coil to β-sheet structure (Serpell, Berriman et al. 2000; el-Agnaf and Irvine 2002), (ii) the ability to form single cylindrical β-sheets (Perutz, Pope et al. 2002), and (iii) the ability to form Aβ-like protofibrils and fibrils (Harper, Lieber et al. 1997; Harper, Wong et al. 1997; Conway, Harper et al. 1998; Harper, Wong et al. 1999; Conway, Lee et al. 2000; Ding, Lee et al. 2002; el-Agnaf and Irvine 2002).

The acidic C-terminal region of αSyn does not seem to associate with vesicles, remaining free and unfolded (Eliezer, Kutluay et al. 2001), despite showing resemblance with cytosolic FABP.

As mentioned earlier, αSyn is intrinsically unstructured in solution but it is unlikely that this is the case in the context of a cell. αSyn binds a number of ligands and proteins, which likely alter its conformation. αSyn also shares some sequence similarity with the signaling chaperone protein 14-3-3 (Yaffe 2002). Like the 14-3-3 proteins, αSyn binds to extracellular regulated kinase (ERK), dephospho-BAD (a Bcl-2 homolog), and protein kinase C (PKC). It also binds to the 14-3-3 proteins themselves. These interactions suggest that αSyn may be involved in the regulation of cell viability in ways that are not fully understood. A yeast two-hybrid screen identified synphilin-1 as an αSyn-interacting protein (Engelender, Kaminsky et al. 1999). Synphilin-1 is a novel protein with little similarity to other known proteins therefore the significance of this interaction is not totally clear. Synphilin-1 may act as an adaptor protein and may be involved in vesicle transport or cytoskeletal function. Its presence in LBs and its connection to parkin suggest it may play a role in PD pathology as well (Engelender, Kaminsky et al. 1999; Wakabayashi, Engelender et al. 2000).
Casein kinases 1 and 2 (CK-1 and CK-2), but not protein kinase A (PKA) or C (PKC), phosphorylate αSyn on serine 129, and to a lesser extent on serine 87 (Okochi, Walter et al. 2000). Phosphorylation of αSyn seems to be tightly regulated and it may influence its binding to lipid membranes or to phospholipase D (Jenco, Rawlingson et al. 1998; Okochi, Walter et al. 2000).

αSyn also binds to the microtubule-associated protein tau, a major constituent of insoluble paired helical filaments found in neurofibrillary tangles and plaque in Alzheimer's disease (Grundke-Iqbal, Iqbal et al. 1986; Grundke-Iqbal, Iqbal et al. 1986; Jensen, Hager et al. 1999). Interestingly, αSyn induces fibrillization of tau,

and αSyn and tau sinergize in fiber formation (Giasson, Forman et al. 2003). αSyn binds Aβ and brain vesicles via the repeat region and binds tau via its C-terminus (Yoshimoto, Iwai et al. 1995; Jensen, Nielsen et al. 1998). It is, therefore, possible that αSyn forms bridges between tau and Aβ. Because αSyn stimulates PKA, which in turn phosphorylates tau influencing tau binding to microtubules, αSyn may modulate tau function.

1.3.1.2.2 Putative Functions of αSyn

αSyn is widely expressed in the brain, in both neuronal and non-neuronal cell types including dopaminergic neurons, cortical neurons, noradrenergic neurons, endothelial cells, and platelets (Hashimoto, Yoshimoto et al. 1997; Abeliovich, Schmitz et al. 2000; Tamo, Imaizumi et al. 2002). The aforementioned structural features and interacting partners of αSyn, together with evidence from other experimental approaches, led to speculation about the normal function of αSyn. The putative functions of αSyn include the binding of fatty acids, physiological regulation of certain enzymes, transporters and neurotransmitter vesicles, as well as roles in neuronal survival. Several other functions have been ascribed to αSyn, some of which seem to lack connections between themselves. This is indicative of how little is known about the function of αSyn, in general. This lack of knowledge may also help explaining the little success researchers have had in developing animal models that fully recapitulate features of PD. Moreover, the ability to delay the onset and progression of PD is also likely to be dependent on the knowledge of the normal function of αSyn and other proteins known to be involved in PD.

The involvement of αSyn in synaptic plasticity is suggested due to its presence in presynaptic regions, where it interacts with brain vesicles and phospholipid membranes, affecting dopamine (DA) storage (Lotharius and Brundin 2002).

Specifically, the involvement of αSyn in synaptic plasticity may be mediated by the inhibition of phospholipase D (PLD) (Jenco, Rawlingson et al. 1998; Ahn, Rhim et al. 2002; Payton, Perrin et al. 2004), since PLD was shown to be implicated in cell growth and differentiation (Klein, Chalifa et al. 1995). PLD is an enzyme that has maintained a high degree of similarity among different organisms, as far as protein domains are concerned (Figure 1.7). Despite showing poor sequence conservation, it hydrolyzes phosphatydylcholine to phosphatidic acid (PA) and choline. αSyn can bind to PA and can, therefore, influence changes in cell morphology (Jenco, Rawlingson et al. 1998). Interestingly, the absence of αSyn in mice only causes mild behavioral changes. Mice lacking αSyn only show subtle changes in DA-dependent behavior and do not seem to be impaired in spatial learning (Abeliovich, Schmitz et al. 2000; Chen, Specht et al. 2002). It is possible that there is some degree of redundancy between αSyn and other members of the synuclein family. Double and triple knock outs should yield useful information about the function of αSyn and the other members of this family of proteins.

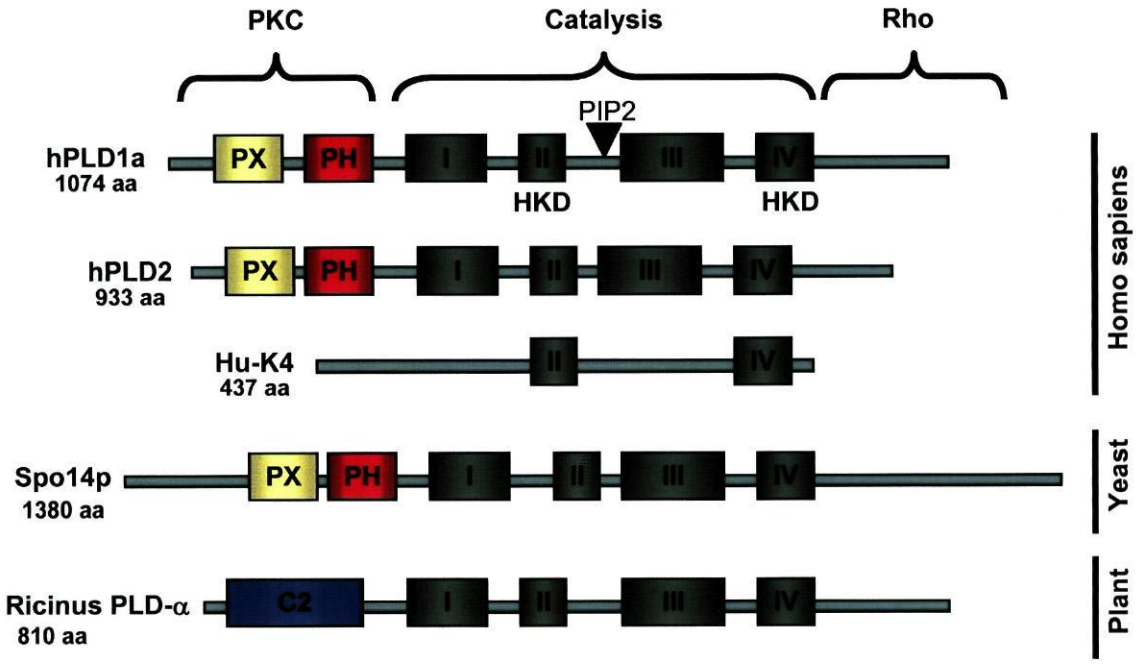


Figure 1.7. Phospholipase D gene structure. Comparison of the domain structure of PLDs from different species.

αSyn has also been associated with the regulation of dopamine release, through electrophysiological studies. αSyn seems to negatively regulate dopamine release but the mechanism for this effect is unclear. Tyrosine hydroxylase (TH), the rate-limiting enzyme in DA biosynthesis, seems to be inhibited by αSyn (Perez, Waymire et al. 2002). αSyn increases the ratio of the non-phosphorylated form of TH over the more active phosphorylated form. αSyn may also modulate the releasable DA pool through its interactions with vesicle membranes and this may also be related to PLD inhibition. Another possibility is that membrane fusion and lysis are inhibited by the membrane-stabilization effects that class A α-helices, such as those formed by αSyn, have on membranes (Davidson, Jonas et al. 1998). Studies in HEK293 cells have also indicated that αSyn facilitates an

increase in the number of plasma membrane dopamine transporters (DAT) (Lee, Hyun et al. 2001).

Expression of αSyn in neuronal cells in culture has yielded a wide variety of results. Whether it has beneficial, adverse, or no effect on cell survival is, therefore, debatable. Pro- and anti-apoptotic effects have been observed and the regulation of cell viability might be mediated by the interaction of αSyn with proteins such as PKC, ERK, or BAD. Some of these effects are likely to be cell-line dependent and caution needs to be taken when interpreting these results. The recent finding of a triplication of the αSyn locus in a family with familial PD (fPD) suggests the levels of expression of αSyn are critical for any toxic effects that might arise (Singleton, Farrer et al. 2003). This idea was confirmed independently using yeast as a model organism (Outeiro and Lindquist 2003) (Chapter 2). Expression of different mutants of αSyn also yielded controversial results but this will be discussed in greater detail in the next section.

1.3.1.2.3 αSyn Mutations and PD

In 1997, the identification of a missense mutation in the PARK1 locus implicated αSyn in PD (Polymeropoulos, Lavedan et al. 1997). The mutation changed a G to an A at position 209 (G209A), resulting in an Ala53Thr (A53T) substitution. The discovery of a second missense mutation in the αSyn gene, G88C, resulting in an A30P substitution, in a German family with autosomal dominant PD (Kruger, Kuhn et al. 1998), reinforced the hypothesis that those mutations were indeed involved in the pathogenesis of PD. It is interesting to note that αSyn in mice and rats has a threonine at position 53, instead of an alanine. Other mutations in the αSyn gene were sought in many families with autosomal dominant PD but it was not until recently that another mutation was found. This

mutation causes an E46K substitution in a conserved area of the protein and causes fPD and dementia with LBs (DLB) (Zarranz, Alegre et al. 2004). The autosomal dominant nature of the inherited mutants is thought to reflect a gain rather than a loss of function in the αSyn proteins. Haploinsufficiency of the WT allele in heterozygotes also seems to play a role in the severity of the PD phenotype (Kobayashi, Kruger et al. 2003). Aggregation of αSyn and the putative gain of toxic function, may, therefore, contribute to haploinsufficiency by entrapping the wild type protein, thus, reducing the amount of available functional αSyn. Our studies in yeast suggest that once αSyn starts to aggregate it may recruit a Syn away from other sites in the cell, and conciliate the gain and loss of function hypotheses (Outeiro and Lindquist 2003) (Chapter 2).
One important aspect of fPD is that only a very small number of families carrying mutations in αSyn have been identified worldwide. Mutations in the αSyn gene account for only very few cases of autosomal dominant PD. The vast majority of PD patients carry WT αSyn.

Several groups described differences in the aggregation kinetics of three different forms of αSyn (WT, A53T and A30P) *in vitro* (Conway, Harper et al. 1998; Narhi, Wood et al. 1999; Ostrerova, Petrucelli et al. 1999; Conway, Lee et al. 2000; Serpell, Berriman et al. 2000; Li, Uversky et al. 2002). Structural differences between these αSyn alleles have also been reported (Bussell and Eliezer 2003). All three forms of αSyn have the ability to form fibers with the typical properties of other amyloids. However, the A30P mutant seems to form fibrils slower than WT αSyn, whereas the A53T fibrillizes faster than WT (Conway, Lee et al. 2000). These findings are suggestive of a toxic role for small αSyn oligomers (not the fibers themselves) in disease. This hypothesis is consistent with the recent finding that oligomers, but not fibers, are toxic to cells in culture (Kayed, Head et al. 2003). The A30P mutant, unlike A53T and WT αSyn, seems to be defective in binding to vesicles and membranes (Jensen, Nielsen et al. 1998; Jo, Fuller et al.

2002). In mammalian cells, fusions of αSyn to the green fluorescent protein (GFP) (GFP at the C-terminus of αSyn) are truncated within GFP, generating inclusions in the cytoplasm (McLean, Kawamata et al. 2001). Interestingly, the A30P mutant does not form the cytoplasmic inclusions that are seen with WT or A53T αSyn. This suggests that the conformation of the A30P mutant differs from that of WT and A53T αSyn (McLean, Kawamata et al. 2001).
αSyn is toxic in HEK293 and SK-N-SH cells in a dose-dependent manner. In these cells the A53T mutant was more toxic than the A30P and both were more toxic than WT αSyn (Ostrerova, Petrucelli et al. 1999). These differences also point at disease mechanisms that might differ depending on the mutant form of αSyn.

The E46K mutant was only found very recently and, thus, has not been examined very extensively. It will be interesting to compare its behavior to that of the other forms of αSyn.

1.3.1.2.4 Animal Models of PD based on Expression of αSyn

Several animal models for PD have been developed. Neurotoxins such as rotenone and MPTP have been used to generate animal models for PD, however, these represent acute models which do not fully recapitulate the cardinal signs of the disease.

Ideal animal models for studying PD-related synucleinopathies would exhibit the following characteristic signs of idiopathic PD: bradykinesia, resting tremor, rigidity, postural impairment, gait disorder, adult onset, selective loss of dopaminergic neurons of the substantia nigra, a good response to DA replacement, and the presence of LBs. Other desirable features would include a

reduced activity of mitochondrial complex I and a high concentration of reactive oxygen species (ROS). In spite of the tremendous effort put into generating and characterizing animal models for PD, the molecular mechanisms that underlie neurodegeneration in PD are not well defined. It is, therefore, important to continue to develop simple model systems amenable to genetic manipulation that may provide important leads for the development of more significant animal models and may ultimately lead to novel neuroprotective agents for the treatment of PD and related synucleinopathies.

For studying the relationship of αSyn to PD and other synucleinopathies, several animal models have been developed. In transgenic and/or viral vector-transduced rodents and flies, overexpression of WT as well as mutant (A53T and A30P) αSyn has led to αSyn pathology with neuronal loss and/or impairment of neuronal function. The outcomes from the different models have been variable, much like the outcomes from the studies with αSyn in mammalian cell culture. Direct comparison of the effects of different αSyn proteins in animals is complicated by the fact that the integration sites, expression levels, and brain cell populations expressing αSyn are not the same.

The different models recreate some, but not all, features of PD. In the fruitfly *Drosophila melanogaster*, an organism that does not contain αSyn or any of the other members of the synuclein family, overexpression of WT or mutant (A53T and A30P) αSyn enabled the study the effect of αSyn on dopaminergic neurons and behavior (Feany and Bender 2000). The flies showed selective depletion of dopaminergic neurons and loss of motor function as measured by a decrease in climbing ability (Feany and Bender 2000). Flies expressing the A30P mutant seemed to show an earlier onset of the motor phenotype but it was not clear whether that was due to a higher toxicity of the mutant αSyn or due to increased expression when compared to lines expressing WT αSyn. Interestingly, the motor impairments in the flies could be reversed by L-DOPA or other drugs commonly

used in PD (Pendleton, Parvez et al. 2002). The degenerating dopaminergic neurons of transgenic Drosophila were shown to contain αSyn positive inclusions, fibrillar in nature, reminiscent of LBs (Takahashi, Kanuka et al. 2003). In the same transgenic flies, co-expression of human Hsp70 alleviated the toxicity of αSyn, despite the continued presence of aggregate pathology (Auluck, Chan et al. 2002).

Transgenic mice have also been widely used to model PD and the other synucleinopathies. Several lines have been generated, using different αSyn variants, different promoters (PDGFβ, Thy1, PrP, TH), and different genetic backgrounds [for a review see (Maries, Dass et al. 2003)]. While all these lines of mice provide some type of useful information about the effects of αSyn, they all seem to fall short in terms of the full spectrum of features known to take place in PD. Some of these differences may be due to organism-specific differences but they are more likely indicative of our general lack of knowledge of the molecular events that dictate the onset and progression of the disease.

Most recently, developments in viral-based expression systems have enabled the design of novel model organisms, such as rats and monkeys. Delivery of αSyn by viral vectors (adeno-associated viruses, AAV, or lentiviruses) has three main advantages over transgenic expression: (i) overexpression can be site-specific; (ii) animals other than mice can be used to express the gene of interest; and (iii) overexpression can be achieved in adulthood, without affecting development or raising issues of compensation.

It is, therefore, important to continue to refine the existing animal models, and to develop new ones until we find the model that best recapitulates PD features.

1.3.1.2.5 αSyn Oligomers: Zooming in on the Toxic Species

The 'amyloid hypothesis' (developed originally for Alzheimer's disease) states that the aggregation of proteins into an ordered fibrillar structure is causally related to aberrant protein interactions that culminate in neuronal dysfunction and ultimately neurodegeneration (Hardy and Selkoe 2002). The nature of the toxic species is presently unknown. Evidence from PD suggests the hallmark inclusions (LBs) may actually be a protective mechanism neurons developed to preclude the accumulation of the pathogenic intermediates, which have been proposed to be αSyn oligomers (Ding, Lee et al. 2002). Molecular crowding could first accelerate the formation of αSyn spherical protofibrils, a process that involves β-sheet formation (Volles and Lansbury 2002) and then chain-like and annular protofibrils (Ding, Lee et al. 2002) (Figure 1.8). *In vitro* studies with purified αSyn demonstrated that protofibril formation and fibril formation require different critical concentrations, suggesting that there are ranges under which one may form preferentially over the other. Annular protofibrils could exert toxicity because of their binding and permeabilization of vesicles. These porelike structures formed by αSyn have been visualized by electron microscopy (Lashuel, Hartley et al. 2002; Lashuel, Petre et al. 2002) and by atomic force microscopy (Ding, Lee et al. 2002) and they are structurally similar to a subset of Aβ protofibrils and other unregulated pore-forming toxins (Volles, Lee et al. 2001; Lashuel, Hartley et al. 2002; Lashuel, Petre et al. 2002; Volles and Lansbury 2002).

Recent findings in cellular systems are consistent with the toxic protofibril hypothesis. The accumulation of prefibrillar aggregates in the membrane fraction, prior to the appearance of αSyn inclusions, was associated with Golgi fragmentation and a reduction in cell viability (Gosavi, Lee et al. 2002). Lysosomes may also be disrupted by αSyn protofibrils in a similar fashion. If

lysosomal degradation of αSyn is critical in αSyn turnover this could start a vicious toxic cycle (Stefanis, Larsen et al. 2001).

In PD flies, the disconnection between pathogenesis and αSyn inclusion formation adds support for roles of αSyn oligomers rather than fibrillar inclusions in toxicity and pathogenesis (Auluck, Chan et al. 2002).

Notwithstanding the recent findings that favor roles for αSyn oligomers in cellular toxicity, it is also likely that fibrillar and/or amorphous αSyn aggregates also contribute to physical damage in neurons (Neumann, Kahle et al. 2002).

Future studies of αSyn neurotoxicity should include careful biochemical characterization of the aggregation state of αSyn.

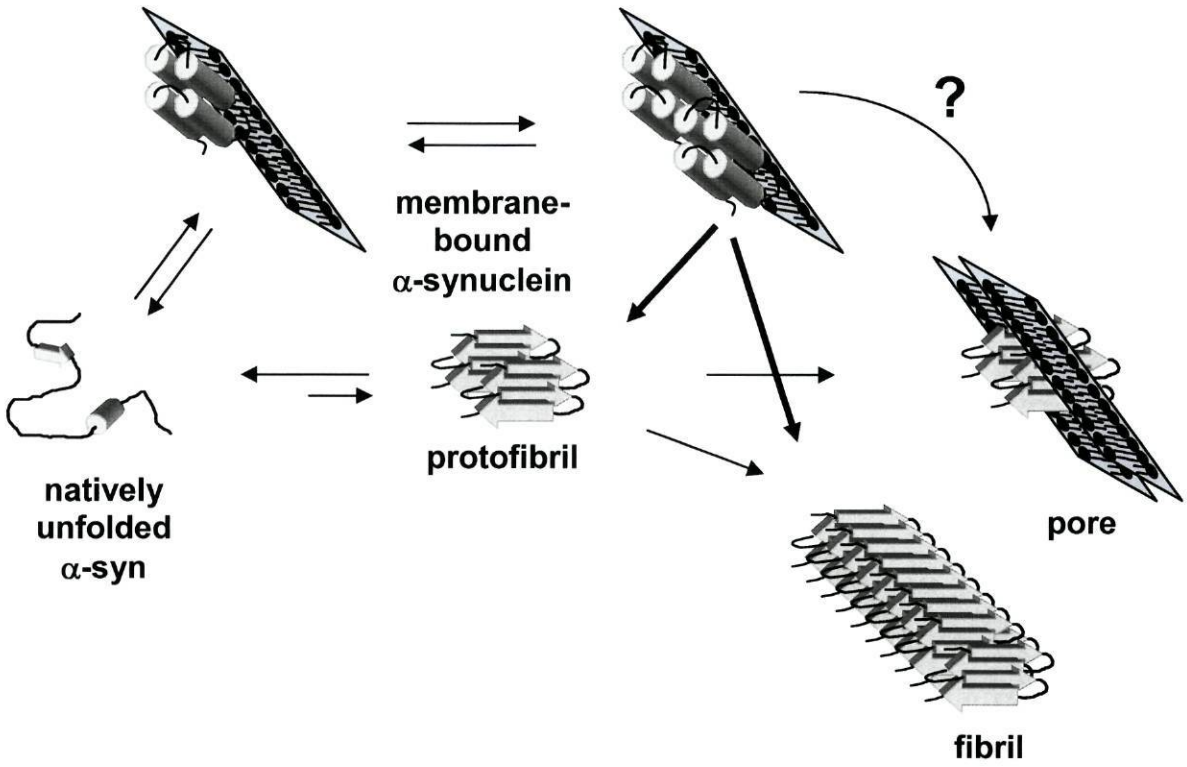


Figure 1.8. Model for αSyn oligomerization. αSyn is a natively unfolded protein that adopts secondary structure upon interaction with membranes (or lipids). It may also adopt alternative folds that enable the formation of protofibrils, intermediate species on the pathway to amyloid fibril formation. Oligomers of αSyn and/or protofibrils may interact with membranes in such a way that enables the formation of pores (Adapted from JC Rochet).

1.3.1.2.6 Selective Neuronal Degeneration in PD: αSyn and Dopamine

The αSyn protein with 140 amino acids is predominantly expressed in the brain but there is also a 112 amino acid splice-variant that is predominantly expressed in the heart, skeletal muscle, and pancreas (Ueda, Saitoh et al. 1994). Why are dopaminergic neurons selectively affected in PD? This is still an open question, but recent studies suggest that dopamine metabolism may explain, at least in part, the selective neurodegeneration. Nigral dopaminergic neurons are particularly exposed to oxidative stress because the metabolism of dopamine generates several potentially toxic molecules, such as dopamine-quinone, superoxide radicals, and hydrogen peroxide (Graham 1978). Recently, dopamine-quinone molecules have been shown to form covalent adducts with αSyn, inhibiting fibrillization and leading to the accumulation of protofibrils (Conway, Rochet et al. 2001). If αSyn protofibrils are toxic, as explained in the previous section, increased levels of cytoplasmic dopamine could lead to the accumulation of the toxic forms of αSyn. Likewise, αSyn protofibrils may themselves increase the levels of cytoplasmic dopamine by binding to and permeabilizing synaptic vesicles through the formation of pores.

While this hypothesis explains the selective degeneration of dopaminergic neurons, it still lacks ultimate proof since the dopamine adducts with αSyn have yet to be found *in vivo*. Moreover, αSyn toxicity has been observed in cells other than just dopaminergic neurons, suggesting events upstream of the putative αSyn -dopamine interaction also contribute to toxicity.

1.3.2 Polyglutamine Diseases

Polyglutamine diseases result from a mutation that results in the expansion of sequences of three nucleotides encoding for the amino acid glutamine. In the human genome, there are repetitive sequences of three nucleotides that above a certain number become pathogenic (Cummings and Zoghbi 2000). Several members of this family, called triplet repeat disorders, have been described, and to date there are close to twenty different known representatives (Heintz and Zoghbi 2000). These disorders are all characterized by somatic and germline instability of the mutation (Zoghbi and Orr 2000). Thus, from generation to generation, there is a tendency towards the expansion of the repeat length, which in turn results in earlier onset and increased severity of the disease, a phenomenon known as anticipation.

The expansion of the repeats can occur either in non-coding sequences or within the coding sequences. The polyglutamine diseases result from the latter. An expansion of CAGs within the coding sequence results in an expansion of consecutive glutamine residues in the translated protein.

In 1991, a polyglutamine expansion was identified as the cause of spinal bulbar muscular atrophy (SBMA), a genetic neurological disease (La Spada, Wilson et al. 1991). The number of diseases identified as polyglutamine disorders has been increasing (Wyttenbach, Sauvageot et al. 2002) (Table 1.3), and the similarities they share suggest a common mechanism of pathogenesis (Fischbeck 2001).

Table 1.3. Polyglutamine expansion diseases

Polyglutamine disorder	Protein	Normal – Pathogenic repeat number	Primary target of neuropathology
Dentatorubropallidoluysian atrophy (DRPLA)	Atrophin 1	7-25 – 49-88	Cerebellum (dentate nucleus), red nucleus, globus pallidus, subthalamic nucleus
Huntington's disease (HD)	Huntingtin	4-34 – 36-180	Caudate nucleus, putamen, globus pallidus
Spinal and bulbar muscular atrophy (SBMA; Kennedy disease)	Androgen receptor	11-24 – 40-62	Motor neuron in anterior horn cells of the spinal cord and brainstem
Spinocerebellar ataxia 1 (SCA1)	Ataxin 1	6-39 – 39-83	Cerebellum, red nucleus, inferior olive, pons, anterior horn cells and pyramidal tracts
Spinocerebellar ataxia 2 (SCA2)	Ataxin 2	15-29 – 34-59	Cerebellar Purkinje cells
Spinocerebellar ataxia 3 (SCA3)	Ataxin 3	13-36 – 55-84	Cerebellar dentate neurons, basal ganglia, brain stem, spinal cord
Spinocerebellar ataxia 6 (SCA6)	Ca^{2+} channel	4-16 – 21-30	Cerebellar Purkinje cells, dentate nucleus, inferior olive
Spinocerebellar ataxia 7 (SCA7)	Ataxin 7	4-35 – 34-300	Cerebellum, brain stem, macula, visual cortex
Spinocerebellar ataxia 17 (SCA17)	TATA-binding protein (TBP)	29-42 – 47-63	Cerebellum, cortex, caudate and putamen

(Adapted from Gusella and MacDonald, 2000; Margolis and Ross, 2001; Nakamura et al., 2001)

1.3.2.1 Huntington's Disease

Huntington's disease (HD), the most prevalent polyglutamine disease, is a devastating neurodegenerative disorder that affects at least 1 in 10,000 people in the United States. The disease was named after George Summer Huntington, who first described it in 1872. The most common symptoms are uncoordinated and uncontrolled involuntary dancing-like movements of limbs and distal muscles and proximal muscles of the trunk, designated as chorea, memory deficits, affective disturbances, change of personality, depression and dementia (Folstein, 1990, Harper, 1996) (Penney, Young et al. 1990). Typically, the first symptoms of the disease appear by 35-40 years of age, progressing inexorably to death, 15-20 years after onset. In juvenile cases (<20 years of age), the disease progresses faster and symptoms are more severe (Vonsattel and DiFiglia 1998).

The motor symptoms in HD can be attributed to the disconnection of the neuronal circuitry between the cortex, striatum and globus pallidus. The striatum is the first and most extensively affected brain structure, by atrophy and neuronal loss in HD (Figure 1.9). Degeneration progresses from dorsal medial regions to ventral and lateral regions (Ferrante, Kowall et al. 1985). Neurodegeneration is accompanied by development of gliosis. The presence of protein aggregates in the brain is one of the hallmarks of HD. N-terminal fragments of mutant huntingtin protein were identified in neuronal intranuclear inclusions and dystrophic neurites in HD cortex and striatum (DiFiglia 1997). Despite intensive research on the significance of these protein aggregates, their function and relevance remain controversial. As in PD and other neurodegenerative diseases with protein inclusions, huntingtin aggregates co-localize with ubiquitin, suggesting that the cell tries to target misfolded huntingtin for proteasomal degradation. A recent study presents evidence for the inability of eukaryotic proteasomes to digest long

polyglutamine sequences, which may accumulate and cause cell death through mechanisms that are not fully understood (Venkatraman, Wetzel et al. 2004).

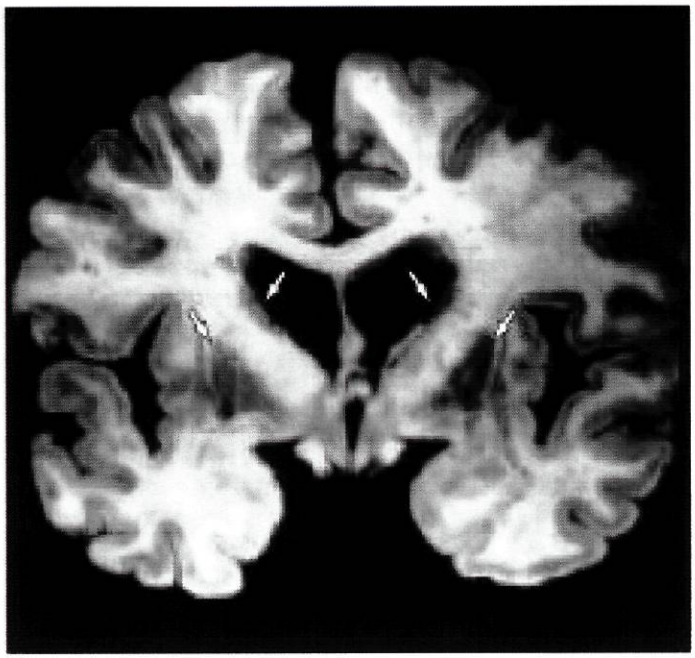


Figure 1.9. Cross section of a brain of an adult with Huntington disease, illustrating marked striatal atrophy. The size of the caudate and putamen (the striatum) (arrows) is dramatically reduced in patients with Huntington's disease. (Adapted from Bradley et al., 1991).

Overall, the relevance of huntingtin inclusions to the pathogenesis of HD remains controversial. While some authors consider inclusions as the direct cause of the disease (DiFiglia 1997; Davies, Beardsall et al. 1998), others propose they are a lateral phenomenon, unrelated to the mechanism of the toxicity or even a detoxification mechanism (Saudou, Finkbeiner et al. 1998).

Another possible mechanism for huntingtin toxicity is the recruitment of transcription factors (also rich in glutamine residues) by mutant huntingtin. This

disrupts gene transcription and therefore contributes to pathogenesis (Luthi-Carter, Strand et al. 2000; Wyttenbach, Swartz et al. 2001). Colocalization of transcription factors with huntingtin aggregates in HD brains has been described, suggesting this may indeed contribute to the pathogenesis (Huang, Faber et al. 1998). Importantly, the transcriptional dysfunction hypothesis does not exclude other mechanisms of pathogenesis because alterations in specific transcripts may potentiate other mechanisms of toxicity, such as apoptosis.

Another attractive explanation for huntingtin toxicity differs from the ones previously described because it assumes a loss of normal function by mutant huntingtin, rather than a gain of toxic function. Wild-type huntingtin up-regulates transcription of brain-derived neurotrophic factor (BDNF), a pro-survival factor produced by cortical neurons that is necessary for survival of striatal neurons in the brain. Mutant huntingtin looses this beneficial activity, resulting in decreased production of BDNF, which eventually leads to cell death (Zuccato, Ciammola et al. 2001). Again, this mechanism for toxicity does not exclude other mechanisms of pathogenesis.

1.3.2.1.1.1 Genetics of HD

The mutation that causes HD is an expansion of CAG repeats [encoding polyglutamine (polyQ)] in the gene *IT-15* (1993). It was isolated in 1993 by a joint effort organized by the Hereditary Disease Foundation, called The Huntington's Disease Collaborative Research Group. The CAG repeat number is polymorphic in the general population, varying between 4-35. Individuals affected by HD have greater than 35 repeats. The mutation is inherited in an autosomal dominant manner. This same mutational mechanism is responsible for a growing number of less common neurodegenerative disorders that include the spinocerebellar ataxias (SCAs) (Table 1.3). In HD, cells in the striatum and cortex are most

affected, resulting in progressive chorea, rigidity, and dementia. The major neuropathological hallmark of HD is the presence of brain lesions composed of intranuclear and cytoplasmic inclusions that contain the protein huntingtin (encoded by *IT-15*). The length of the polyQ expansion in huntingtin correlates directly with kinetics of its aggregation *in vitro*, and inversely with age of onset and severity of the disease in HD patients (Hahn-Barma, Deweer et al. 1998; Sieradzan and Mann 2001). In rare cases, as many as 240 CAG repeats have been identified in HD patients (Nance, Mathias-Hagen et al. 1999). The correlation between age of onset and the size of the repeat is only partial, suggesting that other factors, such as genetic polymorphisms adjacent to CAG repeat, may have significant contributions as well (Vuillaume, Vermersch et al. 1998).

The huntingtin gene contains 67 exons in both mice and humans, spanning over a 200 kb genomic region. The open reading frame encodes a large 350 kDa protein of unknown function. The CAG expansion causing HD is located in the N-terminal region of huntingtin, starting after amino acid 17 (Ambrose, Duyao et al. 1994; Sieradzan and Mann 2001).

HD is an autosomal dominant disorder, which suggests that the disease is caused by a gain of toxic function by the huntingtin protein (Rubinsztein, Leggo et al. 1996). This is also supported by the fact that loss of one allele of the huntingtin gene does not cause HD (Wexler, Young et al. 1987; Myers, Leavitt et al. 1989).

1.3.2.1.2 Model Organisms for Studying HD and Other PolyQ Diseases

Similarly to PD, some animal models based on the administration of neurotoxins inducing HD-like phenotypes have been generated.

The cloning of the huntingtin gene enabled the development of genetic models, which have been essential for the study of HD pathogenesis.
Because of the large size of the huntingtin gene, many models are generated by expressing the exon 1 of huntingtin, where the CAG expansion is located.

Several research groups have recently reported models in yeast to study the folding and behavior of proteins with expanded polyQ tracts (Krobitsch and Lindquist 2000; Muchowski, Schaffar et al. 2000; Meriin, Zhang et al. 2002). Heterologous expression of the first exon of huntingtin in yeast as a fusion to the green fluorescent protein (GFP) results in a polyQ length-dependent aggregation and formation of cytoplasmic inclusions that can easily be detected in living cells by fluorescence microscopy. A simple filter-retention assay can also be used to isolate detergent-insoluble aggregates formed by polyQ proteins with expanded repeats in the disease-causing range. While yeast models were developed ultimately to perform large-scale genetic or chemical genetic approaches to understanding basic mechanisms of protein aggregation and toxicity (see below), studies to date have used a reductionist approach, i.e. studying the effects of one gene family or cellular pathway at a time.

Invertebrate models for polyQ diseases have also been developed in the recent years, offering some experimental advantages (Link 2001). These models recapitulate the protein aggregation and neurodegeneration seen in HD and have enabled the testing of different therapeutic approaches in a timely fashion when compared to other models.

Genetic mouse models have also been an important source of information on different aspects of HD pathogenesis (Nasir, Floresco et al. 1995; Menalled and Chesselet 2002). Knock-out mice for the huntingtin protein demonstrated the essential role of huntingtin in development, because mice show embryonic death

by day 7 {#398). Interestingly, mutant huntingtin rescues the knock-out phenotype, demonstrating its functionality even when mutated and supporting the gain of toxic function hypothesis (White, Auerbach et al. 1997). The first transgenic mouse models of HD, R6 mice, were developed by the group of Gillian Bates and constitute a hallmark in the field of HD research (Mangiarini, Sathasivam et al. 1996). Three lines of these mice were produced but the R6/2 mice are the most commonly used. These mice express a fragment of the human huntingtin protein corresponding to exon 1 with approximately 145 CAG repeats. Although the R6/2 mice present some limitations, particularly the lack of striatal neurodegeneration and non-neurological pathology such as diabetes, they have contributed significantly to HD research. More recently, knock-in mice were generated by gene targeting of the endogenous mouse homologue of the huntingtin gene (Li, Li et al. 2000; Wheeler, White et al. 2000; Lin, Tallaksen-Greene et al. 2001). These knock-in mice are believed to more faithfully reproduce the disease phenotype because the expanded CAG repeat or the full exon 1 replace the homologous region in the mouse genome. In the transgenic mice, the mouse gene is still present and carries the extra copy of the transgene. Although knock-in mice are, at least in theory, better controlled when compared to transgenic mice, they do not completely mimic the human situation because both the huntingtin gene and its promoters differ between the two species.

A conditional mouse model of HD has also been developed, showing a correlation between huntingtin inclusions and motor dysfunction. Motor dysfunction and huntingtin aggregation could be reversed by turning off huntingtin expression (Yamamoto, Lucas et al. 2000).

In HD, similarly to PD, viral vectors have been used to generate animal models (Kirik and Bjorklund 2003). Targeted overexpression of disease-causing genes by recombinant viral vectors provides a new and highly flexible approach for in vivo modeling of neurodegenerative diseases, not only in mice and rats but also in primates, as stated earlier. Importantly, these viral vectors have also been

used in gene delivery approaches for therapeutic intervention in HD, holding great promise for gene delivery/therapy in other neurodegenerative disorders as well (Regulier, Pereira de Almeida et al. 2002; Bensadoun, Pereira de Almeida et al. 2003; Zala, Bensadoun et al. 2004).

While significant efforts have been made to understand the role of huntingtin and the molecular mechanisms that underlie neurodegeneration in HD, a unifying pathogenic mechanism has not been resolved. Published data exist to support the following mechanisms that are not necessarily mutually exclusive: 1) Aggregation of huntingtin into a toxic oligomeric/protofibrillar/fibrillar species that perturbs the structure and/or function of downstream targets; 2) Activation of caspases or other proteases that mediate the cleavage of huntingtin into a toxic species; 3) Impairment of the ubiquitin-proteasome system; 4) Impairment of molecular chaperone function; 5) Localization of huntingtin in the cell nucleus and inhibition of gene transcription, 6) Impairment of mitochondrial function and/or oxidative stress; 7) Impairment of intracellular trafficking pathways and synaptic transmission; and 8) Excitoxicity (Tobin and Signer 2000; Zoghbi and Orr 2000; Ross 2002). If HD does indeed involve multiple disease mechanisms, it will be crucial to understand in a temporal sense the genes and pathways that are of primary significance for disease initiation, progression, and ultimately death. In principle, genetic approaches in yeast could be used to delineate such pathways.

1.4 Yeast as a "Simple" Model Organism for Studying Human Diseases

"Nature is pleased with simplicity"

Isaac Newton

The "genomic revolution" caused by the growing number of completed sequencing projects, of which the human genome constitutes one of the most important, has revealed that the sequence information *per se* is pretty uninformative. More questions have arisen about the complexity of biological systems. This indicates that it is determined not only by the basic units of information (the genes) in the genome, but also by the complex regulatory circuits which control the expression of those genes, synthesis of RNAs and proteins and changes in metabolic pools. Together these allow the cells to respond to environmental changes in a tightly-controlled way. Epigenetic factors are also gaining increasing attention by researchers and are likely to play pivotal roles in most biological processes.

The yeast *Saccharomyces cerevisiae* is one of the simplest eukaryote organisms. Because many of the cellular and biological processes are highly conserved from yeast to humans, it is possible to exploit this unicellular organism, using it as a "living test tube" for studying basic molecular mechanisms underlying human neurodegenerative diseases. This knowledge may then enable the development of better animal models, which more accurately recapitulate the molecular events that lead to the disease phenotypes.

The exploitation of model organisms for the study of complex human diseases is central for a better understanding of the basic mechanisms underlying those diseases, which will then favor the development of novel therapeutics (Figure 1.10).

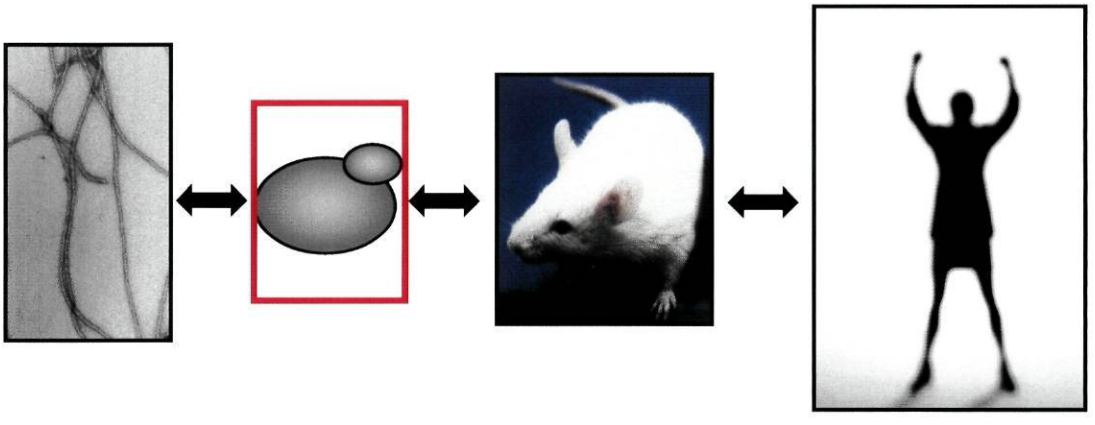


Figure 1.10. Rationale for the project proposed. Yeast will be used to study the molecular mechanisms associated with protein misfolding and amyloid formation. That knowledge should then be taken into animal models, for the development of therapeutic approaches that would ultimately be taken into humans.

1.4.1 The "Awesome Power" of Yeast Genetics

The budding yeast *Saccharomyces cerevisiae*, which has been used as a biotechnological tool for many centuries, is a commonly used model organism because basic cellular mechanisms, such as DNA replication, recombination, cell division, protein folding, intracellular transport, and metabolism are well

conserved between yeast and higher eukaryotes, including mammals. Properties that make yeast an incredibly useful model for molecular genetic approaches include rapid growth on defined media, dispersed cells, ease of mutant isolation by replica plating, well-defined genetic system, and most important, highly versatile DNA transformation system (Sherman 2002). Because yeast can exist in a stable haploid or diploid state, it is an ideal organism on which to perform classical genetics. Its genome of ~14 Mb was the first eukaryotic genome to be fully sequenced. This has enabled more extensive annotation than other sequenced genomes (Goffeau, Barrell et al. 1996). The single greatest advantage of yeast as a model system lies in the ability to rapidly perform genetic manipulations and screen for induced phenotypes. Any single gene can be 'knocked out' or over-expressed with experiments that take a matter of days. In addition, the yeast two-hybrid technique provides a genetic means to identify proteins that physically interact *in vivo*. Due to the "awesome power of yeast genetics", a growing number of molecular biologists use yeast as a primary research tool.

Importantly, the recent sequencing of yeast (Goffeau, Barrell et al. 1996) and human genomes (Lander, Linton et al. 2001; McPherson, Marra et al. 2001) has revealed thousands of yeast proteins that share amino acid sequence similarity with at least one human protein (~2700 at BLAST E-value $<10^{-10}$, ~1100 at E-value 10^{-50}). Several hundred of these involve proteins implicated in human disease. Moreover, several drugs known to target human proteins also inhibit the orthologous protein in yeast (Sinclair, Mills et al. 1997; Hughes, Andrews et al. 2004). These facts illustrate the enormous potential of yeast as a genetic tool for studying complex problems such as those underlying neurodegenerative disorders.

1.4.2 Modeling Protein Aggregation Diseases in Yeast

Although yeast cells cannot be used to directly study neurodegeneration *per se*, numerous studies to date suggest that cell autonomous biological response pathways exist in yeast that are relevant to amyloid diseases. These pathways are beginning to be characterized in yeast and in other model organisms. Indeed, the utility of yeast as a model system for studies relating to basic mechanisms of protein misfolding, aggregation, and toxicity has already resulted in significant insights into pathogenesis of Huntington's disease. It is important to note that the yeast two-hybrid technique has also been used extensively in studies relating to neurodegenerative disorders.

Here I will review genetic and biochemical studies in which yeast has been used as a 'test tube' to study proteins that cause Friedreich's ataxia and Amyotrophic Lateral Sclerosis. This validates the use of yeast for studying Huntington's disease and Parkinson's disease (Table 1.4). I will also describe the rationale behind functional genomics approaches in yeast to study amyloid diseases. The power of yeast models for investigating how conformational changes in proteins affect cell function is illustrated by studies of prion-like factors in yeast that have already yielded major insights into basic mechanisms of prion biology (Lindquist, Krobitsch et al. 2001).

Table 1.4. Human neurologic disorders that have been modeled in yeast.

Disease	Main Protein Involved	Yeast Orthologue	Presence of Inclusions in Disease	References
Alzheimer's disease	APP	No	Yes	(Zhang, Espinoza et al. 1997; Greenfield, Xu et al. 1999)
Parkinson's disease	α-synuclein	No	Yes	(Outeiro and Lindquist 2003)
Polyglutamine expansion diseases (e.g. Huntington's disease)	Several (e.g. Huntingtin)	No	Yes	(Krobitsch and Lindquist 2000; Muchowski, Schaffar et al. 2000; Meriin, Zhang et al. 2002)
Prion encephalopathies	PrP	No, but yeast has prions	Yes	(Ma and Lindquist 1999; Uptain and Lindquist 2002)
ALS	SOD-1	Yes	Yes	(Nishida, Gralla et al. 1994)
Friedreich's Ataxia	Frataxin	Yes	No	(Knight, Kim et al. 1999)

1.4.2.1 Friedreich's Ataxia

One of the first neurodegenerative diseases to be 'modeled' in yeast is Friedreich's ataxia (FRDA), an autosomal recessive disease characterized by progressive gait and limb ataxia, signs of axonal sensory neuropathy, pyramidal weakness of the legs, and dysarthria (Puccio and Koenig 2000). The vast majority of patients with FRDA have a GAA trinucleotide repeat expansion in the gene *frataxin*, which was identified by positional cloning as the gene mutated in this disease (Campuzano, Montermini et al. 1996). The expansion causes reduced expression of the protein. The *frataxin* sequence is similar to a yeast

gene of previously unknown function, subsequently named *YFH1* (yeast frataxin homologue) (Babcock, de Silva et al. 1997). Yeast strains lacking *YFH1* are unable to grow on non-fermentable carbon sources, indicating defective oxidative phosphorylation due to impaired mitochondrial function (Babcock, de Silva et al. 1997; Foury and Cazzalini 1997; Koutnikova, Campuzano et al. 1997; Wilson and Roof 1997). In addition, iron accumulates in mitochondria to high levels in *yfh1* mutants, suggesting that iron accumulation and oxidative damage may play an important role in the etiology of FRDA. Both frataxin and Yfh1p localize to mitochondria. Importantly, wild-type human *frataxin* complements strains lacking *YHF1*, while a point mutant allele from a patient with FRDA only partially complements the yeast mutant (Cavadini, Gellera et al. 2000). Friedreich's ataxia is one of the first neurodegenerative disorders in which a pathogenic mechanism was elucidated directly from studies in yeast, and a more detailed description of these studies can be found elsewhere (Knight, Kim et al. 1999).

1.4.2.2 Amyotrophic Lateral Sclerosis (ALS)

ALS is a devastating neurological disorder that rapidly progresses from mild motor symptoms to severe paralysis and premature death, resulting from the selective death of motor neurons. A significant percentage of patients with familial ALS carry a mutation of *SOD1*, the gene coding for the Cu, Zn superoxide dismutase, an enzyme that catalyzes the disproportion of superoxide radicals to dioxygen and hydrogen peroxide. Interestingly, over 90 mutations in SOD1 have been described thus far. All of the mutations, with one exception, cause familial ALS. SOD constitutes an important defense mechanism against oxidative stress in organisms ranging from microbes to plants and animals (Fridovich 1995). As with frataxin, the human *SOD1* gene can complement yeast *sod1* mutants (Nishida, Gralla et al. 1994; Rabizadeh, Gralla et al. 1995; Corson,

Strain et al. 1998; Corson, Folmer et al. 1999). Studies in yeast and mice indicate that ALS is not due to reduced superoxide dismutase activity, but rather to a dominant gain of function of mutant Sod1 protein (Bruijn, Houseweart et al. 1998; Cleveland 1999; Cleveland and Rothstein 2001). A connection between protein misfolding, aggregation, and disease is less clear cut in ALS than in other neurodegenerative diseases. Nevertheless, Sod1 inclusions and oligomerization have also been observed (Wood, Beaujeux et al. 2003; Kato, Saeki et al. 2004). While the molecular basis of this dominant gain of function is poorly understood, studies to elucidate the molecular mechanisms that underlie ALS are actively being pursued in yeast, neuronal, and animal models.

FRDA and ALS are, therefore, good examples of how powerful the yeast system can be in providing basic information about the molecular mechanisms involved in human diseases.

1.4.3 Functional genomics approaches to studying protein aggregation in yeast

The *Saccharomyces cerevisiae* genome was the first to be sequenced from a eukaryotic organism approximately 8 years ago (Goffeau, Barrell et al. 1996). The availability of well annotated sequence information and the ease with which genetic manipulation can be performed in yeast have quickly led to a number of pioneering studies that utilize genomics and proteomics approaches. For example, the yeast two-hybrid technique has been used successfully to define more than 2700 putative interactions involving at least 2000 different proteins

(Schwikowski, Uetz et al. 2000; Uetz, Giot et al. 2000). In a separate study, the sub-cellular localizations for over 1300 proteins tagged with GFP were determined (Ross-Macdonald, Coelho et al. 1999). The entire set of predicted yeast proteins has also been fused to glutathione S-transferase (GST). The GST fusion proteins can be purified as 64 pools of 96 proteins each, and these pools have been mined for novel biochemical activities (Martzen, McCraith et al. 1999). Other studies take advantage of a collection of gene deletion mutants of *Saccharomyces cerevisiae*, originally developed by the Saccharomyces Genome Deletion Project (Winzeler, Shoemaker et al. 1999; Giaever, Chu et al. 2002). This collection contains 4,850 viable mutant haploid strains, each lacking a single gene. The collection of yeast gene deletion strains (YGDS) has been used successfully to identify new genes and pathways involved in tolerance to radiation (Birrell, Giaever et al. 2001), oxidative stress (Price, Sisodia et al. 1998), human mitochondrial disease (Steinmetz, Scharfe et al. 2002), and to characterize the effects of pharmacological agents (Chan, Carvalho et al. 2000).

In principle, this type of screen could be used to identify genetic modifiers (suppressors or enhancers) (Figure 1.11) for any neurodegenerative disease where a gene product has been linked directly to pathogenesis. In a typical screen, a function or genetic role for the majority of identified genes that modify toxicity has already been determined experimentally or can be predicted (Saccharomyces Genome Database, SGD; http://genome-www.stanford.edu/Saccharomyces/). Using these annotations, genetic modifiers can be grouped into major functional categories (https://www.incyte.com/proteome/YPDsearch-long.html). A typical analysis would be to compare the relative percentages of genes in functional categories obtained in a screen with the percentage of genes in each category across the entire set of genes in the YGDS.

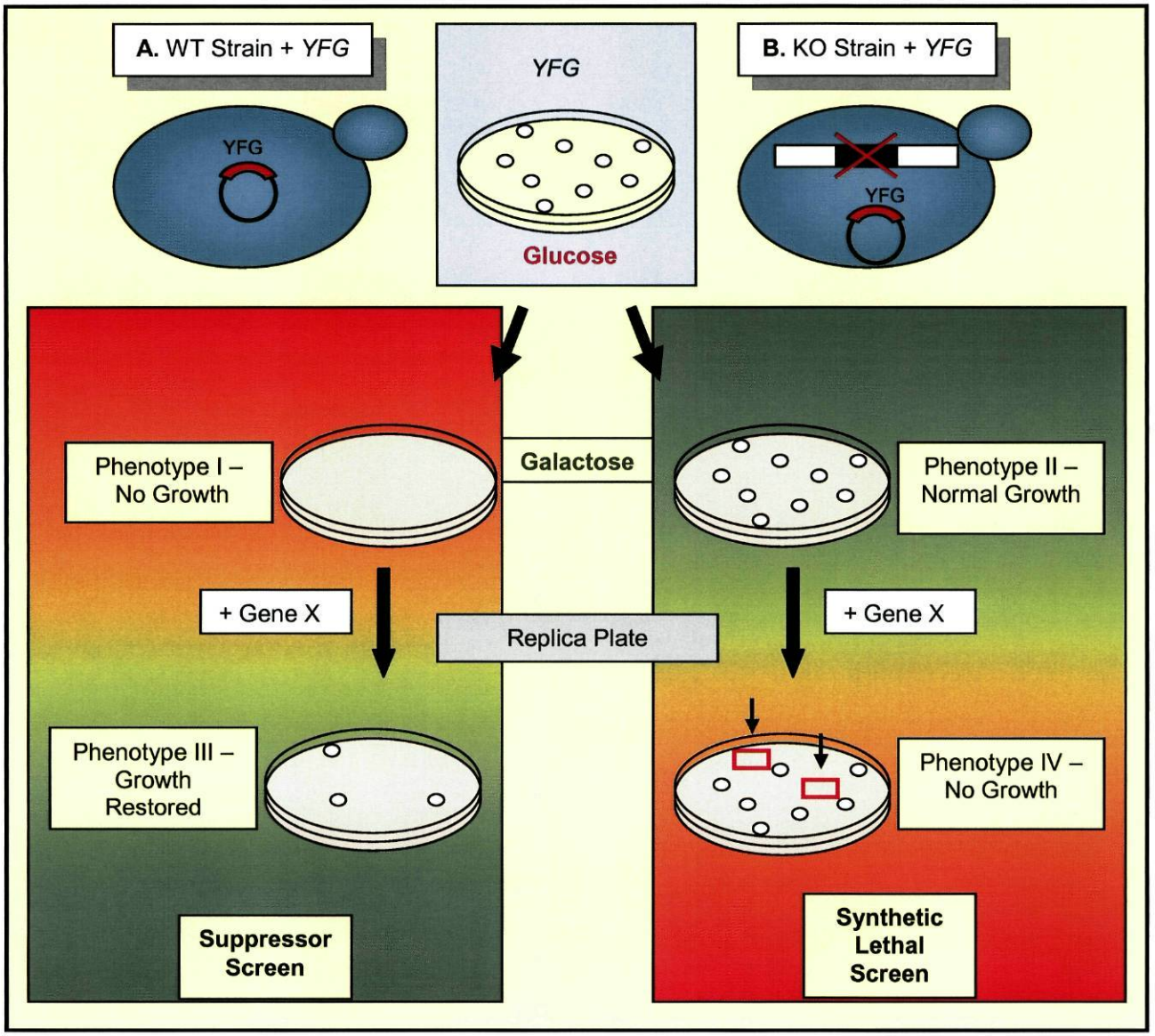


Figure 1.11. An example of two simple genetic screens that can be performed using yeast as a model system. Yeast cells, either WT or bearing gene KO are transformed with plasmids driving the expression of your favorite gene (YFG) from the GAL inducible promoter, and plated onto selective media containing glucose (expression is repressed). Upon transfer to media containing galactose, expression of YFG is induced resulting either in no cell growth (phenotype I) or in normal cell growth (phenotype II). By introducing a second genetic element in the cells the initial phenotypes can give rise to a situation where growth is restored (phenotype III) or there is loss of growth (phenotype IV). These types of genetic screens are called suppressor screens or synthetic lethal screens, respectively.

1.4.4 Limitations of Yeast to Study Neurodegenerative Disorders

It should be acknowledged that yeast as a model system for studies relating to neurodegeneration has its obvious limitations, with a major caveat being simply that some genes important for modulating neurodegeneration may not be present in the yeast genome (such as those that encode caspases involved in apoptosis or growth factors). Despite this caveat, several screens have identified yeast genes with human orthologs. Ultimately, the validity of using yeast as a model organism for studying pathogenic mechanisms of neurodegeneration will only be established by performing complementary approaches in more physiologically relevant models of neurodegeneration.

1.4.5 Yeast and Drug Screens

Large-scale drug screening efforts in yeast models of amyloid formation have also been initiated, to identify therapeutic compounds for the treatment of neurodegenerative diseases (Outeiro and Lindquist, manuscript in preparation). In comparison to mammalian cells, yeast has numerous advantages at the early stages of the drug development process. The low cost of growing yeast cells, the short doubling time, the ease of genetic manipulation, and the high resistance to solvents collectively make *S. cerevisiae* an attractive organism for performing cell-based high-throughput screens. One potential limiting factor for the use of yeast as an assay system is the presence of the cell wall and of several proteins involved in drug export (members of the pleiotropic drug resistance family, Pdr), which may make the cells more impermeable to some organic molecules, or more likely to pump drugs out of the cell. It should be noted, however, that

mammalian cells also possess drug export systems (multiple drug resistance, MDR). Nevertheless, this undesirable attribute of yeast can be eliminated by chemical or genetic means, by increasing the permeability of yeast cells, or by reducing the yeast cells' capability to export drugs.

It is important to stress that drug screening assays in yeast are considered only as primary screening assays that should be complemented by follow-up assays in more physiologically relevant models to allow the successful identification and confirmation of novel lead compounds. The simplicity of *in vitro* screens and screens using cultured cells, while important for the rapid identification of drug-candidates, may also lead to the identification of substances that may be ineffective in whole organisms, due to their much higher level of complexity. For example, it is not uncommon for drugs to bind plasma proteins, reducing or abolishing their activity. *In vivo* studies should be the ultimate proof of the validity and effectiveness of these types of screening efforts.

The use of the simple model organism yeast has an illustrious history of leading to significant advances in our knowledge of human diseases, even in cases in which it was initially difficult to conceive of the utility of this organism (e.g. cancer). The pathogenic mechanisms that underlie neurodegeneration are not well understood, and novel approaches, such as those being performed in yeast, are desperately needed. More importantly, genetic approaches in yeast may identify novel genes not previously known to play any role in neurodegeneration and genes whose products may prove to be attractive therapeutic targets for the treatment of neurodegenerative diseases.

CHAPTER 2

YEAST CELLS PROVIDE INSIGHT INTO ALPHA-SYNUCLEIN BIOLOGY AND PATHOBIOLOGY

CHAPTER 2. YEAST CELLS PROVIDE INSIGHT INTO ALPHA-SYNUCLEIN BIOLOGY AND PATHOBIOLOGY

Abstract

Alpha-synuclein is implicated in several neurodegenerative disorders, such as Parkinson's disease and Multiple System Atrophy, yet its functions remain obscure. When expressed in yeast, alpha-synuclein associated with the plasma membrane in a highly selective manner, prior to forming cytoplasmic inclusions through a concentration-dependent, nucleated process. Alpha-synuclein inhibited phospholipase D, induced lipid droplet accumulation and affected vesicle trafficking. This readily manipulable system provides an opportunity to dissect the molecular pathways underlying normal alpha-synuclein biology and the pathogenic consequences of its misfolding.

2.1 Introduction

Alpha-synuclein (αSyn) is abundant and broadly expressed in the brain, where it interacts with membranes, vesicular structures and a puzzling variety of other proteins (Lucking and Brice 2000). Some PD cases have a genetic basis (Gasser 2001) that implicates protein folding and quality-control (QC) factors, including a ubiquitin ligase (Kitada, Asakawa et al. 1998) and a ubiquitin C-terminal hydrolase (Leroy, Boyer et al. 1998; Liu, Fallon et al. 2002), in αSyn pathology. In mammalian cells αSyn has been reported in the nucleus, in the cytosol, associated with membranes and, in diseased brains, in large cytoplasmic inclusions (Lewy bodies) (Lucking and Brice 2000). Synucleinopathies are now classified as protein misfolding disorders (Singleton, Farrer et al. 2003). Given the strong conservation of protein folding, membrane trafficking and protein QC mechanisms between yeast and higher eukaryotes, we used *S. cerevisiae* to uncover and establish basic aspects of both normal and abnormal αSyn biology.

2.2 Materials and Methods

2.2.1 Plasmid constructions

Alpha-synuclein (αSyn) WT, A53T or A30P sequences (a kind gift from Dr. Peter Lansbury, Harvard University) were cloned into p426GPD (Mumberg, Muller et al. 1995) as *Spe*I-*Hind*III-digested products of PCR amplification (5'GGACTAGTATGGATGTATTCATGAAAGG3' and 5'GGGGAAGCTTTTATTAGGCTTCAGGTTCGTAGTC3'). GFP, CFP and YFP fusions were constructed in the same vector series (Mumberg, Muller et al. 1995) by inserting the NFP (N = G, C or Y) coding sequence as a *Cla*I-*Xho*I-digested PCR product (5'CGATCGATAGCAAGGGCGAGGAGCTG3' and GGTTCTCGAGTTACTTGTACAGCTCGTCC3') in frame with αSyn. Constructs were verified by DNA sequencing. αSyn alone or fused to XFP was subcloned into p416GPD, p423GPD, p425GPD, p426GAL (Mumberg, Muller et al. 1994) or the integrative vectors from the pRS series (pRS306 and pRS304), (Sikorski and Hieter 1989) as *SacI-Kpn*I fragments. Plasmids used in this study are listed in Table 2.1.

2.2.2 Yeast Strains and Genetic Procedures

Genotypes of yeast strains are listed in Table 2.2. Yeast manipulations were performed and media were prepared using standard procedures (1991). Transformations of yeast were carried out using a standard lithium acetate procedure (Ito, Fukuda et al. 1983). Yeast strains carrying the galactose-inducible αSyn constructs were pre-grown in raffinose medium (no repression of

the galactose-inducible promoter) prior to galactose medium, to allow rapid, synchronous induction of expression.

Table 2.1. Plasmids used in this study.

Plasmids	Type of Plasmid	Source or Reference
p42X*GPD	2 μ	(Mumberg, Muller et al. 1995)
p426Gal	2 μ	(Mumberg, Muller et al. 1994)
p416/PQ25	CEN	(Krobitsch and Lindquist 2000)
p416/PQ103	CEN	(Krobitsch and Lindquist 2000)
pRS304	Integrative	(Sikorski and Hieter 1989)
pRS306	Integrative	(Sikorski and Hieter 1989)
p426GPD-GFP	2 μ	This study
p426GPD-CFP	2 μ	This study
p426GPD-YFP	2 μ	This study
p426GPD-αSynWT	2 μ	This study
p426GPD-αSynA53T	2 μ	This study
p426GPD-αSynA30P	2 μ	This study
p42XGPD-αSynWT-N†FP	2 μ	This study
p42XGPD-αSynA53T-N†FP	2 μ	This study
p42XGPD-αSynA30P-N†FP	2 μ	This study
pRS304-αSynWT-GFP	Integrative	This study
pRS304-αSynA53T-GFP	Integrative	This study
pRS304-αSynA30P-GFP	Integrative	This study
pRS306-αSynWT-GFP	Integrative	This study
pRS306-αSynA53T-GFP	Integrative	This study
pRS306-αSynA30P-GFP	Integrative	This study
pYES2-FLAG103Q	2 μ	(Meriin, Zhang et al. 2002)
pPLD1	CEN	(Rudge, Zhou et al. 2002)

*X=3, 5 or 6; †N=C, G or Y

Table 2.2. Strains used in this study.

Yeast Strain	Genotype
W303-1A	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*
sec14ts a	*MAT a his3-D200 lys2-801 sec14-1 ura3-52*
sec14ts α	*MAT α ade2-1D1 his3-D200 trp1-D sec14-1 ura3-52*
sec14ts spo14Δa	*MAT a his3-D200 lys2-801 sec14-1 ura3-52 spo14::KAN*
sec14ts spo14Δ α	*MAT α ade2-1D1 his3-D200 trp1-D sec14-1 ura3-52 spo14::KAN*
cki1Δ	*MAT a his3 ura3 cki1::HIS3*
sec14ts cki1Δ	*MAT a his3 lys2 trp1 ura3 cki1::HIS3 sec14^{ts}-3*

2.2.3 Spotting Experiments

Cells carrying either one or two copies of the αSyn-GFP fusions were grown overnight at 30°C in liquid media containing raffinose until they reached log or mid-log phase. Cultures were then normalized for OD_{600}, serially diluted and spotted onto solid media containing either glucose or galactose, after which they were grown at 30°C for 2-3 days. Cells carrying the 2μ plasmids were grown in synthetic medium lacking uracil, for plasmid selection, serially diluted and spotted onto rich medium (YPD) or synthetic medium lacking uracil.

2.2.4 Plasmid Maintenance Studies

Cells were carrying 2μ plasmids were grown in non-selective medium (YPD) until they reached log or mid-log phase and then plated onto YPD and synthetic drop-out solid media. Plasmid loss was assessed by comparing the number of cells growing on drop-out versus rich medium.

2.2.5 SDS-PAGE and Immunoblottings

Yeast cells expressing αSyn were grown to mid-log phase, harvested, washed with water, and lysed in ethanol with glass beads. The precipitated proteins were spun down at 13,000 rpm for 15 min., at 4 °C, and the pellets resuspended in standard 2 x SDS-sample buffer. Proteins were resolved by SDS-PAGE (12 % gels), transferred to PVDF membranes and immunoblotting was performed

following standard procedures. In Fig. 2.1 results with the Syn-1 antibody (Transduction Laboratories) are shown. Immunoblotting was also performed with antibodies LB509 (Zymed Laboratories) and anti-GFP (Roche). Each stained the same single band.

2.2.6 Microscopy

For analysis of αSyn-GFP distribution, strains carrying either one or two copies of αSyn-GFP were grown overnight in raffinose medium. After normalizing the culture density, expression was induced in galactose for 8 hours, at which time the levels and distribution of αSyn had reached steady state. Cells were then imaged with a Zeiss Axioplan II microscope equipped with MetaMorph (UIC) for acquisition. For analysis of αSyn distribution 2D and/or 3D deconvolution was applied (Huygens Essentials, SVI). Membrane localization precedes the formation of inclusions, neither of which is observed with A30P, supports earlier suggestions that lipids promote αSyn oligomerization (Lee, Choi et al. 2002), a process associated with neurodegeneration (Sharon, Bar-Joseph et al. 2003).

Immunofluorescence (IF) was performed according to standard procedures (1991). In short, cell walls were fixed with formaldehyde (4% final concentration, 1 hour at 30 °C), digested with Zymolyase 100T (Seikagaku Corporation, Tokyo, Japan), and processed for IF. The anti-ubiquitin antibody was from Dako. Alexa-fluor antibodies were from Molecular Probes.

To label lipids, cells grown to mid-log phase were stained with the lipophylic dye Nile Red (final concentration of 0.5 μg/ml) for 5 min (Greenspan, Mayer et al. 1985), washed with PBS and imaged. Images shown in Fig. 2.11 were acquired using the same exposure time.

For FM4-64 staining, cells were grown in raffinose medium overnight, densities of cultures were then normalized and the cultures split in two. One half of the culture was grown in galactose medium while the other was grown in raffinose medium. After 8 hours cells were harvested, washed and stained with FM4-64 dye as previously described (Kranz, Kinner et al. 2001). In this experiment we employed a toxic derivative described previously (Meriin, Zhang et al. 2002), but used a single integrated copy of the gene rather than the 2 μ plasmid previously employed (Meriin, Zhang et al. 2002).

Electron microscopy of yeast cells expressing αSyn was performed as described (Bauer, Herzog et al. 2001), after cell wall digestion with zymolyase 100T (Seikagaku Corporation, Tokyo, Japan).

2.3 Results

To study αSyn dynamics in living cells we created an αSyn-GFP (green fluorescent protein) fusion that was not subject to proteolysis in yeast (Fig. 2.1), as related fusions have been in mammalian cells (McLean, Kawamata et al. 2001). Integrating this construct into the genome under the control of a galactose-inducible promoter allowed routine manipulations in the absence of αSyn expression. Upon induction with galactose, WT αSyn-GFP localized intensely at the plasma membrane, with a smaller quantity in the cytoplasm (Fig. 2.2). By comparison with other GFP fusion proteins, αSyn did not localize to mitochondrial or nuclear membranes (data not shown). Thus, reminiscent of its selectivity for membranes with particular lipid compositions in vitro (Jo, McLaurin et al. 2000), αSyn has a high intrinsic selectivity for particular cellular membranes in vivo.

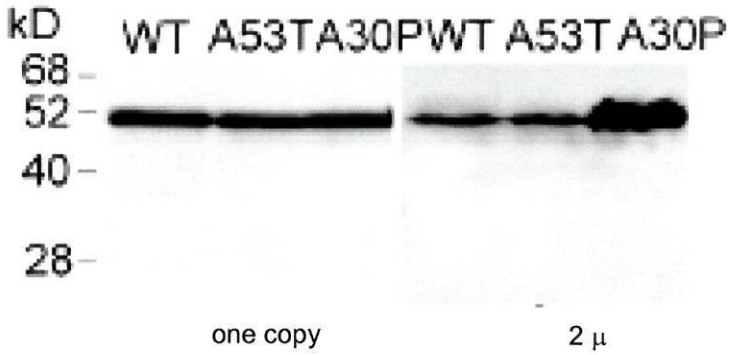


Fig. 2.1. Expression of αSyn in yeast. Immunoblot analysis of cells expressing αSyn-GFP fusions. Single integrated copies (left) of WT, A53T and A30P αSyn produced similar protein levels. 2 μ plasmid (variable copy number) expression levels of WT and A53T were similar but lower than those of A30P αSyn (right).

Two αSyn point mutants (A53T and A30P) are associated with rare forms of early onset familial PD but have distinct physical properties (Polymeropoulos, Lavedan et al. 1997; Kruger, Kuhn et al. 1998). In yeast, each accumulated at the same level as WT αSyn (Fig. 2.1, one copy), but their cellular distributions differed profoundly: like WT αSyn, A53T concentrated at the plasma membrane; A30P dispersed throughout the cytoplasm (Fig. 2.2, one-copy). The effects of the A30P mutation on membrane association in yeast are consistent with its poor membrane-binding capacity in other systems (Jo, Fuller et al. 2002; Bussell and Eliezer 2003).

One hypothesis to explain the late onset of PD (and several other misfolding diseases) is that disease occurs in aging neurons when the capacity of the QC system to cope with accumulating misfolded proteins is exceeded (Taylor, Hardy et al. 2002; Berke and Paulson 2003). It should be possible to mimic this situation, and exceed the QC system of yeast, by increasing αSyn expression. Indeed, simply doubling its expression, by integrating a second copy in the genome, profoundly changed its fate: the vast majority of αSyn appeared in large cytoplasmic inclusions (Fig. 2.2). Notably, inclusions were not formed by excess protein unable to find 'docking sites' at the membrane: 1) much less protein was membrane-localized in two-copy strains than in single-copy strains (Fig. 2.2), and 2) in time courses with two-copy strains, αSyn first accumulated at the membrane and was later recruited away into cytoplasmic inclusions. A53T behaved like WT, but an even smaller fraction associated with membranes. A30P maintained its cytoplasmic distribution (Fig. 2.2, two-copies). In vitro, purified αSyn undergoes nucleated polymerization, with A53T having a greater and A30P a lesser propensity to form large polymers than WT (Conway, Lee et al. 2000). Membrane localization preceded the formation of inclusions, neither of which is observed with A30P, supporting earlier suggestions that lipids promote

αSyn oligomerization (Lee, Choi et al. 2002), a process associated with neurodegeneration (Sharon, Bar-Joseph et al. 2003).

Our studies demonstrate that the recruitment of αSyn to inclusion bodies is a biologically relevant property in living cells, where total protein concentrations are very high (~300 mg/ml) and αSyn concentrations are low (below detection on stained gels).

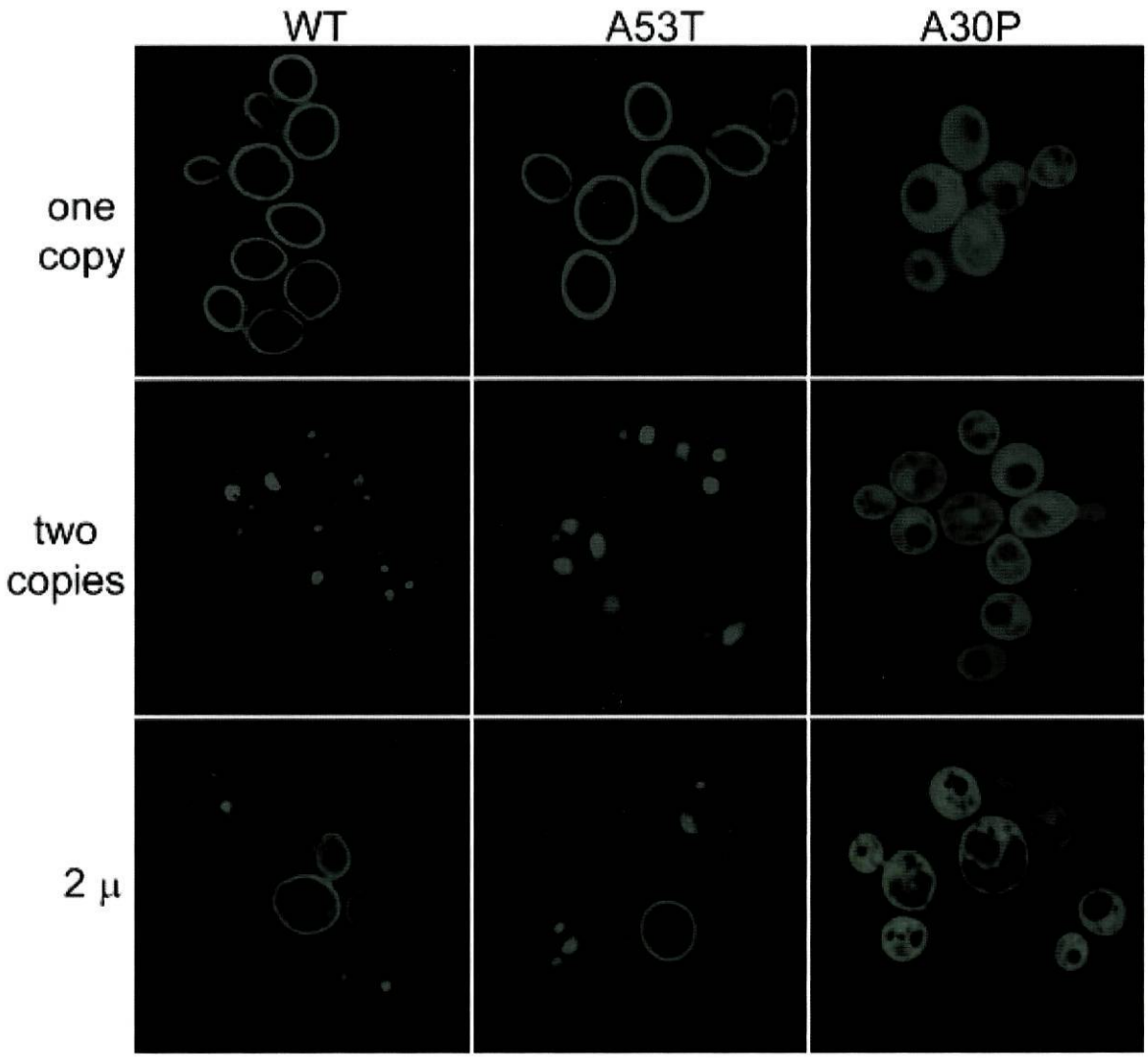


Figure 2.2. Fluorescence microscopy of yeast cells expressing αSyn-GFP. In cells carrying one copy of WT or A53T, the protein concentrated at the plasma membrane with small amounts in the cytoplasm. Cells with two copies of WT or A53T showed cytoplasmic inclusions and reduced membrane localization. When expressed from the 2 μ plasmid, WT and A53T distributions varied from cell to cell, while A30P always had diffuse cytoplasmic distribution.

Under growth conditions used for microscopy, a single-copy of WT or A53T αSyn had no significant effect on growth. Two copies completely inhibited it (Fig. 2.3 and Fig. 2.4). A30P inhibited growth only when expressed from a high copy (2μ) plasmid (Fig. 2.4).

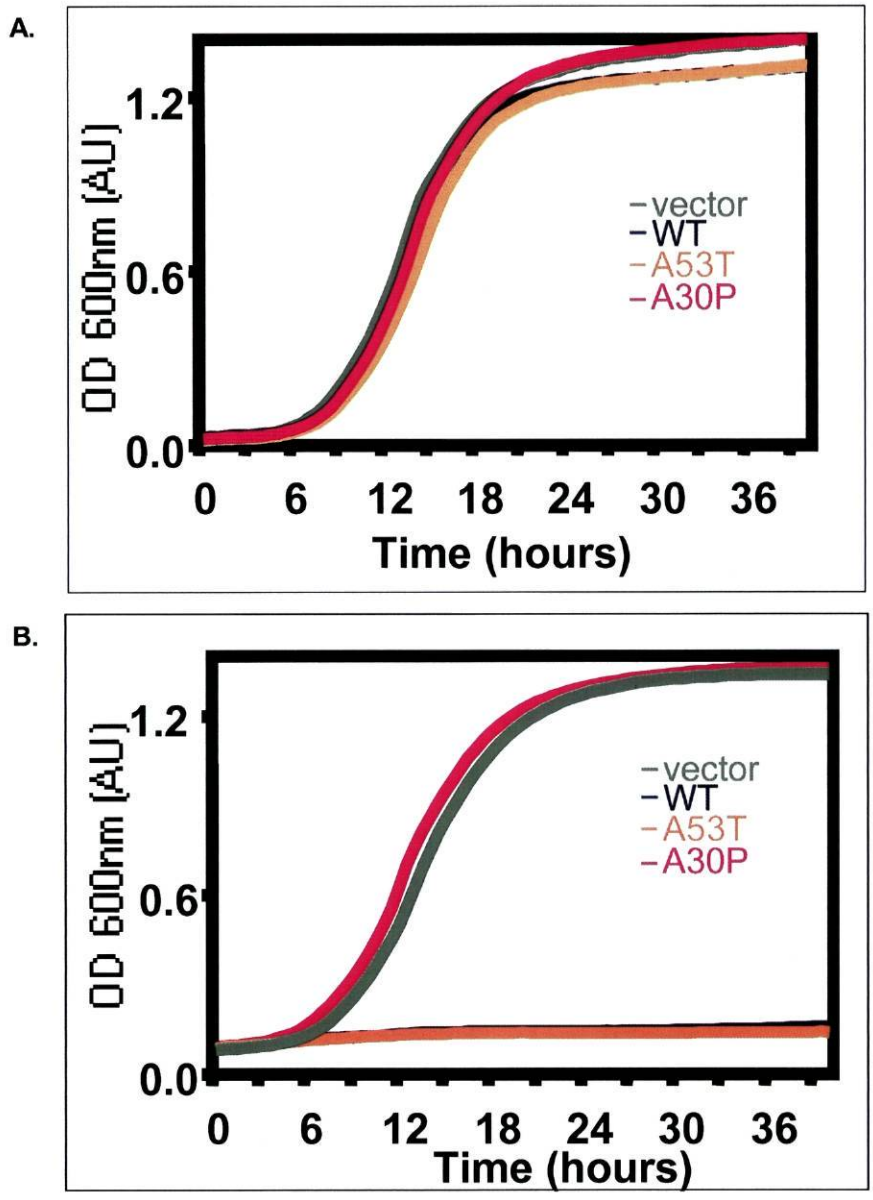


Figure 2.3. αSyn inhibits growth in liquid cultures. **A.** One copy of αSyn WT or A53T had little effect on growth. **B.** Two copies completely inhibited growth.

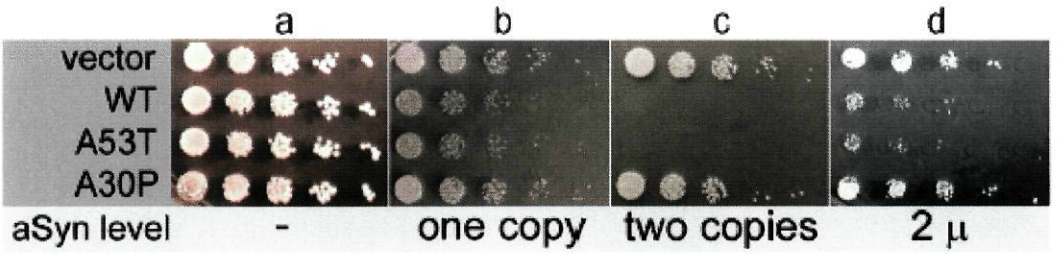


Figure 2.4. αSyn inhibits growth on plates. In liquid cultures, which formed the basis for all the experiments reported, one copy of αSyn had little effect on growth. On plates it slightly inhibits growth. One copy (b) of αSyn WT or A53T had a mild effect on growth whereas two copies (c) completely inhibited it. 2 μ plasmid expression of WT and A53T αSyn caused mild inhibition of cell growth (d). The A30P mutant had no detectable effect on growth on this assay. Cells of all strains grew equally well when αSyn expression was not induced (a). Slight differences in toxicity in liquid and on plates are very useful in genetic screens.

Curiously, cells were able to grow with 2μ plasmids expressing WT and A53T. Further analysis demonstrated that selective pressures had reduced expression by reducing the average copy number of WT and A53T plasmids, confirming the strong concentration-dependence of their toxicity (Fig. 2.1, and data not shown). Cells carrying A30P mutant αSyn maintained the plasmid at higher copy-number, hence the higher levels of expression shown in Fig. 2.1.

In this and all other assays (below and data not shown), similar results were obtained with αSyn constructs not fused to GFP.

At various times after galactose induction, cells were plated on glucose to repress further expression and determine the number of viable cells. A significant number (~50%) retained colony forming ability after 24 hours in galactose (data not shown). This lag between αSyn induction and cell death afforded an opportunity to study the protein's biological effects before toxicity confounded results.

To investigate interactions between αSyn and the QC system, we first stained cells for ubiquitin, a highly conserved polypeptide that is post-translationally attached to misfolded proteins to mark them for degradation (Hershko, Ciechanover et al. 2000). We used the extra-chromosomal plasmids to create mixed populations, perfectly matched for growth and culture conditions but expressing αSyn at different levels in different cells. Direct visualization of αSyn-GFP showed the expected diversity of localization patterns from cell to cell (membranes and cytosolic inclusions) (Fig. 2.2, 2 μ and Fig. 2.5). After fixation and detergent permeabilization (required for immunological detection of ubiquitin) membrane fluorescence was greatly reduced (Fig. 2.6 and Figs. 2.5 and 2.7), demonstrating the advantage of direct localization methods.

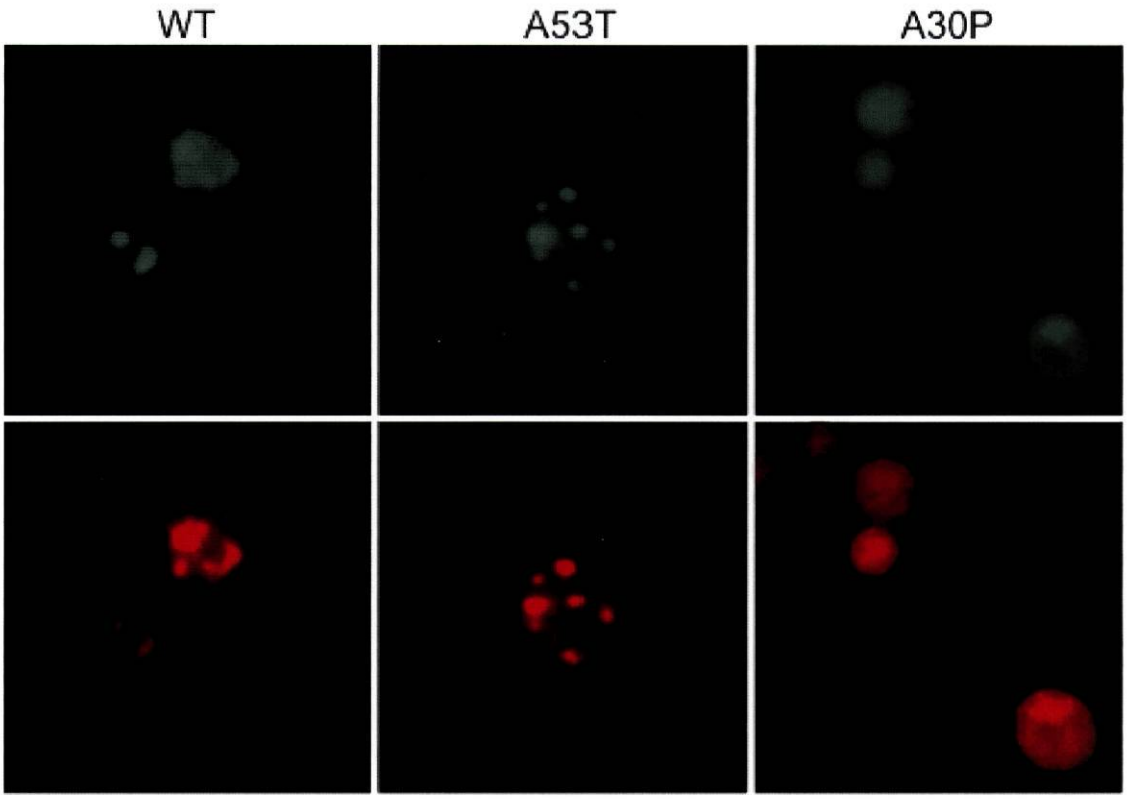


Figure 2.5. αSyn-GFP produces the same pattern of localization when detected by GFP fluorescence or by immunofluorescence (IF) localization with the anti-αSyn antibody Syn-1, confirming that GFP localizations are not an artifact of proteolysis. Cells expressing WT αSyn-GFP, A53T or A30P from a 2 μ plasmid were processed for IF using the Syn-1 antibody followed by an Alexa Fluor 594-conjugated anti-mouse secondary antibody. GFP-positive inclusions (top) overlap with inclusions stained with the Syn-1 antibody (bottom), indicating the presence of full-length αSyn-GFP in the inclusions. The fixation procedure for IF eliminated the ability to assess the highly specific nature of αSyn at the plasma membrane.

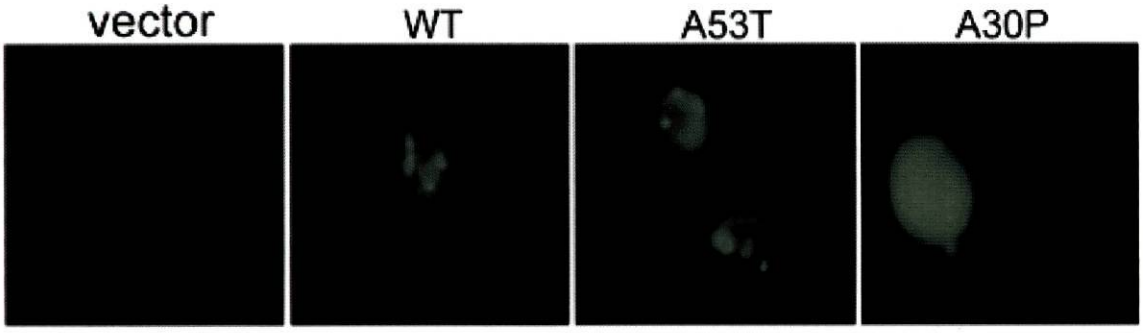


Figure 2.6. αSyn aggregation is not an artifact of fusion to GFP. IF of cells expressing WT, A53T and A30P αSyn from 2 μ plasmids was performed with the Syn-1 antibody followed by an Alexa Fluor 488-conjugated anti-mouse secondary antibody. Inclusions were similar in number, size and distributions to those observed with the 2 μ plasmids of αSyn-GFP fusions, but membrane localization was reduced due to the fixation and permeabilization procedures required for IF.

Nevertheless, the preparations were adequate to unambiguously detect strong accumulation of ubiquitin (Fig. 2.7), and establish that it varied with αSyn expression on a cell-by-cell basis (Fig. 2.7 and data not shown). As in mammalian cells and human brains, some but not all αSyn aggregates stained with ubiquitin antibodies (Stefanis, Larsen et al. 2001; Sampathu, Giasson et al. 2003). Thus, ubiquitylation was neither required for aggregation nor prevented it (Fig. 2.7). We also asked if accumulating misfolded αSyn impaired the capacity of the QC system to degrade other proteins. We used an unstable GFP derivative, GFPu, previously established as a reporter for general proteasome activity and αSyn constructs not fused to GFP (Meriin, Zhang et al. 2003). All three αSyn proteins caused GFPu accumulation, indicating proteasome impairment (Fig. 2.8).

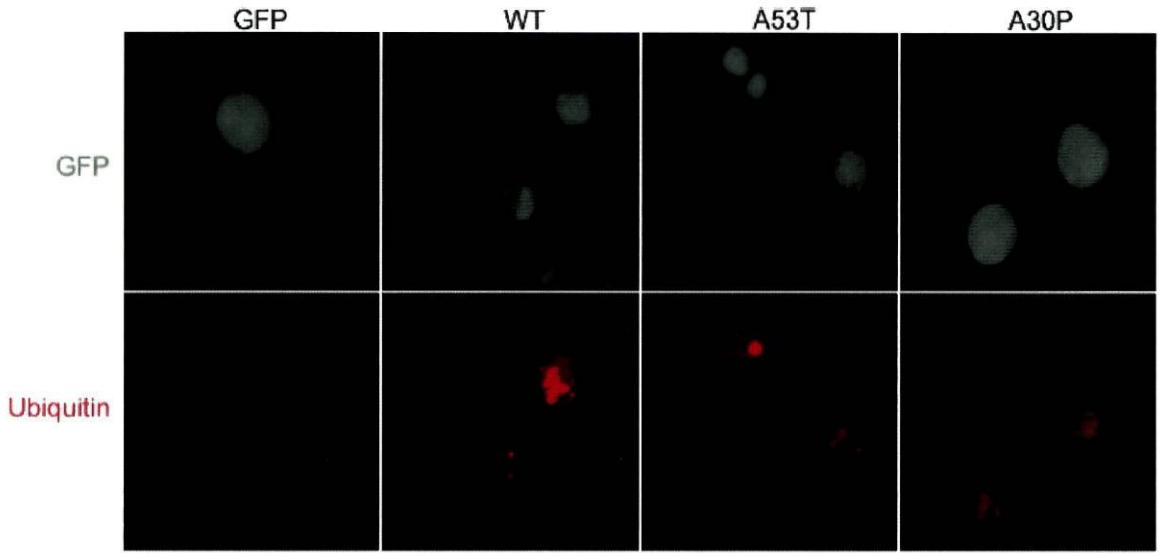


Figure 2.7. αSyn expressing cells accumulate higher levels of ubiquitin/ubiquitinated proteins. Immunofluorescence of cells expressing αSyn-GFP (2 μ) (top) with an anti-ubiquitin antibody (bottom) showed increased levels of ubiquitin accumulation in cells expressing αSyn (WT and both mutants).

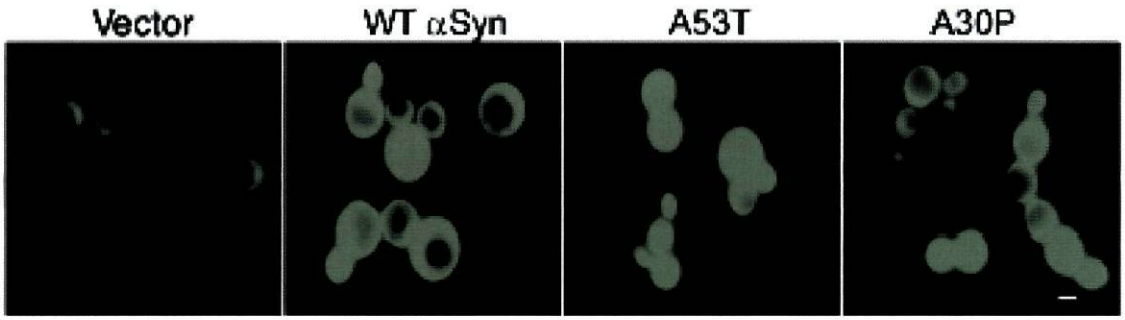


Figure 2.8. Cells co-expressing αSyn and GFPu showed accumulation of the reporter when compared to cells carrying an empty vector control. Scale bar, 1 μ.

Next, we examined properties of αSyn that have been postulated but not directly tested: participation in the regulation of cellular lipid metabolism and vesicle trafficking (Murphy, Rueter et al. 2000; Cole, Murphy et al. 2002). In a search for inhibitors of phospholipase D (PLD) activity, αSyn was serendipitously found to potently and selectively inhibit mammalian PLD in vitro (Jenco, Rawlingson et al. 1998). To determine if αSyn inhibits PLD in vivo we took advantage of a previously established relationship between PLD, Sec14p, a phosphatidylinositol-phosphatidylcholine transfer protein, and Cki1p, a choline kinase (Bankaitis, Aitken et al. 1990). αSyn expressed at a level that had no effect in *cki1*Δ cells, completely blocked growth in *cki1*Δ *sec14ts* double mutants (Fig. 2.9). This suggested that αSyn had inhibited endogenous yeast PLD activity. A53T had the same effect, A30P did not. Cells carrying the *sec14ts* mutation alone can grow at 37°C by acquiring any of several 'bypass mutations' (Rudge, Pettitt et al. 2001). PLD mutations block the ability of bypass mutations to rescue growth; expression of αSyn did the same (Bankaitis, Aitken et al. 1990; Sreenivas, Patton-Vogt et al. 1998) (Fig. 2.10). Overexpressing PLD (from an extra-chromosomal plasmid) restored bypass rescue (Fig. 2.10), confirming that the effects of αSyn in these assays were due to the inhibition of PLD. A mutant huntingtin exon-1 fragment with 103 glutamines (Q103 Htt) did not inhibit PLD (Fig. 2.10). Thus, the serendipitously discovered ability of αSyn to inhibit PLD in vitro is a specific, highly conserved and biologically relevant property of the protein in vivo.

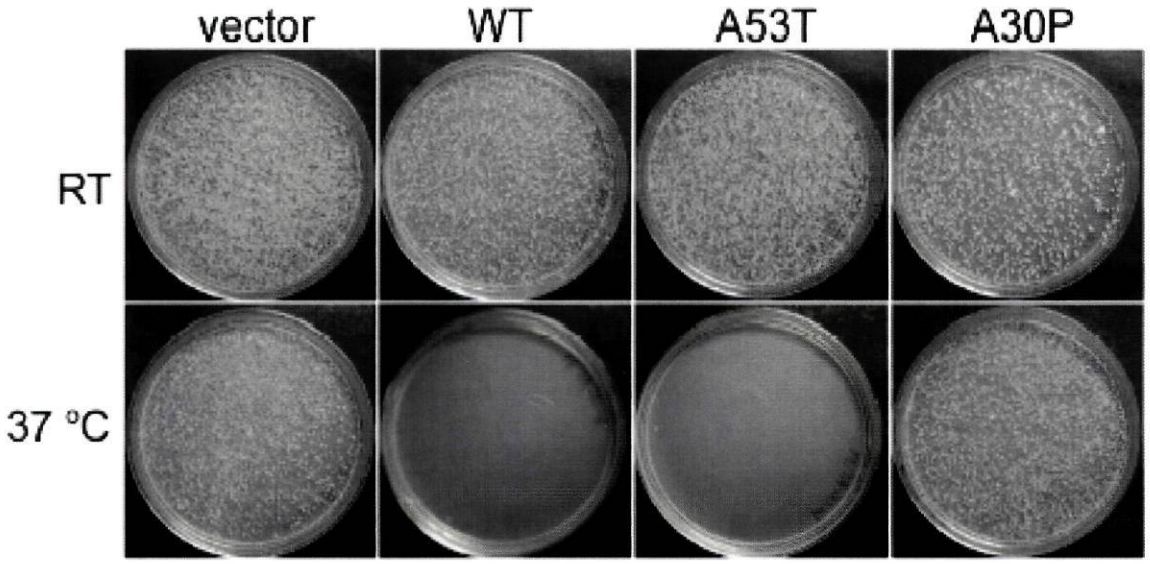


Figure 2.9. αSyn inhibits PLD. A *cki*1Δstrain (top) or a *sec14ts cki*1Δ double mutant strain (bottom) were transformed with 2μ plasmids expressing αSyn and grown at 37°C. (*30*) WT and A53TαSyn blocked growth in *sec14ts cki*1Δ strain, but not the *cki*1Δ strain.

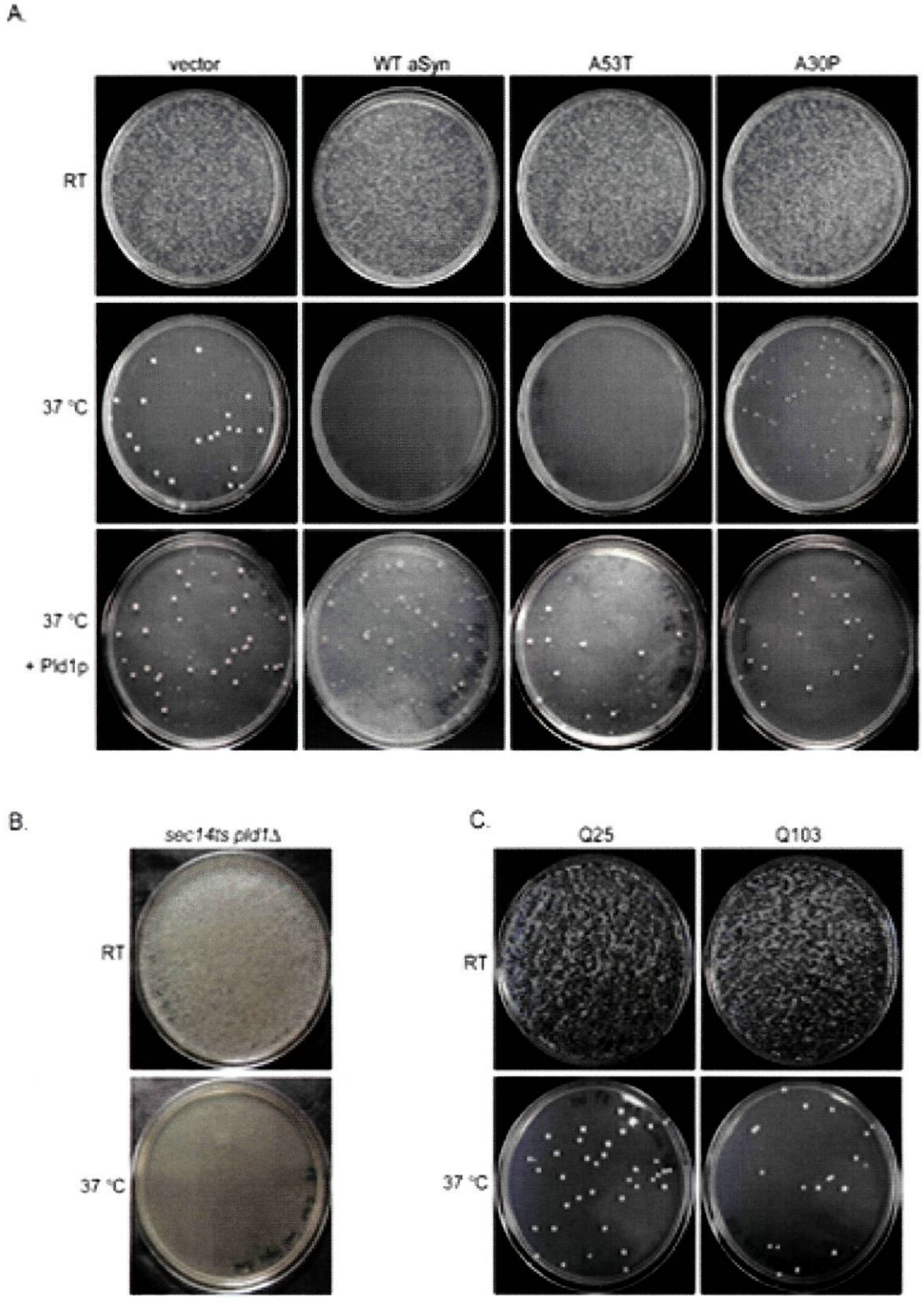

A.
vector
WT aSyn
A53T
A30P
RT
37 °C
37 °C
+ Pld1p
B.
sec14ts pld1Δ
RT
37 °C
C.
Q25
Q103
RT
37 °C

Figure 2.10. αSyn inhibits PLD in *sec14ts* cells. Liquid cultures were grown overnight in synthetic selective medium for the plasmid and equal numbers of cells were plated onto solid selective medium and incubated at either the permissive (30 °C) or non-permissive temperature (37°C). (A) *sec14ts* cells expressing αSyn WT, A53T and A30P from 2 μ plasmids grow normally at room temperature (RT), with those expressing WT or A53T αSyn growing slightly slower than those carrying an empty vector control or those expressing A30P (top and data not shown). When cells are incubated at 37°C PLD activity is required for the accumulation of 'bypass mutations' for growth. Cells expressing WT and A53T αSyn, unlike those expressing A30P or carrying an empty vector, are not able to accumulate those mutations due to PLD inhibition (middle row). Upon introduction of yeast PLD1 from an extrachromosomal plasmid in the same cells, the ability to accumulate 'bypass mutations' was restored in cells expressing WT and A53T αSyn. (B) *sec14ts* cells in which the *PLD* gene is deleted are not able to acquire the 'bypass mutations' required for growth at 37°C. (C) The same *sec14ts* cells expressing normal (Q25) or mutant (Q103) Htt exon-1 show normal growth at RT and acquire the 'bypass mutations' that enable them to grow at 37°C at the same rate as vector controls. Thus, the effect of αSyn on PLD activity is specific.

αSyn shares properties with the broadly distributed family of fatty-acid binding proteins and its oligomerization in vitro is affected by lipids (Sharon, Goldberg et al. 2001; Sharon, Bar-Joseph et al. 2003). In mammalian cells cultured with lipid-enriched medium, αSyn accumulates on lipid monolayers surrounding triglyceride-rich lipid droplets and promotes their accumulation by protecting them from hydrolysis (Cole, Murphy et al. 2002). We used Nile Red, a fluorescent dye that preferentially binds neutral lipids such as triglycerides, to assess the ability of αSyn to influence lipid accumulation. Cells expressing WT αSyn and A53T stained much more strongly than cells expressing A30P, even in the absence of extrinsic lipids (Fig. 2.11, bottom). Electron microscopy established that WT αSyn and A53T caused the accumulation of discrete lipid droplets; A30P did not (Fig. 2.12).

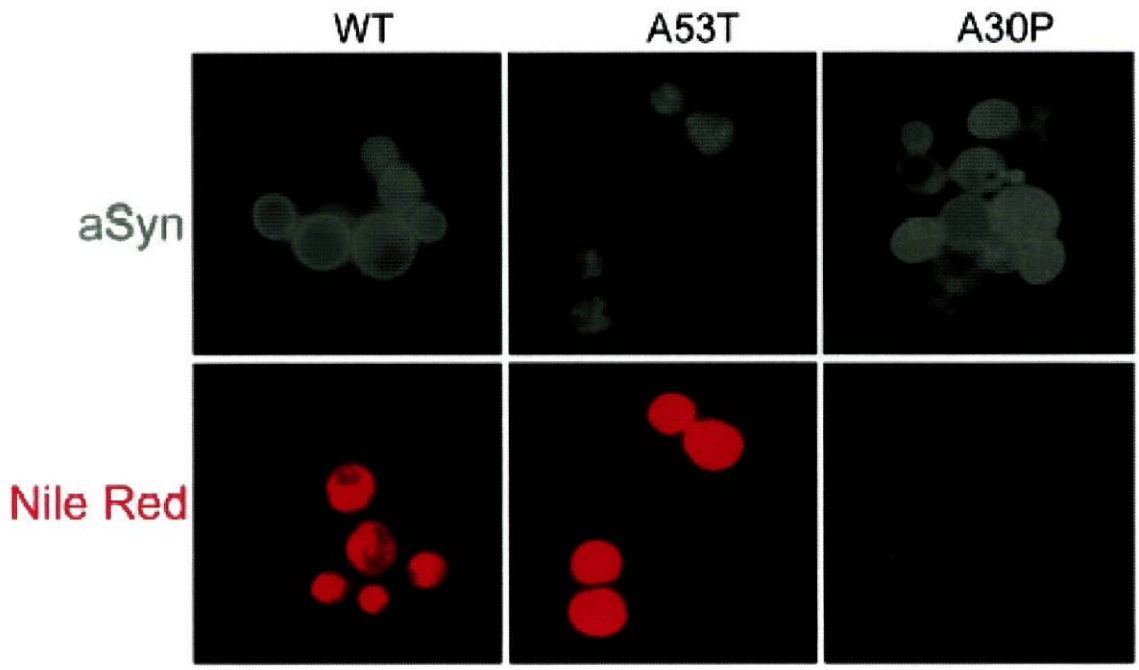


Figure 2.11. Cells expressing αSyn accumulate lipids. Cells expressing either WT or A53T αSyn-GFP (top) were highly reactive for the lipophylic dye Nile Red (bottom); cells expressing A30P were not (*30*).

Finally, we tested αSyn's ability to influence the status of vesicular pools by monitoring internalization of the membrane-binding fluorescent dye FM4-64. As expected, in cells not expressing αSyn FM4-64 was rapidly endocytosed and accumulated at the vacuolar membrane (large ring-like structure; Fig. 2.13, top).

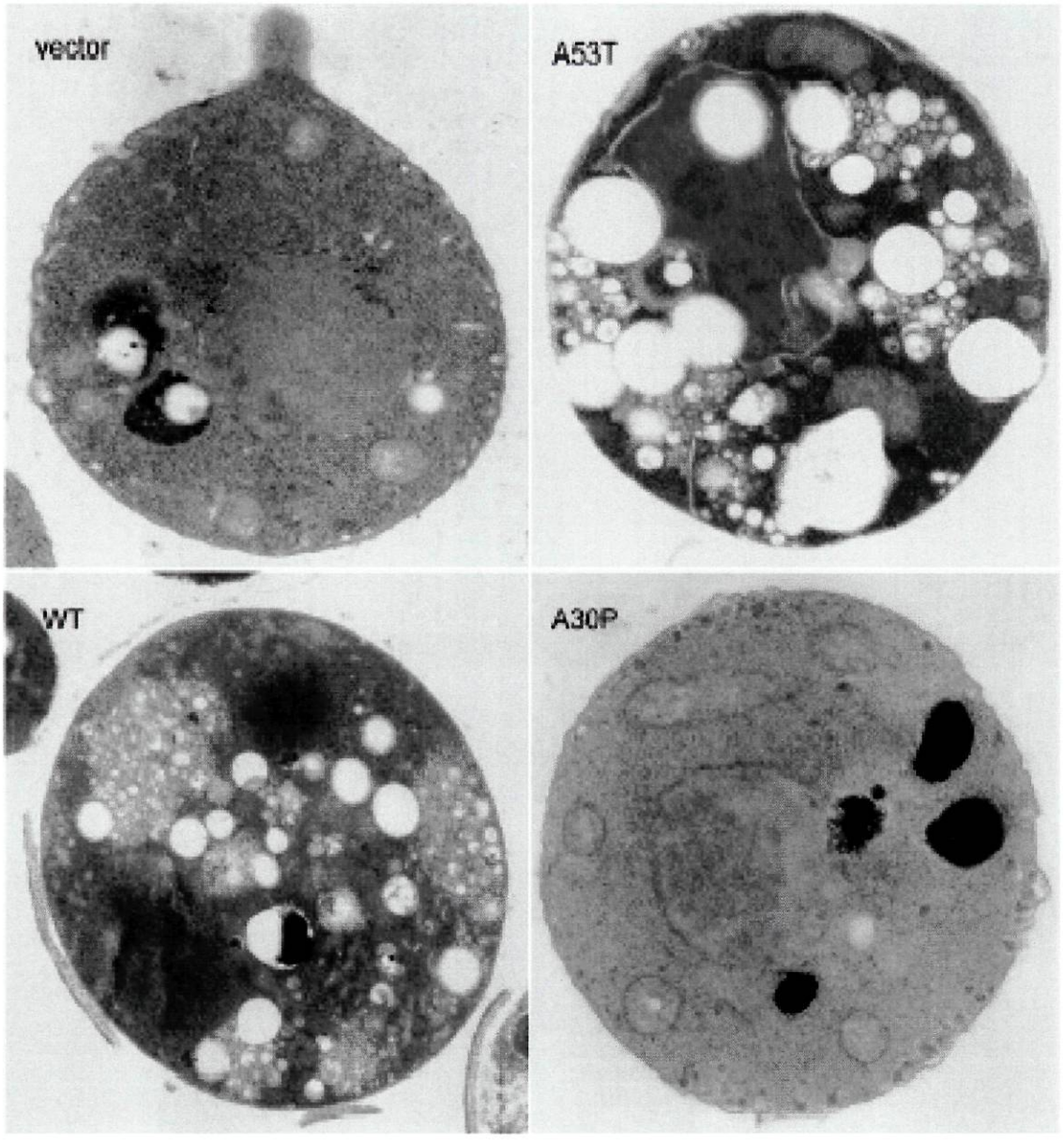


Figure 2.12. Cells expressing αSyn accumulate lipid droplets. Electron microscopy demonstrated the accumulation of lipid droplets (examples indicated by arrow heads) in cells expressing WT or A53T αSyn but not A30P.

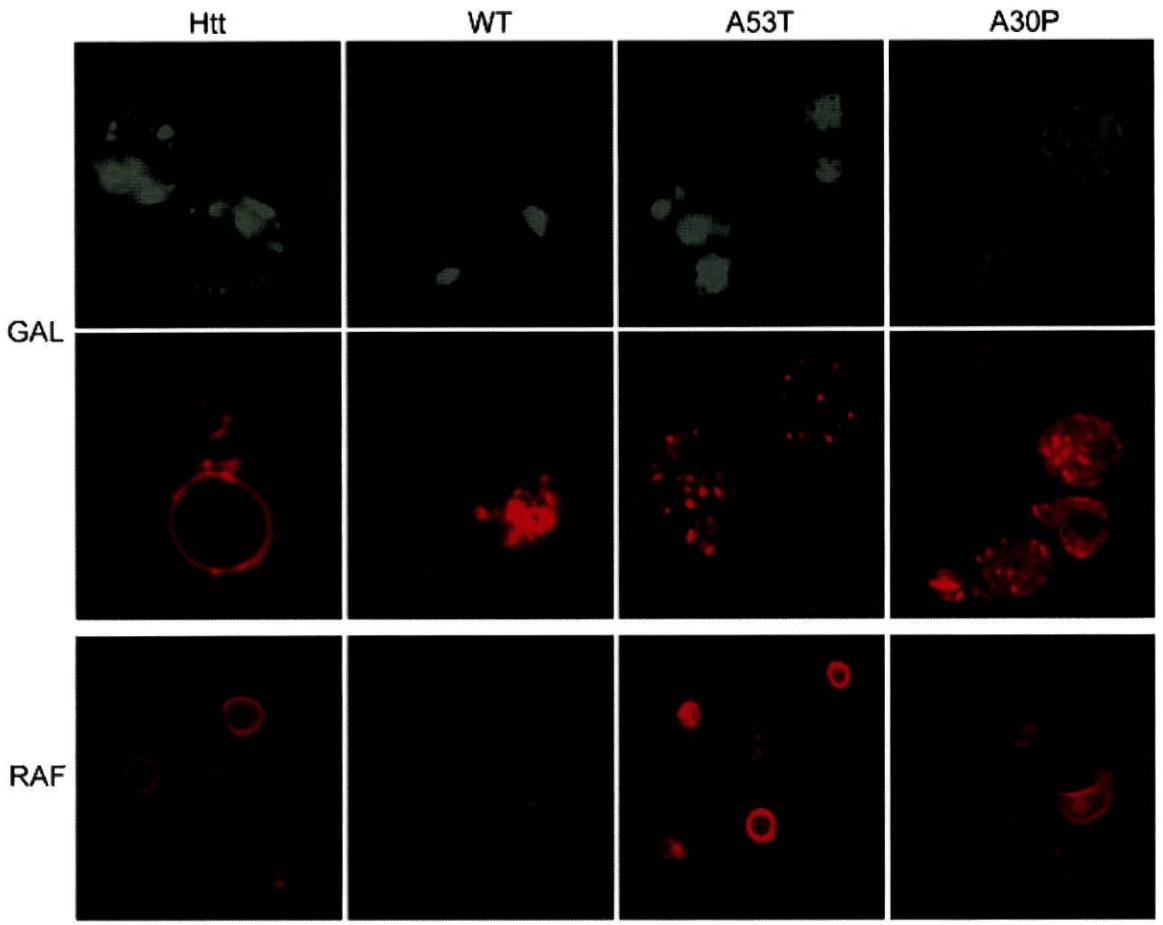


Figure 2.13. αSyn over-expression perturbs the distribution of vesicular pools. Endocytosis of the fluorescent dye FM4-64 (red) was used to monitor the effects of all three αSyn-GFP variants and of Q103 Htt exon 1 (green) on vesicular trafficking. Cells grown in raffinose (uninduced) show normal ring-like vacuolar staining (bottom). Expression of αSyn WT, A53T and A30P dramatically altered FM4-64 distributions (punctate structures in red, middle).

In cells expressing any of the three αSyns (WT, A53T or A30P) FM4-64 distribution was profoundly affected (Fig. 2.13, middle). Again this was not simply the result of protein aggregation or toxicity: the dye accumulated at the vacuolar membrane in cells expressing low, but similarly toxic levels of an aggregation-prone Q103 Htt (Fig. 2.13). Notably, the defect in FM4-64 localization was due neither to PLD inhibition nor to lipid droplet accumulation

because, in contrast with other assays with the same plasmids, A30P affected this phenomenon as strongly as WT and A53T did.

2.4 Conclusions

Expressing αSyn and its mutant derivatives in yeast provides a model for studying normal αSyn function and its misfunction in synucleinopathies. We established that the ability of the protein to inhibit PLD, promote the accumulation of lipids, influence the balance of vesicular pools, associate with membranes in a highly selective manner, induce ubiquitin accumulation, and inhibit the proteasome when misfolded are all intrinsic and biologically relevant properties of the protein that can be uncoupled from each other by the effects of αSyn mutations. Constructs expressing mutant Q103 Htt did not produce similar biological effects and unbiased genetic screens confirmed that distinct pathways are involved in αSyn and Htt toxicities (Willingham, Outeiro et al. 2003). Remarkably, membrane-bound αSyn is in dynamic equilibrium with cytoplasmic forms. Just a two-fold difference in expression was sufficient to cause a catastrophic change in its behavior, inducing nucleated polymerization and recruiting protein previously associated with membranes to cytoplasmic inclusions. This nucleated polymerization process suggests a mechanism by which even small changes in the QC balance of aging neurons could produce a toxic gain of αSyn function concomitantly with a loss of its normal function. Thus, two hypotheses (gain of toxic function or loss of normal function) put forward to explain PD can be reconciled by a single molecular mechanism.

CHAPTER 3

YEAST GENES THAT ENHANCE THE TOXICITY OF A MUTANT HUNTINGTIN FRAGMENT OR α-SYNUCLEIN

CHAPTER 3. YEAST GENES THAT ENHANCE THE TOXICITY OF A MUTANT HUNTINGTIN FRAGMENT OR α-SYNUCLEIN

Abstract

Genome-wide screens were performed in yeast to identify genes that enhance the toxicity of a mutant huntingtin fragment or of α-synuclein. Of 4,850 haploid mutants containing deletions of nonessential genes, 52 were identified that were sensitive to a mutant huntingtin fragment, 86 that were sensitive to α-synuclein and only one mutant that was sensitive to both. Genes that enhanced toxicity of the mutant huntingtin fragment clustered in the functionally related cellular processes of response to stress, protein folding and ubiquitin-dependent protein catabolism, while genes that modified α-synuclein toxicity clustered in the processes of lipid metabolism and vesicle-mediated transport. Genes with human orthologs were over-represented in our screens, suggesting that we may have discovered conserved and non-overlapping sets of cell-autonomous genes and pathways that are relevant to Huntington's disease and Parkinson's disease.

3.1 Introduction

Huntington's disease (HD) is a fatal, inherited neurodegenerative disorder that is characterized by disturbances in movement, cognition and personality. The mutation that causes HD is an expansion of CAG repeats [encoding polyglutamine (polyQ)] in the gene *IT-15* (which encodes the huntingtin protein) (1993). Parkinson's disease (PD) is a major neurodegenerative disorder characterized by muscle rigidity, bradykinesia, resting tremor and postural instability (Goedert 2001). Although the vast majority of cases of PD are idiopathic, a small percentage are caused by missense mutations of the αSyn gene (Polymeropoulos, Lavedan et al. 1997; Kruger, Kuhn et al. 1998). One neuropathological feature shared by HD and PD is the occurrence of ubiquitinated intra-neuronal inclusion bodies in diseased brains. Huntingtin, and/or truncation products of huntingtin, are the major components of cytoplasmic and nuclear inclusion bodies observed in HD, and αSyn is the major component of inclusion bodies (Lewy bodies) in PD. Huntingtin (Scherzinger, Lurz et al. 1997) and αSyn (Rochet, Conway et al. 2000) assemble into fibrillar protein aggregates that display many properties of amyloid in vitro and in vivo. The precise roles of protein aggregation, amyloid formation and inclusion bodies in HD, PD and other amyloid diseases remain controversial, and it is not clear if common pathogenic mechanisms occur in these disorders.

Here, we have used the baker's yeast *Saccharomyces cerevisiae* as a model eukaryotic organism to test the hypothesis that the downstream targets and molecular mechanisms by which a mutant huntingtin fragment and αSyn mediate toxicity are distinct. Similar to neurons, yeast transformed with mutant huntingtin fragments form inclusion bodies in a process regulated by yeast homologs of Hsp40 and Hsp70 (Krobitsch and Lindquist 2000; Muchowski, Schaffar et al. 2000). As in many types of mammalian cells, over-expression of mutant

huntingtin fragments in yeast has no effect on cell viability. This feature allows genetic screens to be performed to identify genes that unmask, or enhance, toxicity. Here, we have used a yeast gene deletion set (YGDS) of 4,850 viable mutant haploid strains (Winzeler, Shoemaker et al. 1999; Giaever, Chu et al. 2002) to identify genes that enhance toxicity of a mutant huntingtin fragment or αSyn.

3.2 Materials and Methods

3.2.1 Yeast screening methods

We obtained the yeast gene deletion set (YGDS) from Research Genetics (Huntsville, AL) as a collection of 4,850 MATa (BY4741) haploid yeast strains frozen in glycerol stocks in 96-well microtiter dishes. These strains were thawed and inoculated individually into yeast extract/peptone/dextrose (YPD) liquid cultures in 96-well microtiter dishes using a Beckman Biomeck 2000 robot, grown to late stationary phase, and then grouped into 4 pools, each containing ~1200 strains. pSAL4-HD53Q (2μ ori) (Muchowski, Ning et al. 2002) or p426GAL-α-syn (2 μ ori) (Outeiro and Lindquist 2003) was used to transform the pooled deletion strains using the Lithium acetate method (1991). For each construct and pool, ~18,000 individual transformants were observed (~15-fold coverage) after plating onto 30 large agar plates that contained synthetic complete media lacking uracil (SC-Ura) to select for the plasmid. This high number of transformants was required to insure that every deletion strain within the pool was transformed and would be screened multiple times. Transformants from each pool were replica plated onto 30 plates containing SC-Ura +copper, or SC-Ura +galactose to induce the expression of HD53Q or αSyn, respectively. Plates were analyzed after 3 days of incubation at 30 ºC. For the HD53Q screen, colonies that grew in the absence of copper but died in its presence (335 colonies out of ~60,000 transformants) were selected for further analysis. Each positive colony was first streaked onto a master plate that contained YPD. From each master plate, at least three independent colonies were re-streaked onto fresh SC-URA -/+ copper plates to identify potential false positives. After re-testing, 68 colonies (20%) remained that demonstrated synthetic sickness or lethality in a reproducible manner. For the αSyn screen, colonies that grew on glucose but died on

galactose (254 colonies out of ~60,000 transformants) were selected for further analysis. After re-testing to identify false positives, 107 colonies (42%) remained that demonstrated synthetic sickness or lethality in a reproducible manner.

3.2.2 Identification of yeast gene deletion strains that are synthetically sick or lethal with HD53Q or αSyn

Colony PCR was used to amplify a DNA sequence that contains a 20 base pair bar code that uniquely identifies each deletion strain. The amplified PCR product was subjected to a DNA sequencing reaction, the results of which were used in a blast search against a database located on the yeast gene deletion web page (http://www.sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) . The results from blast searches give the identities of individual deletion strains. The final number unique gene deletion strains sensitive to HD53Q or αSyn decreased slightly due to several strains being present in multiple copies in the original set of positives (indicating we had saturated our screens), and due to the loss of three strains to copper or galactose sensitivity (see below). All identified strains were streaked out fresh from the original glycerol stocks in 96-well microtiter plates and re-transformed with HD53Q or αSyn for subsequent analysis.

3.2.3 Analysis of viability in yeast gene deletion strains transformed with HD53Q or α-synuclein

Cell viability was determined by serially diluting log-phase cultures onto solid media as described previously (Muchowski, Ning et al. 2002). Viabilities were scored visually according to the growth on solid media. For the HD53Q screen,

two deletion strains out of the original 68 that were isolated (*cup2*Δ and *pmr1*Δ) exhibited copper sensitivity, and were not analyzed further. One strain obtained in the αSyn screen was sensitive to galactose (*gal7*Δ) and likewise was not further analyzed.

3.2.4 Sedimentation analysis of Rnq1p

Total protein extracts were prepared from various *S. cerevisiae* strains and fractionated into soluble and insoluble (pellet) fractions by centrifugation essentially as described by Sondheimer and Lindquist (2000). The resulting protein samples were analysed on 12% SDS-polyacrylamide gels and electrophoretically transferred to PVDF for immunoblotting analysis, employing a polyclonal antibody raised against recombinant *S. cerevisiae* Rnq1p. Anti-rabbit secondary antibody and the ECL reagent (Pharmacia-Amersham) were used to detect bound antibody according to the manufacturer's protocols. Molecular weights of proteins were estimated using pre-stained molecular weight standard markers (BioRad).

3.3 Results

In our model of huntingtin inclusion body formation in *S. cerevisiae* (Muchowski, Schaffar et al. 2000), yeast are transformed with constructs that express exon 1 of the huntingtin gene with a normal (HD20Q) and expanded (HD53Q) polyQ repeat under control of the *CUP1* promoter. The aggregation and inclusion body-forming properties of huntingtin fragments with expanded polyQ tracts can be reproduced faithfully in *S. cerevisiae* (Muchowski, Schaffar et al. 2000). Similarly, over-expression of wild type or mutant (A53T) human αSyn in yeast results in the formation of cytoplasmic inclusion bodies that, at the level of light microscopy, are similar to those formed by mutant huntingtin fragments in yeast (Outeiro and Lindquist 2003).

A collection of 4,850 yeast strains was transformed with constructs that express HD53Q or αSyn under the control of inducible promoters, and was plated onto selective media in the absence of induction. Mutants that were sensitive to a HD53Q or αSyn were isolated by replica plating onto media that contained the appropriate inducer of protein expression. We confirmed putative HD53Q or αSyn -sensitive mutants by re-testing isolated colonies in spotting assays that measure cell viability (Fig. 3.1).

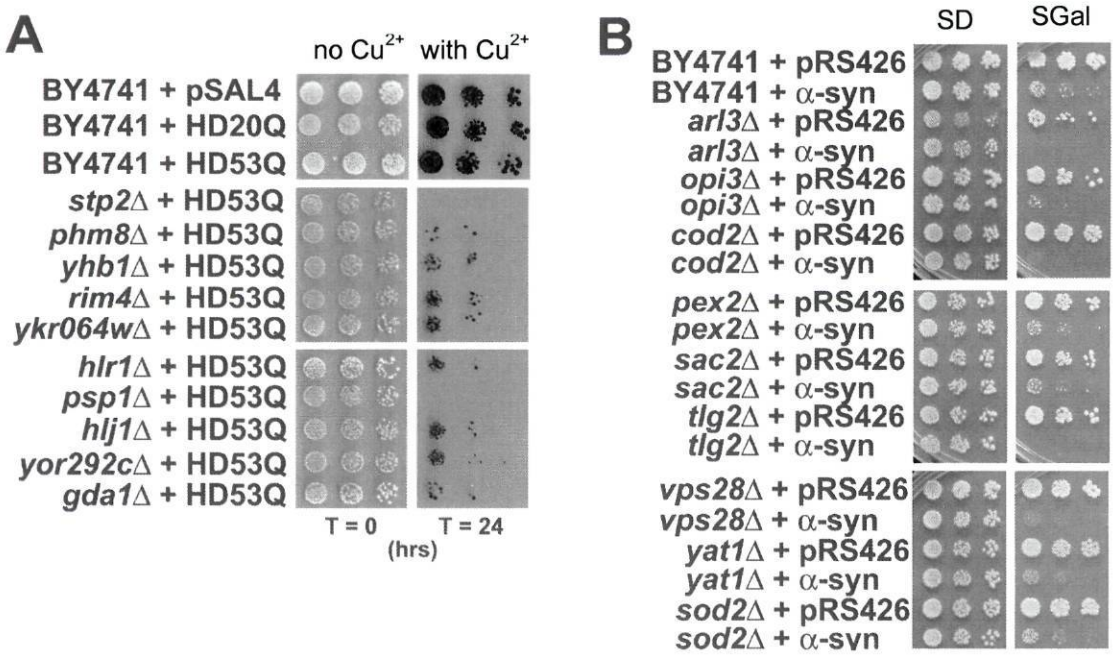


Figure 3.1. Expression of a mutant huntingtin fragment (HD53Q) or αSyn causes synthetic sickness or lethality in yeast gene deletion strains. **(A)** Yeast cells transformed with empty vector (pSAL4), HD20Q or HD53Q were grown in liquid synthetic complete medium lacking uracil (SC-URA) to log-phase and then induced for 24 hours in SC-URA + copper. Aliquots of cells were removed from liquid cultures before (T = 0) and after (T = 24 hrs) copper induction, were spotted on plates containing SC-URA -/+ 400 μM copper, and were incubated at 30 °C for three days. Shown are five-fold serial dilutions starting with equal numbers of cells. Spotting assays derived from single transformants for 10 unique deletion strains and the parental control strain (BY4741) are shown. **(B)** Yeast cells transformed with empty vector (p426GAL) or αSyn (α-syn) were grown in liquid synthetic complete medium lacking uracil (SC-URA + glucose) to log-phase and then induced for six hours in SC-URA + galactose. Aliquots of cells were removed from liquid cultures before (T = 0) and after (T = 6 hrs) galactose induction, were spotted on plates containing SC-URA -/+ galactose, and were incubated at 30 °C for three days. Shown are five-fold serial dilutions starting with equal numbers of cells. Spotting assays derived from single transformants for 9 unique deletion strains and the parental control strain (BY4741) are shown.

Although positive colonies were selected originally because of their complete lack of growth (synthetic lethality) after induction, the re-tests indicated that a sub-lethal effect on toxicity ('synthetic sickness') occurred in many of the deletion strains (Fig. 3.1). Of 4,850 mutants, 52 (~1%) were identified with enhanced toxicity of HD53Q, and 86 (~2%) with enhanced toxicity of wild type αSyn (Tables

3.1 and 3.2). Although over-expression of αSyn caused a modest decrease in cell viability in wild-type yeast (BY4741), all mutant strains that enhanced αSyn toxicity had a phenotype that was more severe than that observed in wild-type yeast (Fig. 3.1B).

Of the HD53Q-sensitive mutants, 77% (40/52) corresponded to genes for which a function or genetic role has been determined experimentally or can be predicted (Table 3.1) (http://genome-www.stanford.edu/Saccharomyces/). 35% (14/40) of these genes clustered in the functionally related categories of response to stress, protein folding and ubiquitin-dependent protein catabolism based on annotations in the Yeast Proteome Database (Fig. 3.2) (https://www.incyte.com/proteome/YPDsearch-long.html). The remaining genes were dispersed among numerous and diverse functional categories (Fig. 3.2). 52% (27/52) of the genes we identified are annotated as having human orthologs (Table 3.1), a value that is significantly higher than the percentage of genes in the yeast genome with mammalian orthologs (~31% based on a P value $\leq 1 \times 10^{-10}$) (Botstein, Chervitz et al. 1997).

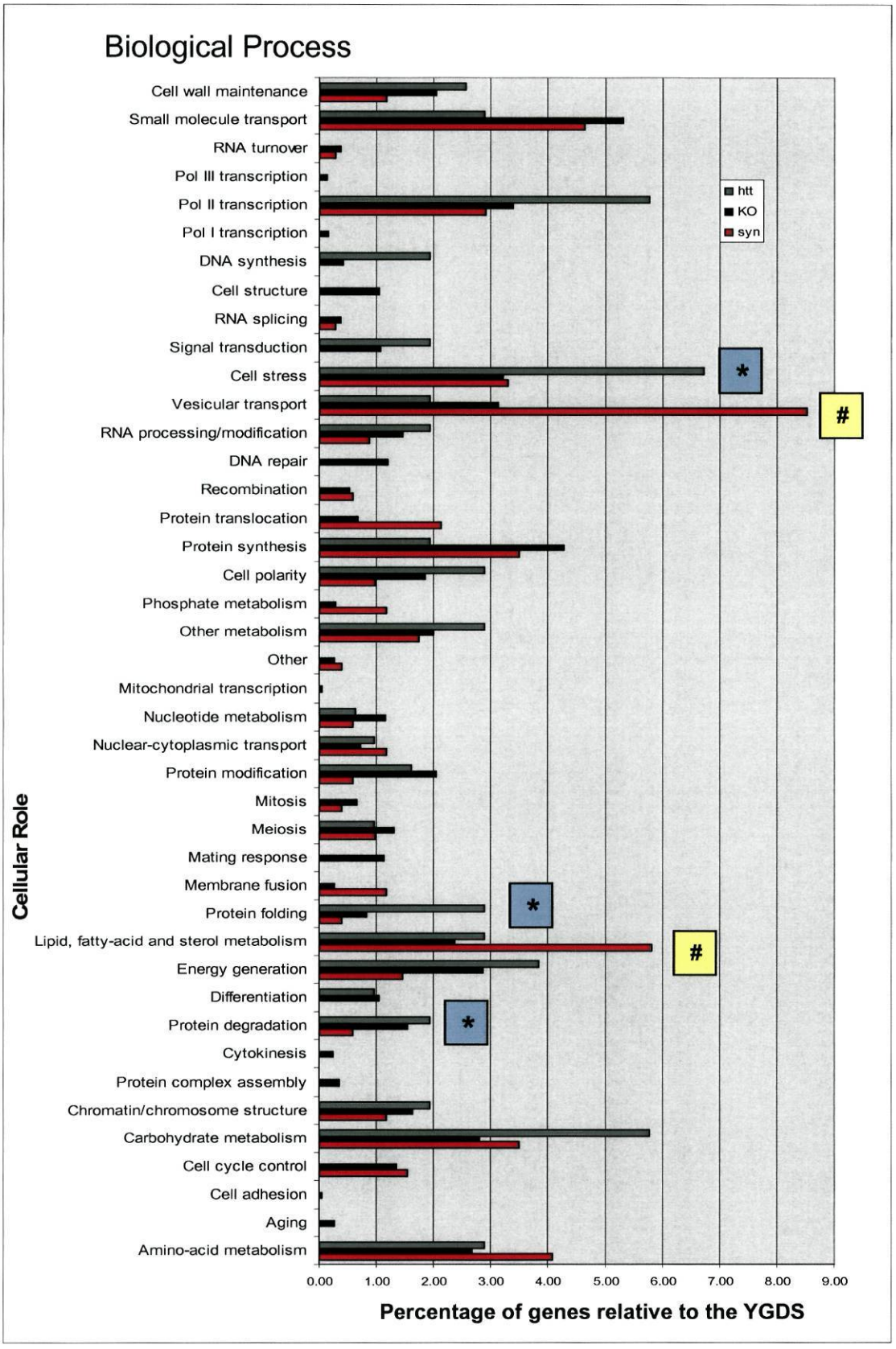

Biological Process
Cell wall maintenance
Small molecule transport
RNA turnover
Pol III transcription
Pol II transcription
Pol I transcription
DNA synthesis
Cell structure
RNA splicing
Signal transduction
Cell stress
Vesicular transport
RNA processing/modification
DNA repair
Recombination
Protein translocation
Protein synthesis
Cell polarity
Phosphate metabolism
Other metabolism
Other
Mitochondrial transcription
Nucleotide metabolism
Nuclear-cytoplasmic transport
Protein modification
Mitosis
Meiosis
Mating response
Membrane fusion
Protein folding
Lipid, fatty-acid and sterol metabolism
Energy generation
Differentiation
Protein degradation
Cytokinesis
Protein complex assembly
Chromatin/chromosome structure
Carbohydrate metabolism
Cell cycle control
Cell adhesion
Aging
Amino-acid metabolism
htt
KO
syn
*
#
*
#
*
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Cellular Role
Percentage of genes relative to the YGDS

Figure 3.2. Comparison of the relative percentage of genes in functional categories for the yeast gene deletion set (YGDS) and the huntingtin/αSyn synthetic lethal screens. 35% (14/40) of genes that enhanced HD53Q toxicity clustered in the functionally related categories of response to stress, protein folding and ubiquitin-dependent protein catabolism (labeled *), while 32% (18/57) of genes that modified αSyn toxicity clustered in the functionally related categories of lipid metabolism and vesicle-mediated transport (labeled #).

Table 3.1. Yeast strains synthetically sick or lethal with HD53Q. The ortholog category indicates yeast genes with human orthologs.

Strain	Ortholog	Function	Strain	Ortholog	Function
1. *apj1Δ*	Yes	Hsp40 chaperone	27. *rim4Δ*	Yes	RNA binding
2. *apm2Δ*	Yes	non-selective vesicle transport	28. *sam2Δ*	Yes	S-adenosylmethionine synthetase 2
3. *ayr1Δ*	Yes	ketoreductase; acylglycerone-phosphate reductase	29. *sas3Δ*	Yes	histone acetyltransferase
4. *cit2Δ*	Yes	citrate synthase, peroxisomal	30. *sdt1Δ*	No	5'-Nucleotidase
5. *cmk1Δ*	Yes	protein histidine kinase	31. *sip18Δ*	No	binds phospholipids
6. *cos111Δ*	No	possibly involved in ubiquitin pathway	32. *sng1Δ*	No	probable transport protein
7. *cps1Δ*	Yes	gly-X carboxypeptidase	33. *stp2Δ*	Yes	transcription factor
8. *dcg1Δ*	No	possibly involved in cell wall biosynthesis	34. *tea1Δ*	No	transcriptional activator
9. *fil1Δ*	No	translation factor	35. *tvp15Δ*	No	possibly involved in vesicular transport
10. *fpr2Δ*	Yes	peptidyl-prolyl cis-trans isomerase	36. *ubp13Δ*	Yes	ubiquitin C-terminal hydrolase
11. *gda1Δ*	Yes	guanosine diphosphatase	37. *vps70Δ*	Yes	possibly involved in vacuolar trafficking
12. *glo2Δ*	Yes	hydroxyacylglutathione hydrolase	38. *yhb1Δ*	Yes	nitric oxide dioxygenase; oxygen transporter
13. *gre2Δ*	Yes	alpha-acetoxy ketone reductase	39. *yrb30Δ*	No	unknown
14. *gsh2Δ*	Yes	glutathione synthase	40. *ybr100wΔ*	No	possibly involved in DNA damage repair
15. *hlj1Δ*	Yes	Hsp40 chaperone in ER	41. *ybr255wΔ*	No	unknown
16. *hlr1Δ*	No	unknown, similar to Lre1 (Pkc1p-MAPK pathway)	42. *ydr215cΔ*	No	unknown
17. *hms1Δ*	Yes	transcription factor	43. *ygr015cΔ*	Yes	alpha or beta hydrolase fold family
18. *ipk1Δ*	No	phosphatidylinositol phosphate kinase	44. *yjr107wΔ*	No	has similarity to acylglycerol lipase
19. *kgd1Δ*	Yes	alpha-ketoglutarate dehydrogenase	45. *ykr017cΔ*	Yes	has a TRIAD composite zinc finger domain
20. *msb1Δ*	Yes	activates Pkc1p-MAPK pathway	46. *ykr064wΔ*	No	transcription factor
21. *mrpl1Δ*	No	protein of the mitochondrial large ribosomal subunit	47. *ylr128wΔ*	Yes	basic helix-loop-helix leucine zipper protein
22. *mup1Δ*	Yes	methionine permease	48. *ymr160wΔ*	No	unknown
23. *pcl6Δ*	No	cyclin-dependent protein kinase	49. *ynl296wΔ*	No	possibly involved in vacuolar trafficking
24. *phm8Δ*	No	possibly involved in phosphate metabolism	50. *yor292cΔ*	Yes	peroxisomal protein
25. *prm5Δ*	No	possibly involved in cell stress	51. *yor300wΔ*	No	bipolar budding and bud site selection
26. *psp1Δ*	No	possibly involved in DNA replication	52. *ypl067cΔ*	No	unknown

We next characterized the effects of HD20Q over-expression in yeast gene deletion strains sensitive to HD53Q. Approximately 77% (40/52) of the deletion strains transformed with HD20Q displayed wild-type viability or exhibited a slight decrease in cell viability (Table 3.3). Although 23% (12/52) of strains transformed with HD20Q displayed a significant loss of viability, in each case the phenotype

was equal to or less severe than that observed with HD53Q (Table 3). HD53Q induced synthetic sickness or lethality observed in yeast gene deletion strains was partially rescued by over-expressing human orthologs (*FKBP2*, *GSS*, *DNAJA2*) of several yeast genes identified in the screen (*FPR2*, *GSH2* and *HLJ1*, respectively) (Fig. 3.3). To determine if a correlation exists between HD53Q aggregation and toxicity in the yeast gene deletion strains, filter-trap assays were conducted on protein lysates from ten strains (Fig. 3.4). No correlation between levels of aggregation and viability was observed in these strains (Fig. 3.4).

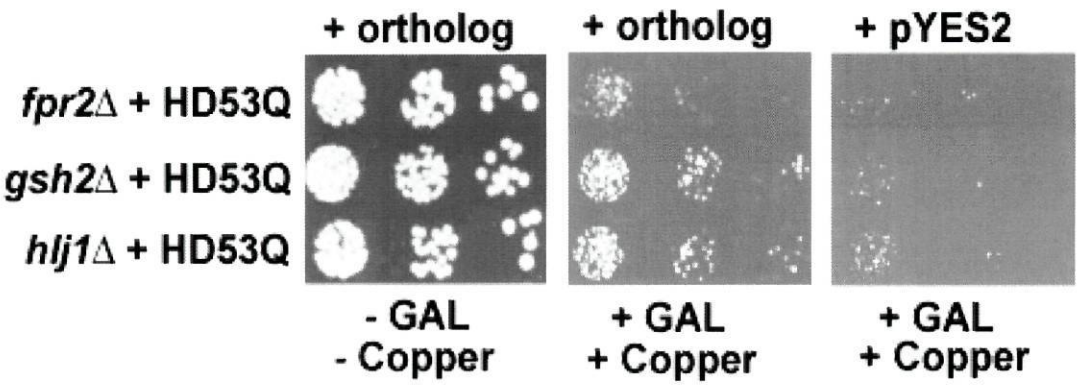


Figure 3.3. Suppression of HD53Q toxicity by human orthologs in yeast gene deletion strains. Yeast deletion strains (*fpr2*, *gsh2*, *hlj1*) were co-transformed with empty vector (pYES2), or vectors that express their respective human orthologs (*FKBP2*, *GSS*, *DNAJA2*) under the control of a galactose-regulated promoter. All cells also contained an expression construct for HD53Q (copper-regulated). Single transformants were streaked on SC-URA-TRP -/+ copper/galactose (GAL). Shown are single transformants for 3 unique deletion strains. The strength of the suppression of toxicity varies depending on the particular human ortholog.

Huntingtin toxicity in yeast has been linked to the presence of a naturally occurring yeast prion called [*RNQ*] (Meriin, Zhang et al. 2002), determined by the protein Rnq1 (Sondheimer and Lindquist 2000). To investigate whether synthetic toxicity was independent of the [*RNQ*] prion we performed sedimentation

analysis of the Rnq1 protein in 6 strains, a method that enables the characterization of the [*RNQ*] status of the cells (Sondheimer and Lindquist 2000). The wild-type strain BY4741 is [*RNQ*$^{+}$], i.e., it contains the [*RNQ*] prion. All strains tested were [*RNQ*$^{+}$] (Fig. 3.5), but synthetic toxicity was independent of the [*RNQ*] prion (data not shown).

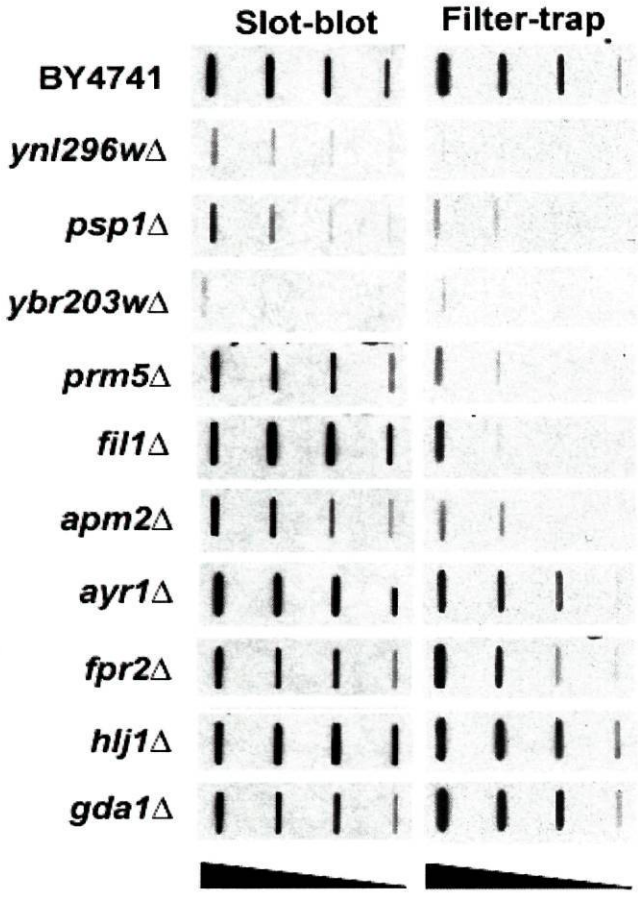


Figure 3.4. Expression and aggregation of HD53Q in yeast gene deletion strains. After six hours of induction with copper at 30 °C, protein lysates were made and analyzed for expression in slot-blots and for SDS-insoluble aggregates in filter-trap assays. Shown are two-fold serial dilutions starting with 10 µg of total lysate. Membranes were probed with an anti-c-myc monoclonal antibody. Viability scores are taken from Table 3.3.

Of the αSyn-sensitive mutants that were identified in the yeast screen, 66% (57/86) corresponded to genes for which a function or genetic role has been determined experimentally or can be predicted (Table 2). 32% (18/57) of these genes clustered in the functionally related categories of lipid metabolism and vesicle-mediated transport (Fig. 3.2). As with the HD53Q screen, the remaining genes in the αSyn screen were distributed among numerous functional categories (Fig. 3.2), and a high percentage of the genes (50% or 43/86) have human orthologs (Table 3.2).

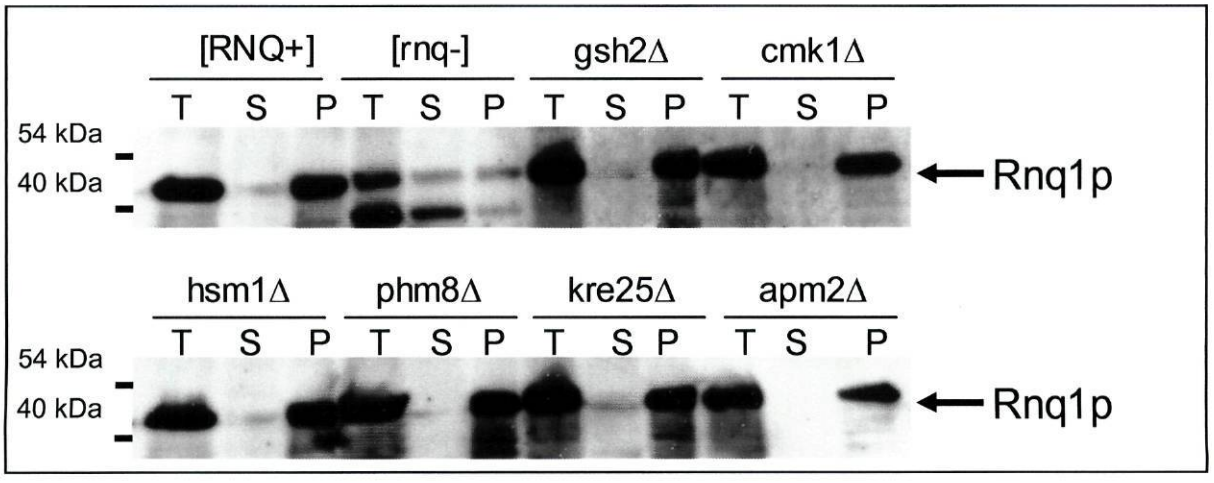


Figure 3.5. [*RNQ*] status in six strains that showed synthetic lethality with HD53Q. Lysates were prepared and centrifuged to separate soluble (S) and pellet (P) fractions. Total fractions (T) were loaded as controls.

Increasing evidence suggests that genes involved in response to stress, protein folding and the ubiquitin-mediated protein catabolism play important roles in HD and the polyQ disorders (Muchowski 2002; Taylor, Hardy et al. 2002). Over-expression of the chaperones Hsp40 and/or Hsp70 in fruitfly and mouse models of polyQ disorders suppresses neurodegeneration, while mutation of genes

involved in ubiquitin-mediated protein catabolism enhance neurodegeneration (Warrick, Chan et al. 1999; Fernandez-Funez, Nino-Rosales et al. 2000; Kazemi-Esfarjani and Benzer 2000; Cummings, Sun et al. 2001).

Table 3.2. Yeast strains synthetically sick or lethal with αSyn. The ortholog category indicates yeast genes with human orthologs.

Strain	Ortholog	Function	Strain	Ortholog	Function
1. *ape2Δ*	Yes	Aminopeptidase	44. *tsl1Δ*	No	alpha,alpha-trehalose-phosphate synthase
2. *arl3Δ*	Yes	ARF small monomeric GTPase activity	45. *ubc8Δ*	Yes	ubiquitin-conjugating enzyme
3. *aro1Δ*	No	arom pentafunctional enzyme	46. *vps24Δ*	Yes	sorts proteins in the pre-vacuolar endosome
4. *cog6Δ*	Yes	involved in vesicular transport to the Golgi	47. *vps28Δ*	Yes	required for traffic to vacuole
5. *crh1Δ*	Yes	cell wall protein	48. *vps60Δ*	No	vacuolar protein sorting
6. *cvt17Δ*	No	lipase	49. *war1Δ*	No	transcription factor
7. *dpp1Δ*	Yes	diacylglycerol pyrophosphate phosphatase	50. *yat1Δ*	Yes	outer carnitine acetyltransferase, mitochondrial
8. *fun26Δ*	Yes	nucleoside transporter	51. *ybr013cΔ*	No	unknown
9. *gip2Δ*	Yes	regulatory subunit for PP1 phosphatase	52. *ybr284wΔ*	Yes	AMP deaminase
10. *glo4Δ*	Yes	hydroxyacylglutathione hydrolase	53. *ybr300cΔ*	No	unknown
11. *gtt1Δ*	No	glutathione transferase	54. *ycl042wΔ*	No	unknown
12. *hbs1Δ*	Yes	~to translation elongation factor EF-1alpha	55. *ycr026cΔ*	Yes	contains type I phosphodiesterase domain
13. *hsp30Δ*	No	heat shock protein for pH homeostasis	56. *ycr050cΔ*	No	unknown
14. *ino4Δ*	No	transcript. factor (phospholipid syn.genes)	57. *ycr051wΔ*	Yes	contains ankyrin (Ank) repeats
15. *mad1Δ*	No	involved in spindle-assembly checkpoint	58. *ycr085wΔ*	No	unknown
16. *mal31Δ*	Yes	maltose transporter	59. *ydl118wΔ*	No	possibly involved in meiotic nuclear division
17. *mei4Δ*	No	required for meiotic recombination	60. *ydr154cΔ*	No	unknown
18. *met17Δ*	Yes	O-acetylhomoserine (thiol)-lyase	61. *ydr220cΔ*	No	unknown
19. *met32Δ*	Yes	transcription factor	62. *yfr035cΔ*	No	unknown
20. *msb3Δ*	Yes	RAB GTPase activator	63. *ygl109wΔ*	No	unknown
21. *nbp2Δ*	Yes	poss. involved in cytoskeletal organization	64. *ygl165cΔ*	No	unknown
22. *nit2Δ*	Yes	nitrilase	65. *ygl226wΔ*	No	unknown
23. *nup53Δ*	Yes	component of nuclear pore complex	66. *ygl231cΔ*	Yes	unknown
24. *opi3Δ*	Yes	phosphatidylethanolamine N-methyltransf.	67. *ygl262wΔ*	No	unknown
25. *pca1Δ*	Yes	P-type copper-transporting ATPase	68. *ygr130cΔ*	Yes	unknown
26. *pex2Δ*	Yes	peroxisomal biogenesis protein	69. *ygr154cΔ*	No	unknown
27. *pex8Δ*	No	peroxisomal biogenesis protein	70. *ygr201cΔ*	Yes	translation elongation factor
28. *pho13Δ*	Yes	4-nitrophenylphosphatase	71. *ygr290wΔ*	No	unknown
29. *pox1Δ*	Yes	acyl-CoA oxidase	72. *yhr199cΔ*	No	unknown
30. *ptk2Δ*	Yes	serine/threonine protein kinase	73. *yjl118wΔ*	No	unknown
31. *rpl41aΔ*	No	structural constituent of ribosome	74. *yjl122wΔ*	No	unknown
32. *rny1Δ*	Yes	endoribonuclease	75. *yjl135wΔ*	No	unknown
33. *sac2Δ*	Yes	involved in protein sorting in the late Golgi	76. *yjr154wΔ*	No	unknown
34. *sap4Δ*	No	serine/threonine phosphatase	77. *ykl098wΔ*	No	unknown
35. *sod2Δ*	Yes	manganese superoxide dismutase	78. *ykl100cΔ*	Yes	unknown
36. *stf1Δ*	No	ATPase inhibitor	79. *ykr023wΔ*	Yes	unknown
37. *stp2Δ*	Yes	transcription factor	80. *ykr035cΔ*	No	unknown
38. *suv3Δ*	Yes	mitochondrial RNA helicase (DEAD box)	81. *ylr365wΔ*	No	unknown
39. *swr1Δ*	Yes	member of Snf2p DNA helicase family	82. *ylr376cΔ*	No	possibly involved in DNA repair
40. *thi7Δ*	No	thiamin transporter	83. *ymr226cΔ*	Yes	oxidoreductase
41. *tlg2Δ*	Yes	syntaxin homolog (t-SNARE)	84. *yml089cΔ*	No	unknown
42. *thr1Δ*	No	homoserine kinase	85. *ymr289wΔ*	No	unknown
43. *tna1Δ*	Yes	nicotinamide mononucleotide permease	86. *ypl136wΔ*	No	unknown

Our yeast screen identified two Hsp40 homologs (Apj1 and Hlj1) that when deleted enhance toxicity of HD53Q. These results indicate that in wild type yeast cells Hsp40 chaperones are necessary to suppress polyQ toxicity. In addition to chaperones, 8 genes (*FPR2*, *GRE2*, *GSH2*, *HLR1*, *PRM5*, *SIP18*, *YHB1*, *YJR107W*) involved in various forms of cellular stress (osmotic, oxidative, nitrosative), and three genes involved in ubiquitin-mediated protein catabolism (*UBP13*, *YBR203W*, *YKR017C*) were identified as enhancers of HD53Q toxicity in yeast.

Table 3.3. Viability scores of yeast deletion strains

Strain wildtype	HD20Q R	HD53Q R	Strain wildtype	HD20Q R	HD53Q R
1. *apj1Δ*	R	S	27. *rim4Δ*	S	SS
2. *apm2Δ*	SS	SS	28. *sam2Δ*	SS	SS
3. *ayr1Δ*	R	SS	29. *sas3Δ*	S	SS
4. *cit2Δ*	R	SS	30. *sdt1Δ*	R	SS
5. *cmk1Δ*	SS	SSS	31. *sip18Δ*	SS	SS
6. *cos111Δ*	SS	SS	32. *sng1Δ*	R	SS
7. *cps1Δ*	S	SS	33. *stp2Δ*	S	SSS
8. *dcg1Δ*	SS	SS	34. *tea1Δ*	R	SSS
9. *fil1Δ*	S	S	35. *tvp15Δ*	R	SSS
10. *fpr2Δ*	S	SS	36. *ubp13Δ*	R	SS
11. *gda1Δ*	SS	SSS	37. *vps70Δ*	S	SS
12. *glo2Δ*	R	SS	38. *yhb1Δ*	R	SS
13. *gre2Δ*	R	SS	39. *yrb30Δ*	S	SSS
14. *gsh2Δ*	S	SS	40. *ybr100wΔ*	R	SS
15. *hlj1Δ*	R	S	41. *ybr255wΔ*	S	SSS
16. *hlr1Δ*	R	SS	42. *ydr215cΔ*	R	SS
17. *hms1Δ*	S	SS	43. *ygr015cΔ*	R	SS
18. *ipk1Δ*	S	SS	44. *yjr107wΔ*	SS	SS
19. *kgd1Δ*	S	SS	45. *ykr017cΔ*	R	SS
20. *msb1Δ*	R	SS	46. *ykr064wΔ*	SS	SS
21. *mrpl1Δ*	R	SS	47. *ylr128wΔ*	R	SSS
22. *mup1Δ*	SS	SS	48. *ymr160wΔ*	SS	SS
23. *pcl6Δ*	S	SS	49. *ynl296wΔ*	SSS	SSS
24. *phm8Δ*	S	S	50. *yor292cΔ*	S	SS
25. *prm5Δ*	R	SS	51. *yor300wΔ*	S	SS
26. *psp1Δ*	S	S	52. *ypl067cΔ*	S	SS

R = resistant (wild type growth)
S = sensitive (partial decrease in cell viability)
SS = super-sensitive (significant decrease in cell viability)
SSS = No viable cells (complete lethality)

Several genes in the functional categories of response to stress and ubiquitin-mediated protein catabolism were also isolated in the αSyn screen (*SOD2*, *GTT1*, *HSP30*, *TSL1*, *UBC8*) (Table 3.2). However, in contrast to the HD53Q screen, the levels of genes in these categories were not increased above background (Fig. 3.2). A recent study demonstrated that Hsp70 over-expression rescues αSyn induced neurodegeneration in a *Drosophila* model for PD (Auluck, Chan et al. 2002). While significant genetic, cell biological and biochemical evidence suggests that genes involved in response to stress, protein folding and ubiquitin-mediated protein catabolism play important roles in the pathobiology of PD, the results from the αSyn yeast screen indicate that genes involved in lipid metabolism and vesicle-mediated transport may also be primary pathways that regulate toxicity of αSyn.

αSyn localizes to nerve terminals and may be associated with synaptic vesicles, based on immunohistochemistry and ultrastructural analyses (Clayton and George 1999). αSyn binds lipid membranes (Davidson, Jonas et al. 1998; Jensen, Nielsen et al. 1998; Perrin, Woods et al. 2000) and can inhibit phospholipase D2 in vitro (Jenco, Rawlingson et al. 1998). αSyn interacts with synphilin-1 (Engelender, Kaminsky et al. 1999), which has been proposed to function as an adaptor protein linking αSyn to proteins involved in vesicular transport. Although the precise function of αSyn is still not clear, this protein has been linked to learning, development and plasticity (George, Jin et al. 1995), and most likely plays a role in synaptic vesicle recycling. Recent in vitro studies suggest that pre-fibrillar intermediates called protofibrils formed by αSyn can bind and permeabilize acidic phospholipid vesicles (Volles, Lee et al. 2001), which has been proposed to lead to defective sequestration of dopamine into vesicles and subsequent generation of reactive oxygen species in the cytoplasm that contribute to neuronal dysfunction and cell death (Lotharius and Brundin 2002). Taken together, these results are consistent with those from our αSyn genetic

screen, and with studies examining the biological and pathobiological effects of αSyn in yeast. αSyn, and not a mutant huntingtin fragment, localized to membranes, caused the accumulation of lipid droplets, and inhibited phospholipase D and vesicular trafficking (Outeiro and Lindquist 2003). The results from the yeast screen are also consistent with recent expression profiling studies in *Drosophila* that over-express αSyn, which showed that lipid and membrane transport mRNAs were tightly associated with αSyn expression (Scherzer, Jensen et al. 2003).

3.4 Conclusions

The results from yeast screens clearly indicate that toxicity mediated by αSyn and a mutant huntingtin fragment is regulated by non-overlapping sets of conserved genes and pathways. The major functional categories enriched in the αSyn genetic screen did not overlap with any of the major categories observed in the HD53Q screen, and only 1/138 genes that enhanced toxicity was found in common to both screens (*STP2*). Collectively, these results suggest that distinct pathogenic mechanisms may underlie HD and PD.

CHAPTER 4

DRUG SCREENING USING YEAST MODELS OF NEURODEGENERATIVE DISEASE

CHAPTER 4. DRUG SCREENING USING YEAST MODELS OF NEURODEGENERATIVE DISEASE

Abstract

Screening collections of small molecules for biological activity, originally developed as a drug discovery tool, has proven to be a powerful approach to understanding biological mechanisms. We utilized two recently developed yeast models of neurodegenerative disease for screening a collection of 1040 compounds. A consortium of 27 laboratories screened the same collection of compounds in other models of disease mechanisms such as protein aggregation, apoptosis and excitotoxicity. The goals of this effort were to investigate relationships among neurodegeneration mechanisms and to identify potential therapies. Our data link at least two clinically approved drug classes to neurodegeneration. Cephalosporin antibiotics and cardiac glycosides affected predominantly ALS-related models. Other bioactive molecules, cannabinoids and celastrol, conferred protection from polyglutamine toxicity. Celastrol's protective activity was linked to heat shock protein levels. Interestingly, we find no compounds active in all assays, as might be expected if a common neurodegeneration pathway exists. The results of these screens provide new perspectives on relatedness of mechanisms, valuable research tools, and promising therapeutic leads for neurodegenerative diseases.

4.1 Introduction

The identification of compounds for the development of therapeutics for numerous so far untreatable neurodegenerative disorders like Huntington's Disease (HD), Parkinson's Disease (PD), Alzheimer's disease (AD), Amyotrophic Lateral Sclerosis (ALS), etc. is of utmost importance. These are devastating conditions, socially disruptive and extremely costly for society, and therefore justify the tremendous efforts being made to address prevention and therapeutics. While these disorders manifest through distinct clinical symptoms, their molecular basis seem to share numerous similarities. They are generally called 'protein misfolding diseases' because the misfolding of one or more proteins is associated with their pathological hallmarks – proteinaceous inclusions. When the cellular quality control mechanisms fail or become overwhelmed, protein misfolding exerts its toxic effects on cells, eventually leading to disease (Wickner, Maurizi et al. 1999). Despite the ultimate lack of knowledge about the toxic species in each disorder, it is generally accepted that intervention might be possible at different levels in the reaction path (Fig. 4.1). It is therefore extremely important to combine *in vitro* and *in vivo* assay systems in order to zoom in on the relevant molecular targets. Each system has its strengths and limitations, therefore a combination of the two types of assays is likely to yield complimentary information.

Currently described model systems for these diseases provide invaluable direct information on neurodegenerative processes, but their manipulation is slow and cumbersome compared to the simple model system provided by yeast, described in the previous chapters. Yeast models offer unparalleled opportunities in terms of genetic screens, but also in terms of chemical genetics.

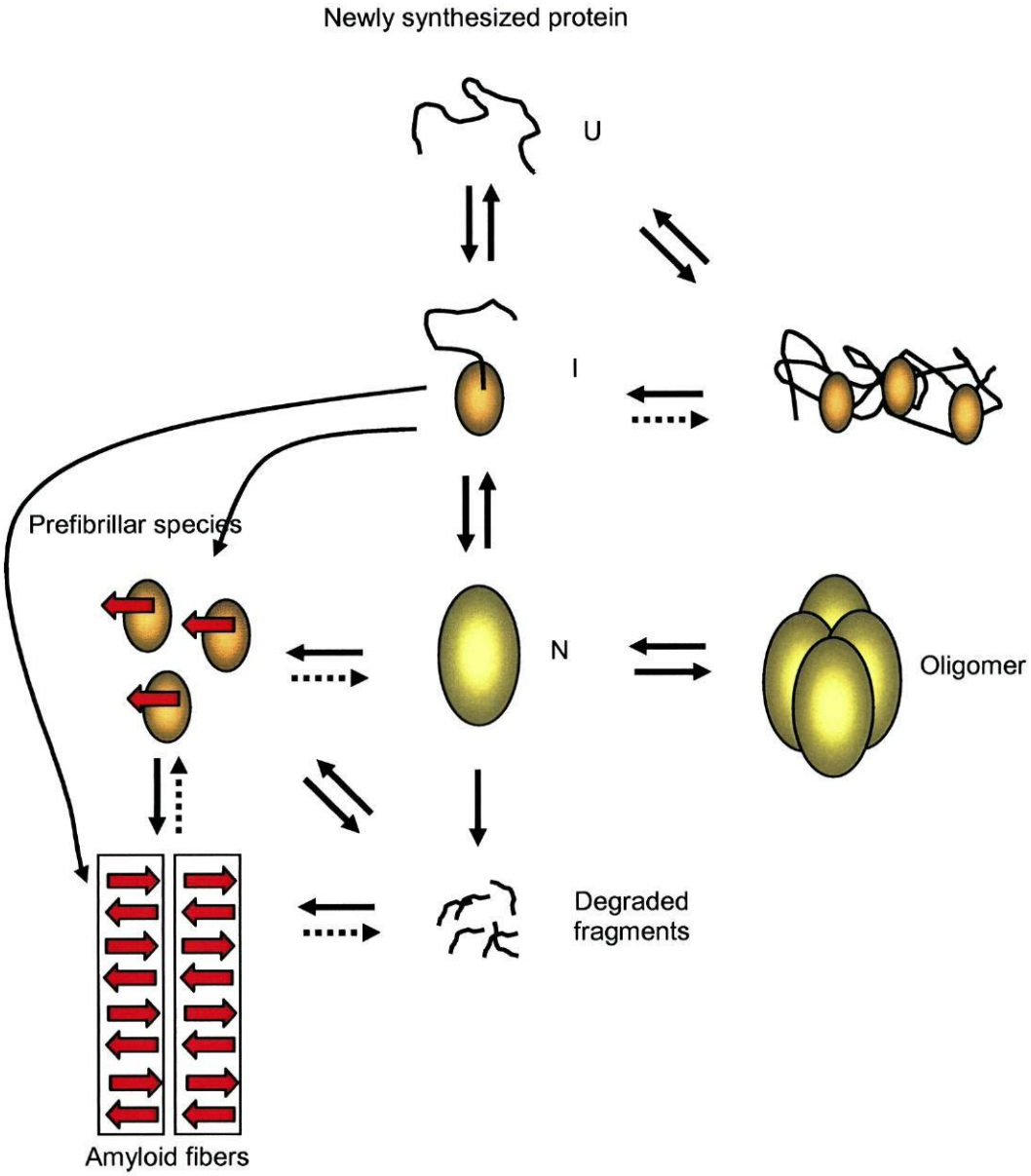


Figure 4.1. Schematic representation of the folding/unfolding/misfolding reactions. Once proteins are synthesized they face a wide variety of challenges in the crowded environment of the cell. Throughout their lives, proteins need to change their conformations, allowing unwanted species to accumulate, which may lead to cytotoxicity. Therapeutic intervention should be possible at different levels along these pathways. U, unfolded; I, intermediate; N, native.

Some of the major advantages of yeast systems are:

a. Screens are much faster, simpler and more direct than in other model systems.
b. Genetic manipulations in yeast are easy to perform.
c. A wide variety of heterologous protein expression systems available facilitate the investigations of protein-protein interactions.
d. Mutations have been identified that greatly enhance drug sensitivity, facilitating the search for therapeutic agents.

Yeast cells have been used in anti-cancer drug screens by the National Cancer Institute (NCI, http://dtp.nci.nih.gov/yacds/index.html), and have proved extremely useful for the identification of novel drug targets.
However, yeast cells also present a major disadvantage: the presence of a rigid cell wall and an efficient membrane efflux pump system composed of several ATP binding cassette transporters (ABC) involved in pleiotropic drug resistance (PDR), which is analogous to the mammalian multiple drug resistance (MDR). A highly complex network of transcriptional regulators controls the expression of these ABC drug efflux pumps (Balzi and Goffeau 1995; Decottignies and Goffeau 1997). These transporters are involved in pleiotropic drug resistance and cellular detoxification, also maintaining low intracellular concentrations of xenobiotics (Kolaczkowski, Kolaczowska et al. 1998). Pdr1p and Pdr3p are closely related transcription factors whose deletion aggravates drug hypersensitivity (Egner and Kuchler 1996). Two approaches can be used to attenuate the problems with permeability and drug resistance: i) introduction of mutations in the efflux pump system itself (transcription factors and ABC transporters) or ii) introduction of mutations in genes involved in ergosterol biosynthesis, an important lipid for membrane stability in yeast.

In this chapter I will describe two drug screens performed using the yeast model for PD, described in chapter 2, and a model for HD (Meriin, Zhang et al. 2002).

4.1.1 Yeast Model for α-Synucleinopathies

The yeast system used for the α–synuclein (αSyn) drug screen consists of cells in which two copies of a galactose-inducible αSyn-GFP fusion are integrated in the genome, resulting in a level of expression that inhibits growth (Fig. 4.2).

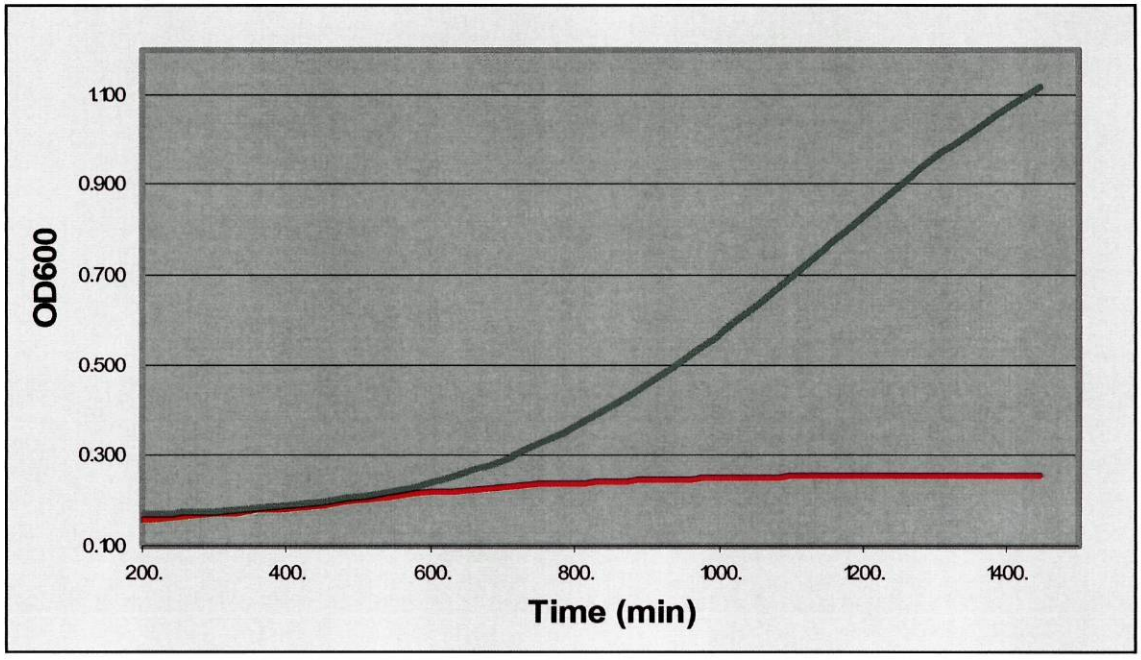


Figure 4.2. Growth inhibition of cells expressing 2 copies of αSyn. Growth curves were generated by measuring OD_{600} of cells growing in SGal medium at 30°C. **Red**, cells expressing αSyn. **Green**, cells carrying empty vectors.

4.1.2 Yeast Model for Huntington's Disease (HD)

Several yeast models for polyglutamine (polyQ) disorders have been developed in recent years (Krobitsch and Lindquist 2000; Muchowski, Schaffar et al. 2000; Hughes, Lo et al. 2001). These models show polyQ-length dependent protein aggregation and insolubility, major characteristics of HD (DiFiglia 1997; Scherzinger, Lurz et al. 1997; Bates, Mangiarini et al. 1998) and other polyQ diseases, but lack polyQ-length dependent toxicity. More recently, Meriin and colleagues described a yeast system in which the length of the polyQ region is associated with aggregation and also with toxicity (Meriin, Zhang et al. 2002). In this system, a version of exon 1 of the huntingtin protein was N-terminally FLAG-tagged and fused to the N-terminus of the green fluorescent protein (GFP) (Fig. 4.3).



Figure 4.3. Schematic representation of the huntingtin construct used to generate the toxicity phenotype used for screening.

Expression of this fragment of the huntingtin protein results in inhibition of growth of yeast cells in a polyQ-length dependent manner. For stable expression, this construct was integrated in the yeast genome under the regulation of a galactose-inducible promoter, enabling routine passaging of the cells without the toxic effects resulting from the expression of the protein.

Cells expressing a 'normal' (not associated with disease) version of this construct (with 25 glutamines) grow just as well as cells carrying empty vectors. Aggregation (Fig. 4.4) and toxicity (Fig. 4.5) increase with increasing the length of the glutamine stretch. This toxicity phenotype (Fig. 4.5) was used for the drug screen.

Drug screens based on the ability of compounds to restore growth present an added advantage, compared to other screens where growth inhibition is the target phenotype, which is the preferential identification of compounds that reduce toxicity without being themselves toxic. Cytotoxic compounds and/or cytotoxic concentrations of toxic compounds are therefore self-eliminated.

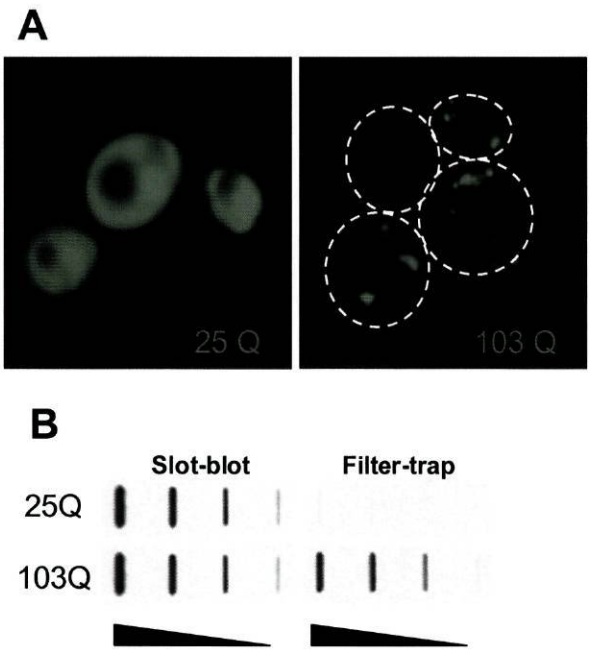


Figure 4.4. PolyQ-length dependent aggregation of exon-1 of the huntingtin protein. **A**, Huntingtin with 103Q forms inclusions observable with epifluorescence microscopy, through a GFP fusion. **B**, Filter-trap and slot-blot assays demonstrating the formation of aggregates that are retained on the membrane upo
n filtration.

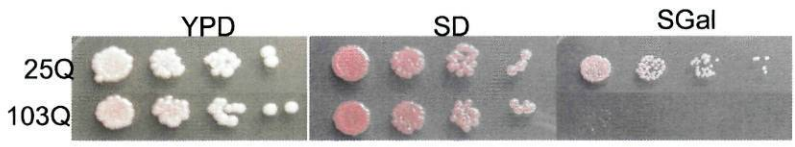


Figure 4.5. PolyQ-length dependent toxicity in yeast. Turning on the expression of the huntingtin constructs (SGal medium) results in growth inhibition in cells expressing the exon 1 of huntingtin with 103 Qs but not 25 Qs.

4.2 Materials and Methods

4.2.1 One-Step Gene Deletions

We targeted *PDR1*, *PDR3* and *ERG6* with the kanMX selection marker, a hybrid gene expressing the bacterial aminoglycoside phosphotransferase under the control of a fungal promoter (Wach, Brachat et al. 1994). This gene confers resistance to the antibiotic geneticin (G418).

The knockout (KO) strategy employed is presented in Fig. 4.6. In short, oligonucleotides A and D (Table 4.1) for each of the genes pdr1, pdr3 and erg6 were used to amplify the KO cassette from yeast strains where these same genes had been knocked out (Yeast Gene Deletion Set – YGDS). This strategy ensured a longer region of homology (~200 bp) for the gene targeting, increasing the efficiency of the homologous recombination event necessary to KO the genes of interest.

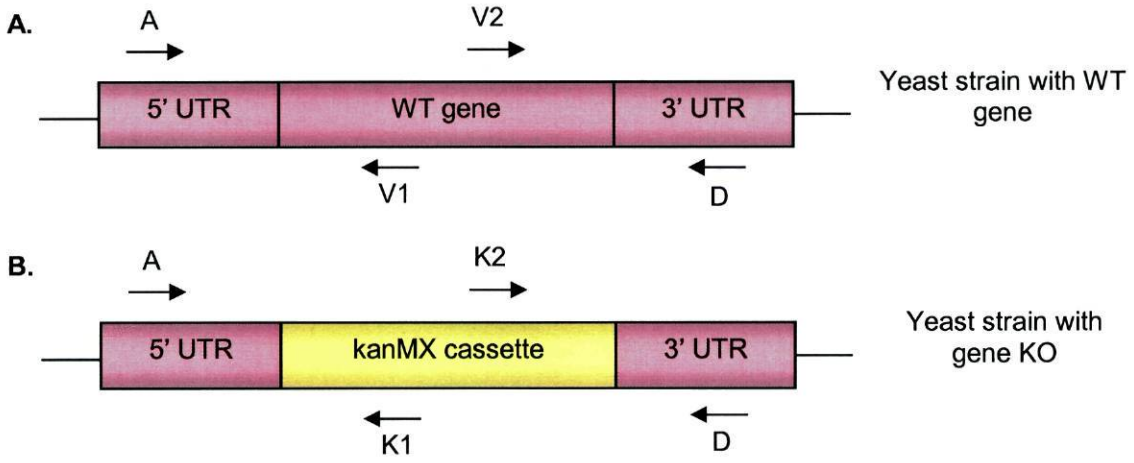


Figure 4.6. Schematic representation of the strategy used for the gene deletions. **A**, in a strain with a WT gene, oligo pairs A+V1 and V2+D will yield amplication products, indicative of the WT gene. **B**, a strain carrying a gene deletion with the kanMX cassette can be detected by the amplification products with oligo pairs A+K1 and K2+D.

Table 4.1. Oligonucleotides used for amplification of gene disruption modules and for verification of disruptions.

Gene	Oligo	Sequence
pdr1	A	5'ACCCTAAATGGAGTTTCTTTTCTTG3'
	V1	5'CACTTCCGCTATTATCACCACTACT'
	V2	5'GTCTGATATGATCAATCCCGATTAC3'
	D	5'CCCATAAGAAATACACCTCATGGTA3'
pdr3	A	5'TTCACTTTTCATTTTCTTCCAGAAC3'
	V1	5'CCAGTTGATTTTGATATTTGGAATC3'
	V2	5'AAGCCAAATTTCTCAGTTGTATGAC3'
	D	5'AATATCTACTGAACAGCTGCATTCC3'
erg6	A	5'CTGTTGCCGATAACTTCTTCATTGC3'
	V1	5'TATCGGTTCTACCATCCCAATTTCTCA3'
	V2	5'TACGTTCAAAACTTAGCTAATTTGGCCA3'
	D	5'GGCCTGCTAGCAATGAACGTGCTA3'
kanMX	K1	5'CTGCAGCGAGGAGCCGTAAT3'
	K2	5'TGATTTTGATGACGAGCGTAAT3'

4.2.2 Yeast Strains, Manipulations and Media

Knockouts were performed according to standard procedures (1998) in the haploid strains listed in table 2 and verified by PCR with the oligo-pairs A+V1 or A+K1 and V2+D or K2+D (Fig. 4.6).
Geneticin plates for selection of successful KOs consisted of YPD medium [1% yeast extract (w/v), 2% bacto-peptone (w/v), 2% glucose (w/v)], 2% agar (w/v) supplemented with 200 μg/ml of geneticin (added after autoclaving).

Strains were crossed to generate diploids, which were then grown on sporulation medium [0.25% yeast extract (w/v), 1% potassium acetate (w/v), 0.05% glucose (w/v)], 2% agar (w/v) for 5 days at 30°C. Tetrads were dissected using a Singer microdissection microscope and the segregation of the markers was followed by passaging on geneticin plates and/or selective medium.

Table 4.2. Yeast strains used in this study.

Strain	Genotype
W303 1-A	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*
W303 1-B	*MAT α can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*
pdr1 Δ	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 pdr1::kanMX*
pdr3 Δ	*MAT α can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 pdr3::kanMX*
erg6 Δ	*MAT α can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 erg6::kanMX*
pdr1 Δ pdr3 Δ	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 pdr1::kanMX pdr3::kanMX*
pdr1 Δ erg6 Δ	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 pdr1::kanMX erg6::kanMX*

4.2.3 Drug Library

We used a library composed of FDA approved drugs and known bioactives, assembled by NINDS and Microsource. This library contains 1040 drugs in DMSO at a concentration of ~10 mM for most drugs.

4.2.4 Screening Strategy

The strategy we used for these screens was to first test all drugs with parental yeast strains at two different concentrations (5 μM and 25 μM), to define the range that would be used for the actual screens. This stage was called 'stage 0' (Fig. 4.7).

The αSyn screen was initially performed at 12.5 μM, and was subsequently repeated at 0.5 μM, 2.5 μM and 62.5 μM. This stage was called 'stage 1' (Fig. 4.7).

The huntingtin screen was performed at 31.25 μM (stage 1, Fig. 4.7).

'Stage 2' would consist in the determination of EC_{50} for each of the hits, by performing dilution series of each compound.

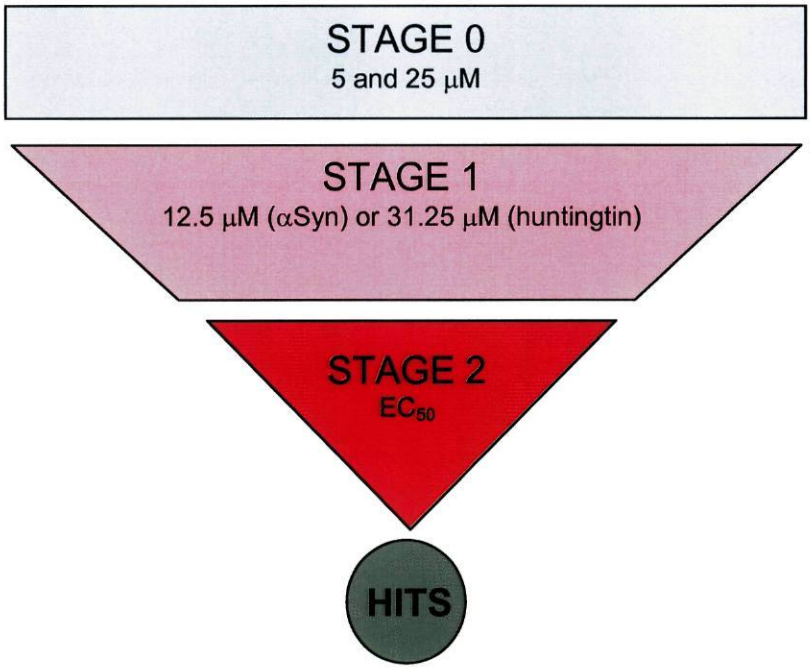


Figure 4.7. The different stages of the drug screens.

4.2.5 Screening Procedures

Yeast cells containing either two copies of αSyn or the toxic huntingtin construct were grown overnight in synthetic medium containing raffinose as the carbon source. Raffinose derepresses the metabolic pathways necessary for utilization of galactose, enabling a stronger induction from the galactose-inducible promoter when cells are shifted to this latter carbon source. Cells were then diluted to an OD_{600} of 0.05 in synthetic medium containing galactose as the sole carbon source.

Synthetic medium containing galactose (350 μl) was dispensed into each well of 100 well honeycomb plates. 40 μl of the diluted cell suspension were then added to each plate. Finally, 10 μl of each drug were dispensed onto each well. The original drug plates were replicated at four different concentrations to enable the use of the same assay setup throughout the screens. Liquid handling was performed by a Multiprobe II liquid handling system from Packard Instruments.

We employed the Bioscreen C plate reader system (Fig. 4.8), from Thermo Labsystems, to monitor the growth of cells over a period of 12 hours. This instrument maintains a constant temperature of 30°C and agitates the plates every 60 seconds. OD_{600} was registered every 30 minutes.

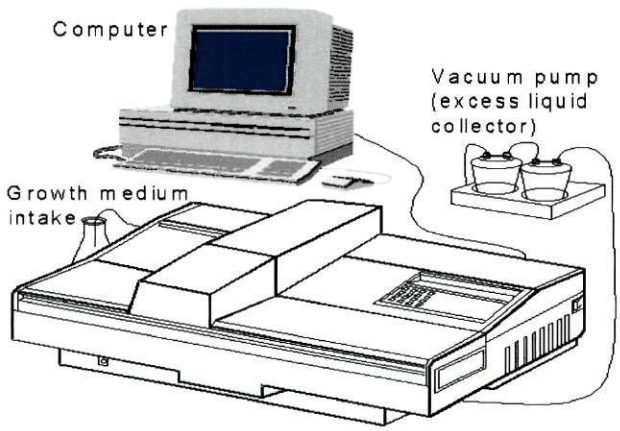


Figure 4.8. Bioscreen-C system for monitoring microbial growth. The plate reader is connected to a computer that registers growth over time.

4.3 Results

One difficulty associated with any drug screen is the identification of the concentration range that is likely to enable the identification of real hits. Using low concentrations of drugs may result in high rates of false negative results, whereas using high concentrations may result in cytotoxicity and therefore also lead to false negative results. Cell-based screens present an additional challenge in comparison to biochemical screens: drugs must enter the cell and must be maintained at high-enough concentrations in the cellular compartment where the target is located.

To identify a concentration range that would maximize the number of hits in these screens (stage 0), we performed a preliminary screen with the parental yeast strain (pdr1Δ pdr3Δ, not expressing αSyn or huntingtin), at two different concentrations (5 and 25 μM). This enabled us to define the concentration range to be used. On stage 0, our goal was to identify a concentration range that would yield ~1 to 2 % of hits, based on the toxicity of the compounds, which would at least give us an indication the compounds were having an effect on the cells.

4.3.1 αSyn Screen

We next performed stage 1 of the αSyn screen, testing the drugs at four different concentrations.

Fig. 4.9A illustrates the reproducibility of the assay for a particular assay plate. Only one compound out of the 1040 tested yielded a false positive (Fig. 4.9B). In this case, we were not able to reproduce the result upon retesting.

After statistical analysis of the results, a ranked activity matrix was generated. Compounds were ranked according to their potencies, from the most potent to

the least potent compound (examples of the most active compounds are shown in Fig. 4.10).

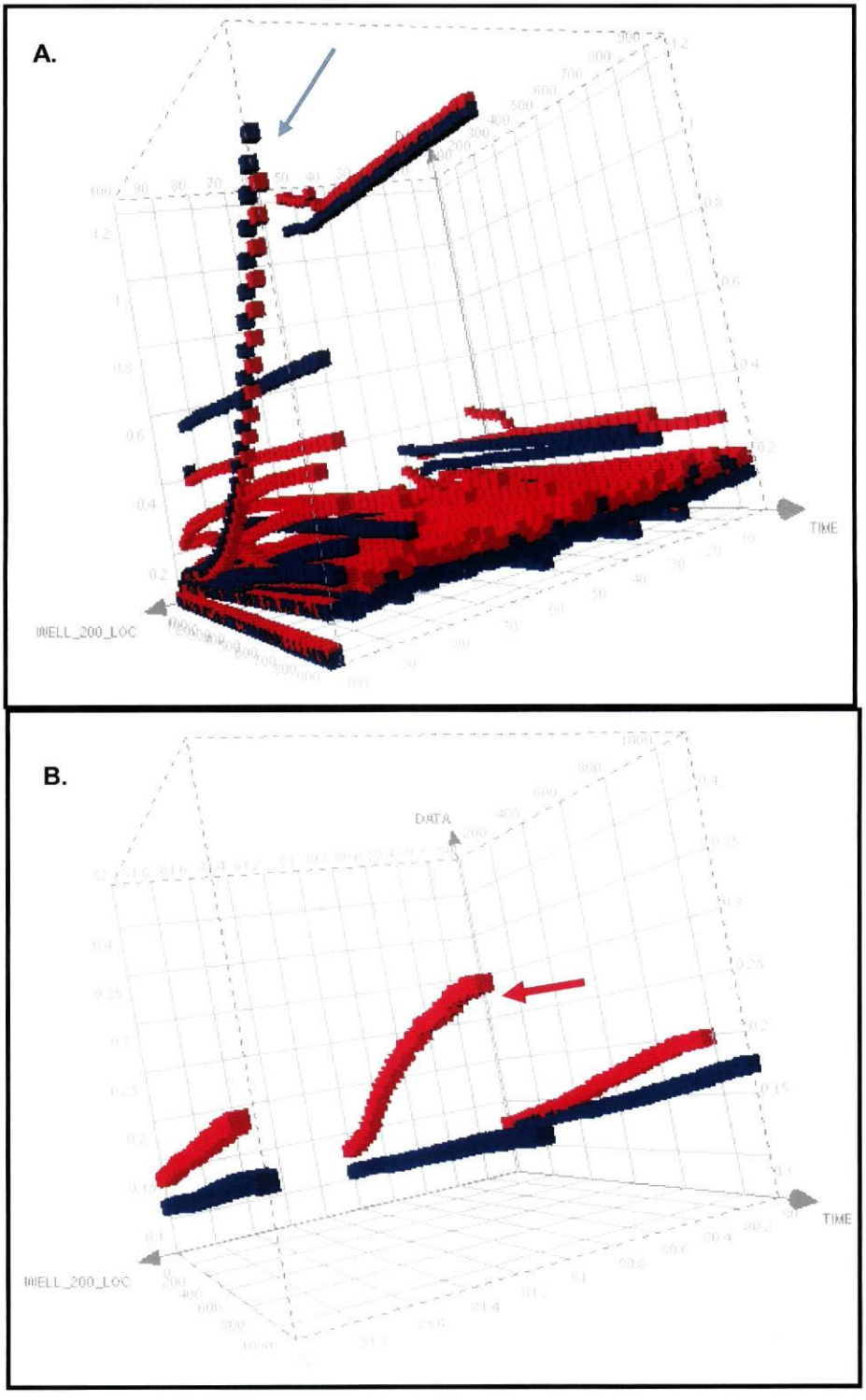


Figure 4.9. Growth of αSyn-expressing cells in the presence of drugs. **A**, growth curves with a set of 96 drugs, ran in duplicate (blue and red). Arrow points to positive controls. **B**, a drug that showed a distinct effect on the αSyn assay (red arrow).

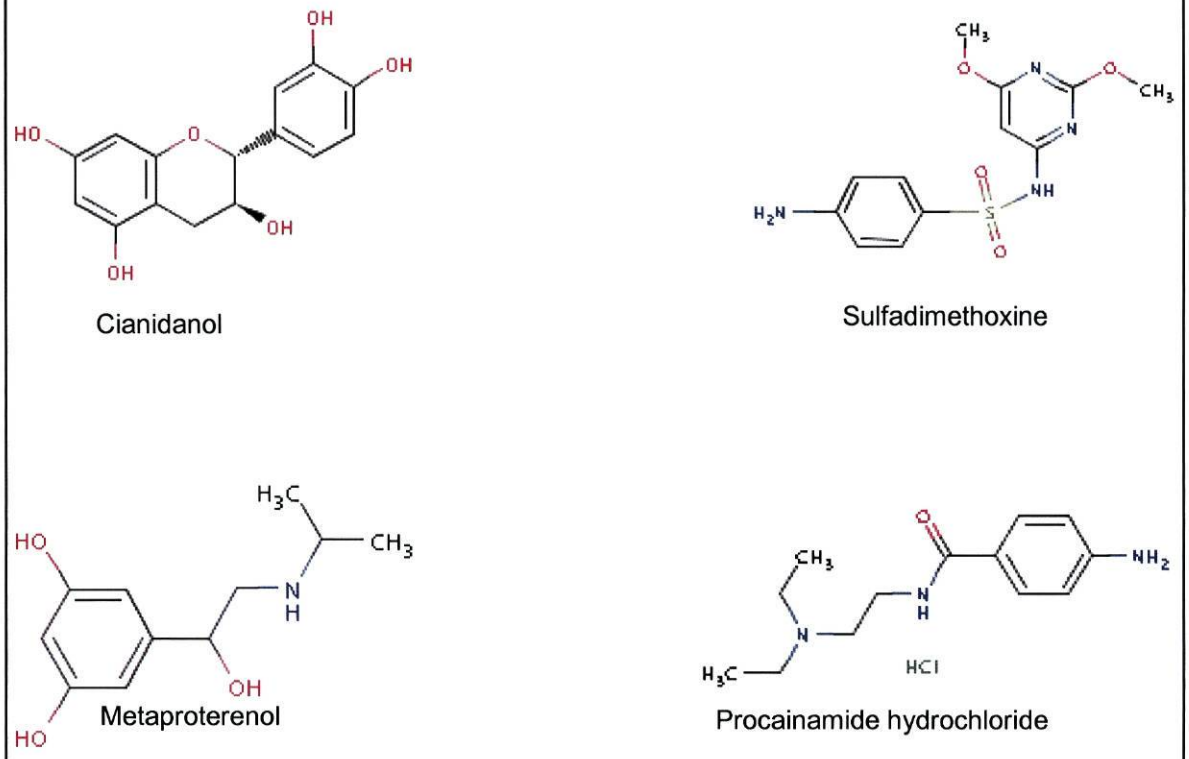


Figure 4.10. Chemical structures of the top active drugs in the αSyn screen.

4.3.2 Huntingtin Screen

The huntingtin screen utilized a similar approach, but in this case we only screened the library at the concentration of 31.25 μM.

The huntingtin screen did not yield any hits in our system (Fig. 4.11). The statistical analysis revealed that no compound had a significant activity.

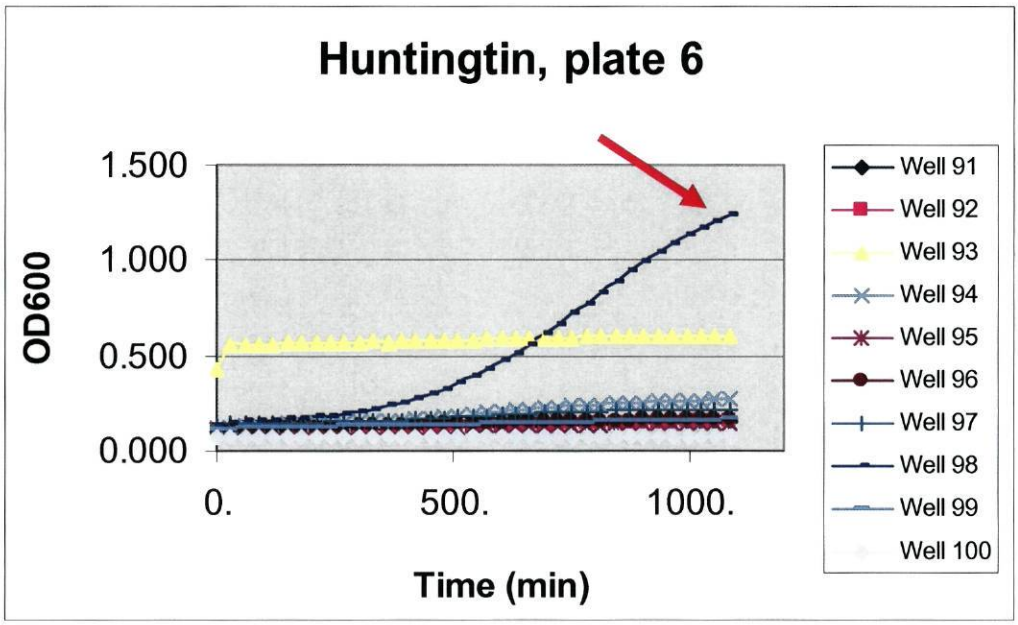


Figure 4.11. Inactivity of a particular set of compounds in the huntingtin screen. After 1200 minutes no significant growth was detected. Red arrow points to the positive control on the plate, showing normal growth.

4.4 Discussion

In the post-genomic era, target-based screening has become an important starting point in the pharmaceutical industry (Bleicher, Bohm et al. 2003; Walters and Namchuk 2003). But in complex diseases, such as the protein misfolding diseases, in which toxicity seems to reflect a gain of function (Sisodia 1998), the molecular targets are often not available. Moreover, the development of cell models for these diseases is a somewhat subjective discipline, also prone to a large number of idiosyncrasies associated with any particular cell line, and choosing between several models is not an easy task.

Biochemical assays, directed towards particular intermediates in the protein folding pathways (e.g. towards the stabilization of the soluble species, or preventing formation of oligomers) (Heiser, Scherzinger et al. 2000; Conway, Rochet et al. 2001) have yielded several candidates, but these have yet to be confirmed in cell and animal models.

Large scale screens cannot be performed in living organisms, as these are costly, time-consuming and not necessarily conclusive.

Our approach was to take advantage of the simple model system yeast, where the expression of proteins that are key players in PD and HD is associated with toxicity phenotypes, and identify drugs that alleviate that toxicity.

The αSyn screen yielded several candidates that can now be tested for efficacy in other assays, including cell-based and biochemical assays, to investigate their effects. Together with yeast genetics, this information may prove invaluable towards the development of novel therapeutics. Cianidanol is an antioxidant flavonoid from woody plants. Mitochrondrial dysfunction and oxidative stress are implicated in the progression of several neurodegenerative diseases, including PD, and several therapeutic approaches include antioxidants (Isacson 2002; Barnham, Masters et al. 2004). Our finding of cianidanol is therefore extremely promising, not only because it validates our yeast system but more importantly

because it may enable the identification of novel antioxidants for treating these diseases. The relevance of the other three top-active drugs is more difficult to understand but, nonetheless, holds significant promise because this was an unbiased approach. Sulfadimethoxine is active against several pathogenic organisms. Metaproterenol is used to prevent and treat wheezing, shortness of breath, and troubled breathing caused by asthma, chronic bronchitis, emphysema, and other lung diseases. It relaxes and opens air passages in the lungs, making it easier to breathe. Procainamide hydrochloride is used to treat cardiac arrhythmias.

The huntingtin yeast screen did not yield any hits. Growth restoration by a drug is generally accepted to be more difficult than growth inhibition. One possibility to explain our results is that there were simply no drugs capable of alleviating the level of toxicity in the huntingtin model. Another possibility is that the concentration range tested was not the correct one to afford any hits, as it is well established that the effective concentration of any drug is dependent on the starting inoculum.

The yeast screens previously described were part of the Neurodegeneration Drug Screen Consortium (NDSC), organized by the National Institute of Neurological Disorders and Stroke (NINDS) in collaboration with The ALS Association (ALSA), the Huntington's Disease Society of America (HDSA) and the Hereditary Disease Foundation (HDF). The results were included in the manuscript presented in the following pages.

4.5 DRUG SCREENING YIELDS NEW APPROACHES TO NEURODEGENERATION

4.5.1 Introduction

The Neurodegeneration Drug Screening Consortium (NDSC) of 27 laboratories was formed through a public-private partnership to examine commonalities among neurodegenerative mechanisms and to identify potential therapeutic agents. The NDSC simultaneously probed multiple neurodegeneration targets with a collection of compounds designed for this purpose, containing clinically approved drugs and other bioactive compounds (Fig. 4.12). Because any single model has inherent limitations in representing a disease process, we aimed to increase the reliability of the results by combining data from testing the same compounds against 29 different neurodegeneration models. Consortium members developed a series of assays of hierarchical complexity, from biochemical assays, to mitochondrial- and cell-based assays, and including *in vivo* assays in *Drosophila* and *C. elegans* (Table 4.3). Each assay was based on a proposed mechanism of neurodegeneration, e.g., protein aggregation, protein toxicity, excitotoxicity, or apoptosis. We probed these mechanisms in a variety of *in vitro* and *in vivo* contexts, seeking cross-validation among closely related screens and examining the relatedness of more disparate ones. We used a collection of known bioactive compounds to probe these models. To optimize the therapeutic potential of the screens, the collection of 1040 compounds contained approximately 750 drugs that are approved for human use in the United States or Europe. The defined human safety profiles of these drugs allow efficient evaluation of the clinical potential of new activities as relevance to the targeted

diseases is validated. The remaining 25% of compounds in the collection were chosen for their known biological activity, and included controlled substances and natural products. Because of their bioavailability and bioactivity, these compounds have a higher probability of activity in novel screens than randomly selected compounds (Root, Flaherty et al. 2003).

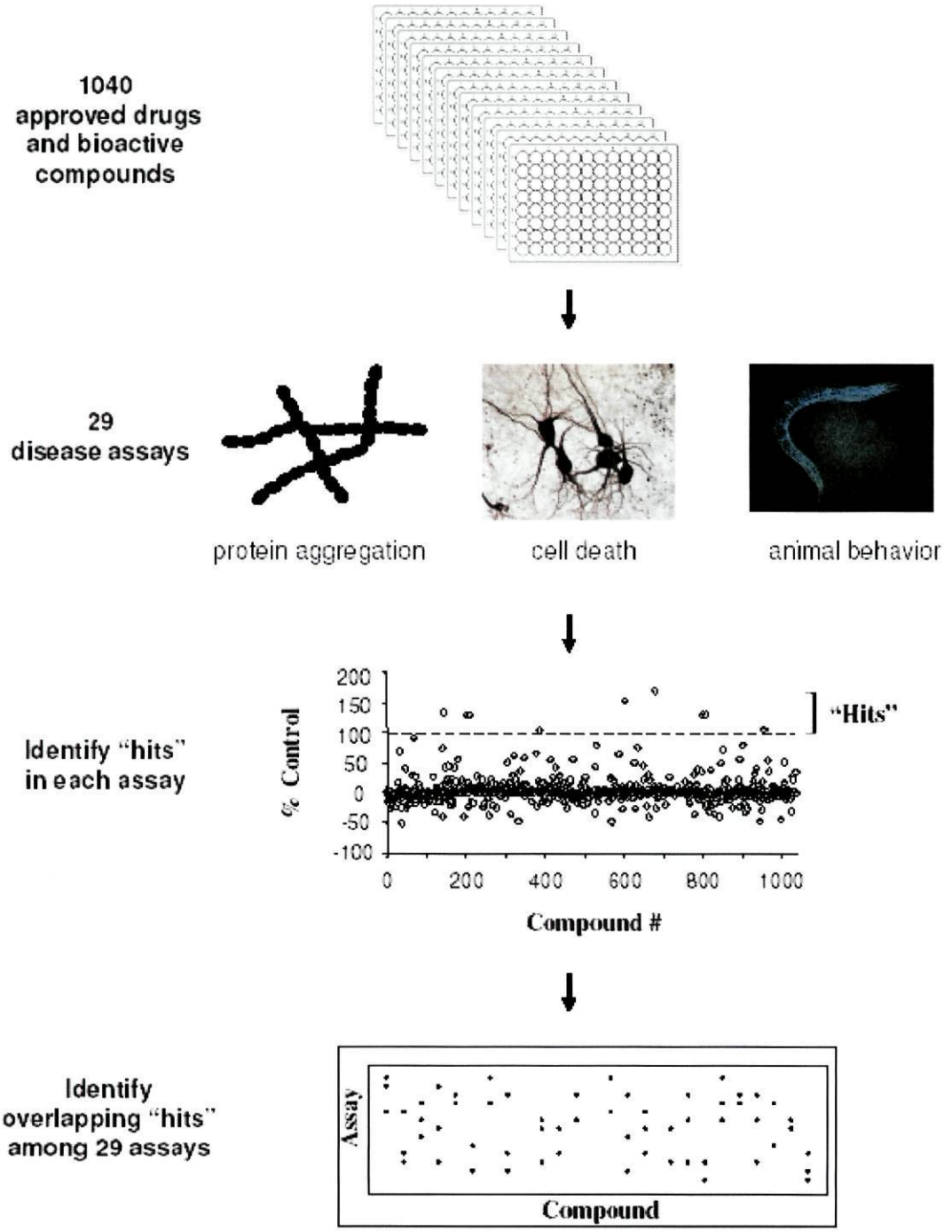


Figure 4.12. NDSC screening strategy. 1040 FDA approved drugs and bioactive compounds were pre-selected for screening in 29 disease-related assays of hierarchical complexity. "Hits", compounds with highest activity, were identified for each assay. Data was compiled into a database to identify hits that overlapped in multiple assays. This database contains complete methods, references and screening results for each assay and is publicly accessible at https://gandalf.wi.mit.edu/nih/index.jsp.

Table 4.3. Models of neurodegeneration screened by the NDSC.

Assay Type	Label	Assay Description*	Database	Investigators
Cytotoxicity (C)	C1	Expanded polyglutamine huntingtin: toxicity in PC12 cells	LDH09-100	Schweitzer, Aiken
	C2	Expanded polyglutamine huntingtin: toxicity in PC12 cells	ROSShtt	Ross, Wang
	C3	Expanded polyglutamine huntingtin: neuronal dysfunction in *C. elegans*	HDELENEU	Nen, Lambert, Parker
	C4	Expanded polyglutamine huntingtin: toxicity in *C. elegans*	CelcO	Morimoto, et al[1]
	C5	Expanded polyglutamine SCA1: neuronal toxicity in *Drosophila*	SCA1FLYpic	Botas, et al[2]
	C6	Mutant SOD1: toxicity in human SHSY5Y neuroblastoma cells	mSOD1	Flanagan, Oberley
	C7	Mutant SOD1: toxicity in mouse N2A neuroblastoma cells	mSODtox	Pasinelli, Trotti, Brown
	C8	Mutant SOD1: toxicity in PC12 cells	ALSOD1	Roos, Ghadge
	C9	Alpha-Synuclein: toxicity in *S. cerevisiae*	YAPD	Lindquist, Outeiro
Excitotoxicity (E)	E1	Glutamate transporter EAAT2 expression in rat spinal cord slice culture	X4_EAAT2	Rothstein, Patel, Lee
	E2	Glutamate uptake in mouse MN-1 motor neuron hybrid cells	GluUPTAKE	Trotti, Williams
	E3	Glutamate-induced death of cultured rat primary motor neurons	MNGlu	Vincent, Feldman
	E4	Kainate-induced death of cultured mouse primary cortical neurons	LDH	Koistinaho, Vartiainen
Apoptotic Signals (A)	A1	Permeability transition in purified rat liver mitochondria	MitoPT	Kristal, Stavrovskaya, Beal
	A2	Cytochrome C release from purified mouse liver mitochondria	CytoCRel	Friedlander, Zhu, Orzata
	A3	Expanded polyglutamine AR**: Caspase-3 activation in HEK cells	Casp3	Fischbeck, Piccioni, Taylor
Protein Association/ Processing (P)	P1	Expanded polyglutamine huntingtin: SDS solubility of in vitro aggregates	QA1	Gusella, Wang
	P2	Thioredoxin-polyglutamine fusion protein: formation of in vitro aggregates	QTHIO	Burke, Brown, Strittmatter
	P3	Synthetic polyglutamine: in vitro elongation of solid phase aggregates	QA3	Wetzel, Berthelier
	P4	Polyglutamine-GFP: aggregation in human SHSY5Y neuroblastoma cells	AGREG	Keller, Dimayuga, Ding
	P5	Polyglutamine-GFP: aggregation in PC12 cells	GFPQ80	Paulson, Bishop
	P6	Polyglutamine-GFP: SDS solubility of PC12 cellular aggregates	SDS	Paulson, Bishop
	P7	Polyglutamine-alpha-tubulin: aggregation-induced lethality in *S. cerevisiae*	75Q-tubH/U	Hughes, Fields, Lo
	P8	Expanded Polyglutamine huntingtin: 2-hybrid formation in *S.cerevisiae*	Qn2Hy	Tartakoff, et al[3]
	P9	Expanded polyglutamine androgen receptor: turnover in mouse MN-1 cells	AR112GFP	Merry, Marinescu
	P10	Mutant SOD1-GFP: aggregation in COS1 cells	GFPSOD1	Liu, Corcoran
	P11	Par4/atypical Protein Kinase C peptides: in vitro protein-protein association	Par4_PKC	Wooten, Seibenhener
RNA Splicing/Transcription (R)	R1	SMN-luciferase splicing reporter: alternative splicing in human C33A cells	SMNLUC	Stockwell, Root, Zhou
	R2	NLS-Polyglutamine-GFP: repression of the *S. cerevisiae* PHO84 promoter	PHO84	Hughes, Fields, Lo
	R3***	Hsp70 promoter-luciferase reporter: promoter activity in human HeLa cells	HSEluc	Morimoto, et al[4]

*Complete results, methods and references are available at https://gandalf.wi.mit.edu/nih/index.jsp

Thus, although the collection was small compared to compound collections typically used in high throughput drug screens, the pre-selection of compounds for biological activity allowed productive screening of more models and models of higher complexity, including *in vivo* models, that could be screened in a high throughput setting. We compiled the data from the screens and methods in a searchable, publicly accessible database available at https://gandalf.wi.mit.edu/nih/index.jsp.

NDSC investigators were blind to the identity of individual compounds during the screening process. We initially screened compounds at a single concentration,

ranging from 1 to 100 μM depending on the requirements of the assay. We retested the most active compounds, usually 10 to 20 in each assay, and analyzed their potency over a range of concentration to determine dose-response relationships. In 26 of the 29 assays tested, at least one compound was identified that had reproducible, concentration-dependent activity. In total, 294 compounds met these criteria in one or more assays. This large number is perhaps not surprising given the number of possible molecular targets in cell-based and *in vivo* assays. However, it suggests that, for any individual active compound, further validation is required to gain confidence in its relevance to neurodegeneration.

One indication of a compound's relevance is reproducible activity in more than one related assay. In screening the same compound collection against many related assays, we expected to identify compounds with overlapping activities. In addition to highlighting the potential importance of individual compounds, our working assumption was that broadly overlapping activities might reveal or confirm relationships among neurodegeneration mechanisms. We found no single compound that was active in a broad range of assays. However, we found that thirteen compounds were reproducibly active in three assays (Table 4.4). In addition, multiple members of three compound classes, the cephalosporin antibiotics, cardiac glycosides and cannabinoids, were represented in two or more assays (Table 4.4). In screening 1040 compounds in 29 assays, one expects some compounds to appear active in multiple screens by chance. However, follow up testing and confirmation removes the element of chance, and, unless otherwise indicated, the activities presented in Table 4.4 and elsewhere in this report were shown to be reproducible and concentration-dependent. Thus, the reliability of the results in each assay suggests testable hypotheses that warrant further evaluation. The value of examining overlapping hits is exemplified below by studies of celastrol, identified in three consortium assays, and further validated in related but independent experiments.

Table 4.4. Overlapping Activities.

Compound	Assays
Acrifavinium HCL	C8, P4, P10
Apomorphine HCL	C1, E4, P4
Avermectin B1	P8, P10, R2
Calcimycin	C1, C3, A1
Celastrol	C1, C3, P1
Citrinin	C3, C5, E2
Ebselen	C1, C3, E2
Ellagic Acid	P3, P10, P11
Gossypol-acetic acid complex	E4, P1, P3
Hycanthone	C1, P3, P10
Protoporphyrin IX	P1, P3, P11
Terfenadine	C1, C3, A1
Thioridazine HCL	E4, A1, A2
Compound Class[1]	
Cannabinoids (4)	C1,C3, E4
Cardiac Glycosides (6)	A3, P10
Cephalosporins (7)	C6, C8, E1, E3[2]

[1]total number of active compounds in class in parentheses
[2]reproducible activity at 1μM, EC_{50} not determined

Celastrol, a naturally occurring quinone methide found in various members of the plant family Celastraceae was initially identified as highly active in three consortium assays (Table 4.4). All three assays were related to polyglutamine diseases (Table 4.3), and two assays measured protection from polyglutamine toxicity. In assay C1, PC12 cells inducibly expressed an N-terminal huntingtin protein fragment containing an expanded polyglutamine repeat of 103 residues (C.T.A. and E.S.S., submitted). Celastrol was able to prevent approximately 50% of polyglutamine induced cell death in this assay (EC50 ~0.5 μM). In assay C3,

transgenic *C. elegans* expressed an N-terminal huntingtin fragment containing a 128 residue polyglutamine repeat in the neurons responsible for the tail touch response (Parker, Connolly et al. 2001). Celastrol protected 50% of animals from polyglutamine-induced loss of touch sensitivity (EC50 ~ 0.4 μM). To verify the protection by celastrol seen in these two assays, we tested its ability to block the induction of caspase-3 activity, a measure of incipient cell death (Jaeschke, Fisher et al. 1998), in the PC12 cell line used in assay C1 (Fig. 4.13a). These experiments showed a significant decrease in caspase-3 activity in the presence of sub-micromolar celastrol, consistent with the observation that celastrol protects PC12 cells from polyglutamine-induced cell death.

Celastrol, however, was not detected in the primary screening of two other cell-based polyglutamine toxicity assays (C2, A3). This may be due to celastrol's toxicity, as compounds were screened at 10μM in these assays. In the PC12 assay C1, celastrol was active in a 0.8μM screen, but was found to be toxic above 2μM.

One potent mechanism of protection from polyglutamine toxicity that has been demonstrated in cellular and animal models of polyglutamine diseases is the activity of heat-shock chaperones such as HSP70 (Warrick, Chan et al. 1999; Jana, Tanaka et al. 2000; Cummings, Sun et al. 2001; Adachi, Katsuno et al. 2003). Chaperones have also conferred protection in models of other neurodegenerative diseases, including Parkinson's disease (Auluck, Chan et al. 2002) and amyotrophic lateral sclerosis (ALS), (Bruening, Roy et al. 1999; Takeuchi, Kobayashi et al. 2002) and, in all of these models, the hypothesized mechanism of protection is clearance of toxic proteins. Identifying a small molecule that induces expression of these neuroprotective chaperones would present a promising therapeutic approach. Therefore, celastrol and the other 293 compounds identified in the original NDSC screens were tested for the ability to activate expression of the human HSP70 promoter in HeLa cells (Figure 4.13b).

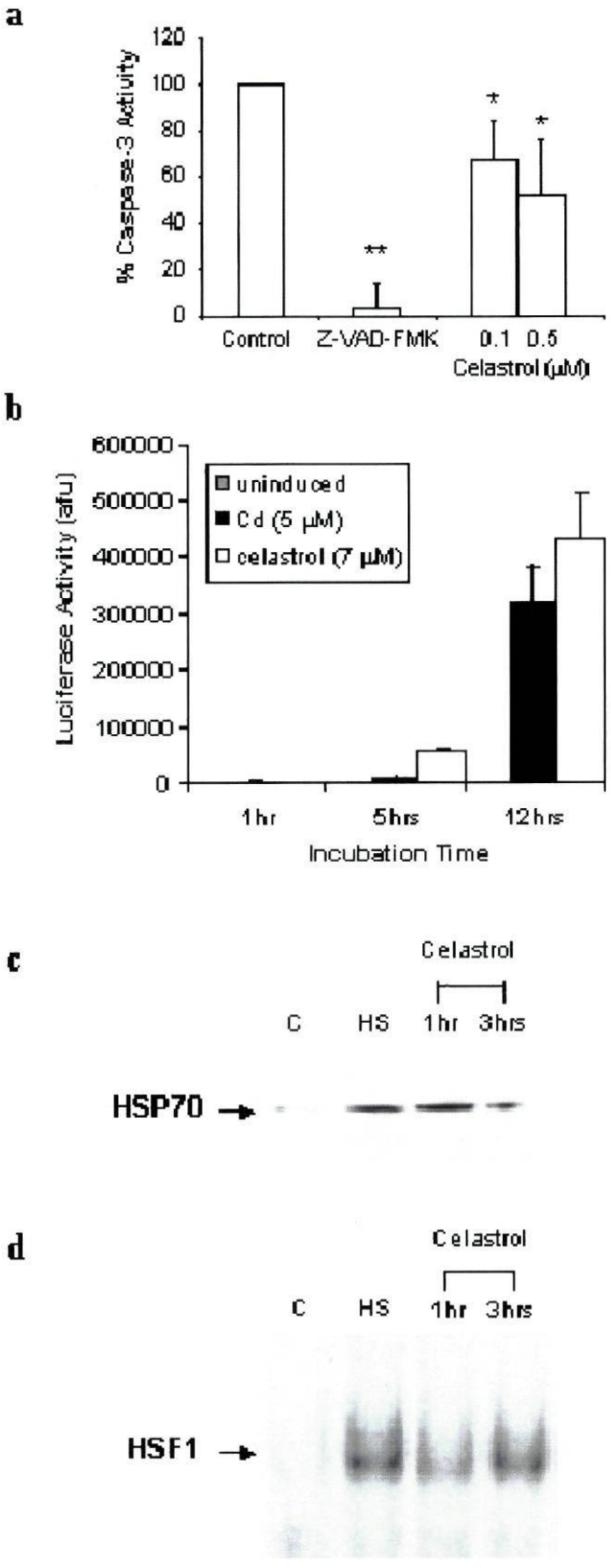

a
% Caspase-3 Activity
120
100
80
60
40
20
0
**
*
*
Control
Z-VAD-FMK
0.1
0.5
Celastrol (μM)
b
Luciferase Activity (afu)
600000
500000
400000
300000
200000
100000
0
uninduced
Cd (5 μM)
celastrol (7 μM)
1hr
5hrs
12hrs
Incubation Time
c
Celastrol
C
HS
1hr
3hrs
HSP70
d
Celastrol
C
HS
1hr
3hrs
HSF1

Figure 4.13. Celastrol mechanisms of action. **a,** Inhibition of caspase-3 activity in PC12 cells expressing expanded polyglutamine huntingtin (cell line described in NDSC database, assay C1). Graph shows the mean +S.D. of caspase activation in response to polyglutamine expression (activity detection methods described in NDSC database, assay A3). Experimental values were normalized to polyglutamine alone (control). The specificity of caspase activity was confirmed by inhibition with 20μM z-VAD-fmk (PharMingen). After 48-hour treatment with celastrol, cells showed a dose-dependent decrease in caspase-3 activity. The differences between control and treatments were found to be statistically significant by Fisher LSD Method (** $p<0.01$, * $p< 0.05$). **b**, Activation of the human HSP70 promoter in HeLa cells. Promoter activation is measured using a luciferase reporter construct (methods described in NDSC database, assay R3). Graph shows the mean +S.D. of luciferase activity expressed in arbitrary fluorescence units (afu). Celastrol induced luciferase expression from the HSP70 promoter with kinetics similar to the positive control, cadmium sulfate (Cd), a potent inducer of the heat shock response (Mosser, Theodorakis et al. 1988). **c**, Expression of endogenous HSP70 protein in HeLa cells. Western blotting with a monoclonal antibody to human HSP70 (Abravaya, Myers et al. 1992) shows that celastrol induces expression of the endogenous HSP70 protein in HeLa cells. C=untreated control cells, HS=30 minute heat shock at 42°C, Celastrol= cells treated with 7μM Celastrol for 1 and 3 hours. **d**, Activation of Heat Shock Factor-1 (HSF-1) in HeLa cells. A complex of activated HSF-1 and a ^{32}P-labelled oligonucleotide containing the heat shock element is detected in a gel shift assay of extracts prepared from HeLa cells (Mosser, Theodorakis et al. 1988). As with heat shock, treatment of cells with celastrol activates DNA binding by HSF-1, suggesting that celastrol activity induces the heat shock response and multiple molecular chaperones. Lanes are labelled as in **c** above.

Celastrol was one of 6 compounds active in this reporter assay (R3, EC50 ~3 μM), and it was also shown to induce the expression of the endogenous HSP70 gene (Fig. 4.13c). Its activation of the HSP70 promoter is comparable to classical inducers of the heat shock response, such as cadmium chloride (Fig. 4.13b), with kinetics similar to heat shock (Fig. 4.13c). Celastrol's ability to activate the heat shock transcription factor HSF1 (Fig. 4.13d) further suggests that celastrol activates expression of multiple chaperones. This suggests that the protective activity of celastrol may result from activating expression of heat shock chaperones.

The third consortium screen in which celastrol was identified was a cell-free assay, based on *in vitro* aggregation of a 58 residue polyglutamine repeat in N-terminal huntingtin (P1) (Huang, Faber et al. 1998). Celastrol prevented the formation of SDS-insoluble polyglutamine aggregates (EC50 ~3.6 μM). However, celastrol was not active in two other *in vitro* polyglutamine aggregation assays

(P2, P3). This discrepancy could reflect the different aspects of aggregation biochemistry measured in the 3 assays, for example, only the P1 assay involved SDS treatment. Still, celastrol's activity, even in a single protein-based assay, suggests that it can interact directly with expanded polyglutamine.

The overlap of results between assays based on specific functions (such as aggregation or heat shock promoter activity) and more broadly targeted screens of cell survival and function may indicate that the biochemical activities are relevant to pathogenesis. In the two cellular toxicity assays (C1 and C3), celastrol could act at any of several points in a pathological cascade, while the heat-shock assay (R3) implies that protein-monitoring pathways may be the source of celastrol's protection. The ability of celastrol to interfere with huntingtin aggregation *in vitro*, however, raises the additional possibility of a direct role for interaction with polyglutamine. One unifying model is that the same complex of celastrol with polyglutamine may both stimulate a protective heat-shock response and prevent the formation of insoluble aggregates. For example, the conformation of polyglutamine or other proteins in complex with celastrol could mimic an endogenous signal for activation of the heat shock response, such as protein misfolding. The conformation of the complex could also render a polyglutamine aggregate more amenable to disruption by SDS treatment *in vitro*. Whether or not further experiments suggest that polyglutamine is the cellular target of celastrol protection, the broad and potent activities of celastrol suggest that it may be a valuable probe for understanding polyglutamine pathogenesis as well as forming the basis of a therapeutic strategy. Because of the neuroprotective function of heat shock chaperones, celastrol's activity may provide a starting point for developing a treatment for polyglutamine and other neurodegenerative diseases, and studies of learning in rats demonstrate its bioavailability in the CNS (Allison, Cacabelos et al. 2001). However, its toxicity raises questions about the feasibility of human use without prior chemical modification.

In addition to single compounds active in multiple assays, there were also three classes of compounds with members active in at least two assays (Table 4.4). The appearance of multiple members of a class, along with their representation in related assays, strongly suggests that these activities warrant further investigation.

One on such class was the approved drug family of cephalosporin antibiotics, compounds of little *a priori* interest for neurodegeneration. Sixteen cephalosporins were included in the NDSC compound collection, and seven of these were active in one or more assay. Our analysis revealed that multiple cephalosporins were active in assays of neuroblastoma cells from SOD1 toxicity (C6, EC50~50-75μM), and a single two distinct pathogenic mechanisms associated with ALS, the cellular toxicity of dominant mutations in the cytosolic copper-zinc superoxide dismutase (SOD1) (Rosen, Siddique et al. 1993; Flanagan, Anderson et al. 2002). Five cephalosporins were found to protect human, and glutamate excitotoxicity 21 cephalosporin was identified in a high concentration screen of SOD1 toxicity in PC12 cells (C8, EC50~12mM). A second proposed mechanism of ALS pathogenesis is glutamate excitotoxicity (Cleveland and Rothstein 2001), and ALS patients show increases in extracellular glutamate and decreased glutamate transport (Rothstein, Martin et al. 1992; Rothstein, Van Kammen et al. 1995). Three of the cephalosporins active in SOD1 assays were also found to increase glutamate transporter expression (E1, EC50~3.5-5 μM). Additionally, excitotoxicity assay E3 revealed modest protection by two cephalosporins at a 1μM screening concentration (A.M.V., C. Backus and E.L.F., submitted). Low micromolar screening concentrations may account for lack of more extensive cephalosporin activity in related ALS assays here, since both C6 and E1 were screened at 100μM, near the EC50 for assay C6, and further testing may reveal more extensive cephalosporin activity among the ALS related assays.

The activity of cephalosporins in glutamate-related assays is supported by a previous study, which showed that the cephalosporin ceftriaxone was neuroprotective in spinal cord cultures exposed to excitotoxic stress (Rothstein and Kuncl 1995). Our results suggest that the mechanism of this protection involves increased glutamate transport. The activities of cephalosporins in the NDSC screens further suggest a pharmacological link between the toxic actions of glutamate and mutant SOD1. An intriguing possibility that relates these findings is that the toxicity of mutant SOD1 results from alterations in the cell's response to glutamate. Consistent with this, increased extracellular glutamate and decreased transport are seen in transgenic SOD1 mice (Trotti, Rolfs et al. 1999). Alternatively, cephalosporins may trigger a more general mechanism that protects against both sources of toxicity. The shared activity of cephalosporins provides a novel pharmacological tool for testing the relationship between glutamate transport, SOD1 toxicity and ALS. In addition, these results raise the possibility that cephalosporins are candidates for clinical testing in ALS. Further validation of these observations will be necessary to judge their promise for patients.

Cannabinoids comprise a second class of compounds that showed activity in multiple assays (Table 4.4). The four cannabinoids contained in the collection all showed activity in the PC12 cell assay of polyglutamine toxicity discussed above (C1, EC50~40-100 μM). One of these, cannabidiol, was also highly active in the *C. elegans* model of polyglutamine toxicity, C3 (EC50~ 20nM). Delta-8-tetrahydrocannabinol (EC50~ 6 μM) and cannabidiol (EC50ND) were also active in an assay of kainate excitotoxicity in primary cortical neurons (E4). These results are consistent with recent experiments in mice demonstrating that the endogenous cannabinoid system can activate neuroprotective molecular cascades, protect against seizure and diminish the degree of neuronal damage induced by kainic acid administration (Marsicano, Goodenough et al. 2003). Taken together, these results suggest that components of the cannabinoid signaling system may be valid targets for intervention in neurodegeneration.

Members of the cardiac glycoside family, six of which were contained in the collection, also showed overlapping activity (Table 4.4). Cardiac glycosides inhibit Na+/K+-ATPase and are widely used in the treatment of congestive heart failure. Five cardiac glycosides were identified in a screen for compounds that inhibit polyglutamine-induced caspase-3 activation, an indicator of apoptosis, in HEK cells (A3, EC50~30nM). These drugs were subsequently shown to prevent polyglutamine-induced cell death by a mechanism that appears to be unrelated to Na+/K+-ATPase inhibition (F.P., B.R. Roman, K.H.F. and J.P.T., submitted). Further, all six of the cardiac glycosides were active in a screen for compounds that inhibit aggregation of mutant SOD1 in Cos1 cells (P10, EC50~0.1-5 μM). This effect also seems to be unrelated to Na+/K+-ATPase activity (L.J.C., T.J. Mitchison and Q.L., in preparation). The novel activity of the cardiac glycosides in these assays may be shared, and the correspondence of activity in an apoptotic assay and an SOD1 aggregation assay suggests a testable link between the aggregation and toxicity of mutant SOD1.

4.5.2 Discussion

Given the many suggestions that common pathogenic mechanisms underlie disparate neurodegenerative diseases (Troy, Stefanis et al. 1997; Taylor, Hardy et al. 2002), it is interesting to note the lack of more general overlap: no universally active compounds emerged from these screens. There were no broadly effective anti-aggregants, proteasome enhancers, antioxidants, NMDA-receptor antagonists, or caspase inhibitors. These results suggest that the individual aspects of the various neurodegenerative diseases may be among the most highly effective targets in the search for disease intervention.

Despite the small size of this compound collection compared to those typically screened in high throughput drug discovery efforts, pre-selection for bioactive compounds was intended to optimize the chances of success while allowing us to test assays that are too cumbersome for large scale screening. Using this broadly accessible screening strategy, the NDSC successfully identified many compounds worthy of further investigation for neurodegeneration—celastrol, cephalosporins, cannabinoids, cardiac glycosides and others, including some of the 294 hits that were active in only a single assay. These appear promising in secondary testing and are actively being pursued. The future identification of the cellular targets of these compounds is expected to reveal new components of neurodegeneration pathways, and allow a more complete understanding of pathogenesis. In addition, these probes are hoped to provide pharmacological inroads into the now uncharted territory of neurodegeneration therapeutics.

CHAPTER 5

BIOLOGICAL EFFECTS OF αSYN EXPRESSION IN YEAST

CHAPTER 5. BIOLOGICAL EFFECTS OF ALPHA-SYNUCLEIN EXPRESSION IN YEAST

Abstract

Alpha-synuclein is a small protein of unknown function that has been linked to Parkinson's disease and other synucleinopathies. A triplication of the alpha-synuclein gene has recently been found to cause familial Parkinson's disease but the mechanism through which increased levels of this protein lead to disease is unknown. Here we investigated the effects of increased levels of alpha-synuclein in yeast cells, and examined the synthetic effects of alpha-synuclein with factors that have been associated with the etiology of Parkinson's disease. Doubling the dose of alpha-synuclein led to an impairment of trafficking between the ER and Golgi, causing the accumulation of an ERAD substrate and increased sensitivity to ER stress. In addition, expression of two copies of αSyn was associated with oxidative stress, causing the cells to accumulate increased levels of ROS. Finally, we showed that further stressing αSyn-expressing with oxidants resulted in increased toxicity, when compared to cells not expressing the protein. Thus, our findings provide the first clues into the pathways affected by increased levels of alpha-synuclein, establish a connection between this protein and factors linked to Parkinson's disease, opening novel avenues for therapeutic intervention.

5.1 Introduction

Alpha-synuclein (αSyn) is a 14 kDa protein of 140 amino acids, first identified as a presynaptic protein in rat brain (Maroteaux, Campanelli et al. 1988). The normal function of αSyn is currently unknown, but it plays a central role in Parkinson's disease (PD) and is implicated in several other neurodegenerative disorders, including Alzheimer's disease (AD), dementia with Lewy bodies (DLB), and Multiple System Atrophy (MSA). Different cell types are affected in each case (Dickson 2001; Mark 2001; Dev, Hofele et al. 2003; Jellinger 2003; Marti, Tolosa et al. 2003), but in all of these diseases αSyn accumulates as misfolded species in cytoplasmic inclusions (Forno 1996). The vast majority of PD cases appear to be sporadic, but a small percentage has a genetic basis, with several genes involved (Gasser 2001; Zarranz, Alegre et al. 2004). Recently, Singleton et al. reported that in a PD family in Iowa a triplication of the αSyn locus is associated with the disease (Singleton, Farrer et al. 2003). Subsequently, an unrelated Swedish-American family with hereditary early-onset parkinsonism with dementia was also shown to carry a triplication of the αSyn gene (Farrer, Kachergus et al. 2004), suggesting increased levels of αSyn may be a more common phenomenon amongst patients with alpha-synucleinopathies.

The biological consequences of increased expression of αSyn are only poorly understood, but an understanding is essential for the development of treatments designed to interfere with the onset and progression of these diseases. Impairment of the ubiquitin proteasome system and mitochondrial dysfunction have important, but as of yet unclear connections to the etiology of PD. Endoplasmic reticulum (ER) stress was also shown to contribute to neuronal death in cellular models of PD (Ryu, Harding et al. 2002). Several Parkinsonian mimetics induce the UPR in neurons (Holtz and O'Malley 2003). Moreover, Parkin, an E3 ubiquitin ligase linked to autosomal-recessive juvenile

Parkinsonism (AR-JP), has been linked to ER stress because one of its substrates, the Pael receptor, accumulates in the brains of AR-JP patients and may cause selective death of dopaminergic neurons (Imai, Soda et al. 2001; Takahashi and Imai 2003; Takahashi, Imai et al. 2003).

Our previous work revealed that αSyn production in yeast affected intracellular trafficking (Outeiro and Lindquist 2003; Willingham, Outeiro et al. 2003) and was related to oxidative stress (Outeiro and Lindquist, unpublished).

The secretory pathway is highly conserved in eukaryotes. Yeast serves as a simplified model of the system, which begins with the entry of proteins into the ER through the Sec61 translocon. In the ER, proteins are folded, glycosylated, form disulfide bonds and oligomerize. The ER quality control system (ERQC) ensures proteins only leave the ER if they fold properly and receive appropriate modifications (Brodsky and McCracken 1999; Sitia and Braakman 2003). Proteins that fail the ERQC are degraded, to avoid any toxic consequences that may result from their accumulation (Kopito 1997; Plemper and Wolf 1999; Plemper and Wolf 1999; Sitia and Braakman 2003). Several diseases may arise due to ERQC problems caused by either the accumulation of misfolded/mutant proteins or by excessive activity of those quality control mechanisms, titrating out the activity of essential proteins. Accumulation of misfolded proteins within the ER induces a highly specific unfolded protein response (UPR) (Kaufman 1999). The UPR activates a signal transduction pathway that induces a specific set of genes which lead to either reduction of ER stress or to death (Mori 2000).

αSyn has also been implicated in oxidative stress, which in turn is intimately associated with PD. The etiology of PD is not fully understood but exposure to oxidizing agents and mitochondrial dysfunction have been linked to the disease (Mizuno, Ohta et al. 1989; Parker, Boyson et al. 1989; Schapira, Cooper et al. 1989; Haas, Nasirian et al. 1995; Gorell, Johnson et al. 1998; Menegon, Board et al. 1998). Additional evidence for mitochondrial impairment in PD comes from

studies with the parkinsonism toxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPP+ (1-methyl-4phenyl-2,3-dihydropyridinium), the active metabolite of MPTP, acts as a complex I inhibitor (Nicklas, Vyas et al. 1985). Moreover, the recently developed rotenone model of PD further substantiates the involvement of pesticide exposure and systemic complex I dysfunction in PD etiology. Importantly, although rotenone causes uniform complex I inhibition throughout the brain, rotenone-treated rats accumulated αSyn-positive nigral inclusions, nigrostriatal dopaminergic degeneration and motor deficits (Betarbet, Sherer et al. 2000; Betarbet, Sherer et al. 2002; Sherer, Betarbet et al. 2002). Understanding how αSyn relates to oxidative stress, and whether increased levels of αSyn may constitute the molecular culprit that links cellular dysfunction to disease is a critical step towards the development of novel avenues for therapeutic intervention.

Here, we investigated downstream cellular consequences of increased expression of αSyn, namely those arising due to impairment of intracellular trafficking.

5.2 Materials and Methods

Chemicals were purchased from either Sigma or Fischer.

5.2.1 Plasmid Construction

pAC375 is a 2μ plasmid harboring the ubiquitin gene expressed by the copper-inducible *CUP1* promoter. This fragment was excised from YEp96 (Ellison and Hochstrasser 1991) (kindly provided by Dr. Mark Hochstrasser) as a *BamHI-PstI* fragment and ligated into Yep351. Plasmids for expressing αSyn in yeast have been previously described (Outeiro and Lindquist 2003).

5.2.2 Yeast Strains and Manipulations

Genotypes of yeast strains are listed in Table 5.1. Yeast manipulations were performed and media were prepared using standard procedures (1991). Transformations of yeast were carried out using a standard lithium acetate procedure (Ito, Fukuda et al. 1983).

Table 5.1. Strains used in this study.

Yeast Strain	Genotype
W303-1A	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*
306	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS306
306-αSynWT-GFP	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS306-αSynWT-GFP
306-αSynA53T-GFP	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS306-αSynA53T-GFP
306-αSynA30P-GFP	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS306-αSynA30P-GFP
304-306	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS304 pRS306
304-306-αSynWT-GFP	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS304-αSynWT-GFP pRS306-αSynWT-GFP
304-306-αSynA53T-GFP	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS304-αSynA53T-GFP pRS306-αSynA53T-GFP
304-306-αSynA30P-GFP	*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS304-αSynA30P-GFP pRS306-αSynA30P-GFP

5.2.3 Growth Rates and Survivorship Assays

Serial dilutions were performed as described (Bi and Broach 1999). Growth curves were generated by growing the described strains overnight in synthetic media containing 2% raffinose at 30°C to log phase and then diluting them to 0.1-0.3 OD_{600nm}. 2% galactose was then added to the raffinose media and subsequent OD_{600nm} readings were taken at the indicated time points. Cell survivorship was determined by growing the described strains overnight in synthetic media containing raffinose to log phase followed by the addition of 2%

galactose to induce expression of αSyn. To maintain wild-type cells in log phase several dilutions were made throughout the time course. At the described time points, 1 OD_{600nm} was harvested, diluted 1:1000 and 300 μl of these cells were plated on synthetic media containing 2% glucose and incubated at 30°C. Colony forming units were then determined.

For the spotting experiments, cells carrying either one or two copies of the αSyn-GFP fusions were grown overnight at 30°C in liquid media containing raffinose until they reached log or mid-log phase. Cultures were then normalized for OD_{600}, serially diluted and spotted onto solid media containing either glucose or galactose, after which they were grown at 30°C for 2-3 days. Cells carrying the 2μ plasmids were grown in synthetic medium lacking uracil, for plasmid selection, serially diluted and spotted onto rich medium (YPD) or synthetic medium lacking uracil.

5.2.4 Growth Curves

Cells were grown in liquid media containing raffinose until they reached log or mid-log phase. Cultures were then normalized for OD_{600}, diluted and dispensed into 100 well-honeycomb plates. Growth was then recorded for 24 or 48 hours on a Bioscreen C instrument (Thermo Labsystems). Whenever applicable, drugs were added to the cultures at the same time of induction of αSyn expression. Each growth curve is the average of at least four replicates.

5.2.5 Protein Preparation, Antibodies, and SDS-PAGE and Immunoblottings

Yeast cells expressing αSyn were grown to mid-log phase, harvested, washed with water, and lysed in ethanol with glass beads. The precipitated proteins were spun down at 13,000 rpm for 15 min., at 4°C, and the pellets were then resuspended in standard SDS-sample buffer. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotting was performed following standard procedures.

5.2.6 GFP Imaging

For analysis of αSyn-GFP distribution, strains carrying either one or two copies of αSyn-GFP were grown overnight in raffinose medium. After normalizing the culture density, expression was induced in galactose for 8 hours, at which time the levels and distribution of αSyn had reached steady state. Cells were then imaged with a Zeiss Axioplan II microscope equipped with MetaMorph (UIC) for image acquisition. For analysis of αSyn distribution, 2D or 3D deconvolution was applied (Huygens Essentials, SVI).

5.2.7 Biochemical Assays and Immunofluorescence

For all assays described, the cells were treated in the following manner: cells were grown overnight in synthetic media containing raffinose to log phase. Galactose was then added to 2% to induce expression of αSyn. Total glutathione levels were measured following three hours of growth in galactose as described (Cuozzo and Kaiser 1999). To control for cell lysis, Bradford assays

(BioRad) for protein concentration were performed. Radiolabel pulse chase experiments to examine both protein degradation and trafficking were performed as described (Caldwell, Hill et al. 2001; Haynes, Caldwell et al. 2002). The secondary antibodies used for immunofluorescence were purchased from Molecular Probes. Immunofluorescence microscopy was performed as described (Hill and Stevens 1994).

5.2.8 ROS Detection

ROS were detected as described (Madeo, Frohlich et al. 1999) using dihydrorhodamine 123 (Sigma). Essentially, cells were grown in galactose media for 10 hours and then dihydrorhodamine 123 was added at 5 μg/ml of cell culture from a 2.5 mg/ml stock solution in ethanol. Cells were viewed without further processing through a rhodamine optical filter after a 2-hour incubation.

5.3 Results

5.3.1 Increased Expression of αSyn is Toxic to Yeast

αSyn had only been directly implicated in PD through two rare mutations (A53T and A30P) (Polymeropoulos, Lavedan et al. 1997; Kruger, Kuhn et al. 1998). More recently, a duplication of the αSyn gene has been linked to familial cases of PD, suggesting the levels of αSyn are critical determinants for the susceptibility for the disease (Singleton, Farrer et al. 2003). To investigate the effect of different levels of αSyn expression in yeast we integrated one or two copies of a αSyn-GFP fusion in the yeast genome, under the regulation of a galactose inducible promoter. As we had previously shown (Outeiro and Lindquist 2003), a duplication in the gene dosage resulted in severe impairment of growth (Fig. 5.1).

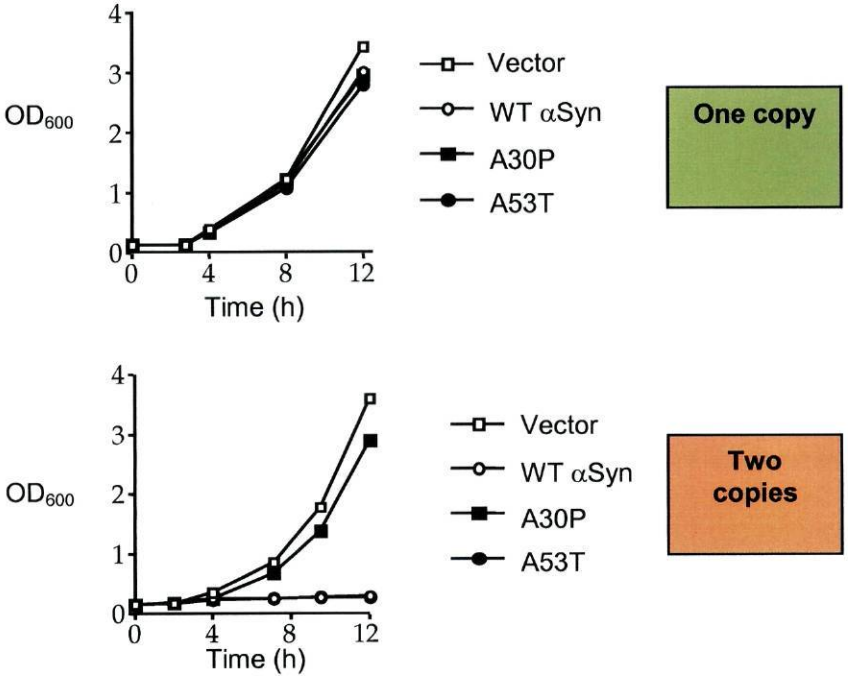


Figure 5.1. Expression of high levels of WT or A53T αSyn is toxic to yeast cells. Cells were grown overnight at 30°C to log phase in synthetic media containing raffinose. Cells were then diluted to 0.1-0.2 OD_{600} and galactose was added to a final concentration of 2%. OD readings were then taken at the described time points. Expression of one copy of aSyn only has a negligible effect on growth whereas higher expression (two copies) of WT and A53T aSyn exacerbates these effects resulting in complete growth inhibition.

Survivorship of cells expressing two-copies of αSyn was drastically reduced 12 hours after induction (Fig. 5.2).

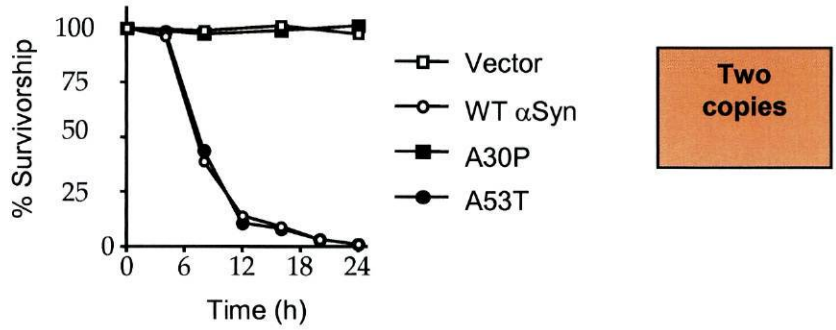


Figure 5.2. Expression of αSyn reduces cell survivorship. Cells were grown overnight in synthetic media with raffinose at 30°C followed by the addition of 2% galactose to induce expression αSyn. To maintain "vector" and A30P cells in log phase, several dilutions were made through out the time course. At the indicated time points, 1 OD_{600} was harvested, diluted 1:1000 and 300μl of these cells were plated on synthetic media with glucose and incubated at 30°C for 48 h. Colony forming units were then determined and converted to relative percentages.

5.3.2 Accumulation of Proteasomal Substrate CPY* but not Sec61-2p nor Cytosolic Substrate

We had previously shown that αSyn inclusions in yeast can be ubiquitin-positive, and also that αSyn accumulation leads to proteasome impairment (Outeiro and Lindquist 2003). To investigate the nature of the proteasome impairment, we examined the degradation of three different types of proteasome substrates: (1) CPY*, which is a mutant form of the vacuolar carboxypeptidase Y (CPY) (Knop, Hauser et al. 1996), a substrate of the ERQC machinery that is retained in the ER; (2) Sec61-2p, which is a mutant form of the Sec61p subunit of the translocon (Biederer, Volkwein et al. 1996) and also a substrate of the ERQC; and (3) a cytosolic chimeric protein that is a proteasomal substrate, Deg1-βGal (Chen, Johnson et al. 1993; Johnson, Swanson et al. 1998). Cells bearing one or two copies of the galactose-inducible αSyn-GFP constructs were grown in the presence of galactose for 6 hours prior to radiolabeling and immunoprecipitations. Fig. 5.3 shows that the degradation of the cytosolic substrate Deg1-βGal is not affected by expression of two copies of αSyn.

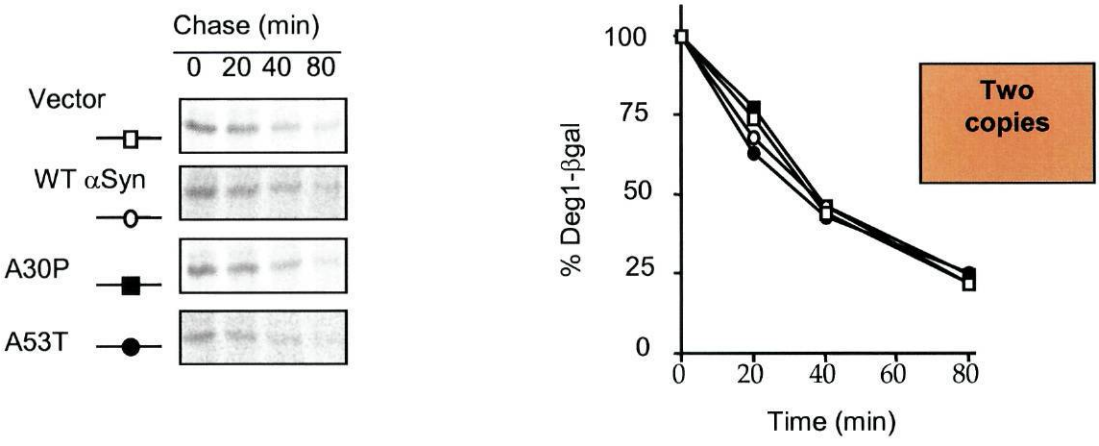


Figure 5.3. Degradation of the cytosolic substrate Deg1-βGal is not impaired in cells expressing αSyn. Cells were grown overnight in raffinose medium and then switched to galactose and grown at 30°C for six hours. Radiolabeling and immunoprecipitation was then performed. The degradation of Deg1-βGal, a cytosolic chimeric protein that is ubiquitinated and degraded by the proteasome, is not inhibited in strains expressing two copies of αSyn.

Degradation of Sec61-2p was also not affected in cells expressing one (not shown) or two copies of αSyn (Fig. 5.4). Interestingly, degradation of CPY* was severely impaired in cells expressing two copies of WT or A53T αSyn, but not the A30P mutant (Fig. 5.5).

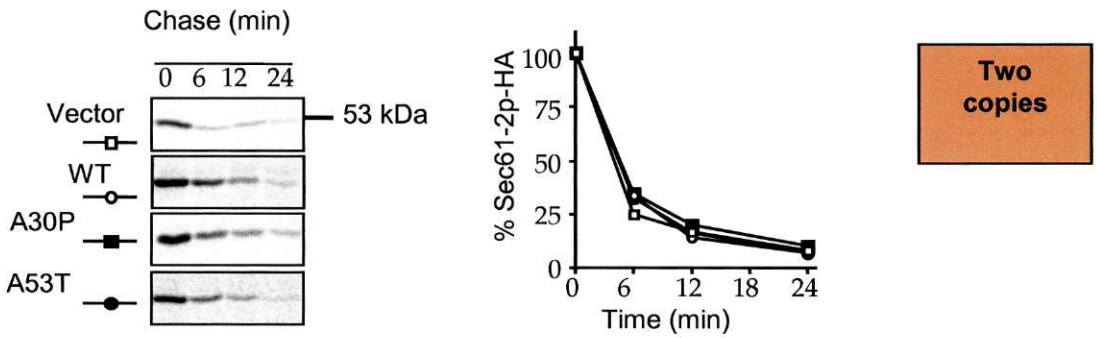


Figure 5.4. Degradation of Sec61-2p is not impaired in cells expressing αSyn. Cells were grown in galactose medium at 35°C (temperature Sec61-2 is an ERAD substrate) for 6 hours prior to radiolabeling. Degradation of this Sec61-2p is not impeded by the presence of the two copies of αSyn.

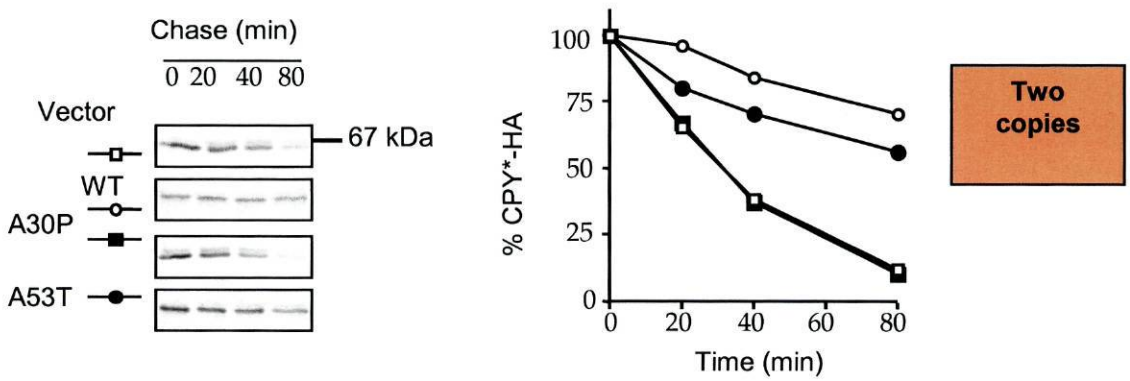


Figure 5.5. Degradation of CPY* is impaired in cells expressing αSyn. αSyn was induced for 6 hours in galactose medium prior to radiolabeling. Degradation of this substrate is severely impeded by the presence of the WT and A53T αSyn (two copies) constructs but not by A30P.

To test whether the growth defects were due to depletion of the pool of free ubiquitin in the cell, we overexpressed ubiquitin in cells expressing two copies of αSyn. Although we could observe a reproducible alleviation of toxicity, the effect was rather small (Fig. 5.6). This result supports the idea that depletion of ubiquitin due to proteasomal impairment is not the sole cause for toxicity.

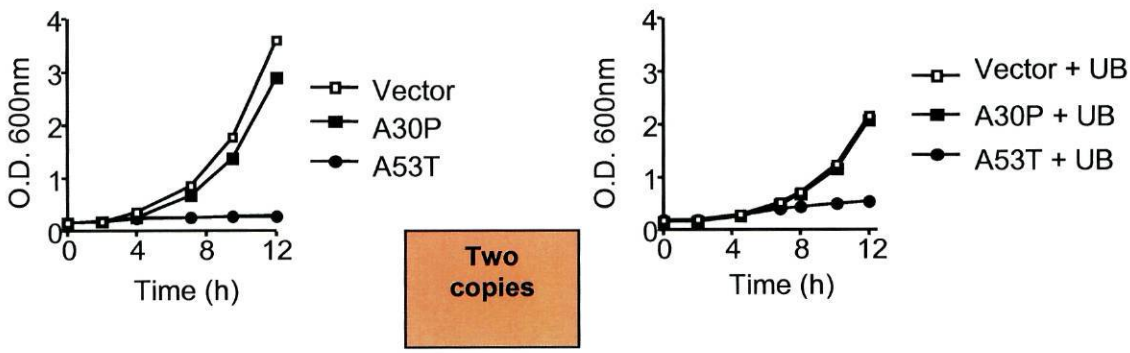


Figure 5.6. Overexpression of ubiquitin alleviates toxicity in cells expressing two copies of αSyn. Strains with two copies of αSyn were transformed with either empty plasmids or plasmids for overexpressing ubiquitin (UB). Growth was monitored in medium containing galactose.

5.3.3 αSyn Impairs Intracellular Trafficking

The function of αSyn is currently not known, but several studies suggest it is involved in the regulation of dopamine storage via effects on neurotransmitter vesicles (Murphy, Rueter et al. 2000; Lotharius and Brundin 2002). We recently showed that endocytosis is impaired in cells expressing two copies of αSyn

(Outeiro and Lindquist 2003). Moreover, a synthetic lethal screen in yeast indicated that a large number of genes involved in vesicular transport causes synthetic lethality/toxicity in cells expressing αSyn (Willingham, Outeiro et al. 2003).

Together with these results, the effect of αSyn on the degradation of CPY* prompted us to ask how increased levels of αSyn affected intracellular trafficking. The trafficking of proteins through the secretory and endocytic pathways is a selective process where proteins are selected and concentrated into distinct vesicle populations, which are then targeted to their specific compartments. The transport of proteins to the yeast vacuole proceeds through two separate pathways: the CPY pathway and the ALP pathway (Burd, Babst et al. 1998).

For these investigations we followed CPY and alkaline phosphatase (ALP) through the secretory pathway to investigate the effects of high levels of intracellular αSyn on trafficking. CPY and ALP are secretory proteins that transit from the ER to the Golgi once their folding and initial glycosylation are complete. In the Golgi, CPY is further glycosylated, and from there it traffics to the vacuole and it becomes an active protease when its prepro- sequence is cleaved off.

Two hours upon induction of αSyn expression from the galactose-inducible promoter, the effect of αSyn on the transport of CPY was already noticeable (Fig. 5.7). Transport was completely inhibited after four hours (Fig. 5.7). This effect on trafficking correlates with the growth impairment (Fig. 5.1). Two hours after induction we did not observe a significant difference in growth, but after four hours these differences became clearer.

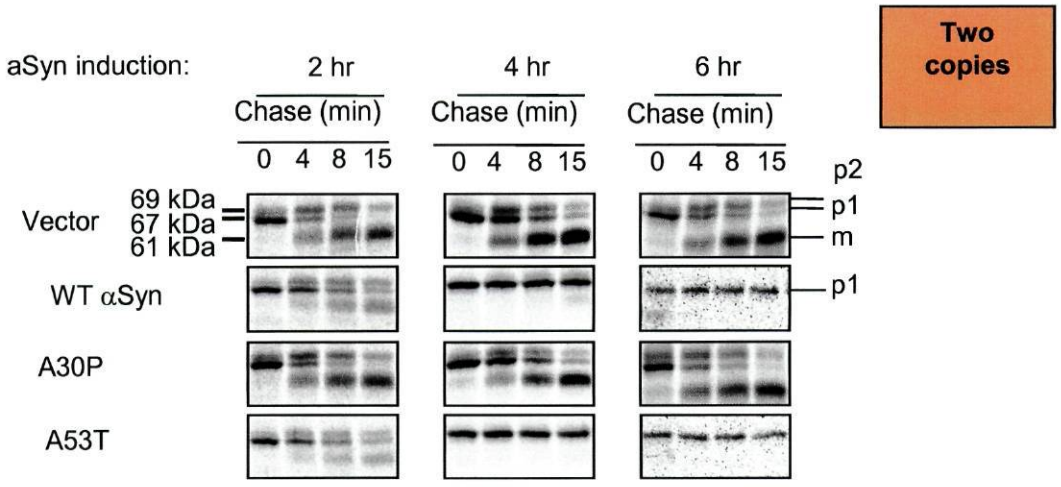


Figure 5.7. Trafficking of carboxypeptidase Y is impaired in cells expressing two copies of αSyn. Cells were grown in galactose at 30°C for 6 hours. CPY enters the ER lumen where it is glycosylated (runs on a gel as the "p1" form). CPY is then transported to the Golgi, where it is further glycosylated (runs on a gel as the "p2" form). From the Golgi, CPY traffics to the vacuole where its prepro sequence is cleaved (the "mature" form on a gel) and it becomes an active protease. Transport of CPY from the ER to the Golgi is severely impeded by the presence of the WT and A53T αSyn. A30P does not affect CPY trafficking.

Next we asked whether ALP trafficking was also affected by high levels of αSyn in the cell. ALP is transported to the membrane through a route that does not involve the vacuolar protein sorting-dependent pathway (Piper, Bryant et al. 1997). ALP transport was also inhibited in cells expressing two copies of WT or A53T αSyn, but not in control cells or cells expressing the A30P mutant (Fig. 5.8). Here, we looked at the effect on ALP trafficking six hours after induction of αSyn expression.

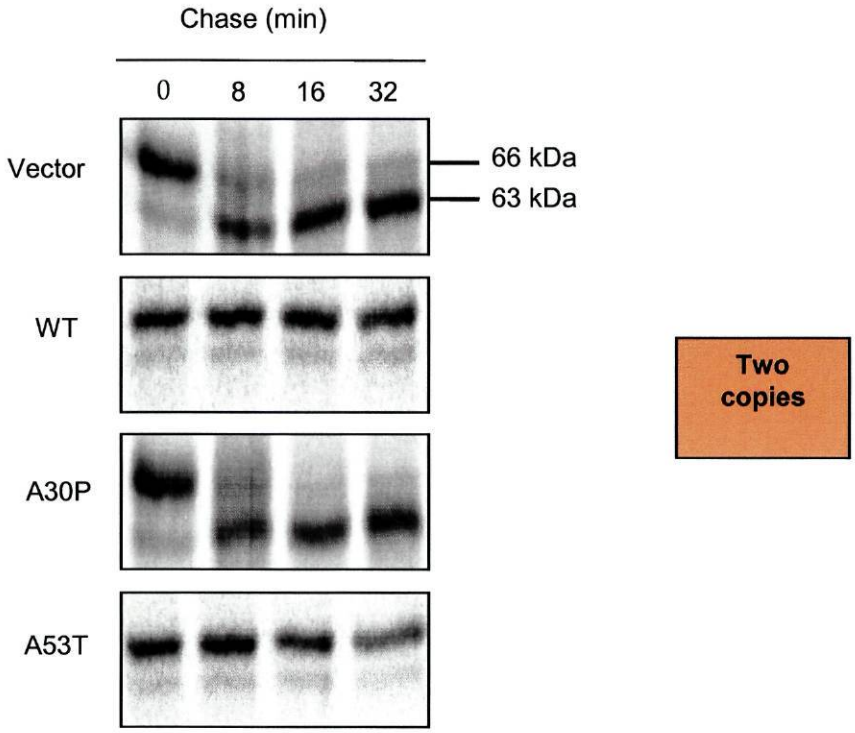


Figure 5.8. ALP trafficking is impaired in cells expressing two copies of αSyn. Cells were grown in galactose at 30°C for 6 hours and ALP trafficking through the secretory pathway was monitored at the indicated times. ALP is a vacuolar protein of ~63 kDa. In the ER-Golgi ALP is ~66 kDa.

These results indicate that αSyn blocks important steps involved in protein exit from the Golgi, irrespective of the pathway utilized for the proteins to reach the vacuole.

5.3.4 αSyn Causes Increased Sensitivity to ER Stress

We next investigated whether the effect of αSyn on ER-Golgi trafficking caused increased sensitivity to ER stress. DTT is s strong reducing agent that does not

allow disulfide bonds to form in the ER, causing the accumulation of misfolded proteins and ultimately, ER stress.

Cells expressing one copy of αSyn show no significant growth defect when compared to control cells (Fig. 5.1 and Fig. 5.9). When DTT is added to these cells, a growth defect can be observed (Fig. 5.9). This suggests the ERQC mechanisms that are normally capable of dealing with low levels of αSyn become overwhelmed and unable to maintain the general ER homeostasis.

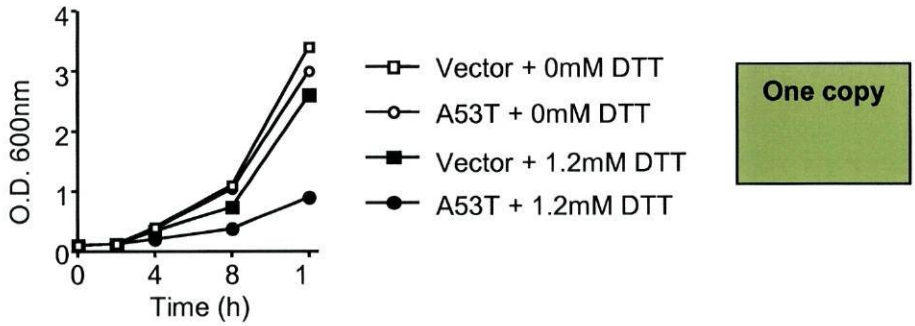


Figure 5.9. Sensitivity to ER stress in cells expressing one copy of αSyn. Cells expressing one copy of αSyn were grown in 2% galactose to log phase and diluted to 0.1 OD_{600} and the indicated amount of dithiothreitol (DTT) was added. The growth rate was then determined. Growth rate of cells expressing A30P αSyn was identical to the control strain (vector). Expression of WT αSyn yielded a growth rate identical to that of cells expressing A53T αSyn.

Tunicamycin is a toxin that blocks N-linked glycosylation of newly synthesized proteins in the ER, therefore causing accumulation of unglycosylated proteins, and consequent failure to pass the ERQC systems.

To verify the synthetic effect of αSyn and ER stress we spotted cells expressing αSyn from a 2μ plasmid onto medium containing tunicamycin (Fig. 5.10). Cells

expressing WT or A53T αSyn showed increased sensitivity when compared to control cells (vector) or cells expressing A30P αSyn.

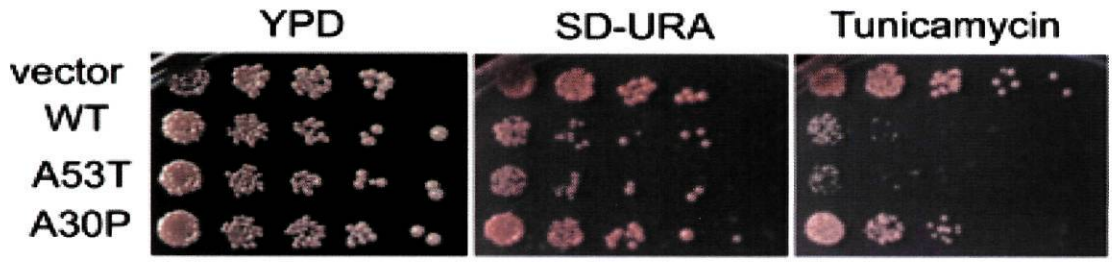


Figure 5.10. Sensitivity to tunicamycin in cells expressing αSyn. Cells expressing αSyn from a 2μ plasmid were grown in liquid, serially diluted and spotted onto solid medium containing tunicamycin. Plates were incubated for 3 days at 30°C.

5.3.5 αSyn Causes ER Breakdown

We and others have previously shown that αSyn interacts with membranes and phospholipid vesicles (McLean, Kawamata et al. 2000; Perrin, Woods et al. 2000; Outeiro and Lindquist 2003), a property that may be important for the formation of oligomeric species and inclusions (Sharon, Goldberg et al. 2001; Lee, Choi et al. 2002; Sharon, Bar-Joseph et al. 2003). Fragmentation of Golgi has been found in several neurodegenerative diseases, including Alzheimer's disease (AD), amyotropic lateral sclerosis (ALS), Creutzfeldt-Jacob disease (CJD) and multiple system atrophy (MSA) (Gonatas, Gonatas et al. 1998; Sakurai, Okamoto et al. 2000; Sakurai, Okamoto et al. 2002). More recently, αSyn was shown to cause Golgi fragmentation in mammalian cells (Gosavi, Lee et al. 2002).

The effects of αSyn on trafficking and the increased sensitivity to ER stress in cells expressing toxic versions of αSyn prompted us to investigate the effect of αSyn on the ER morphology.

We used immunofluorescence microscopy, with an antibody against Kar2p (an ER resident protein), to examine the ER upon induction of αSyn. In cells not expressing αSyn (Fig. 5.11, 0h), ER morphology was normal, appearing as a compact structure around the nucleus. However, after 8 hours of induction of αSyn in galactose-containing medium αSyn inclusions could be observed, as previously reported (Outeiro and Lindquist 2003), and the ER was not distinguishable (Fig. 5.11).

In cells expressing one copy of αSyn the ER appeared normal (data not shown).

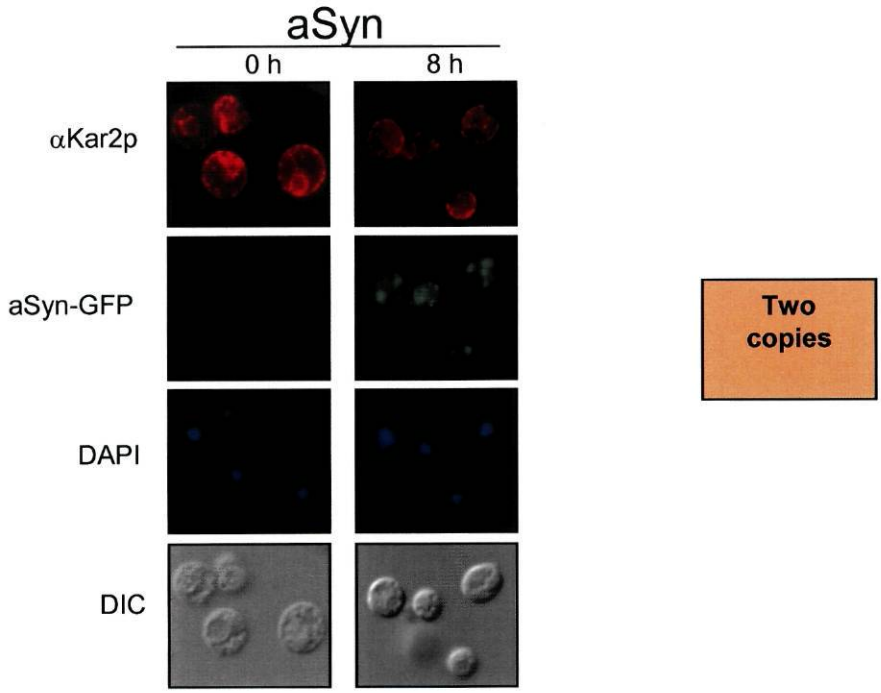


Figure 5.11. aSyn expression causes ER breakdown. Cells were grown in galactose at 30°C for 8 hours. All strains were grown overnight at 30°C to log phase in synthetic media containing raffinose. The cells were diluted to 1.0 OD_{600} and galactose was added to a final concentration of 2%. Cells were harvested and fixed prior to galactose addition (0h) and 8 hours after. The perinuclear ER (not the cortical ER), as judged by antibodies to the ER-localized Hsp70 Kar2p, breaks down/disappears following expression of A53T. This coincides with the appearance of A53T inclusions. The nuclear DNA, as judged by DAPI, appears to be intact.

5.3.6 αSyn Expression Causes Increased ROS Production and Sensitivity to Oxidative Stress

Mitochondrial dysfunction, along with oxidative stress, play an important role in the etiology of PD, but the connection to the levels of αSyn is not clear.

To investigate whether increased expression of αSyn is associated with oxidative stress, we stained cells expressing one or two copies of αSyn with dihydrorhodamine 123, a dye that reacts with hydrogen peroxide (H_2O_2) in the presence of peroxidase, cytochrome C or Fe^{2+}, becoming fluorescent. Cells expressing one copy of αSyn showed similar levels of ROS staining after 10 hours of induction of expression (not shown). In cells carrying two copies of αSyn WT or A53T the levels of ROS detected were substantially higher than in control cells or cells expressing the A30P mutant (Fig. 5.12).

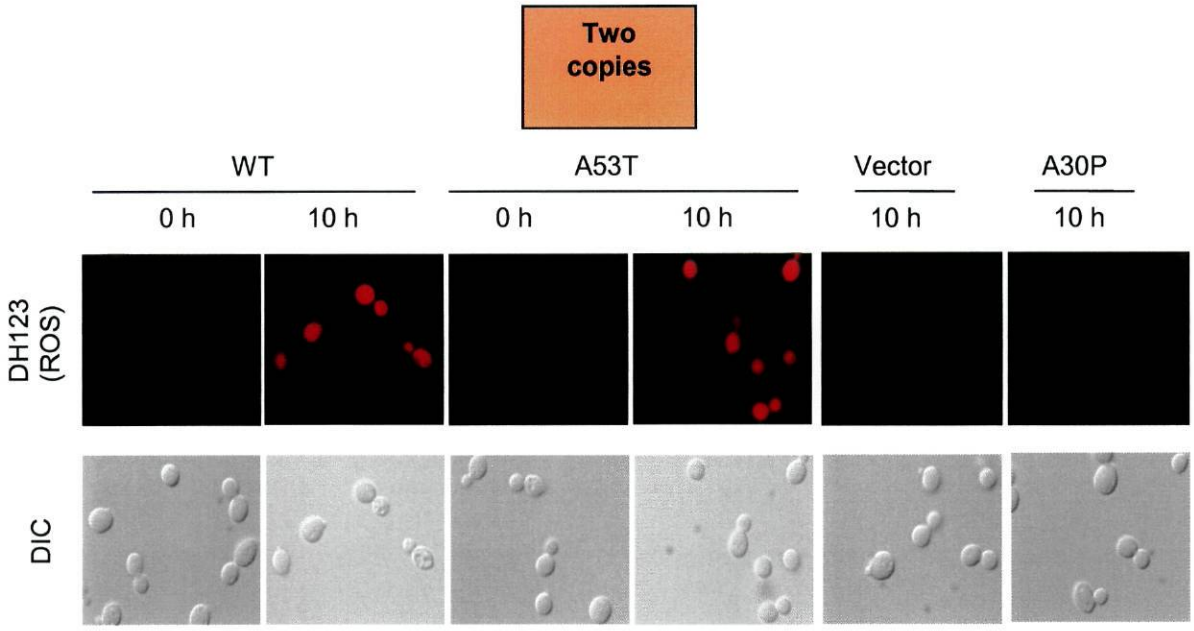


Figure 5.12. Cells expressing αSyn accumulate higher levels of ROS. Cells were grown at 30°C in galactose medium for the indicated times. Cells were harvested every two hours and incubated with dihydroxyrhodamine 123 for an additional two hours. ROS were detected in cells expressing two copies of WT and A53T αSyn but not in those expressing A30P. Cells expressing no αSyn (vector control) did not show ROS accumulation. Microscopy was performed using the rhodamine channel of a fluorescence microscope. The same exposure time was used for all fluorescent images.

We then asked whether forcing the cells to respire, by growing them in non-fermentable carbon sources, would increase αSyn toxicity. Yeast cells grown in glucose typically ferment the sugar, converting it to ethanol, to produce ATP. In non-fermentable carbon sources, such as glycerol or potassium acetate, the cells are forced to use their mitochondria to metabolize the sugars and produce ATP. When cells carrying one copy of αSyn (WT, A53T or A30P) were grown in glucose their growth rates were identical (Fig. 5.13). Interestingly, cells expressing WT or A53T αSyn grew slower than controls or those expressing A30P in glycerol or potassium acetate (non-fermentable carbon sources that

force cells to respire), suggesting that forcing mitochondrial activity in conjunction with αSyn expression was toxic to the cells (Fig. 5.13).

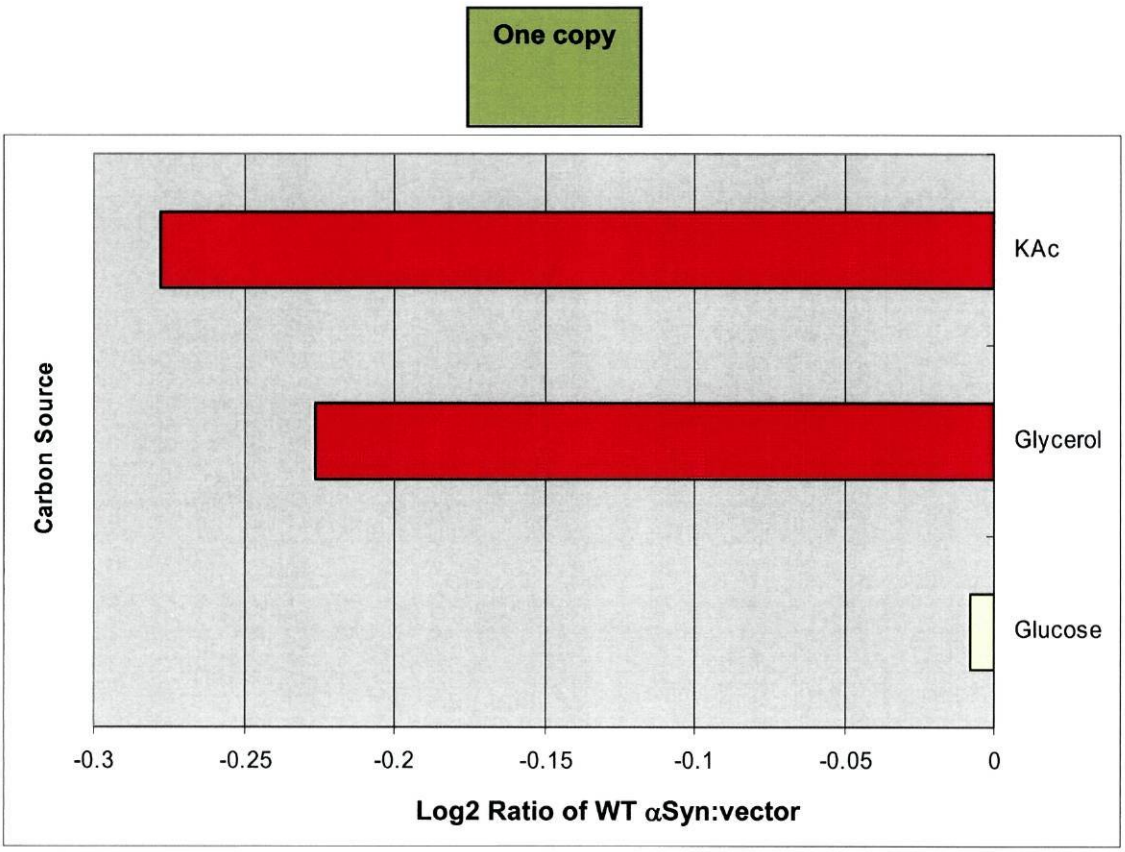


Figure 5.13. αSyn expression causes a growth defect in media containing non-fermentable carbon sources. Cells carrying a CEN plasmid encoding for αSyn under the control of the GPD promoter were grown in synthetic medium containing 2% of KAc, glycerol or glucose as carbon source. Growth was monitored with the Bioscreen C instrument and the log2 of the ratios of the heights of the growth curves of cells expressing αSyn vs. vector were plotted. Results for WT aSyn are shown.

We also tested the sensitivity of cells expressing αSyn to different types of oxidants, known to preferentially affect different cellular pathways (Higgins, Alic et al. 2002; Thorpe, Fong et al. 2004). Interestingly, cells expressing αSyn (WT, A53T and A30P) were less sensitive to paraquat (in glycerol medium) and copper

than control cells (Fig. 5.14). Diamide, menadione and H_2O_2, on the other hand, caused a growth defect on αSyn -expressing cells (Fig. 5.14).

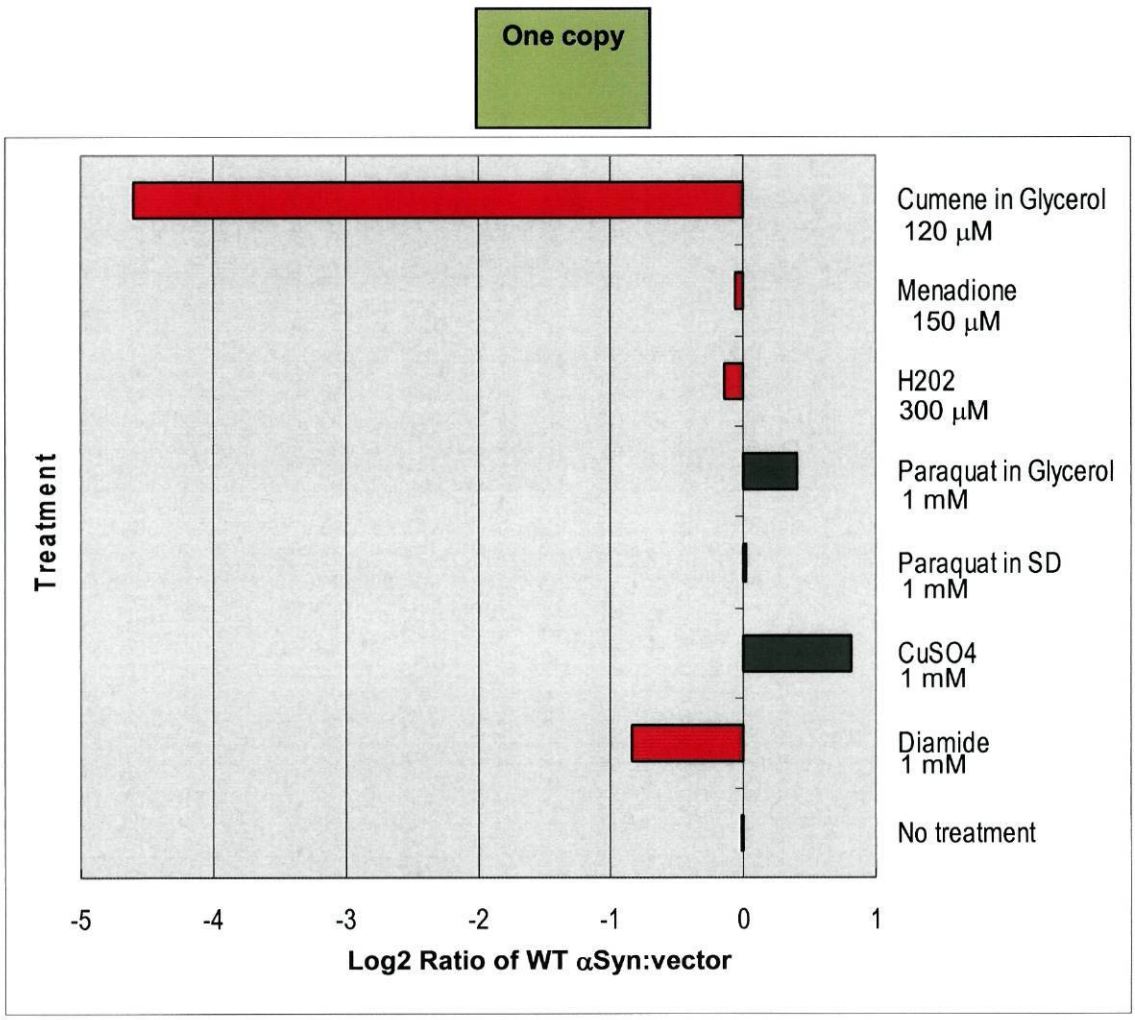


Figure 5.14. Cells expressing αSyn show different sensitivities to oxidants. Cells carrying a CEN plasmid encoding for αSyn under the control of the GPD promoter were grown in synthetic medium containing the different oxidants. Growth was monitored with the Bioscreen C instrument and the log2 of the ratios of the heights of the growth curves of cells expressing αSyn vs. vector were plotted. Results for WT αSyn are shown.

Diamide is a thiol oxidizing agent that rapidly reduces the intracellular pool of glutathione (GSH) (Kosower and Kosower 1995). GSH is an abundant thiol tripeptide that acts as a ROS scavenger, among its other roles in the cell. We next tested whether supplementing the growth media with reduced glutathione would alleviate αSyn toxicity. We did not observe an alleviation of toxicity in cells expressing αSyn when they were treated with glutathione (Fig. 5.15).

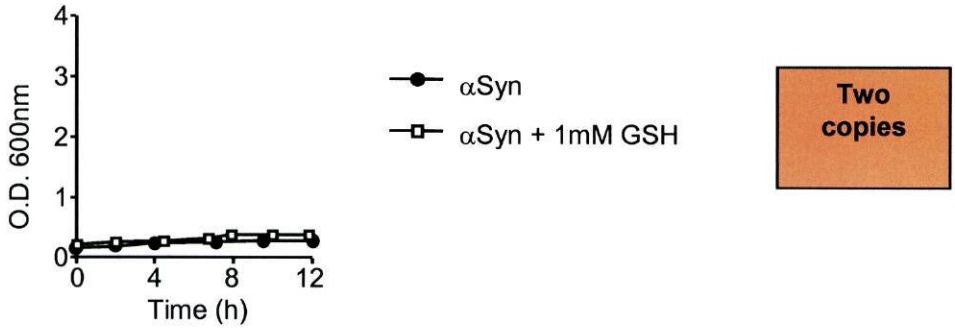


Figure 5.15. Glutathione does not rescue αSyn toxicity. Cells carrying two copies of αSyn were grown overnight in synthetic medium containing raffinose as the carbon source and then switched to galactose-containing medium. At this point, glutathione was added to a final concentration of 1mM and growth was monitored up to 12 hours after induction.

Lastly, we measured total levels of glutathione in the cells expressing αSyn. Cells carrying two copies of αSyn were grown in galactose media for 8 hours prior to harvesting. At this time point viability is still ~50% (Fig. 5.2). Interestingly, cells expressing αSyn WT or A53T accumulated ~4.5-fold higher levels of glutathione than control cells or cells expressing A30P αSyn (Fig. 5.16). This indicates that the cells detect oxidative stress and respond by increasing the levels of glutathione as a defense mechanism.

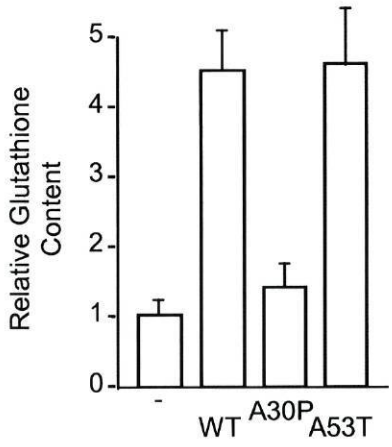


Figure 5.16. GSH levels are increased in cells expressing two copies of αSyn. Strains with two copies of αSyn were grown overnight in raffinose, switched to galactose cells were harvested at 3 hours. Relative levels of total glutathione levels are depicted relative to the level of cells not expressing αSyn. WT and A53T αSyn causes a ~4.5 fold increase in total glutathione levels when compared to control cells.

5.4 Discussion

PD is one of the most common neurodegenerative human disorders with a prevalence of about 2% after age 65. The majority of cases appear to be sporadic in nature (Gasser 2001). PD cases with specific genetic defects may account for fewer than 10% of all PD cases (Gasser 2001). αSyn is a protein of unknown function that is found in Lewy bodies and mutations in this protein cause familial PD. The recent finding that a triplication of the αSyn locus is associated with familial PD indicates the levels of αSyn are likely to be critical for the development of the disease (Singleton, Farrer et al. 2003). However, the molecular consequences of αSyn accumulation in cells have not been studied in detail. In this study we investigated how increased levels of αSyn affected essential processes in yeast, and established a connection between αSyn and exogenous factors that constitute risk factors for PD.

Interestingly, a simple duplication in the levels of αSyn in yeast results in a dramatic growth defect and reduction in survivorship. It is remarkable that the levels of αSyn have such a dramatic effect in the cells, especially because this seems to be conserved from yeast to humans (Singleton, Farrer et al. 2003).

Proteasome inhibition plays an important role in neurodegeneration (Keller, Gee et al. 2002; Dawson and Dawson 2003; Dimcheff, Portis et al. 2003). However, it is still controversial whether intermediates in the protein aggregation pathway lead to proteasomal impairment, or whether proteasomal impairment leads to protein accumulation, aggregation and ultimately to disease. Lewy bodies in PD are immunoreactive for ubiquitin, and proteasomal subunits are also found in Lewy bodies, hinting at a connection between αSyn inclusions and proteasomal inhibition that has since been confirmed by several groups (Lucking and Brice

2000; Rideout, Larsen et al. 2001; Lindersson, Beedholm et al. 2004). We and others showed that increased expression of αSyn may lead to proteasomal impairment. In the present study we highlighted types of proteasomal substrates that accumulate in the presence of higher levels (two fold higher) of αSyn. Notably, we found that the degradation of CPY* was impaired, suggesting an effect on ERAD. The growth defects could not be solely explained due to the depletion of the pool of free ubiquitin. Our data support the idea that dysfunction of the ubiquitin-proteasome system is not the sole cause for toxicity.

The function of αSyn is not known, but it has been implicated in trafficking of synaptic vesicles (Murphy, Rueter et al. 2000). In yeast we had also observed that it interferes with intracellular trafficking (Outeiro and Lindquist 2003; Willingham, Outeiro et al. 2003). Our present data establish that αSyn interferes with ER-Golgi trafficking, an essential process in the cell. This may lead to the problems in ERAD, explaining the deficient degradation of CPY* but not of cytosolic substrates, and may also be the cause of the general growth defects we observe. Our data, at this point, do not enable us to distinguish between problems in ER to Golgi trafficking versus retrograde Golgi to ER transport, but nevertheless, constitute a significant step towards the understanding of the nature of the adverse effects associated with increased intracellular levels of αSyn. Importantly, these data are in good agreement with other downstream consequences such as the increased sensitivity to ER stress and even ER breakdown. Interestingly, our results establish, for the first time, a direct connection between αSyn and ER stress, which may enable the development of novel avenues for intervention in PD.

Further evidence for a link between αSyn and cellular trafficking defects comes from the observation that αSyn causes Golgi fragmentation in Cos-7 cells (Gosavi, Lee et al. 2002). The ER and Golgi are adjacent organelles and our data suggest that the effects of αSyn may actually be exerted on the ER, leading to Golgi fragmentation. Alternatively, this difference could be due to intrinsic

differences between yeast and mammalian cells, because it is known that αSyn preferentially binds membranes rich in acidic phospholipids (Davidson, Jonas et al. 1998).

Oxidative stress is intimately connected with PD and other neurodegenerative disorders, such as AD, HD and even the prion diseases. Postmortem studies provide strong evidence for oxidative stress in the substantia nigra of PD patients. These studies include impaired mitochondrial function, iron accumulation, alterations in the levels of antioxidant defenses and oxidative damage of biomolecules, including αSyn (Dexter, Carter et al. 1989; Riederer, Sofic et al. 1989; Sofic, Lange et al. 1992; Giasson, Duda et al. 2000). Defects in dopamine metabolism are thought to be the main source of ROS in the nigra. αSyn also seems to enhance the susceptibility to oxidative stress (Hsu, Sagara et al. 2000; Ischiropoulos and Beckman 2003), but the pathways through which this protein leads to cell death remain elusive.

A recent study showed that overexpression of αSyn in human dopaminergic cell lines (SH-SY5Y) increases the levels of ROS in the cells (Junn and Mouradian 2002). The effect on ER-Golgi transport may also explain the increased levels of ROS in cells expressing αSyn WT and A53T. The ER provides, under normal circumstances, the appropriate environment for oxidative protein folding. Oxygen is used as the terminal electron acceptor, which can lead to the production of ROS and oxidized glutathione, causing the cells to respond by producing higher levels of glutathione. Alternatively, αSyn oligomers and/or aggregates may accumulate directly in the mitochondrial membrane, causing leakage of ROS and leading to mitochondrial dysfunction, as has been proposed by Hashimoto et al. (Hashimoto, Rockenstein et al. 2003). This would explain the growth defect in non-fermentable carbon sources, and also the increased levels of the scavenger glutathione.

In a recent genome-wide screen aimed at identifying cellular functions needed to maintain cellular viability to ROS, five different oxidants were used in conjunction with the yeast gene deletion set (Thorpe, Fong et al. 2004). This study implicated a large number of genes in ROS resistance, most of which had not been connected to this type of response in the past. Diamide was shown to require vacuolar protein sorting function (Thorpe, Fong et al. 2004), and cells expressing non-toxic levels of αSyn showed increased sensitivity to this oxidant, again establishing a connection between intracellular trafficking and αSyn. Our finding that cells expressing αSyn grew better in the presence of paraquat was intriguing at first, but the recent report that αSyn overexpression protects against paraquat-induced neurodegeneration (Manning-Bog, McCormack et al. 2003) allows us to hypothesize that increased levels of αSyn may actually be protective to this pesticide.

In summary, our results shed light on the molecular consequences of increased levels of αSyn in the cell, and establish important connections between αSyn and several pathways that have been linked to PD by mechanisms that did not involve αSyn. This work emphasizes the importance of αSyn in PD and other synucleinopathies, and opens novel opportunities for therapeutic intervention.

CHAPTER 6

CONCLUSIONS

CHAPTER 6. GENERAL CONCLUSIONS

Protein misfolding and aggregation have been linked to several neurodegenerative disorders but the molecular events that dictate the selective neuronal cell death underlying the clinical symptoms of each disease are unclear. The genes involved in each disease were discovered very recently and not all of them are known as of today. Once the first genes were identified model organisms were developed. While animal models have provided tremendous insight into these diseases, a clear understanding of the molecular mechanisms and cellular pathways involved is still lacking.

The present work establishes the yeast *Saccharomyces cerevisiae* as a model organism for studying the basic biology underlying diseases of protein misfolding, such as Parkinson's disease and Huntington's disease, and provides important insight into cellular pathways involved in these diseases.

Our initial goal was to take advantage of yeast genetics to develop a simplified cellular model for studying αSyn, a major player in PD which is found misfolded in intracellular inclusions called Lewy bodies. Removing the protein from its natural context, i.e., neuronal cells, and introducing it into yeast, a more tractable and manipulable eukaryotic cell type, enabled us to learn about general properties of the protein that are not dependent on cell line-specific partner-proteins. By making use of αSyn-GFP fusions it was possible to monitor its distribution in the cell, *in vivo*, under different experimental conditions. Interestingly, we discovered that a simple two-fold increase in the dosage completely altered the distribution of αSyn in the cell and caused cellular toxicity.

Strikingly, individuals carrying a triplication of the αSyn gene develop familial forms of PD (Singleton, Farrer et al. 2003). This indicates that a slight increase in the levels of αSyn is indeed sufficient to give rise to the toxic events that underlie the onset of the disease. The correlation between our studies in yeast and human cases of PD validates our approach. We also verified that high levels of αSyn expression were associated with proteasomal impairment. The hypothesis that disease takes place when the cellular quality control mechanisms (chaperones and ubiquitin-proteasome pathway) fail or become overwhelmed by doubling the level of expression of αSyn was tested. Under these conditions, αSyn accumulated in cytoplasmic inclusions and was recruited away from the plasma membrane, where it can be found at lower, non-toxic levels. Exploring the power of yeast genetics, we confirmed that αSyn inhibits PLD. This effect on PLD had been proposed a few years back in a search for PLD inhibitors (Jenco, Rawlingson et al. 1998) but its significance was not clear. Our results suggest this effect on PLD may be part of the normal function of αSyn and also that different mutants have different abilities to interfere with PLD activity. We also showed that high levels of αSyn caused accumulation of neutral lipids, in lipid droplets. Recent studies have established a connection between lipid association and oligomerization of αSyn (Sharon, Goldberg et al. 2001; Sharon, Bar-Joseph et al. 2003). Secondary structure-predictions suggest αSyn shares similarity to lipid-binding proteins, such as apolipoprotein A2. Our results allow us to speculate that the interference with PLD activity and the accumulation of lipids may, at least in part, contribute to toxicity. The function of αSyn is unknown but studies by other groups suggest it may be involved in vesicular trafficking to the synapse (Murphy, Rueter et al. 2000). Interestingly, yeast cells expressing two-copies of αSyn had defects in intracellular trafficking. We found that the disease-associated mutant A30P behaved significantly different when compared to WT or A53T αSyn, which behaved very similarly among themselves. The A30P mutant does not associate with membranes to the same extent, does not form

inclusions, does not cause PLD inhibition, and does not cause accumulation of lipids. This different behavior of the A30P mutant suggests it may cause disease through a different mechanism.

We next took advantage of the yeast PD model to perform a synthetic lethal screen to identify genes whose absence exposed αSyn toxicity. In parallel, we performed a similar screen with a yeast model for HD previously established. The aim was to compare the pathways involved in the toxicity of both diseases. Our ultimate goal was to focus on cellular pathways more than on specific genes, as the former are more likely to be conserved between yeast and humans than the latter. Our screens showed that genes involved in intracellular trafficking and lipid metabolism were important modifiers of αSyn toxicity and those involved in protein folding and protein degradation modified huntingtin toxicity. Interestingly, our screens were enriched in genes with human orthologs, suggesting we may have discovered cell autonomous genes relevant for PD and HD. Our results are now being tested in mammalian cell lines (through siRNA) and animal models for these diseases.
An independent study using a Drosophila model of PD has implicated trafficking and lipid metabolism as being involved in the PD-like pathogenesis in the flies (Scherzer, Jensen et al. 2003). Similarly, a study using a Drosophila model of a polyglutamine disease (SCA1) identified genes involved in protein folding and protein clearance as modifiers of ataxin-1 toxicity (Fernandez-Funez, Nino-Rosales et al. 2000). These similar results with the yeast and Drosophila models indicate the molecular basis for cellular toxicity is conserved from yeast to flies, suggesting it may be determined by cell-line independent mechanisms that can be studied in simpler eukaryotic models.

The yeast system enabled us to perform a drug screen to identify suppressors of αSyn or huntingtin toxicity. We screened a small collection of 1040 drugs. A

consortium of 27 laboratories used the same library with different types of assays (in vitro and cellular assays) for different neurodegenerative disorders, with the goal of identifying relationships among neurodegeneration mechanisms since many of these diseases seem to be associated with protein misfolding and aggregation. The αSyn screen identified a set of compounds with marginal activity, and we are currently performing experiments to elucidate their mechanisms of action. The huntingtin screen did not identify any active compounds in the collection but it was only performed at a single concentration, which might explain our results. The simplicity of the yeast system enables adaptation to high throughput screens, in which hundreds of thousands of drugs can be easily assayed. We have already started such screens and the hits will then be tested in the different assays (cellular distribution, lipid accumulation, trafficking, ROS accumulation). The effects of each drug-screen hit can then be categorized and the contribution of each cellular pathway to toxicity can be assessed. Finally, the cross-platform approach identified several bioactive molecules as being active in different models of the same disease, holding great promise for the treatment of these neurodegenerative diseases.

One of the main objectives of this work was to study the biological effects of different levels of expression of αSyn (one copy vs. two copies), especially those relating to the toxicity. Such knowledge should provide novel avenues for therapeutic intervention in PD and other synucleinopathies. αSyn is thought to be involved in several processes, including trafficking to the synapse (Murphy, Rueter et al. 2000). Our studies showed that αSyn impaired ER to Golgi trafficking and caused the accumulation of ERAD substrates. Impairment of ER to Golgi trafficking was also associated with increased sensitivity to ER stressors, such as DTT and tunicamycin. ER breakdown was also observed in cells expressing two-copies of αSyn. Interestingly, Golgi fragmentation is observed in mammalian cells that accumulate αSyn oligomers (Gosavi, Lee et al. 2002),

suggesting similar types of effects also occur in yeast cells. We then investigated whether there was a connection between αSyn accumulation and oxidative stress, as this seems to be intimately related to PD. We found that expression of low levels of αSyn increased the sensitivity of the cells to several types of oxidants, while it protected against others. These effects seem to correlate with recent results from other groups where different cellular pathways were involved in the sensitivity to different oxidants (Thorpe, Fong et al. 2004). In cells expressing two-copies of αSyn we detected an oxidative stress response, with increased accumulation of ROS and higher levels of GSH. This response is perhaps not too surprising since we showed a strong effect of αSyn on ER homeostasis. Oxidative protein folding takes place in the highly optimized environment of the ER where ROS can be readily generated. It will, therefore, be important to dissect the origin of the ROS that accumulate in the presence of two-copies of αSyn. The yeast system enables straightforward experiments to be designed in order to try to address these issues.

In order to develop novel and more effective therapeutic approaches for neurodegenerative diseases associated with protein misfolding, a deeper understanding of the molecular pathways involved is needed. It is also important to investigate at the cellular level what the toxic species really are: the large fibrillar aggregates, oligomeric intermediates, or other as of yet unidentified species. The studies described herein, using yeast models of PD and HD, provide insight into some of those pathways. This knowledge may also enable the development of novel and better animal models for these diseases, which should ultimately contribute to the opening of novel areas for therapeutic intervention.

Our studies of protein misfolding diseases in yeast are by no means an end, but rather constitute a powerful way to begin to dissect the molecular basis of neurodegenerative disorders.

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