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Biochimica et Biophysica Acta 1783 (2008) 1381 – 1395
BBA Biochimica et Biophysica Acta
www.elsevier.com/locate/bbamcr

Review

Protein folding diseases and neurodegeneration: Lessons learned from yeast

Joris Winderickx [a,*], Charlotte Delay [a,b], Ann De Vos [a], Harald Klinger [a], Klaartje Pellens [a], Thomas Vanhelmont [a], Fred Van Leuven [c], Piotr Zabrocki [a]

[a] *Functional Biology, Katholieke Universiteit Leuven, Kasteelpark Arenberg 31, B-3001 Leuven-Heverlee, Belgium*
[b] *Université Lille 2, Faculté de Médecine, Institut de Médecine Prédictive et de Recherche Thérapeutique, Centre de Recherches Jean-Pierre Aubert, Place de Verdun, 59045 Lille, France*
[c] *Experimental Genetics Group, Katholieke Universiteit Leuven, B-3001 Leuven-Heverlee, Belgium*

Received 31 October 2007; received in revised form 23 January 2008; accepted 24 January 2008
Available online 11 February 2008

Abstract

Budding yeast *Saccharomyces cerevisiae* has proven to be a valuable model organism for studying fundamental cellular processes across the eukaryotic kingdom including man. In this respect, complementation assays, in which the yeast protein is replaced by a homologous protein from another organism, have been very instructive. A newer trend is to use the yeast cell factory as a toolbox to understand cellular processes controlled by proteins for which the yeast lacks functional counterparts. An increasing number of studies have indicated that *S. cerevisiae* is a suitable model system to decipher molecular mechanisms involved in a variety of neurodegenerative disorders caused by aberrant protein folding. Here we review the current knowledge gained by the use of so-called humanized yeasts in the field of Huntington's, Parkinson's and Alzheimer's diseases.
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Keywords: Neurodegenerative disease; Huntington; Parkinson; Alzheimer; Yeast; Protein folding

1. Introduction

The baker's yeast *Saccharomyces cerevisiae* has proven its utility for clarifying fundamental cellular and molecular processes. Apart from being a useful toolbox to study both physical and genetic interactions between proteins, this unicellular eukaryote allowed to gain insight in basic cellular mechanisms such as DNA replication, recombination, cell division, protein turnover and vesicular trafficking. It also contributed to our understanding of nutrient- or stress-induced signal transduction, the coordinated regulation of metabolic and cellular adaptations, the cell cycle progression and mechanisms involved in longevity and cell death. The general picture that evolved from these studies shows that key cellular processes are well conserved between yeast and higher eukaryotes, including humans. In addition, upon publication of the yeast and human genomes it became clear that approximately 31% of the yeast genes have a mammalian homologue and an additional 30% of yeast genes show domain similarity [1]. Consistently, about 30% of the genes known to be involved in human diseases may have a yeast orthologue [2,3]. These observations paved the way for a rebirth of the use of yeast in medicinal and medical research. While initial experiments focused on classical complementation assays to elucidate the biological role of human proteins that have a yeast counterpart, a new trend brings the development of yeast cell-based assays or so-called humanized yeast systems to study functional aspects of proteins that do not have a yeast homologue [4].

In this review, we focus on recent contributions made by yeast systems to resolve fundamental questions on the pathogenic role of human proteins in neurodegenerative diseases. To date, several neurological disorders have been modeled in yeast. However, only for Huntington's, Parkinson's and Alzheimer's disease did this consisted of humanized model systems, i.e. expression of human proteins that lack a yeast orthologue (Table 1). The data obtained with these humanized yeast models demonstrate that despite the lack of a nervous system in yeast,

* Corresponding author. Tel.: +32 16 321516; fax: +32 16 321967.
E-mail address: joris.winderickx@bio.kuleuven.be (J. Winderickx).

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doi:10.1016/j.bbamcr.2008.01.020

Table 1
Human neurological disorders modeled in yeast

Disease	Protein involved	Yeast orthologues	References
HD and PolyQ disorders	Huntingtin	No	This paper
PD and synucleinopathies	α-Synuclein	No	This paper
AD and tauopathies	Tau and APP	No	This paper
Amyotrophic Lateral Sclerosis (ALS)	SOD-1	Yes	[166]
Friedreich's Ataxia	Frataxin	Yes	[167]
Batten disease	CLN3 and PPT1	Yes	[168]
Niemann–Pick disease	NPC1	Yes	[169]
Hereditary Spastic Paraplegia (HSP)	AAA-proteases	Yes	[170]

HD, Huntington's disease; PD, Parkinson's disease; AD, Alzheimer's disease.

substantial insights on neurological disease mechanisms were made (Table 2).

2. Humanized yeast models for Huntington's disease and PolyQ disorders

2.1. Characteristics of Huntington's disease and toxic mechanisms in mammalian cells

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder mainly affecting the striatum and cortex of the brain. The disease has a prevalence rate of 5 to 10 persons per 100,000 and is characterized by uncontrolled movements, psychiatric disturbances and cognitive impairments that begin in mid-life and progress to death within 10–20 years of onset. The genetic defect underlying HD is an expansion of a CAG trinucleotide repeat encoding a polyglutamine domain (polyQ) within the N-terminal end of the Huntingtin protein (Htt). In unaffected individuals, the CAG-repeat number in Htt varies between 11 and 34, while in HD this number increases to more than 35 repeats.

Htt is a multidomain protein in which the N-terminal half consists of the polyQ stretch followed by a proline-rich domain and ten HEAT repeats, a characteristic protein–protein interaction motif. The protein plays an essential role in cell survival and is distributed in various subcellular regions where it is believed to fulfill a scaffolding function [5]. Htt was indeed found to interact via its N-terminal segment with a variety of proteins involved in intracellular trafficking, endocytosis, metabolism, and gene transcription [6–8]. Notably, several of the Htt interaction partners have homologous counterparts in yeast [6,9].

Because of the essential function of Htt, the CAG expansion is often described as a loss-of-function alteration. Nonetheless, the expansion also confers a newly gained toxic function resulting in nuclear and cytoplasmic aggregation of the mutated protein and its proteolytic N-terminal fragments that encompass the polyQ domain [10]. It has been proposed that this toxic gain-of-function reflects unusual conformational changes that facilitate oligomer formation and aggregation, as well as altered protein–protein interactions [7,11–13]. However, the mechanisms underlying the neuronal death induced by polyQ expansions remain elusive. Due to the multitude of Htt-interactions, several scenarios have been proposed. Aggregation of Htt was proposed to sequester important transcription factors, such as the CREB-binding protein, CBP, or the neuron-restrictive silencing factor, REST-NRSF, thereby altering the transcription profile in susceptible neurons [10,14]. Others proposed that cell death induced by Htt aggregation mirrors the requirement of Htt for vesicular trafficking, a hypothesis that is reinforced by the recent discovery of a membrane targeting sequence at the very N-terminus and a palmitoylation site at cysteine 214 in Htt [15,16]. An anti-apoptotic function has also been ascribed to Htt and apart from aggregation-induced indirect influences triggering oxidative stress and mitochondrial dysfunction, the protein may also exert a more direct effect since Htt with an expanded polyQ domain is unable to bind to HIP-1, a protein that contains a death box and can activate caspase-8 when released from Htt [17–19].

In addition to the possibility that several pathways may trigger Htt-related cell death, it is still a matter of debate whether soluble oligomers and protofibrils, or the aggregated polyQ peptides are the inducers of toxicity [20,21]. Accumulating evidence favors a toxic role of prefibrillar intermediates and suggests that the fibrillar aggregates are inert and may even have a cytoprotective function. One example related to the latter is a report showing that the mammalian target of rapamycin, mTOR, is sequestered into Htt aggregates, which would lower mTOR activity and thereby induce autophagy, a known clearance pathway for toxic Htt fragments [22].

2.2. Yeast models for Htt-induced toxicity and apoptotic cell death

Several research groups have developed yeast models to study the folding and behavior of proteins with an expanded polyQ domain. These yeast systems usually express polyQ N-terminal peptides of human Htt as fusions or tagged proteins. The first models already demonstrated that heterologous expression of Htt results in a polyQ length-dependent aggregation in yeast [23–25]. Interestingly, in only one of these models the aggregation of Htt-derived fragments triggers toxicity and reduced growth. The reason for this is dual. On one hand, it was shown that toxicity upon expression and aggregation of expanded polyQ peptides in yeast requires the protein Rnq1 in its prion conformation [25]. As such, this observation indicated that the aggregation of Htt fragments is not toxic *per se* and it also suggested that the aggregation of polyQ peptides and of the prion form of Rnq1 share similar features that would allow interference with each other. However, so far no evidence for mixed co-aggregation of polyQ fragments and prions has been provided. Nonetheless, aggregates of Htt and prions appear to have a common β-helical amyloid core structure [26,27] and it has been shown that aggregated expanded polyQ peptides do promote prion formation of Sup35 in yeast [28]. Moreover, a very recently performed compound screen in a zebrafish model of HD led to the identification of two anti-prion compounds as efficient inhibitors of polyQ aggregation [29]. On the other hand, toxicity of aggregated polyQ peptides in yeast strongly depends on the sequence context of the polyQ domain [30]. Particularly, the presence of the endogenous proline-rich

Table 2
Selected articles

Topic	Description	References
Background on the disease		
General	Protein misfolding and neurodegenerative diseases	[21]
	Role of oxidative stress and mitochondrial dysfunction in neurodegeneration	[171]
HD and PolyQ disorders	Molecular pathogenesis of HD and other trinucleotide repeat disorders	[10,59]
PD and synucleinopathies	Genetics and pathways underlying PD	[63,68,75]
AD and tauopathies	Genetic components of AD and the role of protein tau in neurodegeneration	[128,142]
Yeast models		
General	Yeast as a tool for medical and medicinal research	[4]
	Advantages of yeast-based drug discovery screenings	[165]
	The apoptotic machinery in yeast	[164]
Huntington models	Molecular determinants for toxicity of expanded polyQ peptides in yeast	[31]
	Expression of toxic polyQ peptides and apoptotic cell death in yeast	[35]
Parkinson models	Expression of α-syn and blockage of ER-to-Golgi vesicular traffic	[104]
	Functional mitochondria and α-syn toxicity in ageing yeast	[121]
Alzheimer models	Hyper-phosphorylation, conformational changes and aggregation of protein tau in yeast	[135]
	Yeast-based reporter system to monitor the oligomerization of Aβ42-fusions	[162]

domain converts the expanded polyQ peptides into nontoxic proteins, even when present in *trans* on a co-expressed Htt variant with a polyQ domain of normal length [30–32]. This suggests that the proline-rich domain may serve to recruit other proteins that shield Htt from perturbing essential cellular functions.

Recent studies connected Htt-toxicity to apoptotic cell death. Similar to neurons [33], expression of expanded polyQ peptides in yeast was reported to reduce the respiratory capacity due to impairment of the mitochondrial respiratory chain complexes II and III [34]. Consistently, aggregation of polyQ peptides in yeast coincided with apoptotic changes in mitochondria, caspase activation, nuclear DNA fragmentation and apoptotic cell death [35]. This study also showed the accumulation of the expanded polyQ peptides in the nucleus at longer times after induction and interestingly, this nuclear translocation required the functional yeast metacaspase Yca1 [35].

Both toxic and nontoxic polyQ constructs were used to identify proteins that influence Htt aggregation and toxicity in yeast. Several groups showed that aggregation and toxicity of expanded polyQ peptides depends on the activities of the molecular chaperone Hsp104 and family members of Hsp70 and Hsp40 [23,25,32,36,37]. This observation is particularly interesting because Hsp104 is known to recycle proteins from previously formed aggregates, a function it exerts with the assistance of the Hsp70 protein, Ssa1, and the Hsp40 protein, Ydj1 [38]. In agreement, the overexpression of Hsp104 and Ssa1 were found to completely suppress the growth defect associated with expression of toxic polyQ peptides in yeast [32,37]. Although mammalian cells do not encode for an Hsp104 homologue, it is now established that expression of yeast Hsp104 allows for relief of polyQ aggregation and toxicity in mammalian cells as well as in neurons of transgenic mice and rat models of Huntington's disease [39–41]. Moreover, expression of the yeast Hsp104 protein in human leukemic T-cells inhibited heat shock-induced apoptotic signaling [42]. So far, the influence of Hsp104 and its co-chaperones on Htt-induced apoptosis in yeast has not been studied. However, Hsp104 in yeast was reported to be required for replicative life span extension induced by a transient nonlethal heat shock [43] as well as for conformational repair of heat-denatured proteins in the endoplasmic reticulum (ER), which would otherwise cause a sustained unfolded protein response (UPR), oxidative stress and cell death [44–46]. Trehalose, which in concert to Hsp104 allows yeast cells to acquire stress tolerance, is also involved in conformational repair of heat-denatured proteins in the ER [47]. As such, it does not come as a surprise that oral administration of this disaccharide to transgenic mice or expression of the bacterial trehalose synthase genes in mammalian cells not only reduced the number of polyQ aggregates but also exerted beneficial effects on cell viability and life span [48,49]. Most recently, trehalose was shown to act through activation of autophagy in a mTOR-independent manner [50].

Consistent with the observation described above that polyQ fragments of normal length interfere with the toxicity of expanded polyQ peptides, other glutamine-rich proteins also affect polyQ toxicity and are able to convert toxic polyQ peptides into nontoxic ones and vice versa [31]. Interestingly, the glutamine-rich proteins used in this study are not essential for yeast viability and in case the proteins aggregated themselves when overexpressed, the aggregates were not toxic. Hence, the interconversion of a nontoxic to toxic polyQ aggregate cannot solely be explained by sequestration of the glutamine-rich proteins, but must involve other factors and mechanisms.

The yeast models also allowed establishing a connection with endocytosis. In wild type cells, polyQ aggregation led to a rapid cessation of endocytosis, while in mutants affected in early endocytic events, polyQ toxicity is enhanced [51]. A recent follow-up study proposes that the endocytic machinery, at steps of maturation of the forming endocytic vesicle, is itself involved in the process leading to aggregation of polyQ peptides in yeast as well as in mammalian cells [52].

2.3. Yeast-based screenings to identify Htt-toxicity modifier proteins and chemicals

Undoubtedly, the power of yeast lies within its versatility to perform genomic screenings (Fig. 1). Two such screenings have

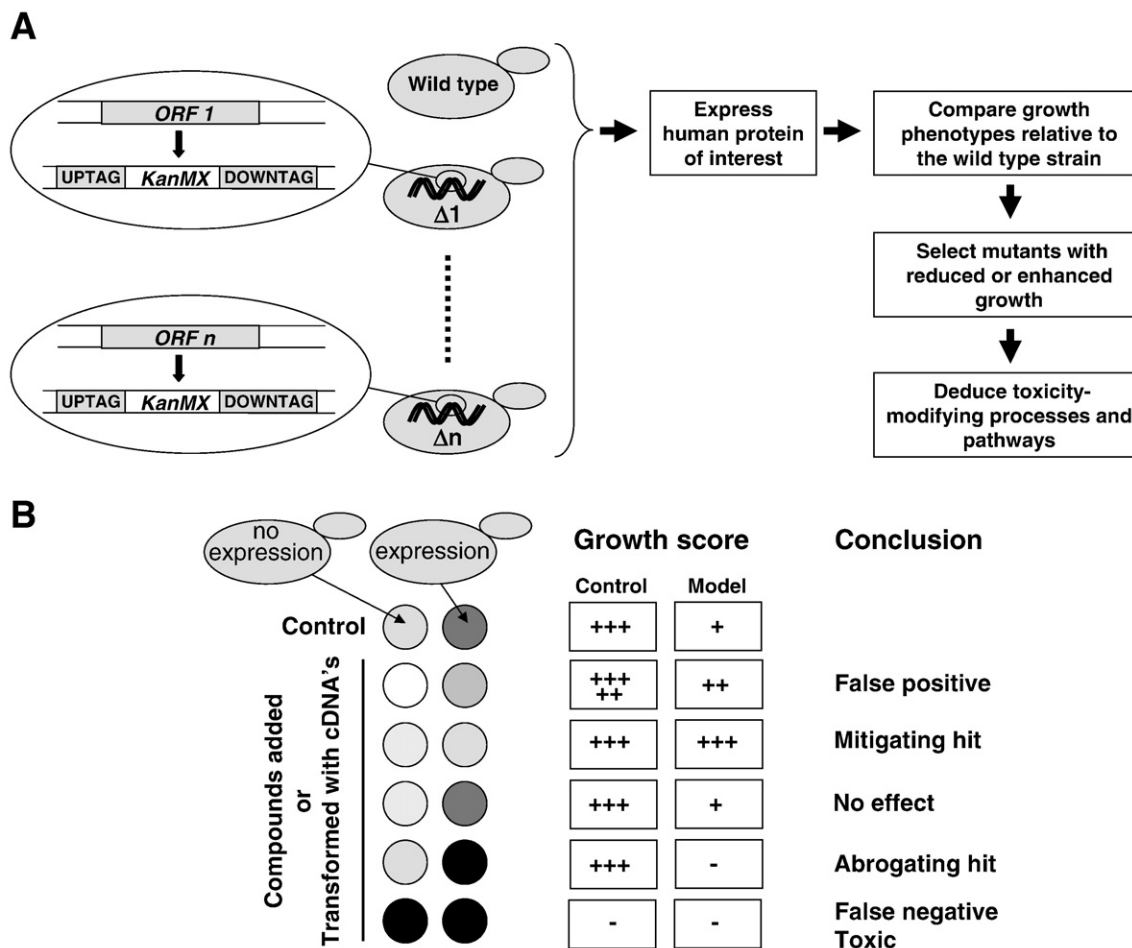


Fig. 1. Global strategies to identify putative targets and lead compounds. A. Synthetic growth-phenotype screening using the genome-wide collection of viable yeast deletion mutants. In this collection, each yeast open reading frame (ORF) has been replaced with a cassette containing unique tags and the selectable marker gene *KanMX*, which confers geneticin resistance. After transformation of the whole collection and the wild type strain with a plasmid allowing for inducible expression of the (toxic) human protein, e.g. Htt, the transformants are grown under non-permissive and permissive conditions. Then, the growth ratios are calculated and a comparison is made relative to the growth ratio obtained for the wild type strain. Mutants are selected that display reduced or enhanced growth as compared to the wild type strain specifically under permissive conditions. This synthetic phenotype suggests a functional relationship between the human protein under investigation and the yeast protein that is absent in the mutant. B. Chemical compound or cDNA screenings can be performed in the wild type or mutant strains using a similar strategy as described above. In this case, chemicals or cDNAs are selected that improve or reduce growth only in cells expressing the toxic human protein under investigation. A compound or a cDNA is considered false positive or negative if it also affects growth to a similar extent in cells that do not express the protein under investigation.

been done. In the first screening, nontoxic expanded polyQ constructs were used to transform the collection of 4850 haploid deletion strains and select mutants with enhanced toxicity [53]. This yielded 52 mutants that, based on their deficiency, revealed a specific enrichment of the functional categories of protein folding and the ubiquitin–proteasome system (UPS) as well as stress response, thereby confirming some of the data described above. Interestingly, this screen also identified strains lacking proteins required for the redox/ROS stress response in yeast, such as glutathione synthase, Gsh2 and the flavohemoglobin, Yhb1. These proteins are known to be induced under conditions that challenge the normal mitochondrial function [54,55]. Hence, in the absence of this defensive response, the otherwise inert polyQ aggregates appear to become toxic. This suggests that the mere presence of nontoxic polyQ aggregates in wild type cells already triggers an increased oxidative stress but to a level that can still be handled by the cellular defense mechanisms. The second genomic screening made use of a toxic expanded polyQ construct and was aiming to identify yeast mutants with reduced toxicity [56]. Twenty-eight strains were identified and interestingly, most of them still contained the polyQ aggregates despite the suppression of toxicity. This is again consistent with the observation of polyQ aggregates not being sufficient to induce a high level of toxicity. The functional categories that were enriched in this screening included vesicular traffic and vacuolar protein sorting, transcription regulation and known or putative yeast prions. Once more, this is in line with data described above. Of particular interest was the identification of the kynurenine 3-monooxygenase, Bna4, in this screen. This enzyme functions in the kynurenine pathway, which is a well conserved route for tryptophan degradation and synthesis of NAD^+ in eukaryotes. Imbalances in the kynurenine

pathway leading to increased oxidative stress have been described in animal HD models and HD patients [57]. Similarly, increased intermediate kynurenine pathway metabolites and enhanced ROS levels were observed upon expression of toxic polyQ peptides in wild type yeast cells, but not in cells carrying a *BNA4* deletion or cells treated with Ro 61-8048, a pharmacological inhibitor of the mammalian kynurenine 3-monooxygenase [56]. This confirms the conservation of the mechanism linking polyQ toxicity to the kynurenine pathway and the generation of ROS between yeast, mammalian cells and individuals with HD.

In addition to genetic screenings, the yeast models have been used as a cell-based high-throughput screening system to find chemical compounds that inhibit polyQ aggregation and toxicity. This led to the identification of a lead compound that is a structural analog of kynurenine 3-monooxygenase inhibitor Ro 61-8048 [58]. Although identified in yeast, this drug also suppresses neurodegeneration in a fly model for HD.

2.4. Other PolyQ disorders

To date, several polyglutamine diseases have been described and the expansion of CAG repeats has been documented in genes such as the androgen receptor, which causes Spinobulbar Muscular Atrophy also known as Kennedy Disease, atrophin-1, which is linked to Dentatorubral-Pallidoluysian Atrophy or DRPLA, and several ataxin genes, which underlie several types of Spinocerebellar Ataxias [59]. Hence, it is clear that progress made in yeast on deciphering the molecular basis of HD will have a direct impact on our understanding of the disease mechanisms involved in these other PolyQ disorders as well. Finally, it should be mentioned that apart from studies linking aggregation of polyQ peptides to toxicity as described above, the yeast system is additionally used to elucidate the mechanisms that lead to expansion of trinucleotide repeats [60,61].

3. Humanized yeast models for Parkinson’s disease and synucleinopathies

3.1. Pathogenesis and genetics of Parkinson's disease

Parkinson’s disease (PD) is the second most common neurodegenerative disease with an age-associated prevalence of approximately 1.6% at 65 years and 4–5% at the age of 85. The disease is clinically characterized by motor deficits such as resting tremor, rigidity, bradykinesia and postural instability. The neuropathological hallmark of PD consists of a progressive degeneration of dopaminergic neurons of the *substantia nigra pars compacta* and the presence of eosinophilic cytoplasmic inclusions called Lewy bodies (LBs) and Lewy neurites [62].

Although environmental factors have been implicated in the development of the disease, accumulating evidence shows that different genetic factors are involved. Tremendous progress has been made over the last few years in the identification of genes underlying rare familial forms of PD. So far, at least 6 genes have clearly been linked to familial PD. Mutations in α-synuclein (α-syn) and LRRK2/dardarin cause autosomal dominant forms of PD, while mutations in parkin, DJ-1, PINK1 and ATP13A2 cause autosomal recessive forms of PD. For other genes, such as the ubiquitin carboxy-terminal esterase L1, UCH-L1, the mitochondrial serine protease Omi/HtrA2 or the synaptic protein Synphilin-1, the association to PD is less clear and often based on single-family reports [63–66]. The different genes function in a number of molecular pathways that link neuronal degeneration, as seen in PD, to protein misfolding, lysosomal and proteasomal protein degradation as well as oxidative stress and mitochondrial dysfunction (Fig. 2) [63,67,68].

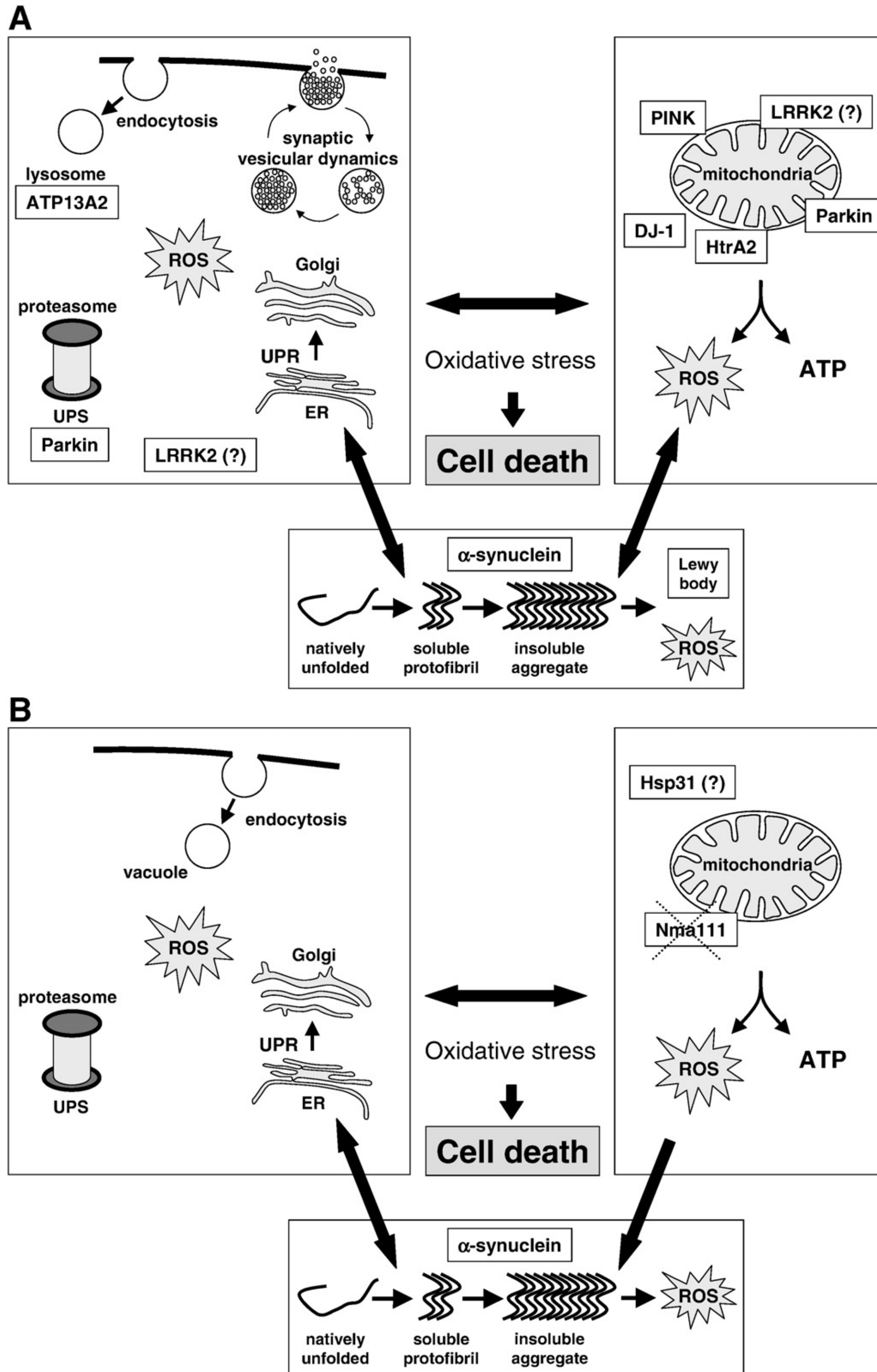
Mutations in parkin cause the most common form of juvenile hereditary PD. Parkin was shown to be an E3 ubiquitin-ligase able to catalyze both poly- and monoubiquitination. Consistently, parkin not only marks specific substrates for degradation, but it can as well control trafficking and sorting of proteins. Moreover, several observations made in cellular and transgenic models suggest a role for parkin in mitochondria, a hypothesis that is strengthened by the finding of an interaction with PINK1, both physically and genetically [69,70]. PINK1 or the PTEN-induced kinase 1 is a serine/threonine kinase located in mitochondria that appears to exert a protective cellular function presumably through phosphorylation of specific mitochondrial proteins. Although the exact pathophysiological role of PINK1 is not yet clear, disruption of this kinase in *Drosophila* was shown to coincide with increased susceptibility to oxidative stress and mitochondrial morphological defects in testis, muscle and dopaminergic neurons. These changes ultimately result in apoptosis of muscle cells and a gradual loss of dopaminergic neurons. Interestingly, the phenotypes induced by loss of PINK1 were found to be rescued by overexpression of parkin, while conversely, phenotypes induced by loss of parkin were not restored by overexpression of PINK1 [70,71]. This confirms that parkin acts as an effector of PINK1 in a linear pathway affecting mitochondrial function. Some studies reported a physical interaction of parkin and PINK1 with DJ-1 [72,73]. DJ-1 is a member of the DJ-1/ThiJ/PfpI superfamily that also includes the yeast protein Hsp31, which was recently shown to confer protection against ROS [74]. The protein has been assigned very diverse functions, but its presumed anti-oxidative properties and chaperone activity are probably the most relevant for PD. However, the exact physiological role of DJ-1 in brain and more specifically in neurodegeneration remains elusive.

Mutations in LRRK2 or dardarin are considered to be the most common known genetic causes of sporadic PD [75]. LRRK2 contains a leucine-rich repeat, a kinase domain, a RAS domain and a WD40 domain. This multidomain structure may explain the pleomorphic pathology associated with the different PD-associated mutations spread across the LRRK2 protein domains. However, despite the vast amount of genetic data linking the protein to PD, almost nothing is known about the exact function or the role of this protein in the pathophysiology leading to PD. Interestingly, mutations localized to the GTPase domain, i.e. R1441C or the kinase domain, i.e. G2019S or I2020T, appear to increase the kinase activity of LRRK2, favoring a gain-of-function [76,77]. Noteworthy, overexpression of the gain-of-function G2019S mutant in rats was reported to enhance apoptosis of dopaminergic neurons [78]. Moreover, LRRK2 localizes at membranous and vesicular structures, including mitochondria [79] and *in vitro* studies further demonstrated an interaction with

parkin [80]. Hence, LRRK2 may also have a role in mitochondrial physiology.

Finally, ATP13A2, encodes for a lysosomal P-type ATPase. PD-associated mutations in ATP13A2 appear to cause a loss-of-function as the protein no longer localizes at the lysosome [67]. The role of this ATPase in neuropathology is not known, but the putative lysosomal dysfunction caused by loss of ATP13A2 may have important consequences for clearance of protein aggregates and organelles, such as damaged mitochondria.

Although the proteins mentioned above are found in Lewy bodies, a major constituent of these inclusions is fibrillated α-syn. To date, three missense mutations (A30P, A53T and E46K)



as well as the duplication and triplication of the α-syn locus are associated with familial forms of PD [81]. α-Syn is a presynaptic protein that is apparently involved in many cellular processes. Although its exact function is still not clear, several observations suggest a role as regulator of dopamine neurotransmission and synaptic vesicular recycling [82]. Most recently, α-syn was suggested to ameliorate complex assembly between plasma membrane and vesicular SNARE proteins [83] and to have a role in vesicle priming at a step before calcium-dependent vesicle membrane fusion [84]. One study reported localization of α-syn at the mitochondrial membrane [85], providing a possible direct link between α-syn, impaired mitochondrial function and increased oxidative stress as seen in PD.

α-Syn is a small protein that contains a N-terminal amphipatic domain with six repetitions of the KTK(E/Q)GV motif, a hydrophobic central region and an acidic C-terminal region. Although the protein is natively unfolded, it exhibits environmentally-induced conformational plasticity and, as such, can assume monomeric and oligomeric α- and β-sheet conformations and form morphologically different types of aggregates, ranging from amorphous to amyloid-like fibrils [86]. The central hydrophobic region of α-syn plays an important role as facilitator of fibril formation, while the C-terminus seems to prevent fibrillation. The N-terminus also plays an important role by allowing formation of two α-helices upon binding to membrane microdomains, known as lipid rafts [86,87]. These helices comprise the residues from Val3 to Val37 and from Lys45 to Thr92. The A30P mutation was shown to prevent the unfolded to folded helix conformational change [88] and, consistently, this mutation also disrupts the raft association and abolishes the normal synaptic localization of α-syn [87]. The E46K and A53T were reported not to disrupt α-helix formation and while the former has a higher tendency to bind liposomes, the latter shows vesicle binding kinetics similar as wild type α-syn (WT-syn) [88]. Interestingly, the capacity of WT-syn and mutant α-syn to bind membranous compounds and form α-helices seems to correlate with the propensity of these proteins to aggregate in transfected SH-SY5Y cells [89].

α-Syn is predominantly non-phosphorylated under normal conditions but was found to be extensively phosphorylated in α-synucleinopathic lesions [90]. Especially phosphorylation of α-syn at Ser129 appeared to be a dominant pathological modification in *Drosophila* and mice models for PD [91–94]. Also in patients suffering from dementia with Lewy bodies or multiple system atrophy, Ser129 phosphorylation is specifically associated to α-syn aggregation [95,96]. Hence, Ser129 phosphorylation of α-syn may result in accelerated oligomerization and fibrillation of α-syn, a hypothesis that was further corroborated by *in vitro* results [90].

Similar as for Htt in HD, there is no consensus whether soluble protofibrils species or matured aggregates of α-syn incite neurotoxicity. The precise molecular events how this protein triggers cellular degeneration also remain elusive. Deregulation of the NMDA subtype glutamate receptor [97], malfunctioning of the ATP-sensitive potassium channels [98], vesicle permeabilization and leakage of dopamine metabolites leading to oxidative damage [99,100], alterations in fatty acid uptake and metabolism [101], binding to mitochondrial membranes and mitochondrial dysfunction [102], deregulation of ER-associated protein degradation (ERAD) and a sustained UPR [103,104], or malfunctioning of the UPS [105] have been suggested. In addition, aggregation of α-syn itself was shown to generate ROS *in vitro* and it was proposed that this could be sufficient to initially trigger the PD pathology *in vivo* [106]. Perhaps most puzzling in this discussion is the specificity by which α-syn induces neurotoxicity as exemplified by the observation that expression of WT-syn in human fetal dopaminergic neurons induces apoptosis, while its expression in non-dopaminergic human cortical neurons protects cells and increases neuronal survival [107].

3.2. Yeast models for α-syn aggregation and toxicity

When expressed in yeast, α-syn faithfully recapitulated some of the aspects previously reported based on studies performed in other model systems or on human brain. Consistent with their lipid and vesicle binding properties, WT-syn and the mutant A53T are delivered to the plasma membrane via the secretory pathway. Once these proteins accumulate at the plasma membrane they start to form inclusions. Also in yeast, inclusion development appeared to be a nucleation-elongation process involving the formation of small seeds at the plasma membrane, which at later stages are displaced but still continue to grow in size in the cytoplasm [108–110]. Moreover, some inclusions were thioflavin-S positive, indicating that they contained β-sheeted amyloidic fibrils [110]. Similar to observations made *in vitro* [111] and cellular systems [112,113], inclusion formation coincided with inhibition of phospholipase D, blockage of ER-to-Golgi transport and retarded endocytosis in yeast cells [104,108,110].

In contrast to WT-syn and A53T-syn, the A30P mutant remained predominantly dispersed throughout the cytoplasm and did not form inclusions [108–110]. This behavior is due to the poor membrane-binding capacity of the mutant since increasing

Fig. 2. Molecular mechanisms leading to cell death in neurons and the yeast PD model. Shown are the main pathways involved in α-syn-mediated cell death, i.e. α-syn aggregation, obstruction of vesicular traffic and protein degradation, and mitochondrial dysfunction. In both neurons (A) and yeast cells (B), aggregation of α-syn, which itself coincides with ROS formation *in vitro*, was reported to hamper vesicle dependent processes, such as endocytosis and ER-to-Golgi traffic, to induce the UPR and to block the UPS. Sustained induction of the UPR and impairment of the UPS in turn trigger the production of ROS. Furthermore, α-syn protofibrils cause vesicle permeabilization leading to disrupted homeostasis of dopamine metabolism, increased cytoplasmic dopamine levels, and enhanced oxidative stress in dopaminergic neurons. The production of ROS by the above described processes has adverse effects on mitochondrial function, and conversely, the oxidative stress induced by mitochondrial dysfunction is known to affect the UPS. In addition, a direct physical interaction of α-syn with the outer membrane of mitochondria was demonstrated and proposed as alternative link explaining mitochondrial dysfunction in dopaminergic neurons. Finally, increased oxidative stress enhances the aggregation of α-syn. Also shown are the different PD-associated proteins and their homologous counterparts in yeast, i.e. DJ-1 and its homologue Hsp31 as well as HtrA2 and its orthologue Nma111. Note that in contrast to the involvement of HtrA2 and DJ-1 in PD, studies in yeast could not confirm a role of Nma111 in α-syn-mediated apoptotic cell death and a protective function of Hsp31 has not yet been demonstrated. In addition, it is not yet known how LRRK2 causes neuronal loss in PD.

the lipid content by treatment of yeast cells with DMSO, allowed A30P-syn to form inclusions. Also, provision of the necessary nuclei by WT-syn allowed inclusion formation of co-expressed GFP-labeled A30P-syn [110]. Hence, these data indicate that the A30P mutant is mainly defective in nucleation because this process requires efficient membrane-binding. Along with the failure to form inclusions under normal growth conditions, the A30P mutant did not affect phospholipase D or endocytosis [108,110]. Possible effects of A30P-syn expression on ER-to-Golgi transport were not studied [104]. Interestingly, a recent study demonstrated the A30P mutant to be targeted by Ypp1 to the vacuole for degradation, while this was not the case with WT-syn or the A53T mutant [114]. Whether the failure of WT-syn and A53T-syn to bind Ypp1 and enter the vacuole directly relates to their obstructing effects on vesicular trafficking routes and the endocytic pathway remains to be clarified.

Although initial experiments established a link between the expression level of α-syn, inclusion formation and toxicity [108], later reports demonstrated that α-syn toxicity is mainly dependent on the genetic background used and that there is no strict correlation between the presence of α-syn inclusions and its effects on growth. As such, *S. cerevisiae* strains can be loaded with α-syn inclusions but display no significant α-syn-induced growth retardation, or conversely, show no signs of inclusions despite a high α-syn-induced toxicity [110,115]. A recent study analyzed the relationship between membrane affinity, fibrillization rate and toxicity of randomly generated α-syn mutants. Based on this analysis it was concluded that α-syn toxicity in yeast solely correlated with membrane-binding and the ability to form α-helices and not with the fibrillization rate of the α-syn mutant proteins [116]. A similar conclusion can be drawn from a study expressing α-syn in *S. pombe*. Unlike in budding yeast, WT-syn and A53T-syn did not target to the plasma membrane and despite extensive aggregation, the proteins were nontoxic to fission yeast cells [117]. In addition, analysis of a mutant deleted for amino acid residues in the hydrophobic central core of α-syn demonstrated this region to be essential to confer α-syn toxicity [118].

3.3. Yeast-based screenings to identify cellular processes associated with α-syn-induced toxicity and cell death

Several groups investigated which factors influence aggregation and/or toxicity of α-syn. The first genetic screening led to the identification of 86 yeast mutants with enhanced α-syn toxicity [53]. As could be expected, many of these mutants were affected in lipid metabolism and vesicular transport. In addition, several mutants were found that lacked functions involved in the ubiquitin–proteasome system, the defense against oxidative stress or mitochondrial activities. Interestingly, with the exception of the mutant lacking the transcription factor Stp2, there was no overlap in mutants retrieved from this screening and a parallel screening performed to identify toxicity modulators for Htt, which led the authors to suggest that distinct pathogenic mechanisms underlie HD and PD. However, as described below, some parallels can be drawn between the processes leading to toxicity of Htt and α-syn expression in yeast.

The link between proteasome inhibition on α-syn behavior was confirmed, as administration of the proteasome inhibiting drug lactacystin was reported to increase α-syn fibrillation [110] and deletion of proteasome cap or barrel proteins was found to enhance α-syn toxicity [115]. One study demonstrated the converse by showing that expression of α-syn induced small changes in proteasome composition resulting in impaired proteasome-mediated protein degradation, an effect more severe during the stationary phase. The same study also revealed α-syn-mediated inhibition of protein synthesis, a phenomenon the authors hypothesized to be linked to ER-stress [119]. ER-stress due to a sustained UPR was reported previously in neural models of PD [103] and was more recently confirmed in yeast as an early toxicity phenotype caused by expression of WT-syn or A53T-syn [104]. Interestingly, the search for modulators of α-syn toxicity led to the identification of several proteins involved in ER-to-Golgi transport, among which was the Rab GTPase, Ypt1. Subsequent examination of invertebrate and mammalian models of PD emphasized the general nature of this finding, since in these models overexpression of Rab1, the mouse homologue of Ypt1, protected dopaminergic neurons against α-syn toxicity [104].

Sustained ER-stress can be a source of ROS accumulation and oxidative stress, next to mitochondria [46]. Given the importance of oxidative stress in the pathology of PD, the yeast system was used to study in detail reciprocal effects between oxidative stress and α-syn toxicity. Induction of free radical generation by addition of ferrous or ferric ions was shown to enhance fibrillation and toxicity of α-syn in yeast [110,118]. In addition, the expression of WT-syn or mutant α-syn rendered yeast cells more vulnerable to peroxide-induced oxidative stress. The reason being that α-syn expression induced the production of ROS, externalization of phosphatidylserine and the release of cytochrome *c* from mitochondria, suggesting that α-syn triggers the apoptotic cell death program in yeast cells [120,121]. This is similar to observations made in fetal dopaminergic neurons [107]. Interestingly, yeast cells can be protected from α-syn-induced ROS accumulation by treatment with the reductant glutathione or the heat shock response activator geldamycin, as well as by a mild heat shock or overexpression of the Hsp70 chaperone Ssa3 [120]. The underlying mechanism for protection by heat shock was not resolved, but seems to involve binding of Ssa3 to α-syn. In addition, or alternatively, the protective heat shock-induced mechanism may involve Hsp104 and its co-chaperones, which as described above for Htt, may relieve cells from ER-stress. The effect of Hsp104 on α-syn toxicity has not been studied in yeast, but it was shown that the protein can decrease α-syn fibrillation *in vitro* [122,123]. In close connection, trehalose, which acts in concert to Hsp104 to provide stress resistance to yeast cells, was also found to shield mammalian cells from α-syn-induced toxicity and apoptosis [50]. As mentioned, trehalose activates autophagy synergistically to rapamycin. In yeast, rapamycin-treatment was reported to induce clearance of α-syn aggregates [110].

Protection against α-syn-induced ROS accumulation was also described to occur upon deletion of the yeast metacaspase, *YCA1* [120]. However, this is still controversial as others observed that deletion of *YCA1* did not ameliorate α-syn toxicity

but instead augmented α-syn-induced growth reduction [118]. Most recently, chronological ageing experiments confirmed that expression of α-syn enhanced apoptotic cell death in yeast independent of Yca1 and even of Omi1/Nma111, the orthologue of human HtrA2 [124], and this despite the clear involvement of mitochondria [121].

The yeast system was also used in a drug discovery program to identify compounds that would reduce α-syn toxicity. This led to the identification of quercetin and (−)-epigallocatechin-3-gallate, two flavonoids with metal chelating and radical scavenging properties [118]. Finally, it should be mentioned that co-expression studies with other PD-associated genes, i.e. DJ-1, parkin, PINK1, UCH-L1 or Synphilin-1, did not uncover significant effects on α-syn toxicity in yeast [116]. Note, however, that several of these proteins were shown to interact, suggesting that it may be necessary to co-express these proteins simultaneously before effects on α-syn toxicity can be observed. Nonetheless, enhanced toxicity was obvious when α-syn was co-expressed with protein tau, a microtubule-associated protein involved in Alzheimer's disease [110].

In conclusion, the yeast models presented above not only allowed researchers to gain fundamental insight on mechanisms of α-syn-mediated cellular degeneration (Fig. 2), but also proved to be valid high-throughput tools for identification of neuroprotective agents. More complex yeast systems should be constructed that incorporate other PD-associated genes in order to establish possible links with α-syn aggregation and toxicity.

4. Humanized yeast models for tauopathies and Alzheimer's disease

4.1. Introduction to tau-dependent neurodegenerative disorders

Tauopathies are multifactorial neurodegenerative disorders clinically characterized by dementia and/or movement dysfunction. Neuropathologically, these disorders are typified by the presence of intracellular inclusions consisting, at least in part, of insoluble aggregated tau. More than 20 tauopathies are known [125], among which Alzheimer's disease (AD) is the most prevalent, with currently an estimated 12 to 17 million affected persons and the projection that this number will double every 20 years [126]. In AD, tau-inclusions are presented as so-called neurofibrillary tangles (NFT) with protein tau assembled into paired-helical filaments (PHF), twisted ribbons or straight filaments [127].

Protein tau is a microtubule-associated protein responsible for the stabilization and spacing of microtubules and as such, important for the regulation of axonal transport. The protein is expressed as six isoforms derived from a single gene by alternative mRNA splicing. These isoforms differ by one or two additional insertions in the N-terminal domain (yielding the 0N, 1N and 2N isoforms, respectively) and by one additional copy of the microtubule-binding repeat (yielding 3R and 4R isoforms, respectively). The N-terminal inserts have as yet no defined function, while the additional C-terminal insert influences microtubule binding since tau-4R isoforms are more efficient in stabilizing microtubules than the tau-3R isoforms.

Binding of tau to microtubules is in the first instance regulated by dynamic phosphorylation via the interplay of various protein kinases and phosphatases. The longest tau isoform, i.e. tau-2N/4R, has 79 putative serine/threonine phosphorylation sites, and to date phosphorylation at about 30 sites has been reported. The study of tau phosphorylation has gained enormous attention ever since the observation that protein tau in PHF and NFT is hyper-phosphorylated [127,128]. A causal relation between hyper-phosphorylation and aggregation of tau is supported by *in vitro* experiments, showing that phosphorylation of recombinant tau with purified kinases or whole brain extracts can induce aggregation of tau. In addition, dephosphorylation by purified phosphatases triggers disassembly of PHF isolated from the brain of tauopathy patients [129,130]. Nevertheless, the exact phosphorylation sites that cause aggregation of tau, and the exact kinases responsible *in vivo*, remain largely elusive. Recent data obtained with a *Drosophila* model indicate that no single phosphorylation site plays a dominant role in controlling tau toxicity, suggesting that different phospho-epitopes cooperate to mediate neurodegeneration *in vivo* [131,132]. An additional complication in deciphering the triggers of tau-fiber assembly is that specific conformational changes and truncations of protein tau also appear to be important [133]. Presumably, the conformational changes occur in response to particular phosphorylation events, but again it is not known which phospho-epitopes are important.

Until recently, there was no direct genetic evidence implicating tau in neuronal loss and dementia. This changed with the discovery that tau-mutations are linked to the pathology in frontotemporal dementias (FTD). Some of these mutations enhance splicing of exon 10 thereby altering the ratio between the 4R and 3R tau isoforms. Other mutations affect the ability of protein tau to interact with microtubules as could be predicted, since they alter amino acid residues in close proximity or within the microtubule-binding repeats. Mutations located in the amino-terminal or the carboxy-terminal region are believed to change the conformation of tau [127,128,134]. So far, however, the effect of mutations on masking and unmasking certain phospho-epitopes has not been studied systematically, leaving the possibility that at least for some tau mutants the described enhanced self-assembly could be a consequence of changes in phosphorylation of tau.

4.2. Yeast models to study fundamentals of the tau phosphorylation–conformation–aggregation cascade

Although there are only few studies on the expression of human tau in yeast, the data obtained so far clearly indicate that the yeast system robustly recapitulates crucial aspects related to the tau pathophysiology. Indeed, human tau-3R and tau-4R isoforms expressed in yeast acquired pathological phospho-epitopes, assumed a pathological conformation and formed aggregates [135]. Importantly, these characteristics were modulated by the yeast protein kinases Mds1 and Pho85, the orthologues of two of the most important mammalian tau-kinases, i.e. GSK-3β and cdk5, respectively. Inactivation of Mds1 decreased tau phosphorylation at epitopes in the C-terminus. Conversely, the

deficiency of Pho85 increased tau phosphorylation at the same epitopes resulting in an increased conformational change and enhanced aggregation of tau. This observation supports the hypothesis that Pho85 in yeast, similar as cdk5 in mammalian neurons [136], does not phosphorylate tau directly but acts indirectly as negative regulator of phosphorylation and thereby conformation and aggregation. Moreover, soluble protein tau, purified from the *pho85* mutant, spontaneously and rapidly formed twisted filaments *in vitro* and upon further fractionation, the monomeric hyper-phosphorylated subfraction was found to seed and accelerate aggregation of protein tau isolated from a wild type yeast strain [135]. When recombinant tau isolated from the different yeast strain was used to monitor the physiological tau function, the results confirmed the inverse correlation between the phosphorylation status and the ability of the protein to bind and stabilize microtubules. Notably, this study also included the clinical mutant tau-P301L, which was demonstrated to actually aggregate onto the microtubules and thereby causing their deformation and bundling [137].

Although wild type and mutant human tau do aggregate in yeast cells, their expression does not affect the exponential phase of growth. However, preliminary data indicate that the expression of human protein tau affects longevity, which may relate to the observation that oxidative stress and mitochondrial dysfunction dramatically enhance tau aggregation in yeast (Vanhelmont and Winderickx, unpublished data). These preliminary results fit nicely with recently reported data obtained in *Drosophila* and mice tauopathy models showing that oxidative stress and mitochondrial dysfunction exacerbate tau-induced neurodegeneration [138,139].

4.3. Aβ deposits as hallmark of Alzheimer's disease

Besides intracellular aggregation of tau into NFT, the AD brain is characterized by the occurrence of extracellular deposits of β-amyloid (Aβ) peptides, known as amyloid or senile plaques. Aβ peptides are generated through sequential proteolytic cleavage of the APP precursor protein by β- and γ-secretases. The alternative non-amyloidogenic α-secretase cleavage of APP occurs in the middle of the Aβ region [140]. APP is an ubiquitously expressed type 1 transmembrane protein with putative functions related to cell adhesion and migration. Cleavage of APP by β-secretase releases a soluble N-terminal ectodomain and a membrane bound C-terminal fragment called C99. The cleavage of C99 by γ-secretase is more variable and generates predominantly peptides Aβ1-40 or Aβ1-42, together with the remaining C-terminal intracellular domain (AICD). The Aβ1-42 peptide is most prone to aggregation and fibril formation and it constitutes the core of the senile plaques. [141]. Aβ1-42 production is increased in familial AD because of mutations in APP or the γ-secretase catalytic subunits presenilin 1 and 2 [142].

While initial research focused on the extracellular aggregates, accumulating evidence suggests that the Aβ peptide may also exert toxicity by accumulation and aggregation within the cell. Like most plasma membrane proteins, APP is synthesized and translocated into the ER, further matured in the Golgi complex, and then transported to the cell surface. A considerable amount of APP is subsequently internalized for recycling. In addition to APP processing at the neuronal plasma membrane, several groups reported APP processing in the secretory pathway and in the endocytic cycle [141,143,144]. How intracellular Aβ peptides cause cellular dysfunction remains unclear, but links to endocytic functions, the ubiquitin–proteasome activity, Ca^{2+} signaling, and synaptic receptor levels have been reported [145–148]. Moreover, some studies link intracellular Aβ accumulation to the formation of ROS, to mitochondrial damage and to apoptosis [149–151]. Full-length APP has also been implicated in oxidative damage and mitochondrial dysfunction because the protein can accumulate in the mitochondria as a transmembrane protein. Interestingly, induction of mitochondrial damage by APP is prevented by cleavage of the protein by the protease HtrA2 in the intermembrane space [152,153]. In addition, HtrA2-dependent cleavage of APP occurs as well in the ER, suggesting a role for HtrA2 in quality control and processing of ER-resident APP and possibly in preventing ER-stress [154].

4.4. Yeast models for APP processing and Aβ oligomerization

Initial studies in yeast focused on the different secretases. The first study demonstrated that APP expressed in yeast is processed by enzymes that possess the specificity of the α-secretases of multicellular organisms [155]. Further analysis revealed that these α-secretases are in fact the Yap3 and Mkc7 proteases, which act on APP in the late Golgi [156]. Yeast cells do not contain endogenous β- or γ-secretase activity, but strains have been engineered that allow cleavage of APP by the human β-secretase and a reconstituted γ-secretase complex [157–159]. Since the proteasome is known to remove aggregation-prone protein derivates, a recent study analyzed processing of the C99 fragment in wild type yeast and a mutant strain lacking proteasome activity. This study not only indicated a role of the proteasome in generating longer Aβ peptides, up to 55 amino acids, but also that the C99 fragment is processed by other enzymes in the absence of an intact proteasome [160].

Yeast cells transformed with a vector encoding the authentic human Aβ1-42 were reported not to produce detectable levels of the peptide [161]. Whether this is due to toxicity of Aβ and reflects counter selection or extremely rapid degradation of Aβ remains to be investigated. However, the Aβ peptide can be successfully expressed in yeast as N- or C-terminal fusion proteins, and studies with such constructs indicated Aβ to lower the growth yield and to induce a heat shock response, indicative that the peptide induces stress in the yeast cells [161]. It would be interesting to analyze whether these phenotypes translate into increased oxidative stress and apoptotic cell death.

Two groups reported yeast cell-based systems that allowed monitoring the oligomerization of Aβ-fusions [162,163]. By the use of these systems it was confirmed that Aβ aggregation is inhibited when point mutations are introduced in regions known to be important for intermolecular Aβ-interaction. In addition, it was found that Hsp104 influences Aβ oligomerization [162], which is consistent with this chaperone playing a central role in protein aggregation as described above. Hence, these yeast systems appear to be very useful tools in the study of Aβ-related

aggregation processes, not only to identify proteins or compounds affecting the aggregation process but also to decipher the molecular pathways triggered by Aβ-aggregation.

5. Conclusions

Humanized yeast models are important tools to investigate protein functions or cellular pathways that mediate misfolding, aggregation and subsequent toxicity of proteins associated to human neurodegenerative disorders. Yeast models confirmed and further extended links to oxidative stress and mitochondrial dysfunction and their consequences on apoptosis demonstrating a high degree of conservation in the eukaryotic kingdom of mechanisms involved in protein-misfolding triggered cell death [164]. As a eukaryote, humanized yeast models provide a relevant biological context, making them ideal models for identification and validation of novel – preferably 'drug-able' – targets, whose modulation may affect disease biology. Also for drug discovery efforts humanized yeast models are preferred systems for high-throughput screening of chemical libraries, not only because compounds are evaluated in a physiologically relevant environment and their use is cost-effective and user friendly, but also because these cell-based assays allow immediate counter selection of toxic and unstable compounds as well as compounds that cannot pass the membrane bilayer or that are substrates for multi-drug resistance pumps [165]. Whatever the use, studies performed in humanized yeast models may constitute important stepping stones for further exploration in the mammalian context. Its genetic tractability and fast-growing properties allow addressing mechanisms of a disease in an unparalleled manner using strategies sometimes not possible in higher eukaryotic systems. Collectively, yeast models of protein-misfolding disorders contribute to the understanding of disease biology and may facilitate strategies aimed to identify therapeutics for treating corresponding diseases.

Acknowledgements

We are grateful to FWO-Vlaanderen, the International Alzheimer's Research Foundation (SAO/FRMA-Belgium), the Marie Curie PhD Graduate School NEURAD and K.U.Leuven for funding and support. We also thank G. Griffioen for critical reading and suggestions.

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