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Oxidative stress and neurodegeneration: the yeast model system

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TABLE OF CONTENTS

1. ABSTRACT

In this chapter we are treating yeast cells as a model for oxidative stress response and the consequences of oxidative stress which are one cause for a number of human diseases, including neurodegenerative diseases, which form the main part of this paper. All such model building depends on orthologous relations between highly conserved yeast and human genes, which are easily recognized by sequence comparisons, but much more difficult to prove functionally. Previously we have treated Friedreich's ataxia, while presently we are describing in detail the neuronal ceroid lipofuscinoses, among them Batten disease. A general overview is given how yeast can aid current research in three of the most devastating and at the same time quantitatively most important neurodegenerative diseases of old age: Alzheimer's, Huntington's, and Parkinson's disease. In the ensuing part of the chapter, we describe yeast as model for metabolic regulation and hence as a model for inborn errors of metabolism that are in some instances very faithfully mirrored by introducing the same point mutations into yeast cells which are known from patients.

2. INTRODUCTION

This introductory section presents an overview of the genetic and biochemical conservation of cellular functions between yeast and human cells. A large number of tools for molecular genetics are now available in the two yeasts, *S. cerevisiae* and *S. pombe* for functional analysis of any gene which tools are, for the most part, unavailable for human cells and which increasingly are used to study the function of yeast orthologues of human genes harbouring disease-causing alleles. Two examples are presented: cell cycle regulation and its involvement in cancer, and frataxin, an essential protein involved in iron homeostasis in mitochondria.

Yeasts are single-cellular eukaryotes that share a remarkable number of cellular processes with higher organism. Of the ~6000 genes in *Saccharomyces cerevisiae*, it is estimated that ~60% have a homologue in humans, and in many cases a human gene inserted into a yeast strain deleted for the homologous gene will complement the defect in the mutant.

Many fundamental cellular processes, such as metabolism, cell division, transcription, translation, secretion of proteins and autophagy are quite highly conserved from these simple ascomycetes to man, and basic research on these has greatly advanced many areas of medical research. This includes analyses of how cells respond to DNA damage as well as to stresses such as those imposed by heat, exposure to reactive oxygen species, hypo- and hyper-osmolarity and starvation. A classic example is the discovery of the fundamental aspects of cell-cycle regulation via cyclin/cyclin-dependent kinase(s) (1) and how elucidating the control at the G1 to S phase boundary has led to major research and a greater understanding in the field of cancer biology. In some cases a specific human gene can complement a yeast mutant lacking a specific homologous gene, which allows more detailed study of function of the gene in a rapid way. For example, the human homologue of the *Schizosaccharomyces pombe (S. pombe) cdc2*$^{+}$ gene (Cdk1) can complement the *cdc2* defect in *S. pombe*. This means that yeast can be a test bed in which to analyze gene functions and metabolite or drug interactions with a human protein.

The genetics, biochemistry and genomics of several yeasts have been developed to a very remarkable degree, providing the opportunity to study very rapidly some cellular processes and function of genes as they relate to medical pathology. For the two yeasts, *S. cerevisiae* and *S. pombe*, there are sophisticated tools available for molecular and cellular biology research. For both organisms there are readily available methods for transcriptomics and proteomics to study gene expression and deletion mutants for every non-essential gene. For *S. cerevisiae,* there is a comprehensive set of deletion mutants available for every non-essential gene (2), and for essential genes under the control of regulated promoters (3), temperature sensitive (ts) conditional alleles , a genome-wide set of genes under regulated promoters for over-expression analyses (4), and substantial data for protein-protein interactions (5-7), protein complexes (8), protein localization (9), synthetic gene array data for the phenotypes of double mutants (10, 11) and genome–wide data on transcription factor binding (12, 13). These are complemented with an impressive array of data on gene function readily available in the SGD database (www.yeastgenome.org/)(14).

Previously we gave an example how yeast genetics and molecular biology helped to identify the function of a highly conserved gene involved in the human hereditary disease, Friedreich's ataxia (15). The human candidate gene was identified by positional cloning before the sequence of the human genome became known (16). This sequence gave no hint to the possible function of the gene. At about the same time, in 1996, the genome sequence of *S. cerevisiae* was published. This was the first complete genome sequence obtained for any eukaryotic organism, and the very high degree of sequence identity of one of the ~ 6000 yeast genes to the human candidate gene was noted (17, 18).

Subsequently, the development of the large compendium of yeast molecular genetic methods stimulated researchers working on other medical problems to use yeast as a model organism in which to elucidate the function of disease genes, even for proteins that do not have a direct yeast orthologue. In this way, yeast research helped to determine the function of proteins involved in neurodegenerative disorders including Huntington's disease (19), Spinocerebellar ataxia type 2 (20), metabolic diseases such as ribose-5-phosphate isomerase deficiency (21) or even cancer (22).

In all of these cases, yeast projects stimulated further research in mammalian cells. In the case of frataxin it became clear that the function of the protein encoded by the human gene, FRDA, and its yeast homolog, frataxin, was involved in iron homeostasis of mitochondria. The physiological function of frataxin was determined to be an iron chaperone acting in the mitochondria. The disease phenotype was caused by down-regulation of the amount of the wild type form of human frataxin, caused by mutations in one intron of the gene sequence. This leads to an excessive radical production, presumable via an excess of ferrous ions in mitochondria and the well-known Fenton and Haber-Weiss chemistry. Based on this knowledge, therapeutic strategies which are showing promise were devised and tested in a mouse model of the disease (23). Functional complementation of frataxins of yeast, mouse and human cells was possible and was a prerequisite for the success of this project. The analysis of this gene, although it began in 1998 (24) is unfinished and presently (2012) still under active research (25). A prerequisite for success was that Friedreich's ataxia, a neurodegenerative disease, is monogenic and recessive and shows little phenotypic broadness. This means that studying one gene is sufficient to unravel the pathological mechanism, since there is little interference by the genetic background and by lifestyle factors in this disease. The same is true for the inherited disease, Batten disease, which again presents with clearly defined neurodegenerative symptoms.

3. THE NEURONAL CEROID LIPOFUSCINOSES (BATTEN DISEASE)

These disorders represent an example that is somewhat similar to frataxins with respect to the disease phenotype, but completely different with respect to the primary function of the gene harbouring the disease-causing mutation. They are all characterised by the accumulation in the lysosome of autofluorescent, insoluble material known as ceroid lipofuscin.

3.1. Nature of ceroid lipofuscin

Section summary: this section provides an overview of the chemical structure, occurrence, and relation to oxidative stress of lipofuscin (age pigment) and the similar ceroid, which are found in neuronal cells of patients suffering from several neurodegenerative diseases, among them most prominently the group of ceroid lipofuscinoses.

It is fascinating to see, how a few and relatively small eukaryotic proteins or protein "modules" are involved in a universe of different metabolic- and signalling pathways. Likely, they are interconnecting them

in order to maintain homeostasis in the cell, and hence, mutations in those genes quite unexpectedly can then lead to cellular - predominantly oxidative - stress. As shown here and in our previous work (26) there are many different types of oxidative stress and, consequently of defences against oxidative stress and these stress situations can have different biochemical consequences for the cell. In ceroid lipofuscinosis, the human neuronal cells form and accumulate ceroid lipofuscin, which is one of the most generally found aging marker in cells and organisms. Aging yeast cells also accumulate this autofluorescent molecule (our own unpublished observations).

Lipofuscin is a synonym for age pigment and is observed in all known model systems of aging under conditions of physiological aging (for review: (27); (28)). Ceroid is a similar pigment found in diseases like the neuronal ceroid lipofuscinoses discussed here. This macromolecule does not show a well-defined chemical structure, but rather varies in different diseases and physiological situations. However, this yellow-brownish pigment on average contains about two-thirds oxidized protein, one-third oxidized lipids, and less than 2% of carbohydrates and transition metals, among which iron is prominent. Lipofuscin is typically located in lysosomes and arises from incomplete or abortive mitophagy (reviewed in (29); (30)). One prominent protein that has been repeatedly reported to be a cross-linked component of lipofuscin, is subunit c of the mitochondrial ATP synthase (Oli1p, mitochondrially encoded). The structural features responsible for autofluorescence are not precisely known, but are probably the result of Schiff's bases produced by the reaction of carbonyl groups of lipid oxidation products like malondialdehyde and 8-hydroxynonenal (HNE) with primary amines, for instance the ε-amino group of lysine residues in proteins.

The production of lipofuscin is clearly secondary to oxidative stress, which is secondary to the lysosomal sorting defect described in the succeeding sections. Considering that chemically different forms of ceroid lipofuscin are found in different forms of the disease, one question seems to be pertinent: is the composition and structure of age pigment found in natural aging different from the ceroid found in young but mortally sick victims of NCL?

3.2. Clinical features of ceroid lipofuscinoses

The eight loci recognized as responsible for this group of neurodegenerative diseases are presented together with the clinical features observed in the patients.

The neuronal ceroid lipofuscinoses (NCLs), collectively termed Batten disease, are a group of autosomal recessive inherited disorders of childhood that share a number of traits. With an incidence of up to 1 in 12.500 births (31), the NCLs are the most frequent childhood onset neurodegenerative diseases. The condition is named after the British pediatrician who described the juvenile form of this disorder in 1903 (32).

According to age at onset and the ultrastructure of the storage material involved, four major subtypes have been classified: infantile (INCL), late-infantile (LINCL), juvenile (JNCL) and adult (ANCL). Recent biochemical and molecular genetic studies resulted in a new classification. According to the presently predicted gene loci, human NCLs are classified into 8 main genetic forms (33, 34). It is crucial to point out that different mutations in a single gene may result in distinct phenotypes, including varying ages at onset. Common mutations that predominate in a certain form of NCL are usually associated with the classic clinical picture, while rare ''private'' mutations may produce a divergent phenotype.

3.2.1. CLN1

This variant may cause four different phenotypes, but the classic INCL (Santavuori-Haltia type) is by far the most prevalent. Affected babies fail to thrive and suffer from decelerated head growth, visual loss, myoclonic jerks and other seizures. Onset is between the age of six months and two years, rapid progression causes death before the age of five.

3.2.2. CLN2

Mutations of the CLN2 gene result in LINCL (Jansky-Bielschowsky type). Onset is usually between the age of two and four years with a variety of epileptic seizures, followed by ataxia, myoclonus, developmental regression and progressive visual loss. Progression is rather rapid, resulting in death between the ages of six and 12 years.

3.2.3. CLN3

Defects in the CLN3 gene usually cause JNCL (Batten-Spielmeyer-Vogt type), which is the most common NCL worldwide. Incidence differs in distinct regions with the highest numbers (up to 7 from 100.000 live births) referred from Scandinavia (35). Many cases, mostly northern European, are due to a common ancestral 1-kb deletion mutation (36). Onset of symptoms is between the ages of four to 10 years. Commonly, the first sign is visual failure, less frequent are seizures. Impairment of vision starts with central visual loss, progressing to complete blindness within two to 10 years. Fundoscopy presents macular and retinal degeneration, optic atrophy and pigment accumulation in the peripheral retina. Postmortem studies show neuronal loss in all retinal layers, absence of photoreceptors at the macula and degenerated rods and cones (37). Patients with the major 1-kb deletion mutation present brisk widespread retinal degeneration, whereas other variants result in decelerated impairment of vision.

Slowly advancing deterioration of cognitive functions usually starts by eight or nine years of age with impairment of short-term memory. Behavioral symptoms include aggressiveness, mood disorders, anxiety and psychosis. The clinical course also covers dysarthric and dysfluent speech with echolalia, usually after the age of 15 years. Seizures usually appear between 7 and 18 years of age and are mostly generalized tonic-clonic or complex partial, myoclonus occurs with the progression of disease (38). Many patients generate signs of Parkinsonism by their mid-teens, resulting in disturbance of mobility and gait (34). Patients usually die before the age of 40 years, but slow-going courses have been described.

Electroencephalography (EEG) usually displays nonspecific abnormalities, electroretinography (ERG) is seriously abnormal (even at early stages) and visual evoked potentials (VEPs) are extenuated or even below limit of detection. After the age of 12 years, progressive brain atrophy, mainly affecting the cerebral hemispheres, is demonstrated by neuroimaging techniques. To substantiate clinical suspicion, vacuolated lymphocytes may be regularly identified on peripheral blood smears, a unique finding in NCLs. Neuropathologic findings include a moderate generalized atrophy and dilatation of ventricles, a reduced thickness of the cortical ribbon and depigmentation of the substantia nigra. Histological workup demonstrates mutable neuronal loss, reactive astrocytic proliferation and microglial activation. Intracytoplasmic accumulation of autofluorescent granular storage material is detected in neuronal perikarya, and the widespread destroyed neuroretina is substituted by scar tissue.

3.2.4. CLN4

CLN4 is reserved for the hypothetical gene implicated in ANCL (Kuf˝s/Parry disease), which is a very infrequent condition. ANCL seems to be genetically heterogeneous, and although autosomal recessive heredity is usually observed, some families with autosomal dominant inheritance have been reported. Symptoms begin before the age of 40 and present with two phenotypes: type A is characterized by progressive myoclonus epilepsy, dementia, ataxia and extrapyramidal signs, type B presents with depression, cognitive decline, ataxia and extrapyramidal signs. Visual impairment is not observed in any type of CLN4. Life expectancy is shortened, and patients decease approximately after a course of 12-15 years.

3.2.5. CLN5, CLN6, CLN7, CLN8, CLN 9, CLN10

The CLN5 variant of the LINCL is restricted to Finland. Motor symptoms as clumsiness and muscular hypotension, mental retardation, visuomotor problems and generalized seizures are the clinical criteria with age of onset between four and six years. Defects in the CLN6 gene result in a variant form of LINCL, also named early juvenile NCL (Lake-Cavanagh), which is found in various ethnicities. The clinical features are similar to those of classic LINCL, with one third of patients showing a slightly later onset and a more protracted course. In NCL7 onset is between 2 and 7 years. Psychomotor regression or seizures may be the presenting signs at onset. A defect of the CLN8 gene causes Northern epilepsy (NE), also named progressive epilepsy with mental retardation, up to now only described in Finland. CLN9 is from a patient cell line, which appears not to have a mutation in the established genetic profiles. The congenital CLN 10 is characterized by encephalopathy, status epilepticus, and death due to respiratory insufficiency in the first days or weeks of life (38).

3.3. Genetic analysis of BTN1, the yeast homolog of CLN3, the gene for JNCL

CLN3 is one of the two known genes harbouring mutations causing ceroid lipofuscinosis for which a closely similar yeast gene has been identified. The human gene can complement the yeast gene deletion defect. The mutation in human and yeast cells causes massive oxidative stress. A mouse model of this form of lipofuscinosis has been created by inserting the most common human mutation into the homologous mouse gene. The neurological symptoms are evident in this mouse.

Like in Friedreich's ataxia, the neurological symptoms start early in life in the CLN3 subtype. The human CLN3 gene is the functional orthologue of the *S. cerevisiae* gene, *BTN1*, also known as *YHC3*. The amino acid sequences of the two proteins show 33.6% identical and 44.3% similarity, the homologous sequence blocks span the length of this 46 kD membrane protein, with few gaps in the alignment (Figure 1). Importantly, the human cDNA can complement the phenotype of the yeast deletion mutant (reviewed in (39)). Btn1 is predicted to be a farnesylated integral membrane protein. It is localized in the yeast vacuole, which is the lytic compartment of the yeast cell and which is closely comparable to the lysosome of human cells where CLN3 is located. Structural models of the membrane orientation of CLN3 were proposed by Phillips *et al.* (40). Depending on the physiological state of the cell, Btn1 can also be shown to be located in the yeast Golgi apparatus (25).

The main reason for discussing this disease and the causative mutation here, is to demonstrate how the yeast system provided functional insights based on the unique methods available in yeast, but not in human cells, and how the methods available in both systems – cultured cells carrying the human mutation and yeast cells deleted for the *BTN1* gene – complemented each other in elucidating the putative function of the protein. Fortunately, highly homologous genes are also found in the mouse and in the worm, *Caenorhabditis elegans*, and these two animal disease models also contributed to the same task. Compared to the yeast and cell culture systems, the mouse and worm model system have the undisputed advantage that one can, in addition to the basic cellular function also test the effect of the mutation on the nervous system of these animals.

Another main reason for discussing the *BTN1* and CLN3 genes is that the recessive gene defect, although not directly related to redox homeostasis and oxidative stress, indirectly leads to massive oxidative stress in the cells, a theme in neurodegeneration that runs through this part of modern neurological science like the red Ariadne's thread in classical mythology. In juvenile Batten disease, neurodegeneration starts early, however the molecular markers, which include accumulation of ceroid lipofuscin and other degradation products (such as protein carbonyls) caused by oxidative stress in the cells, are the same as those found in aging cells and organisms (41, 42) and in neurodegenerative diseases typical of old age, like sporadic Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and many prion diseases (43).

Among the gene defects identified as being responsible for the different subtypes of NCLs (neuronal

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CTN3          1 mggcagsrrrfsdsegEetvpeprlplld--hqgahWknavgfwllglcn     48
                                          :.|  ||...:     |||.||.|
Btn1p         1 -------------------------msdkshqiycy-----fwlfglin     19

CTN3         49 nfsyvvmlsaahdilshkrtsgnQshvdpgptpiphnsssrfdcnsvsta     98
                |..|||:||||.||:              |||              :..:
Btn1p        20 nvlyvvilsaavdiv--------------gpt--------------lpks     41

CTN3         99 avLladilptlvikllaplglhllpysprv--lvsgicaagsfvlvafsh    146
                .||||||.|:|.|||.:|..:..:.||.|:  |::..| .|.| ||:|.
Btn1p        42 lvlladifpslaiklcspffidrikysyriwslitmsc-lgmf-lvsfk-     88

CTN3        147 svgtslcgvvfAsiSSglgevtfLsltafypraviswwssGtGgagllga    196
                ::...|.|:.|||||||.||||||.||.:|.:..::.|||||||||::|.
Btn1p        89 nlfvcllgisfasissgfgevtflqlthyykqislngwssgtggagiigg    138

CTN3        197 lsYlgltqaglspqQtllsmlgipalllasyfll------ltspeaqdpg    240
                .||:.||.....|.:..|.:..:.......||.|      ||....|.
Btn1p       139 asymfltsifkvpvkltllvfsllpfaflfyfklesndtnltyqslqq--    186

CTN3        241 geeeaesaarqplirteapeskpgssssls------------rerwtvf    278
                 .:|||.....|.     |.:...:::|.||            |.|..||
Btn1p       187 -ideaeddqlvpf-----pvafthtnasqslystrqhilqtvkrlrrlvf    230

CTN3        279 kgllwyivplvvvyfaEyfinqglfell--------------ffwntsls    314
                .    |:|||..||..||.|||.:...|              ||::   .
Btn1p       231 p----ymvplttvylfeylinqavaptllfpingdersksmpfffh---k    273

CTN3        315 haqqyrwyqmlyQagVfasRsslrccrirftwalallQclnlvflladvw    364
                :...|..|..|||.|||.|||.....|:|..:.||.||.:||...:...|
Btn1p       274 yrdiyvtygtlyqlgvfisrsfghlmrmrslyilaflqgvnlcitvlqsw    323

CTN3        365 fgflpsiylvfliilyegllggaayvntfhnialetsdehrefamaatci    414
                |....|.:.|.::|.|||.||||:|||||.||..:...:..||||.|..|
Btn1p       324 fyvthspwavmilifyegflggasyvntflnileqedpdetefamgavsi    373

CTN3        415 sDtlgislSgllalplhdflcqls-----------    438
                :|:.|:.|:.||.|.|...||:..
Btn1p       374 adsfgvflaallglglepklcrhqiaddrpwcrme    408
```

Figure 1. Amino acid sequence comparison of human CLN3 and yeast BTN1. The alignment was performed with EMBOSS Needle - Pairwise Sequence Alignment (www.ebi.ac.uk/Tools/psa/emboss_needle/). Identical amino acids (I) and conservative substitutions (:) are indicated. Amino acids shown in red capitals denote point mutations found in patients. They are in most (but not all) cases highly conserved in the sequence. The two sequences are 33.6% identical and 44.3% similar. A number of gaps are necessary to obtain the alignment, however two large blocks of sequence are conserved without gaps.

ceroid lipofuscinoses), two stand out as being located in highly conserved eukaryotic genes: CLN3 (BTN1) and CLN10 (PEP4). Both gene products are biochemically unrelated, both indirectly lead to oxidative stress in the respective cells, and both together can probably teach us more about the disease than each one by itself.

3.4. The important contributions of yeast molecular genetics to unravelling the pathomechanism of Batten disease (JNCL)

Batten disease is caused by a recessive loss of function mutation in CLN3, which is nicely mimicked by analogous mutations in BTN1 in yeast cells. However, the distinct pathomechanism (chain of events) is only incompletely known. The primary defect may cause the unfolded protein response, followed by massive oxidative stress, failure to degrade proteins and whole organelles (primarily mitochondria) in the lytic compartment of the cell, formation of ceroid lipofuscin and finally cell death through apoptosis.

Both the deletion and the overexpression of *BTN1* in *S. cerevisiae* cells led to distinct phenotypes. The GFP fusion protein localized in vacuoles corresponding to localization in lysosomes of HeLa cells (44). Initially the yeast deletion mutant was found to have no apparent phenotype under a variety of growth conditions (45); however, another group discovered that the *btn1* deletion mutant led to resistance to the inhibitor, ANP ("chloramphenicol base") (24, 46). This phenotype could be complemented by the human CLN3 cDNA. Amino acids corresponding to point mutations found in patients were

nearly all conserved in the yeast sequence (Figure 1). The ability of disease alleles of CLN3 to complement the yeast phenotype was related to the severity of the disease. This showed functional homology of the yeast and human genes (47). Another hint towards the pathological mechanism was the pH dependence of the growth inhibition by ANP – the *btn1* deletion caused a defect in vacuolar pH homeostasis and the inhibition of growth by ANP could be reversed by a slightly acidic pH of the medium. This was later shown to be due to restoration of the vacuolar pH under these conditions. Transcriptome analysis comparing WT and the *btn1* deletion mutant revealed that *HSP30*, which is involved in pH control, is overexpressed in *btn1-Δ* pointing to a compensatory mechanism (48).

The idea that altered pH homeostasis in the vacuole and in the cytoplasm was the underlying primary cause of Batten disease, was further investigated by studying the deletion of the functional interactors of *BTN1* (*BTN2* and *HSP30*) and their influence on the plasma membrane H^+ ATPase and on pH regulation in both the cytoplasm and the vacuole (49). In *btn1-Δ* yeast cells the plasma membrane H^+ ATPase was up-regulated (presumably as a compensatory mechanism) and vacuolar pH was considerably lower in the early logarithmic growth phase. The vacuolar H^+ ATPase was up-regulated when either *HSP30* or *BTN2* (encoding a cytoplasmic protein of unknown function) were deleted resulting in poor buffering capacity of the cells and poor growth at low pH. The BTN2 deletion exhibits very similar phenotypes as *btn1-Δ* but affects the retrieval of cargo from late endosomes to the Golgi (25).

Subsequently (50, 51) it was shown that arginine transport into the vacuole was diminished in the *btn1-Δ* mutant, and this could be complemented by the human CLN3 cDNA. This caused the low pH in the vacuole of the mutant, leading to the hypothesis that the primary function of *BTN1* might be transport of basic amino acids. Arginine transport into the vacuole was previously shown to be mediated by an arginine H^+ antiporter. The deletion of *BTN1* also led to low levels of arginine in the cytoplasm, and, importantly, over-expression of *CAN1* (encoding for the arginine transporter in the plasma membrane) was found to be lethal in *btn1-Δ* strains. Subsequent studies in part questioned the above findings and showed (52), that both in yeast and in lymphoblast cell lines from JNCL patients the low arginine level is not due to altered transport or synthesis of arginine, but is the result of regulatory phenomena and it appears that raising arginine levels is toxic to the mutant cells. On the other hand, the combination of *btn1-Δ* with *gcn4-Δ* creates a synthetic arginine auxotrophy. *GCN4* encodes a master regulator of amino acid metabolism, which by itself is not an essential gene and its mutation does not create auxotrophy. It appears, therefore, that the true primary defects of *btn1-Δ* are at least two-fold and possibly inter-related in complex metabolic regulation, in particular in amino acid metabolism. The authors conclude that supplying sufficient arginine to the cells (which was at that time discussed) would not be a viable therapeutic strategy. The other primary function which was identified using the yeast model (25) is regulation of the retrograde vesicular transport from the Golgi apparatus to the vacuole by regulating phosphorylation of the late endosome SNARE, Sed5.

The deletion of *BTN1* was found to down-regulate the vacuolar H^+ ATPase, partially compensating for the pH changes in early log phase growth. In *S. pombe*, deletions of *BTN1* and the gene encoding the Vma1 subunit of the vacuolar ATPase are conditionally synthetic lethal showing strong functional interaction.

More recently, a further functional link of *BTN1* was found that is important because it is conserved between yeast and human cells. In both types of cells, Btn1 (CLN3) is a direct interaction partner for the Schwachman-Bodian-Diamond syndrome protein, SBDS, which is highly conserved in yeast where it is encoded by *SDO1* and whose function as a ribosome–associated protein is in the maturation of the 60s ribosomal subunit (53). In the presence of CCCP, the ionophoric uncoupler of membrane potential, the combination of *BTN1* over-expression with *sdo1-Δ* is lethal. *BTN1* over-expression by itself has uncoupling activity (52).

The low arginine levels observed in *btn1-Δ* yeast cells have prompted an investigation into nitric oxide synthase, for which arginine is a substrate (54). The yeast mutant is severely defective in NO synthesis, which could contribute to the pathology of the disease, however, nitric oxide metabolism has not been studied in cells of the JNCL patients.

Recent investigations (55) revealed further biochemical details that could be tested in human cells and which may be helpful in elucidating the biochemical basis of the disease. The glycosylation state of Btn1 and its localization depend on the pH of the growth medium. Vacuolar localization is observed in “normal“ medium (pH around 6.5), but in more acidic growth medium, Btn1-eSapphire appears punctate and does not co-localize with Vma2-GFP. Interaction of Btn1 and Sdo1 occurs only in those punctate structures, not in the vacuolar membrane. The clarification of the nature of this subcellular compartment awaits further research. It could be pre-vacuolar vesicles, which would not contradict the previous research on this question.

Additional indirect phenotypes of the *btn1-Δ* and the overexpression mutant were found in yeast: These include changes in amino acid metabolism and mitochondrial metabolism (56). One obvious yeast experiment, which has never been performed but would be revealing is to test whether lipofuscin production occurs in yeast cells lacking *BTN1*. The question remains as to how the multiple phenotypes described with the help of the yeast system and other model systems (*C. elegans, D. melanogaster, M. musculus*, various cell cultures derived from patients) can explain the downstream events that are known to cause pathology. We believe that the chain of events would be in this order: unfolded protein response, massive oxidative stress, failure to degrade proteins and

whole organelles (primarily mitochondria) in the lytic compartment of the cell, formation of ceroid lipofuscin and finally cell death through apoptosis. A direct intervention in lysosomal protein degradation by specific mutations in other NCL genes seems to prove the predominant role of this degradation pathway: CLN1, CLN2 and CLN10 are involved in lysosomal protein degradation. In particular, CLN1 is specifically needed for the normal functioning of the metabolically highly active neuronal cells, because recycling of the secretory vesicles is absolutely needed for neuron function and neuron-specific palmitoylated proteins play a major role here (57).

3.5. Relation to animal models of Batten disease (JNCL)

Disease models of JNCL were constructed by deletion of the respective CLN3 homologue in the mouse, C. elegans, and D. melanogaster. While the complete deletion of CLN3 in *C. elegans* showed no phenotype, the mouse model most faithfully reproduced the disease phenotype, and the D. melanogaster model showed a very clear relation to oxidative stress by genetic analysis of revertants.

The still incomplete picture drawn above is not realized completely in every one of the model systems investigated. A triple deletion of the three *C. elegans* genes showing close homology to human CLN3 did not reveal any neuronal pathology and no deposition of ceroid lipofuscin. Similarly, the worm deletion mutant for ppt1 (palmitoyl protein thioesterase) did not show neuronal pathology or deposition of ceroid lipofuscin (39). However, in a mouse model of INCL carrying a homozygous knock out of palmitoyl protein thioesterase, the mechanistic link between a block in lysosomal degradation of palmitoylated proteins (some of them neuronal-specific), the ER unfolded protein response, generation of oxidative stress, and activation of the apoptotic genetic program could be clearly shown (58).

In *Drosophila*, a homozygous deletion mutant of the CLN3 homologue, as well as an overexpression mutant was studied. In addition, a gain of function genome-wide screen for suppressors was performed. The important result is that CLN3 in *Drosophila* closely interacts with the oxidative stress defence system and that the deletion mutant is defective in mounting an appropriate oxidative stress defence. Other forms of stress were tested but were not affected. Overexpression of CLN3 leads to increased oxidative stress resistance. This is to date the clearest result linking Cln3 and the oxidative stress response (59). Defects of mitochondria and of oxidative phosphorylation in dog, sheep and mouse models of NCL subtypes CLN6 and CLN8 have been shown (60).

Finally, in the mouse model of JNCL, the most detailed study of oxidative stress in the brain has been performed (61). The homozygous knock-out of CLN3 seems to be a faithful model of the human disease presenting all major neurological symptoms known from clinical studies. A massive increase in mitochondrial defects, protein carbonyls, lipid peroxidation, and other biochemical markers was found. However, it remains to be discovered how the probable primary defect of vesicular transport to the lysosome in nerve cells leads to the oxidative stress phenotype.

Based on the successful mouse model just described, an attempt at therapy was published recently using a lymphoblastoid cell line derived from a JNCL patient (62). The authors could show that the well-known drug, resveratrol, led to a decrease of the ER stress marker, glucose regulated protein 78, increased SOD-1 (superoxide dismutase) in the cells, and decreased biochemical markers of oxidative stress. This is still far from a pre-clinical therapeutic trial.

3.6. Role of other mutations causing neuronal ceroid lipofuscinosis

Most of the other known gene defects leading to neuronal ceroid lipofuscinoses cause defects in lysosomal degradative processes of proteins or lipoproteins. Only two of these genes have close homologues in the yeast genome, CLN3 and CLN10. The latter gene encodes cathepsin D. The yeast homologue of cathepsin D, Pep4, has not been studied in relation to a NCL disease model.

Not all of the already mentioned CLN genes have been functionally annotated to date. Some sequences have only been identified by positional cloning, and no further data are available. Of the eight CLN genes presently known ((63); OMIM 2012), two display a strong sequence homology to yeast genes and most are related to lysosomal degradation pathways (Table 1). Table 1 summarizes the current knowledge (2012) about clinics, gene symbols, chromosomal locations, gene products, number of different mutations found in patients, and yeast homologues of the neuronal ceroid lipofuscinoses (data modified after Mole *et al.* (63) and NLL Resource – a gateway for Batten disease (www.ncl.ac.uk/ncl/)). The consequences of defects in the lysosomal degradation pathways can be very diverse affecting processes such as mitophagy, the unfolded protein response, retrograde vesicular transport, mitochondrial function, and proteasomal degradation pathway, all of which are also being studied in yeast or could be studied in yeast. However, all of these diverse downstream defects result in oxidative stress with oxygen radical production, the formation of ceroid and ultimately apoptosis in neural cells. Taken together, the data obtained from the other CLN genes support the pathomechanism that we have described for Batten disease (JNCL).

BTN1 has been discussed in detail in this review. The other yeast gene, *PEP4*, encodes for a vacuolar protease highly homologous to the gene coding for the human cathepsin which harbours the CLN10 mutations. Another lysosomal protease which when mutated can cause juvenile onset NCL is encoded in CLN2 (TPP1), a serine tripeptidyl protease with no obvious yeast orthologue. Both proteins are needed for lysosomal degradation of proteins and the loss of this activity leads to increased ceroid formation.

The ceroids are the hallmark of all NCL diseases. Two major protein components of the ceroids specific for

Table 1. Mutations in CLN genes leading to neuronal ceroid lipofuscinosis

Clinical types	Gene symbol	Chromosome	Gene product	Number of mutations	Yeast homologues
INCL, also late infantile, juvenile and adult onset	CLN1	1p32	Lysosomal enzyme palmitoyl protein thioesterase 1	48	−
LINCL, also juvenile onset	CLN2	11p15	Lysosomal enzyme tripeptidyl peptidase I	72	−
JNCL	CLN3	16p12	Transmembrane protein, localizes within the nucleus and along cell membranes and is associated with lysosomal membranes	50	Btn1p
ANCL	DNAJC5 (=CLN4)	20q13	cysteine string protein, member of the J protein family	2	−
LINCL, Finnish variant, also juvenile onset	CLN5	13q31-32	Soluble protein	27	−
LINCL; EJNCL, Indian/Costa Rican/Czech Gypdy variant, also juvenile onset	CLN6	15q21-23	endoplasmic reticulum protein	55	−
LINCL, Turkish variant	MFSD8 (=CLN7)	4q28	lysosomal membrane protein, part of the major facilitator superfamily	23	−
LINCL, Turkish variant and Northern epilepsy, also juvenile onset	CLN8	8p23	localizes to the endoplasmic reticulum and ER-Golgi intermediate compartment	16	−
CNCL	CLN10	1p15	cathepsin D	4	Pep4p

disease subtypes are known: In INCL (infantile NCL, mutations in CLN1), the sphingolipid activator proteins (SAP1 and SAP2, also known as saposins A and D) are found. CLN1 is located on chromosome 1p32 and encodes for palmitoyl protein thioesterase, a lysosomal enzyme needed to hydrolyse the palmitoyl lipid anchors of proteins before they can be degraded in lysosomes. The SAP1 and SAP2 proteins, as well as the GAP-43 protein are palmitoylated and accumulate in cells of the patients leading to ceroid formation. The functional link to neuron physiology is that GAP-43 is neuron-specific and its degradation and recycling is essential for neuron survival. If the undegradable palmitoyl-GAP-43 accumulates, not only ceroid formation, but also the unfolded protein response (due to ER clogging) and consequent apoptosis results. The second known form of ceroid incorporates primarily the hydrophobic subunit c of the mitochondrial ATPase. This indicates that lysosomal mitophagy may be a process affected in NCL. However, mitophagy up to now has not been studied in detail in relation to JNCL and its yeast model.

It is understandable that defects of lysosomal proteolytic enzymes needed for protein degradation would lead to very similar phenotypes in mutant cells, as exemplified by CLN2 (also known as TPP1, located on chromosome 11p15). However, CLN10 (cathepsin D, located also on chromosome 11p15) is highly homologous to the yeast vacuolar protease Pep4. Both human lysosomal proteases when defective lead to disease phenotypes of (late) infantile NCL, and a variant juvenile NCL, respectively. The yeast *pep4* deletion mutant has up to now not been used for phenotypic investigations in relation to NCL.

CLN5, located on chromosome 13q22.3, encodes a lysosomal glycoprotein with both soluble and membrane bound splice isoforms, which was shown to be closely interacting functionally and physically with CLN1 as well as with the membrane protein, CLN3. It is therefore plausible that the pathomechanism should be related to the one displayed by loss of the interactor proteins.

CLN7, located at chromosome 4q28.1, was identified as the gene harbouring causative mutations of the Turkish variant NCL. The gene codes for MFSD8, a member of the major facilitator superfamily with 12 trans-membrane helices, which is located in the lysosomal membrane. This possibly suggests functional interaction with CLN3 (which is also located in the lysosomal membrane and leads to defects in transport of solutes into the lysosome). The protein is highly conserved with one orthologue each in vertebrate species. The yeast genome contains 4 homologues of MFSD8, which have not been studied in detail and the deletion mutations not tested for phenotypic similarity at the cellular level to the NCL.

CLN8, located at chromosome 8p23, was found to harbour mutations in the "Northern epilepsy" variant of NCL. This membrane protein of 286 amino acids is located in the ER and ER to Golgi vesicles and is apparently involved in vesicle recycling between the ER and Golgi apparatus. It does not seem to be directly related to the lysosomal pathomechanism discussed presently.

4. MODELING ELEMENTS OF OTHER COMMON NEURODEGENERATIVE DISEASES IN YEAST

The following discusses the use of yeast models to study additional common neurodegerative diseases. These diseases include Parkinson's disease and alpha-synuclein in yeast; Alzheimer's disease, Abeta and tau toxicity in yeast as well as suppressors of yeast cell killing by these proteins; pharmacological screens; Huntington's disease and polyQ toxicity in yeast; yeast prions; ALS and the genes FUS, TDP-43 and SOD1; and frontotemporal lobar degeneration.

S. cerevisiae provides a relatively simple system to model elements related to complex diseases of humans including neurodegeneration. To date elements of neurodegenerative diseases modelled in yeast have included: Parkinson's (alpha-synuclein); Alzheimer's (amyloid beta); tauopathies (Alzheimer's, Parkinsonism linked to chromosome 17, FTDP-17); frontotemporal

dementia, Pick's disease, progressive supranuclear palsy (protein tau); Huntington's disease (Huntingtin,); Spinocerebellar Ataxia's, and other polynucleotide-expansion disorders, Prion disease (e.g. *PSI*$^{+}$); amyotrophic lateral sclerosis (ALS: TDP-43, FUS, Sod1) and Dystonia (TorsinA). The use of yeast as models for neurodegeneration has been extensively reviewed elsewhere (64-75). A brief commentary on recent contributions made using yeast model systems is provided. For the most part, important features of the above-mentioned neurodegenerative diseases have been recapitulated in yeast models indicating the efficacy of these systems. These yeast models have often highlighted novel genetic or biological associations, identified potential therapeutic targets and/or provided a facile system for screening for drugs/compounds with potential therapeutic benefit. Once efficacy has been demonstrated this has led to a rapid expansion in the number of studies and groups exploiting yeast models for analysis of a human protein associated with neurodegeneration and a concomitant acceleration in the discovery of new leads that have guided further investigation in higher eukaryotic systems.

4.1. Parkinson's disease

While the precise cellular role of alpha-synuclein has yet to be elucidated, its contribution to familial Parkinson's disease has been demonstrated. A S. cerevisiae alpha-synuclein model system was initially reported by Outeiro and Lindquist (2003), who examined the localisation and growth fitness effects in yeast of wild-type alpha-synuclein and two variants (A53T and A30P) associated with early onset PD in humans (69). This study was important since it demonstrated for the first time that alpha-synuclein expression is associated with cell death in a dose-dependent manner. The study also demonstrated the different cytotoxicity of the wild-type, A53T and A30P forms towards yeast cells. In the same issue of Science (76) screening of the genome-wide haploid deletion collection (77) indicated that lipid metabolism and vesicle-mediated transport were important for tolerance of alpha-synuclein toxicity. Extensive work by numerous groups has helped to demonstrate the efficacy of the yeast model system with key features observed in other PD models systems being recapitulated in the fungal system including a dose-dependent increase in the formation of alpha-synuclein inclusions, interactions with lipid rafts and vesicle trafficking defects and triggering of programmed cell death (69, 78-80).

The use of a transcriptomic approach combined with screening a gene overexpression library for modifiers of alpha-synuclein toxicity provided a comprehensive view of the manner in which alpha-synuclein impacts cells and discovery of novel relationships between this protein and pathways involved in basic cellular processes (81). The use of yeast model systems for the study of proteins associated with PD has led to important advances in the field in a relatively short period, and this is likely to continue for some time yet.

4.2. Alzheimer's disease

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder and is characterized by a progressive loss of cognitive functions, particularly those relating to memory. A hallmark trait of Alzheimer's disease is the appearance of extracellular plaques in the brains of affected individuals. The primary component of these plaques is the peptide amyloid beta (Abeta). Another hallmark trait of AD is the presence of intracellular neurofibrillary tangles of the microtubule binding protein, tau. Alzheimer's disease and the role of Abeta and tau in AD have been extensively reviewed (82-85). Here, discussion is restricted to some of the recent uses of yeast in the study of Abeta and tau. The low order oligomeric forms of $Abeta_{42}$ are the most toxic forms of the peptide, however it has yet to be fully resolved whether the dimeric or trimeric oligomeric form is the most neurotoxic (86, 87). Due to the strong implication of Abeta and its oligomeric forms in AD, many researchers have sought to investigate the mechanism(s) of Abeta toxicity and to identify strategies and drugs that influence Abeta-oligomerisation.

Recently Treusch and colleagues (2011) expressed an ER-targeted form of $Abeta_{42}$ in yeast cells as part of a genome-wide screen for high-copy suppressors or enhancers of $Abeta_{42}$ toxicity (88). The genetic screen identified 23 suppressors of $Abeta_{42}$ toxicity including those involved in clathrin-mediated endocytosis and vesicle transport, while the 17 enhancers included genes involved in the cellular response to osmotic stress. Importantly the yeast screen identified a number of the genes that have a homologue in humans that has been identified as a validated risk factor for AD or a gene/encoded protein known to interact with a validated risk factor. For example yeast gene *YAP1802* was identified as a suppressor of Abeta toxicity. *YAP1802* is a homologue of PICALM a gene recently implicated as AD risk factor by a large genome-wide association study (89). Some of the key findings in yeast were further studied in glutamatergic neurons of *C. elegans* and primary rat cortical neurons. The study strongly implicated endocytic trafficking as an AD risk factor.

While the above study used expression of ER-targeted Abeta in yeast, Abeta forms expressed in the yeast cytosol have also been studied. Expression of a cytosolically localised $Abeta_{42}$-green fluorescent fusion protein has also been used to demonstrate that $Abeta_{42}$ expression leads to a growth defect and induction of the heat shock response (90). Bagriantsev and Liebman (2006) also generated an $Abeta_{42}$ fusion protein that provides a facile system to screen for factors (compounds or genetic factors) affecting the level of lower order $Abeta_{42}$ oligomers (91). Recently Liebman and colleagues (92) exploited this system to undertake a high throughput screen of 12 800 compounds to identify those that inhibit $Abeta_{42}$ oligomerisation. The method couples the extent of $Abeta_{42}$ oligomer formation to the growth fitness of yeast cells. The approach identified two "presumptive anti-oligomeric compounds" that were also found to inhibit $Abeta_{42}$ oligomerisation in a subsequent *in vitro* assay. This yeast-based system provides a cheap and powerful platform to screen for compounds that inhibit $Abeta_{42}$ oligomerisation. These studies demonstrate the efficacy of yeast as a model system to study elements of AD.

4.3. Huntington´s disease

Huntington's disease (HD) is an autosomal dominant disorder associated with expansion of polyglutamine (polyQ) repeats in the *N*-terminal region of the Huntingtin protein, whereby the number of the glutamine repeats strongly correlates with the likelihood of disease, age of onset and rate of disease progression (93). Yeast model systems have also proved useful for studying the toxic interactions of polyQ expansions on cells. Importantly two key features associated with HD have been recapitulated in yeast systems. These relate to the influence of the length of glutamine repeat region of fragments of huntingtin on protein aggregation as well as toxicity towards cells. Two separate genome-wide deletion mutant screens highlighted the cellular processes that influence tolerance to polyQ variants expressed in yeast. The first, reported by Willingham and colleagues (2003) identified deletion mutants with increased sensitivity to a polyQ variant (76). The study highlighted the important role of protein folding and catabolic pathways and pathways involved in tolerance to stress in countering polyQ toxicity. These findings were consistent with the view that an important factor in HD is the interaction of mutated huntingtin with other polyQ-containing proteins in cells (94). A second yeast genome-wide deletion mutant screen (95) identified both enhancers (52 genes) and suppressors (28 genes) of the toxicity of a huntingtin fragment and demonstrated the role of kynurenine metabolism, in particular kynurenine 3-monoxygenase, in modulating polyQ toxicity. A recent analysis of *de novo* prion (PSI^+) formation in over 400 yeast deletion mutants identified two distinct classes (I and II) of gene deletions (96). The class I deletions (containing *bug1*, *bem1*, *arf1*, and *hog1*) reduced the efficiency of PSI^+ induction, but formed normal Sup35-GFP fluorescent rings while those of class II (containing *las17, vps5* and *sac6*) inhibited both PSI^+ and ring formation. Interestingly, class II deletions reduced toxicity associated with expression of glutamine repeats of exon 1 of huntingtin while those belonging to class I increased toxicity. In another study using yeast it was proposed that wild-type and mutant polyQ domains associate with the outer mitochondrial membrane leading to mitochondrial dysfunction (97). Overexpression of *HAP4*, encoding the catalytic subunit of the Hap2/3/4/5p transcriptional regulator complex, rescued cells from the toxic effects of polyQ on mitochondrial respiratory function, indicating that polyQ toxicity was linked to altered mitochondrial physiology. Interestingly Sorolla and colleagues (2011) examined the effects of glucose levels and the metabolic state of cells on polyQ toxicity and found that polyQ was more toxic to respiring cells and was associated with increased oxidative stress (98). Whether the increased toxicity and oxidative stress were linked to changes in mitochondrial function reported by Ocampo *et al.* (2010) is unclear (95, 97).

Numerous polyQ variants have been examined in yeast. Some of the different systems facilitating polyQ expression in yeast and for assessing associated toxicity have recently been described (99). Yeast model systems will continue to provide powerful platforms to investigate the cytotoxic effects of polyQ variants on cells. However, yeast models played also an important role to discover first functional links between Huntingtin and other proteins causing trinucleotide-expansion disorders. For instance, a yeast screen identified the wild-type form of both huntingtin and ataxin-2, the disease causing protein in Spinocerebellar Ataxia type 2, to be toxic in yeast cells lacking the actin bundling protein Sac6 (100). This phenotype could be compensated by overexpression of human plastin proteins, indicating that both huntingtin and the SCA2 protein play a role in maintaining the plasticity of the actin cytoskeleton. Nowadays, it is broadly assumed that numerous functional links between polyQ proteins exist (101), and that the polynucleotide expansion is not the only common feature between these complex disorders.

4.4. Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration

ALS is characterized by death of motor neurons of the central nervous system leading to paralysis and death. While the underlying cause of most cases appears sporadic, in recent years a number of genes including FUS (fused in sarcoma), TDP-43 and SOD1 have been implicated as playing a role in the pathogenesis of ALS in certain cases (102). FUS and TDP-43 both appear to be nucleic acid-binding proteins, however their exact roles in the central nervous system and in the mechanism leading to ALS are unclear. While wild-type FUS is predominantly located in the nucleus, mutation of the protein can lead to its translocation to the cytoplasm where it can form inclusion bodies in affected spinal motor neurons of glia. Wild type TDP-43 on overexpression forms protein aggregates in yeast with concomitant efficient killing of the cells (Figure 2).

Expression of FUS in yeast also leads to the formation of cytoplasmic inclusions and associated toxicity. Ju and colleagues (2011) performed a genome-wide overexpression library screen in yeast identifying 11 genes, five of which encode RNA-binding proteins, capable of rescuing FUS toxicity (103). The group also exploited the yeast model system to determine the regions of the protein that are important for nuclear localization. The data obtained in yeast were also used to identify several human proteins that could rescue FUS toxicity when expressed in yeast. Simultaneously Sun and colleagues (2011) reported their findings of FUS and TDP-43 expression in yeast (104). FUS cytosolic aggregation and RNA binding was essential for toxicity, and like in ALS patients FUS aggregates partition to stress granules in yeast. Unlike TDP-43, mutations in FUS linked to ALS did not affect its aggregation. In an analogous manner to Ju *et al.* (2011), Sun and colleagues also undertook a genome-wide gene overexpression screen to identify enhancers and suppressors of FUS toxicity. The screens implicated an important role of genes associated with stress granule assembly and RNA metabolism in FUS but not TDP43 toxicity. These and other findings led the group to propose that while TDP-43 and FUS are both RNA-binding proteins the mechanisms influencing their aggregation and disease promotion are distinct. It has also since been shown that expression of TDP-43 in yeast triggers mitochondrial-dependent programmed cell death (105). TDP-43

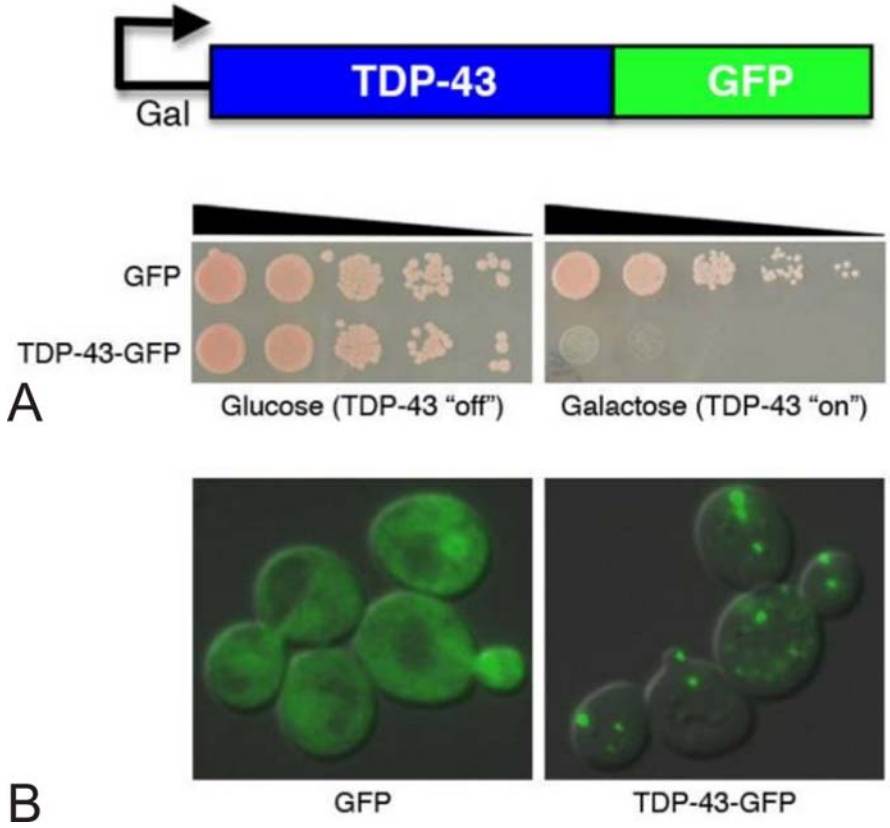

Figure 2. Yeast TDP-43 proteinopathy model. A TDP-43 expression construct under the control of a tightly regulated galactose-inducible promoter was introduced into yeast cells. (A) Spotting assays using five-fold serial dilutions demonstrate TDP-43 is toxic when expressed at high levels in yeast cells (pAG426Gal-TDP-43-GFP).When cells are spotted on plates containing glucose, TDP-43 expression is repressed and induced in the presence of galactose. (B) TDP-43-GFP formed multiple cytoplasmic foci when expressed in yeast cells, whereas GFP alone was diffusely distributed throughout the cytoplasm and nucleus. Taken from Armakola *et al.* with permission (64).

cytotoxicity was dose and age-dependent and TDP-43 aggregates were localized in a perimitochondrial manner. Toxicity of TDP-43 was reduced upon impairment of mitochondrial respiratory capacity. The use of yeast and other model systems for the study of ALS has recently been reviewed (106, 107).

5. YEAST AS A MODEL FOR OXIDATIVE STRESS AND INBORN DEFECTS IN METABOLISM

This section stresses the close connection between inborn errors of metabolism in the central pathways of glycolysis and the pentose phosphate pathway on the one hand, and inherited neurodegenerative diseases on the other. Oxidative stress seems to be a common phenomenon in these enzymopathies. Triose phosphate isomerase deficiency was one of the first enzymopathies in this category. Interestingly, it is not the loss of enzyme activity that causes the disease, but the regulatory properties of this enzyme which depend on dimerization.

Neurodegenerative disorders have strong metabolic components. *Vice versa*, most disorders of central metabolism have neuronal phenotypes and cause mental retardation or cause motor-neuro symptoms. Due to the high conservation of central metabolism among the eukaryotic line, yeast has emerged as a useful model in studying these metabolic defects.

Inborn defects in metabolism, or genetic metabolic diseases, occur with regular frequency due to the natural mutation rate. However, especially enzymopathies that are caused by mutations in central metabolic enzymes regularly occur at a very low frequency; as in most cases, the phenotypes are strong and evolutionarily deleterious. It was almost 50 years ago, that Triosephosphate isomerase (TPI) deficiency, one of the first inherited enzymopathies, was characterized (108). The mutant protein, TPI, is a highly abundant glycolytic enzyme, ubiquitously expressed, and highly conserved. TPI deficiency presents as a recessive systemic disorder, characterized by cardiomyopathy, haemolytic anaemia, susceptibility to infections, severe neurological dysfunction, and, in most cases, death in early childhood (108-110). However, although symptoms are prominent, and molecular diagnosis can easily be conducted by candidate gene re-sequencing, TPI deficiency has been diagnosed less than 100 times worldwide (110).

The frequency of heterozygous TPI null allele carriers predicts a higher incidence of homozygous null allele carriers (111). Transgenic yeast models were developed to compare the different pathogenic TPI alleles in an identical genetic background. These yeast strains were generated by a gene replacement strategy, in which yeast TPI was replaced with its human orthologue, in its wild-type and its pathogenic forms, yielding isogenic models which differed only in the TPI allele (112). Experiments with these yeast strains revealed that the pathogenic TPI alleles were not catalytically dead as was concluded from enzyme assays performed in patient blood cells. Instead, altered formation of the dimeric TPI quaternary structure was detected in pathological allelic combinations (112). Later, this discovery was confirmed by structural analysis of mutant TPI (113). These investigations provided an explanation of the low frequency of TPI deficiency, as only one single point mutation ($TPI^{Glu104Asp}$) could be associated to all but two cases of this disease, and the formation of the pathogenic dimer (110, 112, 113). Thus, TPI deficiency is rarer than predicted from the occurrence of null alleles, as these seem to cause embryonic lethality in the homozygous state, but only cases that exhibit certain TPI structural deficits are viable.

6. YEAST AS A MODEL ORGANISM FOR THE OXIDATIVE STRESS RESPONSES

A further rapid response mechanism occurring under oxidative stress involves pyruvate kinase. Inactivation of pyruvate kinase leads to inactivation of triose phosphate isomerase via accumulation of phosphoenol pyruvate and by this mechanism to increased NADPH production via the pentose phosphate pathway. This mechanism was shown to be active in yeast and in cancer cells exhibiting the Warburg effect.

Interestingly, it was discovered in the above context that another TPI allele ($TPI^{Ile170\ Val}$) (114) conferred resistance to oxidative stress caused by the thiol oxidizing compound diamide (112). Oxidative stress has been found to contribute to age-related disorders, including Alzheimer's disease, cancer, and senescence (115-120), and is defined as a change in the equilibrium of oxidizing over reducing molecules in a cell (121). This disequilibrium can either be caused by a strongly increased production of reactive oxygen species (ROS) in the respiratory chain or other biochemical redox processes such as misfolding of proteins in the ER, overloading the cellular anti-oxidative capacity, or by defects in the latter (121, 122).

The human TPI allele $TPI^{Ile170Val}$ is not directly associated with any disease phenotype, but exhibited a reduced activity to about 30% (112). Interestingly, the enzyme next to TPI in glycolysis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is rapidly inactivated when cells are treated with oxidants (123, 124). It had been speculated that inactivation of this glycolytic enzyme could protect against oxidative stress by activating the pentose phosphate pathway (PPP), a carbon breakdown pathway closely interconnected with glycolysis and which is the major source of NADPH in the cell cytoplasm (123). The identification of the oxidant phenotype of the $TPI^{Ile170Val}$ allele allowed experimental test of this hypothesis: indeed, oxidant treatment and reduced TPI inactivation increased the metabolite concentration in the PPP, and increased the rate of $NADP^+$ reduction to NADPH. Furthermore, all anti-oxidant effects of mutant TPI were abolished in yeast in which the first enzyme of the oxidative PPP, glucose 6-phosphate dehydrogenase, was deleted (125). This oxidative PPP is responsible for NADPH production and is not reversible. NADPH in turn is an essential molecule for detoxification of free radicals and for stabilizing the cellular redox state, as it provides the reducing power for anti-oxidant enzymes and for recycling the antioxidant peptide glutathione (126). Although there are several sources of NADPH in the cell, the $NADP^+$ reducing function of the PPP is of particular importance during the stress response: the $NADPH/NADP^+$ ratio collapses upon an oxidant treatment, when the oxidative PPP is deficient (127) .

Later, it was shown that the boost in PPP activity through GAPDH inactivation is of particular importance allowing cell survival during the first few seconds after a cell contacts an oxidative stressor (128). Whereas transcriptional changes of the stress response become first evident after a couple of minutes (129), metabolite levels change within seconds. This corresponds to the speed of GAPDH inactivation, which has a half-life if the order of seconds (128).

Recently, it has been shown in yeast that a similar reconfiguration of central metabolism prevents oxidative stress when cells switch from fermentative (anaerobic) to oxidative metabolism (aerobic). Here, the enzyme pyruvate kinase (PYK), catalysing the last step of glycolysis, takes centre stage. When yeast cells respire, the activity of PYK is reduced. This causes accumulation of the PYK substrate phosphoenol-pyruvate (PEP), which acts as a feedback inhibitor of TPI. TPI inhibition by PEP was required to prevent oxidative stress and oxidative damage in respiring yeast cells (130). Recently, it was confirmed that this mechanism seems to be of major importance for cancer cell metabolism. Similarly to respiring yeast, cancer cells exhibit reduced pyruvate kinase activity and accumulate PEP (131). Oxidation of pyruvate kinase in cancer cells was found to be responsible for reduced catalytic activity and activation of the PPP. Distortion of this mechanism induced oxidative stress in cancer cells. These cells accumulated ROS, showed increased oxidative damage which finally slowed cancer cell proliferation (132).

7. YEAST MODELS FOR DISORDERS OF THE PENTOSE PHOSPHATE PATHWAY

Enzymopathies based on mutations in three enzymes of the pentose phosphate pathway are described: glucose-6-phosphate dehydrogenase, transaldolase, and ribose-5-phosphate isomerase. Yeast models were established for these enzymopathies and these have helped to get insight into the pathomechanism.

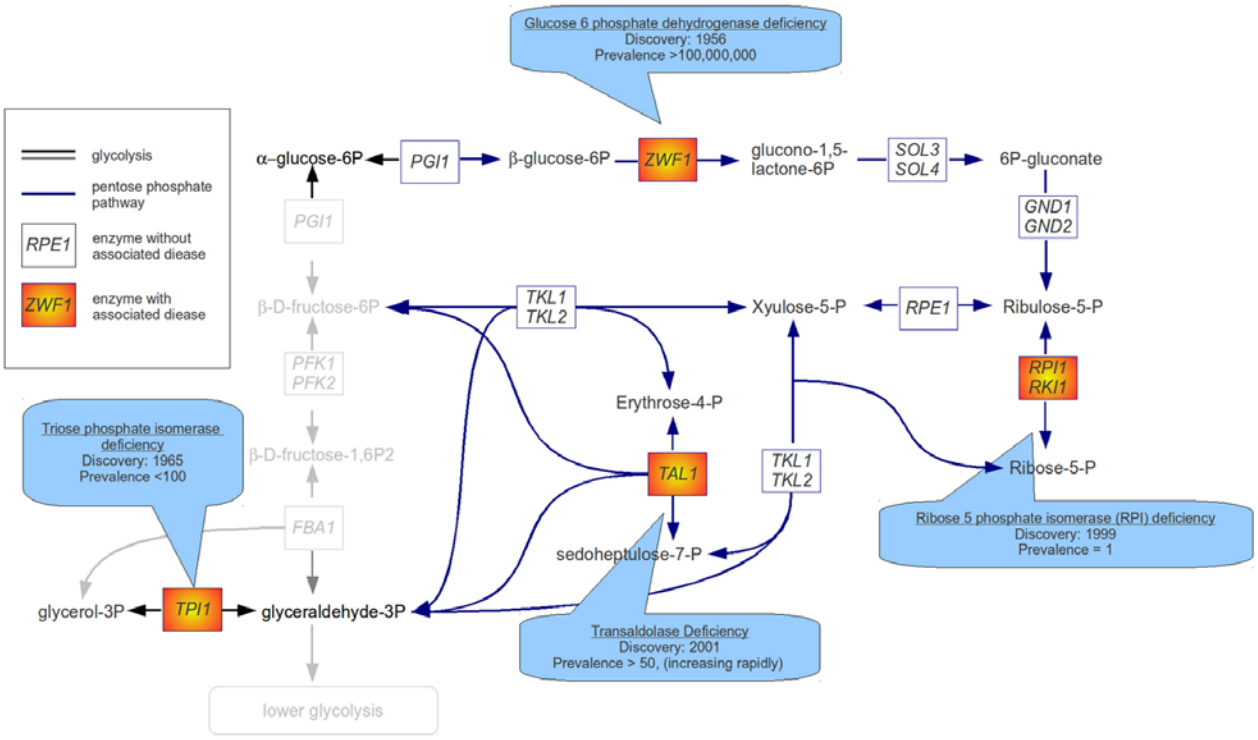

Figure 3. A schematic overview of the pentose phosphate pathway (and parts of the glycolytic pathway). Four of the enzymes involved were found to harbor mutations in patients with mild or severe metabolic diseases, which affect among other organs the central nervous system (see text for details). PGI1, phosphoglucose isomerase; ZWF1, glucose-6phosphate dehydrogenase; SOL3, SOL4, lactonase; GND1, GND2, gluconate-6-phosphate dehydrogenase; PGI1, phosphoglucose isomerase; TKL1, TKL2, transketolase; RPE1, ribulose-5-phosphate epimerase; RPI1 + RKI1, ribose-5-phosphate isomerase; TAL1, transaldolase; PFK1, PFK2, phosphfructokinase; FBA1, fructose-bisphosphate aldolase; TPI1, triosephosphate isomerase.

The role of the PPP in the oxidative stress response is not limited to its function as NADPH donor; it also helps to induce the transcriptional changes that accompany an oxidative burst. Therefore, neither in yeast nor in humans, are the effects of PPP disturbances fully described by changes in NADPH oxidation; upon an oxidative burst, gene expression changes do not follow the wild-type trend when PPP activity is disturbed (133).

The multiple functions of the PPP may be responsible for the variety of phenotypes seen in PPP deficiencies. In humans, mutations in enzymes of the PPP are causative for at least three highly different metabolic syndromes (Figure 3) (134). Interestingly, these comprise both the most frequent as well as the rarest human enzymopathies.

The first disease, Glucose-6-phosphate dehydrogenase deficiency is the most common human enzyme defect and present in more than 400 million people worldwide. Its high frequency may be explained by a selective advantage against parasite infection (135). Glucose-6-phosphate dehydrogenase deficiency does not have a strong clinical phenotype, unless the patient's red blood cells are exposed to certain chemicals or stress conditions.

Transaldolase deficiency, the second defect, is however much rarer, and has a much more severe phenotype. Since its first description in 2001 (136), this disease has meanwhile been diagnosed in more than 30, mostly unrelated patients of Turkish, Arabian, Pakistani and Polish origin (134, 137). Transaldolase deficiency presents with liver cirrhosis and hepatosplenomegaly during early infancy, and develops into a systemic disease during the (dramatically reduced) lifetime.

The third defect, Ribose 5-phosphate isomerase (RPI) deficiency is, with a single diagnosed case, considered to be the rarest human genetic disorder. The unique boy was first diagnosed to suffer from development abnormalities which turned out to be caused by a (neuronal) white matter disorder (138). Systematic metabolic profiling identified increased concentration in open long-chain alcohols (polyols), which originate in PPP intermediates. Resequencing of PPP enzymes finally identified a genetic defect in the RPI enzyme (138). At the time of the discovery of the enzyme defect a complete loss of RPI enzyme activity was suggested to be the molecular cause of the patient's molecular and pathologic phenotype. However, similar to TPI deficiency, a total loss of RPI activity as disease cause would predict a higher frequency: RPI null alleles occur with a certain frequency due to the natural mutation rate.

Here a combination of yeast molecular genetics and biochemical analysis of patient cell lines helped to shed light on the pathomechanism of the rareness of the disorder: the patient was compound-heterozygous for two RPI alleles with a different defect. The first allele, containing a premature stop codon, encoded a non-functional and rapidly degraded RPI form. The defect of the second allele, however, was more complex. Expressed in yeast, it inhibited a residual activity about 20%-30% of that of the wild-type enzyme. This activity was also found in patient lymphoblast- but not fibroblast extracts. Analysis of RPI mRNA and protein levels finally revealed, that this difference results from a cell-type specific expression deficit: in some but not all cell types, the defective allele was expressed, producing enough RPI enzyme allowing survival, but causing a strong pathology.

8. CONCLUSIONS

Here, we have summarized examples for the use of yeast molecular genetics in the elucidation of pathomechanisms of human diseases, with special emphasis on neurodegeneration and oxidative stress. The comprehensive toolset of yeast molecular genetics, genomics, proteomics and metabolomics has helped to uncover the role of human disease proteins, and examples are given where they have made a crucial contribution to understand pathomechanisms of human disease. In particular we described the neuronal ceroid lipofuscinoses which are genetic diseases of the childhood and adult age, then some of the most common and mostly sporadic neurodegenerative diseases, like Alzheimer's disease, and finally enzymopathies of the central metabolic pathways, glycolysis and the pentose phosphate pathway which can cause neurodegeneration and play a role in cancer cells. Obviously, we do not think that the yeast model system in any of these diseases makes other model systems superfluous. On the contrary, we think that model systems based on patients' cell culture, animal models, the yeast model, and clinical data can only together solve the problem of understanding the disease.

With regard to cell cultures, in particular the new and exciting human induced pluripotent stem cells are interesting since they can be induced to differentiate to the required (for instance) neuronal cell types. However, as described here, the yeast model system is superior in certain specialized aspects of molecular genetic research on pathomechanisms. To give one example: large scale screening and selection procedures which help to identify interaction partners of a protein that has been recognized as carrier of the disease-causing mutation can for practical reasons only be performed in a single celled organism like yeast. It is, of course, a prerequisite that an orthologe of the disease protein exists in yeast and that the particular part of cellular physiology that is involved is a genenal eukaryotic one, and not one specific to yeast or other fungi. Thus, in addition to mammalian model systems, yeast models have been established as a toolbox for efficient, economic and reliable biomedical investigations. Currently, new large-scale and proteomic techniques are identifying disease-related molecules at increasing speed. However, understanding their biochemical functions and properties is as challenging as ever in the last decades. Since single celled eukaryotic models are very efficient in assessing biological functions, they are therefore not outdated but more important than ever to create a unified picture of living organisms and of disease processes.

9. ACKNOWLEDGEMENTS

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Abbreviations: NCL: neuronal ceroid lipofuscinosis (a heterogenous group of neurodegenerative diseases), INCL: infantile NCL, LINCL: late infantile NCL, JNCL: juvenile NCL, ANCL: adult NCL, CLN: acronym and gene designation for the genes responsible for NCL, PPP: pentose phosphate pathway, AD: Alzheimr´s disease, PD: Parkinson´s disease, HNE: 8-hydroxynonenal, EEG: Electroencephalography, ERG: electroretinography, VEPs: visual evoked potentials, HD: Huntington's disease, ALS: Amyotrophic lateral sclerosis, TPI: Triosephosphate isomerase, ROS: reactive oxygen species, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, RPI: Ribose 5-phosphate isomerase, PYK: pyruvate kinase, PEP: phosphoenol-pyruvate

Key Words: Review, Ceroid lipofuscinoses, *Saccharomyces cerevisiae,* Yeast, Parkinson's disease, Alzheimer's disease, Huntington´s disease, Amyotrophic lateral sclerosis, Batten disease, Oxidative stress, Pentose phosphate pathway

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