Using yeast models to probe the molecular basis of amyotrophic lateral sclerosis

Emma L. Bastow, Campbell W. Gourlay and Mick F. Tuite
School of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

Abstract
ALS (amyotrophic lateral sclerosis) is a fatal neurodegenerative disease attributable to the death of motor neurons. Associated with ALS are mutations in the genes encoding SOD1 (superoxide dismutase 1), FUS (fused in Sarcoma) protein and TDP-43 (TAR DNA-binding protein-43) each of which leads to aggregation of the respective protein. For example, the ALS-associated mutations in the hSOD1 (human SOD1) gene typically destabilize the native SOD homodimer, leading to misfolding, aggregation and degradation of SOD1. The ALS-associated pathology is not a consequence of the functional inactivation of SOD1 itself, but is rather due to a toxic gain-of-function triggered by mutant SOD1. Recently, the molecular basis of a number of human neurodegenerative diseases resulting from protein misfolding and aggregation, including fALS (familial ALS), was probed by using the baker’s yeast, Saccharomyces cerevisiae, as a highly tractable model. Such studies have, for example, identified novel mutant SOD1-specific interactions and demonstrated that mutant SOD1 disrupts mitochondrial homeostasis. Features of ALS associated with TDP-43 aggregation have also been recapitulated in S. cerevisiae including the identification of modulators of the toxicity of TDP-43. In this paper, we review recent studies of ALS pathogenesis using S. cerevisiae as a model organism and summarize the potential mechanisms involved in ALS progression.

Introduction
As the world’s population continues to increase in age, the prevalence of neurodegenerative disease also rises. A number of these diseases are associated with protein misfolding and subsequent aggregation and include both transmissible (e.g. prion diseases) and non-transmissible (e.g. Alzheimer’s disease and Huntington’s disease) diseases. ALS (amyotrophic lateral sclerosis) is also a non-transmissible neurodegenerative disease and it is estimated that there are currently up to 30,000 ALS patients in the U.S.A., with 5000 new cases being diagnosed each year as reported by the National Institute of Neurological Disorders and Stroke, National Institutes of Health (Bethesda, MD, U.S.A.).

ALS is a fatal neurodegenerative disease affecting the upper and lower motor neurons of the brain and spinal cord. Degeneration of these motor neurons leads to muscle weakness and subsequent atrophy of the surrounding tissues with a prognosis from onset typically between 3 and 5 years. Although most of the ALS cases occur sporadically, 20% have been attributed to a fALS (familial ALS) form. The most common fALS-associated mutation occurs in the gene encoding SOD1 (superoxide dismutase 1), an enzyme that converts superoxide anions into H2O2 and oxygen, thus protecting cells against oxidative damage. However, a number of other genes have been implicated in both the sporadic and familial forms of ALS and include the genes encoding FUS (fused in sarcoma) protein and TDP-43 (TAR DNA-binding protein-43). In each case the mutations cause the protein to show a higher propensity to misfold and aggregate. It remains to be determined why these changes trigger such conformational alterations and how this leads to the disease pathology.

Yeast as a model for studying ALS and other neurodegenerative diseases associated with protein misfolding
To facilitate the analysis of the cause and consequences of disease-associated protein misfolding, a number of ‘neurodegenerative disease models’ have been established in the baker’s yeast, Saccharomyces cerevisiae. S. cerevisiae is a highly tractable but simple eukaryote that nevertheless has many of the protein misfolding and quality control mechanisms that exist in higher eukaryotes. A number of the high-profile human neurodegenerative diseases described to date, including AD (Alzheimer’s disease) [1], PD (Parkinson’s disease) [2] and HD (Huntington’s disease) [3], are being successfully studied in yeast models [4,5]. There has also been recent progress in analysing aspects of fALS in S. cerevisiae by studying the misfolding and cellular consequences of
### Table 1 | Genes implicated in fALS that have been studied in yeast models

<table>
<thead>
<tr>
<th>Gene</th>
<th>Human</th>
<th>Yeast</th>
<th>Gene product</th>
<th>Implication of mutant protein in fALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUS</td>
<td>–</td>
<td>–</td>
<td>75 kDa component of the hnRNP (heterogeneous nuclear RNP) complex</td>
<td>Increased aggregation and cytotoxicity/cell death</td>
</tr>
<tr>
<td>TDP-43</td>
<td>–</td>
<td>–</td>
<td>TDP-43</td>
<td>Cytoplasmic aggregation instead of a nuclear localization/ altered DNA/RNA processing</td>
</tr>
<tr>
<td>SOD1</td>
<td>SOD1 YJR104C</td>
<td>16 kDa SOD1 1, located in cytoplasm/inter-membrane space of mitochondria</td>
<td>Oxidative stress-sensitivity</td>
<td></td>
</tr>
<tr>
<td>ATXN2</td>
<td>PBP1 YGR178C</td>
<td>14 kDa cytoplasmic ataxin-2/7.9 kDa Pab1p-binding protein</td>
<td>Increases toxicity of TDP-43</td>
<td></td>
</tr>
</tbody>
</table>

expressing disease-associated alleles of the SOD1, FUS and TDP-43-encoding genes (Table 1).

The main objective of using a yeast-based model approach to further our understanding of these diseases is to establish the molecular basis of the origin and pathology of this globally important group of diseases. For example, genetically manipulating *S. cerevisiae* to accumulate misfolded proteins allows the molecular mechanisms important in the cellular folding machinery and the response to misfolded proteins to be identified. The factors that trigger misfolding and the generation of the toxic entity associated with this misfolding can also be established in order to identify potential intervention strategies. The value of yeast models in such studies will be illustrated here by reviewing the progress made so far and the prospects for elucidating the molecular basis of fALS. In so doing, we will focus on how mutations in SOD1, FUS and TDP-43 may have an impact on host cell functions and protein homeostasis (Figure 1). For example, various mutations in the hSOD1 (human SOD1) gene lead to aggregation of the encoded mutant SOD1 together with mitochondrial dysfunction and apoptosis. Are there intermediate misfolded protein species that could influence pathogenesis? Does aggregation prevent this intermediate species from further damaging cells; that is, are these aggregates protective or toxic to cells?

Figure 1 | Schematic diagram of potential toxicity pathways identified in studies of yeast expressing mutant SOD1, TDP-43 and FUS proteins

Mutant SOD1 proteins that are unstable and have the propensity to aggregate remain active and retain the ability to scavenge ROS. The instability of SOD1 does, however, lead to the formation of misfolded protein aggregates that are associated with toxicity. Although the mode of action is currently unknown, SOD1-based toxicity may involve the mitochondria. Mutations in SOD1 lead to instability of the protein and subsequent toxic interactions with Bcl-2 that is normally an anti-apoptotic protein. This interaction consequently leads to the release of cytochrome *c* from the mitochondria by causing Bcl-2 to become pro-apoptotic by exposing a toxic BH3 domain. SOD1 mutants also aggregate, causing mitochondrial dysfunction, and are degraded because of their highly misfolded structure. TDP-43 aggregation also leads to apoptosis in yeast in a mitochondrial-dependent manner. Both TDP-43 and FUS lead to cytotoxicity in yeast and the protein encoded by the *PBP1* gene increases the toxicity of TDP-43. It has also been suggested, however, that aggregation of TDP-43 and FUS are protective mechanisms against cytotoxicity and that intermediate species are responsible for their toxic effects on the cell.

The use of *S. cerevisiae* as a model to study fALS associated with SOD1

SOD was first described as a superoxide scavenger in 1967 and was later identified as a potential cause of certain cases of ALS in 1993 [6,7]. The loss of SOD1 activity in humans has not been implicated in ALS progression, suggesting that the potential role of mutant SOD1 in fALS is most likely to be via a toxic gain-of-function rather than through loss-of-function through incorporation into dysfunctional aggregates. This has also been reinforced by the finding that fALS-associated SOD1 mutant proteins show unaltered copper binding and can continue to scavenge oxygen radicals [8].
S. cerevisiae encodes two different SOD enzymes, SOD1, a cytosolic Cu\(^{2+}\)-Zn\(^{2+}\) enzyme that is an orthologue of the fALS-associated hSOD1, and a mitochondrial manganese SOD (SOD2) that serves to protect cells against oxygen toxicity. Yeast cells engineered to lack SOD1 by disruption of the SOD1 gene (sod1\(\Delta\)) show a number of phenotypes that facilitate the analysis of mutant human or yeast SOD1 enzymes. For example, sod1\(\Delta\) mutants are sensitive to oxidative stress induced by the addition of menadione when compared with a SOD1\(^{+}\) wild-type strain (Figure 2). sod1\(\Delta\) strains also show an auxotrophy for lysine in the presence of oxygen that is caused by inactivation of a homoaconitase protein encoded by the LYS4 gene; a mitochondrial matrix protein that catalyses a step in the lysine biosynthetic pathway [9]. It is suggested that the lack of SOD1 probably leads to an increase in superoxide radicals in the mitochondrial inner membrane space and that these then diffuse into the matrix, subsequently inactivating the LYS4-encoded homoaconitase [9]. However, arguing against this hypothesis is the fact that superoxides are unable to cross membranes. The auxotrophy for lysine exhibited by cells lacking SOD1 may therefore reflect a function not directly related to its role as a ROS (reactive oxygen species) scavenger.

One possibility is that SOD1 plays an important role in metabolic signalling processes as evidenced by sod1\(\Delta\) mutants showing an altered metabolism leading to a higher consumption of oxygen and a larger mitochondrial mass. This suggests that, in the presence of glucose, the mitochondria remain functional. This phenotype is partially dependent on the Hap2,3,4,5 complex [10], which functions in transcriptional activation and as a regulator of mitochondrial biogenesis. It is possible therefore that SOD1 plays an important role in the regulation of signalling pathways that control metabolism.

Suppressors of metabolic defects in the sod1\(\Delta\) background have been identified, all of which are associated with mitochondria and include mutations in SSQ1 (stress seventy subfamily Q 1) protein, JAC1 (J-type accessory chaperone) and NFS1 (NiFS-like 1) proteins [11]. As these proteins are all involved in iron–sulfur assembly, the suppression of oxygen toxicity by mutations in these proteins suggests that the mechanism of iron–sulfur assembly in sod1\(\Delta\) increases oxidative damage. Overexpressing the yeast Atx2 (antioxidant 2), a Golgi apparatus-associated protein that is involved in manganese homoeostasis, also rescues the oxygen toxicity of sod1\(\Delta\) strains [12], suggesting that metal homoeostasis contributes to the increase in oxidative damage in cells lacking SOD1. As many enzymes including SOD1 rely on metal binding for their activity, it is important to understand the molecular basis surrounding metal homoeostasis so that potential links with ALS can be established. Such studies can be readily carried out in yeast.

Studies on fALS-associated mutant hSOD1

In addition to exploring the effects that the loss of SOD1 function has in yeast, a number of studies have also examined the consequences of expressing fALS-associated mutant forms of hSOD1 [13]. Although there are over 100 fALS-associated mutations, the hSOD1\(^{G93A}\) mutant has been studied the most extensively. The finding that different hSOD1 mutations affect activity differently [14] reinforces the hypothesis that ALS pathology is a consequence of a toxic gain-of-function rather than a loss of SOD1 function.

As with the yeast enzyme, hSOD1 forms a homodimer carrying copper- and zinc-binding sites that are important for the enzyme’s function. Disruption of these sites affects SOD activity, resulting in an impaired ability to scavenge free radicals [15,16] or alternatively can lead to aberrant catalytic activity that has previously been linked to ALS progression. In addition to its superoxide activity, SOD1 can also exhibit peroxidase activity and generate radicals from H\(_2\)O\(_2\) [17]. In fact the hSOD1\(^{A4V}\) and hSOD1\(^{G93A}\) mutations lead to an increased rate of hydroxyl radical production [18]. However, the hypothesis that such an altered enzymatic activity can lead to ALS has fallen out of favour as mutant forms of SOD1 that cannot bind copper but that carry mutations that promote misfolding still lead to disease in a mouse model [19].

The copper chaperone CCS1 is important in maintaining SOD1 function by inserting a Cu\(^{2+}\) ion into the active site and promoting the formation of a disulfide bond by the targeted oxidation of two specific cysteine residues [20]. The CCS1 protein shows approximately 50% amino acid sequence identity with hSOD1 and the substitution of D200H in CCS1 results in a bifunctional SOD that can self-activate [21]. The presence of GSH and glutathionylation are also essential for SOD1 activation by protecting thiol residues under mild oxidative stress [22]. This process can, however, lead to activation of mammalian SOD1 in a CCS1-independent manner, whereas yeast SOD1 is dependent on CCS1 for activation due to a pair of proline residues at the C-terminus of the protein [23].
The mechanism of CCS1-mediated SOD1 activation has been implicated in fALS in which the immature forms of mutant SOD1, in the absence of copper, are targeted by GRX (glutaredoxin). GRX promotes the reduction of the disulfide bond, subsequently leading to the instability and degradation of mutant SOD1 [24]. The overexpression of the cytosolic GRX, encoded by the GRX1 gene, in neuronal cells leads to solubilization of aggregated mutant SOD1, but does not alleviate the associated mitochondrial dysfunction. The mitochondrial GRX2, however, increases the solubility of mutant SOD1 in mitochondria, resulting in protection against apoptosis [25]. This suggests that the toxicity is mitochondrial-dependent as mitochondrial GSH also increases the survival of NSC-34 mammalian cells expressing SOD1G93A [26]. GSH acts to reduce GRX and is then replenished by GSH reductase, forming a GSH relay system [27]. The distribution of SOD1 in the mitochondria plays an important role in detoxifying ROS produced as a by-product of the electron transport chain. S. cerevisiae expressing a SOD1 variant that has been linked to a targeting sequence for the inner membrane space of mitochondria results in an increased inner membrane space localization that correlates with an increase in protection against oxidative damage [28].

The expression of ALS-linked SOD1G93A leads to an increased proportion of SOD1 in the inner membrane space of mitochondria compared with the wild-type distribution [28]. Surprisingly this increase in SOD1G93A present in mitochondria increases the level of protection against superoxide radicals, indicating a potentially more protective role against oxidative stress than wild-type SOD1.

CCS1 localization determines whether SOD1 enters the mitochondrial inner membrane space through the Mia40 (mitochondrial inner membrane space import and assembly 40)/Erv1 (essential for respiration and viability 1) pathway [29]. This process has been further characterized in S. cerevisiae in which a disulfide relay system between Erv1, Mia40 and CCS1 acts to post-translationally modify apo-SOD1 and retain it in the inner membrane space of mitochondria [30]. The expression of mutant hSOD1 in yeast results in decreased electron transport and haem content in mitochondria, which suggests that mutant SOD1 may also affect the assembly of the electron transport chain complex [31].

Mutant hSOD1 also interacts with the mitochondrial protein Bcl-2. This toxic interaction occurs specifically with the fALS-associated mutant but not the wild-type form of SOD1 in mice and human spinal cords [32]. Bcl-2 is an anti-apoptotic protein that sits on the mitochondrial membrane and interacts with both wild-type (non-toxic) and mutant forms of SOD1 (toxic) [33]. The interaction that occurs with mutant SOD1 exposes a toxic BH3 domain (Bcl-2 homology 3 domain) that changes the protein function from anti-apoptotic to pro-apoptotic. Expression of human Bcl-2 in an S. cerevisiae sod1Δ strain rescues the survival defect in stationary phase cells by preventing apoptosis and functioning as an antioxidant enzyme [34]. The Bcl-2 apoptotic mechanisms have been well characterized in S. cerevisiae, thus reinforcing S. cerevisiae as an ideal model to further our knowledge of the causes and consequences of the toxicity and apoptosis induced by the SOD1 mutant–Bcl-2 interactions.

**TDP-43 and FUS are associated with ALS**

A brain autopsy of an ALS victim typically reveals an accumulation of protein inclusions that consist of non-amyloid ubiquitinated aggregates containing proteins such as SOD1, TDP-43 and FUS. Recent studies in yeast have begun to focus on the latter two proteins and in particular TDP-43. TDP-43 was first identified in 1995 [35] as a protein that binds the TAR DNA sequence motifs of the HIV type 1 provirus and that acts as a transcriptional repressor. However, it was not until 2006 that TDP-43 was first associated with ALS [36]. S. cerevisiae has been used to investigate the cause of TDP-43-linked ALS and studies have reported the recapitulation of the key features of the disease, including the accumulation of cytoplasmic aggregates [37]. More recently, the RNP (ribonucleoprotein) FUS was also associated with ALS. Both TDP-43 and FUS are found in neural inclusions of patients suffering from ALS or FTLD (frontotemporal lobar degeneration) and the two proteins show both functional and structural similarity. Both proteins are associated with RNA processing and are predominantly localized in the nucleus where they act as RNA/DNA-binding proteins. The wild-type and mutant forms of these proteins co-localize and subsequently co-aggregate in the cytoplasm of neurons in which an increase in toxicity is associated with increased expression [38]. There are also distinct domains within TDP-43 and FUS that play an essential role in aggregate formation [39].

A genetic screening in S. cerevisiae identified Pbp1 (Pab1 [poly(A)-binding protein 1]-binding protein) as a factor that modulates TDP-43 toxicity in cells [40]. The yeast PBP1 gene encodes an orthologue of human ataxin-2 and expansions in the numbers of glutamine residues in the ataxin-2 gene (ATXN2) have been associated with ALS [40]. As in the case of other polyglutamine expansion diseases such as Huntington’s disease, the length of these polyglutamine tracts has an impact on the severity of the disease: intermediate expansions of between 27 and 33 glutamine residues increase the susceptibility to ALS, whereas longer repeats correlate more strongly with ALS progression [41]. The use of yeast genetic screens has also led to the identification of five genes encoding DNA/RNA-binding proteins that can suppress the toxicity of human FUS [42].

It remains to be determined whether TDP-43 or FUS protein aggregates are the central cause of disease or whether a novel toxic gain-of-function instigates ALS in a way similar to that seen when mutant SOD1 instigates ALS. Further experiments in S. cerevisiae have, however, shown that TDP-43 triggers PCD (programmed cell death) in a mitochondrial-dependent manner [43]. That study also established that an increased respiratory capacity was associated with enhanced...
Although S. cerevisiae is a unicellular eukaryote, the basic machinery employed to grow, adapt and survive is similar to multicellular eukaryotes. Numerous studies in this non-pathogenic yeast have both aided and complemented other animal models in understanding the mechanisms underlying a range of neurodegenerative diseases [49]. In the present short review, we have summarized recent progress in our understanding of the appearance and associated pathology of ALS using yeast models. Novel interactions with mutant proteins that are not seen with the wild-type protein support the proposal that a toxic gain-of-function is responsible for the manifestation of the disease. Further research into neurodegenerative disease models in yeast and other higher eukaryotes is required in order to establish whether proteins that aggregate are a root cause or a secondary consequence of the respective disease. There is no doubt that yeast models are a very promising tool for helping us to achieve this goal.

The relevance of each novel interaction, the stability of proteins and the effect that mutations have on the activity of proteins in the context of a cellular network require considerable further analysis since it is not yet clear how these processes are linked to each other and lead to ALS. Mitochondria appear to play a vital role in disease progression and this needs to be fully explored. We also know that SOD1 is a critical enzyme for detoxifying cells and releasing H$_2$O$_2$. Is this H$_2$O$_2$ molecule, which is important in cellular signalling, becoming re-distributed as SOD1 gets trapped inside mitochondria? Which cellular processes does this re-distribution have an impact on? Are there intermediate SOD1 species that play a role independently of superoxide scavenging that have the potential to become the toxic gain-of-function instigators? Does SOD1 itself act as a chaperone for the refolding of misfolded proteins or interact with chaperones that carry out this function? These are some of the important questions that need to be answered and addressing these questions in yeast models provides a fast, cheap and safe means through which to decipher the mechanisms underlying neurodegenerative diseases.

**Funding**

E.L.B. is supported by a Ph.D. studentship funded by the Biotechnology and Biological Sciences Research Council. C.W.G. is supported by a Career Development Fellowship [grant number 78573] from the Medical Research Council.

**References**