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Role of *CNC1* gene in TDP-43 aggregation-induced oxidative stress-mediated cell death in *S. cerevisiae* model of ALS

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ABSTRACT

TDP-43 protein is found deposited as inclusions in the amyotrophic lateral sclerosis (ALS) patient's brain. The mechanism of neuron death in ALS is not fully deciphered but several TDP-43 toxicity mechanisms such as mis-regulation of autophagy, mitochondrial impairment and generation of oxidative stress *etc.*, have been implicated. A predominantly nuclear protein, Cyclin C, can regulate the oxidative stress response *via* transcription of stress response genes and also by translocation to the cytoplasm for the activation of mitochondrial fragmentation-dependent cell death pathway. Using the well-established yeast TDP-43 proteinopathy model, we examined here whether upon TDP-43 aggregation, cell survival depends on the *CNC1* gene that encodes the Cyclin C protein or other genes which encode proteins that function in conjunction with Cyclin C, such as *DNM1*, *FIS1* and *MED13*. We show that the TDP-43's toxicity is significantly reduced in yeast deleted for *CNC1* or *DNM1* genes and remains unaltered by deletions of genes, *FIS1* and *MED13*. Importantly, this rescue is observed only in presence of functional mitochondria. Also, deletion of the *YBH3* gene involved in the mitochondria-dependent apoptosis pathway reduced the TDP-43 toxicity. Deletion of the *VPS1* gene involved in the peroxisomal fission pathway did not mitigate the TDP-43 toxicity. Strikingly, Cyclin C-YFP was observed to relocate to the cytoplasm in response to TDP-43's co-expression which was prevented by addition of an anti-oxidant molecule, *N*-acetyl cysteine. Overall, the Cyclin C, Dnm1 and Ybh3 proteins are found to be important players in the TDP-43-induced oxidative stress-mediated cell death in the *S. cerevisiae* model.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder which is characterized by progressive weakening of the muscles, difficulty in movement and respiratory failure that ultimately leads to death [1]. Up to 95% of the ALS cases are found to be sporadic (sALS) while the rest are heritable and familial in nature and manifest mutations in the *TARDBP*, *SOD1*, *FUS*, *C9ORF72*, *NEK1* or several other genes [1–9]. Currently, the therapeutics of ALS remains unrealized. Thus, uncovering the molecular mechanisms responsible for causing neuro-toxicity and cell death would be immensely helpful [2,7,10–12].

TAR DNA binding protein (TDP-43), encoded by the *TARDBP* gene, is a ribonucleoprotein that performs versatile physiological functions in the RNA metabolism including transcription, translation, stabilisation and transport of RNA *etc.* [7,8]. Exhaustion of the TDP-43 pool in the nucleus and the resulting loss of physiological function due to its cytoplasmic mis-localization has been reported in ALS and frontotemporal lobar degeneration (FTLD) disease patients. This has been proposed to lead to pathological hallmarks such as the accumulation of ubiquitinated and hyper-phosphorylated TDP-43 in inclusion bodies, the generation of C-terminal TDP-43 fragments (CTFs) and their deposition as cytoplasmic aggregates [13–17]. In fact, this abnormal mis-localization and aggregation of TDP-43 in the cytoplasm has also been demonstrated to lead to impaired endocytosis, increased mis-localization of TDP-43 to the mitochondria, dysregulation of the ubiquitin proteasome-mediated protein degradation, dysfunctional metal ion homeostasis, disturbances in the chromatin remodelling and oxidative stress *etc.* [2,11,18–24]. Additionally, post-translational modifications such as cysteine oxidation, acetylation, ubiquitination, hyper-phosphorylation and PARylation have also been proposed to have a role in effecting

Abbreviations: ALS, amyotrophic lateral sclerosis; TDP-43, TAR DNA binding protein-43; CTFs, C-terminal TDP-43 fragments; ROS, reactive oxygen species; Cdk8, Cyclin dependent kinase-8; PCD, programmed cell death; PVDF, polyvinylidene difluoride; APP, alkaline phosphatase; PI, propidium iodide; DCFDA, 2′,7′-dichlorofluorescin diacetate; FTLD, frontotemporal lobar degeneration.

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the TDP-43 toxicity [2,25]. However, the molecular events culminating in the cell death upon the TDP-43 aggregation remain to be fully elucidated.

A key sub-cellular centre promising to illuminate the TDP-43 cytotoxicity is emerging to be the mitochondria. As neurons are post-mitotic cells and have high demands for ATP production, any mitochondrial dysfunction can severely affect their survival. Proposedly therefore, the cytotoxicity in many neurodegenerative diseases including ALS, may be mediated *via* the mitochondrial damage [26–30]. TDP-43 expression has also been shown to induce mitochondrial damage, display aberrant increment in the reactive oxygen species (ROS) levels and impair the membrane potential of the mitochondria [31]. In fact, TDP-43 has been shown to localize to the inner membrane of the mitochondria and the inhibition of its mitochondrial localization can significantly reduce the neuronal toxicity [21]. In fact, moderately increasing the expression of TDP-43 has been reported to elicit aggregation of mitochondria and enhance the levels of the mitochondrial fission protein Fis1 [32]. Consistent reports from several model systems, including the ALS patient-derived fibroblasts harbouring TDP-43 mutations, have also indicated damage to the mitochondria upon the TDP-43 expression [20,33–37]. Notably, the overexpression of TDP-43 in the primary motor neurons altered the mitochondrial length and its transport and co-expression of the mitochondrial fusion protein, Mfn2, resulted in the reversal to normal mitochondrial morphology [20]. Also, importantly, TDP-43 and its ALS associated disease-causing mutants were found to localize to the mitochondria and preventing their abnormal localization was found to reduce the TDP-43-mediated neuronal toxicity [21,31]. Additionally, using a small molecule to disrupt the association of the two proteins, Drp1 and Fis1, which are known to cause fission of the mitochondria, was found to mitigate the TDP-43 toxicity [32,38]. Although the mitochondrial damage and the altered expression of the mitochondrial fission and fusion proteins have been consistently observed, the molecular events linked to the TDP-43-induced altered mitochondrial dynamics that initiate and culminate in cell death, remain to be elucidated.

Notably, the list of human proteins that can *in vitro* convert into altered aggregation-prone amyloid-like conformation is ever-increasing [39–42]. The yeast *Saccharomyces cerevisiae* has been extensively used as a eukaryotic model organism to study the aggregations and toxicities of the prion and amyloid proteins of both yeast and human origins [43–47]. Previously, expressing the TDP-43 protein in the *Saccharomyces cerevisiae* model has been shown to cause cytotoxicity and affect the cellular redox status [24,48–51]. Also, high throughput genetic screens in yeast enabled the identification of potential suppressors and enhancers of the TDP-43 toxicity when deleted or overexpressed [52,53]. For example, deletion of the gene *DBR1* encoding for the RNA lariat debranching enzyme, supressed the toxicity of TDP-43 in yeast [52]. Also, using a candidate approach investigation, overexpression of a Hsp40 chaperone Sis1 was found to mitigate the TDP-43 toxicity and restore the proteostasis in yeast [54]. Furthermore, TDP-43 was shown to form peri-mitochondrial aggregates in yeast and the TDP-43 toxicity was significantly reduced in cells without functional mitochondria [24]. Strikingly, respiration was recently shown to enhance the TDP-43 toxicity in yeast but some toxicity was observed even in the absence of respiration [55].

It is also known that mitochondria are major centres for producing reactive oxygen species (ROS) [56]. In addition to the mitochondrial functional dysregulation, oxidative stress has been speculated to play a vital role in ALS pathogenesis. In fact, increasing the intracellular glutathione levels in the motor neuron cells by supplementation with glutathione monoethyl ester, was shown to reduce the TDP-43 aggregation and lower the ROS levels [57]. Conversely, increased aggregation of TDP-43 has been observed to induce enhanced oxidative stress response *via* the anti-oxidant mediator protein, Nrf2 [27]. Also, TDP-43 has been found to interact with the mitochondrial proteins that are important for mitophagy [58]. In addition to the mitochondria, the peroxisome is another key site for the production of reactive oxygen species as it is a dynamic organelle involved in several metabolic pathways such as fatty acid oxidation and nucleic acid catabolism [59]. Although so far, any effect of TDP-43 on peroxisomes is not known, it is noteworthy that the fission proteins of mitochondria, Dnm1 and Fis1, and the adaptor proteins Caf4 and Mdv1, are also involved in the peroxisomal fission [60–63]. Alternatively, peroxisome fission is also regulated by the Vps1 protein independently of the mitochondrial fission machinery proteins [64]. Although the fissions of the mitochondria and the peroxisomes are essential for their inheritance to the daughter cells, these fission events, when not regulated, can affect the yeast cell viability [61,65].

In yeast, the Cyclin C protein can mediate the stress response against various stresses including the oxidative stress, heat shock, osmotic shock, actin depolymerisation and cell wall stress *etc.* [66–69]. Of note, the Cyclin C protein and its partner Cyclin-dependent kinase (Cdk8) are known to associate with the RNA polymerase II and cause repression of the stress response genes. Therefore, it plays a role in the transcriptional regulation as well as in mitochondrial fragmentation (Fig. 1). In the event of oxidative stress, Cyclin C dissociates from Cdk8 as well as its nuclear anchor protein Med13 and moves to the cytoplasm where it associates with the mitochondria [70]. The Ask10 protein is another major component of the oxidative stress signaling pathway which also affects the Cyclin C's dissociation from Cdk8 and Med13 in order to translocate to the cytoplasm [71]. Upon its cytoplasmic localization, Cyclin C interacts with the mitochondria and helps in signalling the recruitment of the mitochondrial fission machinery [70]. The mitochondrial outer membrane receptor, Fis1, recruits the dynamin-like GTPase protein, Dnm1, to the mitochondria *via* two adaptor proteins, Mdv1 and Caf4 [72]. This helps in activating the enhanced mitochondrial fission response which in turn relays into the movement of the pro-apoptotic protein, Ybh3, from the vacuole to the mitochondria that can cause programmed cell death (PCD) (Fig. 1) [73].

We used the *Saccharomyces cerevisiae* yeast model of TDP-43 aggregation and toxicity to examine how the TDP-43 aggregation-induced oxidative stress is relayed to culminate in cytotoxicity and cell death. In yeast, and also in humans, Cyclin C which is predominantly a nuclear protein can regulate the cellular response to the oxidative stress by affecting the transcription of stress response genes and also by translocation to the cytoplasm to affect the mitochondrial fission [73]. Therefore, we examined the TDP-43 toxicity in the yeast deleted for the *CNC1* gene that encodes Cyclin C and also *MED13* gene that encodes the protein involved in the nuclear retention of Cyclin C. Furthermore, we also examined the TDP-43 toxicity in the yeast deleted for the genes such as *DNM1* or *FIS1* that encode proteins which function in the mitochondrial fission pathway. Additionally, we also investigated the TDP-43 toxicity in the yeast strain deleted for the gene *VPS1* that codes for a regulator of the peroxisomal fission which functions independently of the mitochondrial fission response proteins. Furthermore, we also examined the TDP-43 toxicity in the yeast strain deleted for the *YBH3* gene that encodes for a pro-apoptotic protein that gets activated in response to mitochondrial hyper-fission. As Cyclin C has a role in transcriptional regulation as well as mitochondrial fragmentation, making the cells petite [rho^{-}], would be useful to dissect if the role of Cyclin C in mediating the TDP-43 toxicity is mitochondria dependent. Thus we examined TDP-43 toxicity in [rho^{-}] *cnc1*Δ yeast compared to [RHO^{+}] *cnc1*Δ yeast. Finally, we investigated if the oxidative stress caused by the TDP-43 protein aggregation can alter the sub-cellular localization of the Cyclin C protein and whether neutralizing the oxidative stress by the addition of an anti-oxidant can thwart its cytoplasmic localization induced by the TDP-43 co-expression.

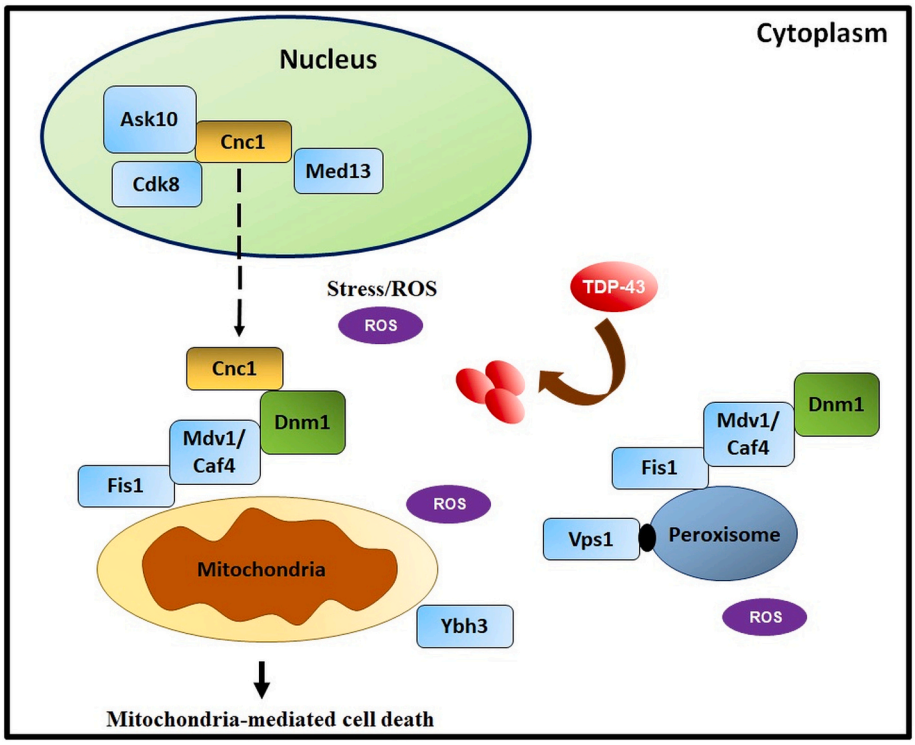

Fig. 1. Schematics of cytoplasmic localization of Cyclin C upon induction of oxidative stress and the protein complexes that function in the fission of mitochondria & peroxisomes in yeast.
Mitochondria and peroxisomes are the sources of reactive oxygen species (ROS) and their fission can regulate cell death and longevity in yeast. Oxidative stress-induced cell death in yeast is mediated *via* mitochondria. Various stress signals including oxidative stress elicit Cyclin C protein-mediated stress response in yeast. Cyclin C protein along with the Cyclin-dependent kinase, CDK8, is predominantly a nuclear protein. Upon oxidative stress, Cyclin C dissociates from CDK8 as well as from its nuclear anchoring protein Med13 and moves to cytoplasm. Ask10 protein activation is caused by increased oxidative stress which also promotes the Cyclin C's movement to cytoplasm. After localizing to cytoplasm, Cyclin C triggers the recruitment of the dynamin-like protein, Dnm1, to the mitochondrial fission protein Fis1 *via* the adaptor proteins Caf4 or Mdv1. This activates mitochondrial fission. Aberrant mitochondrial fission also leads to the recruitment of the pro-apoptotic protein, Ybh3, from the yeast vacuole to mitochondria which thereafter starts the programmed cell death (PCD) pathway. The mitochondrial fission machinery proteins Fis1, Caf4 and Mdv1 are also involved in regulating the peroxisomal fission in a Dnm1-dependent manner. Another protein, Vps1, also regulates the peroxisomal fission in a Dnm1-independent manner.

2. Materials and methods

2.1. Materials

D-galactose, D-raffinose and redox indicator dye, 2′,7′-dichlorofluorescein diacetate (DCFDA) (catalogue no: D6883) were purchased from Sigma (USA). CellRox Deep Red reagent (catalogue no: C10422) was obtained from Thermofisher scientific. For western blot experiments, CellLytic™ Y (Sigma USA, catalogue no: C4482), Bradford reagent (BioRad), blocking buffer for western blot (Himedia India), anti-TDP-43 antibody (Sigma, catalogue no: WH0023435M1), anti-GAPDH antibody (Abcam, catalogue no: ab125247), anti-mouse antibody tagged with alkaline phosphatase (Sigma, catalogue no: A3562), CDP star (sigma, catalogue no: C0712), and molecular weight standards (Thermofisher scientific), were used. Propidium iodide (Sigma, catalogue no: P4170) was used for dead cell staining. For staining the yeast nuclei, DAPI (Sigma, catalogue no: D9542) or Hoechst 33342 (Thermofisher scientific, catalogue no: 62249) were used.

2.2. Yeast strains

Yeast strains BY4742 (*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*) (obtained from GE Dharmacon) and 74-D-694 (*MATa ade1-14 his3-200 leu2-3, 112 ura3-52 trp1-289*) (a kind gift of Prof. Susan Liebman, University of Nevada, USA) were used for the studies [74]. The other yeast strains used including *med13*Δ, *fis1*Δ, *dnm1*Δ, *ybh3*Δ, *vps1*Δ and *cnc1*Δ, were all derivatives of BY4742 and obtained from the yeast *MATα* knock-out clone collection from GE Dharmacon [75,76]. Isogenic [pin^{-}] versions of the *med13*Δ, *fis1*Δ, *dnm1*Δ, *ybh3*Δ, *vps1*Δ and *cnc1*Δ yeast strains were obtained by growing the strains on YPD plates added with 5 mM GuHCl for five passages as patches to eliminate any presence of the [PIN^{+}] prion and then selecting for the [RHO^{+}] cells on media with glycerol as the only carbon source (Supplementary Fig. 3S). All studies were carried out in the [pin^{-}] yeast strains [77]. A *CNC1* deleted derivative of BY4742 was also obtained here by gene disruption with *HIS3*. For this, first, the PCR amplification of the *HIS3* gene along with its promoter was carried out using the plasmid pRS403 as the template. The forward primer, 5′GATAAGGCATCTGAATCTCAAAAGTTAGACGGCCT CCTCTAGTACAC 3′ was used along with the reverse primer, 5′CTTAACGGTTTTTAAATTTATTCTTCGCATGCGGTGTCACTACATAAG 3′. These primers also contained homology to the *CNC1* gene to facilitate homologous recombination. After transformation with the PCR product, the transformants were selected on media lacking histidine and genomic DNA was isolated from the obtained His^{+} colonies and analysed by PCR to screen for the successful disruption of the *CNC1* gene using the forward primer 5′ AAATTAGAAAACAGGTGAAGACCAC 3′ and reverse primer 5′ CACTCAACGATTAGCGACC 3′ that respectively bind in the promoter of *CNC1* and the coding region of the *HIS3* genes (Supplementary Fig. 6S). Likewise, *DNM1* and *YBH3* gene deleted derivatives of BY4742 were also obtained here by PCR-mediated gene disruption with the coding sequence of the *HIS3* gene using the plasmid pRS403 as the template (Supplementary Fig. 6S). The primers used for the *DNM1* disruption were: forward primer, 5′ACCAGCGAATCTAAATACG ACGGATAAAGAATGACAGAGCAGAAAGC 3′ and reverse primer, 5′CTTAACGGTTTTTAAATTTATTCTTCGCATGCGGTGTCACTACATAAG 3′. The primers used for the PCR confirmation of the *DNM1* gene disruption were: forward primer, 5′TAAAACCGCGTAAAAAAGCTAAAG 3′ and reverse primer, 5′CACTCAACGATTAGCGACC 3′. For the *YBH3* gene disruption with *HIS3*, the primers used were: forward, 5′ GCAAATAAAAGGATATAGAATCAAGTTAATATGACAGAGCAGAAAGC 3′ and reverse, 5′ TCGTGTAAATAATTCGATTTTTCCCTTTTTCGG TGTCACTACATAAG 3′. The primers used for the PCR confirmation of the successful disruption of the *YBH3* gene with *HIS3* were: forward primer, 5′ CGTTATGTACATAGCATTTGGCGTG 3′ and reverse primer, 5′ CACTCAACGATTAGCGACC 3′ (Supplementary Fig. 6S).

2.3. Plasmid construction

Plasmids used in the study were *pRS416* (*URA3*) [78], *pAG416 GAL1-ccdB-EGFP* (*URA3*) (Addgene: 14195), low copy TDP-43 expression plasmid *pRS416-GAL1p-TDP43-YFP* (*URA3*) (Addgene: 27447), high copy TDP-43 expression plasmid *pRS426-GAL1p-TDP43-GFP* (*URA3*)

(Addgene: 27467), *pGAL1-TDP-43-DsRed* (*URA3*) and *pGAL1-TDP-43* (*TRP1*) [48,49,54]. Also, a plasmid expressing a C-terminal fragment of TDP-43 (aa: 175-414) as GFP fusion was generated here using Q5 site-directed mutagenesis system (NEB, USA) using the plasmid *pRS426-GAL1p-TDP43-GFP* (Addgene: 27467) as the template following the manufacturer's protocol. The forward primer 5′*ATGTGCAAACTTCC TAATTCTAAGC3*′ was used along with the reverse primer 5′*TTTGTTATCTCCTTCGAAGCCTG3*′ to generate the plasmid (*pRS426-GAL1p-TDP-43-(175-414)-GFP*) to express the C-terminal fragment aa: 175-414 of TDP-43 tagged with GFP. For the plasmid construction, following the *DpnI* digestion of the template DNA, the PCR product was transformed into competent *DH5α E. coli* cells (Invitrogen, USA). Then, plasmids were isolated from the obtained transformants and analysed for their respective desired lengths of DNA by double digestion using restriction enzymes *SpeI* (which cuts before initiation codon of TDP-43 encoding gene) and *XhoI* (which cuts after *GFP* gene) to ascertain the successful generation of the C-terminal fragment encoding plasmid. Similarly, the forward primer, 5′ *ATGGATCAGATTGCGCAGTCTC* 3′ was used along with the reverse primer, 5′*TTTGTTATCTCCTTCGA AGCCTG3*′ to generate the plasmid (*pRS426-GAL1p-TDP-43-(237-414)-GFP*) and the forward primer,

5′*ATGAACTTTGGTGCGTTCAGC* 3′ was used along with the reverse primer, 5′*TTTGTTATCTCCTTCGAAGCCTG3*′ to generate the plasmid (*pRS426-GAL1p-TDP-43-(311-414)-GFP*) in the destination vector as used for the construction of the *pRS426-GAL1p-TDP-43-(175-414)-GFP* plasmid described above. In addition, to generate a Cyclin C-YFP encoding plasmid, a *CNC1* ORF containing plasmid (*pHA-CNC1*) obtained from GE Dharmacon (catalogue no: YSC3867-202329183) [79] was used as the template. The forward primer, 5′*ACTTCTAG AATGTCGGGGAGCTTCTGG3*′ added with *XbaI* restriction site and the reverse primer, 5′*ATGGATCCAATTGCAGATGCTGGTCTAAG3*′ containing BamHI restriction site, were used to PCR amplify the *CNC1* ORF. The plasmid *pRS426-GAL1p-RNQ1-YFP* (Addgene: 18686) was first digested with the restriction enzymes *XbaI* (which cuts before initiation codon of the *RNQ1* gene) and *BamHI* (which cuts before the *YFP* gene). Then the *RNQ1* ORF insert was removed and the vector with *YFP* was used to ligate in the *XbaI* and *BamHI* digested PCR product of the *CNC1* ORF to obtain the *pRS426-GAL1p-CNC1-YFP* plasmid. For gene complementation assay in a *cnc1*Δ yeast, the expression of Cyclin C was done using a URA3-marked plasmid *pHA-CNC1* obtained from GE Dharmacon that expresses Cyclin C from galactose-inducible *GAL1* promoter [79].

2.4. Yeast culture, plasmid transformation and diploid generation

Standard procedures were used for preparing media and cultivating yeast [80]. Complex media (YPD) or synthetic media lacking amino acids essential for the plasmid selection along with either D-glucose (SD) or D-raffinose (SRaf) or D-galactose (SGal) as the carbon source were used. Plasmid transformation in yeast was carried out by the standard method [80]. *GAL1* promoter-controlled genes in plasmids were over-expressed by the addition of 2% galactose unless stated otherwise. For the generation of the diploid strains towards complementation assays, a library yeast strain deleted for a specific gene in the BY4742 background (*MATα*) was mated with the 74-D-694 wild-type strain (*MATa*) on a YPD plate and then diploids were selected on the SD-Lys-Trp plates [74].

2.5. Yeast growth inhibition assay

Yeast cells were first cultured overnight in a plasmid selective, 2% raffinose-containing broth to overcome the glucose repression. Next morning, cells were inoculated to fresh raffinose containing broth and were allowed to grow till mid-log phase OD_{600nm} = 0.5. Then, the cell numbers were normalized and serially diluted ten-fold and 5 μL of the yeast cell suspension was spotted onto appropriate plasmid selective synthetic media plates that contained glucose, raffinose or galactose as their carbon source and incubated at 30 °C for about 48 h before imaging. Any toxicity due to the expression of TDP-43 through the galactose promoter was evaluated by comparing the relative growths on the galactose-containing plates in reference to the appropriate controls.

2.6. Yeast growth curves

Yeast cells were first grown overnight in plasmid selective and 2% raffinose-containing broth to overcome glucose repression. Next morning, cells were inoculated to a fresh raffinose containing broth and were allowed to grow till mid-log phase OD_{600nm} = 0.5. Then, the cell numbers were normalized to OD_{600nm} = 0.1 in plasmid selective 2% raffinose broth supplemented with 2% galactose for the TDP-43 expression. Subsequently, 200 μL of the yeast cell suspension was transferred to a sterile 96 well microplate. The culture's $OD_{600\ nm}$ was continuously recorded in a PerkinElmer Enspire microplate multi-mode reader maintained at 30 °C. The $OD_{600\ nm}$ was read every 5 min and the microplate was shaken for 15 s between the two reads. The absorbance for four independent plasmid transformants was averaged and plotted as a function of time to obtain the yeast growth curve. For comparison, quantification of the growth curves was done by calculating the areas under the curves using the GraphPad Prism software.

2.7. Generation of petite yeast strains

Isogeneic petite [*rho*$^-$] versions of *dnm1*Δ, *ybh3*Δ and *cnc1*Δ were created by allowing the [*RHO*$^+$] strains to grow on YPD supplemented with 0.5 mg/mL ethidium bromide (EtBr) which has been previously reported to result in the respiration-deficient mutant strains termed [*rho*$^-$] or petite strains [81]. Cells from the EtBr plates were then streaked on to YPD plates to obtain single colonies. Several colonies from the YPD plates were screened to obtain the respiration-deficient mutants (*i.e.* those converted to [*rho*$^-$]) by checking for their absence of growth on the complex media, YPG, containing 2% glycerol (non-fermentable) as the only carbon source.

2.8. Western blotting

To compare the levels of expressions of TDP-43-GFP or TDP-43-YFP, cell extracts from the yeast cells grown for 24 h with galactose (2%) were analysed using standard procedure. Cells were re-suspended in CellLytic™ Y buffer and glass beads were added for carrying out lysis of the yeast cells by vortexing at 4 °C and the obtained cellular lysate was pre-cleared by centrifugation at 2000 rpm for 3 min at 4 °C to remove the cell debris. The resulting supernatant was collected and the total concentration of protein was estimated using the Bradford assay. Samples were normalized for equal total protein contents and were separated on a 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. Following the transfer of protein, the PVDF membrane was blocked for 120 min in casein blocking buffer (HiMedia) and then washed with phosphate-buffered saline (PBS) containing 0.1% Tween (pH 7.5). For detection of TDP-43 (TDP-43-YFP and TDP-43-GFP), primary mouse monoclonal anti-TDP-43 antibody (1:2000 dilution) was added. After the primary antibody incubation, the secondary anti-mouse antibody (1:10000 dilution) tagged with alkaline phosphatase (APP) was added. As a loading control, levels of the endogenous expression of GAPDH protein were also concurrently detected. For this, primary antibody, mouse monoclonal anti-GAPDH (1:2000 dilution) followed by incubation with secondary antibody, anti-mouse antibody (1:10000 dilution) tagged with APP, were used. CDP-star was used as the chemiluminescent substrate for APP as per the manufacturer's protocol to probe the TDP-43 and GAPDH protein levels. A molecular weight standard cocktail was also concurrently run and electro-blotted onto the PVDF membrane to assign the molecular weights to the GAPDH and TDP-43 proteins.

2.9. Fluorescence microscopy

Single yeast colony isolates transformed with plasmids were grown at 30 °C in broth containing raffinose for overnight. Then, the yeast cells were suspended in fresh broth added with 2% galactose for the expressions of TDP-43-YFP, TDP-43-GFP or Cyclin C-YFP and then incubated at 30 °C for 6 h before the yeast cells were collected for the microscopy experiments. Using Leica DM-2500 microscope, the fluorescence images were acquired using a 100× objective lens (oil immersion). Pseudo-colouring of the fluorescence images were done using ImageJ software [82]. Detection of the presence of reactive oxygen species (ROS) in yeast upon the TDP-43-YFP expression using CellROX deep red staining and the visualization of the staining of the yeast nuclei by DAPI or Hoechst, were also performed by fluorescence microscopy as detailed in the following sections.

2.10. CellROX deep red staining for ROS detection

For reactive oxygen species (ROS) detection, the yeast cells were stained with the CellROX deep red dye followed by visualization of the cells using RFP filter in a Leica DM-2500 microscope. For this, the yeast cell suspensions expressing the *URA3* marked plasmid-encoded TDP-43-YFP or vector were prepared as described earlier [83]. First, the yeast cells were grown in SRaf-Ura broth and were re-inoculated into SGal-Ura broth for the overexpression of TDP-43-YFP for 24 h. Then, the cells were harvested and re-suspended in 100 μL of water and incubated with 1.5 μM CellROX deep red in dark for 1 h. Fluorescence images were acquired using leica DM-2500 microscope by examining the yeast cells using a 100× objective lens (oil immersion) in bright field and also using RFP filter and then processed using ImageJ software [82].

2.11. DAPI and Hoechst staining for nuclei visualization

To examine the cytoplasmic localization of the TDP-43-YFP aggregates, after 6 h of expression of TDP-43-YFP, the yeast cells were harvested and re-suspended in 70% ethanol and incubated for 30 min at room temperature. Then, the yeast cells were washed and re-suspended in PBS and incubated with 5 μg/mL of DAPI at room temperature for 10 min. Subsequently, the cells were washed thrice with PBS before visualizing the nuclei under the microscope. For examining the nuclear localization of Cyclin C-YFP, the nuclei were stained with the Hoechst dye. For this, Cyclin C-YFP was expressed either alone or co-expressed with TDP-43-DsRed for 18 h before the yeast cells were centrifuged and collected. After washing the cells with PBS, 5 μg/mL of Hoechst was added and followed by incubation at room temperature for 30 min. Then, the yeast cells were washed thrice with PBS before visualizing the nuclei under Leica DM-2500 microscope.

2.12. Flow cytometry

The yeast cells were induced using 2% galactose for 36 h to express TDP43-YFP or EGFP. More than one million cells were collected and washed. Next, the yeast cells were re-suspended in 500 μL of PBS and added with 10 μg/mL of propidium iodide (PI) for staining and incubated in dark for 15 min. Then, 50,000 cells were acquired in AriaIII FACS (BD) and analysed using the filter PI-A (for PI) to calculate the percentage of total dead cell population which get stained with PI. For testing statistical significance, unpaired *t*-test by comparing the vector controls (EGFP) with TDP-43-YFP in the wild-type or *cnc1*Δ yeast strain, was done by the GraphPad Prism software and the p-values were calculated. The data provided is an average of five independent transformants in the wild-type and the *cnc1*Δ yeast backgrounds. For examining the oxidative stress levels in the yeast, CellROX deep red stained population was also quantified by flow cytometry. For this, the yeast cells harbouring TDP-43-YFP or EGFP-encoding *GAL1* promoter-driven plasmids, were induced using 2% galactose for 36 h and then collected and washed with PBS. Next, the yeast cells were re-suspended in 500 μL of PBS and stained with 10 μM of CellROX deep red reagent for 1 h in dark. Then, 50,000 cells were acquired in AriaIII FACS (BD) and analysed using the filter APC-A (for CellROX) to calculate the percentage of the CellROX-stained cells in a given population.

3. Results

3.1. Deletion of CNC1 and some associated mitochondrial pathway genes rescue cells from TDP-43 toxicity

So far, the involvement of mitochondria in mediating the TDP-43 toxicity in the yeast model has been documented but the downstream pathways that lead to cell death remain to be understood. Thus, as the TDP-43 expression causes oxidative stress in the yeast cells, we examined if Cyclin C or its associated proteins which generally respond to oxidative stress and cause mitochondrial fragmentation, are also involved in mediating the TDP-43 toxicity-induced cell death. For this, the respective strains with the deleted genes from the *MATα* deletion library clone collection were first cured of any endogenous prions by passaging five times on 5 mM GuHCl-containing media which is known to eliminate prions such as [PIN^+] that may affect the TDP-43 toxicity [54,84]. The cured strains were ascertained to have functional mitochondria by growth on glycerol medium prior to their usage for the TDP-43 toxicity assay.

First, we performed a serial dilution growth assay in the wild-type yeast which was transformed with a low copy plasmid encoding TDP-43-YFP driven by the *GAL1* promoter and found that the expression of TDP-43-YFP caused cytotoxicity as reported earlier (Fig. 2a) [49]. As the TDP-43-YFP expression is also known to induce oxidative stress in yeast [51], we therefore examined the TDP-43-YFP toxicity in the yeast strains deleted for the Cyclin C encoding gene *CNC1* or the downstream genes that encode for proteins that work in collaboration with Cyclin C. Strikingly, upon the TDP-43-YFP expression, the deletion of *CNC1* exhibited rescue of the TDP-43-YFP toxicity. On the contrary, deletion of the *MED13* gene encoding the Med13 protein which is vital for retaining Cyclin C in the nucleus, did not exhibit any rescue of the TDP-43 toxicity (Fig. 2a). Furthermore, we also examined the toxicity of TDP-43-YFP in the yeast deleted for genes encoding the proteins Dnm1 and Fis1 that are mediators of the mitochondrial fission process. We found that the TDP-43-YFP toxicity was reduced in the d*nm1*Δ yeast strain, whereas, there was no toxicity rescue in the *fis1*Δ yeast (Fig. 2a).

Notably, there are several common key players between the peroxisome fission and the mitochondrial fission processes. These fissions in yeast are mediated by the peroxisomal/mitochondrial membrane-anchored protein Fis1 and the cytoplasmic protein Dnm1 [60]. Independent of the Dnm1 pathway, the Vps1 protein also regulates the peroxisomal fission in yeast [64]. Both mitochondria and peroxisomes are known centres of ROS generation and in the event of mitochondrial or peroxisomal hyper-fission the cellular longevity in yeast is compromised [61,65]. Since, we observed that the TDP-43-YFP toxicity was rescued in *dnm1*Δ, we then also examined the TDP-43-YFP toxicity levels in the *vps1*Δ yeast strains using growth assays and found that the *vps1*Δ strain exhibited the TDP-43-YFP toxicity comparable to that of the wild-type yeast (Fig. 2a).

A previous study has shown that the TDP-43-expressing yeast cells display markedly increased apoptotic markers, however the key proteins involved in the mitochondria-mediated cell death such as the apoptosis inducing factor or cytochrome *c*, were not found to directly mediate the cell death upon the TDP-43 expression [24]. Therefore, considerable interest exists in finding out the events and the key players that culminate the mitochondria-dependent cell death caused by the TDP-43 expression in yeast. It is known that the outer membrane of the yeast is permeabilized by BH3 domain-containing proteins and the yeast BH3 containing protein, Ybh3, is implicated in mediating the mitochondria-mediated apoptosis and the deletion of the *YBH3* gene confers resistance

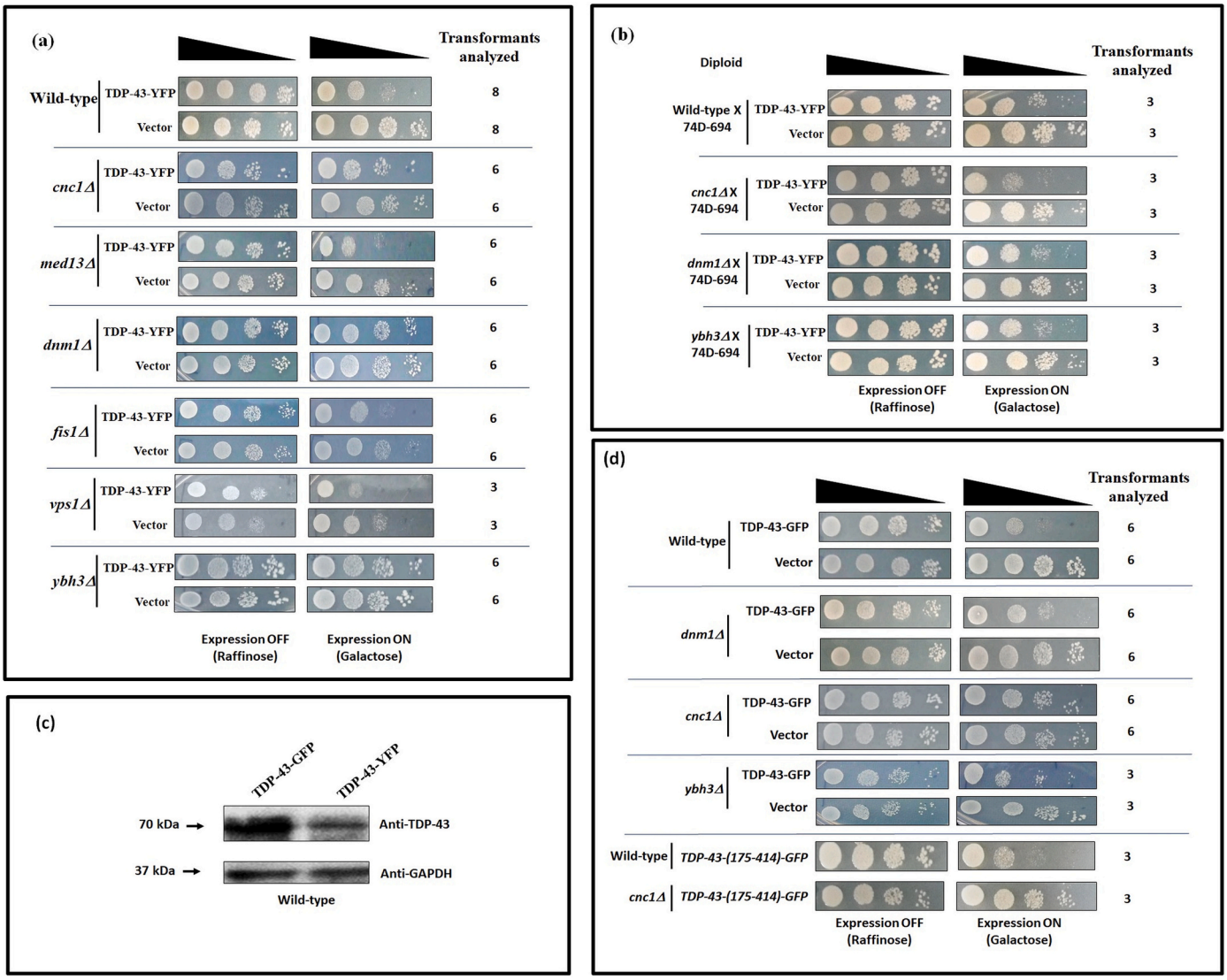

Fig. 2. TDP-43 cytotoxicity in the yeast cells with deletion of the *CNC1* gene or its associated mitochondrial/peroxisomal fission pathway genes.
a. Wild-type yeast (BY4742) was transformed with low copy number, *GAL1* promoter-driven *CEN* plasmid encoding TDP-43-YFP (*pRS416-GAL1p-TDP-43-YFP*, *URA3*) or with an empty vector (*pRS416*). Serial dilution (10-fold) growth assays were performed onto the plasmid-selective galactose-containing media where the TDP-43-YFP expression is turned ON and also, for the cell number controls, onto the raffinose-containing plates where the *GAL1* promoter is switched OFF. Similar serial dilution (10-fold) growth assays were also performed for the TDP-43-YFP plasmid transformants in the BY4742 derivative strains singly deleted for the gene, *CNC1*, encoding the Cyclin C protein or the proteins that function in conjunction with Cyclin C. The yeast strains deleted for *cnc1*Δ, *dnm1*Δ or *ybh3*Δ showed significant rescue of the TDP-43-YFP toxicity. Whereas the yeast deleted for the genes *MED13* or *FIS1* manifested toxicity similar to that of the wild-type strain. Deletion of the *VPS1* gene the encoded protein of which is involved in the peroxisomal fission failed to rescue the TDP-43-YFP-induced cytotoxicity. The numbers of independent transformants examined for a particular deletion strain are indicated. Images were acquired after 48 h of incubation at 30 °C. b. For complementation assay, diploid strains were generated by crossing the haploid *cnc1*Δ or *dnm1*Δ or ybh3Δ or the wild-type yeast in BY4742 background with a haploid wild-type strain (74D-694) of the opposite mating type. In the serial dilution (10-fold) growth assays, the obtained diploid strains pre-transformed and expressing the TDP-43-YFP were examined for cytotoxicity. Images were acquired after 48 h of incubation at 30 °C. c. Two fusion proteins, TDP-43-YFP expressed from a low copy number plasmid (*pRS416-TDP-43-YFP*) or TDP-43-GFP expressed from a high copy number plasmid (*pRS426-TDP-43-GFP*), both under *GAL1* promoter control, were first checked for relative TDP-43 protein expression levels after 24 h of expression in wild-type yeast by using anti-TDP-43 antibody. As a protein loading control, GAPDH protein was simultaneously probed with anti-GAPDH antibody. TDP-43-GFP exhibited many-fold higher expression than TDP-43-YFP. d. To examine if toxicity upon high level expression of TDP-43 can also be rescued, serial dilution (10-fold) growth assays were performed in the wild-type, *dnm1*Δ or *cnc1*Δ or *ybh3*Δ yeast strains expressing either the vector (*pRS416*) or TDP-43-GFP from a high copy number plasmid. In the *dnm1*Δ, *ybh3*Δ and *cnc1*Δ yeast there was a significant rescue of the TDP-43-GFP toxicity. Serial dilution (10-fold) growth assays were also performed in the wild-type and *cnc1*Δ strains expressing a toxic C-terminal fragment (CTF) (aa: 175-414), usually generated by aberrant caspase mediated cleavage of TDP-43 in the ALS patients, which we generated with GFP fusion (TDP-43-(175-414)-GFP) also in a high copy number plasmid under the *GAL1* promoter control. In the *cnc1*Δ yeast, there was a significant reduction in the toxicity of this CTF in comparison with that of the wild-type yeast strain. Images were acquired after 48 h of incubation at 30 °C.

to the apoptosis induction [24]. As our results show that deletions of certain genes involved in the mitochondrial fission pathway rescue the TDP-43 toxicity, we further examined whether *ybh3*Δ yeast, where there is a loss of the pro-apoptotic protein Ybh3, has any effect on the TDP-43 toxicity. The Ybh3 protein, which is generally a vacuolar protein, is translocated to mitochondria upon oxidative stress after which it induces programmed cell death. Interestingly, we found that the deletion of the *YBH3* gene can alleviate the TDP-43-YFP-induced cytotoxicity and cell death (Fig. 2a).

Furthermore, to confirm that the effects observed on the TDP-43-YFP

toxicity were indeed due to these gene deletions, and not due to any non-Mendelian genetic elements such as yeast prions that are known to affect the heterologous amyloid protein aggregation and toxicity in yeast [54,77], we further examined for complementation. For this, we first crossed the haploid *dnm1*Δ, *cnc1*Δ and *ybh3*Δ yeast with a wild-type haploid yeast strain of the opposing mating type to obtain diploid strains and then expressed TDP-43-YFP in these diploids. Notably, the toxicity of TDP-43-YFP reappeared in all the diploid strains, thereby corroborating that the previously observed rescue of the toxicity was indeed due to the *bona fide* effects of the respective gene deletions (Fig. 2b).

As the Dnm1-independent peroxisomal fission modulator Vps1 did not appear to mediate the TDP-43 toxicity, we further examined how the TDP-43 toxicity is affected by *dnm1*Δ and *cnc1*Δ which are involved in the mitochondrial fission and in *ybh3*Δ which is involved in mitochondria-dependent apoptosis. As the toxicity caused by low level expression of TDP-43-YFP encoded from a low copy centromeric plasmid, was rescued in the *dnm1*Δ, *cnc1*Δ and *ybh3*Δ yeast strains, thus we asked whether the toxicity of TDP-43 expressed at high levels from a multi-copy 2 μ plasmid, can also be rescued in these deletion backgrounds. Thus, we first expressed TDP-43-GFP encoded from a 2 μ plasmid driven by the *GAL1* promoter and confirmed using western blotting that the TDP-43-GFP had enhanced expression levels compared to TDP-43-YFP that was expressed from the low copy number plasmid (Fig. 2c and Supplementary Fig. 2S). As reported by the previous groups, the TDP-43-GFP over-expression exhibited enhanced toxicity in comparison with the TDP-43-YFP over-expression in the wild-type yeast strain [85]. Notably, in the *dnm1*Δ and *ybh3*Δ yeast, we observed only a partial rescue of the TDP-43-GFP toxicity upon expression from the high copy number plasmid (Fig. 2d). Expectedly, when there is enhanced expression and higher aggregation of TDP-43, several independent pathways may get involved in rendering the TDP-43 toxic (Fig. 2d). Strikingly however, in the *cnc1*Δ yeast, the toxicity of the overexpressed TDP-43-GFP was observed to be completely rescued. In fact, the TDP-43-GFP toxicity rescue levels were similar to that of the extent of TDP-43-YFP toxicity rescue in the *cnc1*Δ yeast. In summary, Cyclin C appears to be a highly important and key mediator of the TDP-43 toxicity and therefore *cnc1*Δ yeast could rescue the toxicity of TDP-43 when expressed both at low as well as high levels.

Notably, several C-terminal fragments (CTFs) of TDP-43 have previously been found generated, proposedly by aberrant action of caspases, and deposited in the ALS patients [86]. Also, these CTFs have been proposed to be highly aggregation-prone and also projected to even have the capability of seeding the aggregation of the full-length TDP-43 [86,87]. To examine whether the *cnc1*Δ yeast can rescue the toxicity of only the full-length TDP-43 or even that of the caspase generated CTFs of TDP-43, we first generated a *GAL1* promotor-driven high copy expression plasmid encoding one such caspase generated CTF with aa: 175-414, which is generally referred to as the TDP-25 CTF [48,87]. This fragment also encompasses the TDP-43 domains reported to be essential for manifesting toxicity in the yeast model [49]. We observed that the *cnc1*Δ yeast could rescue the toxicity of the overexpressed TDP-43-(175-414)-GFP as observed previously for the full-length TDP-43-GFP (Fig. 2d). This further supports an important role of Cyclin C in mediating the TDP-43 toxicity and also indicates similar mechanisms of the toxicity for the full-length TDP-43 and the TDP-43-(175-414) C-terminal fragment in the yeast cells. Furthermore, we also tested the toxicities of two more CTFs, TDP-43-(237-414)-GFP and TDP-43-(311-414)-GFP both of which we cloned in the same high copy *GAL1* promoter-driven plasmid as that of TDP-43-(175-414)-GFP. However, these CTFs were not toxic in the wild-type yeast and also remained non-toxic in the *cnc1*Δ strain (Supplementary Fig. 8S).

When expressed at low levels in yeast, the TDP-43 protein which harbours a nuclear localization signal (NLS) is known to remain in the nucleus [48]. However, a moderate/high expression of TDP-43 causes aggregation of TDP-43 in the cytoplasm, thus, recapitulating the TDP-43 pathology as also observed in the ALS patients [85]. As the TDP-43-YFP toxicity was rescued in the *cnc1*Δ, *dnm1*Δ and *ybh3*Δ yeast, we therefore examined whether there is any prevention of the aggregation of TDP-43 or its cytoplasmic mis-localization that may be reducing the toxicity. However, we found that TDP-43-YFP formed cytoplasmic aggregates in all the deletion backgrounds similar to that of the wild-type yeast (Fig. 3). Thus, the deletions of *CNC1*, *YBH3* and *DNM1* genes do not seem to modify the TDP-43 toxicity *via* preventing its cytoplasmic aggregation and possibly the rescue of toxicity is a consequence of defects in the downstream relay of the toxicity mechanisms of the TDP-43 aggregates.

To confirm the results from the yeast growth assays and to quantify the level of rescue, flow cytometry was employed to estimate cell death. When the yeast cells expressing TDP-43-YFP or the control vector were treated with propidium iodide (PI) and the dead cell population was counted using flow cytometry, the TDP-43 expression in the wild-type caused significantly increased levels of cell death. On the contrary, and in conformity with the yeast growth assays, the TDP-43 expression in the *cnc1*Δ yeast did not cause any significant increase in the dead cell population in comparison with that of the control (Fig. 4a and Supplementary Fig. 1S).

Furthermore, to rule out the possibility that the rescue of the TDP-43 toxicity observed in the deletion library *cnc1*Δ strain is due to any unknown secondary mutation(s), we made a new strain deleted for *CNC1* in the wild-type BY4742 strain by disrupting *CNC1* with *HIS3* gene and then re-examined if the rescue of TDP-43 toxicity in the *cnc1*Δ yeast can only be observed *via* spot growth assays on the agar medium plates or even when growth curves are recorded in a broth medium (Fig. 4b, Supplementary Fig. 6S). For this, TDP-43-YFP encoding *GAL1* promoter-driven plasmid was transformed into the *cnc1*::*HIS3* deletion strain made in the BY4742 yeast and also into the wild-type BY4742 yeast. Four independent transformants were grown in plasmid selective liquid broth medium in 96-well microplates and the expression of TDP-43-YFP was induced with 2% galactose and the OD_{600nm} was recorded in a microplate reader to examine the growth trends of the transformants (Fig. 4b). When the growth curves (Fig. 4b, left panel) were quantified by calculating the areas under the curves (Fig. 4b, right panel), a significantly slower growth suggesting toxicity, was observed in the TDP-43-YFP-expressing wild-type yeast compared to the vector control. On the contrary, in the *cnc1*::*HIS3* yeast there was no significant difference between the growth trend of the TDP-43-YFP-expressing yeast compared to the vector control (Fig. 4b). This also suggests the rescue of the TDP-43 toxicity in the *cnc1*::*HIS3* yeast which is in conformity with the previous observations using the spot growth assays on the agar medium plates.

Similar to the rescue of the TDP-43 toxicity observed in the deletion library *cnc1*Δ strain (Fig. 2d), we also observed a rescue of the TDP-43-GFP toxicity, expressed from a high-copy plasmid, in the newly made *cnc1*::*HIS3* deletion yeast strain (Fig. 4c). To corroborate the dependence of the observed rescue of the TDP-43 toxicity on lack of the Cyclin C protein, we also examined if the expression of Cyclin C from a plasmid can bring back the TDP-43 toxicity in the deletion library *cnc1*Δ strain. For this, we co-transformed the *cnc1*Δ strain as well as the wild-type yeast with a *GAL1* promotor-driven TDP-43-DsRed encoding plasmid and a *GAL1* promotor-driven HA-tagged Cyclin C expressing plasmid (*pHA-CNC1*). When the expression was switched ON by growth on 0.1% galactose, TDP-43-DsRed caused toxicity in the wild-type yeast which was fully rescued in the *cnc1*Δ strain (Fig. 4d). However, when TDP-43-DsRed and HA-tagged Cyclin C were expressed together in the deletion library *cnc1*Δ strain, the toxicity of TDP-43-DsRed was not fully rescued (Fig. 4d). This suggests that the plasmid expressing HA-tagged Cyclin C is able to mediate and reinstate the TDP-43 toxicity alike the endogenous Cyclin C of the wild-type yeast. However, when TDP-43-DsRed and HA-tagged Cyclin C were co-expressed from their respective *GAL1* promoters using higher concentrations of galactose (2%), opposite effects on the TDP-43-DsRed toxicity reversion, compared to the 0.1% galactose

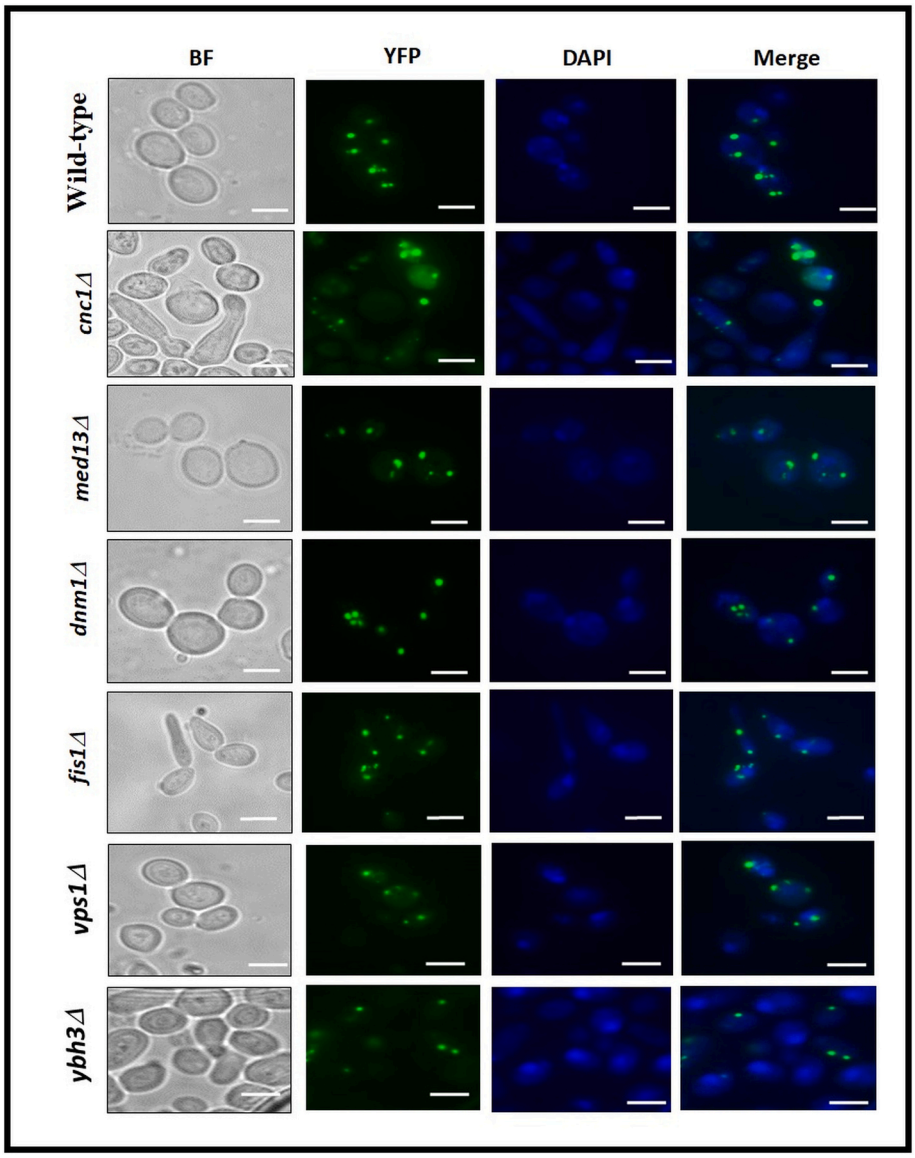

Fig. 3. Cytoplasmic localization of TDP-43 in the yeast cells with deletion of the *CNC1* gene or its associated mitochondrial/peroxisomal fission pathway genes.
To examine if any of the gene deletions caused hindrance to the TDP-43-YFP aggregation or prevention of the cytoplasmic mis-localization of TDP-43-YFP, the TDP-43-YFP protein was expressed with 2% galactose for 6h in various gene deletion strains and the formation of punctate fluorescent foci indicative of the TDP-43-YFP aggregation, was examined under the YFP filter using Leica DM2500 microscope. To determine the sub-cellular location of the TDP-43-YFP aggregates, yeast cells were also treated with DAPI to stain the nuclei and then the localization of the TDP-43-YFP aggregates with respect to the nucleus in a cell was examined. Scale bar is 10 μm.

induction, was observed possibly due to the over-expression of Cyclin C causing other unknown effects (data not shown).

Furthermore, to show that the rescue of the TDP-43 toxicity observed in the library *dnm1*Δ yeast was indeed due to the *bona fide* absence of the *DNM1* gene and not due to any unknown secondary mutations, we created a new deletion strain (*dnm1::HIS3*) by replacing *DNM1* with *HIS3* in the genome of the BY4742 yeast (Fig. 5a, Supplementary Fig. 6S). When we examined the toxicity of TDP-43-YFP expressed from a *GAL1* promoter-driven low copy plasmid in the *dnm1::HIS3* deletion and compared with that of the wild-type BY4742 yeast, upon expression using 2% galactose, the TDP-43-YFP toxicity was fully rescued in the *dnm1::HIS3* deletion strain similar to the deletion library *dnm1*Δ yeast (Fig. 5a). Furthermore, in consistence with our observation on the partial rescue of the toxicity of TDP-43-GFP expressed from a *GAL1* promoter-driven high copy plasmid in the library *dnm1*Δ yeast using 2% galactose (Fig. 5a), the TDP-43-GFP toxicity was also rescued in the *dnm1::HIS3* deletion strain upon expression of the TDP-43-GFP with 0.5% galactose (Fig. 5b). Taken together, the data from the rescue of the toxicities of TDP-43-YFP and TDP-43-GFP show that the *DNM1* gene participates in mediating the TDP-43 toxicity in the yeast model.

Furthermore, to show that the rescue of the TDP-43 toxicity observed in the library *ybh3*Δ yeast was indeed due to the *bona fide* absence of the *YBH3* gene and not due to any unknown secondary mutations, we created a new deletion strain (*ybh3::HIS3*) by replacing *YBH3* with *HIS3* in the genome of the BY4742 yeast (Supplementary Fig. 6S). When we examined the toxicity of TDP-43-YFP expressed from a *GAL1* promoter-driven low copy plasmid in the *ybh3::HIS3* deletion and compared with that of the wild-type BY4742 yeast, we found that, upon expression using 2% galactose, the TDP-43-YFP toxicity was partially rescued in the *ybh3::HIS3* deletion strain (Fig. 5c). Furthermore, alike to the observed partial rescue of the toxicity of TDP-43-GFP expressed from a *GAL1* promoter-driven high copy plasmid in the *dnm1::HIS3* strain, the TDP-43-GFP toxicity was also partially rescued in the *ybh3::HIS3* deletion strain upon expression of the TDP-43-GFP using 0.5% galactose induction (Fig. 5d) which is in agreement with the partial rescue of the TDP-43-GFP toxicity observed in the deletion library *ybh3*Δ yeast (Fig. 2d). Taken together, the data from the rescue of the toxicities of TDP-43-YFP and TDP-43-GFP show that the *YBH3* gene participates in mediating the TDP-43 toxicity in the yeast model (Table 1).

3.2. The cnc1Δ, dnm1Δ and ybh3Δ yeast strains can rescue the TDP-43 toxicity only in the cells with functional mitochondria

Earlier, presence of the functional mitochondria was shown to be

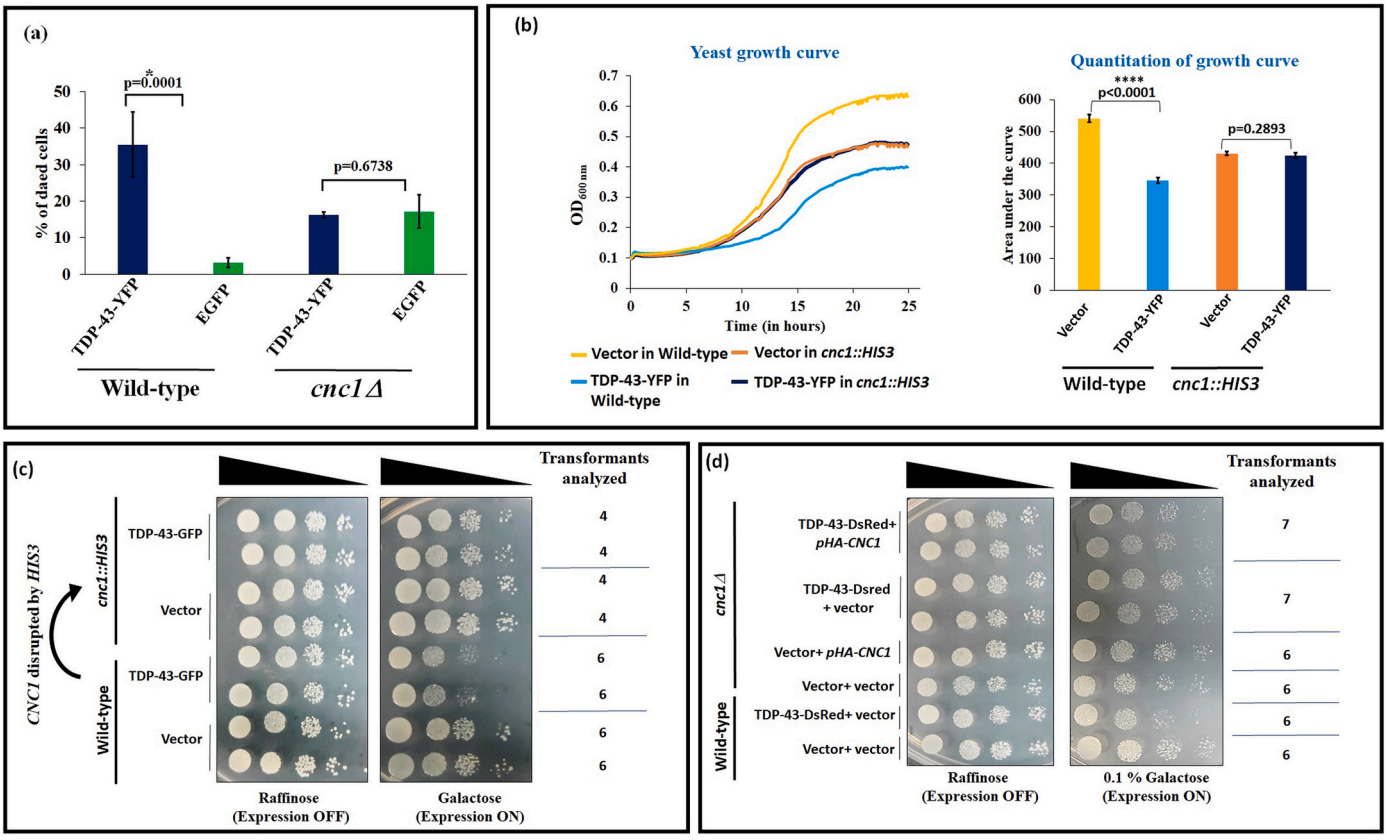

Fig. 4. Deletion of the *CNC1* gene in the wild-type yeast rescues the TDP-43 toxicity and expression of Cyclin C from a plasmid in the *cnc1*Δ yeast reinstates the TDP-43 toxicity.

a. In order to corroborate the rescue of the TDP-43-YFP toxicity observed in the serial dilution growth spot assays, the number of dead cells were counted using flow cytometry for one of the deletions, *cnc1*Δ. For this, the yeast cells expressing TDP-43-YFP or control vector (EGFP) in the wild-type or *cnc1*Δ yeast, after 36 h of expression, were treated with propidium iodide (PI) and the quantitative measurement of the dead cells stained by PI were analysed by flow cytometry using FACS Aria III BD Bioscience machine. The data represent the mean number of dead cells from five independent transformants and the error bars indicate standard deviation. The *p*-values were obtained by using un-paired *t*-test by comparing the vector controls (EGFP) with TDP-43-YFP in the wild-type or *cnc1*Δ yeast strain. The * in the p-value represents statistical significance. b. To investigate whether the observed rescue of the TDP-43 toxicity in the deletion library *cnc1*Δ yeast is indeed due to the lack of the Cyclin C protein or rather due to any unknown secondary mutations, the *CNC1* gene in the wild-type yeast was deleted by disruption with *HIS3* gene and then examined for the rescue of the TDP-43 toxicity. To examine if the rescue of the toxicity of TDP-43 in the *cnc1*Δ yeast can also be observed when growth curves are recorded in a broth medium, the *cnc1::HIS3* deletion strain made in the BY4742 yeast and the wild-type BY4742 yeast were transformed with the TDP-43-YFP encoding *GAL1* promoter-driven plasmid. Then, four independent transformants were grown in liquid plasmid-selective 2% raffinose-containing broth and the expression of TDP-43-YFP was induced with 2% galactose and to examine the growth trends of the transformants, the OD_{600nm} was recorded continuously in a PerkinElmer Enspire multi-mode microplate reader maintained at 30 °C (left panel). Also, the growth curves in the left panel were quantified by calculating the areas under the curves using the GraphPad Prism software. Statistical significance was assessed using *t*-test. The * in the *p*-value represents statistical significance. c. The *cnc1::HIS3* yeast strain was transformed with the TDP-43-GFP encoding high copy plasmid (*pRS426-GAL1p-TDP-43-GFP*). Transformants in the *cnc1::HIS3* yeast, and also in the wild-type yeast for comparison, were spotted by serial dilution (10-fold) on media plates with 2% galactose to induce the expression of TDP-43-GFP from the *GAL1* promoter. Relative growth was compared to assess for the toxicity of TDP-43-GFP. Images were acquired after 48 h of incubation at 30 °C. d. To evaluate whether the TDP-43 toxicity is reinstated in the *cnc1*Δ yeast when the Cyclin C protein is restored, expression of Cyclin C was carried out from a plasmid (*pHA-CNC1*) that encodes HA-tagged Cyclin C driven by the *GAL1* promoter. The deletion library *cnc1*Δ strain as well as the wild-type yeast were co-transformed with a *GAL1* promotor-driven TDP-43-DsRed encoding plasmid and the *pHA-CNC1* plasmid which is a 2 μ high copy plasmid. Transformants were spotted by serial dilution (10-fold) on media where the *GAL1* promoter is either switched OFF (raffinose media) or switched ON (0.1% galactose). Transformants expressing only TDP-43-DsRed in the *cnc1*Δ strain and also into the wild-type yeast were used to compare the toxicity levels. Growth plates were imaged after 48 h incubation at 30 °C.

important for mediating the TDP-43 toxicity in yeast [24]. Recently, respiration was found to enhance the TDP-43 toxicity, but the toxicity was not completely abrogated in the absence of respiration [55]. The Cyclin C protein functions in both the transcriptional regulation as well as the mitochondria-mediated cell death pathway. Thus, to dissect out whether the observed rescue of the TDP-43 toxicity in the *cnc1*Δ yeast is due to the loss of the Cyclin C's function related to the transcriptional regulation or due to the loss of the mitochondria-related role, we generated a petite strain, [*rho*$^-$], of the *cnc1*Δ yeast and for comparison we also generated the [*rho*$^-$] versions of the wild-type and *dnm1*Δ yeast strains (Supplementary Fig. 3S). The [*rho*$^-$] strains have non-functional mitochondria which are compromised for the respiratory capability. Then, we examined the rescue of TDP-43 toxicity in the *cnc1*Δ and *dnm1*Δ yeast with either functional [*RHO*$^+$] or non-functional mitochondria [*rho*$^-$]. For this, we used the *GAL1* promoter-driven low copy number plasmid-encoded expression of TDP-43-YFP in the *cnc1*Δ, *dnm1*Δ, *ybh3*Δ and wild-type yeast strains, which are all derivatives of the BY4742 strain. While several yeast strains such as 74-D-694 when compromised for the mitochondrial respiration do not utilize galactose, the respiration-deficient derivatives of BY4742 are known to successfully utilize galactose [88,89]. Notably, in contrast to the observed reduction in the TDP-43-YFP toxicity in the *cnc1*Δ, *dnm1*Δ and *ybh3*Δ yeast cells containing the respiring and functional mitochondria (*i.e.* [*RHO*$^+$] yeast), in the isogenic yeast strains with non-respiring

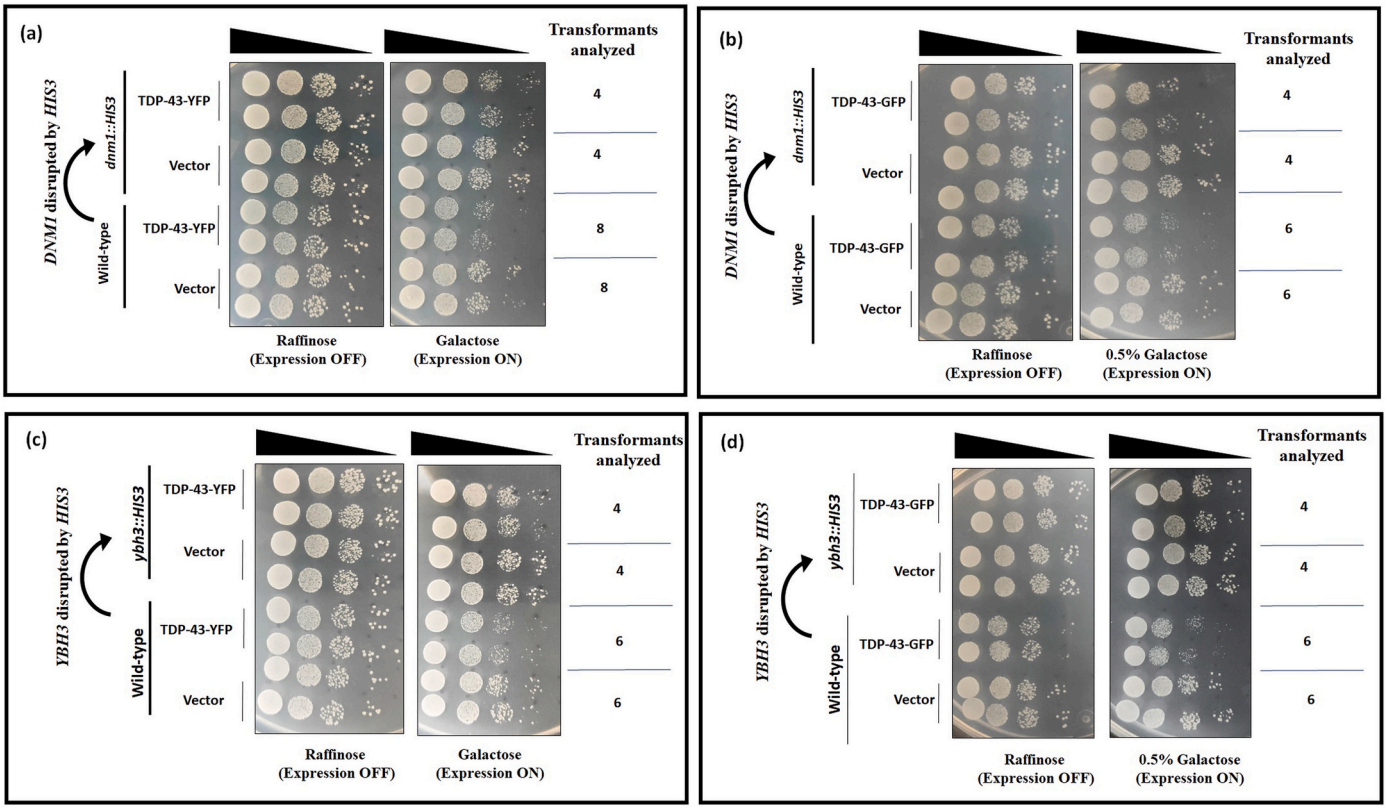

Fig. 5. Deletion of the *DNM1* or *YBH3* gene in the wild-type yeast rescues the TDP-43 toxicity.
a. To examine if the observed rescue of the TDP-43 toxicity in the deletion library *dnm1*Δ yeast is indeed due to the lack of the DNM1 protein or rather due to any unknown secondary mutations, we deleted *DNM1* gene in the wild-type yeast by disruption with *HIS3* and examined the rescue of the TDP-43-YFP toxicity. The obtained *dnm1::HIS3* yeast was transformed with TDP-43-YFP encoding low copy plasmid (*pRS416-GAL1p-TDP-43-YFP*). Transformants in the *dnm1::HIS3* yeast, and also in the wild-type yeast for comparison, were spotted by serial dilution (10-fold) on media plates with 2% galactose to induce the expression of TDP-43-YFP from the *GAL1* promoter or on control media plates with 2% raffinose where the expression is OFF. Relative growth was compared to assess for the toxicity of TDP-43-YFP. Images were taken after 48 h of incubation at 30 °C. b. To further test if the *dnm1::HIS3* yeast can also rescue the toxicity of TDP-43 expressed from a high copy plasmid, the strain was transformed with a high copy plasmid (*pRS426-GAL1p-TDP-43-GFP*) that expresses TDP-43-GFP under the control of the *GAL1* promoter. Transformants in the *dnm1::HIS3* yeast, and also in the wild-type yeast for comparison, were spotted by serial dilution (10-fold) on media plates with 0.5% galactose to induce the expression of TDP-43-GFP. Relative growth was compared to assess for the toxicity of TDP-43-GFP. Images were acquired after 48 h of incubation at 30 °C. c. To examine if the observed rescue of the TDP-43 toxicity in the deletion library *ybh3*Δ yeast is indeed due to the lack of the Ybh3 protein and not due to any unknown secondary mutations, we deleted *YBH3* gene in the wild-type yeast by disruption with *HIS3* and examined for the rescue of the TDP-43-YFP toxicity. The obtained *ybh3::HIS3* yeast was transformed with TDP-43-YFP encoding low copy plasmid (*pRS416-GAL1p-TDP-43-YFP*). Transformants in the *ybh3::HIS3* yeast, and also in the wild-type yeast for comparison, were spotted by serial dilution (10-fold) on media plates with 2% galactose to induce the expression of TDP-43-YFP from the *GAL1* promoter. Relative growth was compared to assess for the toxicity of TDP-43-YFP. Images were taken after 48 h of incubation at 30 °C. d. To further test if the *ybh3::HIS3* yeast can also rescue the toxicity of TDP-43 expressed from a high copy plasmid, the strain was transformed with a high copy plasmid (*pRS426-GAL1p-TDP-43-GFP*) that expresses TDP-43-GFP under the control of the *GAL1* promoter. Transformants in the *ybh3::HIS3* yeast, and also in the wild-type yeast for comparison, were spotted by serial dilution (10-fold) on media plates with 0.5% galactose to induce the expression of the TDP-43-GFP. Relative growth was compared to assess for the toxicity of TDP-43-GFP. Images were acquired after 48 h of incubation of the assay plates at 30 °C.

Table 1
Summary of gene deletions affecting TDP-43-YFP toxicity in [*pin*$^-$] yeast cells with functional mitochondria [*RHO*$^+$].

Yeast gene deleted	Effect on TDP-43 toxicity	Protein function
MED13	No effect	Subunit of the RNA polymerase II mediator complex. Essential for the nuclear retention of Cyclin C.
CNC1	Rescue	Cyclin-like component of RNA polymerase II holoenzyme. Involved in oxidative stress response.
DNM1	Rescue	Dynamin-related GTPase required for mitochondrial fission and inheritance.
FIS1	No effect	Involved in mitochondrial fission and peroxisomal abundance.
YBH3	Rescue	Involved in mitochondria-dependent apoptosis
VPS1	No effect	Involved in peroxisomal fission

mitochondria (*i.e.* [*rho*$^-$] yeast), the TDP-43-YFP expression-caused toxicity was not rescued and in fact displayed marginally enhanced toxicity (Fig. 6). Possibly, toxicity mechanisms independent of the mitochondrial fragmentation dominate and mediate the TDP-43 toxicity in the absence of the functional mitochondria. As Cyclin C has a role in transcriptional regulation as well as mitochondrial fragmentation, making the cells petite help in dissecting the mechanistic role of Cyclin C in mediating the TDP-43 toxicity. The absence of rescue of the TDP-43 toxicity in the [*rho*$^-$] version of the *cnc1*Δ yeast strongly suggests that the transcriptional role of Cyclin C is not important otherwise the same effect, *i.e.* the rescue of the TDP-43 toxicity, would be expected in both the [*rho*$^-$] and [*RHO*$^+$] yeast. So far, there is no report of any interference in the transcriptional role of Cyclin C when the mitochondria are not functional for example in the [*rho*$^-$] yeast. Thus, the observation of the lack of rescue of the TDP-43 toxicity in the [*rho*$^-$] yeast rather implicates the non-canonical, non-transcriptional, functional mitochondria-dependent role of Cyclin C in mediating the TDP-43

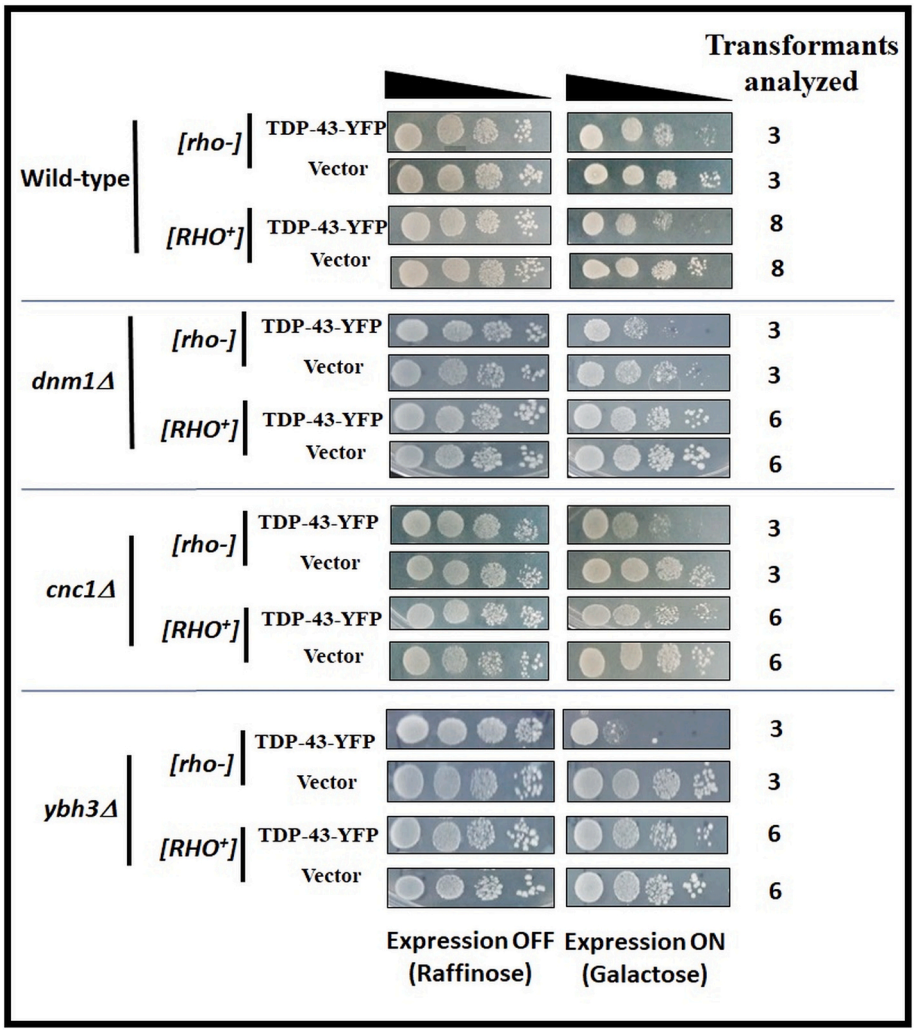

Fig. 6. TDP-43-YFP cytotoxicity in the *dnm1*Δ, *cnc1*Δ and *ybh3*Δ yeast strains containing functional or non-functional mitochondria.
To check whether the effects of *CNC1* gene on the TDP-43 cytotoxicity were mediated *via* the mitochondria-dependent pathway, isogeneic non-respiring [*rho*$^{-}$] versions of the wild-type, *dnm1*Δ, *cnc1*Δ and *ybh3*Δ strains were pre-generated by growing the strains on YPD with 0.5 mg/mL of ethidium bromide and then transformed with TDP-43-YFP encoding plasmid (*pRS416-GAL1p-TDP-43-YFP*). Serial dilution (10-fold) growth assays were performed in the isogenic wild-type, *dnm1*Δ, *cnc1*Δ and *ybh3*Δ yeast strains expressing either the vector (*pRS416*) or TDP-43-YFP in the yeast strains with functional mitochondria [*RHO*$^{+}$] or non-functional mitochondria [*rho*$^{-}$]. Images were acquired after 48 h of incubation of the assay plates at 30 °C.

toxicity in the yeast cells.

3.3. Although the TDP-43 aggregation-induced ROS is present in cnc1Δ and dnm1Δ yeast, cell death is prevented

As the expression of TDP-43 is known to elicit oxidative stress in wild-type yeast [24,50,51], we examined here the levels of reactive oxygen species (ROS) in the *cnc1*Δ and *dnm1*Δ yeast using the cell permeant reporter dye CellROX deep red. This dye usually remains non-fluorescent in the absence of ROS but exhibits bright red fluorescence upon oxidation by ROS. In the wild-type cells, upon TDP-43-YFP expression for 36 h, about 16% of the cells exhibited bright red fluorescence whereas only ~1% of the cells exhibited the CellROX staining in the vector controls. This corroborates the previously documented high ROS generation upon the TDP-43 expression in yeast. Next, we examined whether TDP-43-YFP expression also induces ROS in the yeast deleted for the mitochondrial fission genes, *CNC1* or *DNM1*, or the observed rescue of the TDP-43-YFP toxicity in these backgrounds is due to the absence of ROS generation. When counted using flow cytometry, we found that only around 7% of the TDP-43-YFP- expressing cells and around 1% the of vector transformants exhibited the CellROX red fluorescence in the *dnm1*Δ yeast thereby suggesting an overall reduction in the ROS levels relative to the wild-type yeast but not a complete abrogation of the ROS generation (Fig. 7). Also, the ROS-positive cell numbers were statistically significantly higher in the TDP-43-YFP-expressing *dnm1*Δ yeast in comparison with thosein the vector transformed *dnm1*Δ. Notably, around 17% of cells in the *cnc1*Δ yeast with TDP-43-YFP expression and ~1% of the cells with the vector control exhibited the fluorescence of CellROX, thereby indicating a lack of any overall reduction in the ROS levels in the *cnc1*Δ yeast in comparison with the wild-type yeast (Fig. 7). This data suggests that although TDP-43 expression induces considerable ROS levels in the *cnc1*Δ and *dnm1*Δ yeast, the lack of the Dnm1 or Cyclin C protein prevents the ROS from culminating in cell death.

3.4. TDP-43-induced oxidative stress causes the movement of Cyclin C from the nucleus to the cytoplasm

Cyclin C, a protein predominantly present in the nucleus, has been shown to translocate to the cytoplasm in the event of oxidative stress [73,90]. Cytoplasmic localization of Cyclin C upon stress is observed from yeast to mammals and it results in the induction of the programmed cell death [91,92]. Recently, it has been shown in the mammalian cell lines that the cytoplasmic Cyclin C protein directly stimulates the GTP affinity of the Drp1 protein and promotes mitochondrial fission in response to oxidative stress [91]. As the TDP-43 expression results in the enhanced oxidative stress and we observed that the TDP-43 toxicity is rescued in the *cnc1*Δ yeast, we therefore examined whether there are any alterations in the sub-cellular localization of Cyclin C in yeast upon the TDP-43 expression. In a previous study, to examine the cellular localization of Cyclin C in proximity to the mitochondria upon oxidative stress, the Cyclin C protein tagged with

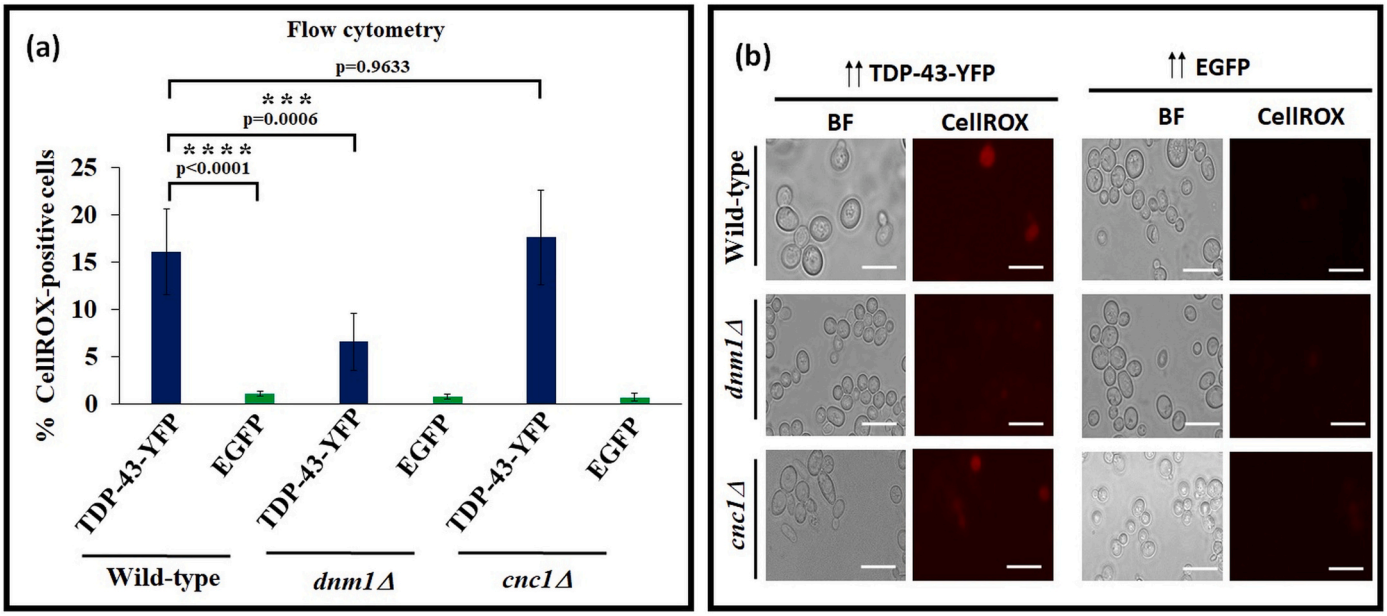

Fig. 7. Assessment of the ROS levels in the yeast cells deleted for the *DNM1* and *CNC1* genes.
To test whether the *cnc1*Δ and *dnm1*Δ yeast that show the rescue of the TDP-43 toxicity fail to accumulate ROS upon TDP-43 expression or rather fail to transmit the effect of ROS into cell death, any presence of ROS in the yeast cells upon the TDP-43-YFP expression was visualized by a ROS-reporter CellROX deep red reagent. CellROX is generally non-fluorescent but converts to bright red fluorescent species in the presence of ROS. The yeast cells were grown for 24h with 2% galactose for expressing the TDP-43-YFP or the vector control (EGFP) before treatment with the CellROX deep red reagent. a. Flow cytometry counting of the number of cells stained with CellROX. The data represent the mean number percentage of the CellROX-positive cells from five independent transformants and the error bars represent standard deviation. Statistical significance was tested to obtain the *p*-values using two-way ANOVA followed by Tukey *Post Hoc* test by comparing the TDP-43-YFP in *cnc1*Δ or *dnm1*Δ using the TDP-43-YFP in the wild-type yeast. The **** on the p-value represents statistical significance between CellROX-positive cells from the TDP-43-YFP expressing cell population or from the vector control cells in wild-type. The *** represents statistical significance between CellROX-positive cells due to the TDP-43-YFP expression in wild-type and *dnm1*Δ. b. Yeast cells expressing TDP-43-YFP or the vector control (EGFP) as described above in the wild-type, *cnc1*Δ or *dnm1*Δ yeast were treated with 1.5 μM CellROX deep red in dark for 1 h and then visualized under the RFP filter using 100× objective lens under Leica DM2500 fluorescence microscope. Images were processed with ImageJ software. Scale bar is10 μm.

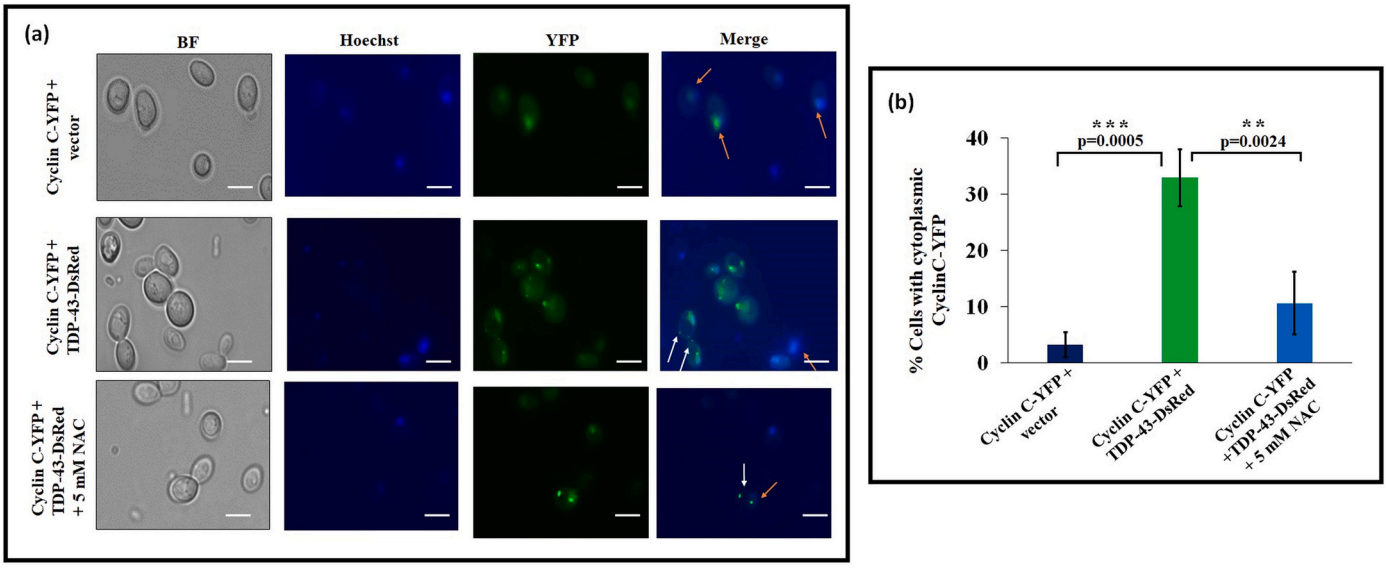

Fig. 8. Oxidative stress-induced cytoplasmic localization of Cyclin C upon TDP-43 co-expression.
a. Cyclin C-YFP and TDP-43-DsRed were expressed from plasmids under *GAL1* promoter using induction with 2% galactose for 18 h in the wild-type BY4742 yeast. The cells were then stained with Hoechst stain for visualizing the nuclei and images were acquired using Leica DM2500 fluorescence microscope. The cytoplasmic or nuclear localization of Cyclin-C-YFP was scored in reference to the Hoechst stained nuclei. Nuclear Cyclin C-YFP is indicated by orange color arrows whereas white color arrows point to the cytoplasmic Cyclin C-YFP. An anti-oxidant, *N*-acetyl-cysteine (NAC) was added in the cells co-expressing both Cyclin C-YFP and TDP-43-DsRed to examine its effect on the cytoplasmic translocation of Cyclin C-YFP. Scale bar is 10 μm. b. Blindly acquired images from three independent transformants were manually counted for cytoplasmic or nuclear localization of Cyclin C-YFP using only the yeast cells that exhibited both the nuclear staining by Hoechst and also the expression of Cyclin C-YFP. The error bars denote standard deviation. The p-values were obtained by performing one-way ANOVA followed by Tukey *post hoc* analysis. **** and *** in the p-values represent statistical significance.

YFP and a mitochondrion localizing protein tagged with DsRed, were used suggesting a lack of interference between these two types of fluorescence [70]. On similar lines, we constructed a Cyclin C-YFP expressing plasmid and used it in conjunction with a plasmid encoding TDP-43-DsRed. In the control sample where the TDP-43-DsRed expression was absent, as expected Cyclin C-YFP was found to be localized mostly to the nucleus and only ~3% of cells exhibited Cyclin C-YFP localization to the cytoplasm (Fig. 8a, 8b and Supplementary Fig. 4S). On the contrary, when TDP-43-DsRed was co-expressed with Cyclin C-YFP, ~30% of cells exhibited cytoplasmic localization of Cyclin C-YFP. Furthermore, we also examined whether an untagged TDP-43 protein's co-expression along with Cyclin C-YFP can also drive the cytoplasmic translocation of Cyclin C-YFP similar to the observations with the TDP-43-DsRed's co-expression. For this, we used 74-D-694 yeast strain and co-transformed it with *pGAL1-TDP-43* (*TRP1*) and *pRS426-GAL1p-CNC1-YFP* (*URA3*). Indeed, a significant percentage of the yeast cells were found to show Cyclin-C-YFP in the cytoplasm when it was co-expressed with the untagged TDP-43 as opposed to the vector co-expression controls (Supplementary Fig. 4S). Recently, it was shown that the TDP-43 toxicity in yeast is significantly reduced in the presence of a reducing agent, *N*-acetyl cysteine (NAC) [55]. We first checked and confirmed that the TDP-43-induced oxidative stress was significantly lowered when cells were grown with the addition of NAC (Supplementary Fig. 5S). Strikingly, when the cells co-expressing Cyclin C-YFP and TDP-43-DsRed were grown in the presence of the anti-oxidant, NAC, the cytoplasmic localization of Cyclin C was significantly reduced (Fig. 8a, b, and Supplementary Fig. 5S). This suggests that the TDP-43 expression-induced ROS-mediated translocation of Cyclin C from the nucleus to the cytoplasm is a potential mediator of the TDP-43-induced cellular toxicity and the consequent cell death in the yeast model.

4. Discussion

Previously, the TDP-43 overexpression in the yeast cell was shown to mimic ALS-like cytoplasmic mis-localization of TDP-43 and cause cytotoxicity. Furthermore, several yeast genes and metabolic pathways have been implicated in mediating the TDP-43 toxicity in the yeast cell [22,49,52,93,94]. Several candidate genes were identified as modifiers of the TDP-43 toxicity in a high-throughput genetic screen [52]. Also, a previous report has shown that the TDP-43 toxicity in yeast is enhanced by the endogenous yeast prion [PIN^+] and over-expression of the yeast Hsp40 chaperone Sis1 can mitigate the TDP-43 toxicity [54]. Also, as certain strains in the deletion library were reported to have acquired [PIN^+] [84], a possibility stays that some modifiers of the TDP-43 toxicity may not have been detected during genetic screens [54]. Overall, several molecular mechanisms have been proposed for causing the TDP-43 proteinopathy such as impaired endocytosis, increased localization of TDP-43 to the mitochondria, dysregulation of the ubiquitin proteasome-mediated protein degradation, prion-like propagation of the TDP-43 aggregates, disturbances in the chromatin remodelling and oxidative stress *etc.* [2,11,18–24,95]. Of note, pathogenic TDP-43 has been associated with an increased mitochondrial localization and consequent disruption of the mitochondrial translation [21]. Additionally, it has been shown that the TDP-43 expression exacerbates the mitochondrial damage *via* the activation of the mitochondrial unfolded protein response pathway and proposedly, reversing or blocking the mitochondrial damage could potentially alleviate the TDP-43-mediated neurodegeneration [21]. In the single cell eukaryotic yeast model, the TDP-43 expression has been shown to cause oxidative stress and mitochondria have been implicated in mediating the TDP-43 toxicity, however the genes/proteins that are the key players, remain to be unravelled [24,50,51]. In fact, deletion of neither the mitochondrial apoptosis inducing factor gene nor the cytochrome *c* gene could relieve the TDP-43 toxicity although the yeast cells expressing TDP-43 displayed apoptotic markers [24]. Thus, here we examined to further learn how the cell death ensues upon the TDP-43 expression by investigating whether the oxidative stress potentiated by the TDP-43 expression or the presence of mitochondrial fission genes or a pro-apoptotic gene, play any role.

Here, we find that the deletion of the *CNC1* gene, which encodes for the Cyclin C protein which is known to be involved in the oxidative stress response and the mitochondrial hyper fission, markedly decreased the TDP-43 toxicity. As the Cyclin C-mediated oxidative stress response is a conserved phenomenon from yeast to humans, the observed rescue of the TDP-43 toxicity in the *cnc1*Δ yeast could be of relevance to the ALS pathology. Cyclin C is associated with the Cdk8 protein in the nucleus and their interaction is essential for the transcriptional repression of the stress response genes in the un-stressed cells. As we observed that the TDP-43 toxicity was rescued in the *cnc1*Δ yeast only when they contained functional mitochondria (*i.e.* [RHO^+]) but not when they contained non-functional mitochondria (*i.e.* [rho^-]), the transcriptional role of Cyclin C in mediating the TDP-43 toxicity may not be implicated. Rather, the data support mitochondria-dependent Cyclin C's role in mediating the TDP-43 toxicity possibly *via* mitochondrial hyper fission-mediated apoptosis, as the role of Cyclin C in mediating the mitochondrial hyper fission is established in the yeast cells [73,90,91].

Recently, it has been shown that Cyclin C, when present in the cytoplasm, directly stimulates the affinity of Drp1 (human homolog of Dnm1) for GTP for mediating the mitochondrial fission [91]. Here, we observed that the deletion of *DNM1* also markedly relieved the TDP-43 toxicity. Alike to the *cnc1*Δ yeast, the presence of functional mitochondria was also essential for the rescue in the *dnm1*Δ yeast. It is noteworthy that an inhibitor of Drp1 and Fis1 interaction was previously found to relieve the TDP-43-induced mitochondrial fragmentation and cytotoxicity [96]. Surprisingly however, the toxicity of TDP-43 was not rescued in the *fis1*Δ yeast, the reason for which is not completely understood. Notably, it is also previously documented that *fis1*Δ yeast is still capable of undergoing mitochondrial fragmentation which may be a reason for the lack of the TDP-43 toxicity rescue in the *fis1*Δ yeast [65]. We also observed here that as expected the deletion of the *MED13* gene that encodes for the Med13 protein which is essential for the nuclear retention of Cyclin C failed to rescue the TDP-43 toxicity [97,98]. Taken together, the factors influencing the cytoplasmic translocation of Cyclin C seem vital in mediating the TDP-43 toxicity.

As there is a considerable overlap in the array of proteins involved in the mitochondrial and peroxisomal fission processes, we also examined the TDP-43 toxicity in the yeast strains deleted for the genes encoding proteins involved in the peroxisomal pathway. We find that the toxicity of TDP-43 is substantially rescued in *dnm1*Δ, which lacks a protein that contributes both to the fission of the mitochondria as well as that of the peroxisomes. On the contrary, the deletion of the *VPS1* gene that encodes for the Vps1 protein which is an independent regulator of the peroxisomal fission, failed to rescue the TDP-43 toxicity. Thus, peroxisome fission seems an unlikely key player in the TDP-43-mediated cytotoxicity and cell death in yeast.

Remarkably, the *cnc1*Δ yeast could rescue the toxicity of TDP-43 when it was expressed at low as well as high levels and irrespective of whether the TDP-43 protein was fused to the YFP, GFP or DsRed fluorescent proteins. Even the toxicity of a highly over-expressed TDP-43's C-terminal fragment (aa: 175-414), TDP-25, was also rescued in the *cnc1*Δ yeast. Thus, the data support that Cyclin C is a *bona fide* key player in mediating the toxicity of TDP-43 in the yeast cells.

Yeast Bcl-2 homology domain (BH3) containing pro-apoptotic protein, Ybh3, similar to its human pro-apoptotic counterpart, is also needed to localize to the mitochondria to initiate the mitochondria-mediated programmed cell death (PCD) [24,73]. Strikingly, we found here that the deletion of the *YBH3* gene that encodes for the Ybh3 protein also rescues the TDP-43-induced cytotoxicity. As this rescue was observed only in the [RHO^+] yeast but not in the [rho^-] yeast, therefore the mitochondria-dependent, Ybh3-mediated PCD appears important for the TDP-43-induced cytotoxicity in yeast.

Furthermore, although the TDP-43-induced oxidative stress was present in the wild-type, *cnc1*Δ and *dnm1*Δ yeast strains, but the cell

death was significantly reduced in the *cnc1*Δ and *dnm1*Δ yeast thereby suggesting a missing relay link between the ROS and the downstream PCD. If Cyclin C was the link to relay the TDP-43-induced ROS effect to culminate in cell death, it would be expected that Cyclin C moves to the cytoplasm upon the TDP-43 expression. Indeed, Cyclin C-YFP was observed to translocate from the nucleus to the cytoplasm upon the TDP-43's co-expression with Cyclin C-YFP. This strongly suggests that the oxidative stress caused by the TDP-43 expression could relay the toxicity and cause cell death *via* the Cyclin C-dependent pathway. In accordance, the addition of an anti-oxidant molecule *N*-acetyl-cysteine (NAC), which has been previously shown to relieve the TDP-43 toxicity in yeast [55], also significantly prevented the cytoplasmic localization of Cyclin C-YFP. Recently, it has been shown that Cyclin C associates with Bcl-2 homology containing protein BAX and tethers it with the stress-induced mitochondrial fission complex in the mammalian cell line. This provides sufficient dwell time on mitochondria for BAX to be recognized by the BH3 containing proteins leading to the initiation of PCD in the mammalian cell line [96]. Since Cyclin C translocation from the nucleus to the cytoplasm is a conserved process from yeast to humans, this finding of the TDP-43-induced Cyclin C translocation in yeast may have direct relevance to the ALS pathology [90,92,96,97,99]. Therefore, targeting the TDP-43 expression-induced oxidative stress and the Cyclin C translocation may have potentially fruitful consequences towards finding of the ALS therapeutic targets.

5. Conclusions

TDP-43 protein is implicated in the pathology of the ALS disease. Several important pathways mediating the toxicity of TDP-43 have been unearthed using various model systems. In this study, we used the yeast model of TDP-43 proteinopathy to investigate which genes are important for mediating the mitochondria-dependent TDP-43 toxicity in response to oxidative stress that is previously documented to ensue in yeast upon the TDP-43 aggregation. We show that the toxicity of TDP-43 is rescued in the *cnc1*Δ, *dnm1*Δ, and *ybh3*Δ yeast strains. We also show that the *cnc1*Δ yeast can even rescue the toxicity of a C-terminal fragment (aa: 175-414) of TDP-43. The *CNC1* and *DNM1* genes encode proteins that function in the mitochondrial fission process while *YBH3* encodes a pro-apoptotic protein. The Cyclin C protein (encoded by *CNC1*) responds to oxidative stress and causes mitochondrial fragmentation leading to mitochondria-dependent cell death in yeast. Consistent with the earlier reports of oxidative stress-induced translocation of Cyclin C from the nucleus to the cytoplasm to mediate mitochondrial fragmentation, we find here that the co-expression of Cyclin C-YFP with TDP-43, which is known to cause oxidative stress in yeast, causes Cyclin C to translocate to the cytoplasm. Thus, the observed rescue of the TDP-43 toxicity in the *cnc1*Δ yeast suggests a role of the mitochondrial fragmentation-dependent cell death pathway in mediating the TDP-43 toxicity in the yeast model.

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CRediT authorship contribution statement

VB and AG performed experiments. BKP conceived the study and analysed data. VB and BKP wrote the manuscript.

Declaration of competing interest

Authors declare that no competing interests exist.

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Appendix A. Supplementary data

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References

[1] J.P. Taylor, R.H. Brown Jr., D.W. Cleveland, Decoding ALS: from genes to mechanism, Nature 539 (7628) (2016) 197–206.
[2] A. Prasad, et al., Molecular mechanisms of TDP-43 misfolding and pathology in amyotrophic lateral sclerosis, Front. Mol. Neurosci. 12 (2019) 25.
[3] D.R. Rosen, Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis, Nature 364 (6435) (1993) 362.
[4] C. Vance, et al., Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6, Science 323 (5918) (2009) 1208–1211.
[5] M. DeJesus-Hernandez, et al., Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS, Neuron 72 (2) (2011) 245–256.
[6] D. Brenner, et al., NEK1 mutations in familial amyotrophic lateral sclerosis, Brain 139 (Pt 5) (2016), e28.
[7] S.C. Ling, M. Polymenidou, D.W. Cleveland, Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis, Neuron 79 (3) (2013) 416–438.
[8] A.N. Coyne, B.L. Zaepfel, D.C. Zarnescu, Failure to deliver and translate-new insights into RNA dysregulation in ALS, Front. Cell. Neurosci. 11 (2017) 243.
[9] S. Rossi, M. Cozzolino, M.T. Carri, Old versus new mechanisms in the pathogenesis of ALS, Brain Pathol. 26 (2) (2016) 276–286.
[10] A. Girdhar, et al., Computational insights into mechanism of AIM4-mediated inhibition of aggregation of TDP-43 protein implicated in ALS and evidence for in vitro inhibition of liquid-liquid phase separation (LLPS) of TDP-43(2C)-A315T by AIM4, Int. J. Biol. Macromol. 147 (2020) 117–130.
[11] L. Guo, J. Shorter, Biology and pathobiology of TDP-43 and emergent therapeutic strategies, Cold Spring Harb. Perspect. Med. 7 (9) (2017) a024554.
[12] M. Bowerman, et al., Pathogenic commonalities between spinal muscular atrophy and amyotrophic lateral sclerosis: converging roads to therapeutic development, Eur. J. Med. Genet. 61 (11) (2018) 685–698.
[13] M. Hasegawa, et al., Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis, Ann. Neurol. 64 (1) (2008) 60–70.
[14] T. Nonaka, et al., Phosphorylated and ubiquitinated TDP-43 pathological inclusions in ALS and FTLD-U are recapitulated in SH-SY5Y cells, FEBS Lett. 583 (2) (2009) 394–400.
[15] M. Neumann, et al., Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis, Science 314 (5796) (2006) 130–133.
[16] D. Dormann, et al., Proteolytic processing of TAR DNA binding protein-43 by caspases produces C-terminal fragments with disease defining properties independent of progranulin, J. Neurochem. 110 (3) (2009) 1082–1094.
[17] D.F. Lázaro, et al., Editorial: protein misfolding and spreading pathology in neurodegenerative diseases, Front. Mol. Neurosci. 12 (312) (2020).
[18] A. Berson, et al., TDP-43 promotes neurodegeneration by impairing chromatin remodeling, Curr. Biol. 27 (23) (2017) 3579–3590 (e6).
[19] T.N. Dang, et al., Increased metal content in the TDP-43(A315T) transgenic mouse model of frontotemporal lobar degeneration and amyotrophic lateral sclerosis, Front. Aging Neurosci. 6 (2014) 15.
[20] W. Wang, et al., The ALS disease-associated mutant TDP-43 impairs mitochondrial dynamics and function in motor neurons, Hum. Mol. Genet. 22 (23) (2013) 4706–4719.
[21] W. Wang, et al., The inhibition of TDP-43 mitochondrial localization blocks its neuronal toxicity, Nat. Med. 22 (8) (2016) 869–878.
[22] G. Liu, et al., Endocytosis regulates TDP-43 toxicity and turnover, Nat. Commun. 8 (1) (2017) 2092.
[23] Q. Xia, et al., TDP-43 loss of function increases TFEB activity and blocks autophagosome-lysosome fusion, EMBO J. 35 (2) (2016) 121–142.
[24] R.J. Braun, et al., Neurotoxic 43-kDa TAR DNA-binding protein (TDP-43) triggers mitochondrion-dependent programmed cell death in yeast, J. Biol. Chem. 286 (22) (2011) 19958–19972.
[25] A. Prasad, et al., The amyloidogenicity of a C-terminal region of TDP-43 implicated in Amyotrophic Lateral Sclerosis can be affected by anions, acetylation and homodimerization, Biochimie 150 (2018) 76–87.
[26] O. Kann, R. Kovacs, Mitochondria and neuronal activity, Am. J. Phys. Cell Physiol. 292 (2) (2007) C641–C657.
[27] A. Verkhratsky, A. Schousboe, V. Parpura, Glutamate and ATP: the crossroads of signaling and metabolism in the brain, Adv. Neurobiol. 11 (2014) 1–12.
[28] M.T. Lin, M.F. Beal, Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases, Nature 443 (7113) (2006) 787–795.

[29] P.H. Reddy, Role of mitochondria in neurodegenerative diseases: mitochondria as a therapeutic target in Alzheimer's disease, CNS Spectr. 14 (8 Suppl 7) (2009) 8–13 (discussion 16-8).
[30] J. Gao, et al., TDP-43 proteinopathy and mitochondrial abnormalities in neurodegeneration, Mol. Cell. Neurosci. 100 (2019) 103396.
[31] P. Wang, et al., TDP-43 induces mitochondrial damage and activates the mitochondrial unfolded protein response, PLoS Genet. 15 (5) (2019), e1007947.
[32] Y.F. Xu, et al., Wild-type human TDP-43 expression causes TDP-43 phosphorylation, mitochondrial aggregation, motor deficits, and early mortality in transgenic mice, J. Neurosci. 30 (32) (2010) 10851–10859.
[33] K. Hong, et al., Full-length TDP-43 and its C-terminal fragments activate mitophagy in NSC34 cell line, Neurosci. Lett. 530 (2) (2012) 144–149.
[34] J. Lu, et al., Mitochondrial dysfunction in human TDP-43 transfected NSC34 cell lines and the protective effect of dimethoxy curcumin, Brain Res. Bull. 89 (5–6) (2012) 185–190.
[35] M. Gautam, et al., Mitochondria, ER, and nuclear membrane defects reveal early mechanisms for upper motor neuron vulnerability with respect to TDP-43 pathology, Acta Neuropathol. 137 (1) (2019) 47–69.
[36] J. Magrane, et al., Abnormal mitochondrial transport and morphology are common pathological denominators in SOD1 and TDP43 ALS mouse models, Hum. Mol. Genet. 23 (6) (2014) 1413–1424.
[37] E. Onesto, et al., Gene-specific mitochondria dysfunctions in human TARDBP and C9ORF72 fibroblasts, Acta Neuropathol. Commun. 4 (1) (2016) 47.
[38] A.U. Joshi, et al., Inhibition of Drp1/Fis1 interaction slows progression of amyotrophic lateral sclerosis, EMBO Mol. Med. (2018) **10**(3).
[39] J.S. Ebo, et al., Using protein engineering to understand and modulate aggregation, Curr. Opin. Struct. Biol. 60 (2020) 157–166.
[40] V. Sivalingam, B.K. Patel, Familial mutations in fibrinogen Aα (FGA) chain identified in renal amyloidosis increase in vitro amyloidogenicity of FGA fragment, Biochimie 127 (2016) 44–49.
[41] V. Sivalingam, et al., Wild-type hen egg white lysozyme aggregation in vitro can form self-seeding amyloid conformational variants, Biophys. Chem. 219 (2016) 28–37.
[42] N. Sharma, S. Vishwanath, B.K. Patel, Recombinant human semenogelin-1 (Sg1) and Sg1 (1–159) form detergent stable amyloid like aggregates in vitro, Protein Pept. Lett. 23 (1) (2016) 87–96.
[43] B.K. Patel, J. Gavin-Smyth, S.W. Liebman, The yeast global transcriptional co-repressor protein Cyc8 can propagate as a prion, Nat. Cell Biol. 11 (3) (2009) 344–349.
[44] B. Panaretou, G.W. Jones, Yeast models for amyloid disease, Essays Biochem. 56 (2014) 85–97.
[45] B.K. Patel, S.W. Liebman, "Prion-proof" for [PIN+]: infection with in vitro-made amyloid aggregates of Rnq1p-(132–405) induces [PIN+], J. Mol. Biol. 365 (3) (2007) 773–782.
[46] M.F. Tuite, G.L. Staniforth, B.S. Cox, [PSI(+)] turns 50, Prion 9 (5) (2015) 318–332.
[47] M. Verma, et al., Q-Rich yeast prion [PSI(+)] accelerates aggregation of transthyretin, a non-Q-rich human protein, Front. Mol. Neurosci. 11 (2018) 75.
[48] B.S. Johnson, et al., A yeast TDP-43 proteinopathy model: exploring the molecular determinants of TDP-43 aggregation and cellular toxicity, Proc. Natl. Acad. Sci. U. S. A. 105 (17) (2008) 6439–6444.
[49] B.S. Johnson, et al., TDP-43 is intrinsically aggregation-prone, and amyotrophic lateral sclerosis-linked mutations accelerate aggregation and increase toxicity, J. Biol. Chem. 284 (30) (2009) 20329–20339.
[50] V. Bharathi, A. Girdhar, B.K. Patel, A protocol of using white/red color assay to measure amyloid-induced oxidative stress in *Saccharomyces cerevisiae*, Bio-protocol 7 (15) (2017), e2440.
[51] V. Bharathi, et al., Use of ade1 and ade2 mutations for development of a versatile red/white colour assay of amyloid-induced oxidative stress in *Saccharomyces cerevisiae*, Yeast 33 (12) (2016) 607–620.
[52] M. Armakola, et al., Inhibition of RNA lariat debranching enzyme suppresses TDP-43 toxicity in ALS disease models, Nat. Genet. 44 (12) (2012) 1302–1309.
[53] H.-J. Kim, et al., Therapeutic modulation of eIF2α-phosphorylation rescues TDP-43 toxicity in amyotrophic lateral sclerosis disease models, Nat. Genet. 46 (2) (2014) 152–160.
[54] S.K. Park, et al., Overexpression of the essential Sis1 chaperone reduces TDP-43 effects on toxicity and proteolysis, PLoS Genet. 13 (5) (2017), e1006805.
[55] S.K. Park, S. Park, S.W. Liebman, Respiration enhances TDP-43 toxicity, but TDP-43 retains some toxicity in the absence of respiration, J. Mol. Biol. 431 (10) (2019) 2050–2059.
[56] M. Ott, et al., Mitochondria, oxidative stress and cell death, Apoptosis 12 (5) (2007) 913–922.
[57] T. Chen, et al., Glutathione monoethyl ester prevents TDP-43 pathology in motor neuronal NSC-34 cells, Neurochem. Int. 112 (2018) 278–287.
[58] S.A. Davis, et al., TDP-43 interacts with mitochondrial proteins critical for mitophagy and mitochondrial dynamics, Neurosci. Lett. 678 (2018) 8–15.
[59] L.M. Sandalio, et al., Role of peroxisomes as a source of reactive oxygen species (ROS) signaling molecules, Subcell. Biochem. 69 (2013) 231–255.
[60] Q. Guo, et al., The mitochondrial fission adaptors Caf4 and Mdv1 are not functionally equivalent, PLoS One 7 (12) (2012), e53523.
[61] A. Aufschnaiter, S. Buttner, Peroxisomal fission controls yeast life span, Cell Cycle 14 (15) (2015) 2389–2390.
[62] S.D. Lefevre, S. Kumar, I.J. van der Klei, Inhibition of peroxisome fission, but not mitochondrial fission, increases yeast chronological lifespan, Cell Cycle 14 (11) (2015) 1698–1703.
[63] K. Kuravi, et al., Dynamin-related proteins Vps1p and Dnm1p control peroxisome abundance in *Saccharomyces cerevisiae*, J. Cell Sci. 119 (19) (2006) 3994.
[64] A.M. Motley, G.P. Ward, E.H. Hettema, Dnm1p-dependent peroxisome fission requires Caf4p, Mdv1p and Fis1p, J. Cell Sci. 121 (Pt 10) (2008) 1633–1640.
[65] Y. Fannjiang, et al., Mitochondrial fission proteins regulate programmed cell death in yeast, Genes Dev. 18 (22) (2004) 2785–2797.
[66] P. Zarzov, C. Mazzoni, C. Mann, The SLT2 (MPK1) MAP kinase is activated during periods of polarized cell growth in yeast, EMBO J. 15 (1) (1996) 83–91.
[67] K.R. Davenport, et al., A second osmosensing signal transduction pathway in yeast. Hypotonic shock activates the PKC1 protein kinase-regulated cell integrity pathway, J. Biol. Chem. 270 (50) (1995) 30157–30161.
[68] H. Martin, et al., Activity of the yeast MAP kinase homologue Slt2 is critically required for cell integrity at 37 degrees C, Mol. Gen. Genet. 241 (1–2) (1993) 177–184.
[69] T. Ketela, R. Green, H. Bussey, *Saccharomyces cerevisiae* mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway, J. Bacteriol. 181 (11) (1999) 3330–3340.
[70] K.F. Cooper, et al., Stress-induced nuclear-to-cytoplasmic translocation of cyclin C promotes mitochondrial fission in yeast, Dev. Cell 28 (2) (2014) 161–173.
[71] T.J. Cohen, et al., Ask10p mediates the oxidative stress-induced destruction of the *Saccharomyces cerevisiae* C-type cyclin Ume3p/Srb11p, Eukaryot. Cell 2 (5) (2003) 962–970.
[72] Y. Zhang, D.C. Chan, Structural basis for recruitment of mitochondrial fission complexes by Fis1, Proc. Natl. Acad. Sci. U. S. A. 104 (47) (2007) 18526–18530.
[73] R. Strich, K.F. Cooper, The dual role of cyclin C connects stress regulated gene expression to mitochondrial dynamics, Microb. Cell 1 (10) (2014) 318–324.
[74] I.L. Derkatch, et al., Genesis and variability of [PSI] prion factors in *Saccharomyces cerevisiae*, Genetics 144 (4) (1996) 1375–1386.
[75] A. Wach, et al., New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*, Yeast 10 (13) (1994) 1793–1808.
[76] E.A. Winzeler, et al., Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis, Science 285 (5429) (1999) 901–906.
[77] I.L. Derkatch, et al., Prions affect the appearance of other prions: the story of [PIN(+)], Cell 106 (2) (2001) 171–182.
[78] R.S. Sikorski, P. Hieter, A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*, Genetics 122 (1) (1989) 19–27.
[79] D.M. Gelperin, et al., Biochemical and genetic analysis of the yeast proteome with a movable ORF collection, Genes Dev. 19 (23) (2005) 2816–2826.
[80] F. Sherman, G.R. Fink, J.B. Hicks, Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1986.
[81] E.S. Goldring, et al., The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid during induction of petites with ethidium bromide, J. Mol. Biol. 52 (2) (1970) 323–335.
[82] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat. Methods 9 (7) (2012) 671–675.
[83] Y. Kamei, et al., The yeast mitochondrial permeability transition is regulated by reactive oxygen species, endogenous Ca(2+) and Cpr3, mediating cell death, Biochim. Biophys. Acta Bioenerg. 1859 (12) (2018) 1313–1326.
[84] A.L. Manogaran, et al., Most, but not all, yeast strains in the deletion library contain the [PIN(+)] prion, Yeast 27 (3) (2010) 159–166.
[85] M. Armakola, M.P. Hart, A. Gitler, TDP-43 toxicity in yeast, Methods 53 (3) (2011) 238–245.
[86] L.M. Igaz, et al., Enrichment of C-terminal fragments in TAR DNA-binding protein-43 cytoplasmic inclusions in brain but not in spinal cord of frontotemporal lobar degeneration and amyotrophic lateral sclerosis, Am. J. Pathol. 173 (1) (2008) 182–194.
[87] Q. Li, et al., The cleavage pattern of TDP-43 determines its rate of clearance and cytotoxicity, Nat. Commun. 6 (1) (2015) 6183.
[88] F. Devaux, et al., Genome-wide studies on the nuclear PDR3-controlled response to mitochondrial dysfunction in yeast, FEBS Lett. 515 (1–3) (2002) 25–28.
[89] O. Matangkasombut, et al., Cytolethal distending toxin from Aggregatibacter actinomycetemcomitans induces DNA damage, S/G2 cell cycle arrest, and caspase-independent death in a *Saccharomyces cerevisiae* model, Infect. Immun. 78 (2) (2010) 783–792.
[90] K.F. Cooper, et al., Oxidative-stress-induced nuclear to cytoplasmic relocalization is required for Not4-dependent cyclin C destruction, J. Cell Sci. 125 (Pt 4) (2012) 1015–1026.
[91] V. Ganesan, et al., Cyclin C directly stimulates Drp1 GTP affinity to mediate stress-induced mitochondrial hyperfission, Mol. Biol. Cell 30 (3) (2019) 302–311.
[92] K. Wang, et al., Cyclin C mediates stress-induced mitochondrial fission and apoptosis, Mol. Biol. Cell 26 (6) (2015) 1030–1043.
[93] H.J. Kim, et al., Therapeutic modulation of eIF2alpha phosphorylation rescues TDP-43 toxicity in amyotrophic lateral sclerosis disease models, Nat. Genet. 46 (2) (2014) 152–160.
[94] C. Leibiger, et al., Endolysosomal pathway activity protects cells from neurotoxic TDP-43, Microb. Cell 5 (4) (2018) 212–214.
[95] T. Nonaka, M. Hasegawa, Prion-like properties of assembled TDP-43, Curr. Opin. Neurobiol. 61 (2019) 23–28.
[96] J. Jezek, et al., Mitochondrial translocation of cyclin C stimulates intrinsic apoptosis through Bax recruitment, EMBO Rep. 20 (9) (2019), e47425.

[97] D.C. Stieg, et al., A complex molecular switch directs stress-induced cyclin C nuclear release through SCF(Grr1)-mediated degradation of Med13, Mol. Biol. Cell 29 (3) (2018) 363–375.
[98] S. Khakhina, K.F. Cooper, R. Strich, Med13p prevents mitochondrial fission and programmed cell death in yeast through nuclear retention of cyclin C, Mol. Biol. Cell 25 (18) (2014) 2807–2816.
[99] E. Krasley, et al., Regulation of the oxidative stress response through Slt2p-dependent destruction of cyclin C in *Saccharomyces cerevisiae*, Genetics 172 (3) (2006) 1477–1486.