Annals of Microbiology (2019) 69:341–352
https://doi.org/10.1007/s13213-018-1422-3

ORIGINAL ARTICLE

Inhibition of TOR signalling in *lea1* mutant induces apoptosis in *Saccharomyces cerevisiae*

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Received: 29 May 2018 / Accepted: 13 December 2018 / Published online: 5 January 2019

Abstract

The target of rapamycin, TOR, maintains cell growth and proliferation under vivid environmental conditions by orchestrating wide array of growth-related process. In addition to environmental conditions, e.g., nutrient and stress, TOR also governs cellular response to varied intracellular cues including perturbed intracellular mRNA levels which may arise due to altered regulation of mRNA processing at splicing or turnover levels. The purpose of this study is to explore the role of TOR signalling in growth of cells with accumulated unprocessed RNA. Growth analysis of *lea1*Δ (splicing deficient) was carried out under varied conditions leading to nitrogen starvation. The expression of TORC1 and TORC2 marker genes was examined in this delete strain. Sensitivity of the *lea1*Δ towards oxidative agents was observed. Apoptosis was analyzed in caffeine-treated *lea1*Δ cells. The hypersensitivity of *lea1*Δ cells towards caffeine is outcome of highly perturbed TOR signalling. The growth defect is independent of PKC pathway. Cells with accumulated unprocessed RNA experience high oxidative stress that induces apoptosis. An inadequate TOR signalling in *lea1*Δ cells substantiates the effect of oxidative stress induced by accumulated RNA to the extent of inducing cell death via apoptosis.

Keywords RNA accumulation · Oxidative stress · Reactive oxygen species · Caffeine · Splicing

Introduction

TOR is essentially a central regulator of growth across eukaryotes (Loewith and Hall 2011). Thorough mechanistic understanding of TOR signalling has always been sought for development of efficient therapeutic interventions against diseases resulting from dysregulated TOR signalling. Under diverse environmental conditions, TOR optimizes cell growth by regulating several key cellular growth-related processes such as transcription, translation, and autophagy (Cardenas et al. 1999; Beck et al. 1999). The precise regulation of cell growth involves its ability to sense and respond to both extracellular and relatively less studied intracellular cues. TOR manages growth homeostasis through two highly conserved complexes TOR complex I (TORC1) and TOR complex II (TORC2) (Wullschleger et al. 2006; De Virgilio and Loewith 2006; Adami et al. 2007). An elaborate understanding of the function of TORC1 complex has been made possible by the use of inhibitors, e.g., rapamycin and caffeine which mimic nutrient starvation-like conditions and display remarkably similar global gene expression profile in *Saccharomyces cerevisiae* (Loewith et al. 2002; Urban et al. 2007; Wanke et al. 2008; Lempiäinen et al. 2009). An activated TORC1 promotes cell growth through active protein and ribosome biosynthesis (Powers and Walter 1999; Morita et al. 2015). Conversely, the inhibition of TORC1 activity leads to sequestration of various transcription factors in the cytoplasm required to induce the expression of nitrogen catabolite response genes (*GAP1*), retrograde pathway genes (*CIT2*), and stress-responsive genes (*SOD2*) (Crespo et al. 2002; Inoki et al. 2005; Loewith and Hall 2011). TORC2 primarily regulates actin cytoskeleton to maintain polarized nature of cell growth (Fadri et al. 2005; Kamada et al. 2005; He et al. 2013; Niles and Powers 2014). Further studies underscore the association of TOR signalling with vesicular trafficking system (Puria et al. 2008; Kingsbury and Cardenas 2016).

In addition, to extracellular conditions, recent studies have appreciated the emanating role of TOR signalling to different

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intracellular conditions. Class C *vps* mutants with defective amino acid homeostasis have highly compromised TOR signalling (Stanfel et al. 2009; Loewith and Hall 2011). Further TOR signalling is also influenced by effectors of cross-talking pathways. Interestingly, the role of TOR signalling has also been seen in cells with perturbed RNA levels which may arise due to defective RNA degradation or processing. TOR signalling controls mRNA turnover in yeast (Albig and Decker 2001; Munchel et al. 2011). However, the mechanism is intricate as the rate of decay of multiple RNAs is highly variable under different stress conditions. During nutrient depletion or upon exposure to rapamycin, multiple RNAs are destabilized but not decayed at the same rate (Albig and Decker 2001). The role of CK2 kinase in mediating stress response at the level of pre-mRNA processing has been demonstrated (Bergkessel et al. 2011). Global protein kinase and phosphatase interaction network in yeast identified several potential substrates/regulators of TOR complex including Sky1, a conserved Ser/Thr kinase that phosphorylates pre-mRNA splicing factors of SR family (Siebel et al. 1999). Stresses that lead to TORC1 inactivation caused rapid loss of RPG pre-mRNA transcripts (Bergkessel et al. 2011). Apparently, since RP genes are rich in introns, TOR signalling may possess a direct role in transcriptional processing of Ribi regulon, genes required for ribosome biogenesis in yeast. Recently, Heintz et al. have shown relationship between TOR signalling and splicing wherein loss of Sfa1 splicing factor suppresses life span extension conferred by decreased TORC1 signalling (Heintz et al. 2016). Since regulation at the level of pre-mRNA processing contributes specifically and dynamically to the regulation of gene expression program under wide range of environmental challenges may have a bigger role in guiding cell growth at the level of pre-mRNA processing.

Previously, genome-wide screen searching for genes that when mutated in combination with *tor1*Δ reduce fitness or render cells inviable resulted in identification of several genes associated with ribosomal and mitochondrial functions, vesicle docking and fusion, protein transport, microautophagy, and vacuolar inheritance, including spliceosome complex gene *LEA1* (Zurita-Martinez et al. 2007). *LEA1* (looks exceptionally like U2A') is highly conserved across metazoans, is a specific component of U2snRNP, and is crucial for efficient first step of splicing in vivo. It plays an essential role in spliceosome assembly with U2 snRNA base pairing with both the pre-mRNA branch point sequence and U6 snRNA. Cells lacking *LEA1* harbored low levels of various mRNA and accumulated pre-mRNA to different levels (Caspary and Séraphin 1998; Dreumont and Séraphin 2013). The anticipated prevalence of unspliced RNAs in *lea1*Δ and its synthetic sick interaction with *tor1*Δ persuaded us to directly investigate impact of unspliced RNA/RNA load on TOR signalling. We envisaged that accumulation of pre-transcripts in *lea1*Δ cells may lead to intracellular stress conditions that require active TOR signalling to support cell growth. Thereby, an inhibition of TOR signalling in *lea1*Δ will reduce cell growth and survival.

Material and methods

Strains, medium, and growth conditions

Strain used in this study was isogenic with *JK9-3d* (*MATa leu2-3,112 ura3-52 rme1 trp1 his4*) (Heitman et al. 1991a). *LEA1* gene was disrupted with *URA3* marker by PCR-based short homology recombination method. *tor1*Δ and *rom2*Δ strains were disrupted with G418. Lithium acetate TE method was used for all transformations in this study (Gietz et al. 1995). Primers used in this study are listed in Table 1.

Yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose), synthetic dextrose (SD) medium (2% glucose, 0.17% yeast nitrogen base (YNB) without amino acids and ammonium sulfate, 0.5% ammonium sulfate), synthetic low ammonia dextrose (SLAD) (2% glucose, 0.17% YNB without amino acids and ammonium sulfate, 0.05 mM ammonium sulfate), and YNB proline medium (2% glucose, 0.17% YNB, and 0.1% proline). SD medium was supplied with various amino acids on the basis of auxotrophies in strains. Medium was supplemented with caffeine (20 mM, 10 mM, 5 mM, and 2 mM), sorbitol (1 M), adenine (20 mM), H_2O_2 (1.5 mM and 2.5 mM), dithiothreitol (DTT) (1 mM), glutathione reduced (GSH) (10 mM), and *N*-acetyl cysteine (NAC) (30 mM) for different experiments conducted in this study. All the chemicals used in this study were purchased from Sigma-Aldrich, HiMedia, and Biochem.

Growth assays

For growth analysis, strains were grown in rich medium and selectable medium until stationary phase and subsequently diluted to $OD_{600} = 0.05$ in a 96-well plate. Growth of cells was measured at 600 nm at intervals of 2 h for 16 h using synergy H1 multimode plate reader (BioTek). For cell viability, after completion of 16 h, cells were serially diluted 10-fold and plated on YPD agar plates lacking caffeine. Cell viability was demonstrated by measuring colony-forming units (CFU) after 3 days of incubation at 30 °C. For spot test, cells were grown overnight in rich medium to $OD_{600} = 1$ and collected at equal density in log phase (~ 0.2 OD) prior to spotting. Subsequently, 5-fold serially diluted cells in liquid-rich medium (YPD) were spotted in 5 μL drops on solid medium with and without caffeine. Cells were allowed to form colonies at 30 °C for 2–3 days prior to image acquisition.

Sensitivity to oxidizing agent hydrogen peroxide (H_2O_2) and reducing agents DTT, GSH, and NAC was determined by growing WT and *lea1*Δ cells for 16 h (H_2O_2) and 48 h

Table 1 Primers used in this study

S. no.	Primer name	Sequence	Purpose
1.	RPLEA1F	AACTACAGGACTTGGAAAATATCAGTTTTT ATAAGCAATAGCGGCATCAGAGCAGATTG	Used in construction of *lea1* mutant
2.	RPLEA1R	TTTTTTTTTAAGTCATTGAACAGTCGCACT AACCAAAAGACGTTTACAATTTCCTGATG CGG	Used in construction of *lea1* mutant
3.	LEA1FC	TCGCATACTCTGCATCCAAC	Used in check PCR of *lea1* mutant
4.	URA3RC	CTGGCGGATAATGCCTTTAG	Used in check PCR of *lea1* mutant
5.	ACT1F	AGTTGCCCCAGAAGAACACC	Used as reference gene in qRT-PCR analysis
6.	ACT1R	TACCGGCAGATTCCAAACCC	Used as reference gene in qRT-PCR analysis
7.	GAP1F	AGGGTTTGCCAAGTGTTTGTC	TORC1 marker gene expression
8.	GAP1R	AACGCAATAAGACCGAATGC	TORC1 marker gene expression
9.	CIT2F	GCCAAAGAAGAGAGCTTCCA	TORC1 marker gene expression
10.	CIT2R	GGCAAAGCGTATTCAGGGTA	TORC1 marker gene expression
11.	SOD2F	GGTTCCAACTCAAGCGCAAG	TORC1 marker gene expression
12.	SOD2R	ACCCGTTCAAACCTGATGCA	TORC1 marker gene expression
13.	RPS26AF	GCCAAAGAAGAGAGCTTCCA	TORC1 marker gene expression
14.	RPS26AR	CTGGCGTGAATAGCACAAGA	TORC1 marker gene expression
15.	CMK2F	GTCTAAACGGCCACCCTGTA	TORC2 marker gene expression
16.	CMK2R	CCTGCAATGCCTTCTTCAAT	TORC2 marker gene expression
17.	DIA1F	TAAGAACGGCAGACGAACCT	TORC2 marker gene expression
18.	DIA1R	TCCAAATCCCTGTGTCCTTC	TORC2 marker gene expression
19.	NCW2F	GAGAGACGTCAAGCGGAATC	TORC2 marker gene expression
21.	NCW2R	GATGAGGATCCCGAAGATGA	TORC2 marker gene expression
22.	MCA1F	CTCCTCCACCTAACCAGCAG	Check apoptosis in *lea1* mutant
23.	MCA1R	GGGTGGACCGTACATATTGG	Check apoptosis in *lea1* mutant

(DTT, GSH, and NAC) in SD medium at 30 °C. SD media was supplemented with 2.5 mM concentration of H_2O_2.

Quantitative real-time PCR

Total RNA from strains was isolated using RNeasy mini kit as per manufacturer's instructions (QIAGEN). Total RNA was quantified at A_{260} and A_{280}/A_{230} ratio with Nanodrop spectrophotometer (Thermo Scientific) and integrity of total RNA was assessed by denaturing agarose gel. One-microgram RNA was used to synthesize cDNA by quantitect reverse transcriptase kit (QAIGEN) and RNase-free DNase was also used to avoid any genomic DNA contamination. Quantitative real-time PCR (qRT-PCR) using a two-step SYBR green reverse transcriptase PCR kit (QAIGEN) with primers listed in the Table 1 was carried in Bio-Rad CFX96™ real-time PCR machine (Bio-Rad Laboratories Inc., New Delhi, India). Melt curve analysis was generated at the end of each PCR to verify that a single DNA species was amplified. The following cycling parameters were used: denaturation at 95 °C for 5 min, followed by 40 amplification cycles denaturing at 95 °C for 10 s, annealing at 60 °C for 30 s. *ACT1* was amplified as the reference gene to calculate fold change for genes of interest and to assure equal loading of the sample. All quantitative PCR experiments were performed in triplicate. Graph pad prism 5.0 was used for further analysis of real-time data.

Microscopy

Vacuolar morphology

FM4-64 (excitation/emission: 488/585–610 nm) dye was used to stain vacuolar membrane of *Saccharomyces cerevisiae* (Vida and Emr 1995). Overnight grown primary culture was diluted into fresh YPD medium and grown up to exponential phase ($O.D_{600nm} = 0.5–0.6$). Harvested cells were suspended in 100 μL SD medium supplemented with FM4-64 (1.6 μM) and incubated at 30 °C for 20 min. Cultures were treated with caffeine and cells were removed and washed at interval of 0 h, 1 h, and 2 h with fresh YPD and resuspended in 25 μL of YNB medium. Cells were placed on poly-L-lysine-coated slides and observed under inverted fluorescence microscope (Nikon eclipse ×60 objective lens) using red filter.

Actin morphology

Actin morphology was visualized by using dye Alexa Fluor 488 phalloidin. Exponential phase ($OD_{600nm} = 0.5–0.6$) cells

were treated with caffeine (20 mM). WT and caffeine-treated cells were taken out after 0 h and 3 h. Cells were harvested, washed with 1X PBS, and fixed with 4% paraformaldehyde for 60 min at 30 °C. After two washes of 1X PBS, cells were resuspended in 100 μL PBS. Glycine (0.1 M) and Triton-100 (0.1%) were used to lyse the cell wall. One microliter of Alexa Fluor 488 phalloidin (excitation/emission: 495/518 nm) was added and incubated for 60 min at room temperature in dark. After one wash of PBS, cells were visualized under Nikon confocal microscope with 100X. All images were analyzed with NIS software 4.0 of microscopy.

Nuclear morphology

Nuclear morphology was envisaged by fluorescence microscopy following DAPI staining. Log phase cells were treated with caffeine (10 mM) for 0 h and 6 h respectively and same log phase cells were treated with H_2O_2 (2.5 mM) for 0 h and 2 h respectively. The cells were stained for 30 min with 5 μg/mL DAPI (excitation/emission (nm): 358/461). The cells were then washed with PBS and stained nuclei were photographed under fluorescence microscope with a 60X objective (Nikon Eclipse microscope).

Propidium iodide staining

Cell permeability and nuclear DNA staining by propidium iodide (PI) staining method were performed as previously described (Ocampo and Barrientos 2011; Zhang et al. 2018). For PI staining, exponential phase cells were treated with caffeine (10 mM) for 4 h and fixed with 4% paraformaldehyde for 30 min at 30 °C. After washing two times with PBS, the same log phase cells (500 μL) were resuspended in PBS. To analyze permeability and viability with PI, these exponential phase cells were incubated with PI (Sigma-Aldrich) for 30 min at 30 °C in the dark. The fluorescence microscope (Nikon Eclipse) was used to observe the cells with 60X objective (excitation 510–70 nm).

Statistical analysis

Averages of data are presented with means ± SD from at least three independent experiments. The p values were calculated using Student's t test, one-way ANOVA. All the mean values of the experiments were analyzed by the post-Tukey test. The significant difference was considered when probability $*p$ between 0.05 and 0.01, $**p \leq 0.01$, and $***p \leq 0.0001$.

Results

lea1Δ exhibits hypersensitivity towards *caffeine*

The mechanism of action of rapamycin and caffeine is significantly different. Inside the cell, rapamycin first complexes with Fkbp12 to specifically bind to FRB domain of Tor kinase and inhibit its activity (Heitman et al. 1991b). However, caffeine directly hits the activity of Tor kinase (Reinke et al. 2004; Kuranda et al. 2006; Wanke et al. 2008). In addition to rapamycin-specific responses, it also affects a diverse array of cellular responses related to cell growth, DNA metabolism, and cell cycle progression, most likely by acting as low-affinity ATP analog (Reinke et al. 2004). Initially to elaborate on TOR signalling in cells lacking *LEA1*, *lea1Δ* strain was exposed to inhibitory concentrations of both rapamycin and caffeine. No any sensitivity of this mutant was observed in rapamycin but a high sensitivity was observed in presence of caffeine in enriched media (YPD) (Fig. 1a). WT cells and *lea1Δ* cells exposed to caffeine were monitored for their growth in 10 mM caffeine for 16 h in YPD and declined survival of *lea1Δ* in caffeine was supported by observed 4-fold reduction in CFU/mL in YPD (Fig. 1b). This sensitivity was also confirmed by dilution spotting of log phase cells in plates supplemented with 10 mM caffeine (Fig. 1c). Similar growth defect was also observed in synthetic dextrose media supplemented with caffeine (5 mM, 8 mM) and rapamycin (5 nM) (Fig. 1d). Up to 8-fold reduction in growth of *lea1*Δ was observed after 16 h of exposure to 8 mM caffeine in defined media (Fig. 1e).

Further decreased growth of *lea1*Δ cells was observed in poor nitrogen conditions. *lea1*Δ showed reduced growth in SLAD and YNB proline (SD-pro.) (Fig. 1f, g). Apparently, lack of Lea1 sensitizes cells to caffeine and nitrogen starvation probably through rapamycin-resistant function of TOR kinase.

lea1Δ cells have altered TOR signalling in caffeine

To investigate the involvement of TOR signalling in *lea1*Δ, we looked at the transcriptional outputs of TOR complexes TORC1 and TORC2 in *lea1*Δ cells treated with caffeine. The TORC1 complex comprises of Tco89, Lst8, Kog1, and either Tor1 or Tor2 and is considered as rapamycin sensitive (Loewith et al. 2002; De Virgilio and Loewith 2006).The TORC2 complex comprises of Tor2, Avo1, Avo2, Avo3, Bit61, and Lst8 and is largely considered as rapamycin insensitive (Jacinto et al. 2004; Wullschleger et al. 2006). In agreement with known inhibitory effects of caffeine on TOR signalling, an induced expression of low-affinity amino acid transporter gene *GAP1*, stress gene *SOD2*, and retrograde response gene *CIT2* was observed in WT strain. Further a reduced expression of ribosome biogenesis gene, *RPS26A*, supported suppression of protein synthesis in cells treated with caffeine (Bonawitz et al. 2007). Intriguingly, *lea1Δ* itself

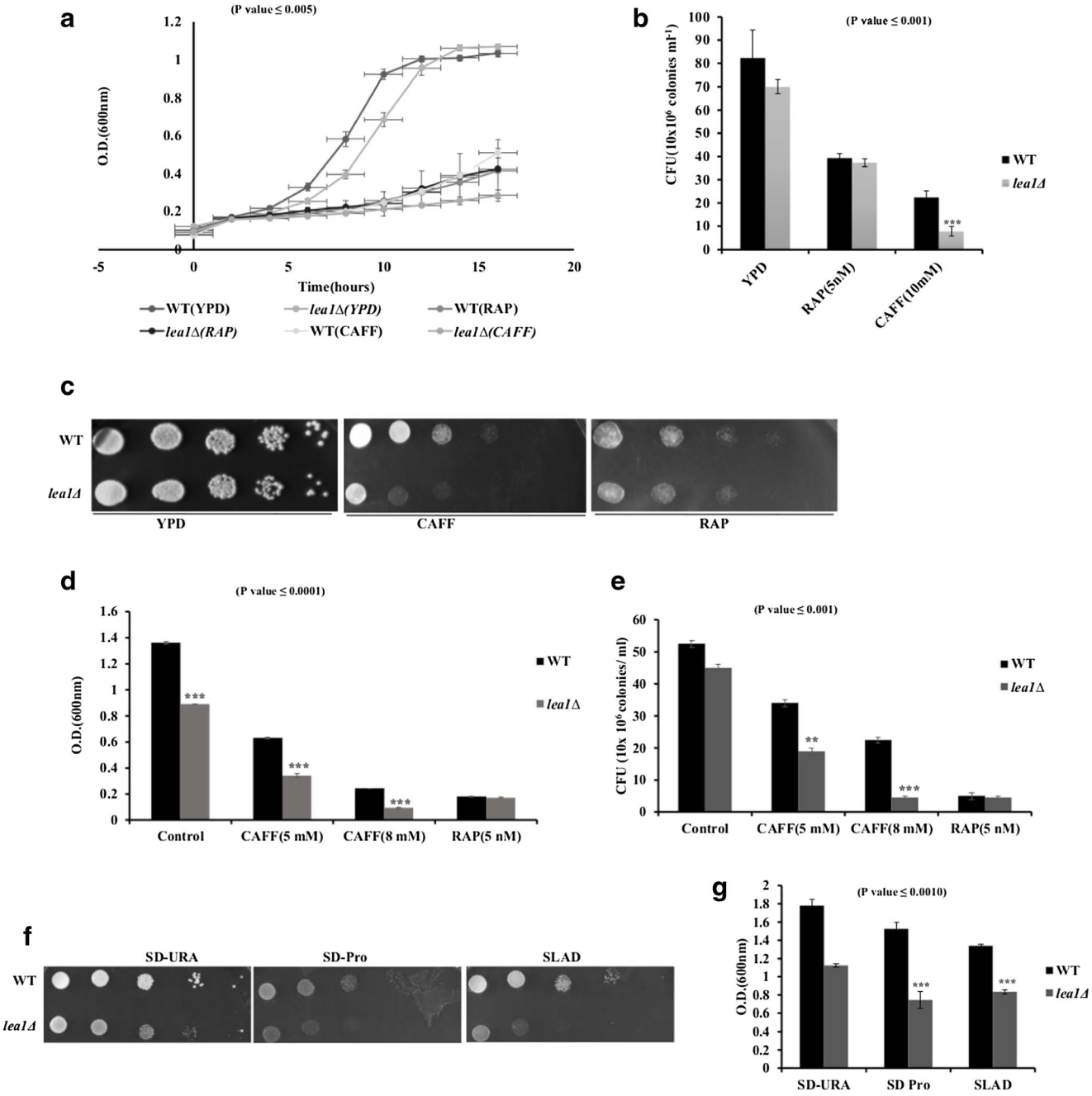

Fig. 1 *lea1Δ* cells are hypersensitive to caffeine. *lea1Δ* cells are sensitive towards caffeine but not to rapamycin in comparison with WT. **a** Growth analysis in YPD, YPD supplemented with caffeine (10 mM), and YPD supplemented with rapamycin (5 nM). **b** Viability of cells exposed to caffeine and rapamycin after 16 h. **c** Dilution spotting of *lea1Δ* and WT strain on YPD alone, YPD plates supplemented with 10 mM caffeine, and YPD plates supplemented with 5 nM rapamycin. **d** Absorbance and **e** viability after 16 h of growth in synthetic dextrose media in caffeine (5 mM and 8 mM) and rapamycin (5 nM). *lea1Δ* exhibits reduced growth in low nitrogen sources. **f** Dilution spotting and **g** absorbance (after 16 h) of WT and *lea1Δ* in synthetic dextrose media without uracil (SD-URA), synthetic low ammonium dextrose (SLAD) media, and synthetic dextrose with proline (SD-pro)

showed activation of stress-induced TORC1-mediated functions (Fig. 2a). While *GAP1*, *SOD2*, and *CIT2* were approximately induced by 2-fold, 2.15-fold, and 1.15-fold, expression of *RPS26A* was reduced by up to 3-fold in comparison to caffeine-treated WT cells. Similar observations were made with TORC2 marker genes. Though caffeine is known to influence TORC1-mediated functions, a highly induced expression of *NCW2*, *DIA1*, and *CMK2* in comparison to WT suggested an affected TORC2 function in caffeine-treated *lea1Δ* cells in our set of conditions (Fig. 2b). All marker genes lack introns except *RPS26A*. Further validation of a highly perturbed TOR signalling as a consequence of removal of

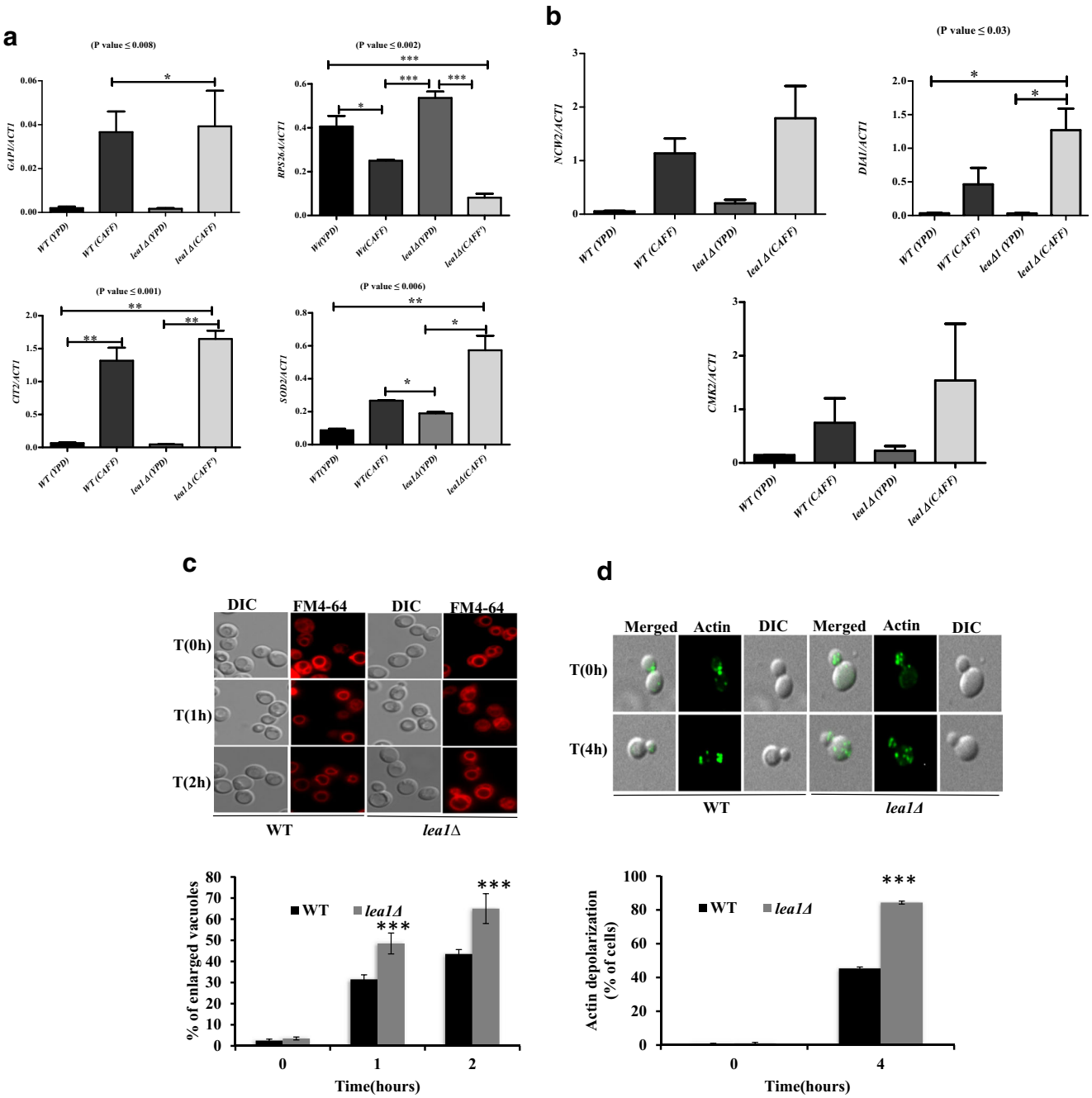

Fig. 2 TOR signalling in *lea1 Δ*. Expression profile of TORC marker genes. **a** Expression of TORC1 marker genes (*GAP1*, *RPS26A*, *SOD2*, and *CIT2*). **b** Expression of TORC2 marker genes (*CMK2*, *DIA1*, and *NCW2*) in WT control and *lea1Δ* cells untreated or treated with 20 mM caffeine for 1 h. **c** Vacuolar morphology by staining with FM4-64 after 1 h and 2 h of growth in presence of caffeine (20 mM). **d** Actin morphology by Alexa Fluor 488 phalloidin after 4 h of growth in presence of caffeine (20 mM), in *lea1Δ* and WT strain

LEA1 was conceded by analyzing vacuolar morphology and actin polarization. For studying the vacuolar morphology, we performed fluorescence microscopy of caffeine-treated and caffeine-untreated cells with lipophytic dye FM4-64 which incorporates into plasma membrane and serves as an endocytic marker that localizes to vacuolar membrane (Vida and Emr 1995; Müller et al. 2007). Around 200 cells were scored after 60′ and 120′ of caffeine treatment. WT and *lea1*Δ cells showed no any significant defect in endocytosis. At T0 h, both WT and *lea1*Δ had 1–3 prominent vacuoles as predominant phenotype. Further, no major difference in vacuolar morphology was observed in WT control cells after 120′ exposure to caffeine. However, in about 60% of caffeine-treated *lea1*Δ cells, vacuoles appeared as single large spheres, as class D *vps* mutants of vesicle trafficking pathway (Fig. 2c). Vacuoles are storehouse of amino acids and large vacuoles

suggest *lea1*Δ are probably more nutrient deprived/stressed compared to WT cells. Next studies were also performed to examine the effect of caffeine on actin morphology of *lea1*Δ strain. In yeast, actin is organized into actin cables and cortical actin patches which are largely accumulated in bud tip to ensure normal daughter cell formation (Martin and Arkowitz 2014). TORC2 is absolute required for maintenance of polarized cell growth in budding yeast (Gaubitz et al. 2015). *lea1*Δ cells treated with caffeine upon staining showed altered actin polarization. Actin was seen scattered on periphery or over whole cell surface in about 80% of cells instead of being clustered in bud region (Fig. 2d). In fact as observed in previous study, an altered chitin distribution was observed in caffeine-treated *lea1* mutant cells (results not shown). Taken together, these results revealed that exposure of *lea1*Δ cells to caffeine results in highly perturbed TOR signalling with impact on both TORC1- and TORC2-mediated functions.

Caffeine sensitivity of *lea1Δ* is largely independent of PKC pathway

Mutants of PKC1 pathway also exhibit sensitivity towards caffeine as *tor1*Δ. However, unlike *tor1*Δ, the sensitivity of these mutants can be rescued by addition of excess sorbitol or adenine. In order to visualize the contribution of PKC pathway in caffeine sensitivity of *lea1*Δ, the growth of *lea1*Δ strain was analyzed in caffeine supplemented with 1 M sorbitol and 20 mM adenine (Fig. 3). WT and *rom2*Δ mutant showed no defect in 5 mM caffeine supplemented with 1 M sorbitol. Whereas *rom2*Δ, GDP/GTP exchange factor, PKC pathway component exhibited improved survival in caffeine in presence of sorbitol and adenine, no significant rescue was observed in growth of *lea1*Δ and *tor1*Δ. These results of *rom2*Δ confirm the previous studies that Rom2p inhibits the Ras/cAMP signalling pathway and activates the Rho1p-Pkc1p signalling pathway (Kuranda et al. 2006). This suggests that caffeine sensitivity of *lea1*Δ is largely independent of PKC pathway.

lea1Δ cells exhibit hypersensitivity to oxidizing agent H_2O_2

*lea1*Δ cells are deficient in splicing, resulting in differential levels of accrual of various RNAs. We randomly selected seven genes with introns and analyzed levels of pretranscripts by amplifying with forward primers from intron and reverse primer from exon region (I/E). Three genes *MUD1*, *VPS29*, and *YSF3* showed substantial increase in levels of pretranscripts in presence of caffeine in *lea1*Δ cells. This suggests that caffeine accrue the intracellular stress in *lea1*Δ through accumulation of unprocessed RNA (data not shown).

Cell exploits elaborate RNA surveillance mechanism to prevent accumulation of aberrant RNAs and ensure integral expression of protein-encoding genes, e.g., increased RNA stability induces reactive oxygen species-dependent apoptosis (Mazzoni et al. 2003). On the basis of prior information and our observation, that strains lacking *LEA1* contains reduced levels of U2SnRNA and they have impaired pre-mRNA splicing that results in accumulation of unprocessed RNA, we envisaged that the persistence of unspliced RNAs in *lea1Δ* cells may sensitize these cells towards oxidizing agent. The increased expression of *SOD2* mentioned above actually supports the enhanced oxidative stress experienced by splicing-deficient *lea1Δ* cells. H_2O_2 is an established oxidative agent and is known to increase ROS production at lower concentrations (Kamada et al. 2005; Pereira et al. 2012). Thus, to investigate the consequences of loss of *LEA1* on oxidative stress tolerance, cell growth was observed in presence of oxidative agent H_2O_2 at 1.5 mM and 2.5 mM concentrations. In

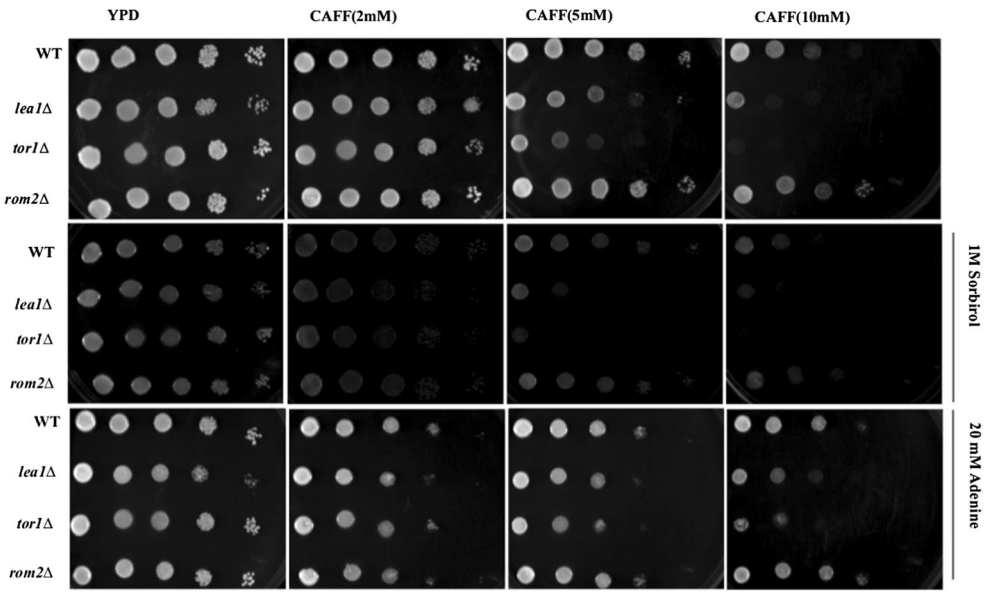

Fig. 3 Effect of addition of sorbitol and adenine on caffeine sensitivity of *lea1*Δ. Serial dilutions of WT, *lea1*Δ, *tor1*Δ, and *rom2*Δ strains were spotted on YPD plates containing 2 mM, 5 mM, and 10 mM caffeine and supplemented with either 1 M sorbitol or 20 mM adenine. Growth was monitored after 2 days at 30 °C

comparison to WT cells, *lea1*Δ cells exhibited hypersensitivity to H_2O_2. Interestingly, *tor1*Δ exhibited insensitivity to H_2O_2 (Fig. 4a). Yeast nuclei exposed to ROS are known to become less compact and appear larger and more diffused. Similar diffused and punctate fragmented nuclei were observed in about 70% of *lea1*Δ cells compared to 50% in control WT population after 2 h of exposure to H_2O_2 (Fig. 4b). Further, no significant nuclear disintegration was observed in *tor1*Δ cells in presence or absence of caffeine. Probably inhibition of TOR1-mediated signalling alone does not lead to oxidative stress sensitizing cells to H_2O_2. Reduced cell growth resulting from increased ROS generated by H_2O_2 can well be prevented by addition of anti-oxidant substances such as dithiothreitol (DTT). As anticipated, the addition of anti-oxidants rescued almost 100% of the cells (Fig. 4c). The mutant cells with higher ROS get quenched by the addition of DTT, L-glutathione-reduced (GSH), and *N*-acetyl cysteine (NAC) which allowed their better survival (Sariki et al. 2016). Further substantiation to our hypothesis on role of oxidative stress in *lea1*Δ growth defect came from combinatorial growth studies of cells exposed to both H_2O_2 and sub-inhibitory concentration of caffeine (Fig. 4d).

Caffeine promotes oxidative stress-induced apoptosis in *lea1*Δ

ROS are key regulators of yeast apoptosis (regulated cell death). Earlier studies have demonstrated the existence of functional apoptotic machinery in unicellular organisms that respond to oxidative stress. Caspase-like protease (Yca1/Mca1) mediates cell death triggered by oxygen stress, salt stress, or chronological aging (Madeo et al. 1997; Farrugia and Balzan 2012). Mca1 is essentially required for apoptosis induced by increased mRNA stability (Mazzoni et al. 2005). Quantitative RT-PCR of *MCA1* in caffeine-treated and caffeine-untreated WT and *lea1*Δ cells unveiled an induced and equivalent expression of *MCA1* in caffeine-treated control and caffeine-untreated *lea1Δ* cells which means that suppression of TOR signalling by caffeine induces oxidative stress to the levels induced in cells lacking *LEA1*. However, the expression of *MCA1* in caffeine-treated *lea1Δ* cells was enhanced (Fig. 5a). An extended support to expression results was obtained by microscopic examination of DAPI-stained DNA of *lea1*Δ cells. Highly fragmented enlarged diffused nuclei indicative of regulated cell death was observed in more than 50% of caffeine-treated *lea1*Δ cells (Fig. 5b). Again, *tor1*Δ did not exhibit any nuclear disintegration suggesting caffeine sensitivity of *tor1*Δ does not result in apoptosis. In support of apoptosis in *lea1*Δ mutant, propidium iodide (PI) staining was also done and % of PI-positive cells was counted after 4-h incubation of caffeine (10 mM). In *lea1*Δ mutant, 70% cells were PI positive as compared to WT where 42% cells were PI positive (Fig. 5c). This result showed that cell death in *lea1*Δ mutant is also contributed by loss of integrity of plasma membrane.

Discussion

The levels of RNA are precisely regulated inside the cell, to safeguard the cell growth. Although role of TOR signalling in cell growth that is regulation is well established, the association of TOR signalling with accumulated unprocessed RNA is known largely through indirect evidences only. Moreover, no direct studies have been performed previously to illustrate role of TOR signalling in growth of cells with defects in RNA splicing.

In the present study, the altered TOR signalling by caffeine in *lea1*Δ clearly demonstrates that cells lacking *LEA1* can sense and respond to environmental conditions. The *lea1*Δ exhibits exacerbated expression of TORC1 marker genes (*GAP1*, *SOD2*, and *CIT2*). This implies role of additional stress generated by deletion of *LEA1* to be responsible for their reduced survival in caffeine compared to wild-type strain. The highly reduced expression of RPG transcripts explains slow growth (splicing deficiency) of *lea1Δ* strain compared to WT strain. *SOD2* is known to have an antioxidant function by catalyzing the disproportionation of superoxide anion to H_2O_2 and is essential for defense against oxygen radicals generated at mitochondrial respiratory chain (Fukai and Ushio-Fukai 2011). Intriguingly, induced expression of *SOD2* gene provided us the initial evidence of the additional oxidative stress faced by splicing deficient *lea1Δ* strain. Noteworthy, recent studies have shown that the role of TORC2 on regulation of actin polarization is mediated through reactive oxygen species (ROS) produced from mitochondrial and non-mitochondrial sources (Niles and Powers 2014). The heightened TORC2 expression along with depolarized actin distribution indicates accumulation of ROS in caffeine-treated *lea1*Δ cells. Thereby, expression of TORC1 and TORC2 marker genes guided our studies towards role of additional oxidative stress experienced by *lea1*Δ.

Lack of *LEA1* remarkably affects accumulation of pre-mRNA. Stabilized RNAs generated in yeast mutants in mRNA decapping pathway such *as lsm1*, *lsm4*, *dcp1*, and *dcp2* trigger apoptosis (Mazzoni et al. 2005; Mazzoni and Falcone 2011; Raju et al. 2015). However, the specific mechanism coordinating RNA stability and apoptosis remains unknown. Further, oxidizing agents, e.g., H_2O_2 and acetic acid at low doses, are also known to induce apoptosis (Liang et al. 2008). No direct link of TOR signalling, accumulated unprocessed RNA, and regulated cell death is known except that yeast protein expression profile generated during acetic acid-induced apoptosis indicates casual involvement of TOR

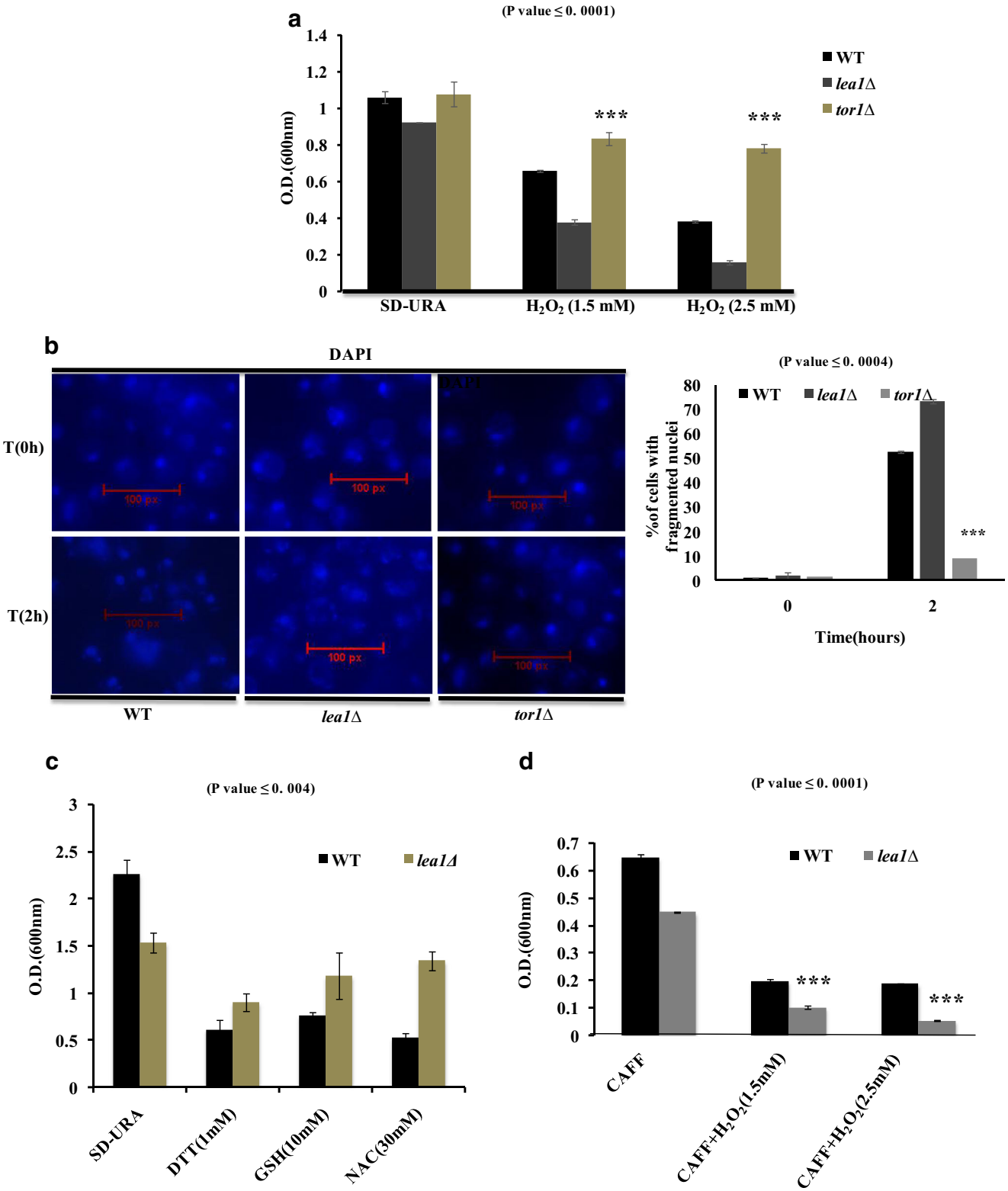

Fig. 4 *lea1Δ* cells are hypersensitive to oxidative stress. **a** Growth curve of WT, *lea1*Δ, and *tor1*Δ in presence of SD-URA and H_2O_2 at 1.5 mM and 2.5 mM concentrations after 16 h. **b** DAPI staining of WT, *lea1*Δ, and *tor1*Δ mutant at 2 mM conc. of H_2O_2 at 0 h and 2 h. Statistical analysis of DAPI-stained cells. **c** Effect of antioxidants 1 mM DTT, 10 mM L-glutathione reduced (GSH), and 30 mM *N*-acetyl cysteine (NAC) on growth of *lea1Δ* in comparison to WT control. **d** Simultaneous exposure to caffeine and H_2O_2 reduces growth of *lea1*Δ

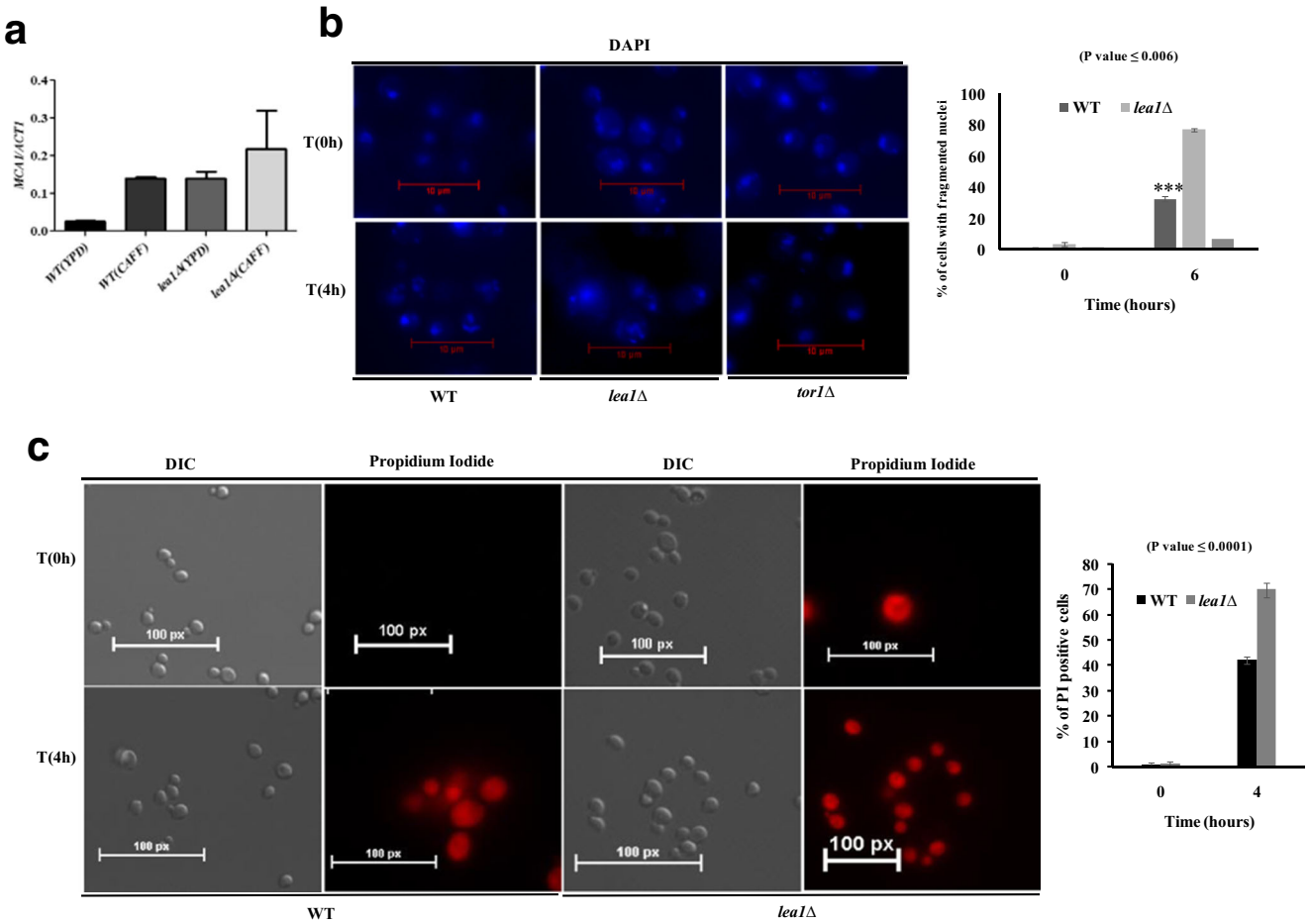

Fig. 5 Caffeine induces apoptosis in *lea1Δ* cells. **a** Induced expression of yeast metacaspase *MCA1*. **b** Nuclear disintegration in WT, *lea1Δ*, and *tor1*Δ after 0-h and 4-h incubation of caffeine. Statistical analysis of cells with fragmented nuclei. **c** Propidium iodide (PI) staining in *lea1*Δ and WT after 4-h incubation of caffeine. Statistical analysis of PI-positive cells in both strains. CAFF caffeine, WT wild type

pathway (Almeida et al. 2009). The hypersensitivity of *lea1*Δ to H_2O_2, the induced expression of *MCA1*, and nuclear fragmentation of *lea1* mutant provided sufficient evidence to conclude that accumulated RNA leads to oxidative stress that subsequently results in caspase-dependent cell death.

The heightened sensitivity of *lea1* mutant cells towards oxidizing agent upon inhibition of TOR signalling via caffeine shows that oxidative stress induced by accumulated RNA is exacerbated in cells with compromised TOR signalling, which ultimately results in reduced growth and survival through induced apoptosis. Interestingly, this mechanism is specifically induced by unprocessed RNA, as *tor1*Δ does not exhibit any nuclear fragmentation suggestive of apoptosis irrespective of presence of caffeine. The positive PI staining in *lea1*Δ cells upon caffeine treatment also shows that caffeine promotes cell death in *lea1*Δ mutant via destroying the integrity of plasma membrane because in dead cells, PI can penetrate the plasma membranes. Our studies demonstrate that role of TOR in RNA processing/splicing may not be limited to direct function of various splicing factors under diverse environmental conditions but also emphasizes on crucial role of TOR signalling in appropriately responding to intracellular stress/s generated by defective splicing. The high conservation of TOR signalling and RNA processing across eukaryotes strongly suggests that mechanistic insights acquired here will be broadly applicable and further investigation can be done to capitulate insights into link of TOR signalling with pre-mRNA processing.

Acknowledgements Initial work contribution of Shubhi Sahni is highly acknowledged. We are highly thankful to Dr. Maria E. Cardenas for providing us *Jk9-3d* strain.

Author contribution This study was designed by PK and RP. Experiments were performed by PK and analyzed along with VN, DK, AKM, and RP. PK and RP wrote the manuscript. PK completed all the figures. All the results and final version of manuscript were reviewed by all the authors.

Funding This work was supported by research grant to RP from SERB, Department of Science and Technology, Govt. of India (grant no. SR/FT/LS-93/2010). Pavan is thankful to SERB for fellowship.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals (if applicable) N/A

Informed consent Yes

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References

Adami A, García-Alvarez B, Arias-Palomo E et al (2007) Structure of TOR and its complex with KOG1. Mol Cell 27:509–516. https://doi.org/10.1016/j.molcel.2007.05.040

Albig AR, Decker CJ (2001) The target of rapamycin signaling pathway regulates mRNA turnover in the yeast Saccharomyces cerevisiae. Mol Biol Cell 12:3428–34383

Almeida B, Ohlmeier S, Almeida AJ et al (2009) Yeast protein expression profile during acetic acid-induced apoptosis indicates causal involvement of the TOR pathway. Proteomics 9:720–732. https://doi.org/10.1002/pmic.200700816

Beck T, Schmidt A, Hall MN (1999) Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. J Cell Biol 146:1227–1238

Bergkessel M, Whitworth GB, Guthrie C (2011) Diverse environmental stresses elicit distinct responses at the level of pre-mRNA processing in yeast. RNA 17:1461–1478. https://doi.org/10.1261/rna.2754011

Bonawitz ND, Chatenay-Lapointe M, Pan Y, Shadel GS (2007) Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. Cell Metab 5:265–277. https://doi.org/10.1016/j.cmet.2007.02.009

Cardenas ME, Cutler NS, Lorenz MC et al (1999) The TOR signaling cascade regulates gene expression in response to nutrients. Genes Dev 13:3271–3279. https://doi.org/10.1101/gad.13.24.3271

Caspary F, Séraphin B (1998) The yeast U2A'/U2B complex is required for pre-spliceosome formation. EMBO J 17:6348–6358. https://doi.org/10.1093/emboj/17.21.6348

Crespo JL, Powers T, Fowler B, Hall MN (2002) The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. Proc Natl Acad Sci U S A 99:6784–6789. https://doi.org/10.1073/pnas.102687599

De Virgilio C, Loewith R (2006) The TOR signalling network from yeast to man. Int J Biochem Cell Biol 38:1476–1481. https://doi.org/10.1016/j.biocel.2006.02.013

Dreumont N, Séraphin B (2013) Rapid screening of yeast mutants with reporters identifies new splicing phenotypes. FEBS J 280:2712–2726. https://doi.org/10.1111/febs.12277

Fadri M, Daquinag A, Wang S et al (2005) The pleckstrin homology domain proteins Slm1 and Slm2 are required for actin cytoskeleton organization in yeast and bind phosphatidylinositol-4,5-bisphosphate and TORC2. Mol Biol Cell 16:1883–1900. https://doi.org/10.1091/mbc.E04-07-0564

Farrugia G, Balzan R (2012) Oxidative stress and programmed cell death in yeast. Front Oncol 2:64. https://doi.org/10.3389/fonc.2012.00064

Fukai T, Ushio-Fukai M (2011) Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxid Redox Signal 15:1583–1606. https://doi.org/10.1089/ars.2011.3999

Gaubitz C, Oliveira T, Prouteau M et al (2015) Molecular basis of the rapamycin insensitivity of target of rapamycin complex 2. Mol Cell 58:977–988. https://doi.org/10.1016/j.molcel.2015.04.031

Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360. https://doi.org/10.1002/yea.320110408

He Y, Li D, Cook SL et al (2013) Mammalian target of rapamycin and Rictor control neutrophil chemotaxis by regulating Rac/Cdc42 activity and the actin cytoskeleton. Mol Biol Cell 24:3369–3380. https://doi.org/10.1091/mbc.E13-07-0405

Heintz C, Doktor TK, Lanjuin A et al (2016) Splicing factor 1 modulates dietary restriction and TORC1 pathway longevity in C. elegans. Nature 541:102–106. https://doi.org/10.1038/nature20789

Heitman J, Movva NR, Hall MN (1991a) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science 253:905–909

Heitman J, Movva NR, Hiestand PC, Hall MN (1991b) FK 506-binding protein proline rotamase is a target for the immunosuppressive agent FK 506 in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 88:1948–1952

Inoki K, Ouyang H, Li Y, Guan K-L (2005) Signaling by target of rapamycin proteins in cell growth control. Microbiol Mol Biol Rev 69:79–100. https://doi.org/10.1128/MMBR.69.1.79-100.2005

Jacinto E, Loewith R, Schmidt A et al (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol 6:1122–1128. https://doi.org/10.1038/ncb1183

Kamada Y, Fujioka Y, Suzuki NN et al (2005) Tor2 directly phosphorylates the AGC kinase Ypk2 to regulate actin polarization. Mol Cell Biol 25:7239–7248. https://doi.org/10.1128/MCB.25.16.7239-7248.2005

Kingsbury JM, Cardenas ME (2016) Vesicular trafficking systems impact TORC1-controlled transcriptional programs in *Saccharomyces cerevisiae*. G3(Bethesda) 6:641–652. https://doi.org/10.1534/g3.115.023911

Kuranda K, Leberre V, Sokol S et al (2006) Investigating the caffeine effects in the yeast Saccharomyces cerevisiae brings new insights into the connection between TOR, PKC and Ras/cAMP signalling pathways. Mol Microbiol 61:1147–1166. https://doi.org/10.1111/j.1365-2958.2006.05300.x

Lempiäinen H, Uotila A, Urban J et al (2009) Sfp1 interaction with TORC1 and Mrs6 reveals feedback regulation on TOR signaling. Mol Cell 33:704–716. https://doi.org/10.1016/j.molcel.2009.01.034

Liang Q, Li W, Zhou B (2008) Caspase-independent apoptosis in yeast. Biochim Biophys Acta Mol Cell Res 1783:1311–1319. https://doi.org/10.1016/j.bbamcr.2008.02.018

Loewith R, Hall MN (2011) Target of rapamycin (TOR) in nutrient signaling and growth control. Genetics 189:1177–1201. https://doi.org/10.1534/genetics.111.133363

Loewith R, Jacinto E, Wullschleger S et al (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell. https://doi.org/10.1016/S1097-2765(02)00636-6

Madeo F, Fröhlich E, Fröhlich KU (1997) A yeast mutant showing diagnostic markers of early and late apoptosis. J Cell Biol 139:729–734. https://doi.org/10.1083/jcb.139.3.729

Martin SG, Arkowitz RA (2014) Cell polarization in budding and fission yeasts. FEMS Microbiol Rev 38:228–253. https://doi.org/10.1111/1574-6976.12055

Mazzoni C, Falcone C (2011) mRNA stability and control of cell proliferation: figure 1. Biochem Soc Trans 39:1461–1465. https://doi.org/10.1042/BST0391461

Mazzoni C, Herker E, Palermo V et al (2005) Yeast caspase 1 links messenger RNA stability to apoptosis in yeast. EMBO Rep 6:1076–1081. https://doi.org/10.1038/sj.embor.7400514

Mazzoni C, Mancini P, Verdone L et al (2003) A truncated form of KlLsm4p and the absence of factors involved in mRNA decapping

trigger apoptosis in yeast. Mol Biol Cell 14:721–729. https://doi.org/10.1091/mbc.E02-05-0258

Morita M, Gravel S-P, Hulea L et al (2015) mTOR coordinates protein synthesis, mitochondrial activity and proliferation. Cell Cycle 14:473–480. https://doi.org/10.4161/15384101.2014.991572

Müller J, Mettbach U, Menzel D, Samaj J (2007) Molecular dissection of endosomal compartments in plants. Plant Physiol 145:293–304. https://doi.org/10.1104/pp.107.102863

Munchel SE, Shultzaberger RK, Takizawa N, Weis K (2011) Dynamic profiling of mRNA turnover reveals gene-specific and system-wide regulation of mRNA decay. Mol Biol Cell 22:2787–2795. https://doi.org/10.1091/mbc.E11-01-0028

Niles BJ, Powers T (2014) TOR complex 2-Ypk1 signaling regulates actin polarization via reactive oxygen species. Mol Biol Cell 25:3962–3972. https://doi.org/10.1091/mbc.E14-06-1122

Ocampo A, Barrientos A (2011) Quick and reliable assessment of chronological life span in yeast cell populations by flow cytometry. Mech Ageing Dev 132:315–323. https://doi.org/10.1016/j.mad.2011.06.007

Pereira C, Bessa C, Saraiva L (2012) Endocytosis inhibition during H_2O_2-induced apoptosis in yeast. FEMS Yeast Res 12:755–760. https://doi.org/10.1111/j.1567-1364.2012.00825.x

Powers T, Walter P (1999) Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in Saccharomyces cerevisiae. Mol Biol Cell 10:987–1000

Puria R, Zurita-Martinez SA, Cardenas ME (2008) Nuclear translocation of Gln3 in response to nutrient signals requires Golgi-to-endosome trafficking in Saccharomyces cerevisiae. Proc Natl Acad Sci 105:7194–7199. https://doi.org/10.1073/pnas.0801087105

Raju KK, Natarajan S, Kumar NS et al (2015) Role of cytoplasmic deadenylation and mRNA decay factors in yeast apoptosis. FEMS Yeast Res 15. https://doi.org/10.1093/femsyr/fou006

Reinke A, Anderson S, McCaffery JM et al (2004) TOR complex 1 includes a novel component, Tco89p (YPL180w), and cooperates with Ssd1p to maintain cellular integrity in Saccharomyces cerevisiae. J Biol Chem 279:14752–14762. https://doi.org/10.1074/jbc.M313062200

Sariki SK, Sahu PK, Golla U et al (2016) Sen1, the homolog of human Senataxin, is critical for cell survival through regulation of redox homeostasis, mitochondrial function, and the TOR pathway in *Saccharomyces cerevisiae*. FEBS J 283:4056–4083. https://doi.org/10.1111/febs.13917

Siebel CW, Feng L, Guthrie C, Fu XD (1999) Conservation in budding yeast of a kinase specific for SR splicing factors. Proc Natl Acad Sci U S A 96:5440–5445

Stanfel MN, Shamieh LS, Kaeberlein M, Kennedy BK (2009) The TOR pathway comes of age. Biochim Biophys Acta Gen Subj 1790:1067–1074. https://doi.org/10.1016/j.bbagen.2009.06.007

Urban J, Soulard A, Huber A et al (2007) Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. Mol Cell 26:663–674. https://doi.org/10.1016/j.molcel.2007.04.020

Vida TA, Emr SD (1995) A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. J Cell Biol 128:779–792

Wanke V, Cameroni E, Uotila A et al (2008) Caffeine extends yeast lifespan by targeting TORC1. Mol Microbiol 69:277–285. https://doi.org/10.1111/j.1365-2958.2008.06292.x

Wullschleger S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. Cell 124:471–484. https://doi.org/10.1016/j.cell.2006.01.016

Zhang N, Fan Y, Li C et al (2018) Cell permeability and nuclear DNA staining by propidium iodide in basidiomycetous yeasts. Appl Microbiol Biotechnol 102:4183–4191. https://doi.org/10.1007/s00253-018-8906-8

Zurita-Martinez SA, Puria R, Pan X et al (2007) Efficient Tor signaling requires a functional class C Vps protein complex in Saccharomyces cerevisiae. Genetics 176:2139–2150. https://doi.org/10.1534/genetics.107.072835