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Calcineurin activation improves cell survival during amino acid starvation in lipid droplet-deficient yeasts

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ABSTRACT

Lipid droplets (LD) are storage sites for neutral lipids that can be used as a source of energy during nutrient starvation, but also function as hubs for fatty acid (FA) trafficking between organelles. In the yeast *Saccharomyces cerevisiae*, the absence of LD causes a severe disorganization of the endomembrane network during starvation. Here we show that cells devoid of LD respond to amino acid (AA) starvation by activating the serine/threonine phosphatase calcineurin and the nuclear translocation of its target protein Crz1. This activation was inhibited by treatments that restore a normal endomembrane organization, *i.e.* inhibition of FA synthesis with cerulenin or deletion of the inhibitory transcription factor Opi1. Activation of calcineurin increased the lifespan of LD-deficient cells during AA starvation. Indeed, deletion of its regulatory or catalytic subunits accelerated cell death. Surprisingly, calcineurin activation appeared to be calcium-independent. An increase in intracellular calcium was observed in LD-deficient cells during AA starvation, but its inhibition by genetic deletion of *MID1* or *YVCI* did not affect calcineurin activity. In contrast, calcineurin activation required the direct regulator of calcineurin Rcn1 and its activating (GSK-3)-related protein kinase Mck1.

1. Introduction

To face variations in nutrient availability, cells can store energy in the form of neutral lipids, in organelles called lipid droplets (LDs). LDs contain a hydrophobic core mainly composed of triacylglycerols (TAG) and steryl esters (STE) surrounded by a monolayer of phospholipids and associated proteins (reviewed in Ref. [1]). These organelles play a key role in the turnover of lipids. Contact sites between LDs and other organelles such as endoplasmic reticulum (ER), mitochondria and peroxisomes control fatty acid (FA) trafficking and metabolism (reviewed in Ref. [2]). The number and size of LDs can vary depending on physiological conditions, as a result of a tight control of the metabolic flux of FAs into TAGs or phospholipids (PLs). Under stress or starvation conditions, cells can store FAs in their LDs. When nutrients are abundant, FAs can be released from LDs and used to initiate PL synthesis for membrane biogenesis [3]. By buffering free FAs, LDs play a role in lowering lipotoxicity. In keeping with their role in FA homeostasis, LDs are involved in a number of diseases affected by lipid imbalances [4].

In *Saccharomyces cerevisiae* a mutant lacking LDs (designed here as «

RS4Δ») has been generated by deleting the genes encoding the four enzymes involved in TAG and STE synthesis: *DGA1*, *LRO1*, *ARE1* and *ARE2* [5]. This yeast mutant is viable, but is highly sensitive to excess unsaturated FAs, in particular oleic acid (OA) [6,7] which causes a severe ER membrane proliferation and mitochondrial necrotic cell death [7,8]. We showed recently that LD-deficient yeasts were unable to undergo autophagy in response to amino acid (AA) starvation [9]. However, the absence of LDs did not have a general impact on the ability of these cells to generate autophagosomes because the RS4Δ mutant was perfectly able to achieve autophagy in response to ammonium starvation or rapamycin treatment [9–11]. Rather, we found that the autophagy impairment during AA starvation was due to a mitochondrial dysfunction [9]. Another set of studies have shown that aberrant respiration and overproduction of ROS by mitochondria could cause the activation of calcineurin (CN) and the inhibition of AA starvation-induced autophagy in yeast [12]. Calcineurin is a calcium calmodulin-dependent serine/threonine phosphatase composed of a catalytic subunit (Cna1 or Cna2 in *Saccharomyces cerevisiae*) and a positive regulatory subunit (Cnb) (reviewed in Ref. [13]). The CNA subunit

Abbreviations: AA, amino acid; CN, calcineurin; ER, endoplasmic reticulum; FA, fatty acids; GFP, green fluorescent protein; GSK-3, glycogen synthase kinase 3; LD, lipid droplet; OA, oleic acid; ROS, reactive oxygen species; STE, steryl esters; TAG, triacylglycerols.

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contains an autoinhibitory domain which is inactivated by interaction with a complex composed of CNB, Ca²⁺ and calmodulin [14]. In yeast, CN is activated in response to various stresses. It dephosphorylates the transcription factor Crz1, which then translocates to the nucleus and activates the transcription of target genes encoding ion pumps, signaling proteins, cell wall biosynthetic enzymes and the regulator of calcineurin Rcn1 [15–17]. In addition to Crz1, CN dephosphorylates a number of other targets including its own regulator Rcn1 [18,19].

In the present study, we show that CN activity is significantly increased in LD-deficient yeasts submitted to amino acid starvation. This activation appeared to occur as a result of the perturbed lipid metabolism and the major ER disorganization that has previously been described in these cells during AA starvation, but it was not the cause of their inability to undergo autophagy. On the contrary, CN appeared to have a positive effect on RS4Δ cells because it improved their survival in AA starvation conditions. Unexpectedly CN activation did not require changes in intracellular Ca²⁺ levels, but it relied mainly on the (GSK-3)-related protein kinase Mck1 and the regulator of calcineurin Rcn1.

2. Materials and methods

2.1. Yeast strains, oligonucleotides, plasmids and growth conditions

All yeast strains described in this study are derived from BY (Euroscarf). BY4742 was used as the wild-type strain. RS4Δ (BY4742 derivative; *MATa are1 are2 lro1 dga1 ura3 trp1 leu2 lys2 his3 met15*) was kindly provided by Dr. Schneider.

Deletion of *CNA1*, *CNA2*, *CNB1*, *CRZ1*, *INO1*, *MCK1*, *MID1*, *OPI1*, *RCN1* and *YVC1*, were performed by PCR-based targeted homologous recombination, replacing the entire ORF with the *URA3* cassette [20]. The oligonucleotides used for these deletions are listed in Table 1. PCR-mediated GFP tagging of Opi1 was performed as described with pYM28 [21].

Plasmid pMR346 containing the *DGA1* gene was isolated from a Yep13 genomic library kindly provided by Dr. Schneider. Plasmid pKK249 expressing GFP-Crz1 [22] was kindly provided by Dr. Martha Cyert. Plasmid pEVP11-AEQ expressing APOAEQUORIN was kindly provided by Dr. Patrick Masson [23]. The two CEN plasmids, pTJK512 expressing *CNB1* (CN regulatory subunit) under control of its own promoter [24] and plasmid pTJK517 expressing *CNA1* (CN catalytic subunit) under control of its own promoter [24] were purchased from Addgene.

Yeast strains were cultured in minimal YNB medium (yeast nitrogen base with ammonium sulfate and 2 % glucose) supplemented with the

appropriate Dropout mix (Takara), or complete YPD medium (1 % yeast extract, 1 % peptone, 2 % dextrose). For amino acid starvation experiments, cultures in selective medium were inoculated in YPD medium and maintained in exponential growth phase for at least two generations; cells were harvested by centrifugation, washed with distilled water and inoculated in YNB medium without amino acid. When indicated, the calcineurin inhibitor FK506 (Santa-Cruz, sc-24649) or the fatty acid synthase inhibitor cerulenin (Santa-Cruz, sc-200827) were solubilized in DMSO and added to the culture medium at a final concentration of 3 μM or 18 μM respectively. Oleic acid (Santa-Cruz, sc-200797) was diluted in DMSO and used at a final concentration of 0.001 % (v/v).

2.2. Fluorescence microscopy

Cells expressing GFP-tagged proteins (Crz1 or Opi1) were fixed for 15 min at 4 °C in 3.7 % formaldehyde and washed twice with PBS before the microscopic observations. In Suppl. Fig. 1, cells expressing GFP-Crz1 were fixed and stained for 20 min at room temperature with 2 μg/ml DAPI (Sigma).

Microscopy was performed on a FV3000 Olympus confocal microscope using a 60X oil immersion lens (NA 1.42), for GFP-Crz1, or a 100x oil immersion lens (NA 1.5), for Opi1-GFP and for GFP-Crz1+DAPI in Suppl. Fig. 1., both coupled to a 2.0X numerical zoom (0.13 μm per pixel for 60X, 0.06 μm per pixel for 100X). The excitation wavelength was set to 488 nm for GFP and 405 nm for DAPI.

For GFP-Crz1, the percentage of cells with a nuclear labelling was estimated with the cell counter tool on the Image J software, in at least 5 random microscopic fields in one optical section, with 50–200 cells per field for each condition. The values in the micrographs (Figs. 1, 2, 4 and 6) are from one representative experiment and indicate the percentage of cells with nuclear GFP-Crz1 labelling (mean ± standard deviation). All the experiments have been repeated at least two times.

2.3. RNA extraction, reverse transcription (RT) and real-time PCR

RNA extraction and reverse transcription were performed as previously described [9]. For each sample, a control reaction was run without reverse transcriptase to estimate the amount of contaminating DNA. Real-time qPCR was performed in a LightCycler® apparatus and SYBR Green mix (Roche Applied Science, Meylan, France), with the primers listed in Table 2. Each primer pair gave a single PCR product by melting curve analysis and agarose gel electrophoresis. The PCR steps were: enzyme activation at 95 °C for 10min followed by 40 cycles (for *ACT1*)

Table 1

Oligonucleotides used for gene knock-out (5' to 3' orientation; sequences from SGD= Saccharomyces Genome Database).

Name	Sequence	SGD reference
CNA1-KO Forward	TCTTTTTTTTTGACGTATTAGCTCAGCTGCCATAAAACACTCTCAACGCCACAGCTGAAGCTTCGTACGC	S000004425
CNA1-KO Reverse	AAGAATTTCGATGTTGCATCTCATAGGAATAAAAAAGAATAAAATAGAGATTAGCATAGGCCACTAGTGGATCTG	S000004425
CNA2-KO Forward	CCTTCCCTCCCATAGAGTACACAGGAGCCAGTACTTCTTCTTGAACCCGCACAGCTGAAGCTTCGTACGC	S000004521
CNA2-KO Reverse	AATGCAACTTACTTACTTATTGAAGTATGTACAGTGGAATAGGAGCTTCTGCATAGGCCACTAGTGGATCTG	S000004521
CNB1-KO Forward	CTTGGAACCTCAATGGTATCAGAATCCATAGAAGCATTTTTATTCTTAAACAGCTGAAGCTTCGTACGC	S000001673
CNB1-KO Reverse	TCTTTTCTTAAAAATATGGCATACCATAAATGAATGAAGTGTCCCCTAGTCGCATAGGCCACTAGTGGATCTG	S000001673
CRZ1-KO Forward	TAGGTTGTATTTTTAGTCTCCGATTTGGAAGTTTCGTCCAGACAGTACAAGGAAGCAGCTGAAGCTTCGTACGC	S000004972
CRZ1-KO Reverse	TATTATATAGAAAAAAATTCCTATTCAAAGCTTAAAAAACAAAAATAAGCATAGGCCACTAGTGGATCTG	S000004972
INO1-KO Forward	CTTGATTTATTCTGTTTCATTCCTTTTTTCCAGTGAAGAAAGAAAGTAAACAGCTGAAGCTTCGTACGC	S000003689
INO1-KO Reverse	TGTTTTTTTTATAGGTAGGCGGAAAAAGAAAAAGAGAGTCGTTGAAATGAGAGCATAGGCCACTAGTGGATCTG	S000003689
MCK1-KO Forward	TCAAATTTCTTTTTATTTCGAAACCCCACTCCATCACATTCTAGTACATCAGCTGAAGCTTCGTACGC	S000005251
MCK1-KO Reverse	TTTTGTTCATTAATTTTCCGAGGGGAAAGAGAAACAAATTAATAGAAAATAGCATAGGCCACTAGTGGATCTG	S000005251
MID1-KO Forward	GTAAAACCCGTAACACTACAATTTCTGTCCAGCGGCAGTATTATTTCTAAGGCAGCTGAAGCTTCGTACGC	S000005235
MID1-KO Reverse	ATGTACTTATGCATTTAAATCGTTCAATTTTTTTTGTGATAGTTCCGGTCTCTTGCATAGGCCACTAGTGGATCTG	S000005235
OPI1-KO Forward	GATTAAGCGTGTGTATCAGGACAGTGTTTTTAACGAAGATACTAGTCAATTCAGCTGAAGCTTCGTACGC	S000001012
OPI1-KO Reverse	GTATAATATTATTACTGGTGAATGCATGAAAGACCTCAATCTGCTCCGGCATAGGCCACTAGTGGATCTG	S000001012
RCN1-KO Forward	GCCAAAAGATCAAGCAATAAACCAACCGATATATAAACACAGAAGTGCAGCAGCTGAAGCTTCGTACGC	S000001642
RCN1-KO Reverse	CAACCACCGTAAGCATTTAAGTCTCTTAAGCCAAACAAATCGCCCTCGCCATCGCATAGGCCACTAGTGGATCTG	S000001642
YVC1-KO Forward	TAAGGCAATAGAATTCAGTTATAAAATATAATATTACTAGAACAGGAGCATTAGCTGAAGCTTCGTACGC	S000005613
YVC1-KO Reverse	ATATATATGGATTTCTGAGAAATTAATTAAGCAGTATTTGAACACATGCTGCATAGGCCACTAGTGGATCTG	S000005613

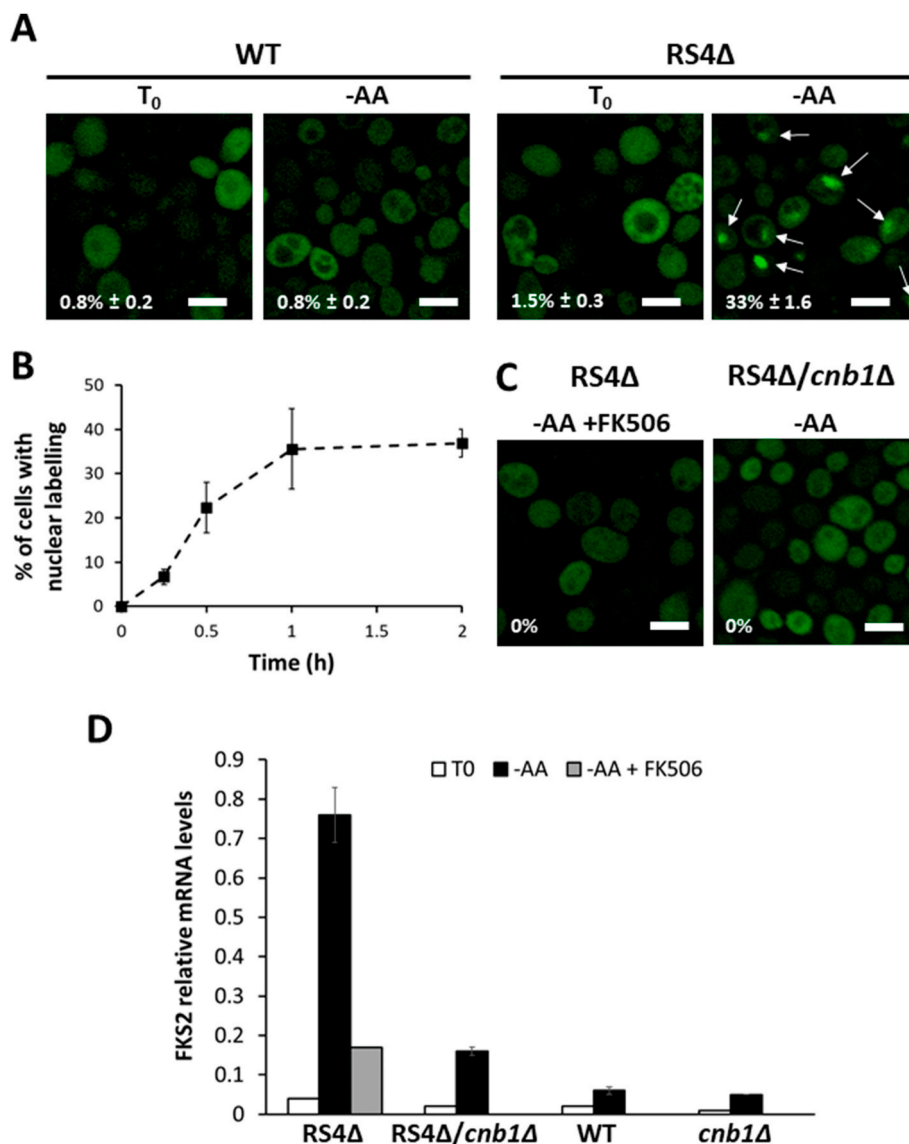


Fig. 1. Crz1 is activated in response to amino acid starvation in lipid droplet-deficient cells. Wild-type (WT) or lipid droplet-deficient cells (RS4Δ) expressing a GFP-Crz1 fusion protein were grown to log-phase (T₀) and switched for 2h in a medium without amino acids (-AA). **A.** The GFP-Crz1 fusion protein was observed by fluorescence microscopy. Arrows indicate nuclear accumulation of GFP-Crz1. **B.** Kinetics of GFP-Crz1 nuclear accumulation during AA starvation. In **C,** AA starvation was performed in the presence of the calcineurin inhibitor FK506 (3 μM) or in a RS4Δ strain with a genetic deletion of *CNB1*. Magnification bar in **A** and **C:** 5 μm. **D.** *FKS2* mRNA levels were measured by real-time RT-qPCR in various strains. Values were normalized to *ACT1* mRNA levels and are expressed as mean ± SD.

or 45 cycles (for *FKS2*, *INO1* and *KAR2*) comprising 10s at 95 °C, 10s at 60 °C (*ACT1*) or 66 °C (*FKS2* and *KAR2*) or 63 °C (*INO1*), and 10s (*ACT1*) or 20s (*FKS2*, *INO1* and *KAR2*) at 72 °C. Each of the PCR products were gel-purified, sequenced and used to establish standard curves with known quantities of each template. We verified that all the PCR sigmoids had a linear distribution ($r \geq 0.99$) with an efficiency of 1.92–1.97. Reverse transcripts (RT) copy numbers were calculated from the standard curves. PCR reactions on non reverse-transcribed RNA samples were used to calculate the copy numbers of contaminant genomic DNA in each sample (always less than 1/100) and these were subtracted from the calculated RT copy numbers. *ACT1* was used as the reference gene to normalize the results. The values indicated in the graphs (Figs. 1 and 4, Tables 3 and 4) are from one representative experiment performed in duplicate (mean ± standard deviation). All the experiments have been repeated at least two times.

2.4. Trypan Blue viability assay

Cells submitted to AA starvation were harvested at intervals and stained for 4 min in an equal volume of 0.4 % Trypan Blue solution (Sigma, T8154). After a brief centrifugation the cells were washed once in PBS and were immediately observed under bright-field microscopy on a Nikon Eclipse Ts2R microscope, using a 100X oil immersion lens. Photographs were taken in at least 10 random microscopic fields (DS-Fi3 Camera coupled to the NIS-Elements Advanced Research software) and the number of blue (dead) and white (alive) cells was counted with the cell counter tool of the Image J software. The experiment was repeated three times with similar results.

2.5. Measurements of cytosolic calcium

To monitor changes in intracellular calcium levels in response to

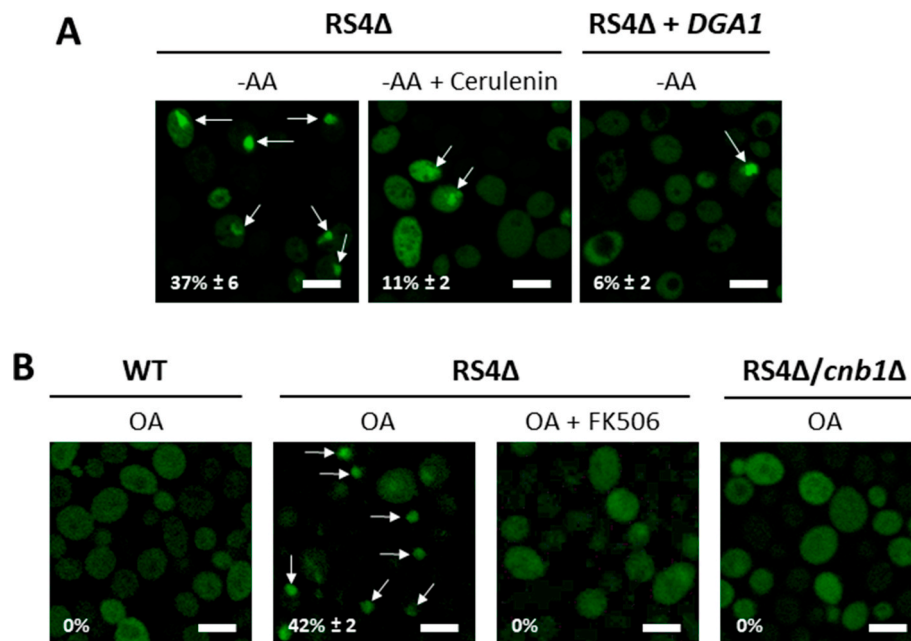


Fig. 2. Calcineurin activation is linked to the absence of lipid droplets and the dysregulation of lipid metabolism. Cells expressing a GFP-Crz1 fusion protein were grown to log-phase in complete medium and were either transferred for 2h in a medium without amino acids (-AA, panel **A**) or kept in the complete medium for 1h with 0.001 % oleic acid (OA, panel **B**). **A:** when indicated, AA starvation was performed in the presence of 4 μg/ml (18 μM) cerulenin or in RS4Δ re-expressing *DGA1*. **B:** when indicated, OA treatment was performed in the presence of 3 μM FK506 or in RS4Δ cells lacking *Cnb1*. **A, B:** The GFP-Crz1 fusion protein was observed by fluorescence microscopy. The percentage of cells with a nuclear labelling (arrows) is indicated (mean ± SD). Magnification bar in **A** and **B:** 5 μm.

Table 2

Oligonucleotides used for qPCR (5' to 3' orientation; sequences from SGD= Saccharomyces Genome Database).

Name	Sequence	SGD reference
ACT1 Forward	CCGGTGATGGTGTACTC	S000001855
ACT1 Reverse	CAAATCTCTACCGCCAAAT	S000001855
FKS2 Forward	GTCGAGGTCTCGTGTACTGG	S000003264
FKS2 Reverse	GCAAATGATGATACGTAAGGTGG	S000003264
INO1 Forward	AAAGGCAACGTAACCACGAGG	S000003689
INO1 Reverse	ACACCTCCAAGATAGATGCTG	S000003689
KAR2 Forward	GGATATCAAGCACTTGCCATTTAA	S000003689
KAR2 Reverse	ACAGTAACGACAGCATGGTA	S000003689

amino acid starvation, cells transformed with the pEVP11-AEQ plasmid were grown to log phase in selective medium lacking leucine and 200 μl aliquots were incubated for 20 min at room temperature in darkness

Table 3

INO1 gene expression measured by real time RT-qPCR, in control RS4Δ or RS4Δ/*opi1Δ* cells (Ctrl) or in cells submitted to AA starvation or treatment with 0.001 % OA. Values indicate the levels of INO1 mRNA normalized to ACT1 mRNA levels. Values in brackets indicate the mean fold-increase compared to control levels. The AA starvation and OA experiments were performed on different days and are presented separately due to variations of basal levels.

Strain	Ctrl	2h -AA	Ctrl	1h OA
RS4Δ	0.0005 ± 0.0001 (1)	0.10 ± 0.03 (204)	0.002 ± 0.000 (1)	0.71 ± 0.01 (356)
RS4Δ/ <i>opi1Δ</i>	0.47 ± 0.00 (942)	0.41 ± 0.06 (826)	1.79 ± 0.66 (895)	2.70 ± 0.08 (1353)

Table 4

KAR2 gene expression was measured by real time RT-qPCR, before (T₀) or after 6h of AA starvation (6h-AA). The strains used were: RS4Δ, RS4Δ lacking the CNB1 gene (RS4Δ/*cnb1Δ*), RS4Δ lacking the CNA1 and CNA2 genes (RS4Δ/*cna1Δ/cna2Δ*), RS4Δ lacking the CNB1 gene but transformed with a plasmid expressing CNB1 (RS4Δ/*cnb1Δ* + CNB1) and RS4Δ lacking the CNA1 and CNA2 genes but transformed with a plasmid expressing CNA1 (RS4Δ/*cna1Δ/cna2Δ* + CNA1). Values indicate the levels of KAR2 mRNA normalized to ACT1 mRNA levels.

	4Δ	4Δ/ <i>cnb1Δ</i>	4Δ/ <i>cnb1Δ</i> + CNB1	4Δ/ <i>cna1Δ/cna2Δ</i>	4Δ/ <i>cna1Δ/cna2Δ</i> + CNA1
T ₀	0.19 ± 0.02	0.18 ± 0.04	0.12 ± 0.04	0.27 ± 0.06	0.15 ± 0.04
6h-AA	0.23 ± 0.01	0.28 ± 0.08	0.09 ± 0.01	0.24 ± 0.01	0.15 ± 0.01

3. Results

3.1. Calcineurin is activated by amino acid starvation in LD-deficient cells

We monitored CN activity by studying the subcellular localization of a GFP-Crz1 fusion protein, by fluorescence microscopy. The transcription factor Crz1 is known to be cytosolic in control conditions, but becomes nuclear after dephosphorylation by CN [16]. When cells were submitted to amino acid starvation (AA starvation), a nuclear translocation of GFP-Crz1 was observed in $\sim 1/3$ of lipid droplet-deficient RS4 Δ cells (mean \pm S.E.M.: 33.5 % \pm 1.6 % in 19 independent experiments), but not in wild-type cells (WT) (Fig. 1A and Suppl. Fig. 1). Contrary to the nuclear translocation of Crz1 in response to high extracellular Ca²⁺, which was rapid but transient in wild-type cells as well as in RS4 Δ cells ([16] and Suppl. Fig. 2), the nuclear accumulation of Crz1 in response to AA starvation was rather slow and steady (Fig. 1B). This activation of Crz1 was completely blocked by the calcineurin-specific inhibitor FK506, or by genetic deletion of either the regulatory subunit Cnb1 (Fig. 1C) or the catalytic subunits Cna1 and Cna2 (Suppl. Fig. 3). Re-expression of the missing subunits (Cnb1 or Cna1) in the respective deleted strains allowed to restore the starvation-induced Crz1 activation (Suppl. Fig. 3). To determine whether the endogenous Crz1 protein was activated in the same way as the plasmid-expressed GFP-Crz1, we measured the expression of *FKS2*, a well-known Crz1 target gene, by RT-qPCR (Fig. 1D). We found that *FKS2* mRNA was indeed increased by AA starvation in the RS4 Δ mutant and this rise was inhibited by FK506 and *CNB1* deletion. A very modest increase in *FKS2* mRNA was observed in wild-type cells as well as in *cnb1* Δ cells. Together, these results indicate that AA starvation selectively activates CN in lipid droplet-deficient cells.

3.2. Calcineurin activation is caused by a dysregulation of lipid metabolism and correlates with a disorganization of the endomembrane system

As demonstrated in previous studies, RS4 Δ cells placed under starvation conditions exhibit increased FA synthesis and alterations in PL composition [10,11,25]. This, combined with their inability to store FA in LDs, causes a major perturbation of the ER and mitochondrial membrane network, which participates to the inhibition of autophagy. Here we show that limiting FA synthesis with a low dose of cerulenin or restoring lipid droplets by re-expressing diacylglycerol acyltransferase (*Dga1*) dramatically reduced the nuclear translocation of Crz1 (Fig. 2A).

On the opposite, incubating RS4 Δ cells with 0.001 % oleic acid (OA) caused an activation of Crz1 similar to the one obtained with AA starvation (Fig. 2B and Suppl. Fig. 1). This OA-induced Crz1 activation was abolished by FK506 or *CNB1* deletion, and was not observed in wild-type cells (Fig. 2B). This demonstrated that CN activation was directly related to the absence of lipid droplets and to the dysregulation of lipid metabolism.

Alterations in PL content have been reported in the RS4 Δ mutant such as a decrease in phosphatidic acid (PA) and an increase in phosphatidylinositol (PI) and diacylglycerol (DAG) [11,25]. Phospholipid synthesis is negatively controlled at transcriptional level by the inhibitory transcription factor Opi1. When PA levels are high, Opi1 is excluded from the nucleus and retained on ER membranes [26]. This causes an increase in the transcription of several genes encoding enzymes involved in PL synthesis, in particular the *INO1* gene which encodes the inositol synthesizing-enzyme Ino1 [26–28]. Here we found that Opi1 epitope-tagged with GFP was located in the nucleus both in wild-type and in RS4 Δ cells grown to log phase in complete medium (Fig. 3). After 1h of AA starvation Opi1 remained nuclear in wild-type cells but became clearly extra-nuclear in the RS4 Δ mutant. In these cells the Opi1 labelling was either associated with the nuclear ER (perinuclear labelling) or appeared in the form of intense spots spread in the cytoplasm. These spots are reminiscent of the tangled ER membranes that have previously been described in the starved RS4 Δ cells [10,11,25]. This suggests that Opi1 could be associated with these disorganized ER membranes. When RS4 Δ cells were treated with cerulenin during AA starvation, the spots were rarely observed and Opi1 remained mostly nuclear (Fig. 3). Since cerulenin decreases FA synthesis, it may have lowered PA levels, thereby increasing the chances of Opi1 to stay in the nucleus. In RS4 Δ cells cultured with OA, Opi1 localization had the same pattern as in starved cells, but with a stronger intensity of the spots and of the perinuclear labelling (Fig. 3). In contrast, Opi1 remained nuclear in OA-treated wild-type cells. In correlation with the subcellular localization of Opi1 in RS4 Δ cells, *INO1* mRNA levels increased ~ 200 -fold after AA starvation and up to 600-fold in response to OA, while it increased only ~ 10 -fold in cells treated with cerulenin during AA starvation (Fig. 4A). We did not observe any strong variations of *INO1* mRNA in wild-type cells grown in the same conditions (Fig. 4B). Although AA starvation caused a major rise in *INO1* expression that correlated with the exclusion of Opi1 from the nucleus, the genetic deletion of *OPI1* in RS4 Δ cells caused a further 4-fold increase in *INO1* mRNA in response to both AA starvation and OA treatment (Table 3).

The massive rise in *INO1* expression was not the cause of CN

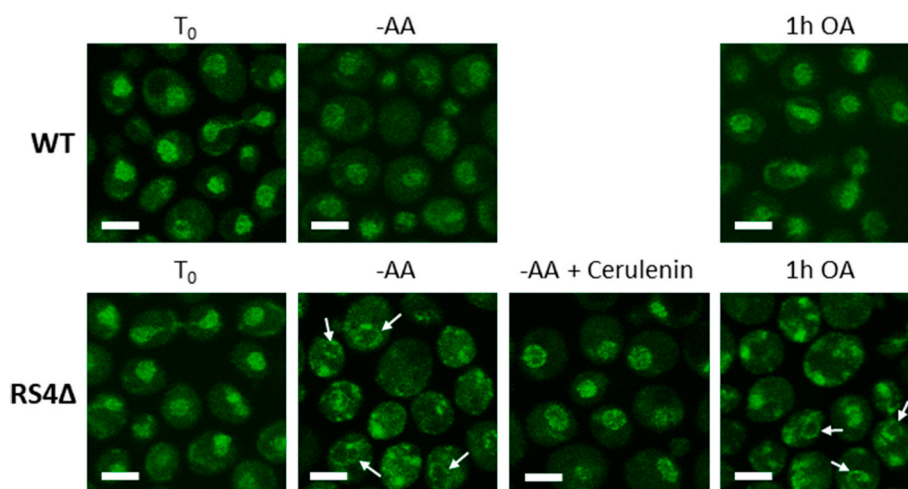


Fig. 3. Opi1 is excluded from the nucleus in lipid droplet-deficient cells submitted to amino acid starvation. Wild-type (WT) or lipid droplet-deficient cells (RS4 Δ) containing an integrated copy of the *OPI1* transcription factor tagged with GFP were grown to log-phase in complete medium (T₀) and switched for 1h in a medium without amino acids (-AA) or kept in the complete medium for 1h with 0.001 % oleic acid (OA). When indicated, AA starvation was performed in the presence of 4 μ g/ml (18 μ M) cerulenin. The Opi1-GFP fusion protein was observed by fluorescence microscopy. Arrows indicate the perinuclear labelling. Magnification Bar: 5 μ m.

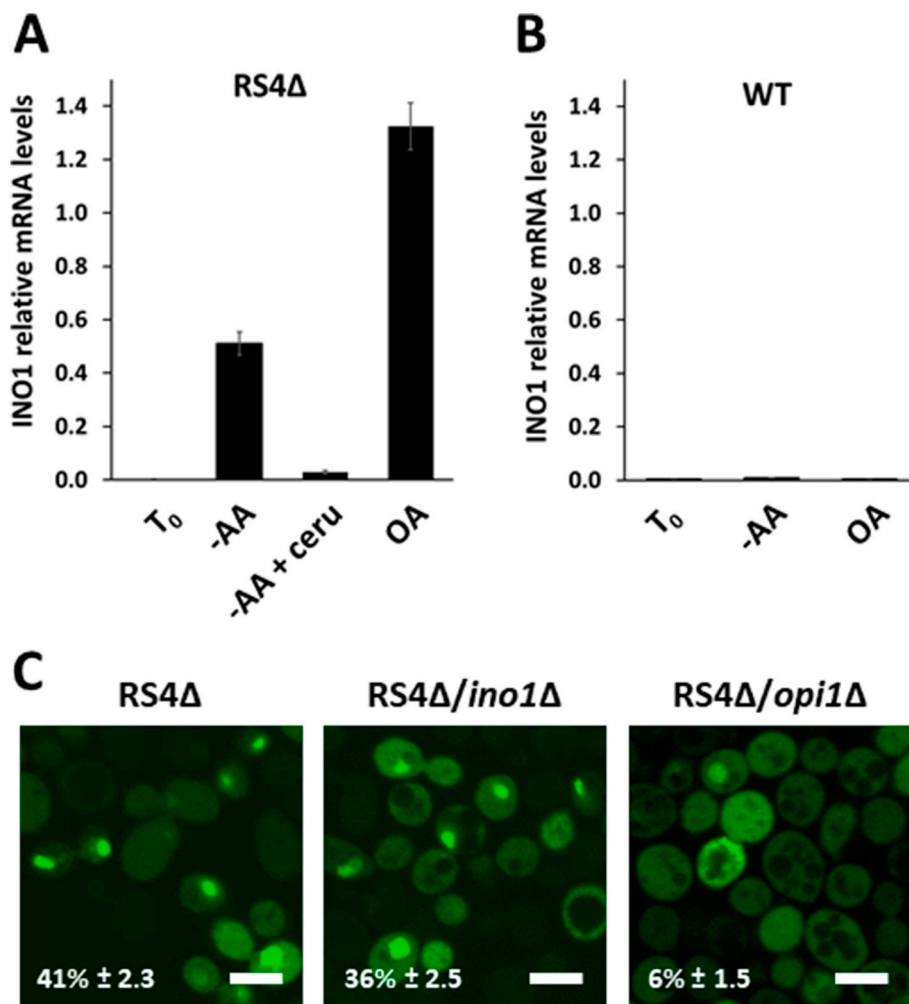


Fig. 4. *INO1* expression is strongly induced by amino acid starvation in lipid droplet-deficient cells, but is not responsible for calcineurin/Crz1 activation. **A, B:** *INO1* mRNA levels were measured by quantitative RT-PCR and normalized to *ACT1* mRNA levels. Lipid droplet-deficient cells (RS4Δ) or wild-type cells (WT) grown to log-phase in complete medium (T₀) were transferred in a medium without amino acids (-AA, 2h, plus or minus 4 μg/ml cerulenin) or kept in complete medium with 0.001 % oleic acid (OA, 1h). **C:** RS4Δ cells (native or lacking the *INO1* or the *OPI1* gene) expressing a GFP-Crz1 fusion protein were grown to log-phase in complete medium and transferred for 2h in a medium without amino acids. The GFP-Crz1 fusion protein was observed by fluorescence microscopy. Magnification bar: 5 μm. Values indicated in the micrographs represent the percentage of cells with a nuclear labelling (mean ± SD).

activation, because the genetic deletion of *INO1* in the RS4Δ mutant did not interfere with the nuclear translocation of GFP-Crz1 in response to AA-starvation (Fig. 4C). On the contrary, CN/Crz1 activation was inhibited by the deletion of *OPI1*, which exacerbated *INO1* over-expression (Fig. 4C).

Together, these results indicate that CN activation in RS4Δ cells occurred in experimental conditions that caused the accumulation of free FAs and the formation of tangled ER membranes, *i.e.* starvation or incubation with external FA [6,7,10,11,25]. On the opposite, CN activation was prevented in situations that allowed to limit free FA accumulation and to maintain a normal ER organization during starvation, *i.e.* partial inhibition of FA synthesis with cerulenin or increase in PL synthesis by *OPI1* deletion [10,11,25].

3.3. Calcineurin activation does not preclude autophagy but improves the survival of lipid droplet-deficient cells during amino acid starvation

To investigate the possible implication of CN in the autophagy blockade, previously observed in these cells [9], we monitored the cleavage of a GFP-Atg8 fusion protein by western blot. We found that the RS4Δ/*cnb1Δ* cells behaved exactly like the RS4Δ mutant, *i.e.* they were able to perform autophagy in response to ammonium starvation, but not

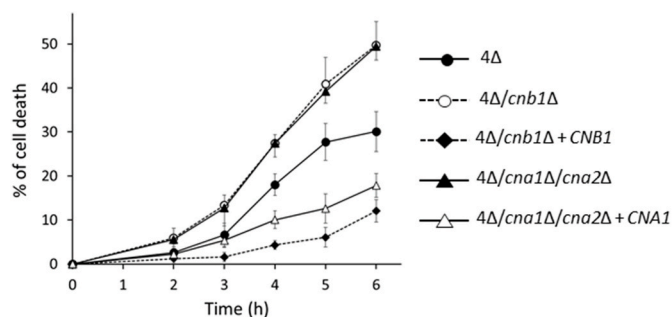


Fig. 5. Calcineurin activation improves LD-deficient cell survival during AA starvation. Yeast cells were grown to log-phase in complete medium, switched to a medium without amino acids and stained with Trypan Blue at different time points. The yeast strains used were: RS4Δ/*cnb1Δ* (RS4Δ cells lacking the *CNB1* gene encoding the regulatory subunit of CN); RS4Δ/*cnb1Δ* + *CNB1* (RS4Δ/*cnb1Δ* cells transfected with a plasmid expressing *CNB1*); RS4Δ/*cna1Δ*/*cna2Δ* (RS4Δ cells lacking both the *CNA1* and the *CNA2* genes encoding the two alternative catalytic subunits of CN); RS4Δ/*cna1Δ*/*cna2Δ* + *CNA1* (RS4Δ/*cna1Δ*/*cna2Δ* cells transfected with a plasmid expressing *CNA1*). Values indicate the percentage of dead (=blue) cells (mean ± SD).

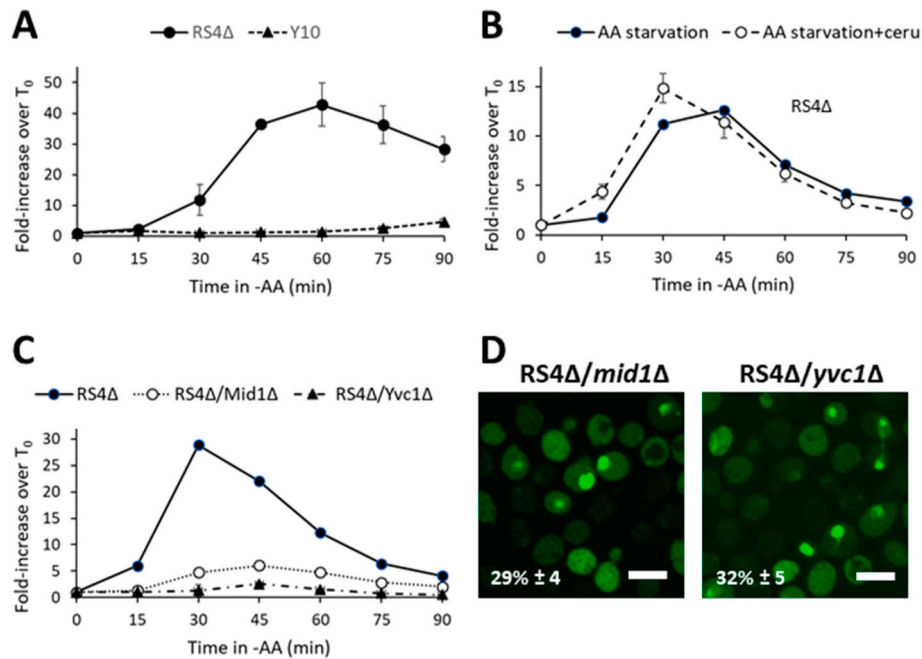


Fig. 6. Calcineurin/Crz1 activation does not rely on a calcium rise. **A, B, C:** Yeasts were grown to log-phase in complete medium and switched to a medium without amino acids (AA starvation). When indicated, AA starvation was performed in the presence of 4 $\mu\text{g/ml}$ cerulenin. Four different strains were used: wild-type (WT), RS4 Δ cells or RS4 Δ lacking the *MID1* or the *YVC1* gene. Intracellular Ca^{2+} levels were measured at different time points (see methods for details). Values are expressed as fold-increase over the T_0 Ca^{2+} levels. **D:** RS4 Δ cells lacking *MID1* or *YVC1* and expressing a GFP-Crz1 fusion protein were grown to log-phase in complete medium and transferred for 2h in a medium without AA. The GFP-Crz1 fusion protein was observed by fluorescence microscopy (magnification bar: 5 μm). The percentage of cells with a nuclear labelling is indicated in each micrograph.

in response to AA starvation (Suppl. Fig. 4). The same results were obtained after deleting the *CRZ1* gene, indicating that neither CN nor Crz1 were responsible for the inability of lipid droplet-deficient yeasts to perform autophagy.

Previous studies reported that CN was activated in response to an ER stress and facilitated the long-term survival of yeast cells under these conditions [29]. Here we found that mortality of RS4 Δ cells during AA starvation was indeed enhanced when either the regulatory subunit (*CNB1*) or the catalytic subunits (*CNA1* and *CNA2*) of CN were deleted (Fig. 5). On the opposite, when these mutant strains were transformed with plasmids to re-express *CNB1* or *CNA1* their survival rate was higher than that of the original RS4 Δ . It seemed unlikely that CN acted by inducing an unfolded protein response (UPR), because expression of the mRNA encoding the Kar2 chaperone did not vary significantly in response to AA starvation and was not dependent on the presence or absence of the CN subunits (Table 4).

3.4. Calcineurin activation induced by AA starvation does not depend on a rise of intracellular calcium

Since calcineurin is known to be activated by Ca^{2+} /calmodulin, we sought to determine whether Ca^{2+} was involved in the response of RS4 Δ cells to AA starvation. Intracellular Ca^{2+} monitored with aequorin did not vary significantly in wild-type cells submitted to AA starvation, while a significant increase was observed in RS4 Δ cells (Fig. 6A). The Ca^{2+} fluctuations varied in amplitude and kinetics between experiments (Fig. 6A–C), but they somehow paralleled Crz1 activation (Fig. 1B). Interestingly, cerulenin which inhibited Crz1 translocation (Fig. 2A) did not have any effect on Ca^{2+} levels (Fig. 6B). In contrast, the Ca^{2+} rise was strongly diminished by the deletion of *MID1* (a membrane channel which is part of the high-affinity calcium uptake system [30]) or *YVC1* (the main channel involved in vacuolar Ca^{2+} release [30]) (Fig. 6C). However, despite their dramatic effect on intracellular Ca^{2+} levels, these deletions did not prevent CN/Crz1 activation (Fig. 6D). Altogether, these results indicated that the CN activation observed in LD-deficient

cells during AA starvation was not functionally connected to this Ca^{2+} rise.

3.5. Calcineurin is activated by the Mck1/Rcn1 pathway

To gain a better insight into the mechanism that led to calcineurin activation in the RS4 Δ mutant, we studied another possible pathway. Calcineurin activity can be regulated by direct interaction with proteins called Regulators of Calcineurin (RCN) [31]. In yeast, Rcn1 was shown to have opposite effects on CN depending on its concentration. Overexpression of *RCN1* inhibits CN, but moderate expression has a stimulatory effect and loss of *RCN1* diminishes CN signaling [32]. The stimulation of CN by Rcn1 in response to Ca^{2+} requires its phosphorylation by the (GSK-3)-related protein kinase Mck1 [18]. Here we found that genetic deletion of *MCK1* or *RCN1* both reduced CN-dependent Crz1 activation following AA starvation (Fig. 7). These data indicate that the CN activation observed in AA-starved RS4 Δ cells relies for a large part on the activation of the Mck1/Rcn1 pathway.

4. Discussion

The present study shows that a dysregulation in lipid homeostasis, that arises in lipid droplet-deficient cells following AA starvation, causes the activation of calcineurin in a calcium-independent manner, through a pathway involving the Mck1 kinase and the direct regulator of calcineurin Rcn1. This activation of calcineurin is beneficial to the lipid droplet-deficient cells because it improves their survival under AA starvation conditions.

Lipid droplets play a major role in lipid homeostasis, not only by storing neutral lipids and steryl esters, but also by communicating with other organelles [2]. Studies conducted by independent groups have reported several anomalies in yeast cells devoid of lipid droplets. One of them is an impairment of their ability to undergo autophagy, in particular in response to AA starvation. The second one is a severe perturbation of the endomembrane organization (ER, Golgi and

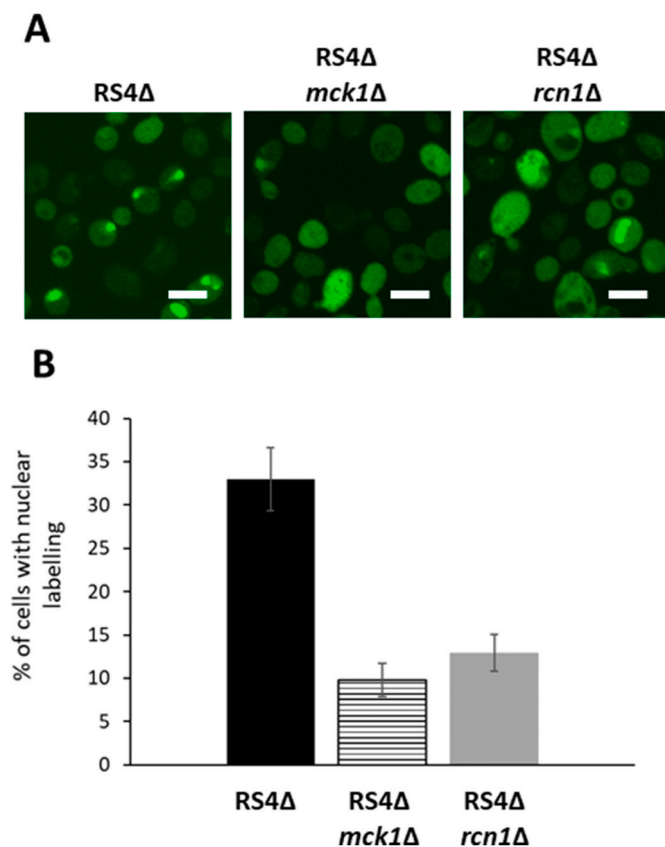


Fig. 7. Calcineurin/Crz1 activation upon amino acid starvation involves the Mck1/Rcn1 pathway. RS4Δ cells lacking *MCK1* or *RCN1* and expressing a GFP-Crz1 fusion protein were grown to log-phase in complete medium and transferred for 2h in a medium without amino acids. The GFP-Crz1 fusion protein was observed by fluorescence microscopy (magnification bar: 5 μm). (A). In B, the percentage of cells with a nuclear labelling was counted in each strain. Histograms represent the mean ± S.E.M. of independent experiments (n = 7 for RS4Δ or RS4Δ/*mck1Δ*; n = 6 for RS4Δ/*rcn1Δ*).

mitochondria), in starvation conditions. These defects are not merely coincidental, because treatments that allow to maintain a normal organization of the endomembrane network (reduction of FA acid synthesis with cerulenin, deletion of Opi1 or stimulation of mitochondrial biogenesis by respiratory conditions) also improve the ability of the lipid droplet-deficient cells to execute autophagy [9–11,25].

The present study provides a new piece of information on the behavior of LD-deficient yeasts during AA starvation, which is the activation of calcineurin and the nuclear translocation of the transcription factor Crz1. Contrary to our first intuition, this event is not responsible for the autophagy blockade previously observed in these cells, but it appears to be related to the same cause: a disorganization of ER membranes. Here we observed that experimental conditions that were previously shown to improve the endomembrane network during starvation, *i.e.* cerulenin treatment and genetic deletion of *OPI1*, also allowed to reduce the activation of CN/Crz1. Cerulenin was shown to have a positive effect on ER membranes organization, because it limited the increase in FA synthesis that occurred in lipid droplet-deficient yeasts during starvation [9,11,25], thereby preventing the accumulation of free FAs in these cells. Opi1, on the other hand, is a transcription factor that represses the expression of genes involved in PL synthesis [26–28]. In wild-type yeasts, its deletion was shown to cause an expansion of ER membranes that helped alleviate an ER stress [33]. This membrane expansion was due to an increase in PL synthesis, because it required the presence of the activating transcription factors Ino2 and Ino4, that are normally inhibited by Opi1 [34,35]. More recently,

studies on LD-deficient cells have shown that the deletion of *OPI1* prevented the appearance of ER lumps under starvation conditions [11,25] and it was assumed that *OPI1* deletion had also caused an increase in PL synthesis in these cells, that allowed to buffer free FA. The data obtained in our study somehow modulate this interpretation, because we showed that AA starvation actually caused the exclusion of Opi1 from the nucleus and a ~200-fold-increase in *INO1* expression in RS4Δ cells. A dramatic increase in *INO1* mRNA was also observed in response to external OA (up to 600-fold), another condition that generates lumps and threads of ER membranes [7]. The deletion of *OPI1* in the RS4Δ cells further increased *INO1* overexpression (~4-fold) during AA starvation or OA treatment, indicating that PL synthesis was not fully derepressed under these conditions. Nevertheless, these data demonstrate that the disorganization of ER membranes can occur in RS4Δ cells, although PL synthesis is strongly activated. This suggests that the role played by Opi1 on ER membrane homeostasis may not merely reflect its ability to repress the transcription of genes encoding PL biosynthesis enzymes.

It was previously shown, in wild-type yeasts, that CN could be activated in response to an ER stress and that it actually facilitated the long-term survival of the cells under these conditions [29]. Here, we also found that CN activation improved the survival of lipid droplet-deficient cells under amino acid starvation, but we detected major differences both in the mechanism causing CN activation and in the mechanism through which CN promotes survival. Regarding the latter issue, the study from Bonilla et al. [29] showed that CN activity was required to sustain the UPR signaling in response to a prolonged ER stress. Here, we did not detect a UPR activation during AA starvation, in spite of the fact that starvation causes severe defects in the ER organization of RS4Δ cells. This absence of correlation between changes in ER organization (tangles or expansion) and UPR activation was already reported in other works on RS4Δ cells [25,33]. Another intriguing aspect of our finding is the non-canonical pathway that causes CN activation during AA starvation and which appears to be Ca²⁺-independent. Indeed, a significant Ca²⁺ rise was actually observed in response to AA starvation, but the deletion of the Ca²⁺ channels Mid1 or Yvc1, which strongly inhibited this Ca²⁺ increase, did not impair CN activation. Conversely, the inhibition of FA synthesis with cerulenin, which was previously shown to prevent the formation of tangled ER membranes in RS4Δ cells [9,11,25] and which strongly decreased CN activation, did not have any effect on the Ca²⁺ rise. Although activation by Ca²⁺ is observed in most examples of CN-regulated events, several studies have reported alternative mechanisms. In *Dictyostelium discoideum*, long chain FAs were shown to directly activate CN by competing with Ca²⁺/Calmodulin [36]. Experiments on purified rat CN demonstrated that the CNB regulatory subunit can bind to and activate the CNA catalytic subunit at a basal level, even in the absence of Ca²⁺ [37]. Finally, in rat cardiomyocytes leptin-induced hypertrophy involves a RhoA pathway which activates CN independently of Ca²⁺ [38]. In our study, the pathway leading to CN activation in the lipid-droplet deficient cells submitted to AA starvation appeared to rely mostly on the direct regulator of calcineurin Rcn1 and on its activating GSK3-related kinase Mck1.

Although the origin of Mck1 activation in the starved RS4Δ cells still remain elusive, a link between Mck1 and lipid metabolism emerged from studies showing that Mck1 was part of the lipid asymmetry signaling pathway [39]. Lipid asymmetry generally refers to the uneven distribution of some PLs in the two leaflets of biological membranes. However, lipid asymmetry can also occur when LDs emerge from the cytosolic leaflet of the ER. Indeed, it was shown that wild-type yeasts grown with 0.1 % OA had an overproduction of LDs that causes a depletion of PLs in the cytosolic leaflet of ER membranes [40]. To compensate this asymmetric usage of PL, ER membranes were reshaped and the cells displayed an aberrant ER morphology in the form of lumps or threads. In RS4Δ cells which cannot produce LDs, a similar ER disorganization was reported at a lower concentration of 0.001 % OA [7]. This suggests that in cells devoid of LDs, an excess of fatty acids, such as the one that occurs in starved RS4Δ cells, may generate an

asymmetry within the ER lipid bilayer which might activate Mck1.

To summarize, our work provides new insights on the events that take place in LD-deficient yeasts during AA starvation. Previous studies have shown that the severe disorganization of their endomembrane network inhibits the autophagic process that should occur in these conditions and consequently causes premature cell death. Here we show that these endomembrane defects also cause the activation of the phosphatase calcineurin, through a non-canonical pathway that does not require calcium but involves the direct regulator of calcineurin Rcn1 and its GSK3-related kinase Mck1. This activation of calcineurin extends the lifespan of the LD-deficient cells during AA starvation. Further investigations will be required to elucidate the precise mechanism involved in this cell survival.

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Data availability statement

The authors agree to make any materials, data and protocols available upon request.

CRediT authorship contribution statement

Marianne Bernard: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Thierry Bergès:** Writing – review & editing, Visualization, Resources. **Stéphane Sebillé:** Writing – review & editing, Visualization. **Matthieu Régnacq:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2024.150670>.

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