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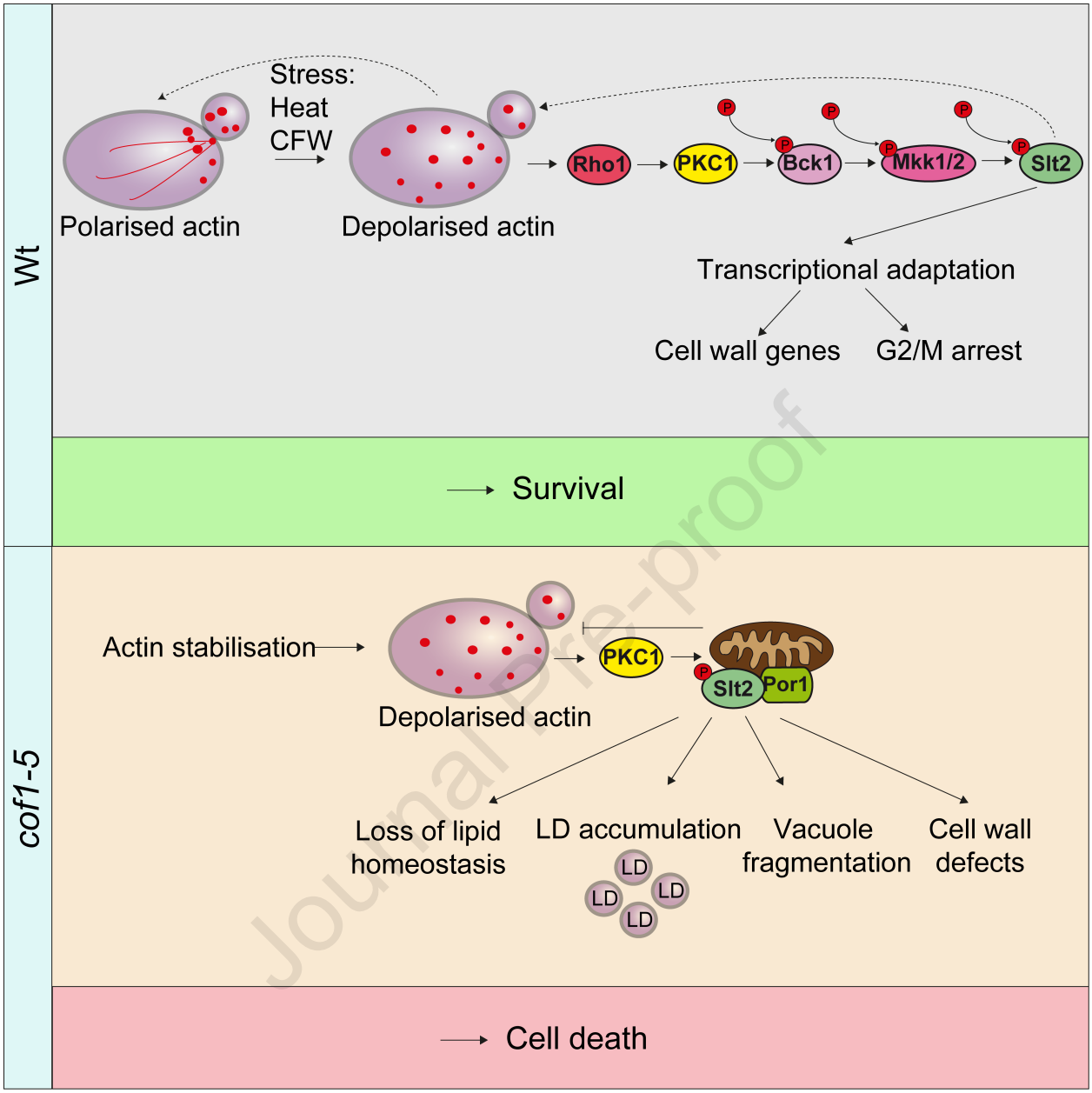

A dynamic actin cytoskeleton is required to prevent constitutive VDAC-dependent MAPK-signalling and aberrant lipid homeostasis.

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Summary

The dynamic nature of the actin cytoskeleton is required to coordinate many cellular processes and a loss of its plasticity has been linked to accelerated cell ageing and attenuation of adaptive response mechanisms. Cofilin is an actin-binding protein that controls actin dynamics and has been linked to mitochondrial signalling pathways that control drug resistance and cell death. Here we show that cofilin-driven chronic depolarisation of the actin cytoskeleton activates cell wall integrity MAPK-signalling and disrupts lipid homeostasis in a VDAC-dependent manner. Expression of the *cof1-5* mutation, which reduces the dynamic nature of actin, triggers loss of cell wall integrity, vacuole fragmentation, disruption of lipid homeostasis, lipid droplet (LD) accumulation and the promotion of cell death. The integrity of the actin cytoskeleton is therefore essential to maintain the fidelity of MAPK signalling, lipid homeostasis and cell health in *S. cerevisiae*.

Introduction

The actin cytoskeleton participates in many cellular processes including cell and organelle architecture, endocytosis, vesicular trafficking, organelle inheritance and communication[1]. As a result, the regulation of actin dynamics is essential and is facilitated by accessory proteins that promote rapid assembly and disassembly of filaments. Perturbations in the control of actin dynamics, instigated either through mutations in actin itself, or as a result of the aberrant activity of actin regulatory proteins, have been shown to trigger cell death in a variety of cell types[2]. In many, if not all, of these cases cell death appears to be underpinned by links between the control of actin dynamics and its effects on mitochondrial function. A good example of this lies with the highly conserved actin regulatory protein cofilin. Cofilin is a member of the ADF/cofilin family of small actin binding proteins found in all eukaryotic cells, which are essential for dynamic polymerisation and depolymerisation of actin[3]. Recent findings suggest that cofilin plays a role in the regulation and maintenance of homeostasis as cells adapt to environmental challenge. For example cofilin has been shown to facilitate the control of permeabilisation of the outer mitochondrial membrane, and so the initiation of apoptosis, in neutrophils at sites of infection in mammalian systems[4]. Cofilin is likely, therefore, to play a major role in innate immune and inflammatory responses. Additionally, aberrant cofilin /actin aggregates, termed ADF/cofilin rods (ACR), which accumulate in normal ageing brains and even more excessively in the hippocampus of Alzheimer's sufferers, are reported to interact with and damage mitochondria under conditions of stress[5]. Cofilin can therefore function within normal cellular responses or, if aberrant, promote disease via its interactions with mitochondria. Subtle changes to the charged surfaces of cofilin have a profound effect on the activity and quality of mitochondrial function. In yeast the regions of cofilin that are involved in controlling mitochondrial function are distinct from the actin binding and regulatory surface[6]. However,

despite the mounting evidence that cofilin can control homeostasis and cell fate via mitochondrial regulation, our understanding as to how this is achieved is lacking.

Cell death can occur in many different regulated or accidental ways which are characterised by their phenotypic features[7]. Importantly, regulated cell death pathways are not limited to multicellular organisms but also occur in single-cell-organisms such as *S. cerevisiae*[8,9]. The different modes and subroutines of cell death in yeast, which include accidental, regulated, and programmed forms of cell death, have been classified based on morphological and biochemical criteria[10]. Importantly yeast has been successfully used to study lipotoxicity/ lipotoxic cell death[11–14].

In this project we wished to gain mechanistic insight into the regulation of cofilin/ actin dependent stress signalling and mitochondrial function using the model yeast *S. cerevisiae*. In order to investigate this we made use of the well-characterised *cof1-5* mutant. In this strain two negatively charged amino acid residues are exchanged for alanine (D10A E11A). These amino acid exchanges do not interfere with actin binding *per se* but reduce cofilin´s actin depolymerisation efficiency, thus stabilising the actin cytoskeleton[15]. We report that cofilin dependent reduction in actin dynamics leads to a number of chronic defects, such as aberrant cell wall construction, vacuole fragmentation and altered lipid metabolism that sensitizes the cells to a necrotic cell fate when they are exposed to additional stress. This “pro-death” cell state is driven by the localisation and constitutive activation of PKC controlled MAPK signalling at the mitochondria.

PKC activation of MAPK signalling is essential for yeast cell survival under a range of stress conditions. In addition to its canonical role in PKC/MAPK cell wall integrity the terminal MAPK Slt2 has been shown to phosphorylate several targets involved in responses to environmental challenge. Slt2 therefore has roles in cell wall, oxidative, heat and calcium stress,

and functions within cellular processes including cell cycle control, membrane trafficking, actin cytoskeleton organisation[16]. Such perturbations to PKC/MAPK signalling are often linked to elevated cell death, highlighting its importance in cell adaption and survival[17,18]. Given the importance of the PKC/MAPK pathway to survive environmental challenge, cells have developed mechanisms to ensure that its activation is tightly controlled[19]. Here we show that a loss of actin regulation can override such controls and leads to the assembly of VDAC/Porin1 dependent MAPK signalling at the mitochondrial compartment. Many of the phenotypes associated with *cof1-5* expression could be rescued by the deletion of *POR1*, suggesting a key and previously uncharacterised role for VDAC in MAPK signalling in yeast. In addition, the deletion of genes *LRO1* or *DGA1*, that control the accumulation of triglyceride in lipid droplets (LDs) was also sufficient to prevent constitutive MAPK signalling and restore cell health in *cof1-5* mutants.

Our data suggests that the integrity of the actin cytoskeleton and the fidelity of PKC/MAPK signalling are inter-connected and that their concerted action is important for cell survival. Actin stabilisation promotes a MAPK signalling module that renders cells vulnerable to environmental challenge. We suggest that this adds to the growing evidence that simple eukaryotes embrace cellular states that ensure cells that cannot respond to environmental cues, such as those with corrupted actin cytoskeleton, are removed from their population.

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Results

1. *cof1-5*-induced actin defects and vacuole fragmentation but not mitochondrial fragmentation are VDAC-dependent.

In mammalian cells cofilin and its binding partner actin have been shown to interact with the mitochondrial outer membrane pore VDAC[4,20]. A primary goal of our study was to determine whether phenotypes associated with changes in actin dynamics that are linked to cofilin function are mediated via VDAC in yeast. In order to investigate this we made use of the well-characterised *cof1-5* mutant.

During cell division cortical actin patches can be observed to polarise to the growing bud[21–24], however in *cof1-5* cells actin patches are observed throughout the mother and daughter[25]. We examined whether the actin depolarisation phenotype observed in *cof1-5* cells was VDAC/Porin1 (Por1) dependent by staining the F-actin cytoskeleton with phalloidin-tetramethylrhodamine B-isothiocyanate (phalloidin-TRITC). Surprisingly, the deletion of *POR1* in *cof1-5* cells resulted in a full rescue of the actin-depolarisation phenotype (Fig. 1A, B) and also reverted the cell size increase as observed in *cof1-5* (Fig. 1A, C). These results suggest that the actin defect observed in dividing *cof1-5* cells is not caused by the action of cofilin on actin, but rather by either a loss of regulation that controls actin patch assembly, or the induction of a cellular stress programme capable of triggering actin depolarisation. The rescue of actin depolarisation upon deletion of *POR1* suggests an interaction between cofilin and/or actin with the mitochondrial compartment.

A further phenotype associated with *cof1-5*-expressing cells is fragmentation of the mitochondrial network[6]. As the fragmentation of mitochondria has been shown to involve F-actin and cofilin in mammalian cells we considered the possibility that *POR1* deletion in *cof1-*

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5 cells may also restore mitochondrial morphology. Deletion of *POR1* lead to the accumulation of mitochondria within a single entity (or maximally two) reminiscent of mutations in the ER-mitochondria encountering structure (ERMES), such as *mdm10*Δ*, mdm12*Δ*, mdm34*Δ and *mmm1*Δ (Fig. 1D)[26]. Mitochondria of *cof1-5 por1*Δ cells also appeared as large spherical structures and identical to those of *por1*Δ (Fig. 1D). As actin has also been linked to vacuole regulation we made use of the fluorescent dye FM4-64 to examine its morphology. Interestingly, *cof1-5* cells showed an aberrant fragmented, or multi-lobed vacuole morphology (Fig. 1E). In *cof1-5* cells 65 % of the cells had multi-lobular vacuoles as compared to only 19 % in wildtype (Fig. 1F). Notably, knock out of *POR1* led to a full rescue of this vacuolar phenotype. Additional electron microscopy confirmed the fragmented vacuole phenotype in *cof1-5* cells (Fig. S1). Altogether, these data suggest that the actin depolarisation and vacuole fragmentation phenotypes observed in *cof1-5* cells are Por1-dependent, whereas mitochondrial fragmentation does not share the Por1-dependency.

2. The *cof1-5* mutation constitutively activates the cell wall integrity pathway (CWI) in a VDAC specific manner.

In addition to changes in growth (Figure 2A) we observed that *cof1-5* mutant cells were prone to flocculation and sedimented rapidly in culture (Figure 2B). Flocculation is a natural phenomenon where cells aggregate in multicellular so-called flocs which increases the chance of survival upon stress[27]. Flocculation involves remodelling of the cell wall and has recently been connected to cell wall integrity (CWI) signalling[28]. The fungal cell wall is composed of glucans, chitin, chitosan, mannans, galactomannans and glycoproteins[29]. Under unstressed conditions chitin is represented at only 2 % of cell wall mass, which can increase up to 20 % upon cell wall stress[30]. This explains the increased reactivity of yeast cells with the chitin-

specific dye Calcofluor White (CFW)[31] upon cell wall stress. Using CFW staining we could indeed confirm that chitin is enriched at the mutant cell wall as compared to the wildtype (Fig. 2C). Examination of the cell wall ultrastructure by electron microscopy revealed that the inner cell wall of the *cof1-5* mutant appeared thicker than in wildtype samples, whereas the electron-dense structures of the outer cell wall were slightly shorter (Fig. 2D). We measured wildtype and mutant cell wall widths and calculated the means for inner- and outer cell walls. Strikingly, we confirmed a substantial increase of inner cell wall width for *cof1-5* (Fig. 2E), whereas the outer cell wall width was slightly reduced (Fig. 2F). Moreover, we observed that these cell wall phenotypes were reversed by additional knock out of *POR1*. These data suggest that VDAC is required for the maintenance of normal cell wall architecture.

3. Actin stabilisation changes expression of genes associated with the plasma membrane compartment, MAPK signalling and regulation of the cell wall.

The *cof1-5* mutation has been shown to lead to a reduction in the dynamic nature of actin filaments[15]. However, as the deletion of *POR1* led to a rescue of the actin patch depolarisation phenotype, we wished to determine whether reduced actin dynamics alone would lead to changes in the cell wall regulation. To achieve this, we made use of a well characterised actin allele, *act1-159*, which reduces actin dynamics by slowing the release of inorganic phosphate following ATP hydrolysis within F-actin filaments[32]. The expression of *act1-159* led to a significant increase or decrease in the expression of 648 and 141 genes respectively during exponential growth phase (Fig. 3A; Table S1). Genes that were upregulated in response to actin stabilisation could be clustered within several cellular processes by Gene Ontology (Fig. 3B; Table S1). The processes controlling transposition, response to pheromone and cell wall biosynthesis, which are controlled by MAPK pathways, were enriched within the upregulated

gene data set (Fig. 3B; Table S1). The changes in gene expression related to MAPK signalling in dividing *act1-159* cells were further exemplified by elevated levels of the membrane pheromone receptor *STE2* and the MAPK *FUS3*, which are normally activated by the presence of extracellular pheromone from cells of the opposite mating type. A MAPK involved in signalling in response to cell wall stresses, *SLT2* (also called *MPK1*) and the upstream MAPKK (MKK1) were also upregulated. This was accompanied by the increase in expression of several genes involved in another MAPK controlled process, cell wall biogenesis (Table S1). Within the genes upregulated for cell wall regulation process were those involved in glucan, chitin and mannan regulation as well as genes encoding several GPI anchored proteins (Table S1). To highlight the effects on cell wall we observed that *act1-159* cells exhibited a strong sensitivity to the cell wall stressor Congo Red (Fig. 3C). Overall these data support the finding that actin stabilisation leads to an increase in intracellular stress, a loss of MAPK regulation, which includes CWI activation, and changes in lipid biosynthesis in a manner akin to that observed in *cof1-5* expressing cells.

4. *cof1-5* mutation triggers activation of the cell wall integrity (CWI) MAPK pathway.

Our data suggest that cell wall changes observed in *cof1-5* cells are dependent on the presence of VDAC/Por1. As cell wall integrity is managed via MAPK signalling, we sought to determine activation of this pathway in *cof1-5* and *cof1-5 por1*Δ cells by immunoblotting for phosphorylation of the terminal MAPK Slt2 at its amino acid residues T190 and Y192[33]. This approach confirmed that Slt2 is constitutively active in *cof1-5* mutant cells and that Slt2 phosphorylation is lost upon deletion of *POR1* in both fermentable (glucose) and non-fermentable (glycerol) carbon source containing media (Fig. 4A, B). Constitutive Slt2 phosphorylation in *cof1-5* cells was largely reduced by addition of the Pkc1 inhibitor

cercosporamide, confirming activation of canonical CWI signalling (Fig. 4C). Expression of *POR1* from a plasmid led to an increase of Slt2 phosphorylation under all tested conditions and was sufficient to compensate for chromosomal loss of *POR1* (Fig. 4D). Unexpectedly, the additional expression of *POR1* from the plasmid on top of basic chromosomal expression in wildtype cells was sufficient to trigger CWI signalling (Fig. 4D). We further used calcofluor white (CFW) stress to trigger CWI signalling. We confirmed Slt2-phosphorylation in response to CFW stress in a concentration dependent manner (Fig. S2 A-C). Basic levels of Slt2-phosphorylation were reduced in *por1*Δ however the strain remained responsive to CFW-induced Slt2-phosphorylation (Fig. S2 D). Since cell wall integrity signalling is a response to stress we wondered whether its activation in *cof1-5* cells was essential to mediate survival upon cell wall stress. We could confirm that while the *cof1-5* mutation led to an increase in the cell population with loss of plasmamembrane integrity, this rose significantly when applying treatment with the Pkc1-inhibitor cercosporamide (Fig. 4E). Combined treatment with CSA and CFW, but not CFW alone, led to a further increase in cells exhibiting plasma membrane permeability in an additive manner, suggesting that Pkc1-activity may promote survival when cofilin/actin-induced stress is experienced. We further tested for cell death sensitivity in a genetic model using gene knock out strains for *POR1* and *SLT2*. In fact, as expected *POR1* deletion provided a rescue from *cof1-5* and CFW-induced cell death, while *SLT2* deletion had the opposite effect (Fig. 4F). In wildtype cells CSA treatment or *SLT2*-deletion increases the PI-positive cell population to 20 %, indicating that under basal conditions the CWI pathway is pro-survival. The *cof1-5* mutant shows increased CWI-signalling and the cell population with loss of plasmamembrane integrity increases concomitantly. It appears that both a loss and gain of Slt2 phosphorylation could result in increased cell death suggesting a non-linear signalling model for Slt2.

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We further conducted chronological ageing experiments with PI staining and survival plating as two independent readouts for cell death (Fig. 4G, H). Importantly, both readouts suggested that *cof1-5* mediated shortening of the chronological lifespan depends on *POR1*. Welch-ANOVA analysis of survival and PI at day 8 of ageing confirmed a significant reduction in survival and PI-negativity in *cof1-5* cells, which was rescued by *POR1*-deletion (Fig. 4I, J).

5. *cof1-5* expression prevents Slt2 localisation to the nucleus and promotes MAPK signalling from the mitochondrial compartment.

Using a wildtype strain expressing Slt2-GFP from its endogenous promoter at its original chromosomal locus we confirmed that, in line with earlier analyses of Slt2-GFP localisation[34], the majority of cellular Slt2 is found in the nucleus (Fig. 5A, B) However, in a small proportion (5 %) of cells Slt2-GFP could be seen to localise to cytoplasmic foci (Fig. 5A, C). In contrast only 1% of *cof1-5* cells showed Slt2 localisation to the nucleus (Fig 5B), while a significant proportion (30 %) of *cof1-5* cells were observed as having foci (Fig. 5A, C), and the remaining cells showed diffuse green fluorescence throughout the cytosol.

Given the porin dependence of the CWI activation in *cof1-5* cells we tested for colocalisation with mitochondria using rhodamine B hexylester. Indeed, the Slt2-GFP foci observed in *cof1-5* largely colocalised with rhodamine B hexylester signal after staining (Fig. 5D, E), whereas no colocalisation of Slt2-GFP foci with LDs was detected (Fig. S3A). We next wanted to assess whether mitochondrial Slt2-GFP foci formation was dependent on Por1. For this purpose, we used an Slt2-GFP expression plasmid. The expression from the plasmid showed a similar result as compared to chromosomal expression, but the percentages of cells with Slt2-GFP-foci and nuclear localisation in *cof1-5* were roughly doubled (Fig. 5F-H). We reasoned that this was an effect of Slt2-GFP overexpression from the plasmid used. Importantly, additional deletion of

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POR1 reduced the fraction of foci-containing cells in *cof1-5* to wildtype levels while at the same time increasing the percentage of cells with nuclear-Slt2-GFP signal to wildtype levels (Fig. 5F-H). The analysis of Slt2-GFP colocalisation with mitochondria further supports the latter finding in that the Pearson coefficient is reduced to wildtype levels in *cof1-5 por1*Δ (Fig. 5I). Additional analysis of Pkc1-GFP showed a similar pattern of foci-formation and colocalization with mitochondria in *cof1-5* as observed when expressing Slt2-GFP (Fig. 5 J-L). The percentage of foci-containing cells was increased by roughly 20 % in *cof1-5* as compared to wild type cells (Fig. 5K) and analysis of the Pearson coefficient of Pkc1 colocalisation with mitochondria was significantly increased from 0.26 to 0.59 (Fig. 5L).

Altogether this suggests that activation of MAPK-signalling in *cof1-5* involves Slt2 and Pkc1 translocation to mitochondria. This further raises the possibility that Slt2-mediated signalling actively occurs at mitochondria.

6. Reduced actin dynamics lead to a Porin-dependent increase in lipid droplet number that are required for CWI activation.

Porin has recently been implicated in mitochondrial lipid import[35]. We therefore determined whether lipid droplet number and their control were altered in *cof1-5* cells. This idea seemed reasonable, since actin and cofilin have been described as regulators of lipid droplet homeostasis in mammalian cells[36–38]. We stained cellular lipid droplets of wt and *cof1-5* mutants having additional gene-knock-out-deletions encoding for the acyltransferases Lro1 and Dga1 using the specific BODIPY 493/503 dye. Lipid droplets of *cof1-5* cells were more abundant than in wildtype cells (Fig. 6A, B). Interestingly, this increase in lipid droplet number was reverted when *LRO1* or *DGA1*, or both in combination, were knocked out in *cof1-5* cells (Fig. 6A and Fig 6B). Basic levels of Slt2-phosphorylation were significantly decreased in

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*lro1*Δ and *lro1*Δ *dga1*Δ DKO phenocopying *por1*Δ (Fig. S4A, B). In addition to that a non-significant trend in reduced susceptibility to CFW treatment was noted (Fig. S4A, B). We further found that the increase in lipid droplet number in *cof1-5* cells was reversed upon deletion of *POR1* or *SLT2* (Fig. 6C, D, S4C). Electron microscopy further supported the hypothesis that LD number is increased in *cof1-5* (Fig. 6E) and as a response to CFW treatment (Fig. S4D). Some LDs as observed in CFW treated cells appear to be surrounded by a membrane (Fig. S4D). Usually the membranes surrounding LDs identify as ER membranes[39], which may suggest an increase of LD-ER-membrane contacts.

7. The acyltransferases Lro1 and Dga1 are required for MAPK-related changes in *cof1-5*.

Given these findings we also investigated whether lipid droplet regulation at the stage of fatty acid esterification, via Lro1 and Dga1, would affect CWI signalling or other downstream effects in *cof1-5*. Actin depolarisation as observed in *cof1-5* could neither be rescued by *LRO1*- or *DGA1*-single-KOs, nor by the double knock out, both of which genes encode for acyltransferases (Fig. 7A). However, the growth defect (Fig. 7B) and the flocculation phenotypes (Fig. 7C) of *cof1-5* were compensated by knock-out of either *LRO1* or *DGA1*. As expected by the rescue of flocculation, the constitutive phosphorylation of Slt2 observed in *cof1-5* cells was turned off upon deletion of *LRO1* or *DGA1* (Fig. 7D). We next tested whether diverse stresses such as hydrogen peroxide (H_2O_2), heat, $CuSO_4$ or calcofluor white (CFW) affected LD abundance. Interestingly, all the stresses triggered an increase in LD abundance with the cell wall stress applied through CFW treatment being strongest (Fig. 7E). In summary, this suggests an active role of CWI-signalling in the control of lipid metabolism and LD homeostasis downstream or independent of cofilin-mediated actin regulation.

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297 **8. Cofilin and Porin are important regulators of lipid homeostasis**

298 With the Porin-dependent changes in lipid droplet quantity as observed in *cof1-5* cells we
299 sought to characterise its effects on global lipid homeostasis. We detected significant changes
300 in *cof1-5* cells for PC, PS, TG (Fig. 8A), PA, PG, lyso-PI (LPI), ceramide (Fig. 8B), sterol
301 esters (SE) and ergosterol (Erg) (Fig. 8C), and the complex sphingolipids MIP2C and MIPC
302 (Fig. 8D) when we compared shotgun lipidomic profiles to wild type (additional data on lipid
303 species are available in Fig. S5 A-E). In all cases, with the exception of LPI, all lipid class levels
304 were restored to wild type levels upon deletion of *POR1*, highlighting the significance of this
305 mitochondrial protein for lipid regulation. The most prominent decrease in *cof1-5* cells was
306 observed for TG. However, additional high performance thin layer chromatography (HPTLC)
307 measurements could only confirm a trend in decrease due to strong variation upon the
308 individual samples (Fig. 8E). The strongest increases were observed for PA, LPI, sterolesters
309 and ergosterol as well as the complex sphingolipids MIP2C and MIPC. The increases in SE and
310 Erg in *cof1-5* as well as the reduction by the additional gene KOs were confirmed by additional
311 HPTLC quantification and can thus be considered as robust (Fig. 8F, G). Since the neutral lipids
312 (SE and TG) are stored in LDs we reasoned that the ratio of SE to TG might correlate with the
313 LD phenotype as observed before. We thus calculated the SE/TG index ($i_{SE/TG}$) and confirmed
314 significant change of the index in *cof1-5* (Fig. 8H) which correlates with the increased amount
315 of LDs as quantified in Fig. 6A-D. Interestingly, gene KOs of *POR1*, *DGA1*, *LRO1 and* SLT2
316 revert $i_{SE/TG}$ back to wildtype levels. Further to this, we noted significant lipidomic changes in
317 the *POR1* deletion strain itself (e.g. PC, TG, PA, Cer, CL, Fig. 8A, B) which confirms that Por1
318 indeed has crucial impact on total lipid homeostasis.

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In summary, these results give evidence that lipidomic changes in *cof1-5* depend on Por1 and further suggest that lipidomic changes in *cof1-5* could be causally linked to CWI signalling.

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Discussion

In a healthy dividing yeast cell the experience of a stressor that perturbs the cell wall results in the depolymerisation of the F-actin cytoskeleton, this is required to assist in both cell cycle arrest (G2/M) and the activation of the cell wall integrity pathway[30]. We have shown that cells expressing the *cof1-5* mutation, which leads to the chronic depolarisation of F-actin patches, exhibit hallmarks of constitutive Pkc1/Slt2 activation, such as flocculation and vacuole fragmentation. Our data also show that this aberrant PKC/Slt2 signalling depends on the mitochondrial outer membrane protein Por1, which is the yeast orthologue of mammalian voltage dependent anion channel (VDAC). Por1 is known to form a pore in the outer mitochondrial membrane thereby facilitating metabolic flux from mitochondria to the cytosol and vice versa[35,40]. Por1 further has been implicated in the control of cell death in response to stress in previous studies[41,42]. However VDAC has also been shown to participate in other cellular processes including the regulation of lipid traffic[35] and in AMPK/Snf1 signalling[43–45]. Here we show that the actin cytoskeleton and mitochondria coordinate MAPK signalling through VDAC and that this plays a role in cell fate. One possibility is the existence of a physical interaction, as was shown in the case of AMPK/Snf1 signalling[44], whereby Slt2 relocates from the nucleus to the mitochondria and docks with VDAC to elicit an alternate signalling response that promotes cell death. In line with this we did observe that the deletion of *POR1* prevented constitutive activation of Slt2 in *cof1-5* cells restored its localisation to the nucleus and reduced the necrotic cell population. Despite a clear correlation between the prevention of Slt2 activation in *cof1-5* cells by deletion of *POR1*, which led to apparent improvement in cell health, we observed the opposite effect when cells were treated with the PKC inhibitor CSA. This result, while unexpected, may be explained by additional off target

effects of CSA, or indeed as a result of functions of PKC that lie outside of the canonical PKC/Slt2 cell wall integrity signalling system. Additional evidence to support a direct link from VDAC to Slt2 activation comes from our finding that the overexpression of *POR1* was also sufficient to activate Slt2. Connections between mitochondria and MAPK signalling are emerging, for example a recent study demonstrated mitochondrial participation in MAPK signalling regulating proteasome granule formation upon carbon starvation[46].

A number of lipid classes were found to show significant change in the *cof1-5* mutant. These included PC, PS, and TG, PA, PG, LPI, ceramide, sterol esters (SE), ergosterol (Erg) and the complex sphingolipids MIP2C and MIPC (Fig. 8A-D). These changes were largely reversed when *POR1* was knocked out suggesting a clear role for VDAC in the control of lipid homeostasis, possibly via the activation of Slt2. The overall increase in PA might also account for the changes in mitochondrial morphology as this phospholipid has been shown to regulate mitochondrial fusion via control of Ugo1 biogenesis[47]. Mitochondrial phospholipids such as PG and CL have been connected to Pkc1/CWI signalling [48,49]. Loss of CL has also been shown to impair CWI signalling which led to defective mitophagy[50]. We also detected changes in the lipid profile of *POR1*-deleted cells as such, which is in line with recently published findings [35,51,52]. This raises the possibility that actin triggers changes in lipid homeostasis with Porin acting as a central point of lipid flux control, or indeed as a component of the signalling system that regulates lipid homeostasis itself. Interestingly the ER-mitochondria encountering structure (ERMES), which can facilitate lipid exchange between these compartments, has been implicated in cell death[53,54].

Our lipidomic results further support the notion that *por1*Δ phenocopies *pgs1*Δ. Pgs1 catalyses the first step of mitochondrial CL synthesis, which is the phosphorylation of phosphatidylglycerol. *pgs1*Δ has reduced levels of beta-1,3-glucans in its cell wall which is thus

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defective[55]. Por1 and Por2 have recently been suggested to mediate mitochondrial phospholipid import succumbing to PG and CL synthesis[35]. Our data are in line with these findings and further support the idea of Por1 as a regulator of mitochondrial phospholipid import.

Intriguingly, we found that two acyltransferases Dga1 and Lro1 were also essential for activation of Slt2 and all the downstream phenotypes with the exception of actin depolymerisation. Given the changes of LD abundance that we observed upon cell wall stress and found in *cof1-5* expressing cells this suggests that CWI signalling involves lipidomic rearrangements and dynamic changes of cellular lipid droplets. Interestingly, the inhibition of actin dynamics through *COF1* depletion has also been shown to disrupt adipogenesis and lipid storage in 3T3-L1 cells[38].

Our findings that actin and mitochondrial functions are linked to lipid homeostasis in yeast may have relevance for antifungal resistance. Mitochondrial function has been linked to changes in cell wall function and to changes in azole sensitivity, a class of drugs that target ergosterol synthesis[56]. Ergosterol-levels are controlled under normal conditions, but conditions of stress (e.g. osmotic or cell wall stress) can require rapid change in ergosterol content. A significant increase in ergosterolesters was observed in *cof1-5* cells (Fig. 8C, F), suggesting that these cells are dealing with an ergosterol overflow, thus detoxifying through esterification and storage in LDs. LDs accumulate upon cell wall stress in *cof1-5* cells and in wildtype cells upon CFW stress, where LDs appear to have strong contacts with ER-membranes (Fig. 6, S4). One possibility is that actin and cofilin are needed to facilitate non-vesicular sterol transport by Osh and Lam proteins[57–59]. Another possibility is that, through activation of the CWI pathway, Pkc1 can phosphorylate Pah1[60,61] (which dephosphorylates PA to yield diacylglycerol) leading to its

degradation by the proteasome[62]. This in turn would increase PA levels and decrease TG at the same time which is what we observe in *cof1-5* expressing cells. Of note, deletion of *pah1* has been described to shorten chronological lifespan[63], which suggests a pro-death trigger upon loss of Pah1 function. Since Pah1 activity has been described to be essential for homotypic vacuole fusion[64], this also offers an explanation for the fragmented vacuole phenotype as we observed in *cof1-5* cells. Indeed, the fragmented vacuoles of *cof1-5* look very similar to vacuoles as observed in *pah1*Δ or propranolol-treated cells, which represents a pharmacological treatment to inhibit Pah1-activity[64]. The increase in ergosterol as well as the increase in complex sphingolipids observed in *cof1-5* expressing cells may represent an adaptation to overcome mechanical stress at the plasma membrane. Generally the integration of complex sphingolipids together with sterols results in increased packaging and thus higher density which renders the plasma membrane more resistant to mechanical stress[65]. Thus, the activation of Slt2 in actin stabilised cells may contribute to the cell's response to stress and so survival in the short term. However, as such cells are clearly sensitive to additional stress, we favour an interpretation that chronic actin depolymerisation in dividing cells promotes necrotic cell death.

Permeabilisation of the vacuole is a feature of regulated cell death in yeast, which through vacuolar release of Pep4, an orthologue of human cathepsin D, into the cytosol succumbs to cell death[66–68]. More recently a molecular pathway of how vacuole membrane permeabilisation is established in yeast was proposed[69]. The vacuolar phenotype as observed in *cof1-5* cells in our study might have similarity to the previously described cell death routines but additionally involves mitochondrial Por1 activity and lipid metabolism. Further investigation will be required to test this possibility.

In summary, our study provides further evidence that the regulation of actin dynamics is crucial for cell fate determination. We show that a reduced ability to regulate actin in dividing yeast

cells leads to a pro-death mode of MAPK signalling under conditions of stress. We would argue that this represents a mechanism by which cells that are unable to regulate actin may be lost within a population of cells, such as a colony or biofilm, and postulate that this may represent a novel cell death mechanism in yeast that helps ensure clonal integrity.

Limitations of the Study

The authors recognise that this study opens a number of questions that arise as a consequence of unrecognised interplay between lipid homeostasis, membrane organisation and cell signalling systems in yeast. Further investigations that we suggest within the discussion section highlight a current need to improve our understanding of lipid regulation within eukaryotic cells. A further limitation lies within our current understanding of yeast cell death as a regulated process. Although there is strong evidence to suggest that different modes of death exist, the experimental tools available to differentiate between programmed and passive cell death in yeast are, in the authors view, limited at time of writing. This led to a conservative interpretation of the data presented as a loss of cellular integrity as opposed to a mechanism of regulated cell death.

Acknowledgements

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Author contributions

Conceptualisation, C.W.G., and P.R.; Methodology, J.D., T.M., M.S., D.S., L.N., J.H., F.B., E.S.M.E., O.K., D.G.,, T.vd.H, C.W.G. and P.R.; Validation, J.D., T.M., M.S., O.K., C.W.G. and P.R.; Formal Analysis, J.D., T.M., M.S., O.K., and P.R.; Investigation, J.D., T.M., M.S., D.S., L.N., J.H., F.B., E.S.M.E., O.K., and P.R.; Data Curation, P.R.; Writing-Original Draft Preparation, C.W.G., and P.R.; Writing-Review and Editing, J.D., T.M., M.S., O.K.; Figure Visualisation, C.W.G. and P.R.; Supervision, Project Administration, and Funding Acquisition, C.W.G. and P.R. All authors have read and agreed to the published version of the manuscript.

Declaration of Interests

The authors declare no competing interests

Inclusion and Diversity

We support inclusive, diverse, and equitable conduct of research.

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Figure Titles and Legends

Graphical abstract. Schematic view of VDAC-dependent MAPK signaling. The actin cytoskeleton of wildtype yeast cells is polarised along the mother-bud axis. Upon cell wall stress such as heat or calcofluor white (CFW) administration the cytoskeleton depolarizes and the CWI MAPK signaling pathway is triggered involving Rho1, Pkc1, Bck1, Mkk1/2 and Slt2. This leads to transcriptional adaptation of cell wall genes and G2/M arrest and has pro-survival character. In *cof1-5* cells actin is stabilised and chronically depolarised. Chronic depolarisation of actin constantly triggers the CWI pathway with its main players Pkc1 and Slt2 localising to the mitochondrion in a Por1-dependent fashion. This leads to cell wall defects, vacuole fragmentation, loss of lipid homeostasis, LD accumulation and finally culminates in cell death.

Figure 1. *cof1-5*-induced actin depolarisation but not mitochondrial fragmentation are VDAC dependent. (A, B) Actin phalloidin (red) and DAPI (blue) staining at exponential growth phase (6h) reveals depolarised actin cytoskeleton in the *cof1-5* mutant, which is rescued by additional *POR1* deletion. Representative microscopy pictures are shown in (A) and cells with polarised actin were quantified in (B). (C) *cof1-5* cells have an increased mean cell diameter (as determined with a CASY cell counter). (D) Fluorescence microscopy pictures at exponential growth phase of wt and *cof1-5* with and without additional *POR1* deletion expressing mitochondrial GFP from a plasmid (pVT100U-mt GFP). (E, F) *cof1-5* mutation leads to Por1-dependent vacuole fragmentation as visualised by FM4-64 staining. Representative microscopy images are shown in (E) and a quantification of cells containing multi-lobular vacuoles is depicted in (F). Statistical significance in (B) (C) and (F) was assessed using ordinary one-way ANOVA. See also Fig. S1.

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Figure 2. *cof1-5* mutation triggers growth defect, flocculation, and cell wall alterations, which depend on *POR1*. (A) Growth performance in liquid culture is reduced in *cof1-5* as compared to wildtype (Wt) but is restored by additional *POR1* deletion. (B) Cultures bearing the *cof1-5* mutation sediment quickly when shaking is stopped (flocculation phenotype). Additional *POR1* deletion prevents flocculation in *cof1-5*. (C) Calcofluor white staining detecting chitin exposure at the cell wall confirms flocculation phenotype of *cof1-5* cells. (D, E, F) The cell wall was analysed by electron microscopy. *cof1-5* mutation is associated with a thicker inner cell wall and thinner outer cell wall. EM-micrographs are shown in D and quantifications of the inner and outer cell wall are plotted in E and F, respectively. ICW, inner cell wall; OCW, outer cell wall; LD, lipid droplet. Statistical significance in (E) was assessed using Kruskal-Wallis test and in (F) Welch ANOVA was performed.

Figure 3. Transcriptional changes in the actin mutant act1-159 suggest involvement of MAPK signalling, flocculation and lipid metabolism. (A) Transcriptional changes of act1-159 vs. ACT1 cells grown to log phase in YPD media were investigated by microarray and plotted in a volcano plot. Gene ontology analysis for the GO-term PROCESS was completed using the GO SLIM mapper function available on the *Saccharomyces cerevisiae* genome database[70] and upregulated genes clustered within enriched cellular processes are depicted in panel (B). (C) Congo red sensitivity of wild type and *act1-159* mutant cells was assessed by a spotting assay using a ten-fold serial dilution series from a starting cell number of 2×10^5. See also Table S1.

Figure 4. *cof1-5* mutation triggers activation of the CWI pathway. (A, B) Immunoblots detecting Slt2 phosphorylation when grown on glucose and glycerol containing media are

shown in A and B, respectively. (C) Pkc1 inhibition by cercosporamide (CSA) administration prevented Slt2 phosphorylation. (D) Porin deletion and overexpression reveal dependence of Slt2 phosphorylation on Por1. (E) *cof1-5* mutation triggers loss of plasmamembrane integrity, as assessed flow cytometrically with PI-positivity at 48 h after inoculation. PI-positivity is exacerbated by additional Pkc1 inhibition using the Pkc1-inhibitor cercosporamide or applying additional cell wall stress with calcofluor white (CFW). Combined treatment with CSA and CFW at the same time shows additive effects. (F) *POR1* deletion rescues from *cof1-5*-dependent loss of viability and loss of plasmamembrane integrity, whereas *SLT2* deletion sensitises to cell death. (G-J) Chronological ageing analysis reveals shortening of chronological lifespan in *cof1-5* cells which depends on *POR1*. Colony forming unit formation based on clonogenic survival is depicted in G and H, whereas PI positivity is shown in I and J. Statistical significance in E, F, H and J was assessed using Brown-Forsythe and Welch-ANOVA test. Asterisks indicate significance based on p-levels of the comparisons to the respective control strains. See also Fig. S2.

Figure 5. Slt2 localisation to the mitochondrial compartment is enhanced in *cof1-5* cells. (A) Slt2, which is mostly found in the nucleus in wildtype cells at stationary phase, forms punctate foci in *cof1-5*, as documented by fluorescence microscopy using chromosomally tagged SLT2-GFP under control of its endogenous promoter. Deconvolved pictures with Hoechst staining for nuclei are shown. (B, C) Cells showing nuclear localisation of Slt2-GFP (B) and foci-forming cells (C) were quantified, plotted and analysed for significant localisation change as compared to wildtype. (D) Representative fluorescence microscopy pictures of Slt2-GFP expressing cells with rhodamine B hexylester staining for colocalization analysis with mitochondria. (E) Colocalisation of Slt2-GFP (green) with the mitochondrial stain rhodamine

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B hexylester was increased in *cof1-5* as shown by significant increase of the Pearson colocalisation coefficient. (F-I) Slt2-GFP expression from a plasmid was used to monitor cellular Slt2 localisation in *cof1-5* cells in dependence of *POR1*. Representative microscopy pictures are shown in (F), Slt-GFP-foci-containing cells are quantified in (G), cells with nuclear Slt2 are quantified in (H) and the Pearson coefficient of Slt2-GFP colocalization with mitochondria is visualised in (I). (J, K) Expression of Pkc1-GFP under control of its endogenous promoter reveals increased PKC1-GFP foci formation in *cof1-5* (J, K). Representative fluorescence microscopy pictures of individual Pkc1-GFP expressing cells with additional rhodamine-B-hexylester staining for mitochondria and autodot staining for LDs are shown in (J) and cells with Pkc1-GFP foci are quantified in (K). (L) The Pearson coefficient was determined as a measure of colocalisation of Pkc1-GFP with mitochondria (rhodamine-B-hexylester). Statistical significance in (B) and (C) was assessed using Welch´s t test, (E, K, L) were analysed using unpaired t test and (G, H, I) by ordinary one-way ANOVA. See also Fig. S3.

Figure 6. Reduced actin dynamics lead to a porin-dependent increase in lipid droplet number that are required for CWI activation. (A, B) Increase of LD number in *cof1-5* depends on Lro1 and Dga1. Mean LD-numbers per cell are plotted in (A) and representative microscopy pictures are shown in (B). Each dot in (A) represents the mean LD number per cell of a single experiment (n=6) with at least 119 cells being evaluated per experiment. (C, D) Gene deletions of *POR1* or *SLT2* are sufficient to prevent LD accumulation in *cof1-5*. Mean LD numbers per cell were assessed by quantifying fluorescence microscopy pictures using Bodipy staining (C). Representative fluorescence microscopy images are shown in (D). (E) Representative EM micrographs supporting the observation of LD-number-increase in *cof1-5*.

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V, vacuole; LD, lipid droplet; N, nucleus. Statistical significance in (A) and (C) was assessed using 2-way-ANOVA with *cof1-5* mutation as first factor and additional KO as second factor. See also Fig. S4.

Figure 7. The acyltransferases Lro1 and Dga1 are required for MAPK-related changes in *cof1-5*. (A) Polarisation of the actin cytoskeleton was assessed using phalloidin (red) and DAPI (blue) staining. The *cof1-5* mutant shows loss of actin polarisation, which is not rescued by gene KO of *LRO1*, *DGA1* or a combined double deletion of the ladder. (B, C) The growth defect (B) and flocculation phenotype (C) as observed in *cof1-5* are restored by additional deletion of the acyltransferases *LRO1*, *DGA1*, or in the double deletion mutant (*lro1*Δ *dga1*Δ). (D) Slt2 phosphorylation in *cof1-5* depends on Lro1 and Dga1 as the KO of either corresponding gene and the double KO prevents Slt2 phosphorylation. (E) Mean LD abundance per cell was quantified in diverse conditions of stress. 150 cells per condition and experiment were quantified with a total of three independent experiments (n=3). Statistical significance in (E) was assessed using ordinary one-way ANOVA.

Figure 8. Lipidomic analysis reveals characteristic Por1-dependent changes in the lipid profile of *cof1-5*. (A, B) Mass spectrometry-assisted lipidomic quantification of highly abundant (A) and less abundant yeast lipids from total cell extracts (B). (C, D) Sterolesters and free ergosterol were quantified separately as shown in (C) and sphingolipids are depicted in (D). (E-H) Lipidomic changes in the neutral lipid classes TG (E), SE (F), Erg (G) and the $index_{SE/TG}$ (H) were further verified by additional HPTLC analysis. Statistical significance in (A-G) was assessed using two-way ANOVA with *cof1-5* mutation as first factor and additional

574 KO as second factor, except for CL, LPC and LPE in (B), which were analysed using Kruskal

575 Wallis test; (H) was analysed by Brown-Forsythe and Welch ANOVA test. See also Fig. S5.

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STAR Methods

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Patrick Rockenfeller (Patrick.rockenfeller@uni-wh.de) and Campbell Gourlay (C.W.Gourlay@Kent.ac.uk).

Materials availability

Plasmids and yeast strains generated in this study are available upon request.

Data and code availability

- Microarray data, original western blot images, lipidomics data and Prism files have been deposited at Mendeley and are publicly available as of the date of publication. doi: 10.17632/bgkscw9ns9.1. Microscopy data reported in this paper will be shared by the lead contacts upon request.
- This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the lead contacts upon request.

EXPERIMENTAL MODEL

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All experiments (except Fig. 3) were carried out using the wildtype (Matα *ura3*-52 *his3*Δ200 *leu2*-3,112 *lys2*-801 *ade2*-101 *COF1::LEU2*) and *cof1-5* mutant (Matα *ura3*-52 *his3*Δ200 *leu2*-3,112 *lys2*-801 *ade2*-101 *cof1-5*::*LEU2*) *S. cerevisiae* strains as generated and described by Lappalainen et al.[15]. Additional gene-knock outs in these two strains for *LRO1* and *DGA1* were generated using the Cre-LoxP system with the KanMX marker for selection[71] using the primers as listed in the key resource table. The *lro1*Δ *dga1*Δ double knock out was generated based on the *dga1*::kanMX single knock out strain using the pFA6a-Ura3-cassette[72] for the additional LRO1 knock out. POR1-, and SLT2-gene knock outs were generated according to the protocols and primer design of Janke et al.[73] using the pFA6a-KanMX6-cassette[74]. See KRT for primer sequences.

For the experiments shown in Fig. 3 the *act1-159* mutant (MATa *act1*-159::*HIS3 his3*D200 *tub2*-101 *ura3*-52 *leu2*-3, 112) which harbours the V159N mutation and has reduced actin dynamics was used together with its corresponding wildtype control strain (MATa *ACT1::HIS3 his3*D200 *tub2*-101 *ura3*-52 *leu2*-3, 112)[32].

Plasmids were propagated in *E. coli* K12 DH5α (see key resource table for details). *POR1* was cloned into pAG416GPD CEN URA by gateway-cloning[75] using pDONR221-*POR1* as a donor-plasmid. pYX122-mtGFP was obtained from Benedikt Westermann[76]. The *SLT2*-GFP expression plasmid was obtained from Matthias Peter.

Slt2-EGFP as shown in Fig. 5A and Pkc1-EGFP as shown in Fig. 5J was expressed under control of their endogenous promoters at their original loci. These strains were generated by PCR and homologous recombination according to established protocols[73] using the plasmid pYM27 and primers as listed in the KRT, except for the experiment shown in Fig. 5F-I, where Slt2-GFP was expressed from a plasmid pRS426-SLT2-GFP[34]. Transformation of yeast cells

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with plasmids or linear DNA for homologous recombination was performed using the lithium acetate method[77]. At least three different clones were tested to rule out clonogenic variations. All experiments (except for ageing experiments as shown in Fig. 4G-J) were carried out in yeast peptone medium with glucose (YPD). YPD medium contains 1% yeast extract (BD), 2% peptone (BD), and 2% glucose. Synthetic complete medium with glucose without Uracil (SCD-Ura), was only used when selection was required for strain construction or to maintain selection for plasmids. SC medium contains 0.17% yeast nitrogen base (Difco), 0.5% $(NH_4)_2SO_4$, and 30 mg/L of all amino acids (except 80 mg/L histidine and 200 mg/L leucine), 320 mg/L uracil, 30 mg/L adenine, and 2% glucose. All media were prepared with ultrapure water (MilliQ) and subsequently autoclaved (20 min, 121 °C, 110 kPa). Amino acid mixtures and glucose were sterilised separately as 10× stocks and added after autoclaving. All yeast cultures were inoculated from a stationary overnight culture to an OD_{600} = 0.1 and then grown at 30 °C and 145 rpm shaking for indicated time points.

METHOD DETAILS

Growth Assays

Strains were inoculated from stationary overnight cultures to an OD_{600} of 0.1 in 24-well plates (Sarstedt, 1 ml total volume per well) in two or three independent experiments, each containing at least three biological replicates. The plate was automatically measured for 24–48 h using a BMG LabTech SPECTROstar^{Nano} plate reader with double orbital shaking at 400 rpm and 30 °C, with OD_{600} measurements every 30 min. Growth curves were plotted in GraphPad Prism.

Analysis of cell viability, density and diameter

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Propidium iodide (PI) staining was used to determine loss of membrane integrity[78,79]. Cells were harvested in 96-well plates at indicated time points and resuspended in 250 µL of 100 µg/L PI in PBS, and incubated in the dark for 10 min at room temperature. After incubation, cells were washed once with 250 µL PBS and analysed via flow cytometry (Beckmann Coulter Cytoflex). A total of 30,000 cells per strain and condition were measured and analysed with CytExpert software. For calcofluor white (CFW) stress experiment in Fig. 4E, wt and *cof1-5* cells were grown overnight and inoculated to an OD_{600} of 0.1, then grown at 30^0C. After 22 h of growth, cells were subjected to either 100 µM cercosporamide, 200 µM CFW or both, and allowed to grow for further 26 h. CFW stress at 200 µM as shown in Fig. 4F and Fig. S4A, B was carried out at exponential growth phase (six hours after inoculation to an OD_{600} of 0.1) and PI was detected 18 hours after stress. For chronological ageing analysis cultures were inoculated to an OD_{600} of 0.1 in SCD medium as mentioned above with additional supplementation of 90 mg/ L lysine (+3x), 10 g/ L myo-inositol (55µM), and 97.8 mg/ L adenine. Samples were measured at indicated days of ageing by clonogenic survival plating and PI staining to determine viability.

For clonogenic survival plating serial dilutions of the main cultures were used to measure cell densities using a CASY cell counter essentially as described before[12]. As an adaptation to former protocols the serial dilutions of all strains were made using ddH_2O containing 10 mM EDTA. The addition of EDTA avoided cell aggregation, which is a characteristic of *cof1-5* cells. Without EDTA treatment reliable estimation of living cells by counting of colony forming units (CFU) was impossible.

Mean cell diameters were determined based on measurement with a CASY cell counter, which calculates mean cell size based on electric currency shifts which are due to the cells acting as electric isolators in conducting salt solution. For these measurements the complex cell mixtures

containing cells at all replicative stages were used, which includes single mother cells, bud-containing mother cells and single daughter cells.

Fluorescence microscopy

S. cerevisiae strains were inoculated to an OD_{600} = 0.1 from overnight cultures in YPD medium and harvested at 6h after inoculuation for analysis at exponential growth phase or 24h for analysis at stationary phase. Cells were immobilised on microscopy slides containing 2% agarose[80]. Fluorescence was detected using a Nikon Eclipse Ni-U fluorescence microscope with a Hamamatsu Orca-Spark C11440-36U monochromatic camera and Nikon Intensilight C-HGFI illumination system. Fluorescence images were captured and saved as z-stacks in a range of 5 µm with 200 nm steps using NIS-Elements BR 4.13.05 64-bit with N-dimensional acquisition. Deconvolution and colocalisation analysis was performed with Huygens essential 21.10 software. Further processing such as maximum intensity z-projection, brightness/contrast adaptation, scale bar inclusion, colour merging was performed in Fiji/ ImageJ[81].

Actin was stained using Phalloidin-Tetramethylrhodamine B-Isothiocyanate (Sigma, P1951) essentially as described before[82]. In brief, approximately 10^7 cells were harvested and fixed with 3.7 % formaldehyde. Cells were washed in PEM buffer (100 mM PIPES, 5 mM EGTA, 5 mM $MgCl_2$, pH 6.9) and stained with 50 µg/ml final concentration of Phalloidin-TRITC in PEM buffer with 25 % methanol. After washing cells were resuspended in PEM buffer containing DAPI at a final concentration of 2.5 µg/ml and mounted on agarose slides for microscopy.

Nuclear staining of live cells in Fig. 5A was performed using Hoechst (bisbenzimide H 33342). Cell wall staining for chitin exposure was performed using calcofluor white fluorescent brightener (Sigma, 910090) at a final concentration of 100 µg/ml[83].

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The vacuole morphology was visualised by staining with SynaptoRed(TM) C2 (equivalent to FM4-64; Biotum BOT-70021) as described before[84].

Rhodamine B hexylester perchlorate (Molecular Probes, Y-7530) was used at a final concentration of 100 nM to stain functional mitochondria. Pearson colocalisation coefficients for Slt2-GFP colocalization with Rhodamine B hexylester signal were determined in Huyghens essential 21.10 using the colocalization analyzer wizard with Gaussian minimum estimation.

Lipid droplets were stained with Bodipy 493/503 or autodot. Bodipy was detected in the FITC channel whereas autodot was detected in the DAPI channel. Quantification of lipid droplets was performed after threshold setting with the "analyze particle" tool and total cell numbers were counted with the "cell counter" plugin.

RNA isolation and Microarray

Total RNA was prepared from wild type and *act1-159* log phase cells from biological triplicate cultures using a Qiagen RNAeasy kit including an on-column DNAse digestion step according to the manufacturer's instructions. Following reverse transcription reactions the cDNA template was hybridised to an Affymetrix Yeast 2.0 GeneChip array. Data was quality controlled and normalised using the Bioconductor plugin, affylmgui[85]. To reduce background noise we used the Robust Multi-Array Average (RMA) algorithm[86]. The affylmGUI package was run using R (version 3.1.0) to generate volcano plots. A significance threshold value for 95% odds of differential expression was chosen, which corresponds to a B statistic of 2.94 and above. We then sorted the significant data into groups for processing using Gene Ontology (GO) Slim Mapper and Yeastmine[87] analysis tools from SGD[70].

Immunoblotting

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For Western blot analysis, cell equivalents of an OD_{600} of 3 were harvested at 24 h after inoculation, and cell extracts were obtained from chemical lysis as described in[88]. Proteins were collected by centrifugation and resuspended in 75 µL 1× loading buffer (125 mM Tris-HCl, adjusted to pH 6.8; 20% glycerol; 3% SDS; 2% DTT; 0.1% bromophenol blue), and heated to 95 °C for 10 min. Samples were centrifuged at 13,000 rpm for 12 s and 10 µL or 15 µL of the supernatant was used for standard SDS-PAGE. Immunoblotting followed standard procedures, with transfer of proteins to a 0.45 µm nitrocellulose membrane and probing with antibodies against phospho-Slt2 (Phospho-p44/42 MAPK, cell signalling, #9101, 1:1000), actin (α-Yeast act1 Goat monoclonal antibody, a kind gift from Prof. John Cooper, Washington University, 1:2000), (glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Life Technologies, MA515738, 1:5000), or VDAC/porin (Abcam, ab110326, 1:5000). As secondary antibodies, we used IRDye goat anti-mouse (Licor, 926–68070, 1:20,000) or IRDye goat anti-rabbit (Licor, 928-40028, 1:20,000) as listed in the key resources table. Signals were recorded with Odyssey Glx, with automatically determined exposure times. Quantitative analysis of western blots was performed using image studio software.

Lipid Extraction and Quantification by Shotgun Mass Spectrometry

Yeast cultures were inoculated from stationary overnight cultures in YPD to fresh YPD medium to an OD_{600} of 0.1. In total, 2 OD_{600} units were harvested after 24 h and homogenised with 0.5 mm zirconia beads in a cooled tissue lyser for 2 × 10 min at 30 Hz in 300 µL IPA. The whole homogenate was evaporated in a vacuum desiccator to complete dryness. Lipid extraction was performed according to[89–91]. In brief, 700 µL internal standard mix in 10:3 MTBE/MeOH was added to each sample and vortexed for 1 h at 4 °C. After the addition of 140 µL H_2O, samples were vortexed for another 15 min. Phase separation was induced by centrifugation at 13,400

rpm for 15 min. The organic phase was transferred to a glass vial and evaporated. Samples were reconstituted in 300 µL 1:2 MeOH/$CHCl_3$. For lipidome, 5 µL of sample were diluted with 95 µL 4:2:1 IPA/MeOH/CHCl3 + 7.5 mM ammonium formate.

Mass spectrometric analysis was performed on a Q Exactive instrument (Thermo Fisher Scientific, Bremen, DE) equipped with a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca, NY, USA) using nanoelectrospray chips with a diameter of 4.1 µm. The ion source was controlled by the Chipsoft 8.3.1 software (Advion BioSciences). Ionisation voltage was +0.96 kV in the positive and −0.96 kV in the negative mode; back pressure was set at 1.25 psi in both modes. Samples were analysed by polarity switching[91]. The temperature of the ion transfer capillary was 200 °C; S-lens RF level was set to 50%. Each sample was analysed for 18 min. FT-MS spectra were acquired within the range of m/z 400–1000 from 0 min to 0.2 min in the positive mode, and within the range of m/z 350–1200 from 6.2 min to 6.4 min in the negative mode at a mass resolution of R m/z 200 = 140,000, automated gain control (AGC) of 3×10^6, and with a maximal injection time of 3000 ms. Ergosterol was determined by parallel reaction monitoring (PRM) FT-MS/MS between 0.2 and 1.7 min. For FT-MS/MS, micro-scans were set to 1, isolation window to 0.8 Da, normalised collision energy to 12.5%, AGC to 5×10^4, and maximum injection time to 3000 ms. t-SIM in positive (1.7 to 6 min) and negative (6.4 to 18 min) mode was acquired with R @ m/z 200 = 140,000; automated gain control of 5×10^4; maximum injection time of 650 ms; isolation window of 20 Th; and scan range of m/z 400 to 1000 in positive and m/z 350 to 1200 in negative mode, respectively. The inclusion list of masses targeted in t-SIM analyses started at m/z 355 in negative and m/z 405 in positive ion mode, and other masses were computed by adding 10 Th increment (i.e., m/z 355, 365, 375, …) up to m/z 1005 in positive mode and up to m/z 1205 in negative mode. All acquired spectra were filtered by PeakStrainer (https://git.mpi-

cbg.de/labShevchenko/PeakStrainer/wikis/home)[92] and stitched together by an in-house-developed script[93]. Lipids were identified by LipidXplorer software[94]. Molecular fragmentation query language (MFQL) queries were compiled for ergosterol, ergosterol esters, PC, LPC, PE, LPE, PI, LPI, PA, LPA, PG, LPG, PS, LPS, TG, DG, IPC, MIP2C, and MIPC lipid classes. The identification relied on accurately determined intact lipid masses (mass accuracy better than 5 ppm) and a signal-to-noise threshold higher than 3. Lipids were quantified by comparing the isotopically corrected abundances of their molecular ions with the abundances of internal standards of the same lipid class. Ergosterol, as well as ergosterol esters, were normalised to the internal cholesterol and internal CE standard, respectively.

Lipid Extraction and Quantification by Thin-Layer Chromatography

Yeast cultures were inoculated from stationary overnight cultures in YPD to fresh YPD medium to an OD_{600} of 0.1. In total, 80 OD600 units were harvested at 24 h after inoculation. Total lipids were extracted with chloroform/methanol 2:1 (*v/v*) according to Folch et al. . Cholesterylformate (Sigma, S448532) was added to each sample before extraction as an internal standard. The organic phase was dried under a stream of nitrogen and dissolved in 1 mL of chloroform/methanol (2:1, *v/v*). For neutral lipid separation a total of 40 µL of lipid extracts was sprayed on HPTLC silica gel 60 plates, 20 x 10 cm (Merck, 1.05641.001) using a CAMAG automatic TLC sampler (ATS4), whereas for phospholipid analysis only 20 µL were applied. Lipid separation was performed using a CAMAG automatic developing chamber (ADC2). Neutral lipids were separated with n-hexane, n-heptane, diethylether, acetic acid (63/18.5/18.5/1 v/v) as mobile phase[96], whereas phospholipid separation was carried out using $CHCl_3$/MeOH/water (32.5:12.5:2) mixture as mobile phase[97–99]. HPTLC plates were derivatized with 0.01 % primuline applied in a CAMAG derivatizer followed by mild heating

to 40°C for 2 minutes on a CAMAG TLC plate heater 3. Developed HPTLC plates were imaged using a CAMAG TLC visualizer 2 with VisionCATS software. Since peak separation of PI and PS was not ideal in all samples we conducted an additional derivatisation step with ninhydrin spray reagent (Sigma Aldrich, N1286), which only stains phospholipids containing free amino groups and thus allows quantification of PS without PI. HPTLC bands were processed into chromatograms and quantified by polynomial regression of standard curves calculated from the applied standards. For phospholipids the standard contained l-α-phosphatidylinositol (840044P), phosphatidylcholine (16:0-18:1; 850457P), phosphatidylethanolamine (16:0-18:1; 850757P), phosphatidylserine (18:1-18:1; 840034P), cardiolipin (18:1-18:1; 710335P), phosphatidic acid (16:0-18:1; 840101P) each at 500 ng/µl all purchased individually from Sigma Aldrich. As a neutral lipid standard we either used a 1:10 dilution of the nonpolar lipid mixture B from Matreya (#1130) which is an equal component mix of cholesteryl-oleate, methyloleate, triolein, oleic acid, and cholesterol (each at 5 µg/µl) additionally supplemented with diacylglycerol (16:0-18:1; Sigma 800815O) or a custom-made neutral lipid standard consisting of a mix of cholesteryl-oleate (700269P), cholesterylformate (S448532), triolein (870110O), diacylglycerol (16:0-18:1; 800815O), oleic acid (O1008) all purchased individually from Sigma Aldrich, and ergosterol from Thermofisher Scientific (117810050) each at 500 ng/µl. The standards were applied at increasing quantities from 250 ng to 15 µg absolute mass.

Electron microscopy high pressure freezing with a Leica EM HPM 100 and freeze substitution

Yeast strains were inoculated from a stationary overnight culture in YPD to an OD_{600} of 0.1. Cells were grown in YPD media and harvested at stationary phase after 24 h of growth and immediately subjected to high pressure freezing. Cell peletts were loaded and frozen using 2000

bar under liquid nitrogen conditions within milliseconds. Freezing was followed by freeze substitution in acetone by adding 2 % osmium tetroxide (OsO_4) and 0.2 % uranyl acetate (UAc) at temperatures below −70°C. After substitution, the samples were embedded in TAAB (Agar Scientific, Essex,GB) epoxy resin[100]. High pressure frozen yeast cells were sectioned (70 nm) with a UC7 Ultramicrotome (Leica Microsystems, Vienna, Austria) and stained with lead citrate for 5 min and platinum blue[101] for 15 min. Images were taken at 120 kV with a Tecnai G2 FEI (Thermo Fisher Scientific) microscope equipped with an Ultrascan 1000 CCD Camera (Gatan). Measurement of cell wall thickness on electron micrographs was performed in ImageJ using the measure tool. For each strain condition at least 117 measurements of the inner and outer cell wall at equally distributed loci around the cells were performed.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were calculated in Graphpad Prism 8. Information on tests for significance is given in each figure. Error bars indicate standard error of the mean (SEM) and asterisks in the figures indicate significant differences, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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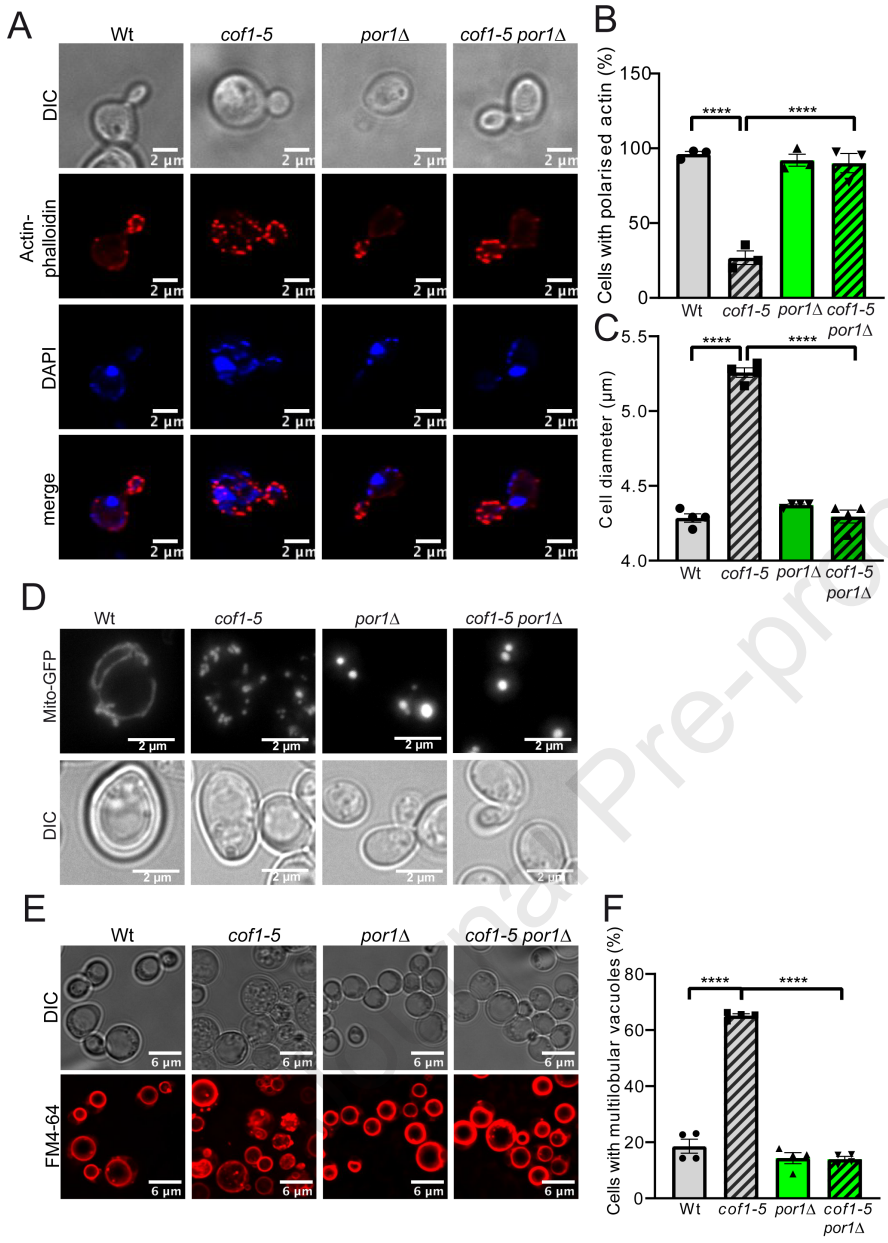
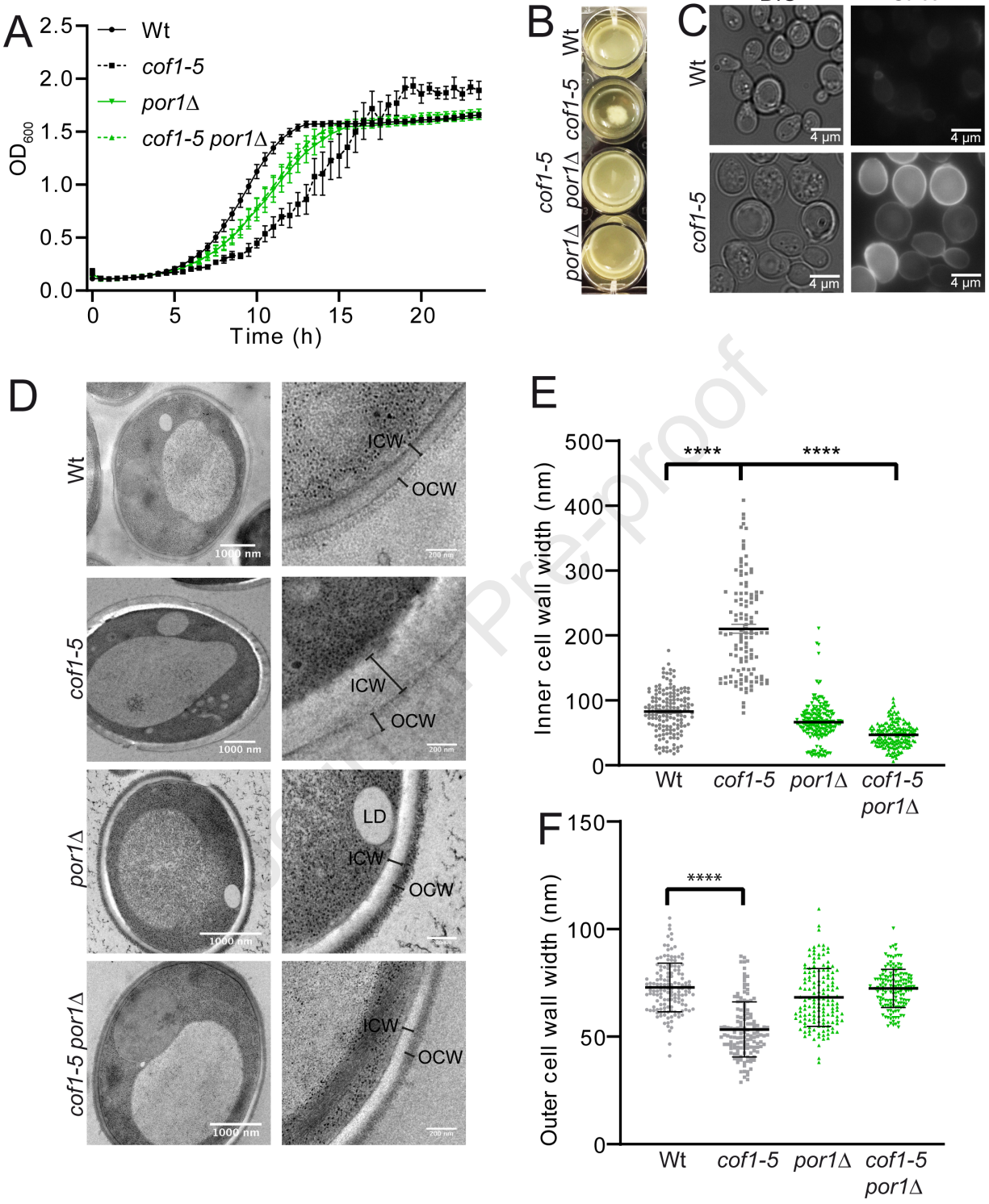
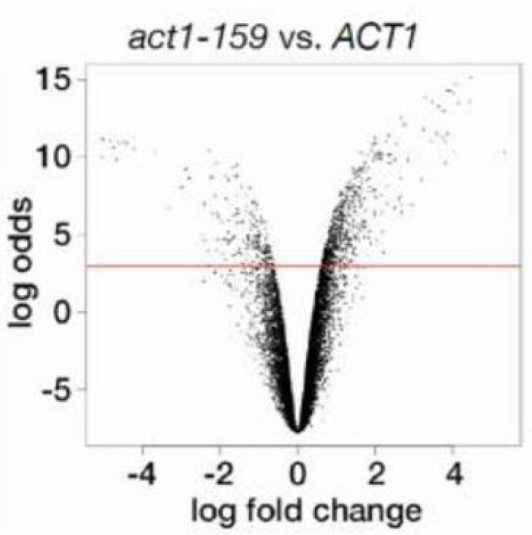
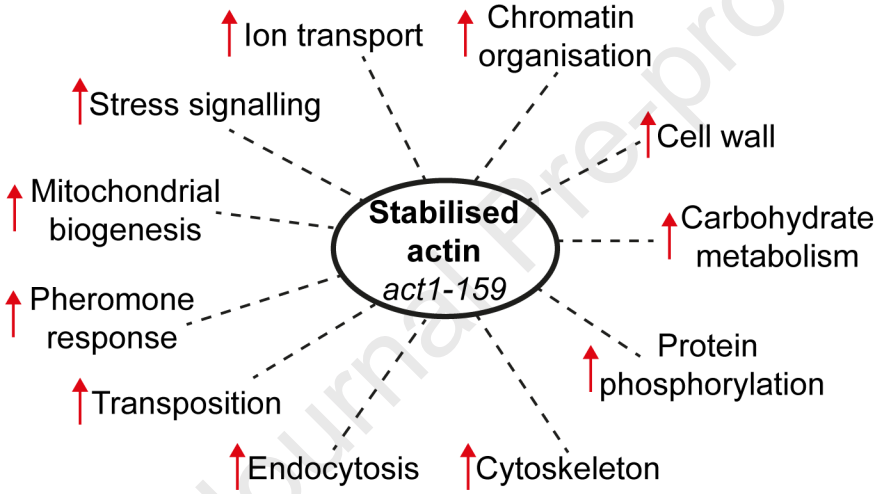

A
Wt
cof1-5
por1Δ
cof1-5 por1Δ
DIC
Actin-phalloidin
DAPI
merge
2 µm
B
Cells with polarised actin (%)
150
100
50
0
****
****
Wt
cof1-5
por1Δ
cof1-5 por1Δ
C
Cell diameter (µm)
5.5
5.0
4.5
4.0
****
****
Wt
cof1-5
por1Δ
cof1-5 por1Δ
D
Wt
cof1-5
por1Δ
cof1-5 por1Δ
Mito-GFP
DIC
2 µm
E
Wt
cof1-5
por1Δ
cof1-5 por1Δ
DIC
FM4-64
6 µm
F
Cells with multilobular vacuoles (%)
80
60
40
20
0
****
****
Wt
cof1-5
por1Δ
cof1-5 por1Δ



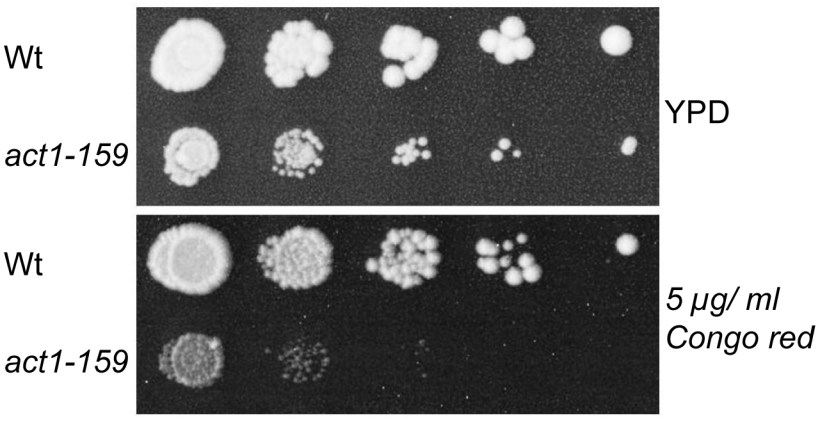
A

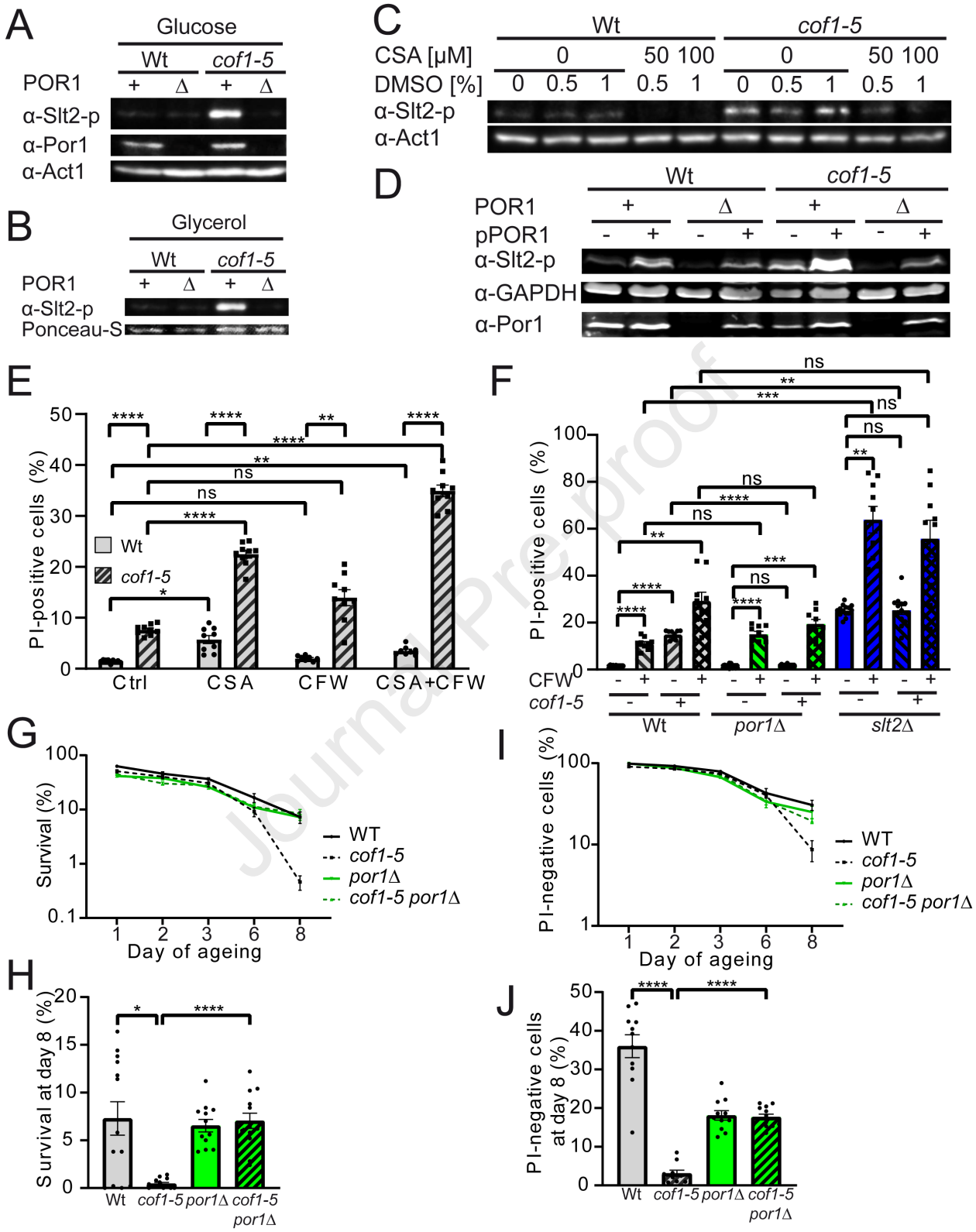


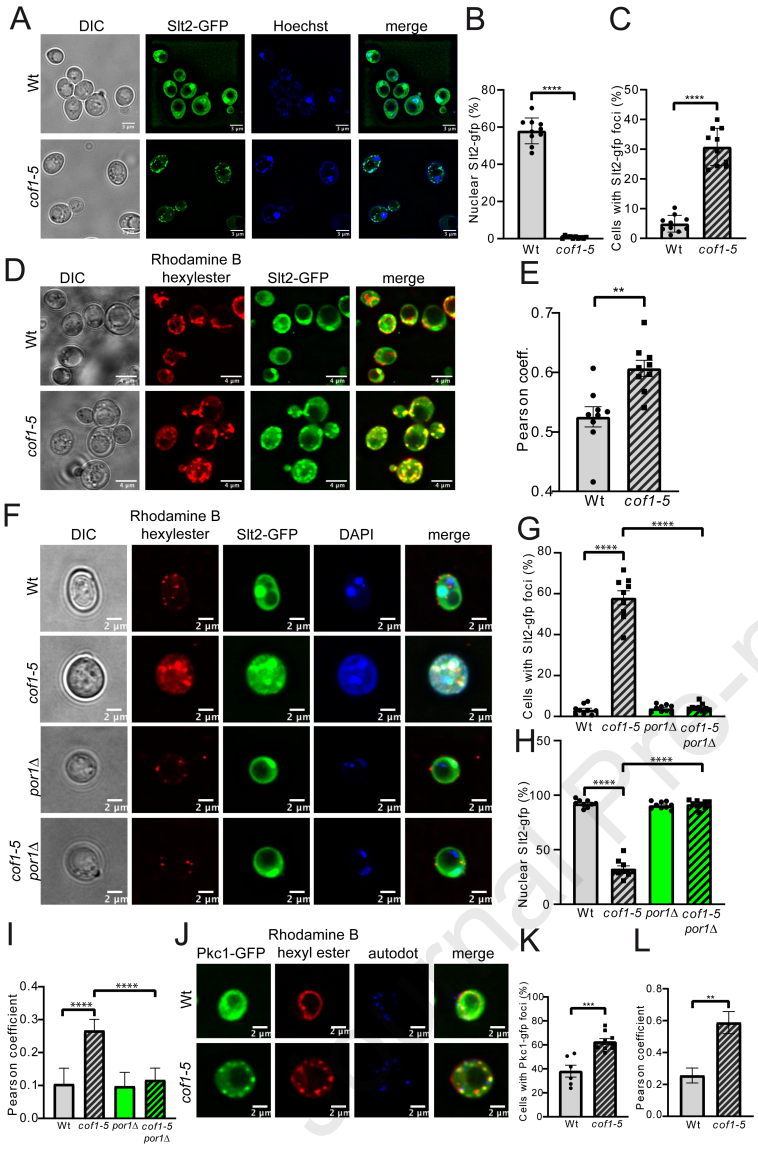
B

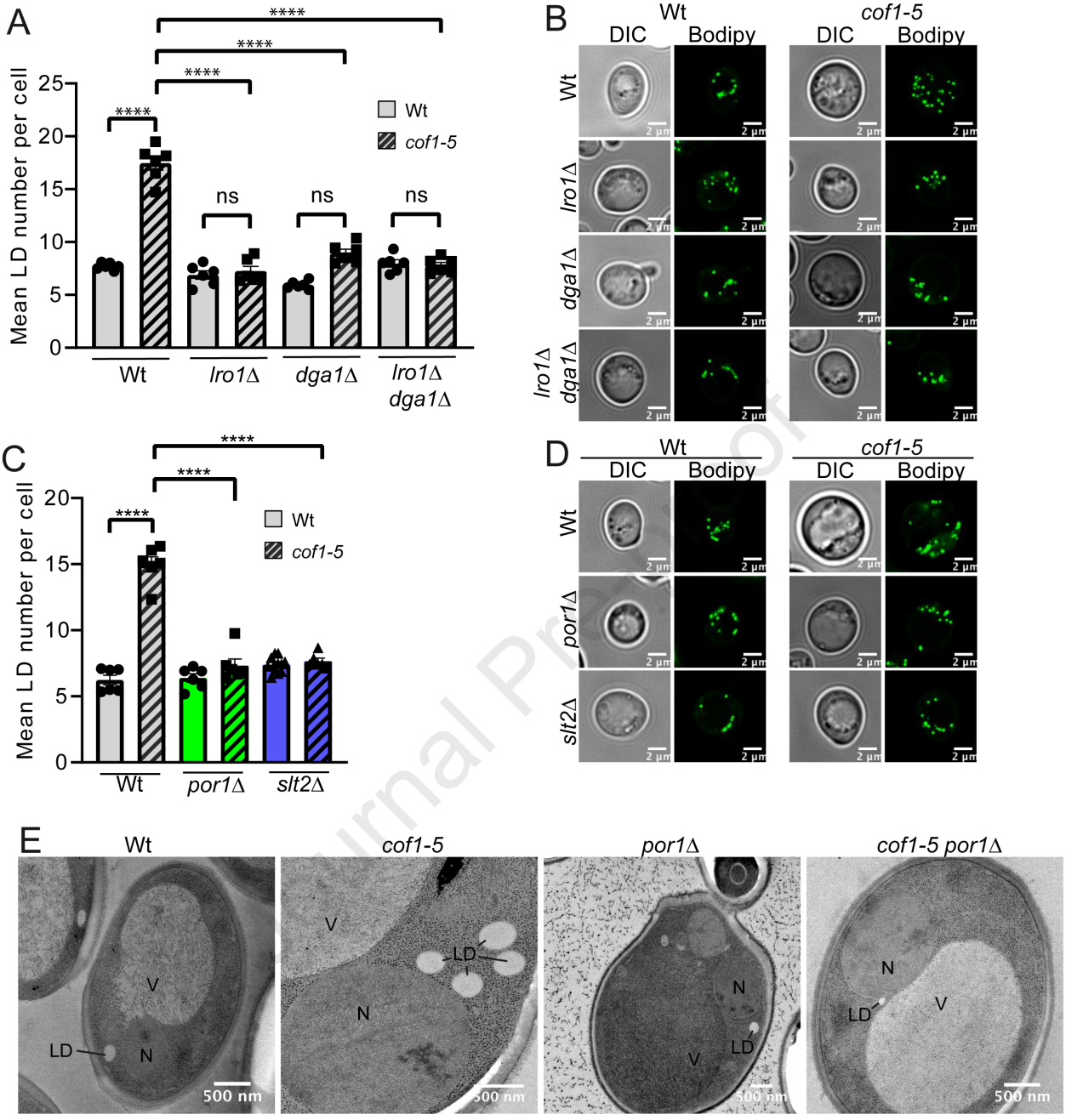


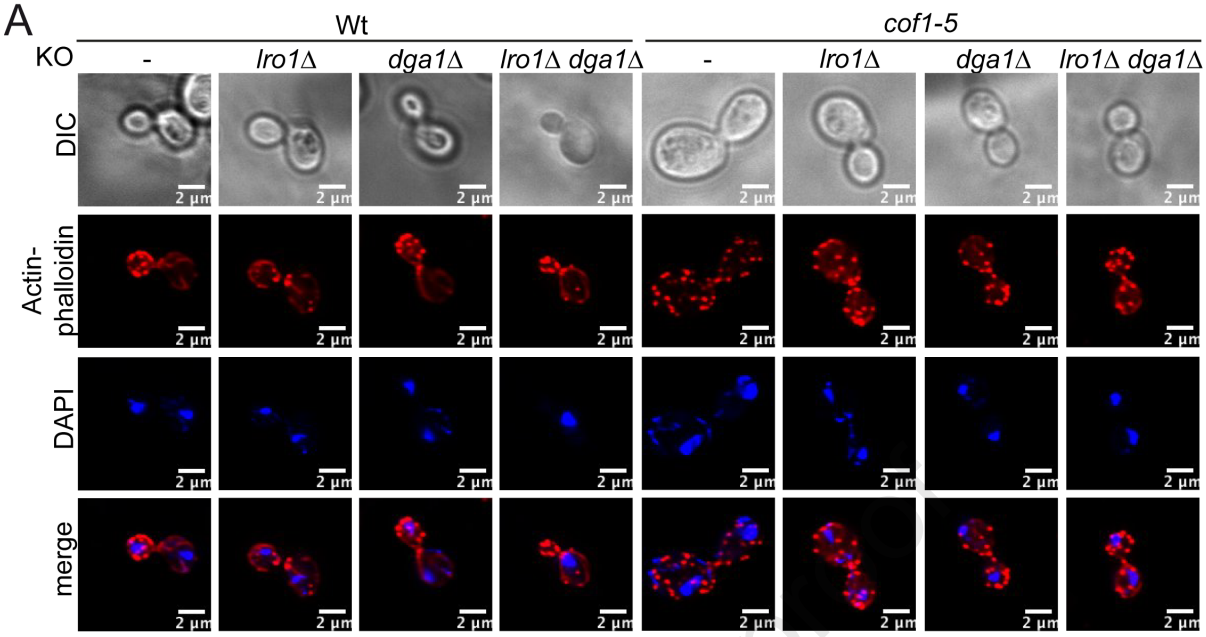
C

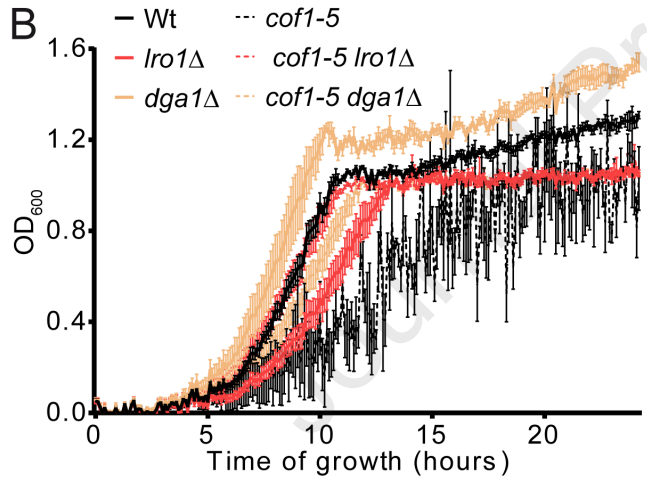


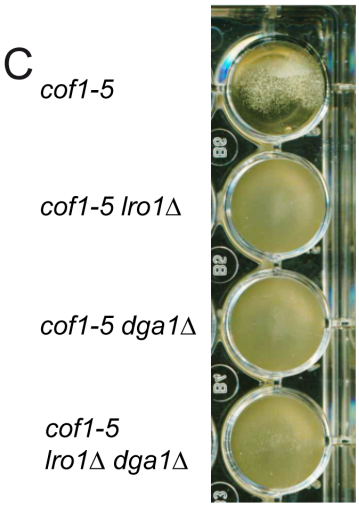

A
Glucose
Wt
cof1-5
POR1
+ Δ + Δ
α-Slt2-p
α-Por1
α-Act1
B
Glycerol
Wt
cof1-5
POR1
+ Δ + Δ
α-Slt2-p
Ponceau-S
C
Wt
cof1-5
CSA [μM]
0
50 100
0
50 100
DMSO [%]
0 0.5 1 0.5 1 0 0.5 1 0.5 1
α-Slt2-p
α-Act1
D
Wt
cof1-5
POR1
+ Δ + Δ
pPOR1
- + - + - + - +
α-Slt2-p
α-GAPDH
α-Por1
E
PI-positive cells (%)
Wt
cof1-5
Ctrl
CSA
CFW
CSA+CFW
F
PI-positive cells (%)
CFW
cof1-5
Wt
por1Δ
slt2Δ
G
Survival (%)
Day of ageing
WT
cof1-5
por1Δ
cof1-5 por1Δ
H
Survival at day 8 (%)
Wt
cof1-5
por1Δ
cof1-5 por1Δ
I
PI-negative cells (%)
Day of ageing
WT
cof1-5
por1Δ
cof1-5 por1Δ
J
PI-negative cells at day 8 (%)
Wt
cof1-5
por1Δ
cof1-5 por1Δ

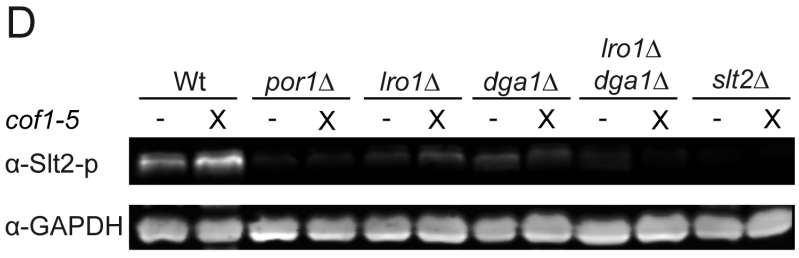

A
DIC
Slt2-GFP
Hoechst
merge
Wt
cof1-5
B
Nuclear Slt2-gfp (%)
****
Wt
cof1-5
C
Cells with Slt2-gfp foci (%)
****
Wt
cof1-5
D
DIC
Rhodamine B hexylester
Slt2-GFP
merge
Wt
cof1-5
E
Pearson coeff.
**
Wt
cof1-5
F
DIC
Rhodamine B hexylester
Slt2-GFP
DAPI
merge
Wt
cof1-5
por1Δ
cof1-5 por1Δ
G
Cells with Slt2-gfp foci (%)
****
****
Wt
cof1-5
por1Δ
cof1-5 por1Δ
H
Nuclear Slt2-gfp (%)
****
****
Wt
cof1-5
por1Δ
cof1-5 por1Δ
I
Pearson coefficient
****
****
Wt
cof1-5
por1Δ
cof1-5 por1Δ
J
Pkc1-GFP
Rhodamine B hexyl ester
autodot
merge
Wt
cof1-5
K
Cells with Pkc1-gfp foci (%)
***
Wt
cof1-5
L
Pearson coefficient
**
Wt
cof1-5

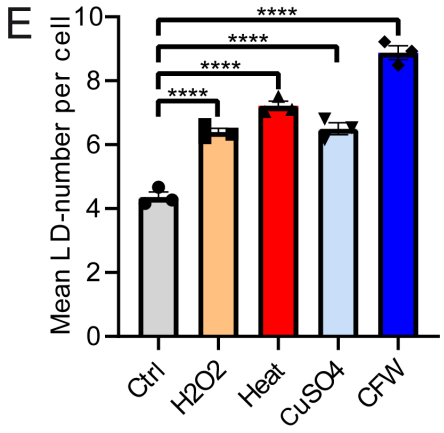


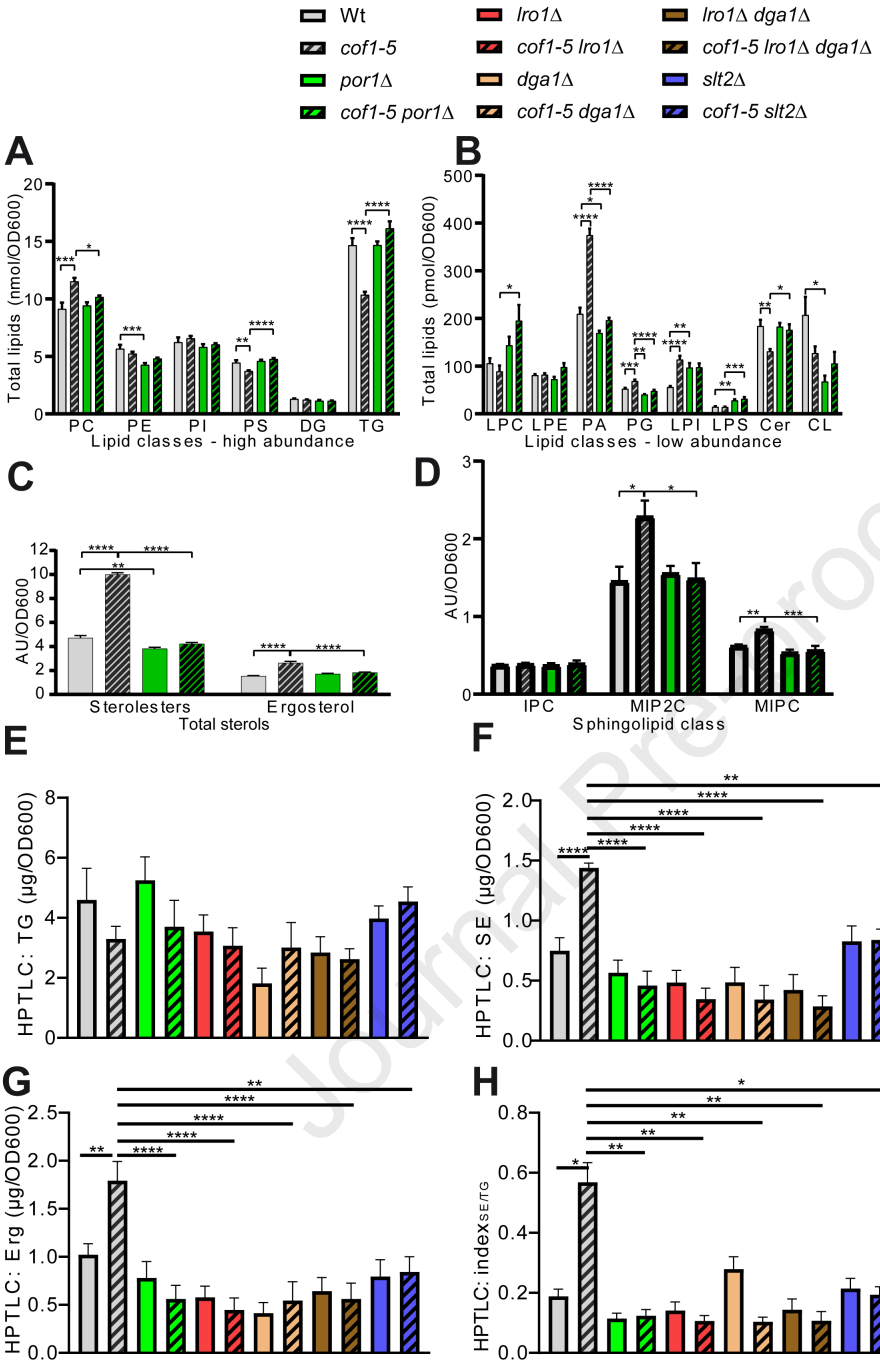

A
Wt
cof1-5
KO
-
lro1Δ
dga1Δ
lro1Δ dga1Δ
DIC
Actin-phalloidin
DAPI
merge
2 µm


B
Wt
lro1Δ
dga1Δ
cof1-5
cof1-5 lro1Δ
cof1-5 dga1Δ
OD600
1.6
1.2
0.8
0.4
0.0
0
5
10
15
20
Time of growth (hours)


C
cof1-5
cof1-5 lro1Δ
cof1-5 dga1Δ
cof1-5 lro1Δ dga1Δ


D
Wt
por1Δ
lro1Δ
dga1Δ
lro1Δ dga1Δ
slt2Δ
cof1-5
- X - X - X - X - X - X
α-Slt2-p
α-GAPDH


E
Mean LD-number per cell
10
8
6
4
2
0
****
****
****
****
Ctrl
H2O2
Heat
CuSO4
CFW



Highlights

1) ***cof1-5* mutation activates CWI-signalling in a VDAC-dependent manner.**
2) ***cof1-5* expression promotes MAPK signalling from the mitochondrial compartment.**
3) **Cofilin and VDAC/ Porin are important regulators of lipid homeostasis.**

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
α-Phospho-p44/42 MAPK	cell signalling	#9101
α-Yeast act1 Goat monoclonal antibody	John Cooper	n/a
α-GAPDH	Life Technologies	MA515738
α-VDAC/porin	Abcam	ab110326
IRDye goat anti-mouse	Licor	#926–68070
IRDye goat anti-rabbit	Licor	#928-40028
Bacterial and virus strains		
pYX122-mtGFP	B. Westermann[76]	pCG44
pRS426-SLT2-GFP	Matthias Peter[34]	PPR A75
pAG418-POR1	This study	YPR B06
pDONR221-*POR1*	DNASU	FLH201444.01X
pFA6a-KanMX6	Addgene.org[74]	#39296; PPR C31
pFA6a-Ura3	Addgene.org[72]	#61924; PPR C29
pYM27	EUROSCARF[73]	YPR B35
Chemicals, peptides, and recombinant proteins		
Cercosporamide (CSA)	Sigma-Aldrich	SML0172
Calcofluor white (CFW)	Sigma-Aldrich	910090
Phalloidin-Tetramethylrhodamine B-Isothiocyanate	Sigma-Aldrich	P1951
SynaptoRed™ C2 (equivalent to FM4-64)	Biotum	BOT-70021
Rhodamine B hexylester perchlorate	Molecular Probes	Y-7530
Bodipy 493/503	Sigma-Aldrich	#490389
Autodot	Abcepta	# SM1000a
Critical commercial assays		
RNAeasy kit	Qiagen	#74004
Yeast 2.0 GeneChip array	Affymetrix	#900553
Deposited data		
Raw and analyzed data	This paper, Mendeley Data	10.17632/bgkscw9ns9.1
Microarray	This paper; Mendeley Data	10.17632/bgkscw9ns9.1
Shotgun lipidomics	This paper; Mendeley Data	10.17632/bgkscw9ns9.1
HPTLC lipidomics	This paper; Mendeley Data	10.17632/bgkscw9ns9.1
Experimental models: Organisms/strains		
S. cerevisiae: Strain background: BY4742 Matα *ura3*-52 *his3*Δ200 *leu2*-3,112 *lys2*-801 *ade2*-101 *COF1::LEU2*	Pekka Lappalainen[25]	YPR K43
cof1-5 COF1::LEU2	Pekka Lappalainen[25]	YPR K44
*por1*Δ	This paper	YPR K45
*cof1-5 por1*Δ	This paper	YPR K46
*lro1*Δ	This paper	YPR K62
*cof1-5 lro1*Δ	This paper	YPR K47
*dga1*Δ	This paper	YPR K63
*cof1-5 dga1*Δ	This paper	YPR K48
*lro1*Δ *dga1*Δ	This paper	YPR K64
*cof1-5 lro1*Δ *dga1*Δ	This paper	YPR K49

slt2Δ	This paper	YPR T29
cof1-5 slt2Δ	This paper	YPRT31
Wt SLT2-EGFP	This paper	YPR T12
cof1-5 SLT2-EGFP	This paper	YPR T15
S. cerevisiae: Strain background: BY4741 MATa *ACT1::HIS3 his3*D200 *tub2*-101 *ura3*-52 *leu2*-3, 112	David Drubin[32]	YPR K50
MATa *act1*-159::*HIS3 his3*D200 *tub2*-101 *ura3*-52 *leu2*-3, 112	David Drubin[32]	YPR K51
Oligonucleotides		
LRO1_fw:CCATTACAAAAGGTTCTCTACCAACGAATTCGGCGACAATCGAGTAAAAACAGCTGAAGCTTCGTACGC	This paper	n/a
LRO1_rev:TTCGCTCTTTGAAATAATACACGGATGGATAGTGAGTCAATGTCGGTCATGCATAGGCCACTAGTGGATCTG	This paper	n/a
DGA1_fw:TACATATACATAAGGAAACGCAGAGGCATACAGTTTGAACAGTCACATAACAGCTGAAGCTTCGTACGC	This paper	n/a
DGA1_rev:AAAATCCTTATTTATTCTAACATATTTTGTGTTTTCCAATGAATTCATTAGCATAGGCCACTAGTGGATCTG	This paper	n/a
POR1-S1: CCAACACGAAACAGCCAAGCGTACCCAAAGCAAAAATCAAACCAACCTCTCAACACGTACGCTGCAGGTCGAC	This paper	A80
POR1-S2: AAGAACGAGCACATATATGGTATATAGTGAACATATATATATTAGATATATACGTATCGATGAATTCGAGCTCG	This paper	A81
SLT2-S1: CTATCAAAATAGTAGAAATAATTGAAGGGCGTGTATAACAATTCTGGGAGATGCGTACGCTGCAGGTCGAC	This paper	B40
SLT2-S2: CTTACATCTATGGTGATTCTATACTTCCCCGGTTACTTATAGTTTTTTGTCCTAATCGATGAATTCGAGCTCG	This paper	B41
SLT2-S3: GCTTCTAGACCTTGAAAAAGAGCTGGAGTTTGGATTAGATAGAAAATATTTTCGTACGCTGCAGGTCGAC	This paper	B42
PKC1-S2: CCGCTTAGATGTTTTATATAAAATTAAATAAATCATGGCATGACCTTTTCTTCAATCGATGAATTCGAGCTCG	This paper	B45
PKC1-S3: GCCAGCAAGAAGAGTTTAGAGGATTTTCCTTTATGCCAGATGATTTGGATTTACGTACGCTGCAGGTCGAC	This paper	B46
Software and algorithms		
Fiji (ImageJ)	https://imagej.nih.gov/ij/[81]	n/a
GraphPad Prism 8.4.3	www.graphpad.com	n/a
R (3.1.0)	www.R-project.org	n/a
Slim Mapper	https://www.yeastgenome.org/goSlimMapper	n/a
Yeastmine	https://yeastmine.yeastgenome.org/yeastmine/begin.do[87]	n/a

Bioconductor plugin affylmgui	[85]	n/a
Robust Multi-Array Average (RMA) algorithm	[86]	n/a
PeakStrainer	https://git.mpi-cbg.de/labShevchenko/PeakStrainer/wikis/home[92]	n/a
LipidXplorer software	[94]	n/a
in-house-developed script	[93]	n/a
Other		
Resource website for yeast genetics SGD	https://www.yeastgenome.org/[70]	n/a