Decrease in plasma membrane tension triggers PtdIns(4,5)P$_2$ phase separation to inactivate TORC2

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The target of rapamycin complex 2 (TORC2) plays a key role in maintaining the homeostasis of plasma membrane (PM) tension. TORC2 activation following increased PM tension involves redistribution of the Slm1 and 2 paralogues from PM invaginations known as eisosomes into membrane compartments containing TORC2. How Slm1/2 relocalization is triggered, and if/how this plays a role in TORC2 inactivation with decreased PM tension, is unknown. Using osmotic shocks and palmitoylcarnitine as orthogonal tools to manipulate PM tension, we demonstrate that decreased PM tension triggers spontaneous, energy-independent reorganization of pre-existing phosphatidylinositol-4,5-bisphosphate into discrete invaginated membrane domains, which cluster and inactivate TORC2. These results demonstrate that increased and decreased membrane tension are sensed through different mechanisms, highlighting a role for membrane lipid phase separation in mechanotransduction.

The plasma membrane (PM) is fundamental for cell survival. Not only does it form a selectively permeable barrier and dynamic interface, but it also serves as a scaffolding platform on which many fate-determining signalling decisions are taken. Furthermore, PM tension, defined as the in-plane counteracting force to surface expansion, plays an important role in transferring and integrating information in cells and within tissues$^{4–6}$. Biological membranes are constantly affected by processes (for example, endo/exocytosis and cell migration) that impinge on their surface area, composition and the activity of attachment proteins. This adds complexity but also provides the cell with multiple options for regulation of PM tension. Indeed, all cell types react, albeit with different kinetics, to counteract perturbations in PM tension, suggesting that this is a tightly controlled biophysical parameter$^{4–6}$.

Despite its established importance, little is known about the mechanisms by which PM tension is sensed and regulated. We recently identified the ‘target of rapamycin complex 2’ (TORC2) as a regulator of cell surface area and PM tension homeostasis$^7$. Specifically, we found that manipulations that presumably increase PM tension, including mechanical stretch of the PM, inhibition of sphingolipid biosynthesis and hypo-osmotic shock, all trigger redistribution of the Slm proteins away from furrow-like invaginations organized by BAR protein assemblies (Bin, Amphiphysin and Rvs) called eisosomes$^{8,9}$, into membrane compartments containing TORC2 (MCTs)$^9$. The signal that triggers Slm protein relocalization remains mysterious, but membrane stretch has been reported to similarly induce mTORC2 signalling in mammalian cells$^{10}$, implying that TORC2 activation by increased PM tension is conserved. Our model does not consider what happens in the case of a decrease in PM tension, although one might assume that Slm1/2 would relocalize away from MCTs and back into eisosomes, inactivating TORC2. Here, we describe a small-molecule modulator of TORC2 signalling, palmitoylcarnitine (PalmC), which acts primarily by reducing PM tension, monitored in live yeast cells with a mechanosensitive probe. Using PalmC and hyper-osmotic shocks as orthogonal approaches to diminish membrane tension, we found that neither treatment acutely affects Slm1 localization, but both induce the phase separation of pre-existing phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$) into pronounced PM invaginations sequestering TORC2, leading to its inactivation.

Results

TORC2 senses hypo- and hyper-osmotic stress through independent mechanisms. Slm1 and 2 have previously been implicated in TORC2 signalling$^{11,12}$ and specifically in the perception of an increase in PM tension upstream of TORC2 (ref. 7). In exponentially growing cells, ~60% of the Slm proteins localize to punctate inclusions called eisosomes$^{14,15}$, with the remainder in MCTs. With increased tension, the eisosomal pool relocates to MCTs, and TORC2 signalling is increased$^7$. To further explore the molecular mechanisms regulating TORC2 activity, we applied osmotic shocks to single cells expressing Slm1-GFP and Lsp1-mCherry, which mark eisosomes. Changes in the co-localization between the two markers were compared with the evolution of TORC2 activity, assessed by monitoring the phosphorylation status of the direct TORC2 substrate T$_{662}$ in Ypk1 (refs. $^{15,16}$). Consistent with our previous work$^7$, following a hypo-osmotic shock, Slm1-GFP moved out of eisosomes and TORC2 was activated with similar kinetics (Fig. 1a–c). However, although TORC2 activity was inhibited under a hyper-osmotic shock, both Slm1-GFP and Lsp1-mCherry distributions remained unaffected (Fig. 1d–f). We noted that hypo-osmotic shock transiently stimulated TORC2 activity in

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an amplitude-dependent manner, but that TORC2 activity went back to steady-state levels after only 5 min, regardless of the amplitude of the shock that was applied to the cells (Supplementary Fig. 1a and c, left panel). Hyper-osmotic shock also caused a transient and amplitude-dependent inhibition of TORC2 activity, but in this case, the return of TORC2 activity to its basal level was slower and amplitude-dependent (Supplementary Fig. 1b and c, right panel). Together, these results demonstrate that hypo- and hyper-osmotic shocks to yeast cells respectively activate and inhibit TORC2 activity, assessed by monitoring Ypk1T662 phosphorylation. Presented blots are representative of results obtained in three independent experiments, and all unprocessed scans are shown in Supplementary Fig. 7.

### Fig. 1 | TORC2 senses hypo- and hyper-osmotic shocks through different mechanisms.

a. d. Top section confocal images of cells expressing Slm1-GFP and Lsp1-mCherry following hypo- (a) or hyper-osmotic (d) shock. The percentage of co-localization between markers is indicated as the mean from \( n > 30 \) cells (\( n = 34 \) cells for hypo-osmotic shock and \( n = 38 \) for hyper-osmotic shock) pooled from three independent experiments. Scale bars, 5 μm. b, e. Hypo- (b) and hyper-osmotic (e) shocks respectively activate and inhibit TORC2 activity, assessed by monitoring Ypk1T662 phosphorylation. Presented blots are representative of results obtained in three independent experiments, and all unprocessed scans are shown in Supplementary Fig. 7. c, f. Correlation between Slm1-GFP/eisosome co-localization and TORC2 activity following hypo- (c) or hyper-osmotic (f) shock. Error bars represent s.d. of mean values from three independent experiments concerning TORC2 activity, or the s.d. to the mean calculated from \( n > 30 \) (\( n = 34 \) cells for hypo-osmotic shock and \( n = 38 \) for hyper-osmotic shock) cells pooled from three independent experiments in the case of the co-localization between Slm1 and eisosomes. Source data are provided in Supplementary Table 3.

### TORC2 signalling affects PM tension.

We entertained the idea that PM tension per se might be what is sensed upstream of TORC2. Hypo- and hyper-osmotic shocks are assumed to increase and decrease PM tension by increasing and decreasing turgor pressure, respectively. To verify that osmotic shocks can be used to manipulate PM tension in yeast, we used the recently developed mechanosensitive ‘FliptR’ (fluorescent lipid tension reporter) probe20 (Supplementary Fig. 2a). This twisted push–pull fluorophore stably integrates into the PM of cells where its planarization and polarization, and thus its fluorescence properties, are sensitive to mechanical forces acting on the membrane (Supplementary Fig. 2b).

Specifically, its fluorescence lifetime, which can be determined by fluorescence lifetime imaging microscopy (FLIM), changes linearly with PM tension in model membranes and in mammalian cells11, making it a valuable tool to monitor PM tension changes in situ. Consistently, hypo- and hyper-osmotic shocks to yeast cells respectively triggered an increase and a decrease in the FliptR lifetime,
A decrease in PM tension leads to clustering of TORC2 to PtdIns(4,5)P₂-enriched PM domains. We first investigated the effect of a PaltmC-induced decrease in PM tension on TORC2 localization. As previously observed, TORC2, visualized through a GFP tag fused to its Avo3 subunit, localized to many small foci distributed along the PM. Following PaltmC treatment, these foci clustered into fewer, brighter puncta (Fig. 4a). We measured and plotted the size distributions of these fluorescence maxima for untreated and PaltmC-treated cells and found that they had very little overlap. This allowed us to define a fluorescent intensity threshold that separates small foci from larger puncta (Supplementary Fig. 4a). Puncta become noticeable after a few minutes of treatment, crested in number after 30 min, when they were present in 84% of the cells, and persisted for up to 90 min. The size and kinetics of assembly and disassembly of these structures were dose-dependent. Notably, this timing correlated with both the kinetics of inhibition and reactivation of TORC2 activity and with a decrease and increase in PM tension as read out by the FlipTR probe (Fig. 4b). Thus, TORC2 clustering into puncta is correlated with its inactivation.

Decreasing PM tension with a hyper-osmotic shock mimicked the PaltmC effect and triggered TORC2 puncta formation; however, direct inhibition of TORC2 activity by the ATP-competitive small molecule NVP-BHS345 did not (Supplementary Fig. 4b). Together, these results suggest that clustering into puncta is potentially the cause of TORC2 inactivation in response to a decrease in PM tension, and not a consequence of its inactivation.

As with hyper-osmotic shock, the cellular distribution of Slm1-mCherry remained largely unaffected during the first 20 min after PaltmC treatment, but these proteins then also began to cluster (Fig. 4c). We used the same threshold method to quantify Slm1 puncta (Supplementary Fig. 4c) and observed that they crested after 60 min of treatment. Interestingly, the kinetics of Slm1 puncta formation tracks with the kinetics of TORC2 reactivation (Fig. 4b). Inhibition of TORC2 results in an increase in PM tension (Fig. 2b), and we assume that once cells have restored a sufficient amount of tension in the PM, the Slm proteins reassociate with TORC2, leading to its reactivation and dissociation of the TORC2 puncta. Consistent with this model, anchoring of Slm1 to eisosomes during PaltmC recovery prevented TORC2 reactivation (Supplementary Fig. 4d,e).

TORC2 is recruited to the PM via the pleckstrin homology (PH) domain of its Avo1 subunit which binds PtdIns(4,5)P₂ (ref. 19). Also, tor2 mutants interact genetically with Mss4 (ref. 27), which encodes the sole PtdIns(4)P-5-kinase in yeast. Thus, we speculated that decreased PM tension might affect the cellular distribution of this lipid, in turn affecting TORC2 activity. To investigate this, we examined the cellular distribution of PtdIns(4,5)P₂ using the biosensor GFP-2xPHFLG (ref. 20). In basal conditions, PtdIns(4,5)P₂ was distributed uniformly along the PM, in accordance with previous studies. Following PaltmC treatment or hyper-osmotic shock, we observed a fast and striking redistribution of GFP-2xPHFLG at the PM, whereas direct inhibition of TORC2 did not trigger this lipid rearrangement (Fig. 4d). Staining cells expressing the GFP-2xPHFLG biosensor with FM4-64 showed that the clusters appearing with decreased PM tension are truly enriched in PtdIns(4,5)P₂, as evidenced by the ratiometric comparison of the images acquired in the two channels (Fig. 4e). These PtdIns(4,5)P₂-enriched structures (PEs) quickly disassemble on PM stretching induced by a hypo-osmotic shock (Fig. 4f), further supporting the idea that a decreased PM tension constitutes the primary cause of their formation.

PM buckling into large invaginations has been observed when cells possess an excess of PM, for example, after hyper-osmotic shock. Interestingly, PaltmC-treated, but not control cells, presented large folded PM invaginations, enriched in PtdIns(4,5)P₂, when observed by electron microscopy after anti-GFP immunogold labeling in cells expressing the PtdIns(4,5)P₂ biosensor (Supplementary Fig. 4f). Similar structures were previously observed in cells deleted proportional to the intensity of the shock (Fig. 2a). This confirms that such shocks can be used to manipulate PM tension in yeast and that the FlipTR probe can be used to qualitatively report on changes in PM tension.

We had previously proposed that TORC2 is part of a homeostatic feedback loop that maintains PM tension. The FlipTR probe now allows us to test this model directly. We chose to inhibit TORC2 using a recently described chemical–genetic approach, where TORC2, but not TORC1, is specifically inhibited by rapamycin-FKBP12. Rapamycin treatment of these cells caused a progressive increase in the lifetime of the fluorophore (Fig. 2b), indicating an increase in PM tension. Conversely, enhanced TORC2 signalling in a strain expressing a hyperactive Ypk2 allele lowered PM tension, evidenced by a reduced lifetime of the fluorophore (Fig. 2c). Together, these data add strong support to the model that TORC2 works in a functional feedback loop to maintain PM tensile homeostasis.

**PaltmC, a small-molecule tool to inhibit TORC2 and manipulate PM tension.** In parallel to these studies, we launched a high-throughput screening campaign to identify small molecules that specifically interfere with TOR signalling in W7 yeast cells (described in the Methods, Supplementary Fig. 3a,b and Supplementary Tables 4 and 5). This effort revealed that PaltmC (Supplementary Fig. 3c) is an inhibitor of TORC2, but not TORC1, signalling. Specifically, our screen revealed that the toxicity of PaltmC is reduced in cells expressing hyperactive Ypk2 (Supplementary Fig. 3d). As this allele suppresses the lethality of TORC2 mutants, this result suggested that PaltmC is toxic specifically because it interferes with TORC2 signalling. Consistently, treatment of cells with PaltmC induced a fast, transient and dose-dependent inhibition of TORC2 activity, but not TORC1 activity, assessed by monitoring the TORC1-specific hydrophobic motif phosphorylation of Sch9 (Fig. 3a,b). Inclusion of PaltmC in vitro kinase assays had no effect on TORC2 kinase activity (Fig. 3c) suggesting that it targets a factor upstream of TORC2. Sensors of the Hog and CWI pathways were again dispensable for the response of TORC2 to PaltmC (Supplementary Fig. 3e), and Hog1 was not activated by PaltmC treatment (Supplementary Fig. 3f), suggesting that these pathways are not implicated in the PaltmC mode of action.

Based on the amphiphilic properties of PaltmC we hypothesized that its effects could be due to modification of the biophysical properties of the PM, particularly tension. Consistent with this idea, we observed a fast and dramatic decrease in the lifetime of the FlipTR probe after PaltmC treatment (Fig. 3d), demonstrating that the drug induces a decrease in PM tension. If this is the cause of TORC2 inhibition, we would expect that hypo-osmotic shock would suppress this inhibition. To test this prediction, we used fps1Δ cells that cannot efficiently export the osmo-protectant glycerol and thus are slow to recover from hypo-osmotic shock. Simultaneous application of a hypo-osmotic shock to these cells largely negated PaltmC-induced TORC2 inactivation (Fig. 3e), whereas hyper-osmotic shock displayed an additive effect with PaltmC on TORC2 inhibition (Fig. 3f). Similar structures were previously observed in cells deleted...
for the genes encoding inositol-5-phosphatases, and thus contain elevated levels of PtdIns(4,5)P₂ (ref. 33). These structures are reminiscent of failed endocytic events34; however, endocytosis requires an intact actin cytoskeleton in yeast35, and pretreatment with latrunculin A (LatA), an actin depolymerizing agent, did not prevent PES formation (Supplementary Fig. 3g). Additionally, LatA treatment did not affect PES dissolution, implying that endocytosis is not essential to resolve these structures (Supplementary Fig. 4h).

As TORC2 clusters and PES appear after PalmC treatment with similar kinetics, we asked whether or not these structures are related. Indeed, we found that Avo3-GFP and mCherry-2xPH PLC₅ puncta co-localized (Fig. 4g, 60.3 ± 14.6% co-localization). Together, these results suggest that the relocalization and clustering of TORC2 into PES is part of a common mechanism to transiently inhibit TORC2 activity following a decrease of PM tension. At later time points, Slm1-mCherry puncta also co-localized with PES (Fig. 4h, 65.4 ± 10.4% co-localization), probably to re activate TORC2 as described above.

PtdIns(4,5)P₂ is crucial for TORC2 clustering and inhibition following a decrease in PM tension. We postulated that the interaction between the PH domain of Avo1 and PtdIns(4,5)P₂ constitutes the driving mechanism responsible for TORC2 clustering. Both the localization and activity of TORC2 are lost following the shift of thermosensitive mss4-103 cells to non-permissive temperature (Supplementary Fig. 5a,b). To prevent the detachment of TORC2 from the PM following loss of PtdIns(4,5)P₂, we replaced the PH domain of Avo1 with a CAAX motif, to enable the PtdIns(4,5)P₂-independent, yet fully functional, PM recruitment of TORC236. The localization of TORC2CAAX in mss4-103 cells was assessed at permissive (30°C) and non-permissive (37°C) temperatures, before and after PalmC treatment. At the permissive temperature, TORC2CAAX failed to cluster at the non-permissive temperature (Fig. 5a). Growth at 37°C per se did not alter the PalmC-induced relocalization of TORC2. These observations demonstrate that PtdIns(4,5)P₂ is necessary for TORC2 clustering following PalmC treatment in a manner that does not solely involve the PH domain of Avo1.

We next queried whether the remodelling of the PM itself upon PalmC treatment was also PtdIns(4,5)P₂-dependent. The absence of PtdIns(4,5)P₂ in mss4-103 cells precluded the use of the GFP-2xPHPLC₅ biosensor so, for these experiments, changes in the PM structure were visualized using FM4-64. At the permissive temperature, in both WT and mss4-103 cells, FM4-64 puncta appeared following PalmC treatment and

**Fig. 2** | **TORC2 regulates PM tension in a homeostatic feedback loop manner.** **a**, Osmotic shocks impact PM tension in a dose-dependent, linear fashion. Cells were submitted to a range of osmotic shocks and the lifetime of the FliptR probe was determined by FLIM. Note that different control values for the hyper- and hypo-osmotic shocks are due to the different initial growth conditions: synthetic complete (SC) versus SC + 1 M sorbitol. Error bars represent propagated error of mean values for three independent experiments (with = 20 cells). Source data are provided in Supplementary Table 3.

**b**, Elevated TORC2 signalling lowers PM tension following a decrease in PM tension. We postulated that the interaction between the PH domain of Avo1 and PtdIns(4,5)P₂ constitutes the driving mechanism responsible for TORC2 clustering. Both the localization and activity of TORC2 are lost following the shift of thermosensitive mss4-103 cells to non-permissive temperature (Supplementary Fig. 5a,b). To prevent the detachment of TORC2 from the PM following loss of PtdIns(4,5)P₂, we replaced the PH domain of Avo1 with a CAAX motif, to enable the PtdIns(4,5)P₂-independent, yet fully functional, PM recruitment of TORC236. The localization of TORC2CAAX in mss4-103 cells was assessed at permissive (30°C) and non-permissive (37°C) temperatures, before and after PalmC treatment. At the permissive temperature, TORC2CAAX failed to cluster at the non-permissive temperature (Fig. 5a). Growth at 37°C per se did not alter the PalmC-induced relocalization of TORC2. These observations demonstrate that PtdIns(4,5)P₂ is necessary for TORC2 clustering following PalmC treatment in a manner that does not solely involve the PH domain of Avo1.

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co-localized with PES (Fig. 5b). FM4-64 clusters were not observed in mss4-103 TORC2$_{CAAX}$ cells following PalmC treatment at the restrictive temperature (Fig. 5b), demonstrating that PtdIns(4,5)P$_2$ is required for the remodelling of the PM that follows a decrease in PM tension. Furthermore, although the basal activity of the TORC2$_{CAAX}$ variant is relatively low in mss4-103 cells (Supplementary Fig. 5c), this remaining activity is largely resistant to PalmC treatment when cells are incubated at the non-permissive temperature (Fig. 5c). This indicates that PtdIns(4,5)P$_2$-mediated TORC2 clustering is necessary for TORC2 inhibition. Yeast cells growing in the wild must
A decrease in PM tension triggers PtdIns(4,5)P₂ phase separation of cells experiencing a sudden decrease in PM tension. TORC2, and that inhibition of TORC2 signalling is an essential intermediate in the sensing of decreased PM tension upstream of mss4-103 of PM tension. Indeed, inactivation of mss4-103, but not the temperature shift per se, impaired regrowth following transient PalmC treatment. Clustering was assessed in three independent experiments, each including 50 cells. TORC2 activity was monitored by Ypk1-T662 phosphorylation and error bars represent s.d. of three independent experiments. PM tension was monitored through the FlpR probe lifetime and error bars represent propagated error of mean values of three independent experiments (n = 20 cells).

**Fig. 4 | Decreased PM tension causes TORC2 to cluster into PtdIns(4,5)P₂-enriched structures.** a, PalmC induces rapid TORC2 (Avo3-GFP, a) and delayed Slm1-mCherry (c) clustering. The percentage of cells displaying clusters is indicated as the mean of three independent experiments including 50 cells. Yellow arrows indicate TORC2 puncta (a) and Slm1 puncta (c). b, Correlation of TORC2 clustering (dark blue), Slm clustering (light blue), TORC2 activity (black) and PM tension (red), following PalmC treatment. Clustering was assessed in three independent experiments, each including 50 cells. TORC2 activity was monitored by Ypk1-T662 phosphorylation and error bars represent s.d. of three independent experiments. PM tension was monitored through the FlpR probe lifetime and error bars represent propagated error of mean values of three independent experiments (n = 20 cells).

da, In a decrease in PM tension, but not direct inhibition of TORC2, triggers PtdIns(4,5)P₂ redistribution. Time lapse of cells expressing the GFP-2xPHPLC biosensor following a 1 M hypo-osmotic shock. Time lapse of cells expressing the GFP-2xPHPLC biosensor upon PalmC treatment, hyper-osmotic shock or rapamycin treatment. e, Membrane domains formed following decreased PM tension are enriched in PtdIns(4,5)P₂. Cells expressing the GFP-2xPHPLC biosensor and labelled with FM4-64 were mock treated or treated with PalmC for 5 min. The last column presents the ratiometric images of the two channels, constructed using the ImageJ image calculator tool. f, An increase in PM tension induces fast disassembly of the PES. Time lapse of cells expressing the GFP-2xPHPLC biosensor and pretreated with PalmC for 15 min following a 1 M hypo-osmotic shock. g, h, TORC2 (g) and Slm1 (h) clusters co-localize with PES after PalmC treatment. The percentage of co-localization between markers is the mean calculated from 10 cells pooled from two independent experiments ± s.d. All images are maximum projections of 0.5-μm-spaced z-planes of the cells, and representative of results obtained in at least two independent experiments. Scale bars, 5 μm. Source data are provided in Supplementary Table 3.

respond to stresses that affect plasma membrane tension such as osmotic shock, and we wondered if TORC2 inactivation contributes to the ability of cells to tolerate such stresses. To test this, we questioned whether depletion of PtdIns(4,5)P₂ would affect the ability of cells to survive and/or recover from stresses that trigger acute loss of PM tension. Indeed, inactivation of mss4-103, but not the temperature shift per se, impaired regrowth following transient PalmC exposure or hyper-osmotic shock (Fig. 5d and Supplementary Fig. 5d). These observations indicate that PtdIns(4,5)P₂ is a crucial intermediate in the sensing of decreased PM tension upstream of TORC2, and that inhibition of TORC2 signalling is an essential response of cells experiencing a sudden decrease in PM tension.

A decrease in PM tension triggers PtdIns(4,5)P₂ phase separation. Quantification of GFP-2xPHPLC⁺ signal after PalmC treatment shows PtdIns(4,5)P₂ clustering into distinct puncta, but no global increase in PtdIns(4,5)P₂ (Supplementary Fig. 6a,b). Mss4 co-localizes to PalmC-induced PES, cresting at 15 min and disappearing by 90 min (Fig. 6a), suggesting that decreased PM tension might act through Mss4 to create PES.

To challenge this hypothesis, we tested whether PES would still form in ATP-depleted cells, in which PtdIns(4,5)P₂ synthesis cannot occur. Following ATP depletion, PtdIns(4,5)P₂ is rapidly turned over through the action of several phosphatases (Inp51, 52 and 53) and the GFP-2xPHPLC⁺ biosensor dissociates from the PM within a minute (Fig. 6b, top). Deletion of INP51 and INP52 prevents this loss of PtdIns(4,5)P₂. Remarkably, even after ATP depletion, PalmC treatment still rapidly induces the formation of PES in inp51Δ inp52Δ cells (Fig. 6b, bottom). These results demonstrate that decreased PM tension triggers PES formation via a redistribution of pre-existing PtdIns(4,5)P₂, and not via de novo synthesis. PtdIns(4,5)P₂ present within PES is not selectively shielded from
Fig. 5 | PtdIns(4,5)P₂ is crucial for TORC2 inhibition and cell survival following an acute decrease in PM tension. a. PtdIns(4,5)P₂ is required for TORC2 clustering with a decrease in PM tension. Avo3-GFP localization following PalmC treatment, in WT and mss4-103 TORC2CAAX cells grown at the indicated temperatures for 90 min. Yellow arrows indicate TORC2 puncta. b. PtdIns(4,5)P₂ is required for PM remodelling with decreased PM tension. FM4-64 PM labelling in WT and mss4-103 TORC2CAAX cells expressing GFP-2xPHPLC and grown at the indicated temperatures for 90 min, following PalmC treatment. c. PtdIns(4,5)P₂ is required for TORC2 inhibition with a decrease in PM tension. Evolution of Ypk1₆₆² phosphorylation with PalmC or Wortmannin (Wort, 2 µM, 5 min) treatment, in WT and mss4-103 TORC2CAAX cells grown at the indicated temperatures for 90 min. Unprocessed scans of blots are shown in Supplementary Fig. 7. Error bars represent s.d. of mean values of three independent experiments, and source data are provided in Supplementary Table 3. d. TORC2 inhibition is necessary for efficient survival of an acute decrease in PM tension. WT or mss4-103 TORC2CAAX cells were grown at the indicated temperature for 30 min before being treated with 10 µM PalmC for 60 min or 2 M sorbitol for 15 min. Serial dilutions were then spotted onto yeast extract–peptone-dextrose (YPD) plates and cell regrowth was monitored 24 h later. All images are maximum projections of 0.5-µm-spaced z-planes of the cells, and are representative of results obtained in at least three independent experiments. Scale bars, 5 µm.

phosphatases because the GFP-2xPHPLC probe is also rapidly relocalized from PES to the cytosol following ATP depletion in WT, but not inp51Δ inp52Δ cells (Supplementary Fig. 6c). From these results we conclude that Mss4 relocalization ensures that PtdIns(4,5)P₂ levels remain high in PES while PM tension is low and that ATP depletion per se does not trigger PES disassembly.

Finally, we wondered whether the formation of PES could be due to a lipid phase separation within the PM. To assess this, we used the lipophilic dye Laurdan and subsequent calculation of the generalized polarization (GP) as a readout for lipid order. Laurdan partitions equally into liquid-disordered (Ld) or liquid-ordered (Lo) membranes and is not associated to specific lipids, so GP values reflect the overall organization of the PM, being high in Lo phases and low in Ld phases. The pixel histogram obtained from the GP images of untreated PM followed a normal distribution, meaning that the PM globally displays a homogeneous organization (Fig. 6c). After PalmC treatment, we observed the appearance of a second population of pixels displaying higher GP (Fig. 6c), indicating the presence of a more ordered phase. Using cells expressing the mCherry variant of the PtdIns(4,5)P₂ biosensor, we confirmed that the domains of high GP correspond to PES (Fig. 6d). Moreover, we observed the appearance of high-GP puncta upon PalmC treatment in ATP-depleted inp51Δinp52Δ, but not WT, cells (Fig. 6c). This confirms the necessity of PtdIns(4,5)P₂ in the phase separation process. Endocytic BAR domain proteins such as Rvs161 or its dimerization partner Rvs167 could conceivably drive lipid phase separation. However, this seems unlikely as Rvs167-GFP does not redistribute to PES following decreased PM tension (Supplementary Fig. 6d). Collectively, our results support the model that PES formation is the result of a spontaneous phase separation of PtdIns(4,5)P₂.
Here, we have demonstrated that decreased PM tension triggers a spontaneous, energy-independent PtdIns(4,5)P$_2$ phase separation. Cryo-electron microscopy revealed that TORC1 clusters are regulated through fundamentally different mechanisms (Fig. 6f). TORC1 and TORC2 are actually a giant helix of regularly assembled TORC1 dimers, organized such that the kinase active site is physically occluded and thus not accessible to substrate. Given the structural similarities between TORC1 and TORC2, we anticipate that the TORC2 clusters that we report here will represent an analogous, higher-order TORC2 assembly responsible for its inactivation.

This result presents the interesting possibility that PtdIns(4,5)P$_2$ phase separation is the primary molecular sensor of decreased PM tension. PtdIns(4,5)P$_2$ patches are well suited to pattern the localized events required for the regulation of a given signalling pathway, by recruiting and activating a range of effector proteins. For example, PES-like structures have also been observed in higher eukaryotes.
where they function to organize CD44–Ezrin interactions linking the PM to the cytoskeletal actin network\(^a\), and to promote exocytosis by concentrating soluble N-ethylmaleimide-sensitive factor attachment-protein receptor (SNARE) proteins\(^b,c\). Interestingly, local PtdIns(3,4)P\(_2\) production following growth factor deprivation was very recently implicated in the inhibition of mTORC1 residing on lysosomes and late endosomes\(^d\). Collectively, these observations suggest that protein sorting by lipid phase separation could constitute a currently underappreciated mechanism of signal transduction regulation in general and regulation of TOR signalling in particular.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41556-018-0150-z.

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Author contributions

M.R. and K.N.-S. carried out most of the experiments, with the exception of the GUV experiments (N.C.), electron microscopy experiments (B.K. and V.M.) and the screening campaign that led to the identification of PalM (M.S.) and S.S.M designed and synthesized the FlippR probe, whose properties were characterized by A.C. Finally, M.R., K.N.-S., A.R. and R.L. designed experiments, interpreted results, and wrote the manuscript with contributions from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Yeast strains and plasmids. All strains and plasmids used in this study are listed in Supplementary Tables 1 and 2. Yeast strains were generated either by homologous recombination of PCR-generated fragments as previously described or by crossing, sporulation, and subsequent dissection of the spores. All primers used for the generation of the strains are listed in Supplementary Table 6. Strains were confirmed by PCR and sequencing. Cloning and site-directed mutagenesis were performed following standard procedures, and plasmids were verified by sequencing. All tagged proteins are functional and expressed from their endogenous promoter.

Yeast culture. Yeast cells were grown according to standard procedures at 30°C or 37°C as indicated in eitherYPD or SC medium lacking appropriate amino acids required for plasmid selection to an optical density at 600 nm (OD600) of 0.6–0.8. For the hypo-osmotic shocks, cells were grown to OD600 = 0.8 in SC containing 1 M NaCl, before being diluted to the indicated sorbitol concentration by addition of pre-warmed SC medium. For hyper-osmotic shocks, cells were grown to OD600 = 0.6 in SC medium, before adding the appropriate volume of SC + 2 M sorbitol to reach the desired final sorbitol concentration.

Chemicals and drugs. Rapamycin (LC Laboratories) was dissolved in DMSO at 5 mg ml−1 and used at a final concentration of 2 μM. Cycloheximide (Sigma) was dissolved in H2O at 10 mg ml−1 and used at 2 μg ml−1. NVP-BHS345 (Novartis) was dissolved in DMSO at 10 mM and used at a final concentration of 1 μM. Palmitic acid was dissolved in DMSO at 10 mM and used at 10 μM, unless otherwise stated. The Flipping probe, dissolved in DMSO, was used at a final concentration of 2 ng ml−1. The Laurdan dye (6-dodecanoyl-2-dimethylaminonaphthalene) was maintained at a 2.5 mM stock solution in DMSF and used at 2.5 μM. The FM1-43 dye (11-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide, ThermoFisher) was dissolved at 10 mM in DMSO and used at a final concentration of 10 μM. For ATP depletion, cells were treated for 10 min with a combination of 3 mM NaN3 and 50 mM 2-deoxy-D-glucose.

High-throughput drug screen. Both TORC1 and TORC2 perform essential functions that can be genetically bypassed following the introduction of mutations that constitutively activate downstream effector pathways. Specifically, the lethality caused by loss of TORC1 activity can be suppressed by expression of a constitutively active variant of the Sch9 kinase and deletion of TPI1, which encodes a protein phosphatase regulator; lethality associated with the loss of TORC2 activity is suppressed by expression of a hyperactive variant of the Ypk2 kinase. To identify small molecules that potentially interfere with TORC1 and/or TORC2 signalling we screened 89,850 molecules from the Rockefeller HTSRC compound library23 and sought out compounds that are more toxic to WT than to bypass cells (Supplementary Fig. 3a,b and Supplementary Table 4). A total of 89,850 unique compounds from the Rockefeller University High Throughput and Spectroscopy Resource Center library were screened. They were part of the following commercially available libraries: ChemDiv (San Diego, CA), Cerep (Poitiers, France), ChemBridge (San Diego, CA), AMRI (Albany, NY), Greenpharma (Orléans, France), BioFocus (Charles River, Wilmington, MA), Prestwick Chemical (San Diego, CA) and LOPAC (Sigma, Carlsbad, CA). All strains and plasmids used in this study are listed in Supplementary Tables 1 and 2. Yeast strains were generated either by homologous recombination of PCR-generated fragments or by crossing, sporulation, and subsequent dissection of the spores. In addition to standard yeast culture conditions, lethality caused by loss of TORC1 activity can be suppressed by simultaneous inhibition of TORC2 with the mechanistic target of rapamycin (mTOR)-inhibitor Rapamycin, both strains, was kept in eight wells in column 24 and used as a positive control.

Drugs were used at a final concentration of 2 ng ml−1. Wortmannin (LC Laboratories) was dissolved in DMSO at 5 mg ml−1 and used at 10 μM. Cells were grown at 30 °C in SC medium to OD600 = 0.2% DMSO was kept in column 23 and used as a negative control. Rapamycin, ATP, 4,2 mM MgCl2 and 100 μg/ml gamma-32P-ATP. They were incubated for 10 min at 30 °C in a thermomixer rotating at 800 r.p.m., and terminated with the addition of 6 μS–PAGE sample buffer and a 10 min incubation at 80°C. The kinase reactions were performed following standard procedures, and plasmids were verified by sequencing. All tagged proteins are functional and expressed from their endogenous promoter.

Chemicals and drugs. Rapamycin (LC Laboratories) was dissolved in DMSO at 5 mg ml−1 and used at a final concentration of 2 μM. Cycloheximide (Sigma) was dissolved in H2O at 10 mg ml−1 and used at 2 μg ml−1. NVP-BHS345 (Novartis) was dissolved in DMSO at 10 mM and used at a final concentration of 1 μM. Palmitic acid was dissolved in DMSO at 10 mM and used at 10 μM, unless otherwise stated. The Flipping probe, dissolved in DMSO, was used at a final concentration of 2 ng ml−1. The Laurdan dye (6-dodecanoyl-2-dimethylaminonaphthalene) was maintained at a 2.5 mM stock solution in DMSF and used at 2.5 μM. The FM1-43 dye (11-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide, ThermoFisher) was dissolved at 10 mM in DMSO and used at a final concentration of 10 μM. For ATP depletion, cells were treated for 10 min with a combination of 3 mM NaN3 and 50 mM 2-deoxy-D-glucose.

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Fluorescence microscopy. Cells were grown at 30°C in SC medium to OD600 = 0.6, mounted on coverslips coated with Concanavalin A (Sigma) and immediately imaged with a spinning-disc microscope assembled by 3i (Intelligent Imaging Innovation) and Nikon (Eclipse CI1) using a x100 objective (NA=1.3, Nikon). Laurdan dye integrated into cell membranes was excited along the 405nm laser line and emission read at ~430–470nm (representing the liquid-ordered phase) or ~490–550nm (representing the liquid-disordered phase). For microfluidics experiments, a concanavalin A-coated coverslip was bonded to the bottom surface of a flow chamber (sticky-slide V1.04, Ibidi) with one entry connected to a syringe pump (Aladdin, World Precision Instrument) and the other left open for sequential injection of different solutions. The flow chamber was primed with SC medium before the loading of cells. Loaded cells were washed several times with SC medium, and then subjected to the appropriate treatments. Images were taken as either single focal planes or captured as z-series to generate 2D maximum intensity projections. For FLMJ, cells were grown overnight in SC medium to OD600 = 0.05–0.1, concentrated by spinning and incubated for 1 min with 2 ng ml−1 of the FlpFIR probe before imaging using the SynchroTime 64 software.

Electron microscopy. Fixed cells (2.5% glutaraldehyde in phosphate buffer 0.1 M for 2h) were further treated with 2% osmium tetroxide and immersed in a solution of uranyl acetate 0.25% overnight. The pellets were dehydrated in increasing concentrations of ethanol followed by pure propylene oxide, then embedded in Epon resin. Thin sections were stained with uranyl acetate and lead citrate and observed in a Tecnai 20 EM.

Image processing and quantification. For quantification of the co-localization of fluorescent markers (Figs. 1a,d and 4g,h), z-stacks of cells were recorded and deconvoluted. The same threshold was used for each channel in all tested conditions to create binary images. Binary masks were overlaid to an RGB image and yellow (co-localizing marker; ≥4 pixels) and green (non-co-localizing; ≥4 pixels) foci were counted using the ImageJ plugin-cell Counter (National Institutes of Health, https://www.nih.gov/). The percentage of foci containing co-localizing markers was calculated with Excel (Microsoft). Ratomic images (Fig. 4e) were constructed using the image calculator tool from Image.

Quantification of protein foci intensities (Supplementary Fig. 4a,c) was performed using ImageJ.

For FLIM analysis (Figs. 2, 3d and 4b), we used the SynchroTime 64 software to fit the data according to a 2-exponential convolution model and calculate the lifetime of the FlpFIR probe.
To study the evolution of PtdIns(4,5)P₂ concentration (Supplementary Fig. 6a), single focal plane images of the cell middle were taken, a 2-pixel-wide line was drawn around the cortex of cells using ImageJ, and fluorescence intensity values were plotted for each channel. For quantification of cellular total GFP-2xPHPLCδ fluorescence intensity (Supplementary Fig. 6b), 0.5-μm-spaced z-plane series of cells were taken with identical laser power and exposure time. The cytoplasmic mean fluorescence intensity was subtracted from the total cellular fluorescence intensity (both measured using ImageJ) for each focal plane before adding the obtained values.

GP values (Fig. 6c) were calculated for each pixel of a yeast PM according to the following equation:

\[ GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \]

where \( I_{440} \) and \( I_{490} \) represent the intensity of pixels in the areas of interest in the image acquired in the ordered and disordered spectral channels, respectively.

**Statistics and reproducibility.** The sample sizes and statistical tests were selected based on previous studies with similar methodologies. All experiments were repeated at least three times, giving similar results. The results of independent experiments are presented as mean values; error bars represent s.d., or the propagated error when the value of each experiment was itself calculated as a mean of individual cells. Statistical significance was tested using the two-tailed Student's t-test.

**Data availability.** Source data for Figs. 1–6 and Supplementary Figs. 1, 2, 5 and 6 are provided in Supplementary Table 3. All other data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Methods

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Unique biological materials

Policy information about availability of materials

Obtaining unique materials: All strains and plasmids can be obtained upon request. The FLipTR probe will be available commercially in the future.

Antibodies

Antibodies used: goat anti-Ypk1 1:1000 (Cell Signaling, no longer produced); mouse anti-phospho-Ypk1-T662 1:500 (see ref 7); rabbit anti-phospho-Sch9-T737 1:250 (see ref 45); rabbit anti-anti-Phospho-Ypk1-T662 1:10000 (Santa Cruz Technology sc-9079); rabbit anti-Hog1 1:1000 (Santa Cruz Technology sc-9079); and the appropriate infrared dye-coupled secondary antibodies used at a 1:10000 dilution (alexa Fluor 680-conjugated secondary anti-mouse and anti-rabbit Ab from Li-Cor Biosciences 926-68072 and 926-68073 respectively, and IRDye® 800 conjugated secondary anti-goat Ab from Rockland Bioconcept 605-732-125).

Validation: Cell Signaling anti phospho p38 (ref 92115). Antibody detects endogenous levels of p38 MAPK only when activated by phosphorylation at threonine 180 and tyrosine 182 [tested by Western Blot]. This antibody does not cross-react with the phosphorylated forms of either p42/44 MAPK or SAPK/INK. Polyclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues around Thr180/Tyr182 of human p38 MAPK. Antibodies are purified by protein A and peptide affinity chromatography. Species reactivity: Human, Mouse, Rat, Monkey, D. melanogaster, Pig, S. cerevisiae.

Santa Cruz anti Hog1 sc-9079: Hog1 Antibody (y-215) is a rabbit polyclonal IgG; 200 μg/mL. epitope corresponding to amino acids 221-435 mapping at the carboxy terminus of Hog1 of Saccharomyces cerevisiae origin, validated by western blot.
Anti-GFP Ab290 from AbCam: polyclonal IgG. This antibody is provided as whole antiserum. It is not possible to determine the exact antibody concentration, since whole serum contains many other host serum proteins besides the antibody of interest. On Western blot the antibody detects the GFP fraction from cell extracts expressing recombinant GFP fusion proteins and has also been shown to be useful on mouse sections fixed with formalin. In Immunocytochemistry, the antibody gives a very good signal on recombinant YES-GFP chimeras expressed in COS cells (McCabe et al. 1999 and figure below). It is routinely used in Immunoprecipitation (IP) and IP-Western protocols and has been used successfully in HRP Immunohistochemistry at 1:200 on whole-mount mouse embryos.

The anti-Ypk1 ab is not produce by cell signaling anymore.

See ref 7, 45, and 21 for the validation of the custom-made ab.