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Flippase-Mediated Phospholipid Asymmetry Promotes Fast Cdc42 Recycling in Dynamic Maintenance of Cell Polarity

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Abstract

Lipid asymmetry at the plasma membrane is essential for such processes as cell polarity, cytokinesis and phagocytosis¹⁻³. Here we identify the lipid flippase complex, composed of Lem3, Dnf1 or Dnf2⁴, to play a role in the dynamic recycling of the Cdc42 GTPase, a key regulator of cell polarity⁵, in yeast. By using quantitative microscopy methods, we show that the flippase complex is required for fast dissociation of Cdc42 from the polar cortex by the guanine nucleotide dissociation inhibitor (GDI). A loss of flippase activity, or pharmacological blockage of the inward flipping of phosphatidylethanolamine (PE), a phospholipid with a neutral head group, disrupts Cdc42 polarity maintained by GDI-mediated recycling. PE flipping may reduce the charge interaction between a Cdc42 C-terminal cationic region with the plasma membrane inner leaflet, enriched for the negatively charged lipid phosphatidylserine (PS). Using a reconstituted system with supported lipid bilayers, we show that the relative composition of PE versus PS directly modulates Cdc42 extraction from the membrane by GDI.

Establishing stable cell polarity is crucial for cellular morphogenesis and differentiation. The Cdc42 small GTPase localizes to the site of polarized growth in yeast and orchestrates structural and signaling events required for budding and mating^{5,6}. Previous work showed that the polarity of Cdc42 distribution is maintained dynamically through two colocalized recycling pathways to counter Cdc42 diffusion⁷⁻⁹. Whereas a slow pathway works through actin-based membrane trafficking, a fast pathway is mediated through Rdi1, the yeast GDI for Rho family GTPases¹⁰. GDI is known to extract Rho proteins from the membrane by forming a complex involving both protein-protein contacts and binding of GDI to the prenyl lipid anchor at the COOH terminus of these proteins^{11,12}. When actin polymerization is inhibited, Rdi1-mediated Cdc42 recycling is required for the polarization of Cdc42 at the presumptive bud site⁹.

In order to gain insights into the molecular mechanisms regulating the location and rate of Rdi1-mediated Cdc42 recycling, we performed a suppressor screen taking advantage of the fact that over-expression of Rdi1 under the Gal1 promoter represses yeast growth, likely due to over-extraction of Cdc42 and other essential Rho family GTPases from membrane

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AUTHOR CONTRIBUTIONS

A.D. and R.L. designed the experiments; A.D. performed all experiments and prepared the manuscript, with help from B.D.S. and J.R.U.; R.A. and D.B. assisted in the whole genome screening; B.R. assisted in data analysis; R.L. conceived and supervised the project and revised the manuscript.

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compartments¹³ (also see Fig. 1). We reasoned that deletion of a gene encoding a protein that facilitates Cdc42 extraction by Rdi1 might rescue the Gal-Rdi1 induced growth defect. As there are many possible mechanisms for the suppression of the growth defect, the candidates would be further narrowed down to those encoding proteins colocalizing with Cdc42 at the site of polarization. A centromeric plasmid expressing Rdi1 from the Gal1 promoter was transformed into each strain of the yeast haploid non-essential deletion library¹⁴. Growth of each strain over-expressing Rdi1 was quantified and normalized relative to its growth without Rdi1 over-expression. This screen revealed ~ 277 initial candidates rescuing the over-expression growth defect, among which 15 encode plasma membrane-localized proteins (Supplementary Information, Fig. S1b). Among these 15 genes, *LEM3* encodes a protein polarized at the site of polarization (Fig. 1a; Supplementary Information, Fig. S1a, S2a). Lem3 is a Cdc50 family protein forming a heterodimeric complex with P-type ATPases Dnf1 or Dnf2, which flips aminophospholipid from the outer to inner plasma membrane leaflet^{4,15,16}. Dnf2 was a weak rescuer from the screen (Supplementary Information, Fig. S1a). Both Dnf1 and Dnf2 also localize to the site of polarization, like Lem3 (Supplementary Information, Fig. S2a-c). This observation and their reported involvement in cell polarity¹⁷ led us to perform an in-depth study on the possible role for aminophospholipid flipping in regulating Cdc42 recycling at the polar cortex. To avoid heterogeneity in polarization stages, all the microscopy analyses in this work were performed with unbudded polarized cells with a Cdc42 polar cap at the incipient bud site.

To test if $\Delta Lem3$ reduces the ability of Rdi1 to extract Cdc42, we first used fluorescence correlation spectroscopy (FCS)¹⁸ to measure the mobile cytosolic concentration of GFP-Cdc42 expressed under the *CDC42* promoter^{7,9} in wild-type (Wt) and $\Delta Lem3$ cells with or without Rdi1 over-expression. FCS records fluorescence intensity fluctuations using a confocal microscope equipped with a photon counter. The autocorrelation curve of this data can be fitted to extract the molar concentration of the diffusing specie from the amplitude of the curve (G_0) and the diffusion time (τ_D) from the shape of the curve¹⁸. Autocorrelation curves of GFP-Cdc42 fitted poorly with a single-component diffusion model but significantly better ($p < 1 \times 10^{-10}$) with a two-component diffusion model (Fig. 1b). This suggests the existence of at least two pools of mobile Cdc42 population, 64% displaying fast diffusion ($\tau_D = 2.9 \pm 0.3$ ms) and 36% displaying slow diffusion ($\tau_D = 70 \pm 8$ ms) (Fig. 1c, d). The fast pool was significantly reduced in the $\Delta rdi1$ background and for the Cdc42^{R66E} mutant, defective in binding Rdi1¹⁹ (Fig. 1c, d). The vast majority (> 95%) of Rdi1 exists in a fast diffusing population ($\tau_D = 2.2 \pm 0.1$ ms) (Fig. 1e). The slow pool of Cdc42 was diminished by the *cdc42*^{C188S} mutation (Fig. 1c, d), which prevents prenylation. This analysis, along with the observed *in vivo* cross-correlation of Cdc42 and Rdi1⁹, suggests that the fast pool of Cdc42 consists largely of the Cdc42-Rdi1 complex, while the slow pool is dependent on prenylation and likely to be membrane vesicle-associated.

Induction of Rdi1 over-expression resulted in a time-dependent increase in total mobile Cdc42 concentration in the cytosol (Fig. 1f). This increase is accompanied by a similar increase in Cdc42 concentration in the fast but not slow pool, consistent with extraction of Cdc42 by Rdi1 from the plasma membrane into the soluble pool (Fig. 1f). Consistently, depletion of Cdc42 from the plasma membrane, especially from the polar cortex, can be observed (Fig. 1g, h). In $\Delta Lem3$ cells, the Cdc42 polar cap was more prominent than in Wt cells prior to Gal-Rdi1 induction (Fig. 1g, h), accompanied by a smaller mobile Cdc42 pool (Fig. 1i). Whereas Rdi1 overexpression reduced the concentration of Cdc42 in the polar cap of Wt cells, in $\Delta Lem3$ cells a nearly Wt pre-extraction level remained (Fig. 1g, h) even after 150 minutes of Rdi1 induction. The cytosol Cdc42 pool also remained constant (Fig. 1i). As the mean fluorescence intensity of GFP-Cdc42 over the entire cell was slightly reduced in $\Delta Lem3$ compared to that in Wt (by ~15%, Supplementary Information, Fig. S2d), the reduced Cdc42 soluble pool after 150 min Gal induction in $\Delta Lem3$ (by ~40%, Fig. 1f, i) can

be explained as a combined effect of a slightly reduced expression and the reduced extraction from the polar cortex.

We next performed fluorescence recovery after photobleaching (FRAP) experiments to examine the recycling of GFP-Cdc42 at the polar cortex⁹ and analyzed this data using a previously established mathematical model for Cdc42 recycling that allows extrapolation of the rates for Cdc42 dissociation and association from FRAP data⁹ (Fig. 2a, b; Supplementary Information, Fig.S2e-g). Model computation found that $\Delta lem3$ most significantly reduces m , the rate of Cdc42 dissociation from the polar cap region, but only slightly reduced h , the rate at which Cdc42 is targeted to the polar cap, while n , the internalization rate outside the cap remained similar (Fig. 2c-e). Under such scenario the model also predicts a slightly more pointed polar cap⁹, which could be observed in $\Delta lem3$ cells (Supplementary Information, Fig. S2h, i). The reduced m in $\Delta lem3$ was confirmed with inverse FRAP (iFRAP), which directly measures the rate of internalization of GFP-Cdc42 at the polar cap⁹ (Fig. 2f, g). For comparison, the Cdc42 internalization was not significantly affected by the $\Delta pil1$ mutation (iFRAP rate of $0.19 \pm 0.028 \text{ s}^{-1}$ compared to $0.21 \pm 0.01 \text{ s}^{-1}$ in wild-type), which disrupts the eisosomes and normal ergosterol distribution in the plasma membrane²⁰.

To determine whether $\Delta lem3$ specifically slows down Rdi1-mediated Cdc42 internalization, iFRAP was performed in the presence of latrunculin-A (LatA), which disrupts endocytosis²¹. LatA further slowed down Cdc42 dissociation in $\Delta lem3$ to an extent comparable to LatA's effect on Wt cells (Fig. 2f, g). By contrast, the combined effect of $\Delta rdi1$ and $\Delta lem3$ on Cdc42 dissociation rate was negligible compared to $\Delta rdi1$ alone (Fig. 2g). These results suggest that Lem3 selectively promotes Rdi1-mediated Cdc42 dissociation from the polar cortex. LatA treatment also led to drastic reduction of polarity in $\Delta lem3$ cells (Fig. 2h), consistent with a role for Lem3 as an important regulator of Rdi1-mediated Cdc42 recycling in the maintenance of cell polarity.

To test if the flippase complex is involved in fast Cdc42 dissociation from the polar cortex, we performed iFRAP on $\Delta dnf1$ and $\Delta dnf2$ mutant cells. Cdc42 dissociation was significantly slowed in these mutant cells (Fig. 3a). We further tested if acute inhibition of lipid flipping had the same effect by using the tetracyclic peptide Ro09-0198 (Ro, also called cinnamycin), which specifically binds to PE exposed on the outer plasma membrane leaflet when added to the culture media^{1,22}. Ro peptide at $50 \mu\text{M}$, which did not cause significant cell death compare to solvent control ($5.6 \pm 0.29\%$ compared to $4.3 \pm 0.7\%$), slowed down Cdc42 dissociation from polar cortex in Wt cells but not in $\Delta lem3$ cells (Fig. 3b). These results show that PE flipping is required for fast Cdc42 dissociation from the polar cortex.

Consistent with a previous report²³, a defect in PE flipping in $\Delta lem3$ correlated with an increase in the negatively charge phospholipid PS at the inner membrane leaflet of the polar cortex, as shown by using the PS biosensor GFP-Lact-C2² (Fig. 3c, d, g; Supplementary Information, Fig. S3a, b). The distribution of PI(4,5)P₂, monitored with the biosensor GFP-2xPH-PLC δ ²⁴, was not significantly different between Wt and $\Delta lem3$ cells (Fig. 3e, f, h). As Cdc42 contains a highly conserved cationic tail immediately adjacent to the prenylation site, the slowed Cdc42 dissociation in $\Delta lem3$ may be due to an enhanced charge interaction between Cdc42 and membrane lipids due to PS enrichment. To test this, we increased the Cdc42 polycationic charge by mutating S185 to lysine (KKS¹⁸⁵KK to KKK¹⁸⁵KK) and performed iFRAP on Wt and $\Delta lem3$ cells expressing Cdc42^{S185K}. In Wt cells, the Cdc42^{S185K} dissociation rate from the polar cortex was significantly slower than that of Cdc42, approaching the same slow rate of Cdc42 dissociation in $\Delta lem3$ (Fig. 4a). In $\Delta lem3$, Cdc42^{S185K} also exhibited a moderate but significant decrease in dissociation rate

compared to that of Cdc42 (Fig. 4a). As serine185 might possibly act as an electrostatic switch through phosphorylation²⁵, we also mutated this residue to alanine, but the Cdc42^{S185A} did not exhibit a reduced iFRAP rate compared to Cdc42 in Wt cells and displayed even a slightly increased iFRAP rate compared to Cdc42 in $\Delta lem3$ (Fig. 4a). We also mutated the S185 residue to the negatively charged aspartic acid, and the resulting mutant protein (Cdc42^{S185D}), unlike Cdc42^{S185K}, was poorly localized to the polar cap in both Wt and $\Delta lem3$ cells (Fig. 4b, c). These observations support the notion that charge interaction between the Cdc42 polycationic tail and the phospholipids at the polar cortex regulates Cdc42 dissociation from the plasma membrane.

To test if the above regulation is direct, we employed an *in vitro* system involving supported lipid bilayers (SLB). SLBs were assembled from liposomes with two different lipid compositions, one enriched for PS (referred to as 40% PS, 15% PE, see Methods), and the other for PE (referred to as 40% PE, 15% PS). Prenylated Cdc42 with an N-terminal tetra-cysteine motif (TC-Cdc42) was purified from a yeast plasma membrane fraction and labeled with the biarsenical dye FIAsh²⁶ (Supplementary Information, Fig. S4; see Supplementary Methods). The labeled TC-Cdc42 was added to the SLB and appeared as a fluorescent layer with some bright foci when observed with total internal reflection fluorescence microscopy (TIRF), and its binding to the SLB was dependent on the prenyl group of Cdc42 (Supplementary Information, Fig. S4c-e). In the SLB with 40% PS, 15% PE, the fluorescence intensity of the SLB-bound TC-Cdc42, observed with TIRF, decreased spontaneously and slowly in the presence of the bovine serum albumin (BSA) control, and this decay was not enhanced by added purified, bacterially-expressed Rdi1 (Fig. 5a, c). In the SLB with 40% PE, 15% PS, addition of Rdi1 stimulated the dissociation of Cdc42 from the membrane in a dose-dependent manner (Fig. 5b, c), confirming that PE strongly facilitates Rdi1-mediated Cdc42 dissociation from the membrane.

Based on the data presented above we propose a model whereby Lem3-Dnf1/2 flippase complex acts as an electrostatic switch to promote fast Cdc42 dissociation from the plasma membrane by Rdi1 (Fig. 5d). At the steady state, the polar cortex region is enriched for PE in the outer leaflet and PS in the inner leaflet^{2,17}. Such lipid asymmetry facilitates the localization of Cdc42 to the polar cortex²⁷ possibly by enhancing the charge interaction between the polycationic tail of Cdc42 and the negatively charged membrane surface. A transient increase in neutral lipids such as PE as a result of flippase activity destabilizes the charge interaction of Cdc42 with the inner membrane leaflet, increasing the chance of exposure of the prenyl moiety on Cdc42, thus promoting its capture by the cytosolic Rdi1 protein and fast internalization.

A previous study implicated the Lem3 flippase activity in regulating the activity of the GTPase activating proteins (GAP) for Cdc42 and proposed this regulation to be important for the apical to isotropical switch of cell growth at G2/M¹⁷. Two lines of considerations suggest that the regulation of Cdc42 dynamics observed in our study by the flippase complex is not a result of GAP regulation. First, the conclusion that PE flipping regulates GAP activity in the previous work was based on the *in vitro* demonstration that PE and PS stimulated the GAP activity of Rga1 toward Cdc42, contrasting the inhibitory effect of PIP₂. However, as shown in this work, the PE flipping defect in $\Delta lem3$ is accompanied by an increased enrichment of PS, not PIP₂, at the inner membrane leaflet of the polar cortex. As PS and PE are equally effective in stimulating Cdc42 GAP *in vitro*¹⁷, the reciprocal enrichment of PS and PE at the inner and outer membrane leaflet in the mutant, respectively, would predict little net effect on the GAP activity. Second, in the previous study the key *in vivo* result supporting the authors' conclusion was that over-expression of the GAP Rga1 rescued the elongated bud morphology in $\Delta lem3$, leading to the idea that lipid flipping regulates GAP activity *in vivo*. However, in budded cells beyond the nascent-bud stage,

Rga1 is localized at the bud neck²⁸, while Lem3 at the bud tip¹⁶. Thus, it is unlikely that a direct regulation of the GAP by bud tip localized flippase activity underlies the observed genetic interaction. Consistently, Rga1 over-expression does not rescue the defect in Cdc42 dynamics in $\Delta lem3$ but shows synthetic growth defect with $\Delta lem3$ (Supplementary Information, Fig. S5a, b). Our model, on the other hand, is based on the change in the charge property of the inner membrane leaflet as a result of phospholipid flipping, and the *in vitro* data supports a direct effect of the lipid composition on Cdc42 dissociation by Rdi1. Thus, our study reveals a mechanism by which dynamic lipid asymmetry regulates a highly conserved signaling process during cellular morphogenesis.

METHODS

Yeast strains and genome-wide screening for suppressors of Gal-Rdi1

A detailed list of yeast strains used in this study is provided in Supplementary information, Table 1. The yeast haploid non-essential deletion library¹⁴ was transformed with a centromeric plasmid containing Rdi1 under a galactose inducible promoter (CEN-pGAL-RDI1:His). Cells were grown for 2 days at 30°C so that all the strains reach maximum growth with approximately similar optical density (OD). At this point strains were diluted (1:100) and spotted in quadruplicates on Sc+2% galactose+2% raffinose (non-transformed control) or Sc-His+2% galactose+2% raffinose agar (transformed experimental strains). A His⁺ Wt control strain was included on every plate. A Wt strain (RLY2530; Wt-control) on non-selective raffinose plates and Wt strain with Gal-Rdi1 (RLY3811; Wt-experimental) were included in several plates throughout the library to obtain the Wt reference value. The plates were incubated at 23°C for 98 hr prior to saturation. Growth of each spot from the scanned images was quantified as the area and averaged over 4 spots to obtain growth for each strain. Area values were normalized with respect to the His⁺ Wt control strain on the same plate. A growth-ratio for each deletion strain was then obtained by dividing the normalized growth on the experimental plate by that on the control plate. The growth-ratio for the Wt control obtained by averaging from several plates was 0.26. Based on the distribution shown in Figure S1a, strains with a growth-ratio equal to or above 0.56 were considered as rescuer candidates. Based on gene annotation in the *Saccharomyces* genome database (SGD), genes encoding proteins involved in galactose-regulated transcription pathway and gene expression were excluded.

Live cell imaging and data analysis

FRAP and iFRAP experiments and data analysis were performed as described⁹. High signal to noise for the still images were obtained by summing 20 middle confocal slices of short movie of 20 frames. Image analysis and data extraction were performed using imageJ software, statistical analysis and data plotting were performed using OriginLab Pro software.

Fluorescence correlation spectroscopy

FCS experiments were performed as described by Slaughter *et al*²⁹. In brief, live yeast cells were mounted on coverslips and FCS measurements were taken in the cytosol. GFP-Cdc42 was excited with the 488nm laser line of a Zeiss Confocor 3 through a HFT 488/561 dichroic. HFT565 was used as an emission dichroic and BP 505-540 as an emission filter to collect fluorescence data. Autocorrelation curves generated from the data were fitted to a two component diffusion model³⁰ with a triplet blinking component of 250 μ s³¹. Fitting was performed using weighted non-linear least squares³². The autocorrelation curves from many short (4 second) data acquisitions were averaged for analysis. Curves that demonstrated bleaching or non-diffusive dynamics as judged by visual inspection of the binned trajectories and individual correlation traces were eliminated from the analysis. Weights were estimated from the standard error in the mean at each time lag point. Errors in the fitted

parameters were estimated by the Monte Carlo method as the standard deviation in fits of 500 simulated correlation curves with Gaussian random errors corresponding to the estimated weights and the observed χ^2 parameter³². These errors represent the estimated standard error in the fit parameters. An F-test was performed to compare the one component and two component diffusion model³². The two component model represented a strong improvement in the quality of the fit as judged by the F-test ($p < 1 \times 10^{-10}$) and the visual inspection of quality of the fit and residuals (Fig.1b). The FCS data obtained from Rdi1-mCherry was also fitted with one and two-component models. In either case, the autocorrelation curve for Rdi1 is dominated by a rapidly diffusing component (more than 95% of population). An F-test between the models preferred the two-component fit with a p-value of less than 1×10^{-10} . However, given the small amplitude of the slow component, we conclude that it represents a relatively insignificant portion of the Rdi1 population.

For measurements of fast and slow pool concentrations in the presence of Gal-Rdi1, it is reasonable to assume that fast and slow diffusion times will remain unchanged. Therefore the diffusion times were fixed to the values obtained for the Wt strain at the 0 time point for Gal induction. Indeed, the curves were reasonably well fit by this model in every case. This allows for unbiased estimation of fast and slow pool concentrations as a function of Gal induction time.

From the fitting we obtained G_0 , amplitude of correlation function at zero time lag, and the average diffusion time τ_D required for the molecules to traverse the focal volume. The average number of molecules (N) present in the focal volume was calculated from the following equation 1

$$N = \gamma / G_0 \quad (1)$$

where γ , is the shape factor of the focal volume. For one photon excitation, the γ factor is 0.36³³. Focal volume was calculated using fluorescein in 0.1 M NaOH with standard method³³⁻³⁶.

Modeling

For each cell, the mathematical model was applied for the analysis of the FRAP movie. Model parameters and assumptions are as described previously⁹. Considering the overlapping window size between the actin and Rdi1 based recycling mechanisms, we measured the window size from the formin Bni1 distribution in $\Delta lem3$ cells (Supplementary Information, Fig. S2e-g) and found it similar to the distribution of Bni1 in Wt cells⁹.

Recombinant protein purifications

We constructed a plasmid with a “pGAL1-GST-HA-TC-Cdc42” sequence (referred to as TC-Cdc42). We used a genetically encoded tetracysteine motif (HRWCCPGCKTF) (TC)³⁷ that binds efficiently to the biarsenical dye FIAsh²⁶ and absorbs at 488nm and emits at 525 nm. The tagged Cdc42 was over-expressed in yeast to maximize the amount of protein at the plasma membrane and purified from a plasma membrane fraction. Cell fractionation to prepare plasma membrane and protein purification was carried out as previously described^{12,38} with modifications. After homogenization of spheroplasts, unlysed cells were pelleted at 400 x g for 10 min. The pellet (P1) obtained at this step was rewashed with 5mL lysis buffer and the supernatants were pooled (S1). The plasma membrane-containing S1 pool was centrifuged twice at 158,420 x g for 45 min to pellet plasma membrane fractions (P2). P2 was resuspended in solubilization buffer (50mM Tris [pH7.5], 300mM NaCl, 5mM MgCl₂, 1% Triton X-100 and 0.8M sorbitol), briefly homogenized 15 times on ice and the resultant solution was incubated under shaking for 45 min at 4°C. The

solution was further centrifuged at 9000 x g for 20min and the supernatant (S3) was loaded on glutathione-agarose beads (Sigma, G4510) pre-equilibrated with solubilization buffer. The beads were washed with several column volumes of wash buffer (50mM Tris [pH7.5], 300mM NaCl, 5mM MgCl₂, and 0.1% CHAPS) supplemented with PI, PMSF and DTT, followed by the elution buffer (Tris-HCl [pH8.0] and 0.1% CHAPS). TC-Cdc42 was eluted with elution buffer containing 10mM reduced glutathione (Sigma-Aldrich, G4251). Recombinant non-prenylated Cdc42 was purified from bacteria using a GST tag. For Rdi1 purification, yeast Rdi1 was subcloned into the pET-28a vector with a C-terminal hexahistidine tag. Protein purification was done essentially as previously described¹² with minor modifications.

Liposome Preparation

To prepare liposomes we purchased chloroform solutions of porcine brain PE (catalog # 840022C), porcine brain PS (catalog # 840032C), and porcine brain PC (catalog # 840053C) from Avanti Polar lipids, Inc. Cholesterol was purchased from Nu-Chek prep, and dissolved in chloroform. Lipids were mixed in a clean glass tube and dried under a gentle stream of nitrogen gas and further dried under vacuum for 1hr. The dried lipid mixture was hydrated and resuspended with the TBSM buffer to a final concentration of 2mM³⁹ and bath sonicated for 30 min at 45°C to produce the final liposome solution⁴⁰.

Cdc42 extraction assay using TIRF microscopy

Supported lipid bilayers were prepared on No. 1.5 glass coverslips. Coverslips were made hydrophilic with a rinse with a concentrated H₂SO₄ and H₂O₂ (3:1) mixture and thoroughly washed with ultrapure water and dried with a stream of nitrogen gas. Liposomes with the composition 40% PS, 15% PE, 5% PC and 40% cholesterol, or 40% PE, 15% PS, 5% PC and 40% cholesterol, were added to this coverslip and incubated for 20 min and washed thoroughly with TBSM buffer. Prenylated Cdc42 with the TC FAsH binding motif was labeled with 166.6μM FAsH dye (Invitrogen, Carlsbad, CA catalog # T34561) for 1hr at room temperature and added to the lipid bilayer at 63-126 nM final concentration, followed by 10 min incubation. Unbound and loosely bound protein was thoroughly washed from the lipid bilayer with TBSM buffer. TIRF imaging were performed using a Carl Zeiss (Jena, Germany) Axiovert 200 M inverted microscope equipped with the Laser TIRF accessory and a Plan-Apochromat 100X, 1.46 NA objective, and a C9100-13 EM-CCD digital camera from Hamamatsu Photonics (Japan). To quantify Cdc42 extraction, rectangular areas excluding the bright foci of Cdc42 aggregates were sampled from each experiment. The fluorescence intensity (I) decay for each area was fitted for to an exponential decay model, $I = A_0 + A_1 \exp(-\alpha t)$, where α is the exponential decay rate using OriginLabPro software.

Statistical analysis

Statistical differences between two sets of data other than FCS data (see above) were analyzed with a two-tailed unpaired Student *t*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. $\Delta lem3$ suppresses the growth and Cdc42 localization defects due to $RDI1$ overexpression

(a) Images of Wt and the $\Delta lem3$ mutant (four replicate spots) grown on solid media with or without Rdi1 over-expression ($pGal-RDI1$) from the genome-wide screen. (b) FCS experiments were performed in live yeast cells (wild type or $\Delta rdi1$) expressing GFP tagged Cdc42, Cdc42^{R66E}, or Cdc42^{C188S}. Example auto-correlation curve of GFP-Cdc42 in Wt cells under its endogenous promoter. FCS data was averaged over 13 cells and fitted with a one-component or two-component diffusion model. Weighted residuals obtained from each fitting are shown in the inset. (c) Autocorrelation curves were fitted to a two-component model to extract percentages of slow and fast diffusing molecules. Error bars represent the estimated standard errors in the parameters determined from fitting an average autocorrelation curves obtained from 12, 10, 13, 22 cells from left to right correspond to each of the Cdc42 mutants (see Methods). (d) Molar concentration (nM) of soluble Cdc42 calculated from the FCS data as described in c (see Methods). (e) The average autocorrelation curve obtained from FCS experiments in live cells (n=12) expressing Rdi1-mCherry was also fitted with one and two-component models in comparison with the Cdc42 curve. Inset shows the distribution of weighted residuals obtained from each fitting. (f) Mobile Cdc42 concentrations (nM) of different pools (as indicated) determined at different times of $pGal1$ -Rdi1 induction in Wt cells. Shown are means and estimated standard errors from the analysis of average autocorrelation curves obtained from 21, 26, 27 cells (from left to right). (g) Representative images of GFP-Cdc42 at the polar cap in unbudded Wt and $\Delta lem3$ cells at different times of Rdi1 induction (as indicated above each column). Scale bar: 2 μm . (h) Fluorescence intensity profiles of GFP-Cdc42 along the perimeter of Wt or $\Delta lem3$ cells at different time points of $Gal-RDI1$ induction. Fluorescence traces from 10-12 cells were first peak-aligned and then averaged to yield each of the profiles shown. (i) Same analysis and display as in f for $\Delta lem3$ cells (14, 21, 23 cells from left to right).

Figure 2. Lem3 regulates Rdi1-mediated Cdc42 internalization and polar cap morphology
(a) Schematic representation of the mathematical model as previously described⁹. Gray horizontal bar represents the overlapping Rdi1 and actin-based delivery window (χ) approximately 25% of the perimeter (Supplementary information, Fig. S2e-g)⁹; d_f , lateral diffusion rate of Cdc42 in the membrane; m and n , internalization rates inside and outside the delivery window, respectively; h , delivery rate inside the window. **(b)** Example normalized FRAP curves of GFP-Cdc42 from Wt and $\Delta lem3$ cells. Solid lines represent the exponential fit of the FRAP data. **(c-e)** Extracted model parameters from FRAP data. Each point represents a value from a single cell. The small square is the mean; the box range is SEM; whiskers represent standard deviation (SD); line is median. **(f)** Examples of normalized iFRAP curves from cells expressing GFP-Cdc42. **(g)** iFRAP rates (1/s) from cells expressing GFP-Cdc42. Box plots are as described in **c-e**. **(h)** Example kymographs obtained from bleach corrected movies (300s-385s long) along the perimeter of $\Delta lem3$ cells expressing GFP-Cdc42 in the presence of solvent control (DMSO) or 100 μ M LatA (3 examples shown). Scale Bar: 2 μ m

Figure 3. The Lem3-Dnf1/Dnf2 complex regulates the polar Cdc42 dynamics and phosphatidylserine distribution

(a) iFRAP rates of GFP-Cdc42 in polarized cells with single or double deletion of *DNF1* and *DNF2* in box plots as described in Figure 2 legend. (b) iFRAP rates displayed as in a for GFP-Cdc42 in Wt and $\Delta lem3$ cells after treatment with 50 μ M Ro-peptide for 30 min at room temperature. Solvent control was with the same volume of water-acetonitrile (1:1). (c) Representative confocal images of Wt and $\Delta lem3$ cells expressing GFP-Lact-C2, a PS biosensor. White arrows point to polar cap. Scale bar: 2 μ m. (d) Representative GFP-Lact-C2 fluorescence intensity traces along the cell perimeter. White area represents total fluorescent intensity in the half of the plasma membrane containing the polar cortex; the opposite half is shown in gray. (e) Confocal images of wild-type or $\Delta lem3$ cells expressing GFP-2xPH-PLC (a PI(4,5)P₂ biosensor). (f) Representative GFP-2xPH-PLC fluorescence intensity traces along the cell perimeter as shown in d. (g) GFP-Lact-C2 fluorescence intensity ratios of the half of the plasma membrane containing the polar cap (white area in d) over the rest. Small Square is the mean; box range shows SEM; whiskers are standard deviation (SD); line is median. (h) GFP-2xPH-PLC fluorescence intensity ratio is measured and shown as in g.

Figure 4. The influence of the charge property of a Cdc42 COOH-terminal region on Cdc42 dynamics

(a) Internalization rates of Cdc42, Cdc42^{S185K} and Cdc42^{S185A} at the polar cortex measured with iFRAP in Wt and $\Delta lem3$ cells, box plots as described in Figure 2 legend. (b-c) Representative confocal images of Wt and $\Delta lem3$ cells expressing GFP-tagged Cdc42^{S185K} (b) or Cdc42^{S185D} (c). White arrows point to polar cap. Scale bar: 2 μ m.

Figure 5. Lipid composition directly regulates Rdi1-mediated Cdc42 extraction

(a) Example traces of fluorescence intensity decay due to dissociation of FIAsh labeled TC-Cdc42 from the SLB containing 40% PS and 15% PE as measured by TIRF microscopy. Fluorescence intensity values (dots) were normalized to the maximum intensity of each trace and fitted to an exponential decay model (lines). (b) Same experiment and data presentation as in a but from SLB containing higher concentration of PE (40%) relative to PS (15%). (c) Rates of TC-Cdc42 dissociation from the SLB determined from the TIRF movies, for 40% PE, 15% PS ($n = 10, 10, 18, 18$ SLB areas from left to right) and for 40% PS, 15% PE ($n = 10, 10, 15, 10$ SLB areas from left to right). Bar graphs show mean and SEM. (d) A schematic model depicting that, by changing the charge property of the inner membrane leaflet, the flippase complex regulates Rdi1-mediated dissociation of Cdc42 from the plasma membrane. On the left, the Cdc42 prenyl group (red) is inserted in the inner leaflet of the plasma membrane at the polar cortex with electrostatic interaction between Cdc42 cationic C-terminal region (+) and the negatively charged phospholipid head groups (blue circles) providing stability for this association. The flippase complex mobilizes phospholipids with neutral head groups such as PE from the outer to inner leaflet, reducing the electrostatic interaction between Cdc42 and the membrane and enhancing the access of Rdi1 to the Cdc42 prenyl group, and thus facilitating the formation of cytosolic Cdc42-Rdi1 complex.