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Supplemental Information

Changes in Cell Morphology Are Coordinated with Cell Growth through the TORC1 Pathway

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Supplemental Figures and Legends

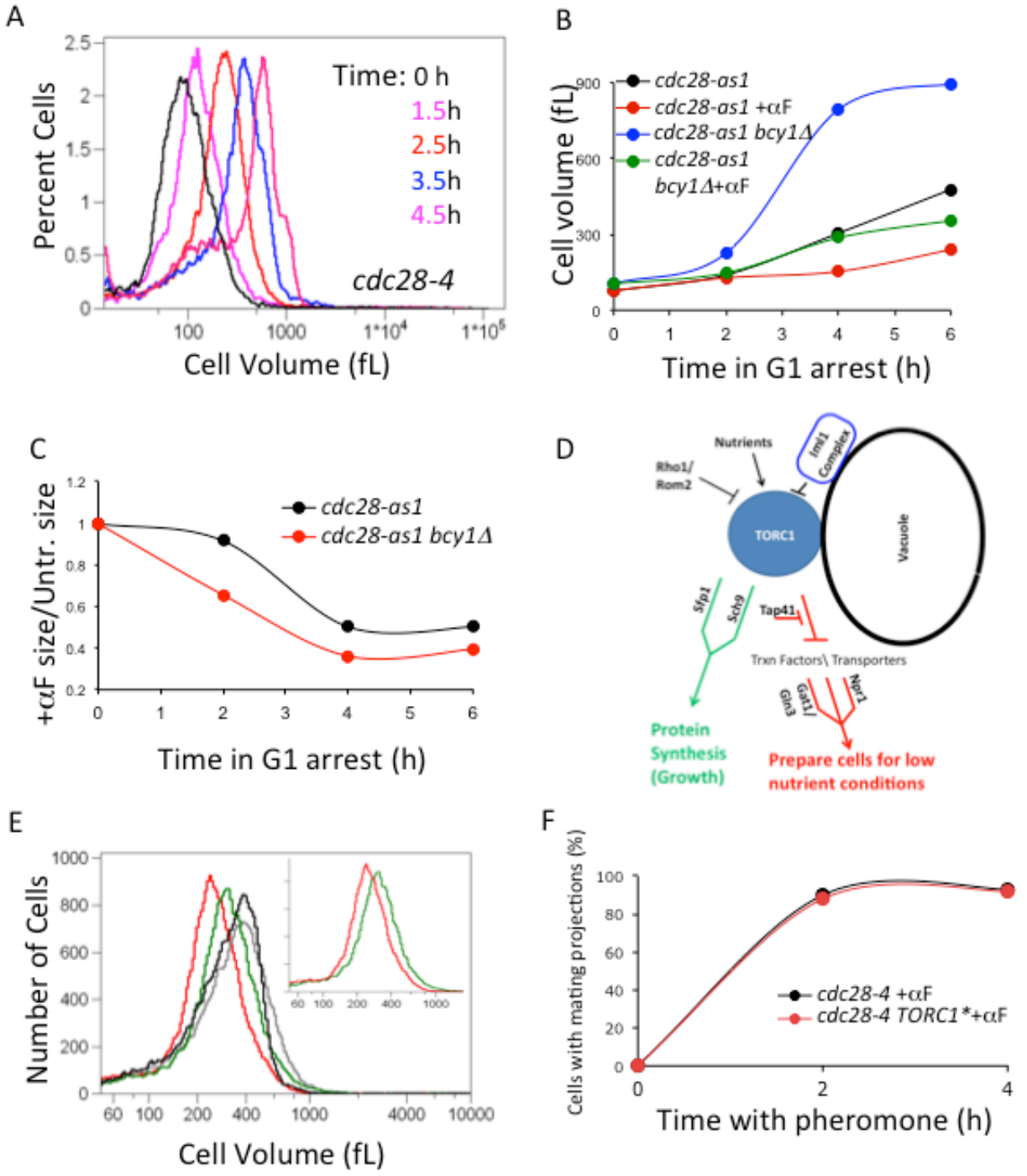


Figure S1. Involvement of RAS and TORC1 pathways in the growth control of pheromone treated cells.

(A) Method for measuring cell growth**.** *cdc28-4* cells (A17132) were grown in YEPD at 25°C. At time 0h cells were shifted to 34°C and the distribution of cell size was determined as described in the Experimental Procedures at the indicated time points.

(B, C) *cdc28-as1* (A4370) and *cdc28-as1 bcy1*Δ (A20223) cells were grown in YPD at 30°C and arrested in a G1- like state by addition of CDK inhibitor. Then half of the culture was treated with pheromone (20μg/ml) and cell volume was measured at the indicated times. In (C) data from (B) were plotted as the ratio of pheromone treated and untreated samples.

(D) Schematic representation of the TORC1 pathway. Mutants in the TORC1 pathway used in the study are illustrated.

(E) Cell size distribution of the 4 hour time point of the experiment presented in Figure 1B. The cell size distribution was determined with a Coulter Counter for *cdc28-4* cells arrested in G1 at 34°C in the presence (red line) or absence (black line) of pheromone and compared to the *cdc28-4 TORC1** strain grown in parallel in the presence (green line) or absence (gray line) of pheromone. Inset shows only the comparison of the pheromone treated samples for both strains (*cdc28-4* in red; *cdc28-4 TORC1** in green) for clarity.

(F) Actin polarization of the pheromone treated cells from the experiment described in Figure 1B. Percent of cells with a mating projection (an indication of actin polarization) for *cdc28-4* (black symbols) and *cdc28-4 TORC1** (red symbols) is shown for indicated time points (n=100 for each sample).

Goranov_Sup_Fig2

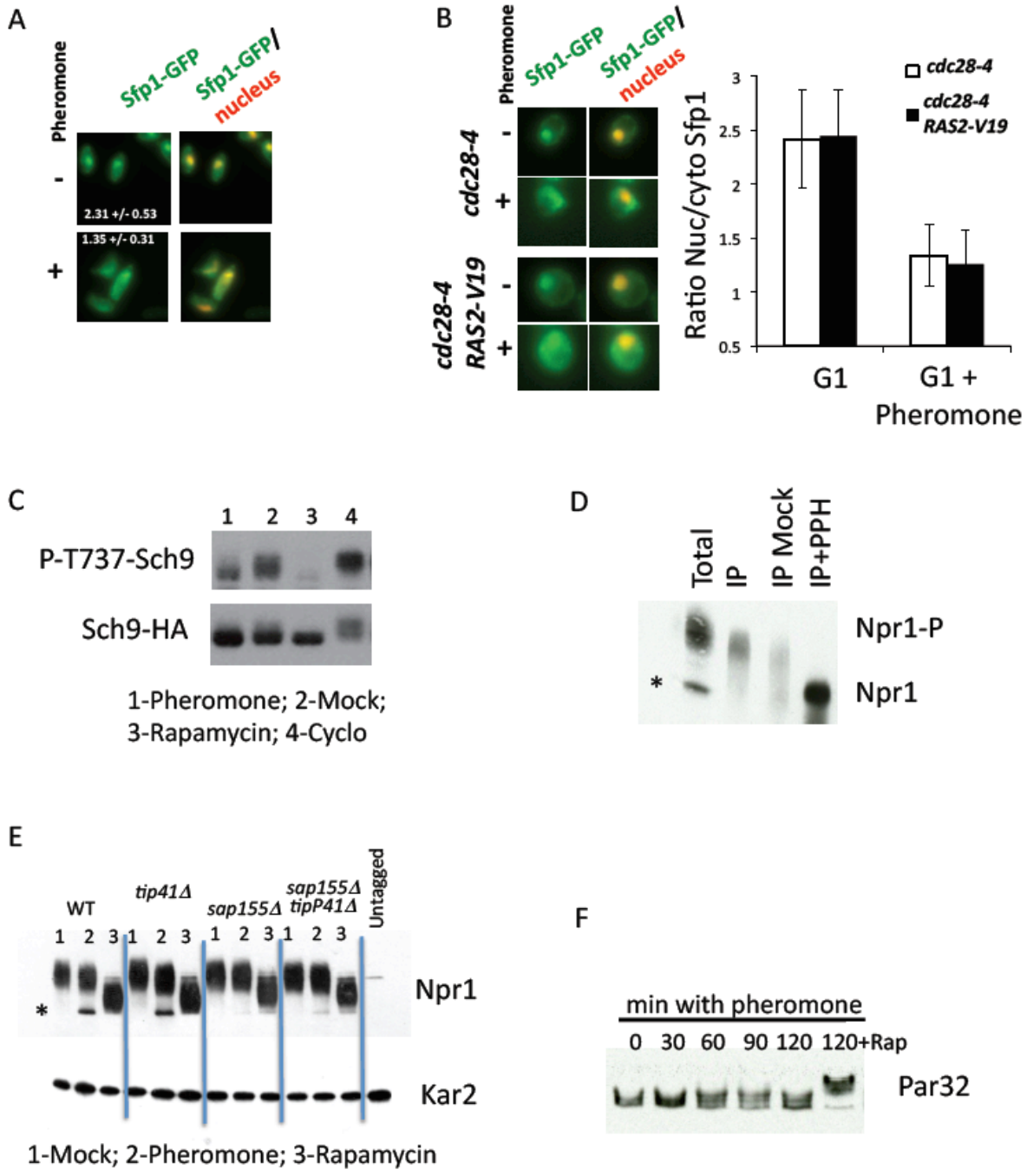


Figure S2: Pheromone causes changes in the localization and phosphorylation of TORC1 targets.

(A) A *MATa bar1Δ cdc28-as1* strain carrying the *SFP1-GFP* allele and a NLS-mCherry marker (A27149) was grown at 30°C in YEPD. Part of the culture was treated with pheromone

(20μg/ml) for 1.5h and part was left untreated. CDK was not inactivated in this experiment. Samples from both conditions were fixed and imaged as described in Experimental Procedures. Representative images for pheromone treated or untreated cells are shown either for the Sfp1 signal alone (green) or together with NLS-mCherry (red). The nuclear to cytoplasmic ratio of Sfp1-GFP signal is indicated in the Sfp1-GFP panels (+/- St. Dev., $p<0.00001$ Student T-test). N is greater than 24 for each sample. Reduction in nuclear to cytoplasmic ratio in the presence of pheromone is similar to that reported for rapamycin (~1.5; [1]).

(B) *MATa cdc28-4 SFP1-GFP NLS-mCherry* strains carrying a WT *RAS2* allele (A31566), or an additional *RAS2-V19* allele (A33665) were arrested at 34°C for 90min, at which point pheromone (20μg/ml) was added to half of each culture for 45min. At this point samples from all cultures were collected, fixed and analyzed as in A. Representative images for each strain are shown. Ratio of nuclear to cytoplasmic Sfp1-GFP signal is plotted on the right (+/- St. Dev.). n>15 for untreated cells, and n>25 for pheromone treated samples.

(C) A *cdc28-as1* strain carrying the *SCH9-3HA* allele (A26485) was grown in YPD medium at 30°C, and treated with CMK inhibitor to arrest cells in a G1-like state. After 60min, the culture was split and cells were treated for 30min with pheromone, rapamycin, cycloheximide, or mock-treated and the presence of the presence of Sch9-T737 phosphorylation and total Sch9 protein was assessed.

(D) Faster migrating forms of Npr1 are hypophosphorylated. *MATa bar1Δ cdc28-as1 NPR1-3HA* cells (A23200) were arrested in G1 with mating pheromone (20μg/ml) for 2h at which time cells were collected (Total) and Npr1 was immunoprecipitated (IP) as described in Experimental Procedures. A portion of IP-ed Npr1 protein was incubated with alkaline phosphatase (IP + PPH; 2 μL), another was mock treated (IP Mock). Samples were resolved on SDS-PAGE and Npr1 detected by Western blotting. Asterisk indicates the hypophopsphorylated Npr1 form.

(E) *cdc28-as1* (A23200), *cdc28-as1 tip41Δ* (A23836), *cdc28-as1 sap155Δ* (A23723), *cdc28-as1 sap155Δ tip41Δ* (A23838) cells all carrying a *NPR1-3HA* fusion were grown in YEPD at 30°C, treated with CDK inhibitor and split into three aliquots: 1: Untreated, cells were collected 2h after receiving vehicle only; 2: cells were treated with pheromone (20μg/ml) for 2h; 3: cells were treated with rapamycin (1μM final) for 30min. Samples were collected and Npr1 mobility was analyzed. Kar2 was used as a loading control in Western blots. *MATa bar1Δ cdc28-as1* (A4370) cells were used as the untagged control sample.

(F) *MATa bar1Δ cdc28-as1 PAR32-3HA* (A29187) cells growing at 30°C were treated with CDK inhibitor (5μM) to arrest cells in a G1-like state. After 90min, pheromone was added to the culture and samples were collected at the indicated times. A sample of cells grown in the presence of pheromone for 120min also received Rapamycin (1μM; "120+Rap") for 15min and was processed with the other samples for Western blot analysis. Samples were resolved on SDS-PAGE containing Phos-Tag (25μM) and Par32-3HA was detected by Western blotting with anti-HA antibody.

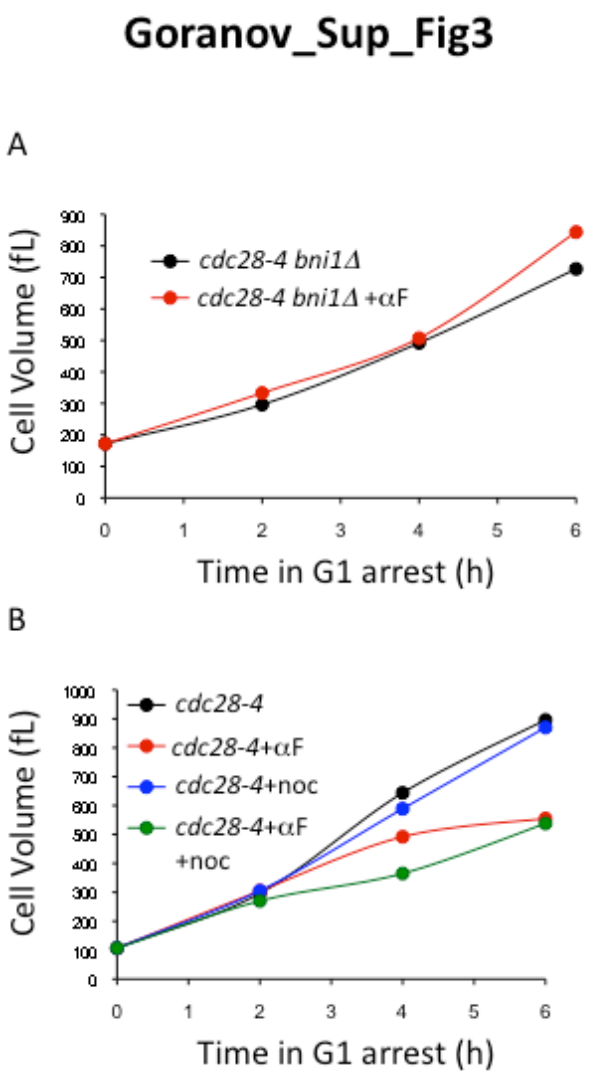


Figure S3. Microtubule depolymerization does not affect growth during G1, but preventing actin cytoskeleton polarization does.

(A) *cdc28-4 bni1*Δ (A18238) cells were grown and treated as described in Figure 1A. At the time of shift to 34°C, one half of the culture was treated with pheromone (20μg/ml) and cell size was measured at the indicated times.

(B) *cdc28-4* cells (A17132) were grown as described in Figure 1A. At the time of shift to 34°C the culture was split into 4 parts. One was mock treated ("Unt", black circles), one received nocodazole (15μg/ml; "noc", blue circles), one received pheromone (20μg/ml "αF", red circles), and one received both pheromone and nocodazole ("αF+noc", green circles)

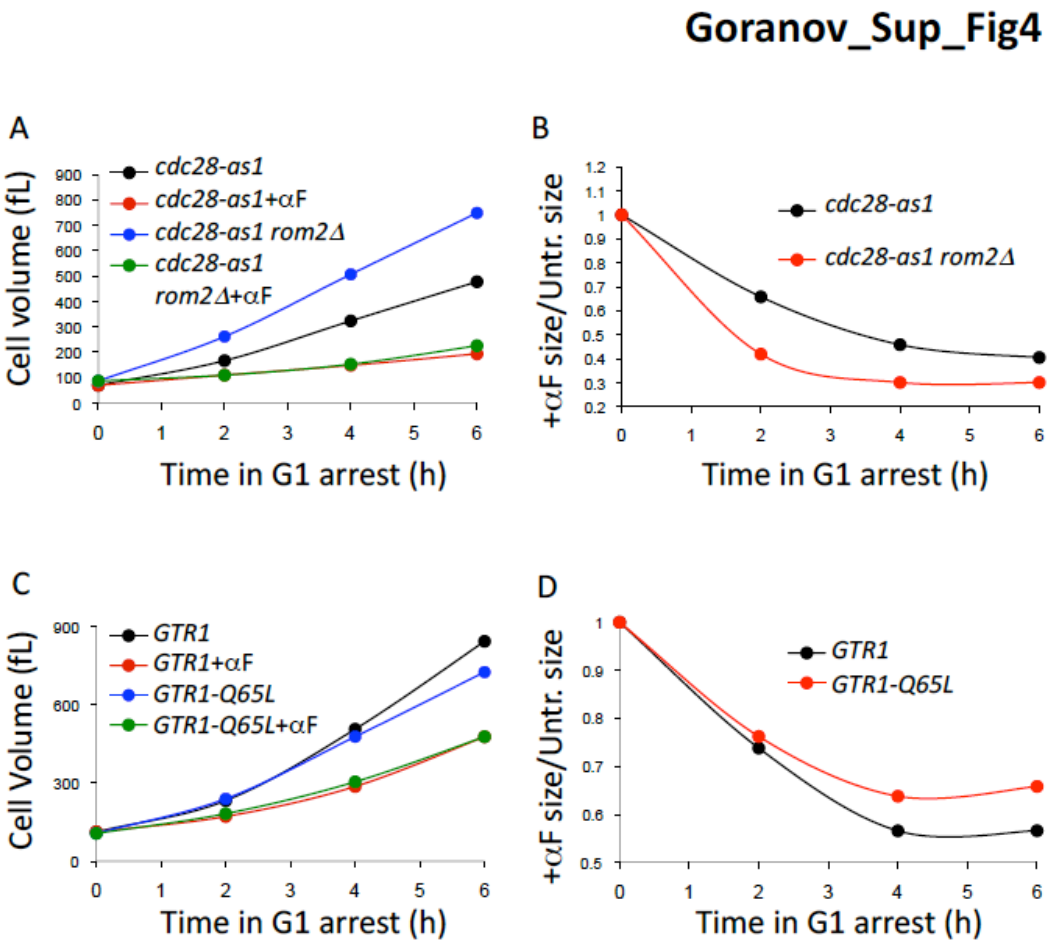


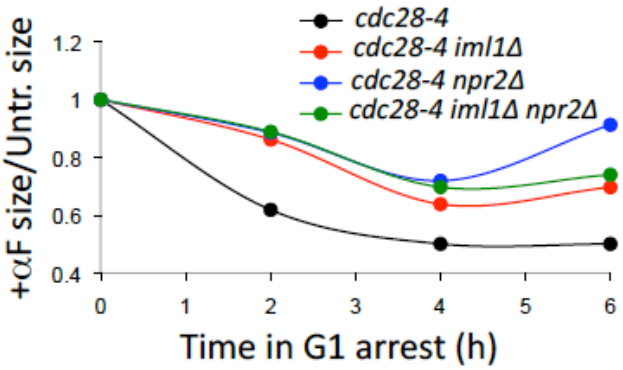
Figure S4. *ROM2* does not mediate pheromone –induced growth inhibition, while Gtr1-Q65L has a subtle effect.

(A, B) *cdc28-as1* (A4370) and *cdc28-as1 rom2*Δ (A24864) cells were grown as in Supplemental Figure 1B and cell volume was measured at indicated time points. Data from (A) were plotted as a ratio between pheromone treated and untreated cells for each strain in (B).

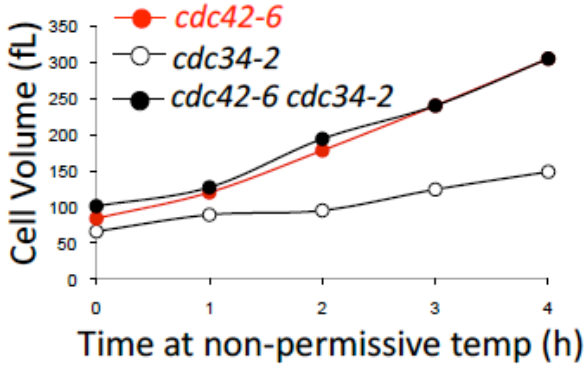
(C, D) *cdc28-4* (A32867) and *cdc28-4 GTR1-Q65L* (A32868) were grown as in Figure 1A. Cell volume was measured at the indicated times (C) and plotted as a ratio between pheromone treated and untreated cells for each strain (D).

Goranov_Sup_Fig5

A



B



C

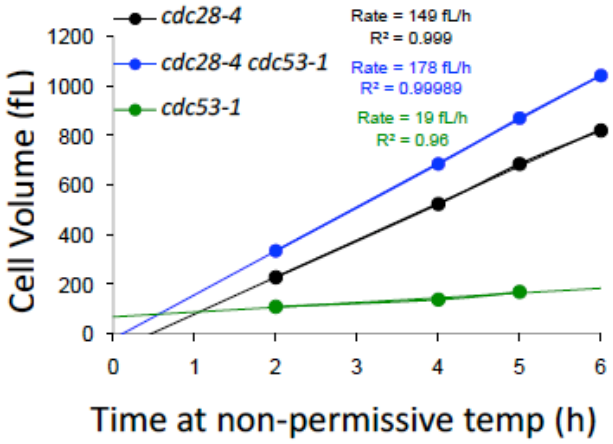


Figure S5. Preventing polarization of the actin cytoskeleton restores the ability of SCF mutants to grow.

(A) *cdc28-4* (A17132), *cdc28-4 iml1Δ* (A33017), *cdc28-4 npr2*Δ (A31346) and *cdc28-4 npr2*Δ *iml1*Δ (A33020) cells were grown and treated as in Figure 1A, and cell volume was measured at the indicated times. Data were plotted as a ratio between pheromone treated and untreated cells for each strain.

(B) *cdc42-6* (A18222, red circles), *cdc34-2* (A1467, black open circles), and *cdc34-2 cdc42-6* (A33019, black closed circles) cells were grown at room temperature in YEPD and were shifted to 37°C at time 0h. Cell volume was measured at the indicated time points.

(C) *cdc28-4* (A17132, black circles), *cdc53-1* (A1469, green circles), and *cdc28-4 cdc53-1* (A31348, blue circles) cells were grown at 25°C and shifted to 37°C at time 0h and cell volume was determined at the indicated time points. Data were fitted to linear functions and slope and R^2 are indicated in appropriate color for each curve.

Goranov_Sup_Fig6

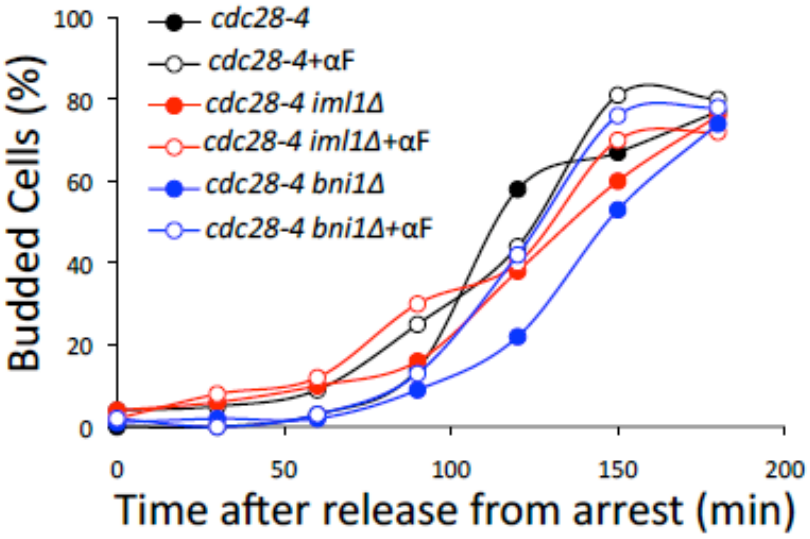


Figure S6. Cell size does not affect the kinetics of cell cycle entry.

cdc28-4 (A17132, black symbols), *cdc28-4 bni1*Δ (A18238, blue symbols), and *cdc28-4 iml1*Δ (A33017, red symbols) cells were arrested in G1 at 34°C in the absence (filled symbols) or in the presence of pheromone (αF, open symbols) for 6h, at which point they were washed and released at room temperature and budding (an indication of cell cycle re-entry) was assessed.

Supplemental Tables

Table S3. *Saccharomyces cerevisiae* strains used in this study

W303	*ade2-1, leu2-3, ura3, trp1-1, his3-11,15, can1-100, GAL, ssd-1d, psi+*
A800	*MATalpha, trp1::GAL-SIC1*(5 copies)::TRP1
A1467	*MATalpha, cdc34-2*
A1469	*MATalpha, cdc53-1*
A4370	*MATa, bar1::HisG, cdc28-as1*
A17132	*MATa, cdc28-4*
A18222	*MATalpha, cdc42-6, URA3*
A18238	*MATa, cdc28-4, bni1::URA3*
A20223	*MATa, cdc28-as1, bcy1::KanMX*
A22358	*MATa/MATalpha cdc28-4/cdc28-4 (diploid)*
A23200	*MATa, bar1::HisG, cdc28-as1, NPR1-3xHA::KanMX*
A23441	*MATa, bar1::HisG, cdc28-as1, NPR1-3xHA::KanMX, bni1::URA3*
A23723	*MATa, bar1::HisG, cdc28-as1, NPR1-3xHA::KanMX, sap155::HisMX*
A23836	*MATa, bar1::HisG, cdc28-as1, NPR1-3xHA::KanMX, tip41::TRP1*
A23838	*MATa, bar1::HisG, cdc28-as1, NPR1-3xHA::KanMX, tip41::TRP1, sap155::HisMX*
A24515	*MATa, cdc28-4, npr3::KanMX*
A24864	*MATa, bar1::HisG, cdc28-as1, rom2::KanMX*
A26485	*MATa, bar1::HisG, cdc28-as1, SCH9-3xHA::KanMX*
A26785	*MATa, bar1::HisG, cdc28-as1, SCH9-3xHA::KanMX, bni1::URA3*
A27149	*MATa, bar1::HisG, cdc28-as1, ura3::NLS-2x-mCherry::URA3, SFP1-GFP::HIS3*
A27150	*MATa, bar1::HisG, cdc28-as1, ura3::NLS-2x-mCherry::URA3, SFP1-GFP::HIS3, bni1::URA3*
A27195	*MATalpha, cdc34-2, SCH9-3xHA::KanMX*

A27196	*MATalpha, cdc34-2, cdc28-as1, SCH9-3xHA::KanMX, ADE2*
A28466	*MATalpha cdc34-2, ura3::NLS-2x-mCherry::URA3, SFP1-GFP::HIS3*
A29187	*MATa, bar1::HisG, cdc28-as1, PAR32-3xHA::KanMX*
A30573	*MATa, cdc53-1, iml1::HisMX*
A31265	*MATa, trp1::GAL-SIC1* (5 copies)::TRP1, *iml1::HisMX*
A31346	*MATa, cdc28-4, npr2::KanMX*
A31348	*MATa, cdc28-4, cdc53-1*
A31414	*MATa, cdc28-4, SFP1-GFP::HIS3*
A31415	*MATa, cdc28-4, iml1::HisMX, SFP1-GFP::HIS3*
A31566	*MATa, cdc28-4, SFP1-GFP::HIS3, ura3::NLS-mCherry::URA3*
A31567	*MATa, iml1::HisMX, cdc28-4, SFP1-GFP::HIS3, ura3::NLS-mCherry::URA3*
A31570	*MATa, cdc28-4, p1048 (RAS2-V19, TRP1)*
A32379	*MATa, cdc28-4, iml1::HisMX, SCH9-3HA::KanMX*
A32867	*MATa, cdc28-4, gtr1::KanMX, p1895 (GTR1, URA3)*
A32868	*MATa, cdc28-4, gtr1::KanMX, p1897 (GTR1-Q65L, URA3)*
A33017	*MATa, cdc28-4, iml1::HisMX*
A33018	*MATa, cdc28-4, tip41::TRP1, sch9::KanMX, gat1::NatMX, gln3::HphMX, ura3::GAL-SFP1-HIS3, p1910 (SCH9-2D3E, URA3, cen)*
A33019	*MATalpha, cdc42-6, cdc34-2*
A33020	*MATa, cdc28-4, iml1::HisMX, npr2::KanMX*
A33021	*MATa, cdc28-4, SCH9-3HA::KanMX*
A33665	*MATa, cdc28-4, SFP1-GFP::HIS3, ura3::NLS-2x-mCherry::URA3, p1048 (RAS2-V19, TRP1)*

Supplemental Experimental Procedures

Strains, Strain Construction, and Growth Conditions.

All strains used in this study are constructed in the *Saccharomyces cerevisiae* W303 background. Strains used are listed in Supplemental Table 3. Gene deletions and epitope tags were generated by a single step gene replacement method [2]. Strains were grown in YEP medium supplemented with 2% glucose (YEPD) or with 2% raffinose and 1% galactose as indicated, and with adenine to 0.055mg/ml final concentration. Pheromone (yeast α-factor; αF) was prepared by resuspending dry, synthetically synthesized peptide pheromone in water to make a 20mg/ml stock solution. Pheromone was used at a final concentration of 20μg/ml of OD_{600} 0.2 culture (unless indicated otherwise) and was re-added every 2h during experiments. Strains carrying plasmids were grown in selective media prior to the experiment, placed in appropriate media (YEPD or YEP Raf/Gal) for at least one doubling before the start of the experiment. Rapamycin (1mM stock in ethanol) was used at a 1μM concentration, cycloheximide (10mg/ml stock solution) at 0.33mg/ml. To inhibit the Cdc28-as1 allele we added CDK inhibitor 1-NM-pp1 (4-Amino-1-tert-butyl-3-(1'-naphtylmethyl)-pyrazolo[3,4-d]pyrimidine, Toronto Research Chemicals, Inc) to a final concentration of 5μM from 5mM stock in DMSO.

Cell volume measurements.

Volume increase of arrested cells was measured as previously described [3]. A 400μl aliquot of cells was briefly sonicated to break clumps and cell volume distribution was assessed with a Coulter Counter Multisizer 3, adjusting cell concentration not to exceed 15% saturation, usually 1:100 dilution into Beckman Isoton II diluent. The mode of the distribution was used to represent the cell size at a given time point.

Western Blot Analysis.

Cells were collected and mixed with an equal volume of cold 10% TCA. After at least 15min incubation at 4°C, cells were washed twice with acetone and dried. Cells were lysed by bead beating in 50mM TE pH 7.5 with 2.75mM DTT, mixed with SDS-PAGE loading buffer (from 4x stock), and heated to 95°C for 10min. Equal amounts of total protein were loaded on SDS-PAGE gels. Usually 10% SDS-PAGE gels were utilized, with the exception of Npr1 and Par32 detection when 6% and 12% SDS-PAGE gels, respectively, were used. Phos-tag (NARD Institute, Ltd) was used at 25μM according to the manufacturer's protocol. For Phos-Tag samples, care was taken to avoid EDTA in the cell breakage buffer. Resolved samples were transferred onto nitrocellulose membrane using a semi-dry Hoeffer apparatus, blocked in 3% milk and probed with appropriate antibodies listed below. NTCB cleavage of Sch9-3HA, was performed as described by Urban *et al.* [4]. Antibodies were prepared in PBS 0.1% Tween-20, 1.5% milk, 1.5% BSA the following dilutions: monoclonal mouse anti-HA was used at a 1:1000 (16B12 Covance), a rabbit anti-phospho MAPK Thr 202/204 (#9102 Cell Signaling Technology) at a 1:1000, a mouse polyclonal anti-Pgk1 (Invitrogen) at a 1:20000, a mouse monoclonal anti-GFP antibody (JL-8, BD Biosciences) at a 1:1000 and a mouse anti-Kar2 polyclonal antibody used at a

1:200 000 dilution. A secondary sheep α-mouse HRP conjugated antibody (GE Healthcare) was used at a 1:5000 dilution and a secondary donkey anti-rabbit HRP conjugated antibody (GE Healthcare) at a 1:5000 dilution. Detection of P-T737 of Sch9 was performed as described previously [5].

Cell Buoyant Mass Measurements.

Cells were grown in YEPD at 25°C until they reached an OD_{600} of 0.15. Cells were then shifted to 34°C and half of the culture was treated with pheromone. Four hours after pheromone addition, 1ml of cells was collected for each sample, cells were immediately treated with sodium azide (0.1% final concentration) to halt further growth during buoyant mass measurement, and were sonicated to break up clumps. Buoyant mass was measured using a suspended microchannel resonator (SMR) employing the method outlined in (Burg et al. 2007). Briefly, cells suspended in growth medium were injected into the SMR using pressure-driven flow. As individual cells transit through the cantilevered resonator a downward shift in its resonant frequency is observed; the minimum of this frequency shift is proportional to the buoyant mass of the cell. 12μm NIST polystyrene beads were used to calibrate the device prior to the measurement. The cross-sectional dimensions of the cantilever microchannel used in this measurement were 25μm x 25μm. To verify that there did not exist a bias in the size distribution sampled by the device, cell samples were run through a Coulter counter before and after the SMR measurement.

Microscopy

1000μL of live cells grown at the appropriate conditions were collected and sonicated briefly to disperse clumps. Cells were imaged with a Zeiss Axioplan 2 microscope equipped with a Hamamatsu ORCA-ER C4742-80 digital CCD camera and processed with Openlab (Improvision) software. For assessing Sfp1-GFP localization, cells were collected at the indicated times, fixed with 4% para-formaldehyde (v/v), 3.2% sucrose for 5min, washed twice in phosphate buffer, and resuspended in 1M sorbitol, 50 mM potassium phosphate buffer pH 7.5. Cells were analyzed within a day of fixation. To assess polarization of the actin cytoskeleton, cells were fixed with 3.7% formaldehyde for 15min, washed twice with phosphate buffer, and resuspended in 30μl PBS-BSA buffer. Phalloidin-Alexa Fluor 568 (Invitrogen; in 100% methanol) was added to cells (1μM final) and incubated in the dark for 30min with occasional mixing. Cells were washed with PBS-BSA and imaged. Polarization of the actin cytoskeleton was defined as actin patches and cables being concentrated at or near a single region of the cell membrane. Quantitative ratios of Sfp1-GFP cellular localization were obtained by imaging of cells with a DeltaVision microscope. 18 Z-stacks (0.2μm step) were obtained per field for both the nuclear marker and Sfp1-GFP. Average projection was calculated with DelataVision Software and the average GFP signal intensity minus background was obtained for the nucleus and cytoplasm.

Npr1 Denaturing IP protocol

Collected cells were incubated in 5% tri-chloro-acetic acid (TCA) for 30min at 4°C, washed with acetone and air dry. Pellets were resuspended in 180 μl breakage buffer (50mM Tris-HCl pH 7.5, 1mM EDTA, 15mM PNP, 60mM β-glycerophosphate, 50 mM DTT, 0.1mM Na_3VO_4, protease inhibitor cocktail (Roche)) and 80μl glass beads were added. Cells were disrupted on a FastPrep

FP120 (Thermo Savant) machine 3-times with 45sec cycles at 4°C with one min on ice between cycles. SDS was added to 1% final concentration and samples were boiled for 5min. 9 volumes NP40 buffer were added with 2 mg/ml BSA and mixed vigorously. Samples were spun down 2-times at 13 000 rpm for 10min at 4°C and supernatant was transferred to fresh tubes. Samples were split into 3 aliquots and anti-HA antibody (mouse monoclonal Covance 16B12) at 1:50 dilution was added to all three samples. Samples were incubated at 4°C with rotation for 2 hours. To these samples 40μl Protein G Sepharose beads (Amersham) preincubated with 5mg/ml BSA were added. Samples were incubated at 4°C for 2 hours with rotation, washed 2-times with NP40 buffer, once with NP40, 1% β-mercaptoethanol (BME), 2-times NP40, 1%BME, 2M urea, and once with 10mM Tris-HCl pH 7.5. One aliquot was saved for "IP" control. For the other two samples, all supernatant was removed, and beads were resuspended in 34μl 50mM Tris-HCl pH 7.5, 4 μl NEB buffer #3. 2μl water was added to one ("Mock") and 2μl Alkaline phosphatase (Roche, 20U/ml) to the other ("+PPH"). Beads were incubated at 37°C for 30min. SDS-PADE loading buffer was added to beads, boiled for 5 min and run on 5% SDS-PAGE gel. Npr1-3xHA was detected by Western blotting.

Phospho-Proteomic Screen

Reductive dimethylation with deuterated or natural formaldehyde and sodium cyanoborohydride was used to generate mass tags for relative quantification. We identified and quantified 24,178 phospho-peptides (0.2% false-discovery rate (FDR)) from 1,380 proteins (1% FDR) representing at least 4,612 unique phosphorylation sites. An analysis of unphosphorylated peptides yielded protein abundance ratios based on >=2 quantified peptides for 1,706 proteins including 620 of the phosphorylated proteins. Where possible, the ratio of phospho-peptide abundance in the treated and untreated samples was corrected for changes in protein abundance and is summarized in Supplemental Table 1. The full set of identified peptides is shown in Supplemental Table 2.

Growth conditions:

Strain A4370 was grown in YEP (2% glucose) at 30°C with aeration to OD_{600} ~0.3. CDK inhibitor 1-NM-pp1 was added (5μM) and the culture was spit into two 250ml cultures. One culture received pheromone to 20μg/ml the other was mock treated with water. After a 2h incubation at 30°C with aeration, 140 ml from each culture (OD_{600} ~0.55) were collected washed and flash-frozen in liquid N_2.

Protein digestion, peptide labeling, and phosphopeptide enrichment

Disulfide bonds were reduced by adding dithiothreitol to a final concentration of 2.5 mM and incubating at 56°C for 40 min. The extract was allowed to cool to room temperature and the reduced cysteines were alkylated by adding iodoacetamide (7.5 mM) and incubating extracts for 40 min in the dark at room temperature. Alkylation was quenched with an additional 5 mM dithiothreitol. Equal amounts of total protein were diluted 2.5-fold into 25 mM Tris-HCl (pH 8.8) and digested by the addition of lysC (Wako; 10 ng/μl) with gentle agitation overnight at room temperature. Digested peptides were acidified with formic acid and loaded onto pre-wet 200 mg tC18, reverse-phase solid phase extraction cartridges (Waters). The columns were washed

with 6 ml of 1% formic acid followed by 3 ml of phosphate/citrate buffer (227 mM Na2HPO4, 86 mM $NaH_2C_6H_5O_7$, pH 5.5). Peptides from the pheromone treated samples were labeled by reductive dimethylation [6] with 6 ml of "light" reductive dimethylation reaction mix (0.8% formaldehyde (Sigma), 120 mM NaCNBH3 (Sigma), in phosphate/citrate buffer). Peptides from G1 arrested (no pheromone) samples were labeled with 6 ml of "heavy" reductive dimethylation reaction mix (0.8% D2-formaldehyde (Isotec), 120 mM NaCNBD3 (Isotec), in phosphate/citrate buffer). The columns were washed with 6ml of 1% formic acid and the peptides were eluted with 1 ml of 70% acetonitrile, 1% formic acid. Small aliquots of each sample were taken off and combined pair-wise to test mixing ratios. Samples from "heavy" (G1 arrested cells) and "light" (pheromone treated cells) peptides were combined and dried in a speed-vac.

Phosphopeptides were enriched using a modified version of the two-step, SCX-IMAC protocol [7] employing step elution from self-packed solid-phase extraction SCX cartridges as described [8] with some changes. Peptides were resuspended in 1 ml SCX buffer A (7 mM KH_2PO_4, pH 2.65, 30% ACN) and loaded onto pre-equilibrated syringe-barrel columns packed with 500 mg of 20 μm, 300 angstrom, polysulfoethylA resin (poly LC). Peptides were eluted by the sequential addition of 3 ml of SCX buffer A containing increasing concentrations of KCl. Six fractions were collected after elution with 0 (flow-through), 10, 25, 50, 100, and 150 mM KCl. All fractions were freeze-dried, resuspended in 1 ml of 1% formic acid, and desalted on 50 mg Sep-paks. Peptides were eluted with 500 μl of 70% ACN, 1% formic acid and dried once again. IMAC enrichment was performed as described [7].

Mass spectrometry

Samples were analyzed on a LTQ Orbitrap Velos or LTQ Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific) equipped with an Accela 600 quaternary pump (Thermo Fisher Scientific) and a Famos microautosampler (LC Packings, Sunnyvale, CA). Nanospray tips were hand-pulled using 100 μm I.D. fused-silica tubing and packed with 0.5 cm of Magic C4 resin (5μm, 100 Å, Michrom Bioresources, Auburn, CA) followed by 20 cm of Maccel C18AQ resin (3μm, 200 Å, Nest Group, Southborough, MA). Peptides were separated using a gradient of 5% to 26% ACN in 0.125% FA over 150 min or 120 min expt. at a flow rate of ~300-500 nl/min. Ions were detected using a data-dependent Top20-MS2 (Velos) method or a Top10 method (Discovery). For each cycle, one full MS scan of m/z = 300-1500 was acquired in the Orbitrap, followed by the selection of the most intense ions, up to 10 or 20, for CID and MS2 analysis in the LTQ. Ions selected for MS2 analysis were excluded from re-analysis for 90 seconds.

Peptide identification and filtering

MS2 spectra were searched using SEQUEST v.28 (rev. 13) against a composite database containing the translated sequences of all predicted open reading frames of *Saccharomyces cerevisiae* (http://downloads.yeastgenome.org, downloaded 10/30/2009) and its reversed complement, using the following parameters: a precursor mass tolerance of ±20 ppm; 1.0 Da product ion mass tolerance; lysC digestion; up to two missed cleavages; static modifications of carbamidomethylation on cysteine (+57.0214), dimethyl adducts (+28.0313) on lysine and peptide amino termini; and dynamic modifications of methionine oxidation (+15.9949), heavy

dimethylation (+6.0377) on lysine and peptide amino termini, and phosphate (+79.9663) on serine, threonine, and tyrosine.

Peptide spectral matches were filtered to 1% FDR using the target-decoy strategy [9] combined with linear discriminant analysis (LDA) [10, 11] using several different parameters including Xcorr, ΔCn', precursor mass error, observed ion charge state, and predicted solution charge state. Linear discriminant models were calculated for each LC-MS/MS run using peptide matches to forward and reversed protein sequences as positive and negative training data. Peptide spectral matches within each run were sorted in descending order by discriminant score and filtered to a 1% FDR as revealed by the number of decoy sequences remaining in the data set. The data were further filtered to control protein level FDRs. Peptides from all fractions in each experiment were combined and assembled into proteins. Protein scores were derived from the product of all LDA peptide probabilities, sorted by rank, and filtered to 1% FDR as described for peptides. The FDR of the remaining peptides fell dramatically after protein filtering. Remaining peptide matches to the decoy database were removed from the final dataset. The Ascore algorithm [12] was used to assess the probability of correct site assignment. Sites with an Ascore >=13 (p<=0.05) were considered localized.

Protein ratios were calculated using the median $\log_2$ ratio of all peptides for each protein. For proteins represented by a single quantified peptide, a minimum signal-to-noise ≥ 5 was required for both "light" and "heavy" signals. The ratios were normalized to recenter the distribution at 1:1 ($\log_2 = 0$).

Phosphorylation site abundance ratios were calculated as the median $\log_2$ ratio of all peptides containing each site.

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