Supplemental material

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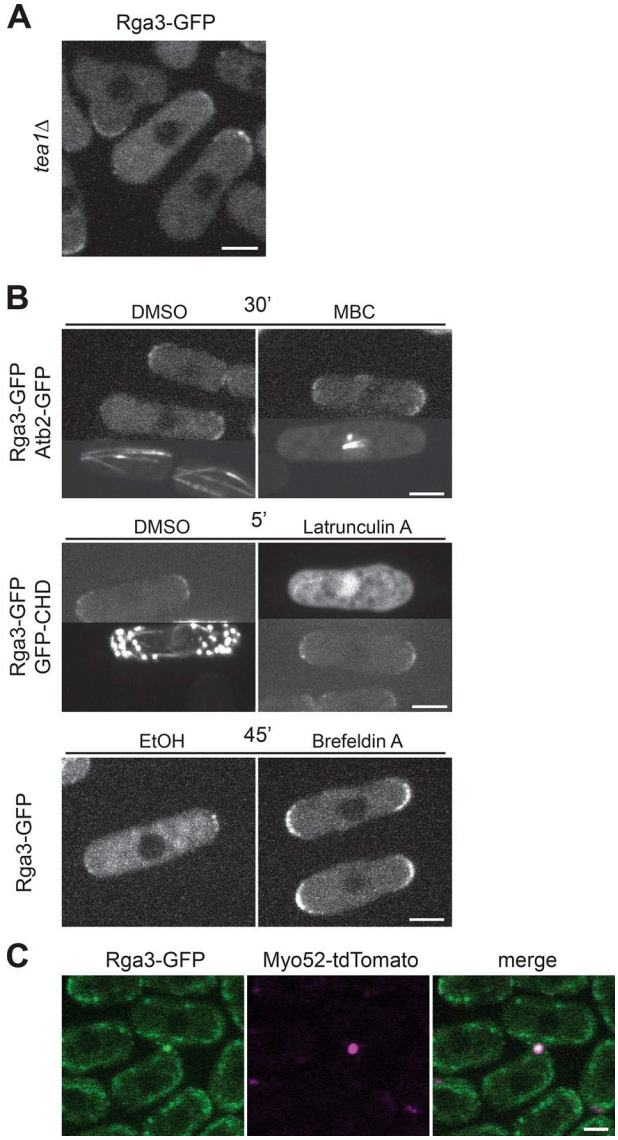


Figure S1. **Localization of Rga3 to sites of polarity independent of the cytoskeleton. (A)** Rga3-GFP at the single growing cell pole of tea1Δ cells. **(B)** Rga3-GFP in cells treated with MBC (top), latrunculin A (middle), or brefeldin A (bottom). Efficiency of MBC and latrunculin A was confirmed by coimaging cells carrying GFP-labeled Atb2 (α-tubulin) or GFP–calponin homology domain (CHD), which labels F-actin, respectively. Images are contrasted differently for these markers. **(C)** Localization of Rga3-GFP (green) at the site of cell–cell fusion as labeled by the Myo52 fusion focus (purple). Bars, 3 µm.

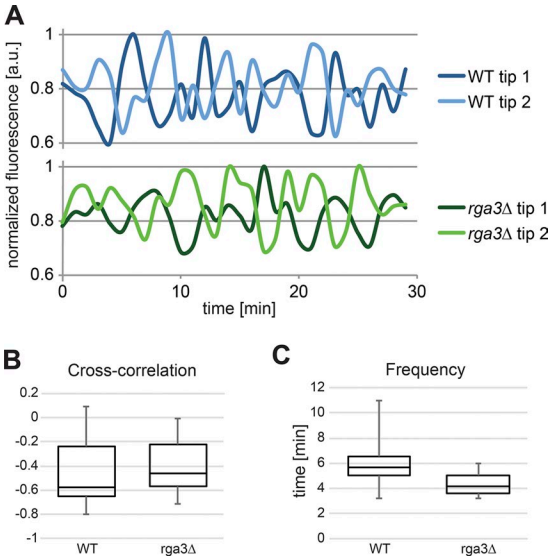


Figure S2. **Minor changes in Cdc42-GTP oscillations in *rga3Δ* cells. (A)** Relative fluorescence intensity of CRIB-GFP at the two cell poles of a single WT and *rga3Δ* cell over 30 min. **(B)** Values for cross-correlation between the fluorescence intensities at the two cell poles in cells as in A showing anticorrelation in both WT and *rga3Δ*. **(C)** Frequency of oscillation derived from traces as in A. The graphs show median values and upper and lower quartiles. Error bars indicate maximum and minimum values. *n* = 20 cells.

Table S1. **Strains used in this study**

Figure	Strain	Genotype
Fig. 1 (A and B)	YSM3130	h- wt ade6- leu1- ura4+
Fig. 1 (A and B)	YSM3131	h- rga3Δ::kanMX ade6- leu1-32 ura4-D18
Fig. 1 (A and B)	YSM1404	h- rga4Δ::ura+ ade6- leu1-32 ura4-D18
Fig. 1 (A and B)	YSM3132	h- rga6Δ::kanMX leu1- ura4+
Fig. 1 (A and B)	YSM3133	h- rga3Δ::kanMX rga4Δ::ura+ ade6- leu1-32 ura4-D18
Fig. 1 (A and B)	YSM3134	h- rga6Δ::kanMX rga4Δ::ura+ leu1-
Fig. 1 (A and B)	YSM3135	h- rga3Δ::HPH rga6Δ::kan ade6- leu1- ura4
Fig. 1 (A and B)	YSM3136	rga6Δ::kanMX rga3Δ::HPH rga4Δ::ura+ leu1-
Fig. 1 (A and B)	YSM3137	h- ade6-M210 leu1-32 ura4-D18 + pSM1354
Fig. 2 (A and B)	YSM3138	h+ cdc42-mCherry-SW-term-kanMX-3′UTR CRIB-GFP-ura4+ leu1-32
Fig. 2 (A and B)	YSM3139	h+ cdc42-mCherry-SW-term-kanMX-3′UTR CRIB-GFP-ura4+ rga6Δ::kanMX rga3Δ::HPH rga4Δ::ura+ leu1-32
Fig. 2 B	YSM3164	h+ rga3Δ::HPH cdc42-mCherry-SW-term-kanMX-3′UTR CRIB:GFP3:ura4+ ade6- leu1-
Fig. 2 C	YSM3140	h- rga3Δ::hph rga4Δ::ura+ rga6Δ::kan scd2-mCherry-NAT
Fig. 2 D	YSM3141	cdc42-mCherry-SW-term-kanMX-3′UTR ade6- leu1-32 ura4-D18
Fig. 2 D	YSM3142	rga3Δ::HPH cdc42-mCherry-SW-kan ade6- leu1-32 ura4-D18
Fig. 2 D	YSM3143	rga4Δ::ura+ cdc42-mCherry-SW-kan ade6- leu1-32 ura4-D18
Fig. 2 D	YSM3144	rga6Δ::KAN cdc42-mCherry-SW-term-kanMX-3′UTR ade6- leu1-32 ura4-D18
Fig. 2 D	YSM3145	rga6Δ::kanMX rga3Δ::HPH rga4Δ::ura+ cdc42-mCherry-SW-term-kanMX-3′UTR
Fig. 3 A	YSM1965	rga4-RFP-kanMX rga3-GFP-kanMX ura4-D18
Fig. 3 B	YSM3146	h+ rga3-GFP-kanMX cdc42-mCherry-SW-term-kanMX-3′UTR
Fig. 3 C	YSM3147	h+ rga3-GFP-kanMX + pSM1138 ade6-M216 leu1-32 ura4-D18
Fig. 3 C	YSM3148	h+ rga3-GFP-kanMX + pSM1358 ade6-M216 leu1-32 ura4-D18
Fig. 3 F	YSM1232	h+ rga3-GFP-kanMX ade6- leu1-32 ura4-D18
Fig. 3 F	YSM3149	h- rga3(-c1)-gfp-kanMX ade6- leu1- ura4+
Fig. 3 G	YSM3150	rga6Δ::kanMX rga4Δ::ura+ rga3-gfp-kanMX
Fig. 3 G	YSM3151	rga3(-c1)-gfp-kanMX rga6Δ::kanMX rga4Δ::ura+
Fig. 4 (A–C)	YSM2042	h- scd2-mCherry-natMX
Fig. 4 (A–C)	YSM3152	h+ scd2-GFP-hphMX ade6+ leu1+ ura4-D18
Fig. 4 (A–C)	YSM3153	h+ rga3Δ::HPH scd2-GFP-hphMX ura4-D18
Fig. 4 (B and C)	YSM3249	h+ rga3(-c1)-gfp-kanMX scd2-GFP-hphMX ura4+
Fig. 4 (B and C)	YSM3250	h+ rga3(-c1)-gfp-kanMX scd2-GFP-hphMX ura4+
Fig. 4 (B and C)	YSM3251	h+ rga3(ΔGAP)-natMX scd2-GFP-hphMX ade6+ leu1+ ura4-D18
Fig. 4 (B and C)	YSM3252	h+ rga3(ΔGAP)-natMX scd2-GFP-hphMX ade6+ leu1+ ura4-D18
Fig. 4 D	YSM3154	h- sxa2Δ::kanMX scd2-GFP-hphMX ade6- leu1- ura4+
Fig. 4 D	YSM3155	h- rga3Δ::kanMX sxa2Δ::kanMX scd2-GFP-hphMX ade6- leu1- ura4+
Fig. 4 E	YSM2042	h- scd2-mCherry-natMX
Fig. 4 E	YSM2764	h+ scd2-mCherry-natMX
Fig. 4 E	YSM3156	h+ ura4-pfus1-dVenus-ura4+ ade6+ leu1+
Fig. 4 E	YSM3157	h+ rga3Δ::hphMX ura4-pfus1-dVenus-ura4+ ade6+ leu1+
Fig. 4 F	YSM3158	h+ wt ade6- leu1- ura4+
Fig. 4 F	YSM3130	h- wt ade6- leu1- ura4+
Fig. 4 F	YSM3159	h+ rga3Δ::kanMX ade6- leu1-32 ura4-D18
Fig. 4 F	YSM3131	h- rga3Δ::kanMX ade6- leu1-32 ura4-D18
Fig. 4 (F and G)	YSM1396	h90 ade6+ leu1+ ura4+
Fig. 4 (F and G)	YSM1996	h90 rga3Δ::kanMX ade6+ leu1+ ura4+

Table S1. **Strains used in this study *(Continued)***

Figure	Strain	Genotype
Fig. 4 H	YSM1232	h+ rga3-GFP-kanMX ade6- leu1-32 ura4-D18
Fig. 4 H	YSM3160	h+ rga3Δ::HPH ade6- leu1- ura4-
Fig. 4 H	YSM952	h- myo52-tomato-natMX ade6-M216 leu1-32 ura4-D18 his7+
Fig. 5 A	YSM3161	h- rga3-gfp-kanMX myo52-tomato-natMX ade6-M216 leu1-32 ura4-D18
Fig. 5 A	YSM3162	h+ rga3-gfp-kanMX myo52-tomato-natMX ade6-M216 leu1-32 ura4-D18
Fig. 5 (B and C)	YSM3154	h- sxa2Δ::kanMX scd2-GFP-hphMX ade6- leu1- ura4+
Fig. 5 (B and C)	YSM3155	h- rga3Δ::kanMX sxa2Δ::kanMX scd2-GFP-hphMX ade6- leu1- ura4+
Fig. 5 D	YSM2042	h- scd2-mCherry-natMX
Fig. 5 D	YSM3153	h+ rga3Δ::HPH scd2-GFP-hphMX ura4-D18
Fig. 6 (A and B)	YSM1371	h+ wt ade6+ leu1+ ura4+ his7+
Fig. 6 A	YSM2042	h- scd2-mCherry-natMX ade6+ leu1+ ura4+
Fig. 6 (A and B)	YSM3140	h- rga3Δ::hph rga4Δ::ura+ rga6Δ::kan scd2-mCherry-NAT
Fig. 6 A	YSM3163	h- rga3Δ::hph rga4Δ::ura+ rga6Δ::kan scd2-mCherry-NAT
Fig. 6 C	YSM1372	h- wt ade6+ leu1+ ura4+ his7+
Fig. 6 C	YSM3139	h+ cdc42-mCherry-SW-term-kanMX-3′UTR CRIB-GFP-ura4+ rga6Δ::kanMX rga3Δ::HPH rga4Δ::ura+ leu1-
Fig. S1 A	YSM1234	h+ tea1::ura4+ rga3-GFP-kanMX ura4-D18
Fig. S1 B	YSM1232	h+ rga3-GFP-kanMX ade6- leu1-32 ura4-D18
Fig. S1 B	YSM1960	h+ SV40-GFP-Atb2-leu+ leu1- ura4-
Fig. S1 B	YSM1273	h- nmt41-GFP-CHD-leu1+ ade6-M216 leu1-32 ura4-D18
Fig. S1 C	YSM3161	h- rga3-gfp-kanMX myo52-tomato-natMX ade6-M216 leu1-32 ura4-D18
Fig. S1 C	YSM3162	h+ rga3-gfp-kanMX myo52-tomato-natMX ade6-M216 leu1-32 ura4-D18
Fig. S2 (A–C)	YSM3138	h+ cdc42-mCherry-SW-term-kanMX-3′UTR CRIB-GFP-ura4+ leu1-32
Fig. S2 (A–C)	YSM3164	h+ rga3Δ::HPH cdc42-mCherry-SW-term-kanMX-3′UTR CRIB:GFP3:ura4+ ade6- leu1-

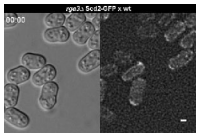
Table S2. **Plasmids used in this study**

Plasmid number	Plasmid name	Plasmid construction	Source
pSM1358	pREP41-cdc42-mCherrySW-Q61L		Bendezú et al., 2015
pSM1414	pGEX-KG-shk1CRIB		Tatebe et al., 2008
pSM1397	pMAL-TEV-cdc42	Amplification of cdc42 from genomic DNA with primers osm2335 (5′-AAGGAAAAAAGCGGCCGCCATGCCCACCATTAAGTGTGTC-3′)-osm2337 (5′-GCTCTAGATTACAGTACCAAACACTTTGAC-3′), digestion with NotI-XbaI, and cloned into pSM819 (pMAL-TEV) digested with the same enzymes	A gift from L. Merlini
pSM1429	pGEX-4T-1-Rga3	Rga3 amplified from pSM1337 (see below) with primers osm2321 (5′-CGGGATCCATGATATTTCGAAAAAGTATTTCC-3′)-osm2323 (5′-AAGGAAAAAAGCGGCCGCCTACAGATTATGAAGAATCTTATCC-3′), digested BamHI-NotI, and cloned into psM394 (pGEX-4T-1) digested with same enzymes.	This study
pSM880	pGEX-4T-1-Rga4		Lab stock
pSM1433	pGEX-4T-1-Rga6	Rga6 fragment amplified from gDNA with primers osm2327 (5′-GGAATTCATGACCTTAAATCCTTCGACTC-3′)-osm2329 (5′-AAGGAAAAAAGCGGCCGCTTAAGATTTCTTCTTGTTTGATCG-3′), digested EcoRI-NotI, and cloned into psM394 (pGEX-4T-1) digested with same enzymes.	This study
pSM1546	pGEX-4T-1-Rga3(R816G)	Mutagenesis PCR on pSM1429 with osm2551 (5′-GAGGTTGAAGGTATTTATgGGATATCTGGCAGTGCTTCT-3′)-osm2552 (AGAAGCACTGCCAGATATCCcATAAATACCTTCAACCTC-3′)	This study
pSM1545	pGEX-4T-1-Rga4(R783G)	Mutagenesis PCR on pSM880 with osm2553 (5′-GATTTTGAAGGTCTGTATgGCAAATCAGGGGCAACTTCT-3′)-osm2554 (5′-AGAAGTTGCCCCTGATTTGCcATACAGACCTTCAAAATC-3′)	This study
pSM1547	pGEX-4T-1-Rga6(R354G)	Mutagenesis PCR on pSM1433 with osm2555 (5′-CATGTACCGGGAATTTTCgGCATTAGTGGTTCTGGTCCC-3′)-osm2556 (5′-GGGACCAGAACCACTAATGCcGAAAATTCCCGGTACATG-3′)	This study
pSM1138	pREP41-Cdc42-mcherry		Bendezú et al., 2015
pSM1358	pREP41-cdc42-mCherrySW-Q61L		Bendezú et al., 2015
pSM1337	pREP41-Rga3-GFP	Amplification of Rga3 from genomic DNA with primers osm1827 (5′-GGAAGATCTATGATATTTCGAAAAAGTATT-3′)-osm1829 (5′-CTGGGATCCCAGATTATGAAGAATCTTATC-3′), digestion with BglII-BamHI, and cloned into pSM621 (pRep41-EGFPc) digested with the same enzymes	This study
pSM1338	pREP41-Rga3(1-790aa)-GFP	Amplification of Rga3 from genomic DNA with primers osm1827 (5′-GGAAGATCTATGATATTTCGAAAAAGTATT-3′)-osm1830 (5′-CTGGGATCCCCCTTCAATTTTCAATTGATT-3′), digestion with BglII-BamHI, and cloned into pSM621 (pRep41-EGFPc) digested with the same enzymes	This study
pSM1339	pREP41-Rga3(1-686aa)-GFP	Amplification of Rga3 from genomic DNA with primers osm1827 (5′-GGAAGATCTATGATATTTCGAAAAAGTATT-3′)-osm1831 (5′-CTGGGATCCAGGATCGGCAGACTTTGATTT-3′), digestion with BglII-BamHI, and cloned into pSM621 (pRep41-EGFPc) digested with the same enzymes	This study
pSM1341	pREP41-Rga3(1-450aa)-GFP	Amplification of Rga3 from genomic DNA with primers osm1827 (5′-GGAAGATCTATGATATTTCGAAAAAGTATT-3′)-osm1833 (5′-CTGGGATCCTGATTGTGCTGGAGTTGAAGA-3′), digestion with BglII-BamHI, and cloned into pSM621 (pRep41-EGFPc) digested with the same enzymes	This study
pSM1408	pREP41-Rga3(288-969)-GFP	Amplification of Rga3 fragment from pSM1337 with primers osm2274 (5′-GGAAGATCTATGTTTCACAAACAATCTTTTACTCCT-3′)-osm1829 (5′-CTGGGATCCCAGATTATGAAGAATCTTATC-3′), digestion with BglII-BamHI, and cloned into pSM621 (pRep41-EGFPc) digested with the same enzymes	This study
pSM1409	pREP41-Rga3(288-686)-GFP	Amplification of Rga3 fragment from pSM1337 with primers osm2274 (5′-GGAAGATCTATGTTTCACAAACAATCTTTTACTCCT-3′)-osm1831 (5′-CTGGGATCCAGGATCGGCAGACTTTGATTT-3′), digestion with BglII-BamHI, and cloned into pSM621 (pRep41-EGFPc) digested with the same enzymes	This study

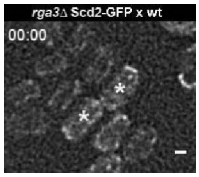
Table S2. **Plasmids used in this study *(Continued)***

Plasmid number	Plasmid name	Plasmid construction	Source
pSM2200	pREP41-Rga3(288-790)-GFP	Amplification of Rga3 fragment from pSM1337 with primers osm5062 (5′-CATGGT ACCAGATCTATGTTTCACAAACAATCTTTTACTCCTG-3′)-osm5063 (5′-CATACCCGGGGA TCCTTCAATTTTCAATTGATTTTCTAACG-3′), digestion with BglII-BamHI, and cloned into pSM621 (pRep41-EGFPc) digested with the same enzymes	This study
pSM2198	pREP41-(Rga3)C1 × 3-GFP	Rga3 C1 domain in three copies was ordered as a Gblock. It was subsequently cloned into pSM621 previously digested with XhoI by In-Fusion method	This study
pSM2187	pJK211-pUra4-AfeI-pfus1-dVenus-terminatortdh1	pfus1-dVenus fragment obtained from pSM1328 (derived from S. Pelet's gift of pSP10; University of Lausanne, Lausanne, Switzerland) digested with KpnI-XhoI and cloned in pSM2185 (pJK211-pUra4-AfeI-terminatortdh) digested with the same enzymes	

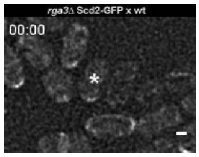
Restriction enzymes are underlined; point mutageneses are lowercase bold.



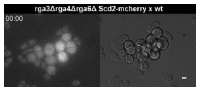
Video 1. **Exacerbated growth phenotypes of *rga3Δ* cells during mating.** Differential interference contrast and Scd2-GFP time-lapse images of h⁺ *rga3Δ scd2-GFP* cells mated to h⁻ WT. From the two *rga3Δ scd2-GFP* cells in the middle of the image, the top one extends a long shmoo and mates with a WT cell, while the bottom polarizes growth predominantly at the top cell pole but also intermittently at the bottom one without finding a partner cell. Time is in minutes. The video is shown at 10 frames/s. Bar, 3 µm. See also Fig. 4 A.



Video 2. **Example of *rga3Δ* cells with patch-wandering behavior.** Time-lapse images of Scd2-GFP in h⁺ *rga3Δ* cells mated with h⁻ WT. Two rga3Δ scd2-GFP cells, marked with asterisks in the first time point, form patches at cell poles that then move laterally toward the mating point with the partner cell. Time is in minutes. The video is shown at 10 frames/s. Bar, 3 µm.



Video 3. **Example of *rga3Δ* cells with patch assembly–disassembly behavior.** Time-lapse images of Scd2-GFP in h⁺ *rga3Δ* cells mated with h⁻ WT. Example of an rga3Δ scd2-GFP cell (marked with asterisk in the first time point) that assembles and disassembles cortical patches before mating with a partner cell on the side. Time is in minutes. The video is shown at 10 frames/s. Bar, 3 µm. See also Fig. 5 D, bottom cell.



Video 4. **Cells lacking Rga3, Rga4, and Rga6 retain polarization ability during mating.** Time-lapse images of Scd2-mCherry in h⁻ *rga3Δrga4Δrga6Δ* cells mating with unlabeled h⁺ WT. See also Fig. 6 B. Time is in minutes. The video is shown at 10 frames/s. Bar, 3 µm.