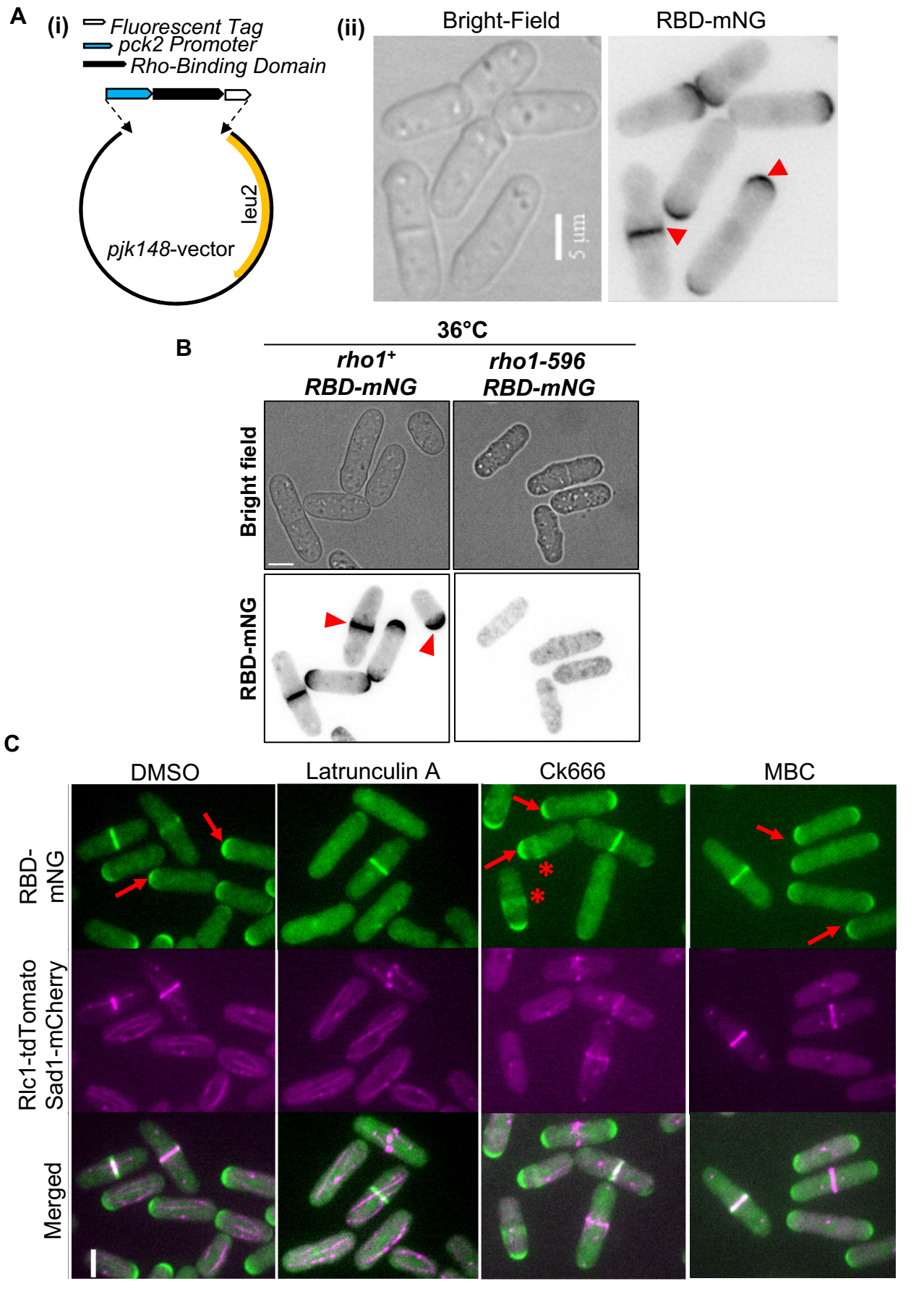


Supplemental Figure 1

Fig. S1. The Rho-probe, (RBD-mNG) detects Rho1 activation at cell tips and the division site. A (i). An Illustration of the Rho-probe design. **(ii).** Rho-probe (RBD-mNG) detects Rho activation at the division site and cell tips in wildtype cells (red arrowheads) **B.** Rho-probe (RBD-mNG) fails to detect active Rho1 in *rho1-596* thermosensitive mutant incubated at 36°C for 2 hours. Red arrowheads point to active Rho1 (RBD-mNG) at cell division site and cell tips. **C.** Effect of cytoskeleton depolymerization on Rho probe (RBD-mNG) localization in live cells that were treated with DMSO, Latrunculin A, Ck666, and Methyl benzimidazol-2-yl-carbamate (MBC), Red arrows point to active Rho1 (RBD-mNG) at cell tips, and asterisks mark ectopic Rho1 activation [Scale Bars, 5μm].

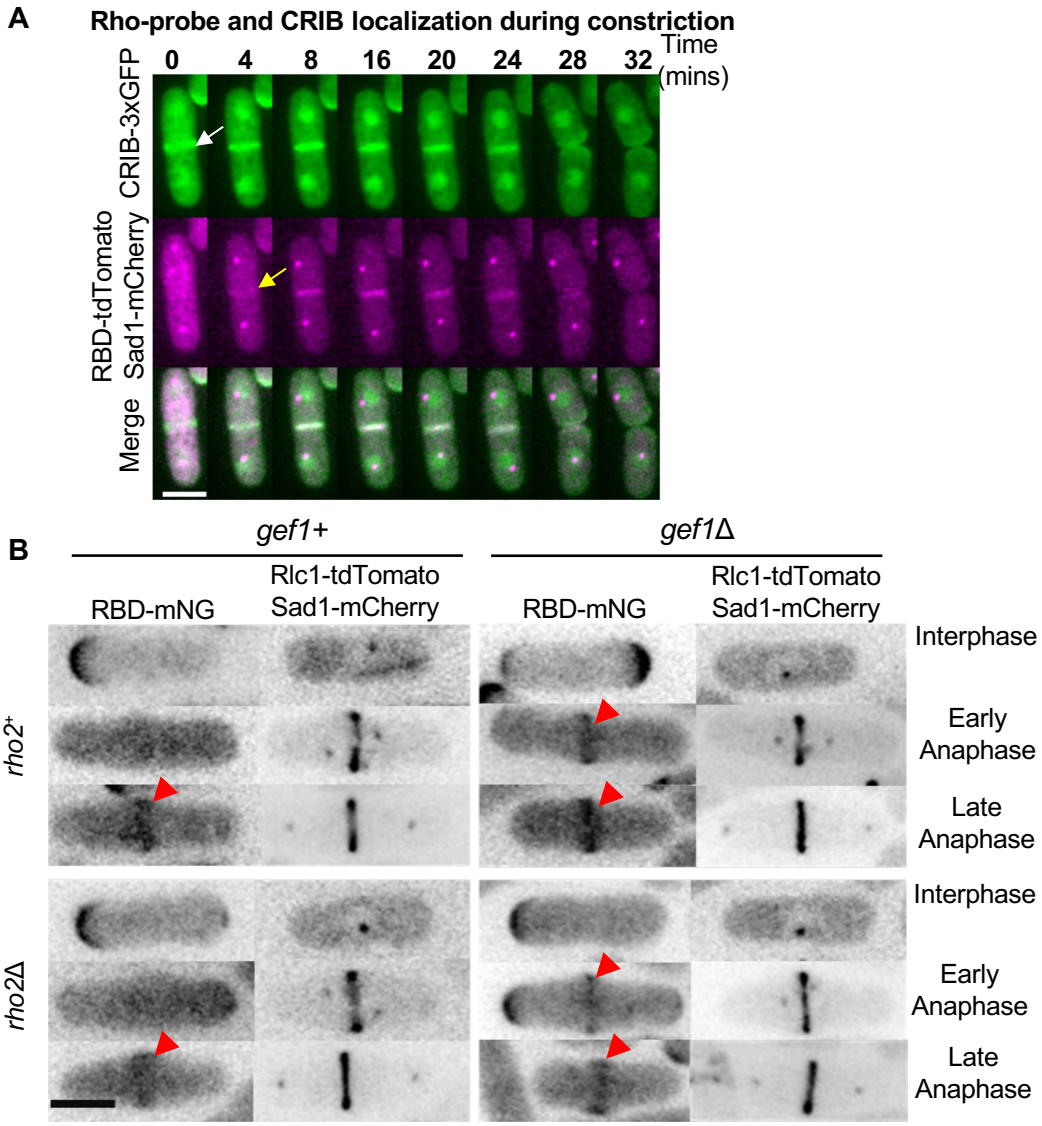


Fig. S2. Rho-probe, RBD-mNG, detects Rho1 activation in cells

A. Time-lapse montage of a representative cell showing Rho1 (RBD-tdTomato, yellow arrow) and Cdc42 activation (CRIB-3xGFP, white arrow) at the division site during ring constriction. **B.** Sum projection of z-stack images showing active Rho1 (RBD-mNG) in *gef1+ rho2+* and *gef1Δ rho2Δ* strains. Red arrowheads point to active Rho1 localization in dividing cells. **C.** Outcome plot shows the SPB distances at which active Rho1 is observed at the division site in the mentioned strains, n=103 cells per indicated strains. [Scale Bar 5μm]

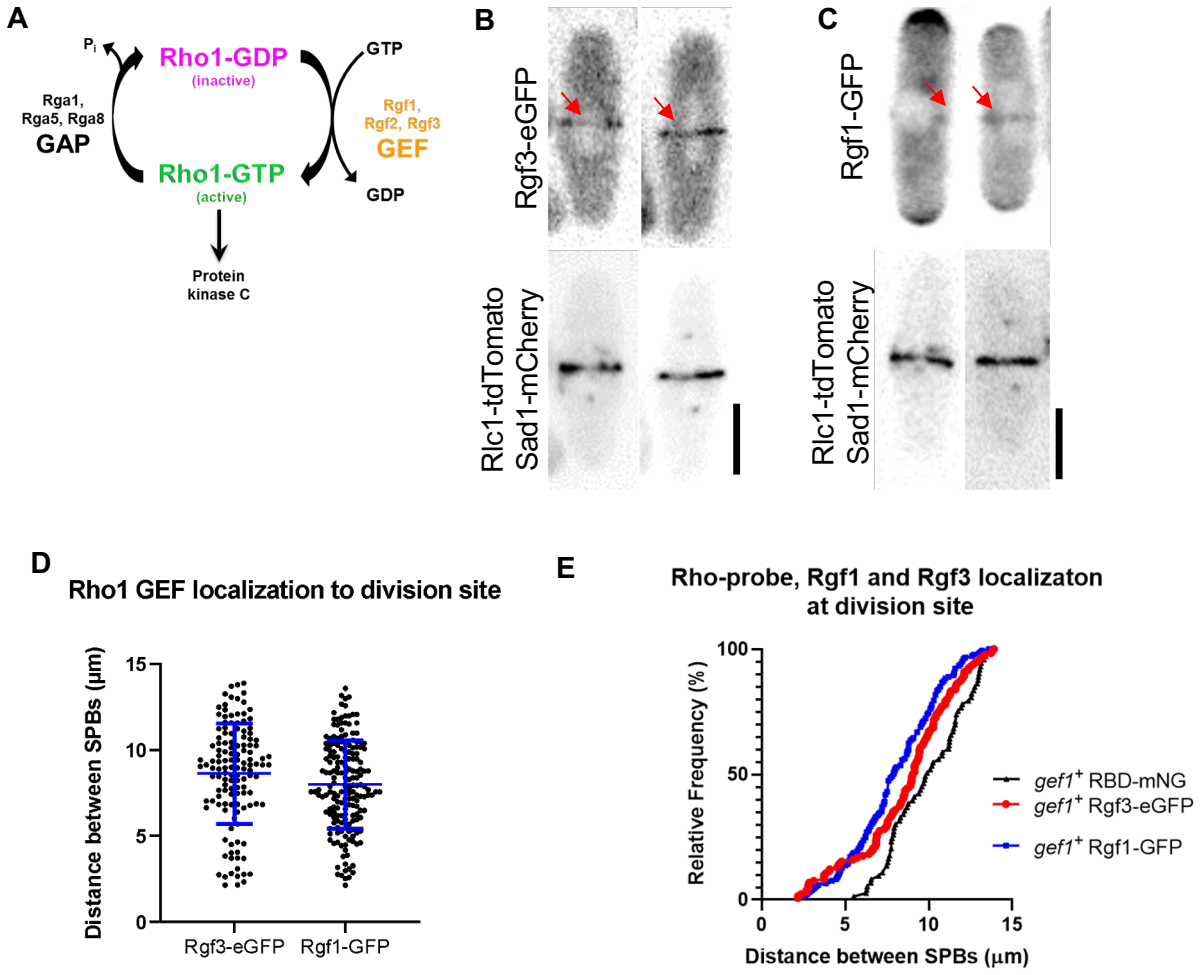
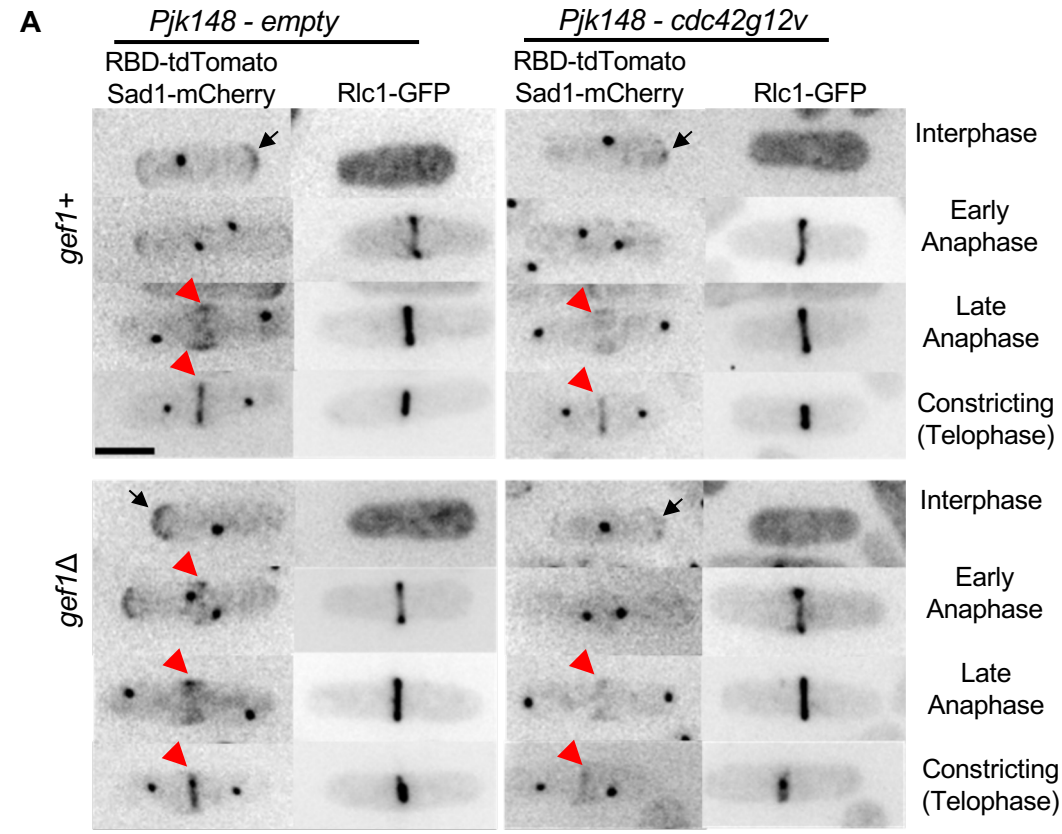
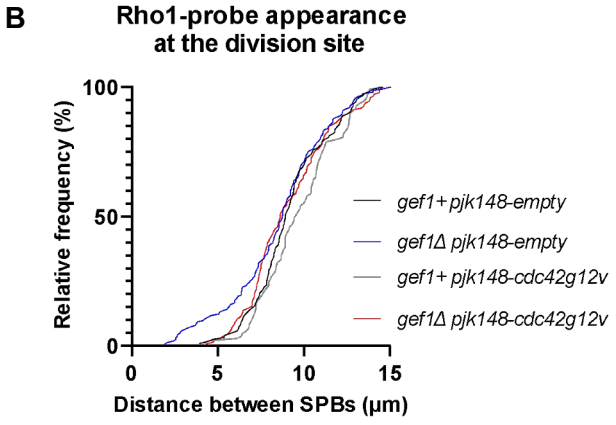
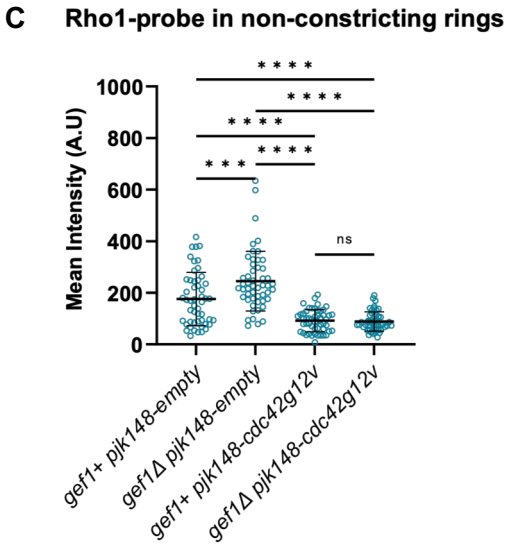


Fig. S3. Rho1-GEFs Rgf1 and Rgf3 localize to the division site early during cytokinesis

A. A schematic of the Rho1-GTPase activation cycle showing the known GEFs and GAPs. **B.** Localization of Rgf3-mEGFP and **C.** Rgf1-GFP to the division site (Red arrows) [Scale Bar, 5µm]. **D.** Quantification of the distance between the spindle pole bodies (SPBs) for strains as indicated, [n≥94 cells for each genotype quantified; Error bars represent standard deviation]. **E.** Frequency plot of the shortest SPB distances at which Rgf3 and Rgf1 are present at the division site. Shorter SPB distances represent an earlier time-point during cytokinesis. [n≥90 cells for each genotype quantified].







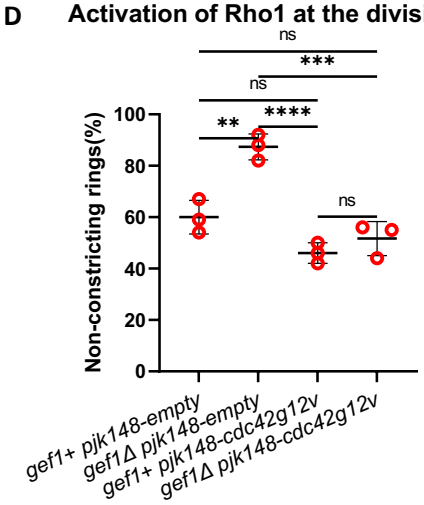
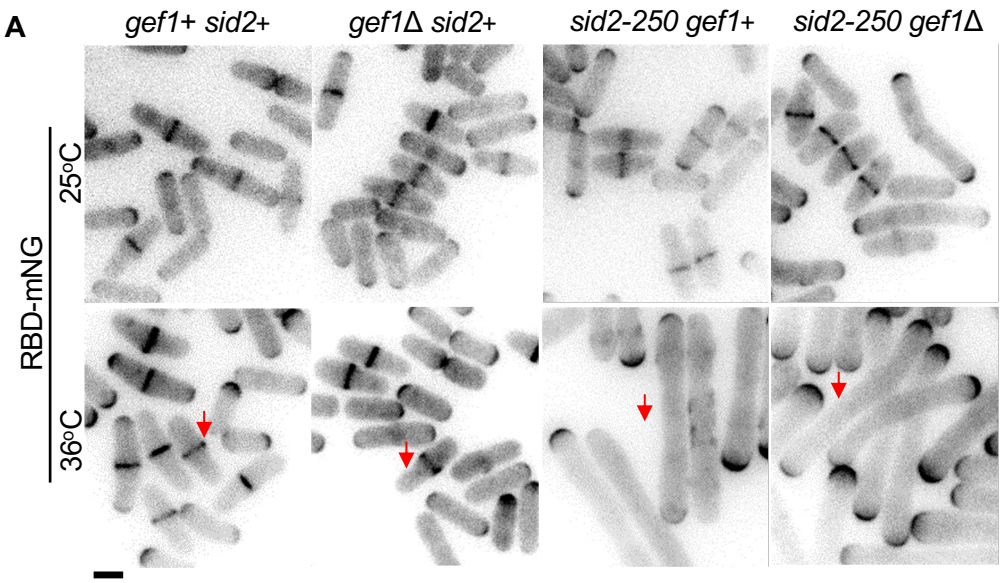


Fig. S4. Constitutively active *cdc42* mutants rescue early Rho1 activation in *gef1Δ* mutants

A. Rho1 activation (RBD-tdTomato) at the division site (red arrowheads) and cell tips (black arrows) of representative *gef1*$^{+}$ and *gef1Δ* cells, transformed with the empty vector pJK148, or expressing constitutively active *cdc42G12V* [Scale Bar, 5μm]. **B.** Outcome plot shows the frequency distribution of SPB distances for which active Rho1 is observed at the division site in the indicated strains, [n=160 cells per strain indicated]. **C.** Quantification of the mean fluorescence intensity of the Rho-probe (RBD-tdTomato) in strains [n≥60 cells per strain]; Statistical significance determined with one-way ANOVA, with Tukey's multiple comparisons post hoc test, ***p≤0.0002 ****p≤0.0001; n.s - not statistically significant; Error bars represent standard deviation]. **D.** Quantification of the percentage of non-constricting rings in strains as indicated. [N=3 experiments; Statistical significance between strains determined by one-way ANOVA followed by Tukey's HSD test, **p≤0.0016 ***p≤0.0003, ****p≤0.0001; n.s - not statistically significant; Error bars represent standard deviation.



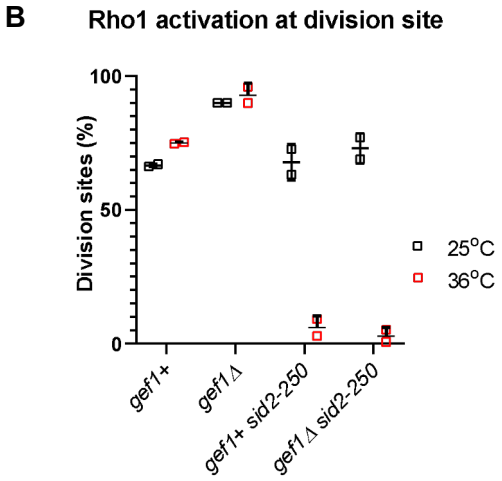
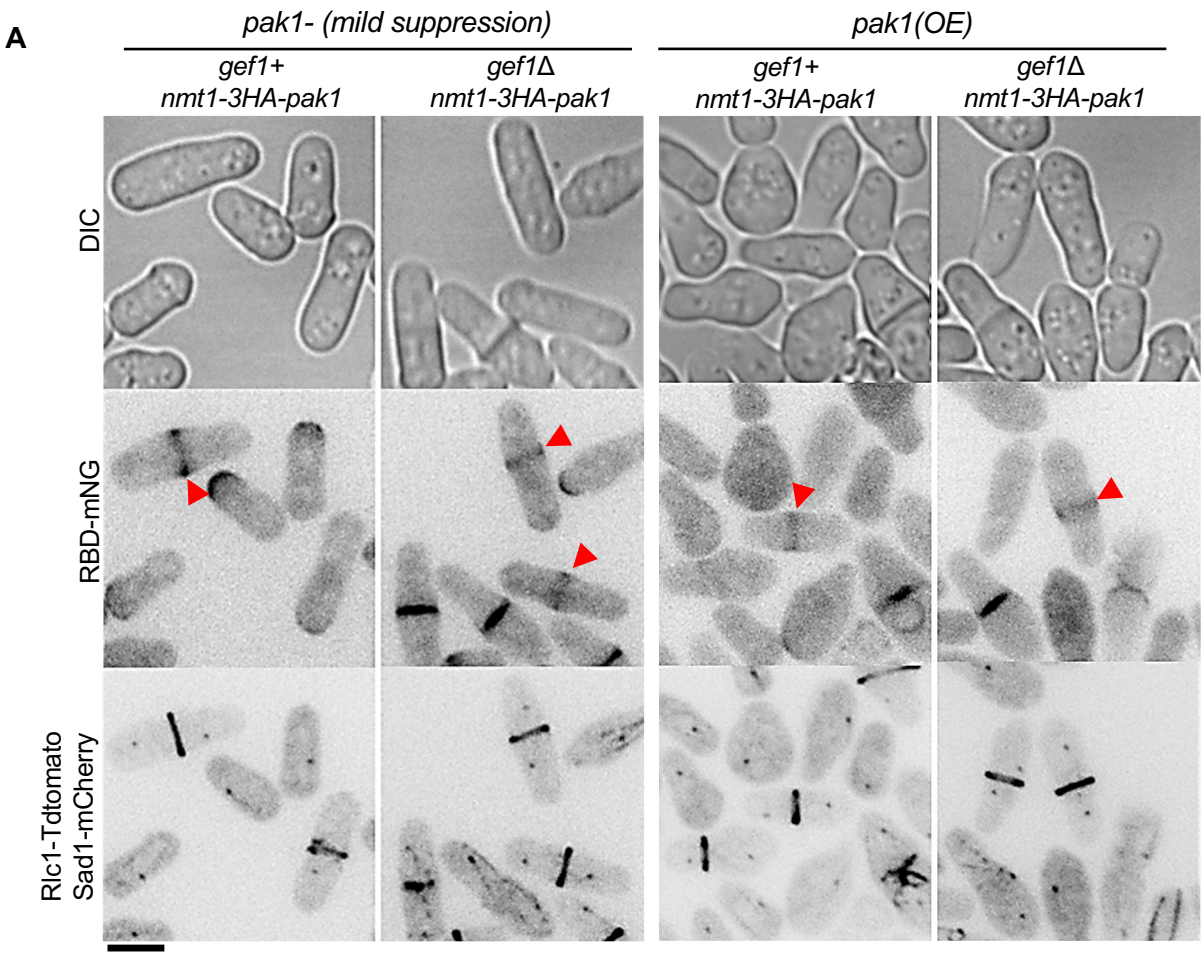
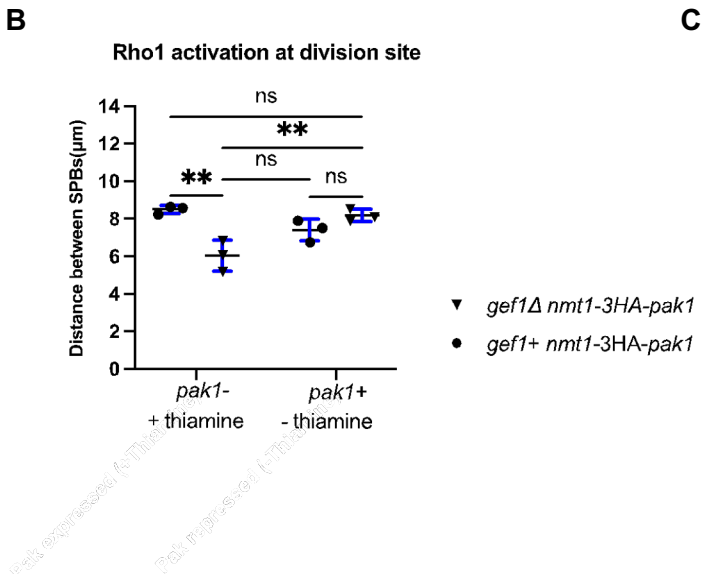


Fig. S5. The SIN pathway is required for Rho1 activation at the division site

A. Rho1 activation (RBD-mNG) during cytokinesis in *sid2-250* strains incubated for 4 hours at the permissive temperature (25°C) and restrictive temperature (35.5°C) [Scale Bar 5μm]. Red arrows point to division sites. **B.** Quantification of the fraction of division sites with RBD-mNG localization [N= 2 experiments, (≥100 division sites analyzed per strain for each experiment); Error bars represent standard deviation].





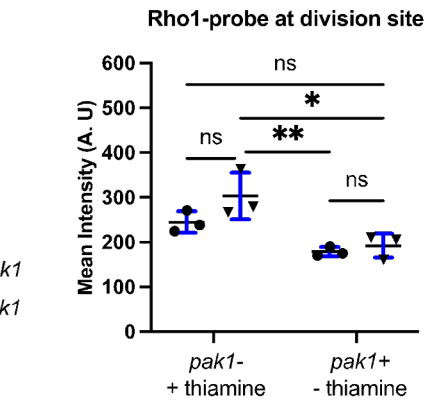


Fig. S6. Overexpression of *pak1* (*pak1*OE) rescues early Rho1 activation in *gef1Δ* cells

A. Rho1 activation (RBD-mNG) during cytokinesis in *gef1+* and *gef1Δ* cells expressing nmt1-3HA-*pak1*, in thiamine repressing (*pak1*-), or overexpressing (*pak1*OE) conditions (see methods). Red arrowheads point to division sites displaying the active Rho1-probe [Scale Bar 5μm]. **B.** Quantification of the cells with active Rho1-probe at the division site during cytokinesis progression as indicated by the SPB distance. Data points on graph represent first quartile of measurements obtained from N= 3 replicate experiments. [Statistical significance between strains determined by one-way ANOVA followed by Tukey's HSD test, **p≤0.005; n.s - not statistically significant; Error bars represent standard deviation]. **C.** Quantification of the mean fluorescence intensity of active Rho1-probe localized to the division site of indicated strains and conditions. [N= 3 replicate experiments; Statistical significance between strains determined by one-way ANOVA followed by Tukey's HSD test, *p≤0.01, **p≤ 0.006; n.s- not statistically significant; Error bars represent standard deviation].

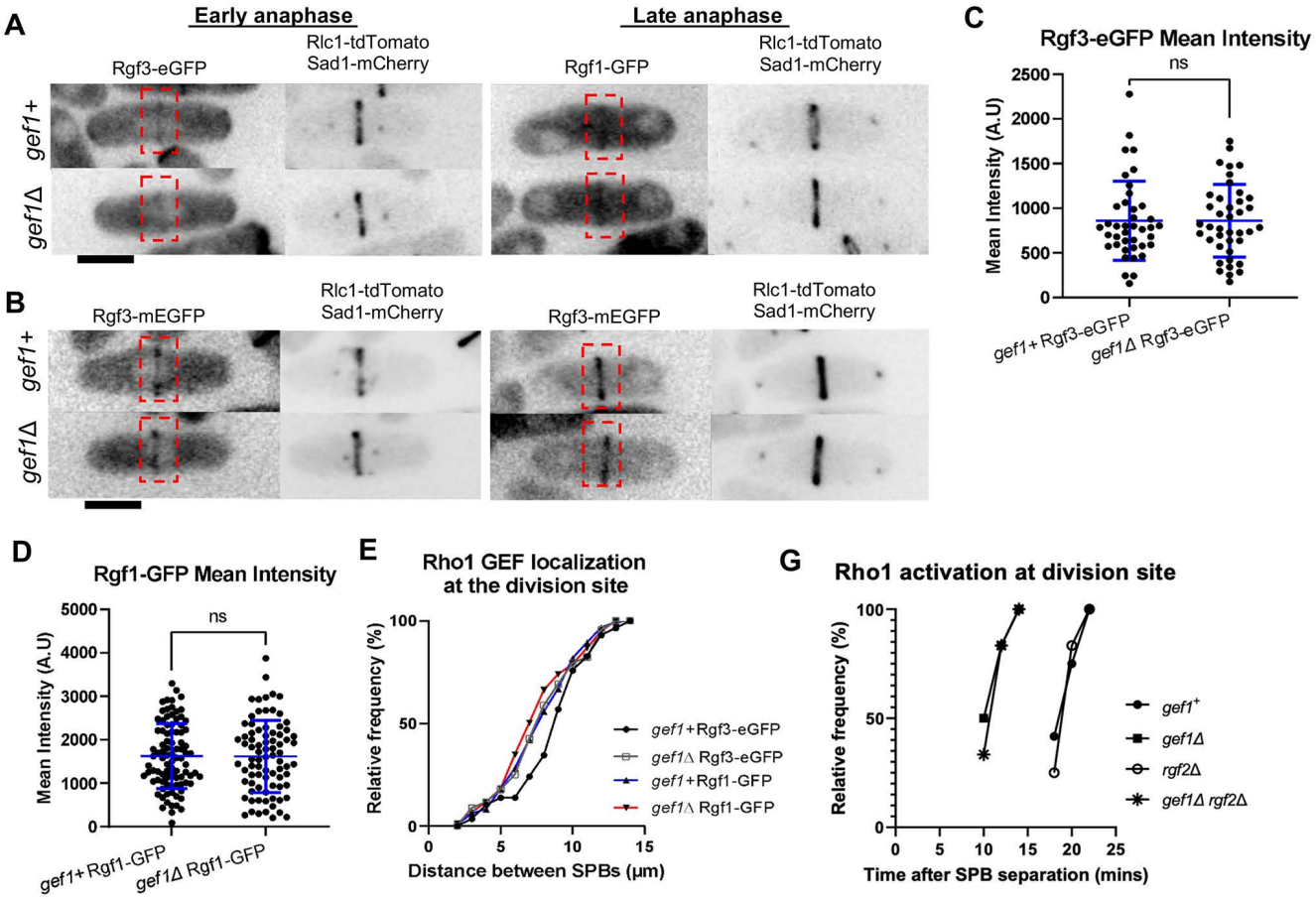


Fig. S7. Localization of Rho1 GEFs is similar in *gef1+* and *gef1Δ* cells Sum projections of cells showing localization of Rgf3-eGFP **(A),** and Rgf1-GFP **(B)** to the division site during early and late cytokinesis (red boxes) in the *gef1+* and *gef1Δ* cells. Quantification of mean fluorescent intensities of Rgf3-mE-GFP **(C),** and Rgf1-GFP **(D),** at the division site of indicated strains, [n ≥80 division sites analyzed per strain; Statistical significance between strains determined by Mann-Whitney test, Rgf3 p=0.774, Rgf1 p=0.936, n.s - not statistically significant; Error bars represent standard deviation]. **E.** Outcome plot shows the distance between the SPBs at which Rgf3 and Rgf1 localization are observed at the division site, n≤190 cells per indicated strains. **F.** Loss of *rgf2* does not rescue Rho1 activation in *gef1* mutants. Time-lapse of representative *gef1+ rgf2+*, and *gef1Δ rgf2Δ* cells shows the time of Rho1 activation (RBD-mNG) at the division site during cytokinesis (yellow box) [Scale Bar 5μm] Time=0 marks the time of SPB separation, and onset of cytokinetic events. **G.** Outcome plot shows the frequency of Rho1 activation over time at the division site during cytokinesis [n=12 cells per strain].

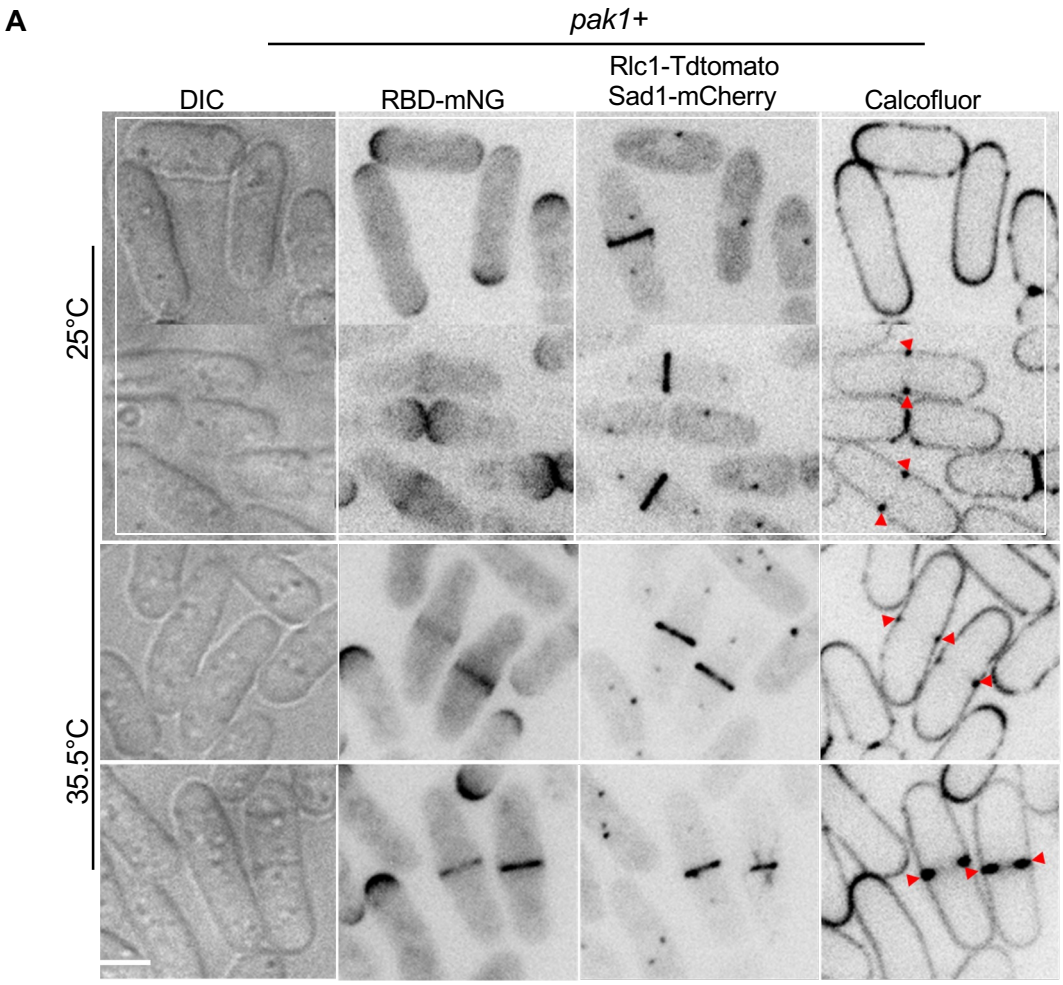


Fig. S8. Hypomorphic *pak1* mutant show early Rho1 activation in cells during cytokinesis

A. Rho1 activation (RBD-mNG) in *pak1+* (*orb2+)* strains grown at permissive (25ºC) and restrictive temperatures (35.5ºC). Septum deposition at the division site (red arrowheads) is visualized with calcofluor staining [Scale Bar 5µm].

Table S1. Strain list

Strain	Genotype	Origin
PN975 YMD493	*h+ ura4-D18 leu1-32 ade6-704*	P. Nurse
YMD527	*Rlc1-tdTomato-NATr Sad1-mCherry: kanMx ade6-M21X leu1-32 his7+ ura4-D18*	This study
YMD1062	*leu2: pck2:RBD-Neon Green leu+ Rlc1-tdTomato-NATr Sad1-mCherry: kanMx*	This study
YMD 2111	*rho1-596:NatMx6- leu2:pck2:RBD-mNeonGreen:leu+*	This study (*rho1-596* – Gift from P.Perez)
YMD 2113	*rho1::ura4/p41xRho1- leu2: pck2:RBD-Neon Green leu - Rlc1-tdTomato-NATr Sad1-mCherry: kanMx*	This Study (*p41xRho1- Gift from* P.Perez)
YMD1099	*gef1Δ::ura+ leu: pck2:RBD-Neon Green leu+ Rlc1-tdTomato-NATr Sad1-mCherry: kanMx*	This study
YMD1394	*orb2-34 (pak1-ts) leu:pck2:RBD-Neon Green leu+ Rlc1-tdTomato-NATr Sad1-mCherry: kanMx*	This study
YMD1706	*Rgf1-GFP:KanMx Rlc1-tdTomato: NATr Sad1-mCherry:KanMX*	This Study
YMD1697	*gef1Δ::ura+ rgf2Δ::KanMx leu2: pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: KanMx*	This Study
YMD1729	*rgf2Δ::Kan leu: pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry:KanMx*	This Study
YMD1699	*gef1Δ::ura+ rgf1Δ::KanMx leu2: pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: KanMx*	This Study
YS733	*h- rgf1Δ::KanMx*	Gift from Y.Sanchez
YS2147	*h- rgf2Δ::KanMx*	Gift from Y.Sanchez
PPG0378	*h- rga5Δ::ura+ leu1-32*	Gift from P.Perez
YMD1728	*rgf1Δ::KanMx leu: pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry KanMx*	This Study
YMD1786	*p3-nmt1-3xHA shk1:G418 leu2: pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: KanMx*	This Study
YMD1801	*pak2Δ::Kan leu: pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: KanMx*	This Study
YMD1775	*gef1Δ::ura+ p3-nmt1-3xHA shk1:G418 leu: pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: kanMx*	This Study
VT88	*rgf3Δ(nmt81-rgf3+)*	Gift from Y.Sanchez
YMD1823	*rgf3Δ(nmt81-rgf3+) leu:pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: kanMx*	This Study

YMD1717	*gef1Δ::ura+ rgf3Δ(nmt81-rgf3+) leu:pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: kanMx*	This Study
YMD1714	*nmt41-cdc42g12v:leu+ RBD-tdTomato:ura+ Rlc1-GFP:Kan Sad1-mCherry:KanMx*	This Study
YMD1715	*gef1Δ::ura+ Rgf1-GFP:Kan Rlc1-tdTomato: NATr Sad1-mCherry:KanMX*	This Study

YMD1827	*rho2Δ::ura+ leu:pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: KanMx*	This Study
YMD1826	*gef1Δ::ura+ rho2Δ::ura+ leu:pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: kanMx*	This Study
YMD1628	*Mob1-mEGFP: KanMx Rlc1-tdTomato: NATr*	This Study
YMD1629	*Sid2-mEGFP: KanMx Rlc1-tdTomato: NATr*	This Study
YMD1636	*gef1Δ:ura+ Mob1-mEGFP:KanMx Rlc1-tdTomato:NATr*	This Study
YMD1635	*gef1Δ:ura+ Sid2-GFP:KanMx Rlc1-tdTomato:NATr*	This Study
YMD1632	*h+ gef1Δ:ura+ nmt41-pjk148-empty: leu+ gef1Δ:ura+ RBD-tdTomato:ura+ Rlc1-GFP:KanMx Sad1-mCherry:KanMx*	This Study
YMD1602	*nmt41-pjk148-empty: leu+ gef1Δ:ura+ RBD-tdTomato:ura+ Rlc1-GFP:KanMx Sad1-mCherry:KanMx*	This Study
YMD1616	*gef1Δ:ura+ nmt41-cdc42g12v: leu+ gef1Δ::ura+ RBD-tdTomato:ura+ Rlc1-GFP:KanMx Sad1-mCherry:KanMx*	This Study
YMD1045	*leu2:pck2:RBD-mNeonGreen:leu+*	This Study
YMD1493	*sid2-250 leu2:pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: KanMx*	This Study
YMD1491	*gef1Δ::ura+ sid2-250 leu:pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: KanMx*	This Study
YMD1119	*Rgf3-mEGFP:leu+ Rlc1-tdTomato: NATr Sad1mCherry:KanMX*	This Study
YMD1121	*gef1Δ::ura+ Rgf3-mEGFP:Kan Rlc1-tdTomato: NATr Sad1-mCherry:KanMX*	This Study
YMD764 (MBY3451)	*h- nmt1-3xHA-pak1*	Loo et al., 2008
YMD1708	*rga5Δ leu:pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: KanMx*	This Study
YMD1652	*gef1Δ::ura+ rga5Δ:: ura+ leu:pck2:RBD-mNeonGreen:leu+ Rlc1-tdTomato-NATr Sad1-mCherry: KanMx*	This Study