Construction of Plasmids and Strains

Yeast strains and plasmids used in this study are listed in Table 1 and Table S1, respectively.

Plasmids and strains for GFP-Rho1: To construct a plasmid for GFP-Rho1 expression, first, NotI site was introduced just after the start codon of RHO1 as follows: The 685-bp DNA fragment covering the region upstream of the start codon was amplified by PCR using YEp24-RHO1 (OZAKI et al. 1996) as template and primers oRHO13 (5'-GAACAAGCTTCTCCCTAT
AATGCGGTAGCATTGG-3') and oRHO18 (5'-GAAGCGGCGACATCTTTCAGATAATTTTTAAAGTTCC-3'). In addition, a 1.23-kb fragment covering the RHO1 ORF from the start codon was amplified by PCR using primers oRHO16 (5'-GAAGCTCGGCCACGTTATCAATGCTCGC-3') and oRHO17 (5'-GAACGCGCCGCTACAAACAGTGG
TAACAGTATC-3'). After digestion of the 685-bp fragment with HindIII and NotI (sites included in the primers) and the 1.23-kb fragment with NotI and XhoI (sites included in the primers), these two fragments were cloned into the pRS426* (pHP1476 = pRS426 lacking NotI) (SINGH et al. 2008) digested with HindIII and XhoI, yielding pRS426*-RHO1 (pHP1697). Next, a 720-bp NotI fragment encoding GFP<sup>567T, V163A, S117G</sup>, isolated from YCp-GFP-RSR1 (pHP767) (PARK et al. 2002), was inserted into the NotI site of pHP1697, yielding pRS426*-GFP-RHO1 (pHP1698). The correct orientation of the GFP insertion in pHP1698 was confirmed by digestion with PvuII.

To construct an integrating plasmid pRS306-GFP-RHO1, pHP1698 was digested with HindIII and XhoI, and the resulting 2.6-kb fragment containing the GFP-RHO1 sequence was cloned into pRS306 (SIKORSKI and HIETER 1989) digested with HindIII and XhoI, yielding pRS306-GFP-RHO1 (pHP1699). To express GFP-Rho1 from the chromosome, pHP1699 was linearized with BglII (which is located at 420 bp downstream of the stop codon of the RHO1 ORF) and integrated into the appropriate strains (see Table 1), and then stable integrants were isolated.

Plasmids and Yeast Strains for BiFC: To construct a strain expressing YCF1 tagged with the N-terminal fragment of Venus (VN) at the C-terminus, a DNA fragment carrying VN-kanMX6 was amplified by PCR using pFA6a-VN-KanMX6 (SUNG and HUN 2007) as template and primers oYCF11 (5'-TTGTTTCTTTACCTGACATGGGAGCTGTTTGTCAATGAAAT
CGGATCCCCCGTTAATTAA-3') and oYCF12 (5'-CTACGTACCAGATTGTCGCCGGACAGTTTTATTAGTTTC
ACAGTGAATTGACGCTGTTTAAAC-3'). The resulting PCR product was transformed into NY2284 by one-step-replacement method (ROTHSTEIN 1991), yielding HPY1710. Correct targeting was confirmed by colony PCR using primers oYCF13 (5'-AGCGAGTTGTGACTCCGGGGCAGAATGTTG-3') and oYCF14 (5'-GCACCTGTTCCTCCGAGAAATGTTG-3').

To express Rho1 fused to the C-terminal fragment of YFP (YFP<sup>C</sup>) at its N terminus, first, the 252 bp NotI fragment of YFP<sup>C</sup> generated from pRS304-YFP<sup>C</sup>-RSR1<sup>K18N</sup> (pHP1678) (KANG et al. 2010) was cloned into the Not1 site of pRS426*-RHO1 (pHP1697),
yielding pRS426-YFP<sup>C</sup>-RHO1 (pHP1737). To express YFP<sup>C</sup>-Rho1 from a CEN plasmid, the 2.1 kb HindIII-Xhol fragment (carrying YFP<sup>C</sup>-RHO1 sequence) from pHP1737 was cloned into pRS316 digested with HindIII and Xhol, yielding pRS316-YFP<sup>C</sup>-RHO1 (pHP1765).

The RHO1<sup>GSRL</sup> and the RHO1<sup>T24N</sup> mutations were generated by PCR-based site-directed mutagenesis using pHP1737 as template and primer pairs oRHO19 (5′-GGGATAGGATACCACTGATAGATTATGAT AGACTAAG-3′) and oRHO110 (5′-CTTTAGTCATGATTTTACTACCGAGGATATCGCAGGC-3′); and oRHO111 (5′-GGGATAGGATACCACTGATAGATTATGAT AGACTAAG-3′) and oRHO112 (5′-GGGATAGGATACCACTGATAGATTATGAT AGACTAAG-3′) and oRHO112 (5′-GGGATAGGATACCACTGATAGATTATGAT AGACTAAG-3′). The resulting PCR product was used to delete the chromosomal TUS1 gene in pHP1710 by one-step gene disruption (ROTHSTEIN 1991), yielding pHP1737. Correct targeting was confirmed by colony PCR using primer pairs, oTUS17 (5′-CATACGACTGAGTAAGGG-3′) and oTRP11 (5′-GTTCACCTGTCGCGAC-3′).

**Plasmids and Strains for Integrated Membrane Yeast Two-Hybrid (IMYTH):** IMYTH construct generation and assays were carried out as previously described (PAUMI et al. 2007; SNIDER et al. 2010) using four THY AP4 MYTH reporter strains—YCF1-CT expressing the C-terminally Cub-LexA-VP16 tagged Ycf1; ArBT-CT expressing Cub-LexA-VP16 tagged artificial bait control construct comprised of the yeast mating factor alpha signal sequence (‘MFaSS’) fused to the transmembrane domain of the human T-cell surface glycoprotein CD4 (‘CD4tm’); and two tus1Δ strains, YCF1-CT ∆T and ArBT-CT ∆T, derived from YCF1-CT and ArBT-CT, respectively. To construct the TUS1 deletion strains in the MYTH bait backgrounds, the NatR resistance cassette was amplified by PCR using primers containing 5′ region homologous to 45 bp upstream (‘forward’ primer) or downstream (‘reverse’ primer) of the TUS1 gene. This PCR product, consisting of the NatR cassette flanked on either side by sequence homologous to the TUS1 gene region, was used to delete the TUS1 gene by one-step gene disruption (ROTHSTEIN 1991).

Construction of Rho1 prey constructs was carried out as follows. The RHO1 gene was amplified from purified *Saccharomyces cerevisiae* genomic DNA and cloned into either the pPR3N or pPR3C MYTH prey vectors (Dualsystems Biotech).
using the classical ‘gap-repair’ homologous recombination method in yeast (Ma et al. 1987). For pPR3N cloning, PCR was carried out using the R3NF (5’-atccagcagtgatcaacgctgccattacggccATGTCACAAACAAGTTGGTGAACATC-3’) and R3NR (5’-tcatgtgtgcaggtcgtcataaatctgtgatatCCTATAACAAGACACTTCTTCTTTC-3’) primers. For pPR3C cloning, the R3CF (5’-gcacatatttcgaatccataactcaactcaatctcaACTCAACAAGTTGGTGAACATC-3’) and R3CR (5’-gtctgtatctcgagagggccggccgcagcatTAACAAGACACTTCTTCTTCTTC-3’) primers were used.

Construction of ycf1 deletion mutants and the YCF1-GFP strain: To construct YCF1 deletion in the NY2284 background, a DNA fragment (2.08 kb) carrying ycf1Δ::KanMX4 was amplified by colony PCR using HPY1904 (an ycf1Δ:: KanMX4 strain obtained from Open Biosystems) and primers, oYCF15 (5’-CTCCTGTTGATGCTTGGGCGGTG-3’) and oYCF14 (5’-GCACCTGTCTCCGGAGAAATGTG-3’). The resulting PCR product was used to delete the chromosomal YCF1 gene in NY2284 and NY2287 by one-step gene disruption (Rothstein 1991), yielding HPY1738 and HPY1739, respectively. Colony PCR was performed using primers oYCF14 and oKanC (5’-CGAGTGATTTTGAACGCGGT-3’) to confirm the correct deletion, which generated a 0.8-kb DNA fragment. The phenotype of ycf1Δ was confirmed by checking growth on a plate containing 30 μM CdCl2.

To construct a strain expressing Ycf1 fused to GFP at its C terminus, a DNA fragment encoding GFP-TRP1 was amplified by PCR using pFA6a-GFP(S65T)-TRP1 (Longtine et al. 1998) as template and primers oYCF11 and oYCF12. The resulting PCR product was transformed into NY2284 by one-step-replacement method, yielding HPY1955. Correct targeting was confirmed by colony PCR using primers oYCF13 and oTRP11.