Supplementary Information

Plasmid Design and Cloning

pRB415A-HA was generated by excising the GFP module from pRB415A and re-ligating with a PCR product consisting of three tandem copies of the HA-tag with XbaI and BamHI overhangs at the 5- and 3- ends, respectively. pRB415A::*FAB1* and pRB415A::*fab1*$^{FYVE(C/S)}$ encodes an Adh-driven GFP-Fab1 and GFP-Fab1$^{FYVE(C/S)}$. It was cloned by a three-step cloning strategy: *i*) ligation of a PCR-amplified N-terminal *FAB1* fragment flanked with a 5' engineered BglII site and ending at position 164 containing a unique AatII; *ii*) this was followed by ligation of a AatII-NruI *FAB1* fragment [or AatII-NruI *fab1*$^{FYVE(C/S)}$ fragment]; *iii*) and completed by inserting a PCR-amplifed C-terminal *FAB1* fragment from the NruI site (nt 6509) and ending with an engineered BspEI site. pRB415A::*FAB1* was internally cut with HinDIII to produce an N-terminal deletion of *FAB1* (pRB415A::FAB1$^{\Delta HinDIII}$). The reading frame was corrected with a linker. Deletion of the kinase domain was done by excision of a NheI-BspEI fragment (pRB415A::FAB1$^{\Delta KIN}$). Deletion of the CCT was done by PCRing and ligating together an N-terminal fragment from position 1-2803 and a C-terminal fragment from 3070 to the stop codon (pRB415A::FAB1$^{\Delta CCT}$). pRS416::*FAB1* and its plasmid-based mutants were tagged with GFP by inserting a fragment amplified from *FAB1*-GFP genomic DNA from the NruI site (nt 6509) to the 3' end of GFP with an engineered NotI site.

pRB415A::FYVE^{Fab1}, pBP74A::GroL and pRB415A-HA::GroL were made by amplifying and ligating 5' HinDIII/3'XhoI flanked fragments. The FYVE^{Fab1} and the GroL-region are encoded by nucleotides 622-951 and 2245-4214 of *FAB1*, respectively. Nucleotides 1-399 of ALP were amplified and inserted into the NheI and SalI sites of

pRB415A-HA and pRB415A to generate pRB415A-HA::ALP and pRB415A::ALP, respectively. The GroL encoding fragment was then inserted in-frame into the HindIII and NheI sites to generate HA-GroL-ALP and GFP-GroL-ALP chimeras.

pRB415A-HA::VAC14 was cloned by amplifying the *VAC14* ORF flanked with engineered 5' BglII and 3' NheI sites. pGEX4T3::*VAC14* and pET23d(+)::*VAC14* were made by PCR-amplifying the *VAC14* ORF flanked with 5'EcoRI and 3'XhoI sites. pET23d(+):*FIG4* was made by amplifying the *FIG4* ORF flanked with 5' BamHI and 3' HindIII sites. pGEX4T3::GroL was made by inserting the fragment encoding the GroL domain into EcoRI and XhoI sites.

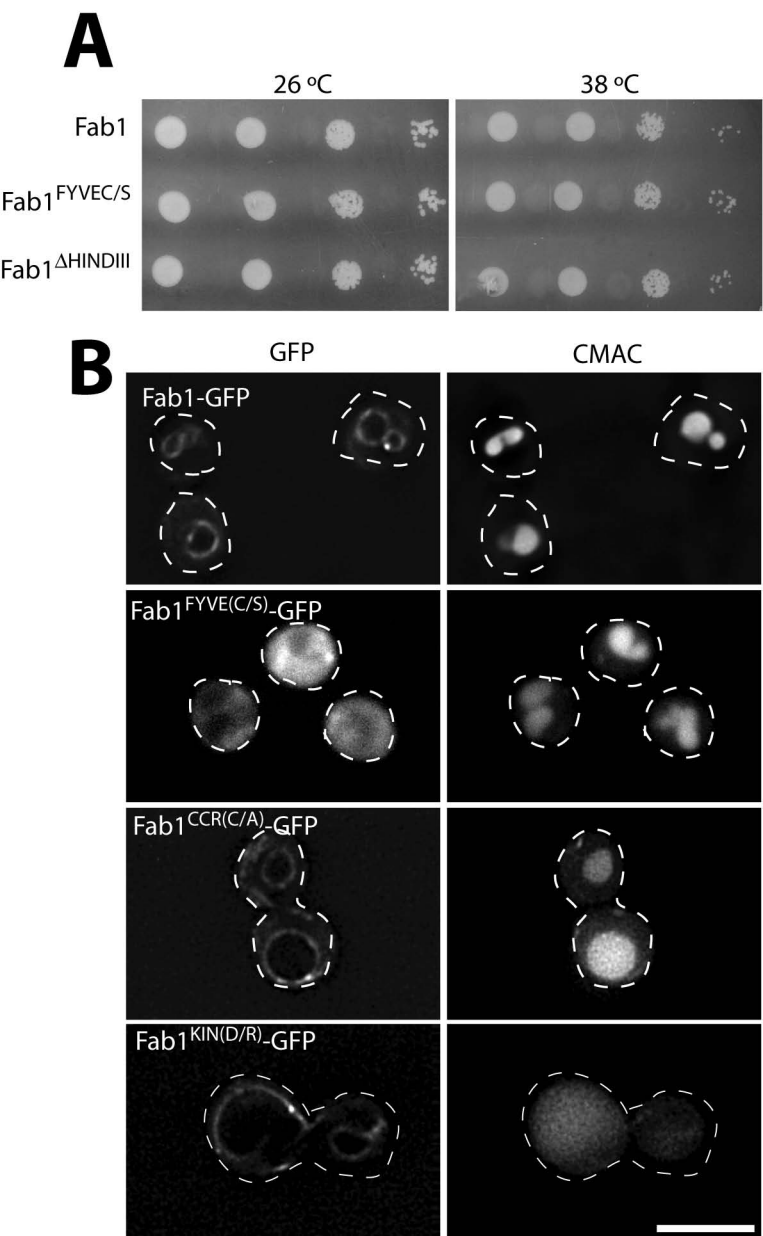
Supplementary Figure Legends

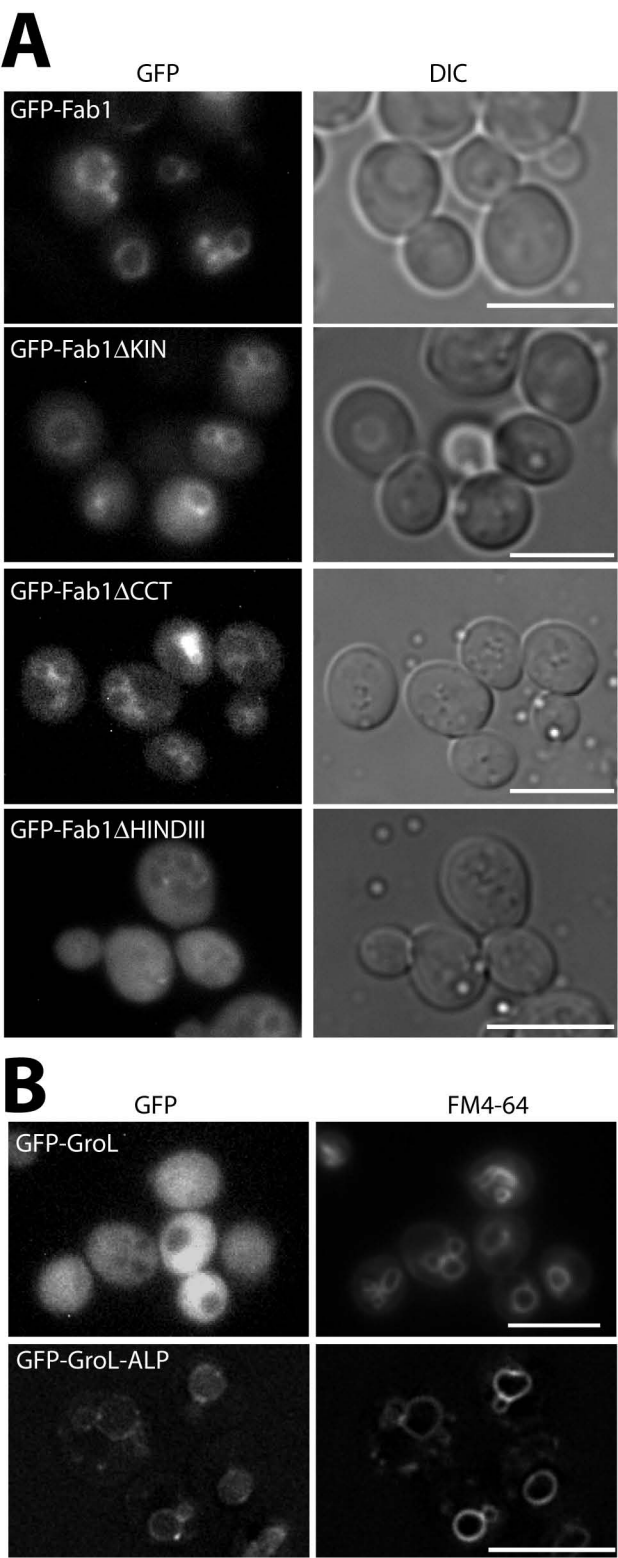
Figure S1: Temperature-sensitivity, localization and vacuole size phenotype for several Fab1 mutants. A. Serial dilutions of *fab1*Δ cells expressing GFP-tagged Fab1, Fab1$^{FYVE(C/S)}$ and Fab1$^{\Delta HindIII}$ at equal levels and grown at 26 °C or 38 °C for three days. **B.** GFP fusion of Fab1 and its mutants expressed in *fab1*Δ cells at endogenous levels. *fab1*Δ cells expressing Fab1-GFP, Fab1$^{FYVE(C/S)}$-GFP, Fab1$^{CCR(C/A)}$-GFP and Fab1$^{KIN(D/R)}$-GFP and stained with CMAC. With the exception of Fab1$^{FYVE(C/S)}$-GFP, these micrographs were subject to deconvolution protocol. Scale bar = 5 μm.

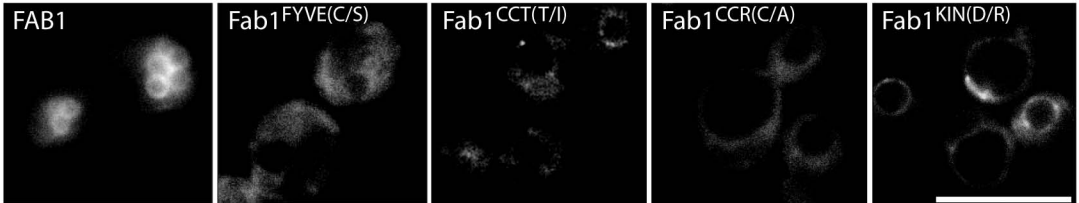
Figure S2: Localization of GFP-Fab1 truncations and of the GroL-like region. **A.** 6210 cells expressing N-terminal GFP fusion proteins of wild-type Fab1 and truncated mutants: ΔKIN= truncation of the kinase domain; ΔCCT= truncation of the CCT domain; ΔHINDIII= truncation of the first ~600 residues of Fab1, which eliminates the FYVE domain but retains the GroL-like region. The ADH promoter drove expression of these proteins. The corresponding DIC images are shown. **B.** 6210 cells expressing a GFP fusion of the GroL-like region and the GFP-GroL-ALP chimera. Vacuoles were labeled wit FM4-64. GFP-GroL-ALP and corresponding FM4-64 has been deconvolved. Scale bar = 5 μm

Figure S3: Behavior of Fig4-GFP in the presence of Fab1 mutants. *fab1*Δ *FIG4-GFP* cells were transformed with vectors expressing wild-type Fab1, the FYVE point mutant Fab1$^{FYVE(C/S)}$, the CCT domain point mutant Fab1$^{CCT(T/I)}$, the CCR domain point mutant Fab1$^{CCR(C/A)}$ and the kinase-dead mutant *FAB1*$^{KIN(D/R)}$. Scale bar = 5 μm

Figure S4: Salt-shock effect on Fab1 complex. **A**. Cells expressing Fab1-Myc were exposed to 0.9M NaCl for 0, 8 or 25 min, followed by glass-beating lysis at 4 °C. After processing, Fab1-Myc was immunoprecipitated and samples were separated by SDS-PAGE and blotted for Fab1-Myc and Fig4 as before. Untagged cells were used to control anti-Myc IP. Inputs represent 20% of total material employed during the IP. **B**. Ratio of immunoprecipitated Fig4 and Fab1-Myc. Non-saturated signal was quantified with ImageJ by obtaining background-subtracted intensity integrated to area. Results are representative of three experiments.

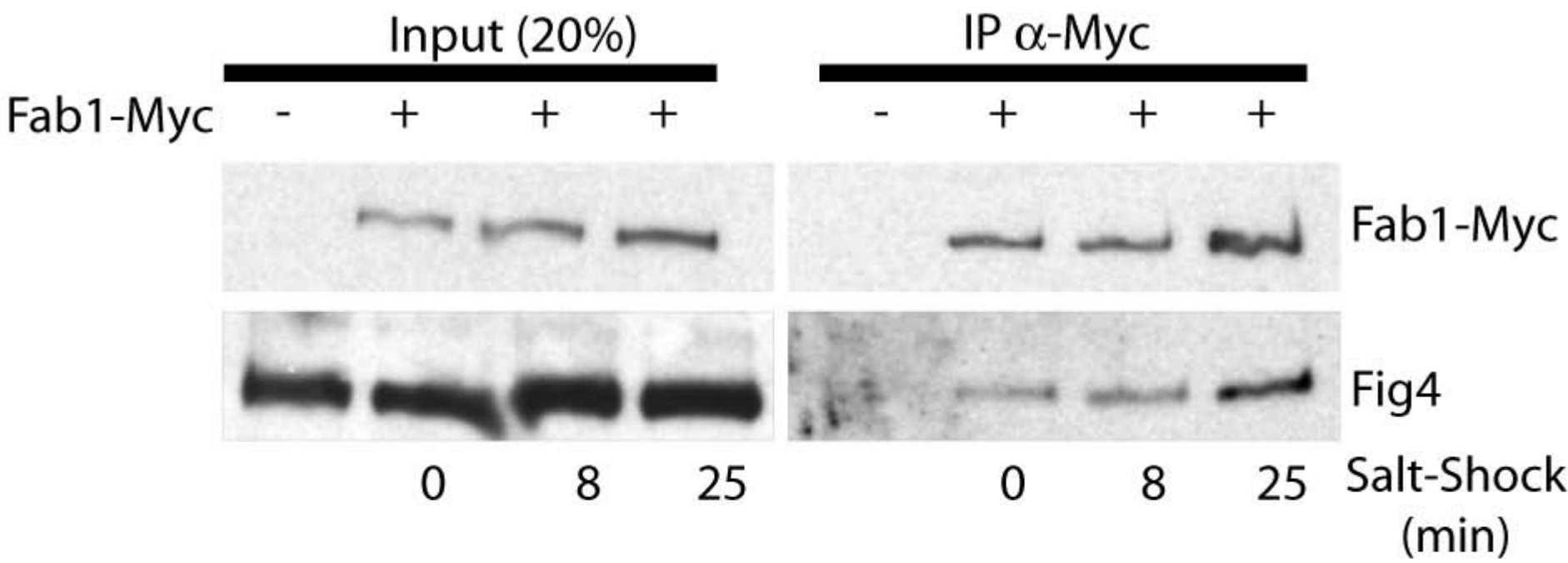

A
26 °C
38 °C
Fab1
Fab1FYVEC/S
Fab1ΔHINDIII
B
GFP
CMAC
Fab1-GFP
Fab1FYVE(C/S)-GFP
Fab1CCR(C/A)-GFP
Fab1KIN(D/R)-GFP


A
GFP
DIC
GFP-Fab1
GFP-Fab1ΔKIN
GFP-Fab1ΔCCT
GFP-Fab1ΔHINDIII
B
GFP
FM4-64
GFP-GroL
GFP-GroL-ALP



*fab1*Δ Fig4-GFP

A


Input (20%)
IP α-Myc
Fab1-Myc
- + + +
- + + +
Fab1-Myc
Fig4
0 8 25
0 8 25
Salt-Shock (min)

B

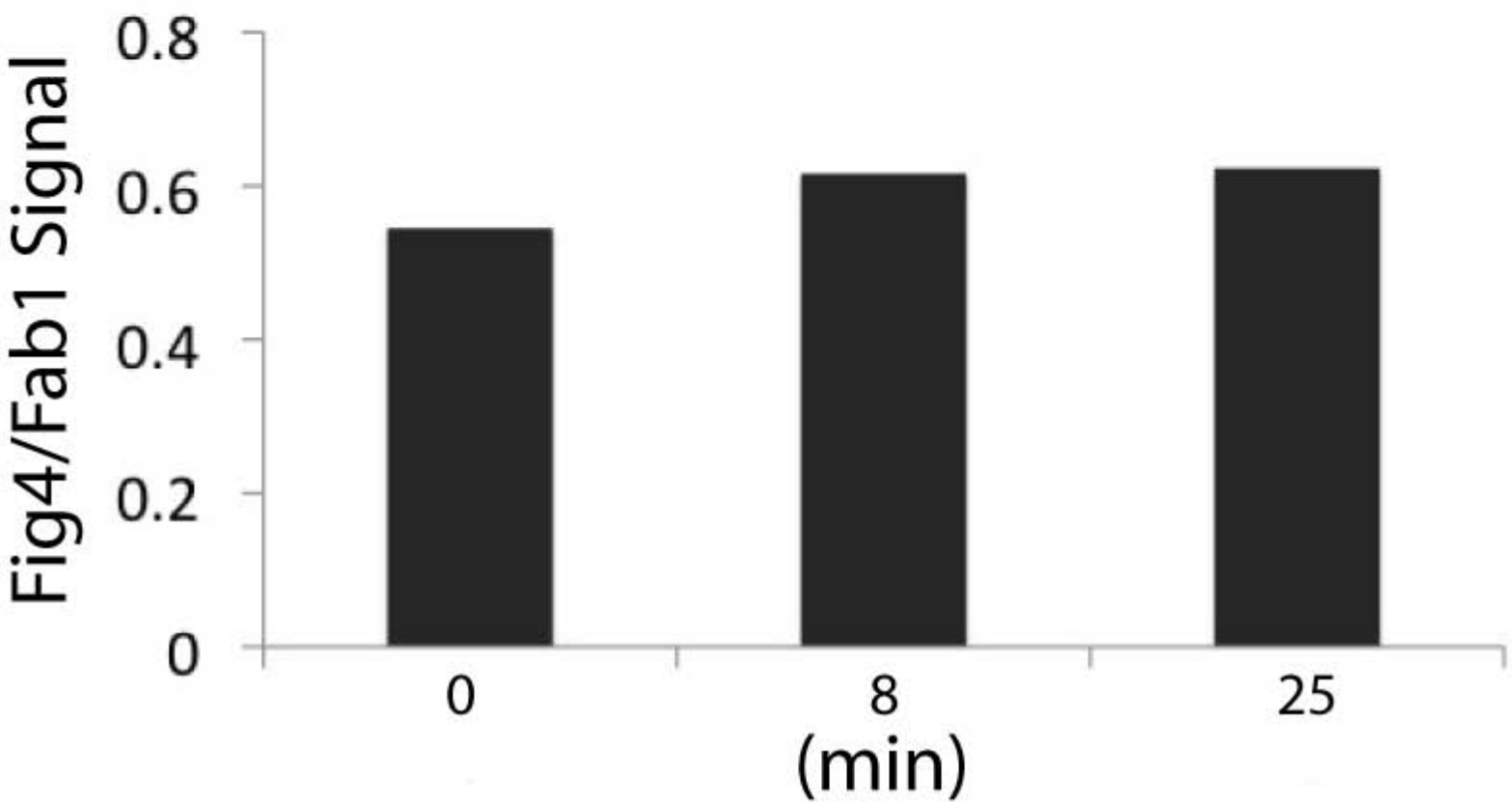


Fig4/Fab1 Signal
0.8
0.6
0.4
0.2
0
0
8
25
(min)