Briguglio et al., http://www.jcb.org/cgi/content/full/jcb.201305086/DC1

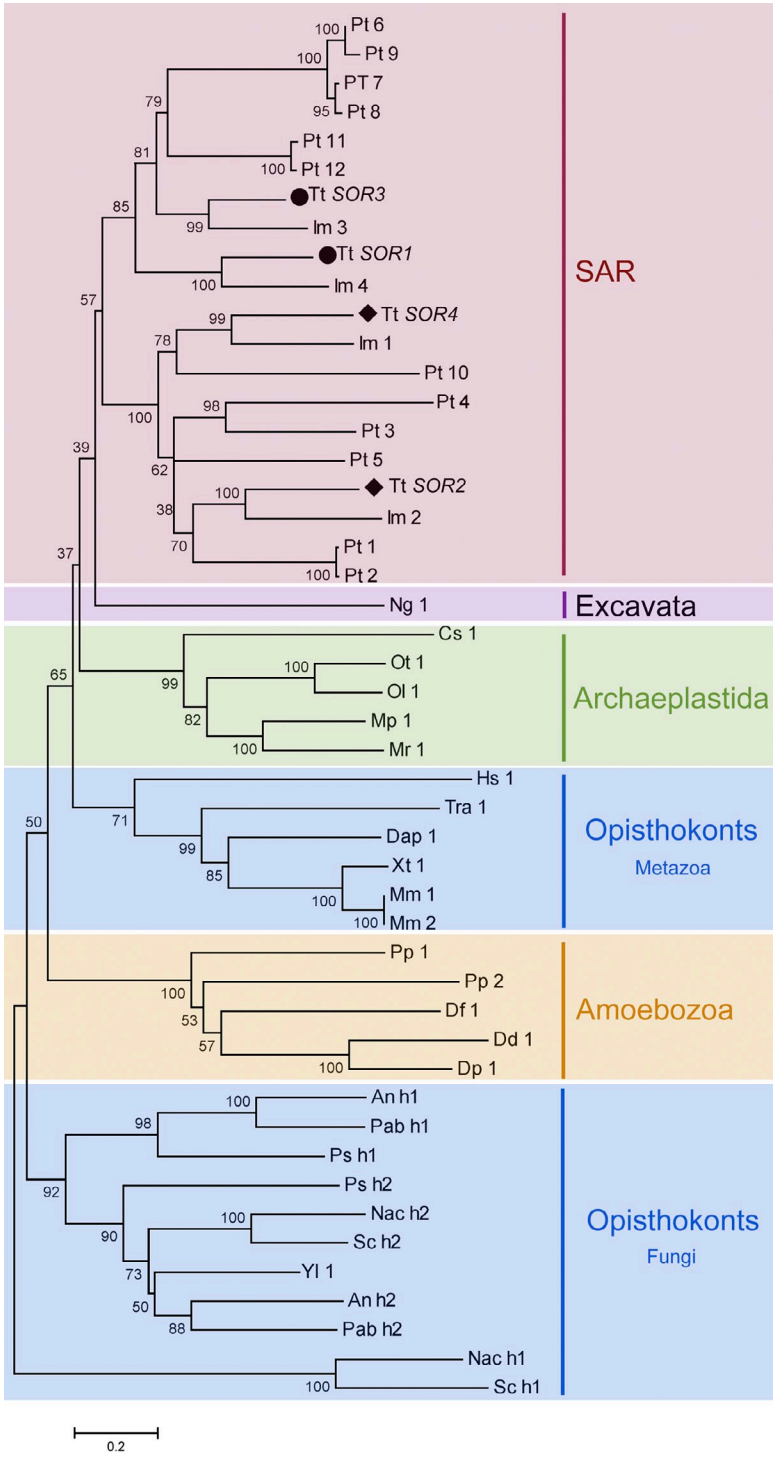


Figure S1. **The *T. thermophila* sortilins fall into two major groups.** Phylogenetic analysis of the VPS10 domains presented in Fig. 1, but here using the Neighbor-Joining method, also infers a similar evolutionary history for VPS10 domains from the major eukaryotic lineages. In some fungi, VPS10 domains are present as tandem repeats, depicted as h1 and h2. Species are abbreviated as follows: *A. nidulans* (An), *C. subellipsoidea* (Cs), *D. pulex* (Dap), *D. discoideum* (Dd), *D. fasciculatum* (Df), *D. purpureum* (Dp), *H. sapiens* (Hs), *I. multifiliis* (Im), *M. pusilla* (Mp), *Micromonas sp. RCC299* (Mr), *M. musculus* (Mm), *N. gruberi* (Ng), *N. castellii* (Nac), *O. lucimarinus* (Ol), *O. tauri* (Ot), *P. brasiliensis* (Pab), *P. tetraurelia* (Pt), *P. pallidum* (Pp), *P. strigosozonata* (Ps), *S. cerevisiae* (Sc), *T. thermophila* (Tt), *T. adhaerens* (Tra), *X. (Silurana) tropicalis* (Xt), *Y. lipolytica* (Yl). See Table S1 for a list of accession numbers for the sequences used to assemble this phylogeny.

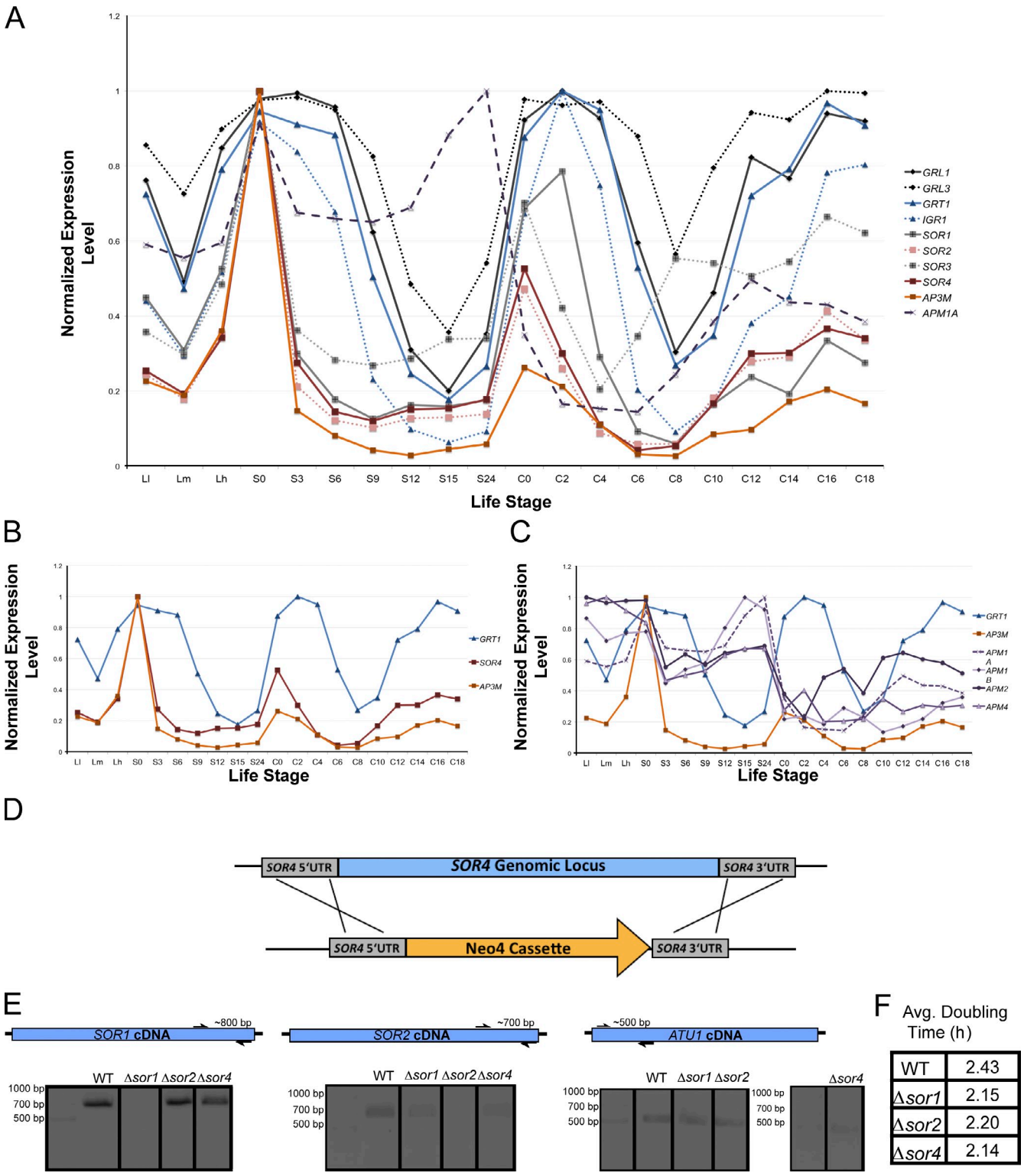


Figure S2. **Expression profiling identifies genes associated with mucocysts, including sortilins.** Expression profiles are derived from the Tetrahymena Functional Genomics Database, with each profile normalized to that gene's maximum expression level. Points on the x axis correspond to successive time points and represent growing, starved, and mating cultures, including three different culture densities (low [LI], medium [Lm], and high [Lh]), 7 samples taken during 24 h of starvation, and 10 samples subsequently taken during the 18 h after conjugation. (A) A composite image of all the expression profiles in Fig. 2 A with the addition of AP-3 and AP-1A expression profiles. (B) The medium subunit of the AP-3 adaptor is coexpressed with genes encoding mucocyst contents and the four *T. thermophila* sortilins (only *GRT1* and *SOR4* are depicted for reference). (C) The medium subunits of the remaining adaptor complexes (AP-1A and -1B, AP-2, and AP-4) are not coexpressed with mucocyst-associated genes or the *T. thermophila* sortilins (*GRT1* and *SOR4* shown for reference). (D) Schematic of the *SOR4* knockout construct. Replacement of the entire *SOR4* genomic locus with the neo4 drug resistance cassette is facilitated by homologous recombination. An identical strategy was used for the remaining sortilins. A detailed description of the construction and use of the *SOR1-4* knockout constructs can be found in the Materials and methods section. (E) Independent cDNA prepared from the sortilin knockouts, as in Fig. 2 C, also shows that knockout cells lack the expected transcript, without affecting transcript abundance of other sortilin genes. The unrelated α-tubulin (*ATU1*) cDNA serves as a loading control. The lanes shown were derived from a single gel, but their order has been rearranged for this figure. (F) Knockout of nonessential sortilins does not impair cell growth. Shown is a table of the mean doubling times calculated from five measurements of culture density (three measurements for *Δsor1*), each after 3-h periods of growth, indicating similar growth rates for the wild-type and mutant lines.

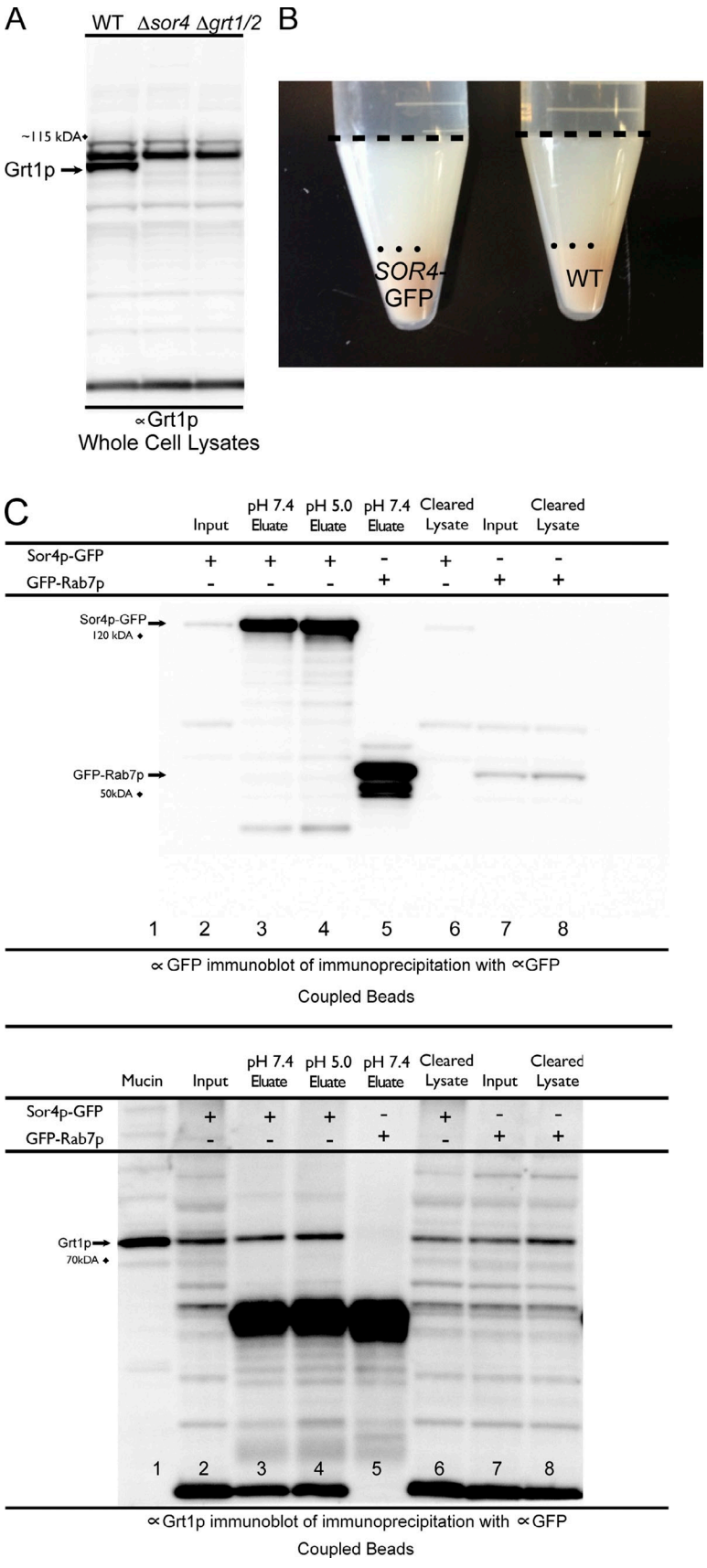


Figure S3. **Sor4p-GFP interacts with Grt1p, and is required for the transport of Grt1p to mucocysts.** (A) Uncropped version of Western blot shown in Fig. 3 C. Grt1p is present as a single band of 80 kD, whereas the additional bands represent cross-reactive proteins that are also present in the *Δgrt1/2* lysate. (B) The addition of a C-terminal GFP tag to endogenous Sor4p does not impair Sor4p function, as judged by mucocyst secretion. As in Fig. 4 B, identical numbers of wild-type and *SOR4*-GFP cells were stimulated with dibucaine and immediately centrifuged. A two-layer pellet was observed for both samples, consisting of an upper flocculent layer (below the broken line) and lower cell pellet (below the dotted line). Similar amounts of flocculent are secreted by the *SOR4*-GFP and wild-type lines, demonstrating that *SOR4*-GFP cells do not show a *Δsor4* phenotype and that Sor4p-GFP provides Sor4p function in mucocyst formation. (C) As in Fig. 3 D, Sor4p-GFP was immunoprecipitated using anti-GFP antiserum from lysates of cells expressing Sor4p-GFP from the endogenous *SOR4* locus, and that were actively synthesizing new mucocysts. Immunoprecipitated samples were analyzed by Western blotting with anti-GFP antiserum (top, lanes 3 and 4), confirming that full-length Sor4p-GFP is expressed, and with anti-Grt1p antiserum (bottom, lanes 3 and 4) to show coprecipitation of Grt1p. Lane 4 demonstrates that the interaction between Sor4p and Grt1p persists at pH 5. The specificity of the Sor4p-Grt1p interaction is further demonstrated by a sample in which GFP-Rab7p was immunoprecipitated with anti-GFP antiserum from lysates of cells expressing GFP-Rab7p, as described in Bright et al. (2010), that were actively synthesizing new mucocysts (top, lane 5). Grt1p was not coprecipitated with GFP-Rab7p (bottom, lane 5). Also shown are Input and cleared lysate lanes, each contain ~30,000 cell equivalents, from the lysate before and after bead addition, respectively.

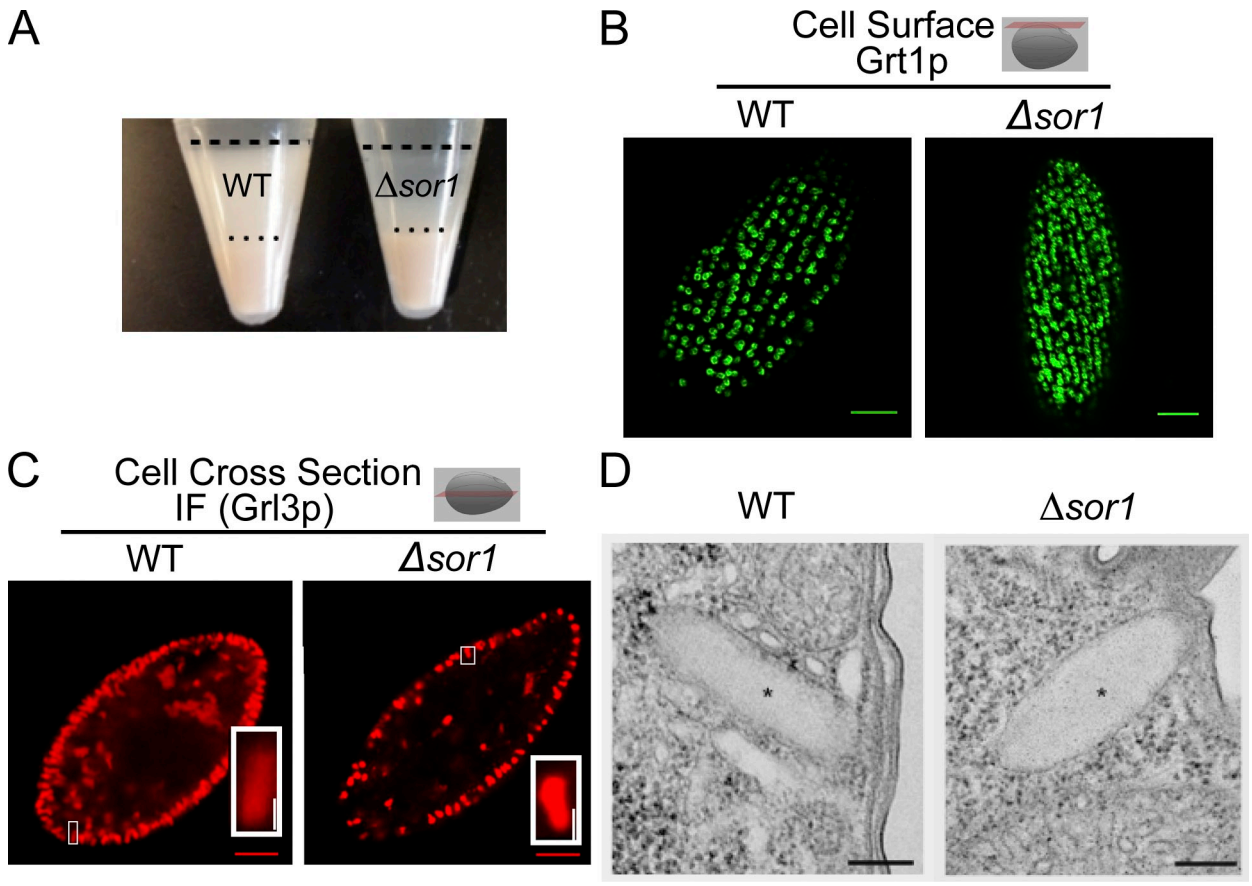


Figure S4. ***Δsor1* is not essential for mucocyst formation and secretion.** (A) A semiquantitative assay, as in Fig. 4 B and including the same wild-type control, was used to evaluate mucocyst discharge. Identical numbers of wild-type and Δ*sor1* cells were stimulated with the secretagogue dibucaine, and immediately centrifuged. The wild-type culture produces a two-layer pellet, in which a thick layer of flocculent (below the broken line) resulting from mucocyst discharge sits atop of the packed cells (below the dotted line). Similar to the wild type, stimulated *Δsor1* cultures still produce a prominent flocculent layer. (B) As in Fig. 3 B, Grt1p properly localizes to mature mucocysts. Localization of Grt1p by indirect immunofluorescence shows the expected array of docked mucocysts at the surface (illustrated by the red plane in the diagram shown at the top) of wild-type (left) and *Δsor1* cells (right). Bars, 5 µm. (C) As in Fig. 5 B and including the same wild-type control, Grl3p localizes to mature mucocysts. Visualization of mucocysts in wild-type and *Δsor1* cells by IF using mAb 5E9 against Grl3p, one of a family of proteins that assemble to form the mucocyst core. As seen in cross section, the mucocysts of *Δsor1* cells appear similar to the wild type. Insets show enlarged views of the indicated regions. Bars: (main images) 5 µm: (insets) 0.5 µm. (D) As in Fig. 5 C and including the same wild-type control, *Δsor1* cells produce mucocysts similar to those in wild type. Electron micrographs of docked mucocysts (labeled with asterisks) in wild-type (left) and *Δsor1* (right) cells. Bars, 200 nM.

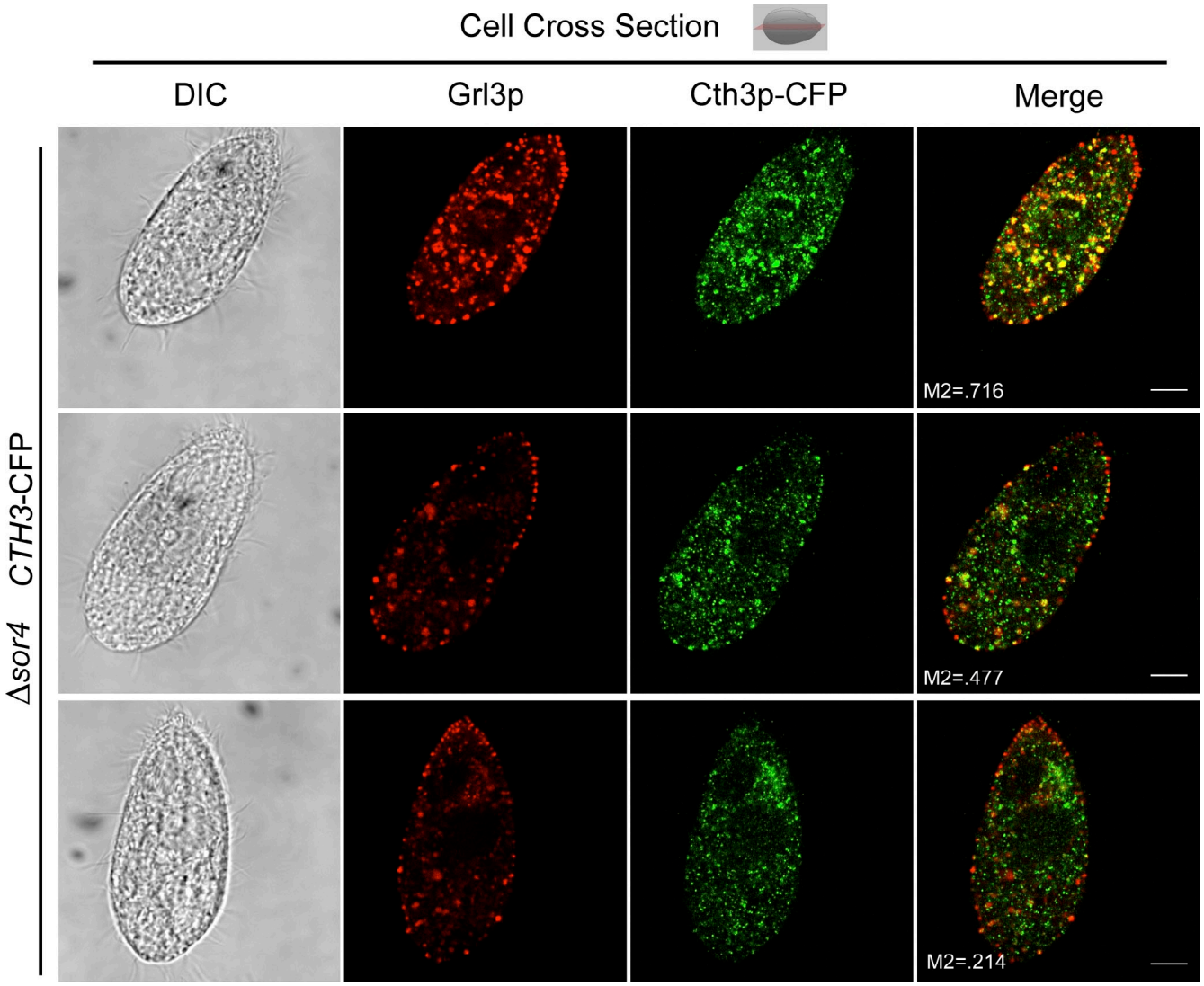


Figure S5. **Sorting efficiency of the exogenously expressed mucocyst protease Cth3p-CFP is reduced in *Δsor4* cells.** As in Fig. 6, Cth3p-CFP was inducibly expressed with 0.75 µg/ml $CdCl_2$ for 2 h. Cth3p-CFP was localized in fixed, permeabilized cells using a polyclonal anti-GFP antibody, and endogenous Grl3p was immunolocalized using mAb 5E9. Colocalization was quantified in 15 *Δsor4* cells, using the Manders' correlation coefficient M2, and a mean M2 value was determined for the population from this sample. The mean M2 value at the cell periphery for the population is 0.373 with an SEM of 0.054. A range of sample M2 values is shown as indicated in the bottom left corner of the merged image. Bars, 5 µm.

Table S1. **Accession numbers from which VPS10 domain sequences were obtained to create the phylogeny in Fig. 1 and Fig. S1**

Eukaryotic supergroup	Subgroup	Organism	Abbreviation	GenPept accession number
Archaeplastida	Green plants	*M. pusilla CCMP1545*	Mp_1	XP_003057805
		O. tauri	Ot_1	XP_003080385
		O. lucimarinus	Ol_1	XP_001418954
		Micromonas sp. RCC299	Mr_1	XP_002500131
		C. subellipsoidea C-169	Cs_1	EIE24395.1
	Rhodophyta	n/a		
	Glaucophytes	n/a		
Opisthokonts	Fungi	*A. nidulans FGSC A4*	An_1	CBF84749
		P. strigosozonata HHB-11173 SS5	Ps_1	EIN06327
		N. castellii CBS 4309	Nac_1	XP_003675013
		Y. lipolytica CLIB122	Yl_1	XP_503576
		P. brasiliensis Pb18	Pab_1	C1FZI7
		S. cerevisiae	Sc_1	AAA18831
	Metazoa	*T. adhaerens*	Tra_1	XP_002118075
		M. musculus	Mm_1	BAE33011
		D. pulex	Dap_1	EFX89061
		X. (Silurana) tropicalis	Xt_1	XP_002941683
		M. musculus	Mm_2	EDL25537
		H. sapiens	Hs_1	CAA66904
Amoebozoa		*P. pallidum PN500*	Pp_1	EFA78916
		D. discoideum AX4	Dd_1	XP_635902
		D. fasciculatum	Df_1	EGG20045
		D. purpureum	Dp_1	XP_003287439
		P. pallidum PN500	Pp_2	EFA75804
Chromalveolates/SAR	Rhizaria	n/a		
	Alveolates	Ciliates from Fig. 2 B/Table S2		
	Stramenopiles (NCBI taxonomy accession no. 33634)	n/a		
	Hacrobia	n/a		
Excavata	Malawimonadidae (accession no. 136087)	n/a		
	Euglenozoa (accession no. 33682)	n/a		
	Heterolobosea (accession no. 5752)	*N. gruberi* strain *NEG-M*	Ng _1	XP_002677529
	Jakobida (accession no. 556282)	n/a		
	Parabasalidea (accession no. 5719)	n/a		
	Fornicata (accession no. 207245)	n/a		
	Preaxostyla, oxymonads (accession no. 66288)	n/a		
	Preaxostyla, trimastix			

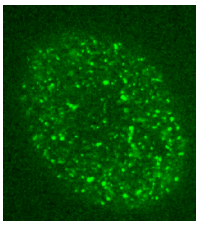
n/a, not applicable.

Table S2. **Accession numbers from which VPS10 domain sequences were obtained to create the phylogeny in Fig. 2 B**

Organism	Abbreviation	GenPept accession number
T. thermophila	Tt_4	XP_001033494
I. multifiliis	Im_1	EGR31810
P. tetraurelia strain d4-2	Pt_1	XP_001453349
P. tetraurelia strain d4-2	Pt_2	XP_001441753
T. thermophila	Tt_2	XP_001020814
I. multifiliis	Im_2	EGR30373
P. tetraurelia strain d4-2	Pt_3	XP_001452914
P. tetraurelia strain d4-2	Pt_4	XP_001461439
P. tetraurelia strain d4-2	Pt_5	XP_001456736
P. tetraurelia strain d4-2	Pt_6	XP_001440903
P. tetraurelia strain d4-2	Pt_7	XP_001443051
T. thermophila	Tt_3	XP_001025035
P. tetraurelia strain d4-2	Pt_8	XP_001443174
P. tetraurelia strain d4-2	Pt_9	XP_001433415
P. tetraurelia strain d4-2	Pt_10	XP_001437890
P. tetraurelia strain d4-2	Pt_11	XP_001460540
P. tetraurelia strain d4-2	Pt_12	XP_001441123
I. multifiliis	Im_3	EGR32320
T. thermophila	Tt_1	XP_001033316
I. multifiliis	Im_4	EGR29891
Other alveolate hits		
Toxoplasma gondii GT1	Tg_1	EEE22595
P. marinus ATCC 50983	Pm_1	XP_002767067
N. caninum Liverpool	Nc_1	XP_003881207
C. muris RN66	Cm_1	XP_002142105
C. hominis TU502	Ch_1	XP_667982
P. vivax Sal-1	Pv_1	XP_001615871
P. knowlesi strain H	Pk_1	XP_002260355
P. cynomolgi strain B	Pc_1	GAB68068
P. yoelii yoelii 17XNL	Py_1	XP_729406
P. falciparum 3D7	Pf_1	XP_001348667
B. microti strain RI	Bm_1	CCF75142
P. marinus ATCC 50983	Pm_2	XP_002781944
T. orientalis strain *Shintoku*	To_1	BAM38545
T. annulata strain *Ankara*	Ta_1	XP_954685
T. parva strain *Muguga*	Tp_1	XP_765543

Table S3. **Master primer list**

Name	5′–3′ sequence	Target
067A	CATTAGGATCTTTAGATAATTTTGATTGAGTAGATAAGTAG	A207
067B	CTACTTATCTACTCAATCAAAATTATCTAAAGATCCTAATG	
093A	ATGCGCTAGCGGATCGATTTAAGAATGGTCTAAGGATCTTGTGAATG	Sortilin 4 C terminus minus stop codon
093B	TAGAAACCATGGATCCAAGCATATCTGCATCGTATTCATCATTG	
094A	ATTCGATATCAAGCTTATCATTTAAAAAATTACTTGAGAGCATAAGC	Sortilin 4 3′ genomic flank
094B	CGGTATCGATAAGCTCTGATTGTTAAATGTTGAAAGAGTATTTTTATGAGAAG	
109A	TAGAGCATGCGCTAGCGTAAAACGCTGTTTATTTTCATATCTGTTATGTTAAATG	Sortilin 4 5′ genomic flank
109B	TGTATATCGAATTCCTGCAGCTAAAAAGATTGCTTTTTGTAATTTCTATGATTTTAAGAG	
084B	TAGAAACCATGGATCCATCATAATGACTCTCTTCATCTTGGTTATCAG	Sorilin 1 C terminus minus stop codon
087	ATGCGCTAGCGGATCAACAAGAATCTGTTTGCTTAAATGGAGAAG	
088A	ATTCGATATCAAGCTTAATTAACTAATTGATTTTTTGTTTTTTCATAAATTTCTTTGTAG	Sortilin 1 3′ genomic flank
088B	CGGTATCGATAAGCTTGATTTACGACAAATTCAATATGCCATTTC	
089A	ATGCGCTAGCGGATCGGAATGTGACTTCGGTTTCTACAG	Sorilin 2 C terminus minus stop codon
089B	TAGAAACCATGGATCCATCGTAATCTTCTTAATAATAGTAATCTTCTTGATCTC	
090A	ATTCGATATCAAGCTTATTAAAATAACAAATTTCTTCATTTTAATTAATTTGTAAG	Sortilin 2 3′ genomic flank
090B	CGGTATCGATAAGCTAACTAGAATATTAATTGCTAAAGTCAAAAATCT	
091A	ATGCGCTAGCGGATCTGCTAAAGACGATAGCAAAGAAAGAAC	Sorilin 3 C terminus minus stop codon
091B	TAGAAACCATGGATCCATTTCTAGGATCAAAAGGATCTTCTTCAC	
092A	ATTCGATATCAAGCTTAAAATAATTGACTGAATAATATTGCTAATTTATTTTTTTAC	Sortilin 3 3′ genomic flank
092C	CGGTATCGATAAGCTGAAGATAAATTATTGCTTCAATCATTTGCTCAG	
106A	TAGAGCATGCGCTAGCCTCAGTCTGGATTAGGCAGCTAG	Sortilin 1 5′ genomic flank
106B	TGTATATCGAATTCCTGCAGAAGATATTTAATCACTTAATAACTAAGTCTGTTTCTCATG	
107A	TAGAGCATGCGCTAGCGTATTCTAATTGAAGAATAAGTAAATTCCTTTTTATCATAACAC	Sortilin 2 5′ genomic flank
107B	TGTATATCGAATTCCTGCAGCAGACTTCCTCTGATTTCCTAACAAAGTAATTC	
108A	TAGAGCATGCGCTAGCTTCCACTTTTATGATTGGATAATTGTATAGAAGAATTATG	Sortilin 3 5′ genomic flank
108B	TGTATATCGAATTCCTGCAGATCTATTTTTTCTCTCAATTATTTTCTTTTCAAGGAT TTG	
S001	CACCATGAGAAGAAGCTTGCTTACAGTAG	CTH3 ORF minus stop codon
S002	ATGTCTTGCCAAAGCAAAACC	



Video 1. **Sor4p localizes to mobile cytoplasmic puncta and not to mature mucocysts docked at the plasma membrane.** Real-time live imaging of immobilized *T. thermophila* expressing Sor4p-GFP at the endogenous *SOR4* locus as a complete gene replacement. Images were acquired with a 100-ms exposure (Marianas Yokogawa type spinning disk inverted confocal microscope; 3i) and displayed in the video at 10 frames/s. There is no detectable Sor4p-GFP in docked mucocysts.

An ImageJ macro that selects a band at the cell periphery for colocalization analysis with the JACoP plugin using user defined thresholds and a variation on the 3 × 3 2D Median Hybrid Filter ImageJ plugin are available for download as a ZIP file.

Reference

Bright, L.J., N. Kambesis, S.B. Nelson, B. Jeong, and A.P. Turkewitz. 2010. Comprehensive analysis reveals dynamic and evolutionary plasticity of Rab GTPases and membrane traffic in *Tetrahymena thermophila*. *PLoS Genet.* 6:e1001155. http://dx.doi.org/10.1371/journal.pgen.1001155