Bacterial tail anchors can target to the mitochondrial outer membrane

Güleycan Lutfullahoğlu Bal, Abdurrahman Keskin[1], Ayşe Bengisu Seferoğlu, and Cory D. Dunn

Department of Molecular Biology and Genetics

Koç University

Sarıyer, İstanbul, 34450

Turkey

[1] Current affiliation:

Department of Biological Sciences

Columbia University

New York, NY 10027

Corresponding author:

Dr. Cory D. Dunn

Koç Üniversitesi

Fen Fakultesi

Rumelifeneri Yolu

Sarıyer, İstanbul 34450

Turkey

Email: cdunn@ku.edu.tr

Phone: +90 212 338 1449

Fax: +90 212 338 1559

MANUSCRIPT TEXT

During the generation and evolution of the eukaryotic cell, a proteobacterial endosymbiont was refashioned into the mitochondrion, an organelle that appears to have been present in the ancestor of all present-day eukaryotes [1]. Mitochondria harbor proteomes derived from coding information located both inside and outside the organelle, and the rate-limiting step toward the formation of eukaryotic cells may have been development of an import apparatus allowing protein entry to mitochondria [2]. Currently, a widely conserved translocon allows proteins to pass from the cytosol into mitochondria [3], but how proteins encoded outside of mitochondria were first directed to these organelles at the dawn of eukaryogenesis is not clear. Because several proteins targeted by a carboxyl-terminal tail anchor (TA) appear to have the ability to insert spontaneously into the mitochondrial outer membrane (OM) [4-6], it is possible that self-inserting, tail-anchored polypeptides obtained from bacteria might have formed the first gate allowing proteins to access mitochondria from the cytosol [7]. Here, we tested whether bacterial TAs are capable of targeting to mitochondria. In a survey of proteins encoded by the proteobacterium *Escherichia coli*, predicted TA sequences were directed to specific subcellular locations within the yeast *Saccharomyces cerevisiae*. Importantly, TAs obtained from DUF883 family members ElaB and YqjD were abundantly localized to and inserted at the mitochondrial OM. Our results support the notion that eukaryotic cells have the ability to utilize membrane-targeting signals present in bacterial proteins obtained by lateral gene transfer, and our findings make plausible a model in which mitochondrial protein translocation was first driven by tail-anchored proteins.

During the integration of an α-proteobacterial endosymbiont within the eukaryotic cell, genes transfered to the (proto)nucleus were re-targeted to mitochondria, allowing these organelles to remain the location of crucial cellular processes. In addition, other

polypeptides that evolved within the eukaryotic lineage or were acquired through lateral gene transfer from other organisms were directed to mitochondria. Across eukaryotes, the β-barrel Tom40 protein forms a pore by which proteins pass through the OM [3,8,9]. However, the Tom40 polypeptide seems to require already existing TOM complexes for mitochondrial insertion [10,11], giving rise to a "chicken or the egg" dilemma when considering how the TOM complex may have evolved.

An early translocation pore that was self-inserting at the mitochondrial surface might have allowed mitochondria to begin to import proteins, permitting the evolution of an improved translocon found in eukaryotes today [7]. Current evidence suggests that the self-insertion of tail-anchored proteins at the mitochondrial OM is possible [4-6], making such a scenario tenable. At the inception of mitochondria, such tail-anchored proteins would likely have been derived from prokaryotes, particularly if mitochondria were required for the generation of the stereotypical compartmentalized structure of eukaryotes.

We focused our attention upon a single aspect of this hypothesis: can TAs obtained from bacterial proteins be inserted into the mitochondrial OM when expressed within a eukaryotic cell? Toward this goal, we selected for analysis 12 *E. coli* proteins predicted to harbor a solitary α-helical TM domain at their carboxyl-terminus (Extended Data Fig. 1), then fused mCherry to the amino-terminus of these predicted *E. coli* TAs and examined their location in *S. cerevisiae* cells by fluorescence microscopy. mCherry-ElaB(TA) (Fig. 1a) and mCherry-YqjD(TA) (Fig. 1b) were readily detectable at mitochondria, as reported by sfGFP fused to the TA of the *S. cerevisiae* Fis1 polypeptide, a protein playing a role in yeast mitochondrial division. A lesser fraction of mCherry-ElaB(TA) and mCherry-YqjD(TA) was localized to the endoplasmic reticulum (Extended Data Fig. 2). ElaB and YqjD are members of the DUF883 family of proteins. Little is known about the function of DUF883 family members, but YqjD may recruit ribosomes to the *E. coli* plasma membrane during stationary phase [12]. Although negligible fluorescent signal was detectable, mCherry-TcdA(TA) was also localized to

mitochondria (Extended Data Fig. 3a). TcdA (also called CsdL) catalyzes the modification of *E. coli* tRNAs [13].

Other predicted TAs derived from the Flk, YgiM, RfaJ, DjlB, FdnH, NrfR, and YmiA proteins appeared to allow at least partial localization of mCherry to various locations associated with the endomembrane system (Extended Data Fig. 4). However, no convincing localization to mitochondria was apparent after fusing any of these TAs to mCherry. Moreover, mCherry-YhdV(TA) appeared to be distributed throughout cytosol and nucleus, indicating failure to target efficiently to any membrane. mCherry-YgaM(TA) was not detectable, suggesting its degradation.

Since our results suggest that the ElaB and YqjD TAs may be competent for insertion at the mitochondrial OM, we tested whether these TAs can functionally replace the TA of Fis1p, a domain whose sole purpose is to allow this protein's insertion at the mitochondrial OM [14,15]. Because Fis1p is required for mitochondrial division in *S. cerevisiae*, *fis1Δ* mutants manifest a highly interconnected network of mitochondria due to unbalanced mitochondrial fusion [16]. As expected, expression of wild-type Fis1p restored normal mitochondrial distribution in this genetic background, while Fis1p prevented from insertion by a A144D substitution within the Fis1p TA [15] could not restore normal mitochondrial morphology (Figs. 2a and 2b). Strikingly, replacement of the Fis1p TA with the ElaB or the YqjD TA within the context of full length Fis1p polypeptide could successfully promote mitochondrial division and restore normal mitochondrial morphology, while a control TA from the *E. coli* YgiM protein, which is not trafficked to mitochondria, could not support Fis1p activity. A Fis1-TcdA(TA) protein could not functionally replace the Fis1p TA in this microcopy-based assay (Extended Data Fig. 3b), suggesting insufficient expression, poor mitochondrial insertion, or meager functionality.

Next, we sought further evidence for functional insertion of the ElaB and YqjD TAs at mitochondria using an assay based on cell proliferation [15]. In brief, expression of

functional Fis1p in a genetic background initially lacking Fis1p and removed of the mitochondrial fusogen Fzo1p can lead to unchecked mitochondrial fragmentation, loss of functional mitochondrial DNA (mtDNA), and a corresponding abrogation of respiratory competence. As previously reported [15], expression of wild-type Fis1p in a *fzo1Δ fis1Δ* genetic background led to an inability to proliferate on nonfermentable medium, while expression of the poorly inserted Fis1(A144D) variant did not prompt mtDNA loss (Fig. 2c). The ElaB and YqjD TAs fused to the cytosolic domain of Fis1p allowed sufficient fission activity to prompt mitochondrial genome deletion from the same genetic background, again indicating successful ElaB TA and YqjD TA insertion at the mitochondrial OM. Even the Fis1-TcdA(TA) protein provoked mtDNA loss in *fzo1Δ fis1Δ* cells (Extended Data Fig. 3c), suggesting some minimal level of OM insertion. The YgiM TA again appeared unable to recruit Fis1p to mitochondria (Fig. 2c).

Our findings, in which several predicted TAs obtained from *E. coli* can target to and function at the mitochondrial OM of *S. cerevisiae*, make plausible a scenario in which tail-anchored bacterial proteins contributed to the formation of the earliest mitochondrial translocon. The structural characteristics of the TAs of ElaB and YqjD, a helical TM domain rich in glycines followed by a positively charged patch ending in di-arginine (Extended Data Fig. 1), are evocative of the Fis1p TA, suggesting a similar, potentially spontaneous mechanism for insertion at mitochondria, although unassisted insertion of the ElaB and YqjD TAs at the mitochondrial surface has yet to be demonstrated. Notably, several conserved members of the current TOM complex are also tail-anchored [17], raising the possibility that at least some of these proteins could be "hold-overs" from an early, self-inserting mitochondrial translocon, although we note that these subunits cannot currently self-insert at mitochondria.

Could the DUF883 family of proteins have contributed to an ancestral mitochondrial OM translocon? While YqjD has been reported to recruit ribosomes to the *E. coli* inner membrane during stationary phase [12], a role in line with promotion of co-translational

protein import into mitochondria [18,19], the DUF883 family is not readily identified in eukaryotic genomes. One might expect, however, that once a more proficient TOM complex centered around the Tom40 pore evolved, a previous translocon would have been lost, or even selected against if it were to interfere with more rapid protein import through an improved OM translocation machinery. Moreover, an inordinate focus on DUF883 family members when seeking components of the earliest mitochondrial translocon may not be warranted in any case, since the structural characteristics likely required for TA insertion at mitochondria might be easily generated from random open reading frame fragments containing a transmembrane domain. Analogously, random sequences from bacteria are readily able to act as amino-terminal mitochondrial targeting sequences [20-22]. If TAs are easily evolved and might recruit other functional domains to the mitochondrial surface, then identifying orthologs of initial tail-anchored translocon components from existing prokaryotic sequences might be difficult, since an untold number of TAs might be predicted among putative open-reading frames. Supporting the idea that mitochondrial TAs might be easily generated from sequences not actually functioning in membrane targeting within their native bacterial environment, we demonstrated limited mitochondrial targeting and partial functionality of the computationally predicted TcdA TA in yeast, even though TcdA is unlikely to be membrane-inserted in *E. coli* [23].

If conversion of endosymbiont to mitochondria were the rare and essential event required for generation of eukaryotes, and if insertion of bacteria-derived, tail-anchored proteins at the OM to form an ancestral translocon were necessary for this conversion, then the question of how hospitable an environment the early mitochondria OM might have been for bacteria-derived TAs comes to the fore. Indeed, the membrane into which tail-anchored proteins are inserted can be at least partially determined by their lipid environment [4], and lipids utilized by many characterized archaea are fundamentally different in structure from bacterial and eukaryotic lipids [24]. However, recent evidence indicates that archaeal clades potentially related to the last eukaryotic common ancestor might have been characterized by membranes more similar to those

of bacteria than of those membranes more typically found in archaea [25]. This finding raises the possibility that the protoeukaryote's specific cohort of lipids was crucial to the ability to form complexes of bacteria-derived tail-anchored proteins at the mitochondrial OM that would allow full integration of mitochondria within the ancestral eukaryote.

Finally, we have not examined in detail the trafficking of *E. coli* TAs that appeared to localize to the endomembrane system during our initial survey. However, the diverse organellar locations to which these TAs were localized supports previous data indicating that eukaryotes may derive organelle targeting information from newly acquired prokaryotic proteins or protein fragments, perhaps even from amino acid sequences previously unselected for targeting proficiency [20-22,26,27]. Lateral gene transfer promotes the evolution of novel functions in prokaryotes [28] and was certainly present in the form of endosymbiotic gene transfer during early eukaryogenesis. Indeed, proficiency in making use of cryptic or explicit targeting information in order to direct newly acquired, nucleus-encoded proteins to the distinct subcellular locations where they might be best utilized might have provided a significant selective advantage to the early eukaryote. Such a scenario may be particularly relevant if some amount of cellular compartmentalization already existed in a pre-eukaryotic host cell before conversion of pre-mitochondrial endosymbiont to organelle [29,30].

METHODS

Yeast strains, plasmids, and culture conditions. Culture conditions are as described in [15], and all experiments have been carried out at 30˚C.

Microscopy. Microscopy was performed on logarithmic phase cultures as in [15], with exposure times determined automatically. mCherry fusions are driven by the *ADH1* promoter and universally contain Fis1p amino acids 119-128 (not necessary or sufficient for mitochondrial targeting) linking mCherry to each TA, and genetic assessment of Fis1p variant functionality was performed as described in [15]. The brightness of all images of mCherry expression was adjusted in Adobe Photoshop CS5 (Adobe, San Jose, California) to an equivalent extent. Scoring of mitochondrial morphology was performed blind to genotype.

Proliferation-based assessment of Fis1p functionality. Genetic tests of Fis1p functionality in cells lacking Fis1p and Fzo1p were performed as in [15].

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EXTENDED DATA FIGURES

Extended Data Figure 1 | A list of predicted TAs examined in this study. FASTA sequences from the *E. coli* proteome were retrieved from UniProt [31] and subjected to analysis using the TMHMM 2.0 server [32]. Polypeptides with a single predicted TM domain (denoted by purple line), harboring 15 or less amino acids carboxyl-terminal to the TM domain, and containing more than 30 amino acids amino-terminal to the TM domain were selected for further analysis. Charged amino acids are also denoted. The UniProt accession number and names of selected proteins are provided. For purposes of sequence comparison, the relevant portion of the *S. cerevisiae* Fis1p TA is also shown.

Extended Data Figure 2 | The predicted ElaB and YqjD TAs can also be visualized at the endoplasmic reticulum. Cells were analyzed as in Figure 1, except BY4741 was transformed with plasmid pJK59, expressing Sec63-GFP, before mating.

Extended Data Figure 3 | The predicted TcdA TA allows minimal localization to, and function at, the mitochondrial outer membrane. a, The predicted TcdA TA can be visualized at mitochondria. Strain BY4741, harboring plasmid b294 (sfGFP-Fis1p), was mated to strain BY4742 carrying mCherry-TcdA(TA)-expressing plasmid b281 and the resulting diploids were imaged by fluorscence microscopy. Scale bar, 5 μm. **b,** Fis1p with its own TA replaced by the predicted TcdA TA cannot promote normal mitochondrial morphology. *fis1Δ* strain CDD741, expressing mitochondria-targeted GFP from plasmid pHS12, was transformed with empty vector pRS313 or plasmids expressing wild-type Fis1p (b239), Fis1(A144D)p (b244), or Fis1-TcdA(TA)p (b319) and mitochondrial morphology was assessed by fluorescence microscopy. Scale bar, 5μm. **c,** Fis1-TcdA(TA)p can allow mitochondrial division. Strain CDD688 was transformed with the plasmids used in (**b**) or a plasmid expressing Fis1-YgiM(TA)p (b316) and examined as in Fig. 2c, except that culture on medium counter-selective for Fis1p activity was carried out for 5d.

Extended Data Figure 4 | Not all predicted *E. coli* TAs are localized to mitochondria in *S. cerevisiae*. Strain CDD961 was transformed with plasmids expressing (**a**) mCherry-Flk(TA) (b273), (**b**) mCherry-YhdV(TA) (b277), (**c**) mCherry-RfaJ(RA) (b278), (**d**) mCherry-DjlB(TA) (b280), (**e**) mCherry-FdnH(TA) (b331), (**f**) mCherry-NrfF(TA) (b332), (**g**) mCherry-YmiA(TA) (b333) and examined by fluorescence microscopy. (**h**) Strain BY4741, carrying plasmid b311 expressing sfGFP fused to the enhanced PTS1 sequence [33], was mated to strain BY4742, containing the mCherry-YgiM(TA)-expressing plasmid b274, and the resulting diploids were imaged.

Supplementary Information 1 | Strains, plasmids, and oligonucleotides used during this study.

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AUTHOR CONTRIBUTIONS

C.D.D. designed the study, wrote the manuscript, and performed experiments. G.L.B., A.K., and A.B.S. performed experiments, generated reagents, and provided manuscript critiques.

AUTHOR INFORMATION

The authors declare no competing financial interest and have no known conflict of interest affecting the outcome or interpretation of this study. Correspondence and requests for materials should be addressed to C.D.D. (cdunn@ku.edu.tr).

FIGURE LEGENDS

Figure 1 | The predicted ElaB and YqjD TAs localize to mitochondria. Strain BY4741, harboring plasmid b294 (sfGFP-Fis1p), was mated to strain BY4742 carrying mCherry-ElaB(TA)-expressing plasmid b275 (**a**) or strain BY4742 carrying mCherry-YqjD(TA)-expressing plasmid b279 (**b**). The resulting diploids were visualized by fluorescence microscopy. Scale bar, 5μm.

Figure 2 | Mitochondria-localized bacterial TAs can functionally replace the TA of Fis1p. a, The ElaB and YqjD TAs can replace the Fis1p TA in promoting normal mitochondrial morphology. *fis1Δ* strain CDD741, expressing mitochondria-targeted GFP from plasmid pHS12, was transformed with empty vector pRS313 or plasmids expressing wild-type Fis1p (b239), Fis1(A144D)p (b244), or Fis1p with its own TA replaced by that of ElaB (b317), YqjD (b318), or YgiM (b316). Cells were examined by fluorescence microscopy. Scale bar, 5μm. **b,** Quantification of mitochondrial morphology of the transformants from (**a**) was performed blind to genotype. White bar represents cells with fully networked mitochondria, grey bar represents cells with mitochondria not fully networked, but networked to a greater extent than wild-type cells, and black bar represents cells with normal mitochondrial morphology. Quantification was repeated three times (n>200 per genotype), and a representative experiment is shown. **c,** Genetic assessment of Fis1p variant functionality. Strain CDD688 was transformed with the plasmids in (**a**) and proliferation was assessed without selection against Fis1p activity (YPALac medium for 2d) and following counter-selection for cells carrying functional Fis1p (SLac-His+CHX medium for 4d).

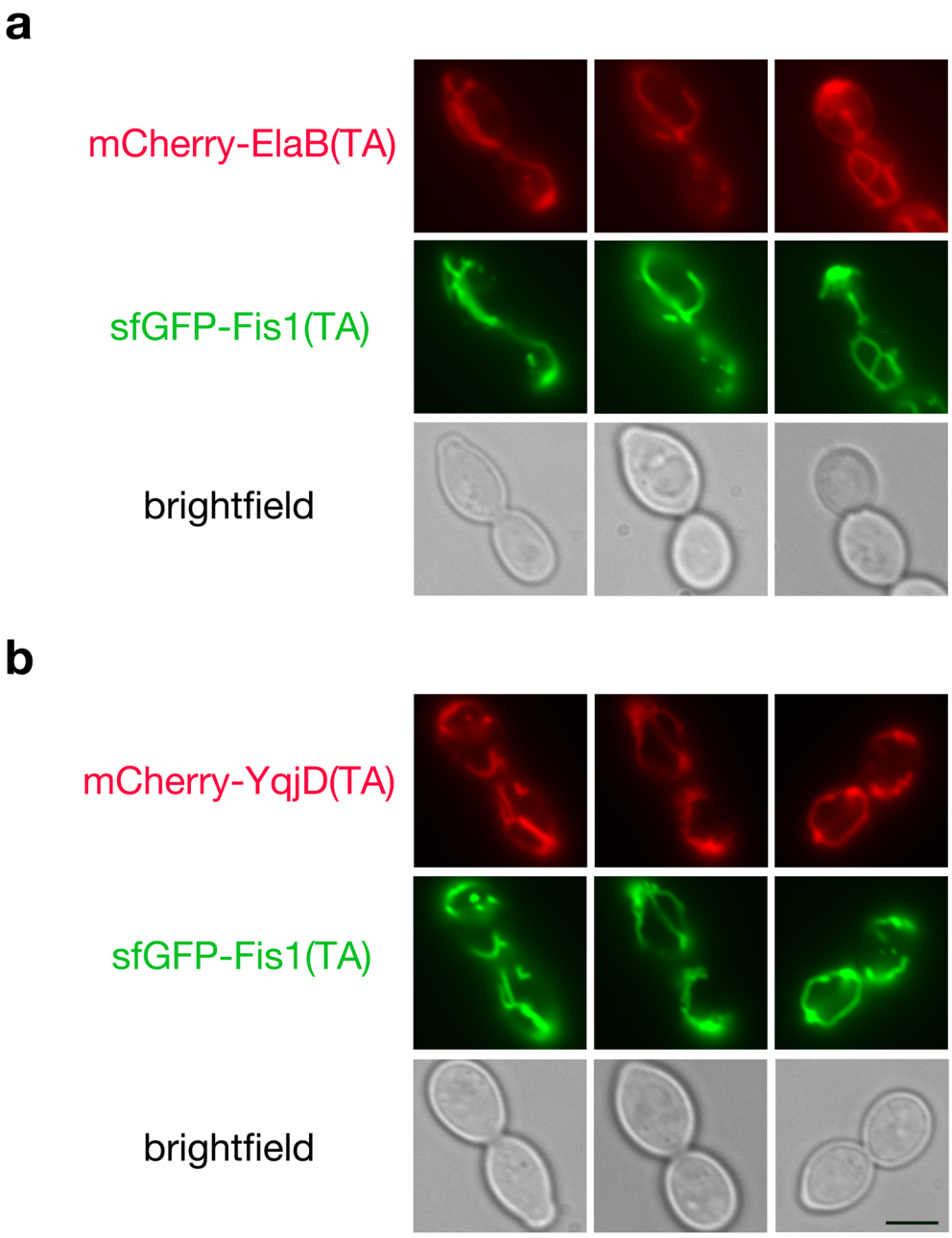
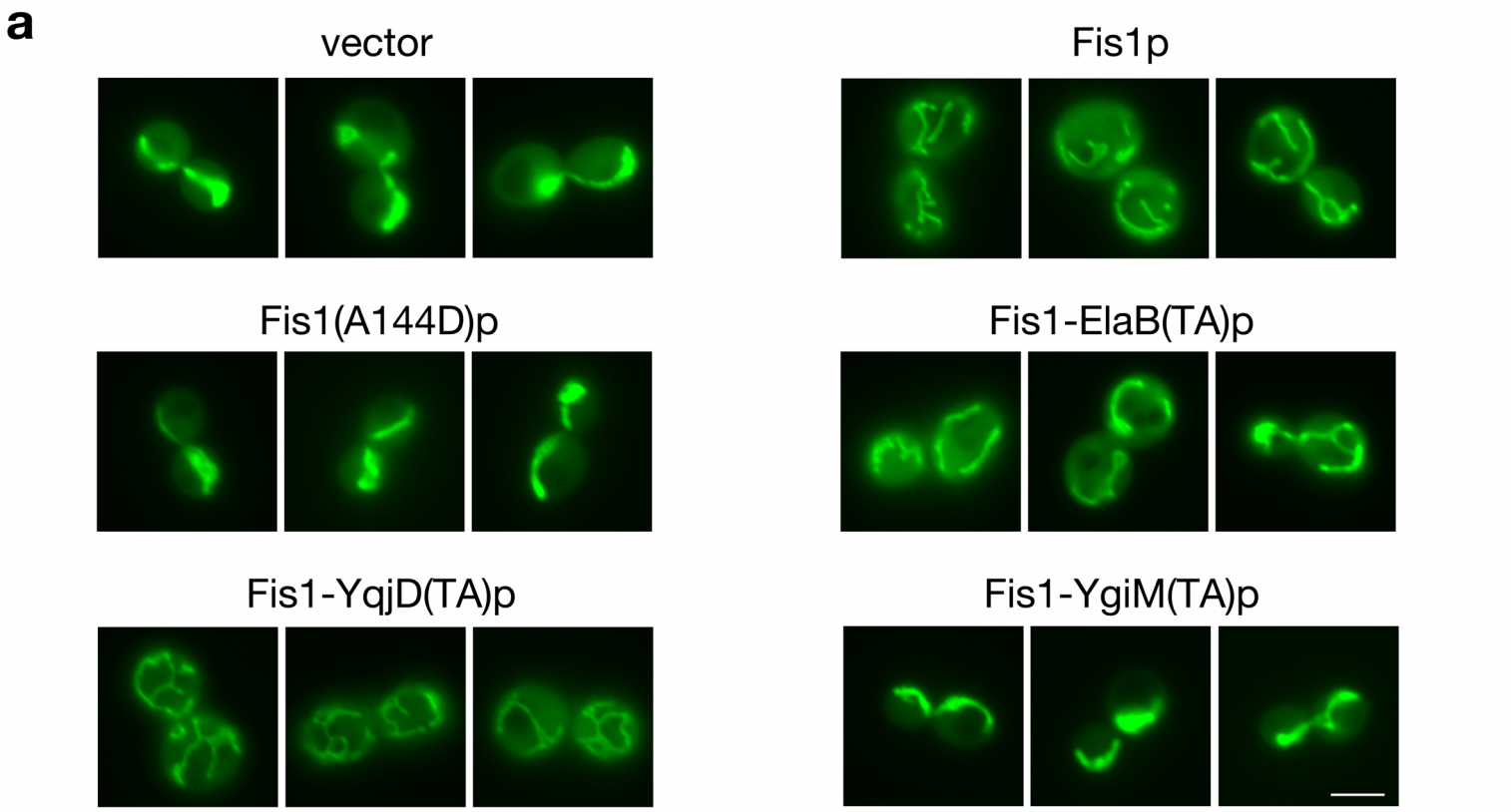
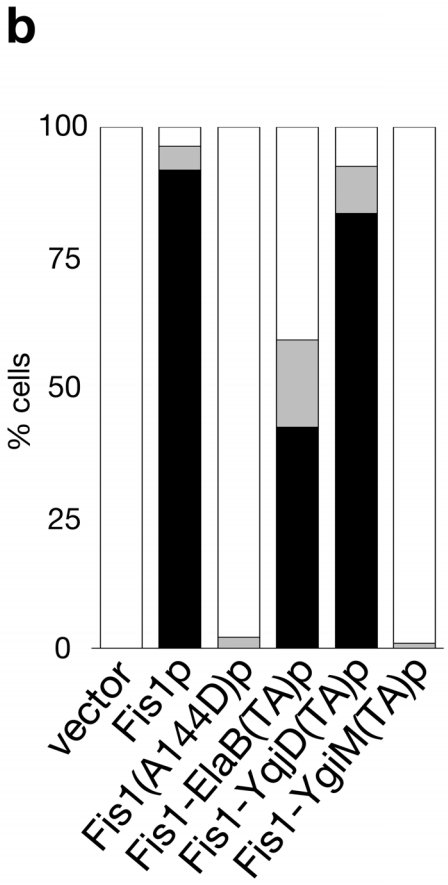


Fig. 1





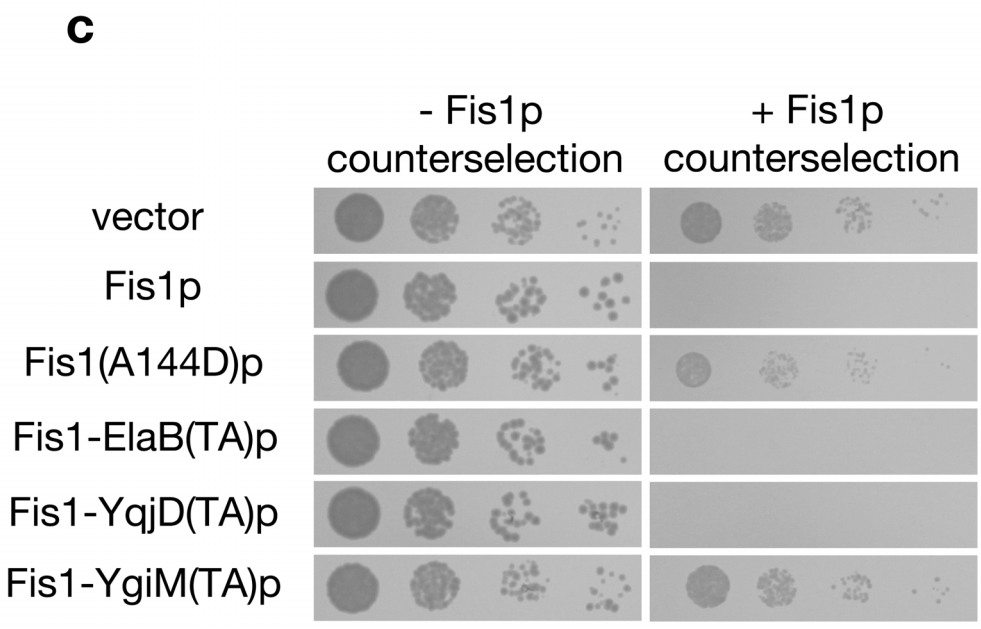
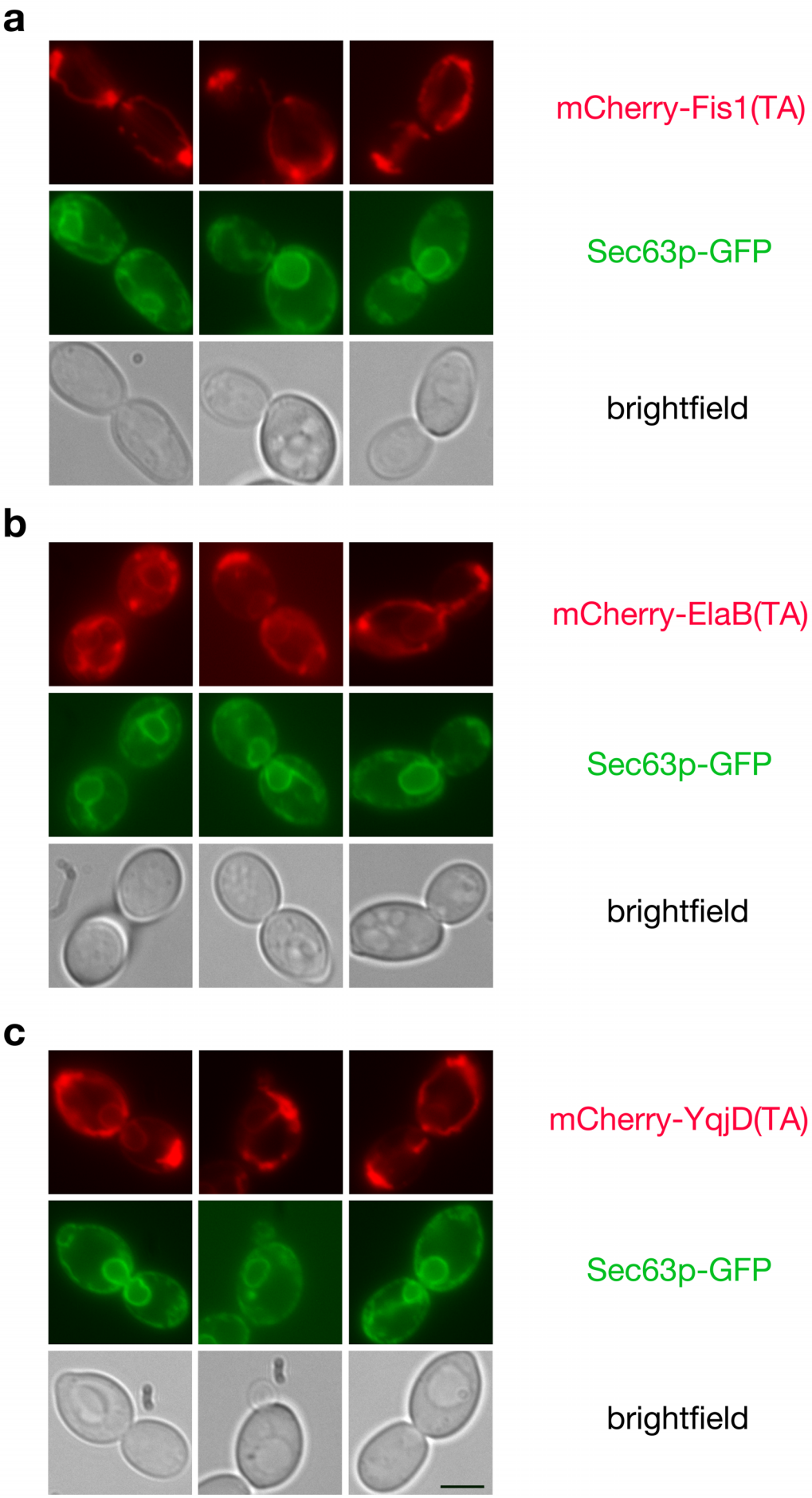


Fig. 2

Extended Data Fig. 1



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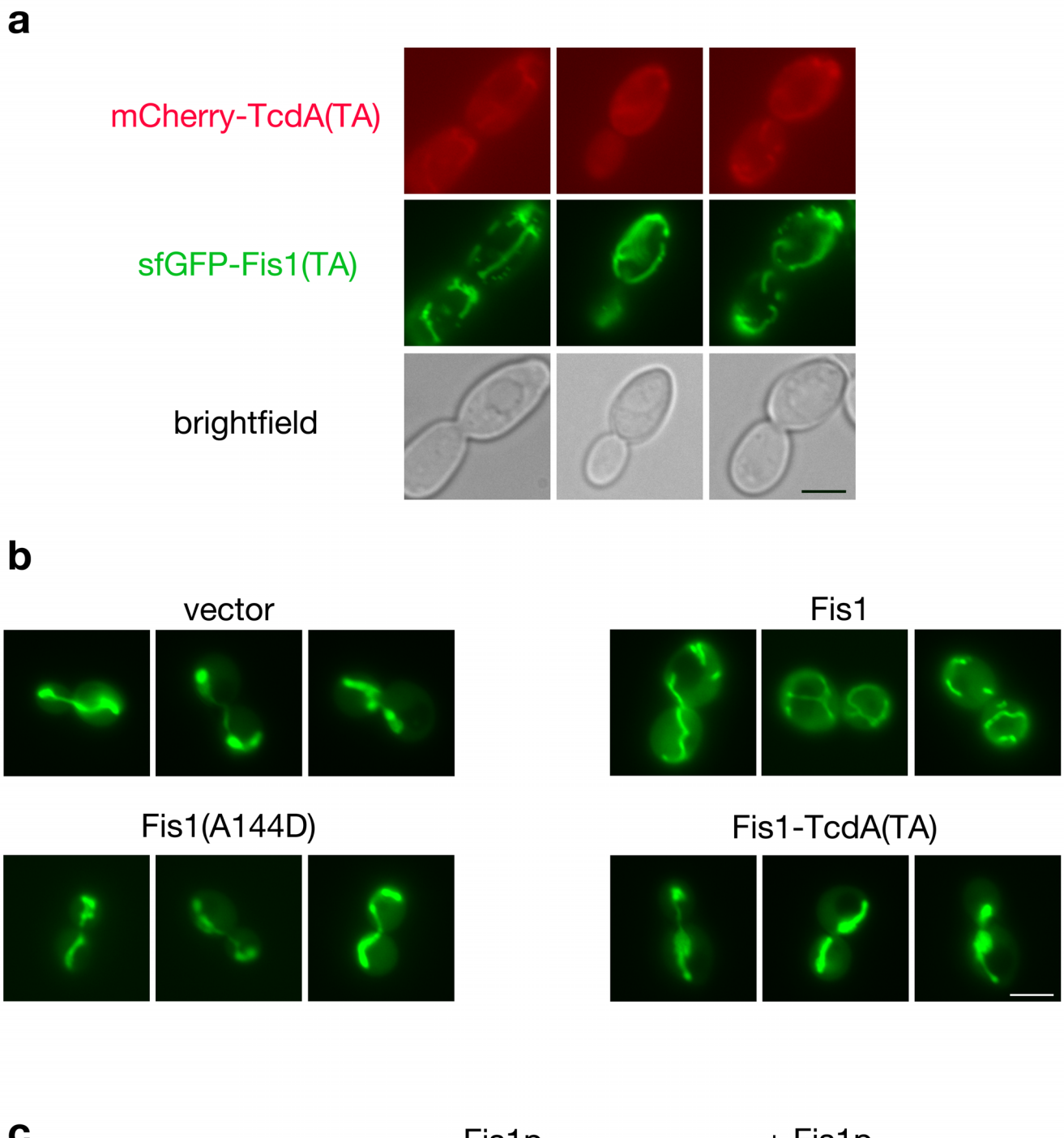

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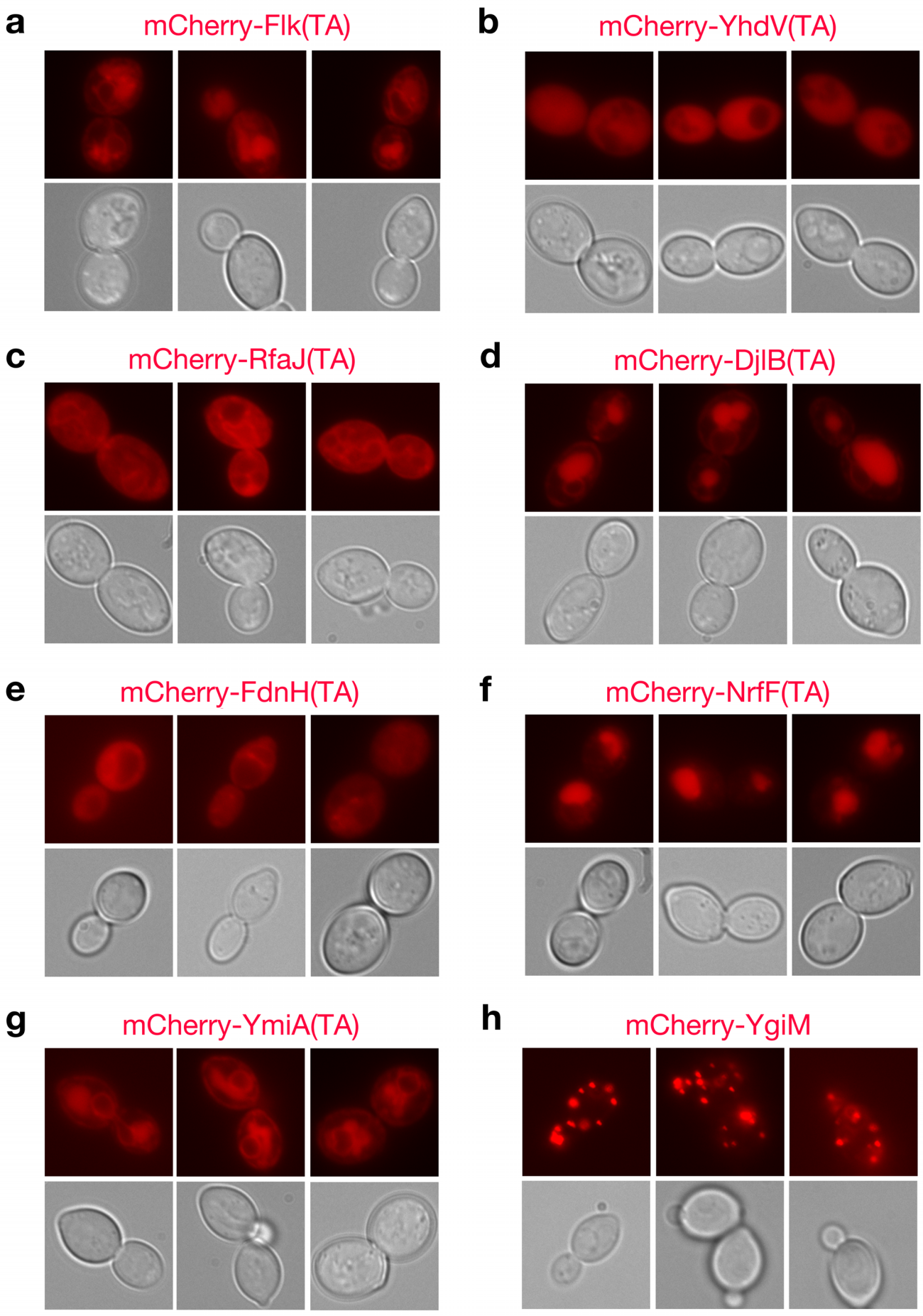

Extended Data Fig. 4