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Bacterial tail anchors can target to the mitochondrial outer membrane

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ABSTRACT

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. 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. Currently, a widely conserved translocon allows proteins to pass from the cytosol into mitochondria, 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), 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. 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 are able 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.

KEYWORDS

protein targeting, membrane insertion, eukaryogenesis, organelle biogenesis, endosymbiosis

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29 **BACKGROUND**

30

31 During the integration of an α-proteobacterial endosymbiont within the eukaryotic cell,
32 genes transferred to the (proto)nucleus were re-targeted to mitochondria, allowing
33 these organelles to remain the location of crucial cellular processes [1-3]. In addition,
34 other polypeptides that evolved within the eukaryotic lineage or that were acquired
35 through lateral gene transfer from other organisms were directed to mitochondria [4-6].
36 Across eukaryotes, the β-barrel Tom40 protein forms a pore by which proteins pass
37 through the OM [7-9]. However, the Tom40 polypeptide seems to require already
38 existing TOM complexes for mitochondrial insertion [10,11], giving rise to a "chicken or
39 the egg" dilemma when considering how the TOM complex may have evolved.

40

41 Several narratives might be proposed for how mitochondria first evolved the ability to
42 transport proteins from the cytosol. In one scenario, an early translocation pore that
43 was self-inserting at the mitochondrial surface might have allowed mitochondria to
44 begin to import proteins, permitting the subsequent evolution of the translocon found
45 in eukaryotes today [12]. Current evidence suggests that the self-insertion of tail-
46 anchored proteins at the mitochondrial OM is possible [13-15], and some tail-anchored
47 pro-apoptotic proteins appear to have the ability to generate membrane pores at
48 mitochondria [16,17], making tenable such a scenario for the evolution of mitochondrial
49 protein import. At the inception of mitochondria, such tail-anchored proteins would
50 likely have been derived from prokaryotes, particularly if mitochondria were required for
51 the generation of the stereotypical compartmentalized structure of eukaryotes.

52

53 We focused our attention upon a single aspect of this hypothesis: can TAs obtained
54 from bacterial proteins be inserted into the mitochondrial OM when expressed within a
55 eukaryotic cell? Indeed, our results demonstrate insertion and function at the
56 mitochondrial OM for predicted TAs encoded by the proteobacterium *E. coli*, and we
57 describe the relevance of our findings to the concept of lateral gene transfer during
58 eukaryogenesis.

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59 **RESULTS**

60

61 Bacterial Tail Anchors Can Localize to Mitochondria

62

63 To test whether predicted bacterial TAs might have the capacity to be inserted at the
64 mitochondrial OM, we identified 12 *E. coli* proteins predicted to harbor a solitary α-
65 helical transmembrane (TM) domain at the polypeptide carboxyl-terminus (Fig. S1),
66 then fused mCherry to the amino-terminus of these TAs and examined their location in
67 *S. cerevisiae* cells by fluorescence microscopy. mCherry-ElaB(TA) (Fig. 1A) and
68 mCherry-YqjD(TA) (Fig. 1B) were readily detectable at mitochondria, as reported by co-
69 localization with superfolder GFP (sfGFP) [18] fused to the TA of the *S. cerevisiae* Fis1
70 polypeptide, a protein playing a role in yeast mitochondrial division. A lesser fraction of
71 mCherry-ElaB(TA) and mCherry-YqjD(TA) was localized to the endoplasmic reticulum
72 (Fig. S2). ElaB and YqjD are members of the DUF883 family of proteins. Little is known
73 about the function of DUF883 family members, but YqjD may recruit ribosomes to the
74 *E. coli* plasma membrane during stationary phase [19]. Although negligible fluorescent
75 signal was detectable by microscopy or flow cytometry (C. Dunn, unpublished results),
76 mCherry-TcdA(TA) could also be visualized at mitochondria (Fig. S3A). TcdA (also
77 called CsdL) catalyzes the modification of *E. coli* tRNAs [20].

78

79 Other predicted TAs derived from the *E. coli* Flk, YgiM, RfaJ, DjlB, FdnH, NrfR, and
80 YmiA proteins appeared to allow at least partial localization of mCherry to various
81 locations associated with the endomembrane system (Fig. S4). However, no
82 convincing localization to mitochondria was apparent after fusing any of these TAs to
83 mCherry. Moreover, mCherry-YhdV(TA) appeared to be distributed throughout cytosol
84 and nucleus, indicating failure to target efficiently to any membrane. mCherry-
85 YgaM(TA) was not detectable, suggesting its degradation.

86

87

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88 **Bacterial Tail Anchors Can Insert into Membranes in a Eukaryotic Cell**

89

90 Previously, we developed an assay in which membrane insertion of proteins might be
91 examined by a proliferation-based assay [21]. In brief, the Gal4 transcription factor is
92 linked to a protein of interest that is thought to be membrane inserted outside of the
93 nucleus. Failure of this fusion protein to insert at its target membrane can allow the
94 Gal4-linked fusion protein to access the nucleus and activate Gal4-responsive
95 promoters that drive proliferation under selective conditions. As previously
96 demonstrated [21], while a membrane-sequestered Gal4-sfGFP-Fis1 fusion protein did
97 not lead to a proliferation defect on non-selective medium (SC-Trp), cells carrying this
98 construct could not survive on medium requiring activation of a Gal4p-driven *HIS3*
99 gene (SMM-His+20mM 3-AT) (Fig. 2). Deletion of the Fis1p TA, or the presence of a
100 A144D charge substitution within the Fis1p TA, led to a failure of membrane insertion
101 at mitochondria, translocation to the nucleus, and Gal4-dependent proliferation on
102 selective medium. When the TA of Fis1p, a domain whose sole purpose is to allow this
103 protein's insertion at the mitochondrial OM [21,22], was replaced with the TA of either
104 ElaB or YqjD, cells were unable to proliferate on medium selective for histidine
105 synthesis, consistent with ElaB and YqjD TA insertion at the mitochondrial OM.

106

107 **Bacterial Tail Anchors Can Function at the Mitochondrial Outer Membrane**

108

109 As these findings indicated that the ElaB and YqjD TAs may be competent for
110 mitochondrial insertion, we tested whether these TAs can functionally replace the
111 membrane-bound TA of Fis1p, thereby allowing Fis1p to promote mitochondrial
112 division. Because Fis1p is required for mitochondrial fission in *S. cerevisiae*, mutants
113 lacking this protein manifest a highly interconnected network of mitochondria due to
114 unbalanced mitochondrial fusion [23-25]. As expected, expression of wild-type Fis1p
115 restored normal mitochondrial distribution in this genetic background, while Fis1p
116 prevented from mitochondrial insertion by a A144D substitution within the Fis1p TA

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117 [21] could not restore normal mitochondrial morphology (Figs. 3A and 3B). Strikingly,
118 replacement of the Fis1p TA with the ElaB or the YqjD TA within the context of full
119 length Fis1p polypeptide could successfully promote mitochondrial division and
120 restore normal mitochondrial morphology. A control TA obtained from the *E. coli* YgiM
121 protein, which is not trafficked to mitochondria, could not support Fis1p activity. In
122 addition, a Fis1-TcdA(TA) protein could not functionally replace the Fis1p TA in this
123 microcopy-based assay (Fig. S3B), suggesting insufficient expression, poor
124 mitochondrial insertion, or meager functionality.
125
126 We then sought further evidence for functional insertion of the ElaB and YqjD TAs at
127 the mitochondrial OM using an assay based on cell proliferation [21]. Expression of
128 functional Fis1p in a genetic background initially lacking Fis1p and removed of the
129 mitochondrial fusogen Fzo1p can lead to unchecked mitochondrial fragmentation, loss
130 of functional mitochondrial DNA (mtDNA), and a corresponding abrogation of
131 respiratory competence [26-29]. As previously reported [21], expression of wild-type
132 Fis1p in a *fzo1Δ fis1Δ* genetic background led to an inability to proliferate on
133 nonfermentable medium, while expression of the poorly inserted Fis1(A144D) variant
134 did not prompt mtDNA loss (Fig. 3C). The ElaB and YqjD TAs fused to the cytosolic
135 domain of Fis1p allowed sufficient fission activity to prompt mitochondrial genome loss
136 from the same genetic background, again indicating successful ElaB TA and YqjD TA
137 insertion at the mitochondrial OM. Even the Fis1-TcdA(TA) protein provoked mtDNA
138 loss in *fzo1Δ fis1Δ* cells (Fig. S3C), suggesting some minimal level of OM insertion, and
139 the YgiM TA again appeared unable to recruit Fis1p to mitochondria (Fig. 3C).
140 Together, our results demonstrate insertion of the bacterial ElaB and YqjD TAs at the
141 mitochondrial surface of a eukaryotic cell.

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142 **DISCUSSION**

143

144 Our findings, in which several predicted TAs obtained from *E. coli* can target to and
145 function at the mitochondrial OM of *S. cerevisiae*, make plausible a scenario in which
146 tail-anchored bacterial proteins contributed to the formation of the earliest
147 mitochondrial translocon. The structural characteristics of the TAs of ElaB and YqjD, a
148 helical TM domain rich in glycines followed by a positively charged patch ending in di-
149 arginine (Fig. S1), are evocative of the Fis1p TA, suggesting a similar, potentially
150 spontaneous mechanism for insertion at mitochondria, although unassisted insertion of
151 the ElaB and YqjD TAs at the mitochondrial surface has yet to be demonstrated.
152 Notably, several conserved members of the current TOM complex are also tail-
153 anchored [30], raising the possibility that at least some of these proteins could be
154 "hold-overs" from an early, self-inserting mitochondrial translocon, although we note
155 that these subunits cannot currently self-insert at mitochondria.

156

157 Could the DUF883 family of proteins have contributed to an ancestral mitochondrial
158 OM translocon? While YqjD has been reported to recruit ribosomes to the *E. coli* inner
159 membrane during stationary phase [19], a role in line with promotion of co-translational
160 protein import into mitochondria [31,32], the DUF883 family is not readily identified in
161 eukaryotic genomes. One might expect, however, that once a more proficient TOM
162 complex centered around the Tom40 pore evolved, a previous translocon would have
163 been lost, or even selected against if it were to interfere with more rapid protein import
164 through an improved OM translocation machinery. Moreover, an inordinate focus on
165 DUF883 family members when seeking components of the earliest mitochondrial
166 translocon may not be warranted in any case, since the structural characteristics likely
167 required for TA insertion at mitochondria might be easily generated from random open
168 reading frame fragments containing a transmembrane domain. Analogously, random
169 sequences from bacteria are readily able to act as amino-terminal mitochondrial
170 targeting sequences [33-35]. If TAs are easily evolved and might recruit other

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171 functional domains to the mitochondrial surface, then identifying orthologs of initial tail-
172 anchored translocon components from existing prokaryotic sequences might be
173 difficult, since an untold number of TAs might be predicted among putative open-
174 reading frames. Supporting the idea that mitochondrial TAs might be generated from
175 sequences not actually functioning in membrane targeting within their native bacterial
176 environment, we demonstrated limited mitochondrial targeting and partial functionality
177 of the computationally predicted TcdA TA in yeast, even though TcdA is unlikely to be
178 membrane-inserted in *E. coli* [36].
179
180 If conversion of endosymbiont to mitochondria were the rare and essential event
181 required for generation of eukaryotes, and if insertion of bacteria-derived, tail-anchored
182 proteins at the OM to form an ancestral translocon were necessary for this conversion,
183 then the question of how hospitable an environment the early mitochondria OM might
184 have been for bacteria-derived TAs comes to the fore. Indeed, the membrane into
185 which tail-anchored proteins are inserted can be at least partially determined by their
186 lipid environment [13], and lipids utilized by many characterized archaea are
187 fundamentally different in structure from bacterial and eukaryotic lipids [37]. However,
188 recent evidence indicates that archaeal clades potentially related to the last eukaryotic
189 common ancestor might have been characterized by membranes more similar to those
190 of bacteria than of those membranes more typically found in archaea [38]. This finding
191 raises the possibility that the protoeukaryote's specific cohort of lipids was crucial to
192 the ability to form complexes of bacteria-derived tail-anchored proteins at the
193 mitochondrial OM that would allow full integration of mitochondria within the ancestral
194 eukaryote.
195
196 Finally, we have not examined in detail the trafficking of *E. coli* TAs that appeared to
197 localize to the endomembrane system during our initial survey. However, the diverse
198 organellar locations to which these TAs were localized supports previous data
199 indicating that eukaryotes may derive organelle targeting information from newly
200 acquired prokaryotic proteins or protein fragments, perhaps even from amino acid

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201 sequences previously unselected for targeting proficiency [33-35,39,40]. Lateral gene
202 transfer promotes the evolution of novel functions in prokaryotes [41] and was
203 certainly present in the form of endosymbiotic gene transfer during early
204 eukaryogenesis. Indeed, proficiency in making use of cryptic or explicit targeting
205 information in order to direct newly acquired, nucleus-encoded proteins to the distinct
206 subcellular locations where they might be best utilized might have provided a
207 significant selective advantage to the early eukaryote. Such a scenario may be
208 particularly relevant if some amount of cellular compartmentalization already existed in
209 a pre-eukaryotic host cell before conversion of pre-mitochondrial endosymbiont to
210 organelle [42,43].
211
212 **CONCLUSIONS**
213
214 We have demonstrated that TAs from bacteria can localize to and insert within the
215 mitochondrial OM. Our results make plausible the suggestion that tail-anchored
216 proteins acquired by bacteria could have formed an initial translocon at the
217 mitochondrial outer membrane, and our findings indicate that membrane-bound
218 proteins acquired by horizontal gene transfer could have easily found their way to
219 diverse locations within eukaryotic cells at which they might provide a selective
220 advantage. Further efforts will be necessary to determine whether self-inserting
221 proteins or peptides may have generated the initial mitochondrial translocon.

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222 **METHODS**
223
224 **Yeast strains, plasmids, and culture conditions**
225
226 Culture conditions are as described in [21], and all experiments have been carried out
227 at 30°C. Strains, plasmids, and oligonucleotides used in this study are found in
228 Supplementary Information 1.
229
230 **Selection of *E. coli* tail anchors subject to investigation**
231
232 FASTA sequences from the *E. coli* proteome were retrieved from UniProt [44] and
233 subjected to analysis using the TMHMM 2.0 server [45]. Polypeptides with a single
234 predicted TM domain (denoted by purple line), harboring 15 or less amino acids
235 carboxyl-terminal to the TM domain, and containing more than 30 amino acids amino-
236 terminal to the TM domain were selected for further analysis.
237
238 **Microscopy**
239
240 Microscopy was performed on logarithmic phase cultures as in [21], with exposure
241 times determined automatically. mCherry fusions are driven by the *ADH1* promoter and
242 universally contain Fis1p amino acids 119-128 (not necessary or sufficient for
243 mitochondrial targeting) linking mCherry to each TA, and genetic assessment of Fis1p
244 variant functionality was performed as described in [21]. The brightness of all images of
245 mCherry expression was adjusted in Adobe Photoshop CS5 (Adobe, San Jose,
246 California) to an equivalent extent. Scoring of mitochondrial morphology was
247 performed blind to genotype.
248
249 **Proliferation-based assessment of Fis1p insertion and functionality**
250
251 Genetic tests of Fis1p insertion and functionality were performed as in [21].

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252 **LIST OF ABBREVIATIONS**

253

254 TA - tail anchor

255 mtDNA - mitochondrial DNA

256 OM- outer membrane

257 TM - transmembrane

258 sfGFP - superfolder GFP

259

260 **DECLARATIONS**

261

262 *Ethics approval and consent to participate*

263 None required.

264

265 *Consent for publication*

266 None required.

267

268 *Availability of data and material*

269 All data generated or analysed during this study are included in this published article

270 and its supplementary information files.

271

272 *Competing interests*

273 The authors declare that they have no competing interests.

274

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282 *Authors contributions*

283 C.D.D. designed the study, wrote the manuscript, and performed experiments. G.L.B.,
284 A.K., and A.B.S. performed experiments, generated reagents, and provided manuscript
285 critiques. All authors read and approved the final manuscript.

286

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419 **FIGURE LEGENDS**

420

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

426

427 **Figure 2. A proliferation-based assay suggests that the ElaB and YqjD TAs are**
428 **membrane inserted.** Strain MaV203, containing a Gal4-activated *HIS3* gene, was
429 transformed with plasmids expressing Gal4-sfGFP-Fis1p (plasmid b100), a variant
430 lacking the Fis1p TA (plasmid b101), a mutant containing the A144D charge
431 substitution in its TA (b180), or the Gal4-sfGFP-Fis1p construct with the Fis1p TA
432 replaced with that of either ElaB (b313) or YqjD (b314). MaV203 was also transformed
433 with empty vector pKS1. Transformants were cultured in SC-Trp medium, then,
434 following serial dilution, spotted to SC-Trp or SMM-His + 20 mM 3-AT and incubated
435 for 2 d.

436

437 **Figure 3. Mitochondria-localized bacterial TAs can functionally replace the TA of**
438 **Fis1p.** (A) The ElaB and YqjD TAs can replace the Fis1p TA in promotion of normal
439 mitochondrial morphology. *fis1Δ* strain CDD741, expressing mitochondria-targeted
440 GFP from plasmid pHS12, was transformed with empty vector pRS313 or plasmids
441 expressing wild-type Fis1p (b239), Fis1(A144D)p (b244), or Fis1p with its own TA
442 replaced by that of ElaB (b317), YqjD (b318), or YgiM (b316). Cells were examined by
443 fluorescence microscopy. Scale bar, 5μm. (B) Quantification of mitochondrial
444 morphology of the transformants from (A) was performed blind to genotype. White bar
445 represents cells with fully networked mitochondria, grey bar represents cells with
446 mitochondria not fully networked, but networked to a greater extent than wild-type
447 cells, and black bar represents cells with normal mitochondrial morphology.
448 Quantification was repeated three times (n>200 per genotype), and a representative

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449 experiment is shown. (C) Genetic assessment of Fis1p variant functionality. Strain
450 CDD688 was transformed with the plasmids in (A) and proliferation was assessed
451 without selection against Fis1p activity (YPALac medium for 2 d) and following
452 counter-selection for cells carrying functional Fis1p (SLac-His+CHX medium for 4 d).
453

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454 **SUPPLEMENTAL FIGURE LEGENDS**
455

456 **Supplemental Figure 1. A list of predicted TAs examined in this study.** The UniProt
457 accession number and names of selected proteins are provided, along with the
458 sequences of the predicted TAs. Charged amino acids are also denoted. For purposes
459 of sequence comparison, the relevant portion of the *S. cerevisiae* Fis1p TA is also
460 shown.
461

462 **Supplemental Figure 2. The predicted ElaB and YqjD TAs can also be visualized at**
463 **the endoplasmic reticulum.** Cells were analyzed as in Figure 1, except BY4741 was
464 transformed with plasmid pJK59, expressing Sec63-GFP, before mating.
465

466 **Supplemental Figure 3. The predicted TcdA TA allows minimal localization to, and**
467 **function at, the mitochondrial outer membrane.** (A) The predicted TcdA TA can be
468 visualized at mitochondria. Strain BY4741, harboring plasmid b294 (sfGFP-Fis1p), was
469 mated to strain BY4742 carrying mCherry-TcdA(TA)-expressing plasmid b281 and the
470 resulting diploids were imaged by fluorscence microscopy. Scale bar, 5 µm. (B) Fis1p
471 with its own TA replaced by the predicted TcdA TA cannot promote normal
472 mitochondrial morphology. *fis1Δ* strain CDD741, expressing mitochondria-targeted
473 GFP from plasmid pHS12, was transformed with empty vector pRS313 or plasmids
474 expressing wild-type Fis1p (b239), Fis1(A144D)p (b244), or Fis1-TcdA(TA)p (b319) and
475 mitochondrial morphology was assessed by fluorescence microscopy. Scale bar, 5µm.
476 (C) Fis1-TcdA(TA)p can allow mitochondrial division. Strain CDD688 was transformed
477 with the plasmids used in (B) or a plasmid expressing Fis1-YgiM(TA)p (b316) and
478 examined as in Fig. 2C, except that culture on medium counter-selective for Fis1p
479 activity was carried out for 5 d.
480

481 **Supplemental Figure 4. Not all predicted *E. coli* TAs are localized to mitochondria**
482 **in *S. cerevisiae*.** Strain CDD961 was transformed with plasmids expressing (A)
483 mCherry-Flk(TA) (b273), (B) mCherry-YhdV(TA) (b277), (C) mCherry-RfaJ(RA) (b278), (D)

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484 mCherry-DjlB(TA) (b280), (E) mCherry-FdnH(TA) (b331), (F) mCherry-NrfF(TA) (b332), (G)
485 mCherry-YmiA(TA) (b333) and examined by fluorescence microscopy. (H) Strain
486 BY4741, carrying plasmid b311 expressing sfGFP fused to the enhanced PTS1
487 sequence [46], was mated to strain BY4742, containing the mCherry-YgiM(TA)-
488 expressing plasmid b274, and the resulting diploids were imaged.
489
490 **Supplementary Information 1. Strains, plasmids, and oligonucleotides used**
491 **during this study.**

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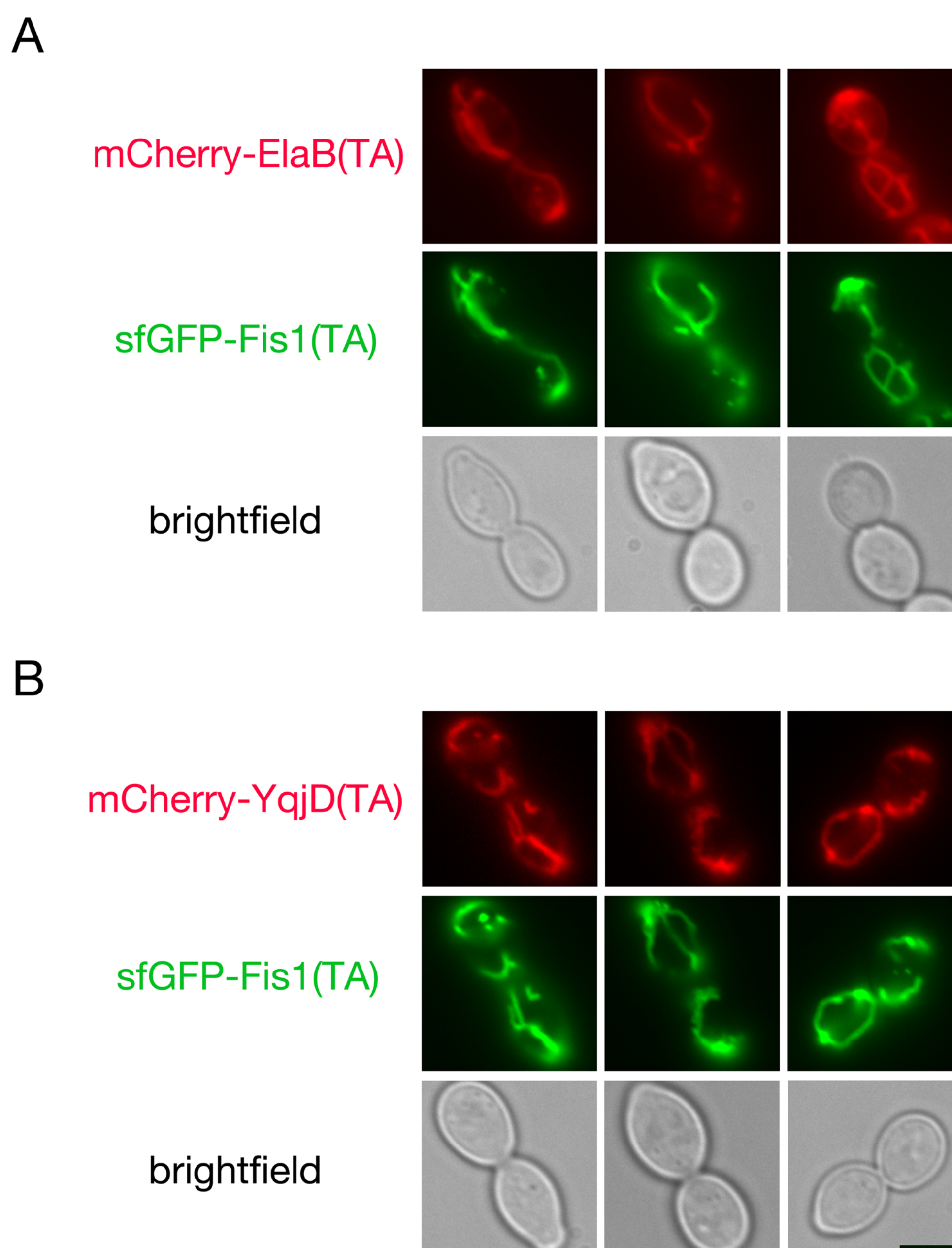

Figure 1

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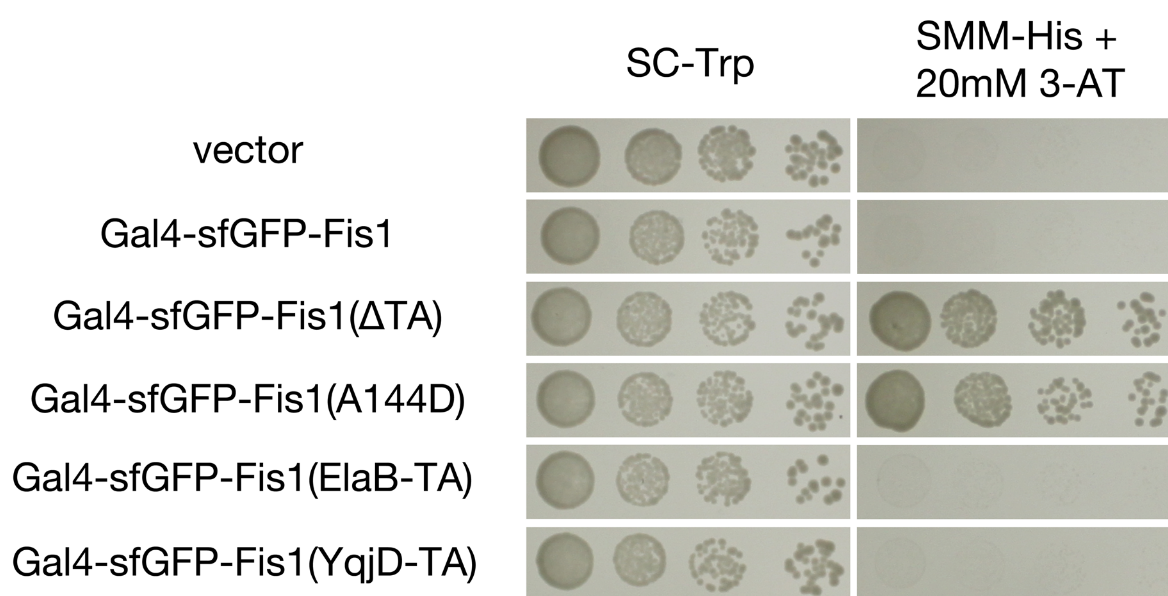

Figure 2

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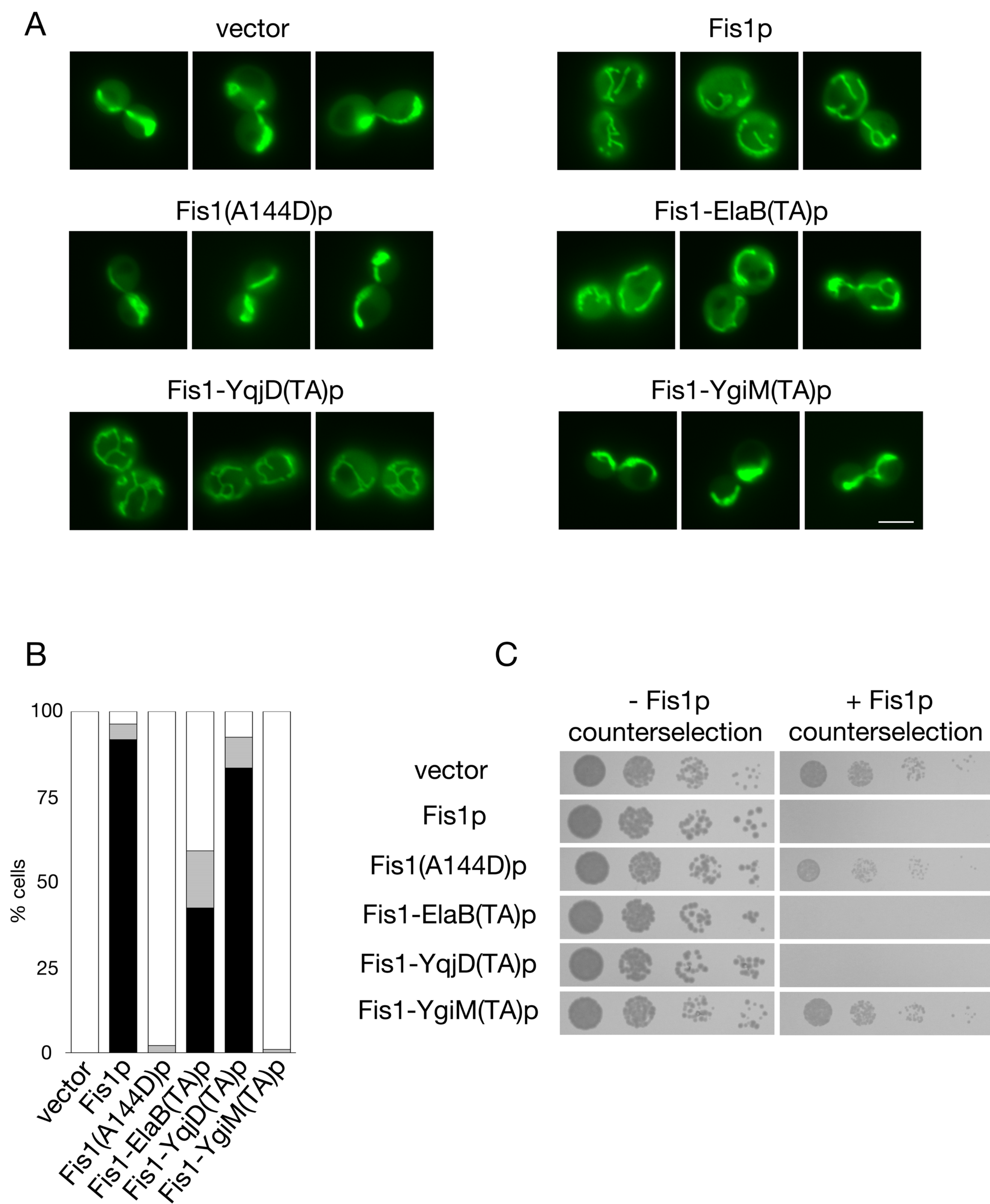

Figure 3

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P15286 Flk PALWILLVAIILMLVWLVR

P64622 YhdV TAGAIAGGAAAVAGLTMGIIALSK

P27129 RfaJ LVQHHYISGIIAGVCYLCRKYYRK

P77381 DjlB LGIIKIIFYIFIFAGLIGKILHLFG

P0AAJ3 FdnH LAAAGFIATFAGLIFHYIGIGPNKEVDDDEEDHHE

P32711 NrfF TGQTLVLWALPVVLLLLMALILWRVRAKR

P0CB62 YmiA WLAVFLGSALFWVVVALLIWKVWG

P0ADT8 YgiM WFMYGGGVLGLGLLLGLVLPHLIPSRKRKDRWMN

P0ADQ7 YgaM GTAAAVGIFIGALLSMRKS

Q46927 TcdA ASGFGAATMVTATFGFVAVSHALKKMMAKAARQG

P0AEH5 ElaB PWQGIGVGAAVGLVLGLLLARR

P64581 YqjD WTGVGIGAAIGVVLGVLLSRR

P40515 Fis1 LKGVVVAGGVLAGAVAVASFFLRNKRR

Supplemental Figure 1

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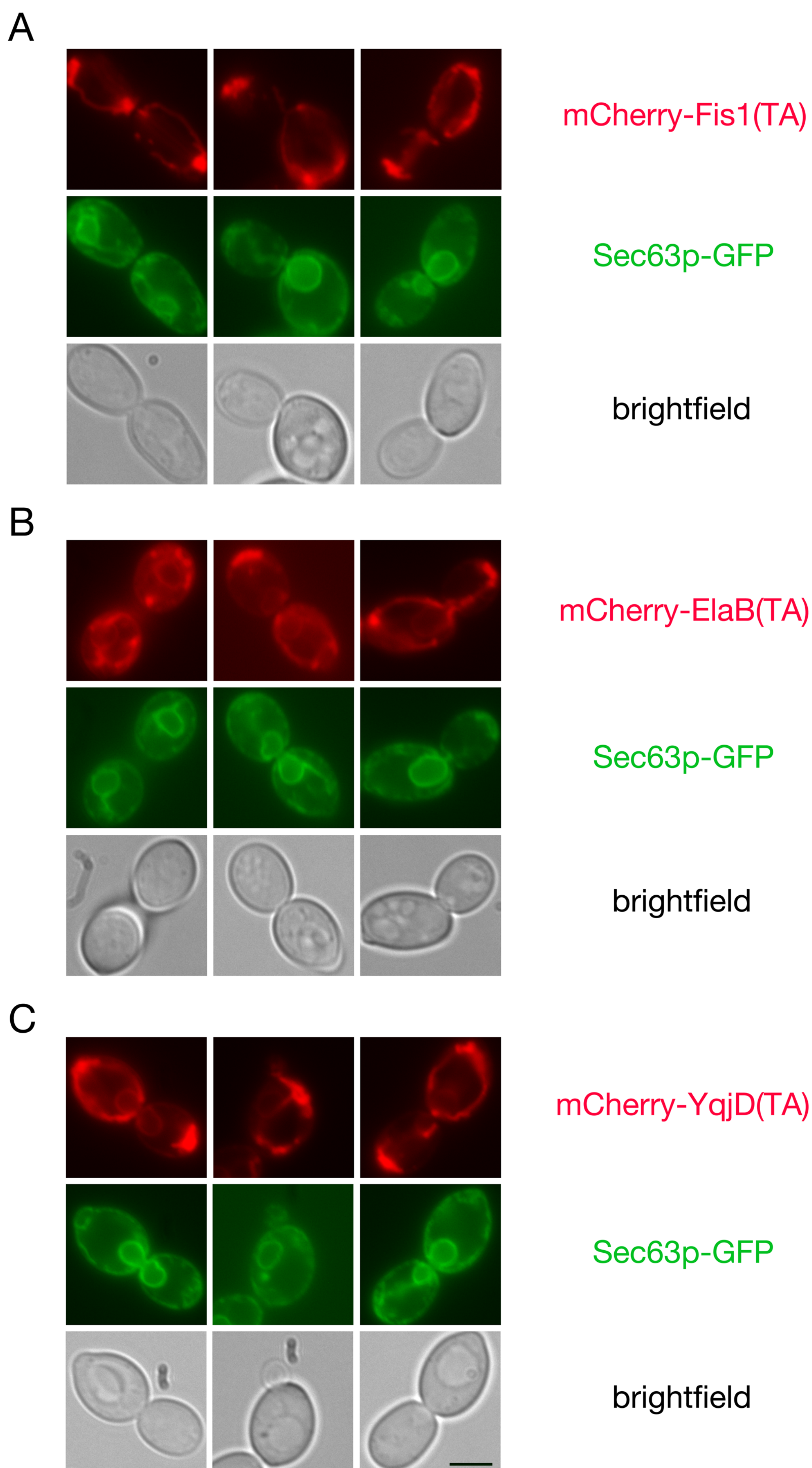

Supplemental Figure 2

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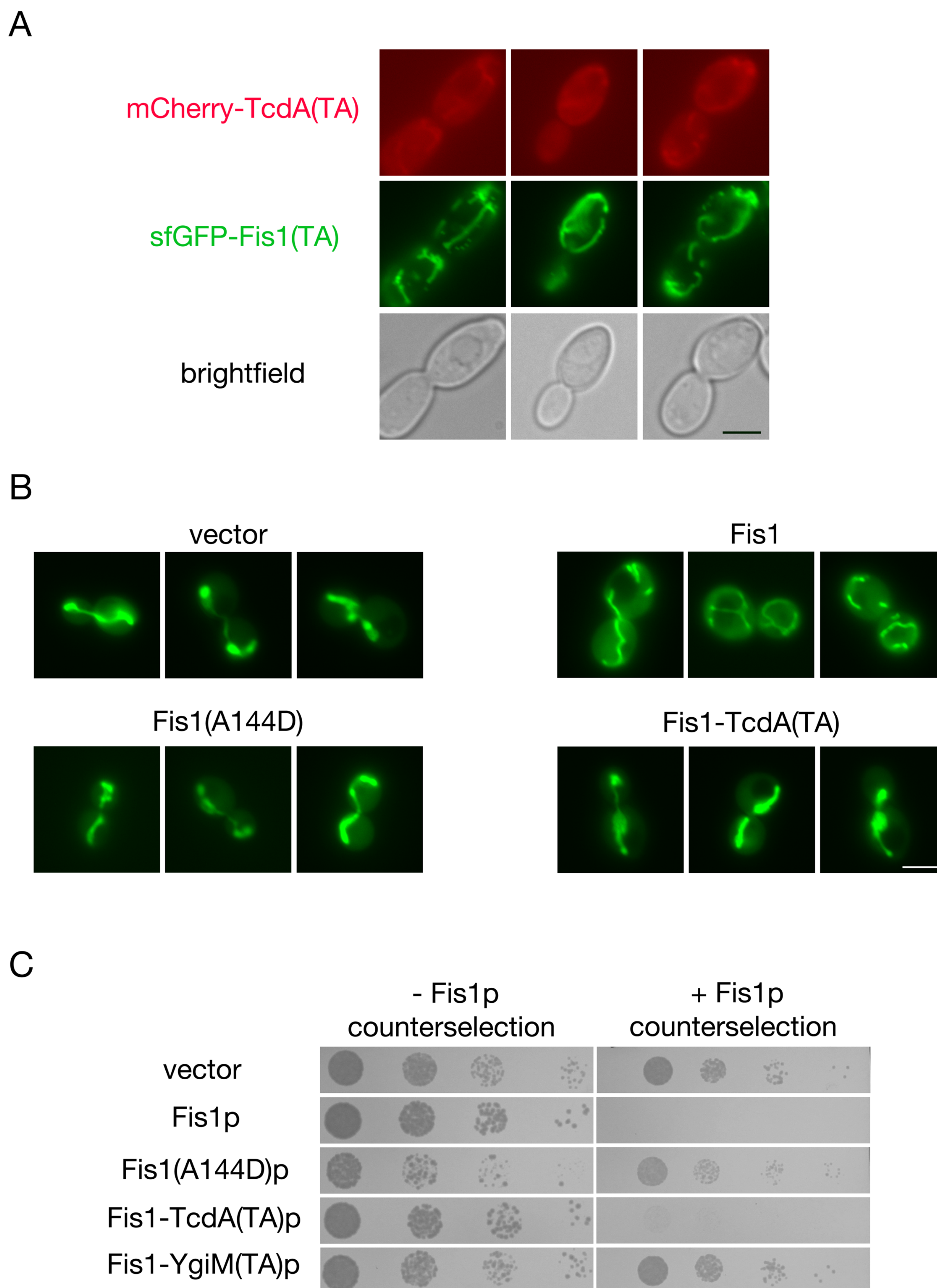

Supplemental Figure 3

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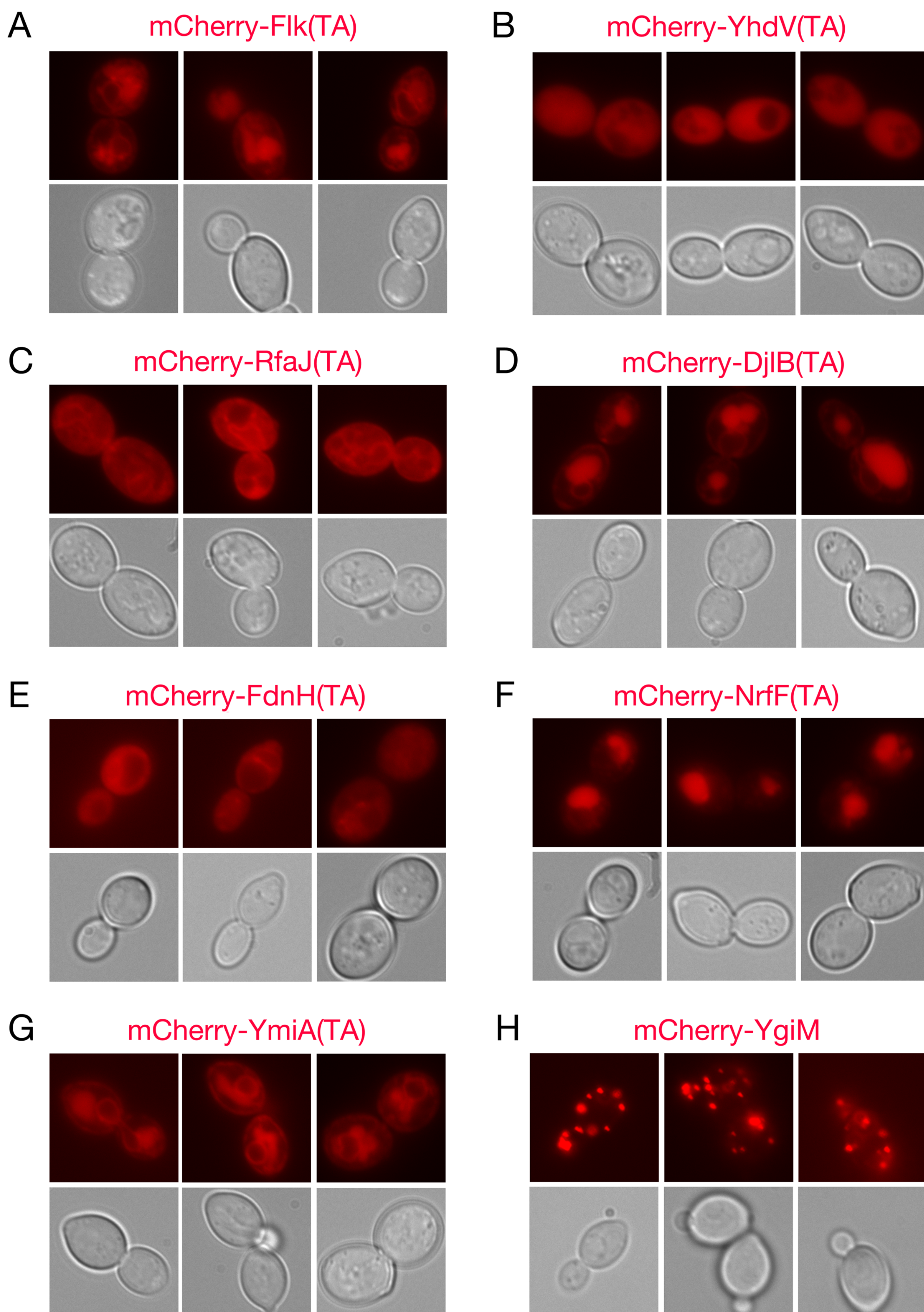

Supplemental Figure 4