

1      **Full Title: Selective transport of fluorescent proteins into the phage nucleus**

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3                      Short title: Protein sorting into the phage nucleus

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23      **Abstract**

24        Upon infection of *Pseudomonas* cells, jumbo phages 201Φ2-1, ΦPA3, and ΦKZ  
25    assemble a phage nucleus. Viral DNA is enclosed within the phage-encoded  
26    proteinaceous shell along with proteins associated with DNA replication, recombination  
27    and transcription. Ribosomes and proteins involved in metabolic processes are  
28    excluded from the nucleus. RNA synthesis occurs inside the phage nucleus and  
29    messenger RNA is presumably transported into the cytoplasm to be translated. Newly  
30    synthesized proteins either remain in the cytoplasm or specifically translocate into the  
31    nucleus. The molecular mechanisms governing selective protein sorting and nuclear  
32    import in these phage infection systems are currently unclear. To gain insight into this  
33    process, we studied the localization of five reporter fluorescent proteins (GFP<sup>+</sup>, sfGFP,  
34    GFPmut1, mCherry, CFP). During infection with ΦPA3 or 201Φ2-1, all five fluorescent  
35    proteins were excluded from the nucleus as expected; however, we have discovered an  
36    anomaly with the ΦKZ nuclear transport system. The fluorescent protein GFPmut1,  
37    expressed by itself, was transported into the ΦKZ phage nucleus. We identified the  
38    amino acid residues on the surface of GFPmut1 required for nuclear targeting. Fusing  
39    GFPmut1 to any protein, including proteins that normally reside in the cytoplasm,  
40    resulted in transport of the fusion into the nucleus. Although the mechanism of transport  
41    is still unknown, we demonstrate that GFPmut1 is a useful tool that can be used for  
42    fluorescent labelling and targeting of proteins into the ΦKZ phage nucleus.

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#### 44      **Introduction**

45              Protein targeting within a cell is essential in all organisms. Generally, eukaryotes  
46    use a sorting sequence to target proteins to specific organelles, such as a nuclear  
47    localization signal to send proteins to the nucleus or an N-terminal signal peptide to  
48    target proteins to the endoplasmic reticulum. These signal sequences are usually  
49    highly conserved, even among different species (1, 2). Though bacterial cells lack the  
50    membrane-bound organelles of eukaryotes, they still utilize a number of protein sorting  
51    strategies to target proteins either extracellularly or to specific intracellular locations (3-  
52    5). For example, secretion of unfolded proteins from the cytoplasm requires a signal  
53    sequence, which directs proteins to the SecYEG pore where secretion is powered by  
54    the ATPase SecA and the proton motive force (4, 6). In contrast, the TatA system  
55    exports fully folded proteins across the cytoplasmic membrane after recognizing a pair  
56    of arginine residues at the C-terminus (5). The Sec and Tat pathways are highly  
57    conserved in all domains of life (3). In addition to these general secretory systems,  
58    many additional systems (Type I - VI) transport specific cargo across the inner and  
59    outer bacterial membranes (3). These transport systems all utilize a beta-barrel  
60    channel that spans the membrane but are widely divergent in most other aspects (3).

61              Protein targeting is essential for establishing and maintaining subcellular  
62    organization as well as for viral replication. We recently described the phage nucleus  
63    assembled by jumbo phages 201Φ2-1 (7, 8), ΦPA3 (9), and ΦKZ (10) in *Pseudomonas*  
64    cells (11, 12). In the early stages of infection, the phage assembles a nucleus-like  
65    structure in the cell and positions it at midcell using a dynamic bipolar tubulin-based  
66    spindle (11-16). Phage proteins synthesized by bacterial ribosomes in the cytoplasm

67 appear to be sorted to specific subcellular destinations based on their biological  
68 functions. Much like in a eukaryotic cell, proteins involved in DNA replication, repair, and  
69 transcription localize inside the nucleus, while proteins involved in metabolic processes  
70 and protein synthesis localize in the cytoplasm outside the nucleus (11, 12). Time-lapse  
71 microscopy experiments show that phage proteins, expressed in our heterologous  
72 system, are synthesized before phage are introduced, then accumulate in the nucleus  
73 as infection occurs, suggesting that a mechanism exists for posttranslational nuclear  
74 protein transport (12). However, no known eukaryotic nuclear localization signals or  
75 bacterial sorting sequences were encoded by the phages. In addition, we have not  
76 identified any homology to bacterial transporters or nuclear pore proteins in the phage  
77 genomes. The mechanisms of protein sorting and intracellular transport are still  
78 unknown.

79 One of the barriers to understanding the details of *Pseudomonas* jumbo phage  
80 replication is the inability to specifically target proteins, such as gene editing enzymes or  
81 other effectors, to the phage nucleus versus the cytoplasm. Here, we report a  
82 technique for targeting proteins into the  $\Phi$ KZ nucleus. Although the nucleus of  $\Phi$ KZ  
83 appears to be largely similar to that of phages  $\Phi$ PA3 and 201 $\Phi$ 2-1, surprisingly, we  
84 found that it imports the fluorescent protein GFPmut1, but not any of the other tested  
85 fluorescent proteins. In addition, any protein fused to GFPmut1 also localized to the  
86  $\Phi$ KZ nucleus. Thus, we have serendipitously discovered a reliable method for  
87 delivering specific proteins into the  $\Phi$ KZ nucleus.

88

89 **Results**

90        During comparative protein localization of cells infected with one of three different  
91    phages ( $\Phi$ PA3,  $\Phi$ KZ, 201 $\Phi$ 2-1), we noticed a discrepancy in localization of the  
92    fluorescent proteins themselves. All fluorescent protein controls (GFPmut1, GFP<sup>+</sup>,  
93    sfGFP, mCherry, and CFP) were localized in the cytoplasm of  $\Phi$ PA3 and 201 $\Phi$ 2-1 as  
94    expected (Fig 1A). Four of these proteins, GFP<sup>+</sup>, sfGFP, mCherry, and CFP also  
95    localized in the cytoplasm of  $\Phi$ KZ infected cells (Fig 1A). However, GFPmut1 localized  
96    inside the  $\Phi$ KZ nucleus even though it was excluded by the nucleus of 201 $\Phi$ 2-1 in *P.*  
97    *chlororaphis* and that of  $\Phi$ PA3 in *P. aeruginosa* (Fig 1B). Our results suggest that  
98    differences exist among the fluorescent proteins that affect their ability to be transported  
99    into the  $\Phi$ KZ nucleus.

100

101 **Fig 1: Fluorescent protein localization during phage infection. Most fluorescent**  
102 **proteins localize to the bacterial cytoplasm and are excluded by the phage**  
103 **nucleus but GFPmut1 is transported into the  $\Phi$ KZ nucleus. Scale bar = 1 micron**

104

105    A. SfGFP, GFP<sup>+</sup>, mCherry, and CFP are excluded by the phage nucleus in *P.*  
106    *chlororaphis* cells infected with 201 $\Phi$ 2-1 and *P. aeruginosa* cells infected with  
107     $\Phi$ PA3 or  $\Phi$ KZ.

108

109    B. GFPmut1 localizes inside the  $\Phi$ KZ phage nucleus but is excluded from both the  
110    201 $\Phi$ 2-1 nucleus and  $\Phi$ PA3 nucleus.

111

112 C. Alignment of fluorescent protein sequences showing key differences in bold letters.

113 Key differences occur at F99, M153, and V163 of GFPmut1 compared to other  
114 fluorescent proteins.

115

116

117 We reasoned that studying nearly identical fluorescent proteins with strikingly  
118 different localizations might provide insights into nuclear targeting. Comparison of the  
119 protein sequences (17-21) of these fluorescent proteins revealed several amino acid  
120 differences that could be responsible for the discrepancy in localization of GFPmut1 (Fig  
121 1C). We identified three amino acids where GFPmut1 differed from the other proteins  
122 tested: F99, M153, V163 (Fig 1C, bold letters, Fig S1) (17, 19, 22). In the 3-dimensional  
123 protein structure of GFP, phenylalanine (F99) and methionine (M153) are both surface  
124 exposed, extending outward from one face of the GFP, while valine (V163) is along the  
125 same surface but facing inward toward the beta barrel (Fig 2A) (19, 22-24). To  
126 determine which of these mutations might influence import into  $\Phi$ KZ, we used site-  
127 directed mutagenesis to individually mutate each amino acid of GFPmut1 to that found  
128 in alternate versions of GFP, specifically those that remained in the cytoplasm during  
129 infection (sfGFP and GFP<sup>+</sup>) (17, 19, 25). At 60 minutes post-infection, GFPmut1 with  
130 the V163A mutation (n=82) retained the same phenotype as the unaltered GFPmut1  
131 (n=111), localizing to the nucleus (Fig 2B, 2C). However, the F99S mutation completely  
132 changed the phenotype so that the fluorescent protein was localized in the cytoplasm  
133 and excluded by the nucleus in 100% of cells (n=177) (Fig 2B, 2C). The M153T  
134 mutation partially altered GFP localization creating a mixed phenotype among cells in

135 the population (n=115) which we quantitated by plotting normalized pixel intensity  
136 profiles of fluorescence signals along a line through the center of the long axis of the  
137 cell (Fig 2C). Individual tracings (Fig 2C, blue) show there is significant variability  
138 among the population of M153T cells, ranging from fully excluded (minimum intensity at  
139 the center) to fully nuclear localized (maximum intensity at the center). In contrast, both  
140 the individual tracings (Fig 2C) and average tracings (Fig 2C, D) of GFPmut1 and  
141 V163A were fully imported while F99S was fully excluded. These results suggest that  
142 the amino acids on the surface of GFPmut1 contribute to its selective import into the  
143  $\Phi$ KZ nucleus.

144

145 **Fig 2: Identification of amino acid residues that alter the nuclear localization**  
146 **of GFPmut1 during PhiKZ infection.**

147

148 A. Three amino acid residues in the GFPmut1 sequence distinguish it from other  
149 fluorescent proteins. The mutations are all located on the beta-barrel but two  
150 (F99, M153) are on the outer surface while V163 is inside the barrel.

151 B. Three amino acids within GFPmut1 were individually mutated and localized  
152 during phage infection. Changing V163 to alanine (V163A) results in nearly 100%  
153 localization inside the phage nucleus similar to the unaltered GFPmut1.  
154 Changing F99 to serine (F99S) results in nearly 100% cytoplasmic localization  
155 during phage infection. M153T appears to localize inside and outside the  
156 nucleus in equal measure. Scale bar = 1 micron

157 C. Normalization of GFP intensity in these versions of GFPmut1 was used to  
158 quantify the localization of these point mutations in comparison with unaltered  
159 GFPmut1. GFPmut1 (n = 111), F99S (n=177), M153T (n=115), V163A (n=82).  
160 Each cell expressing GFPmut1 is represented with one black line, showing 100%  
161 inclusion into the nucleus. An almost identical phenotype is seen with the red  
162 lines representing cells with the V163A mutation. GFPmut1 F99S, shown with  
163 green lines, displays GFP intensity outside the nucleus, indicating 100%  
164 exclusion. M153T, represented by the blue lines, exhibits both nuclear import  
165 and exclusion.

166 D. A plot showing the averages of the individual cells graphed in the left plot.  
167 GFPmut1 in black and V163T in red indicate overall inclusion into the nucleus.  
168 F99S is represented by green line indicating exclusion. The blue line showing the  
169 average of M153T localization profiles is at baseline, showing that on average  
170 the protein localizes both inside and outside the nucleus.

171  
172 We wished to ensure that there were no major structural differences in the  $\Phi$ KZ  
173 nucleus that might explain the differences in permeability. Therefore, we used cryo-EM  
174 to visualize the nuclear structures in all three phages. *P. aeruginosa* was infected for 60  
175 minutes with  $\Phi$ PA3 or  $\Phi$ KZ, plunge frozen in liquid ethane, and processed for FIB  
176 milling and cryo-ET. The process was also performed on *P. chlororaphis* cells infected  
177 with 201 $\Phi$ 2-1. We found that the subcellular organization and phage nucleus structure  
178 of cells infected with  $\Phi$ KZ infected cells (Fig 3A, B) were identical to cells infected with  
179 201 $\Phi$ 2-1 (Fig 3C) and  $\Phi$ PA3 (Fig 3D). The protein shells of all three nuclei formed an

180 unstructured, largely continuous border with a thickness of approximately 5nm (Fig 3).  
181 Phage at various stages of maturation were observed, including capsids attached to the  
182 side of the nucleus that were either empty or filled with viral DNA, as well as phage tails,  
183 some of which were attached to capsids. Bacterial ribosomes were clearly excluded  
184 from the phage nucleus as in cells infected with  $\Phi$ PA3 and 201 $\Phi$ 2-1. These results  
185 confirm and extend our previous microscopy experiments (11, 12). Despite the  
186 differences in their ability to import GFPmut1, we could discern no obvious differences  
187 in the structure of the shell or replication and assembly pathway between these three  
188 phages.

189

190 **Fig 3: Cryo-EM Tomogram of a *Pseudomonas aeruginosa* cell infected with  $\Phi$ KZ.**

191

192 A. Slice through a tomogram of cryo-focused ion beam–thinned  $\Phi$ KZ phage-  
193 infected *P. aeruginosa* cell at 60 minutes post infection. Scale bar, 200nm.

194 B. Segmentation of the  $\Phi$ KZ tomogram shown in (C). The phage nucleus border is  
195 shown in darker blue. Bacterial ribosomes are yellow. Phage capsids are green  
196 and phage tails are cyan blue. The bacterial cell membrane is shown as red and  
197 pink.

198 C. Slice through a tomogram of cryo-focused ion beam–thinned 201 $\Phi$ 2-1 phage-  
199 infected *P. chlororaphis* cell at 60 minutes post infection. Scale bar, 200nm.

200 D. Slice through a tomogram of cryo-focused ion beam–thinned 201 $\Phi$ PA3 phage-  
201 infected *P. aeruginosa* cell at 60 minutes post infection. Scale bar, 200nm.

202

203 Knowing that GFPmut1 alone was transported into the  $\Phi$ KZ nucleus, we  
204 attempted to test the ability of this fluorescent protein to ferry other proteins into the  
205 compartment. As shown previously using cryo-EM and fluorescence microscopy, host  
206 bacterial ribosomes are excluded from the nucleus, including the ribosomal subunit L28  
207 tagged with mCherry (12) (Fig 4A). However, tagging the same ribosomal protein with  
208 GFPmut1 resulted in its localization inside the nucleus (Fig 4A). Cryo-EM indicated that  
209 the  $\Phi$ KZ tails localized in the cytoplasm (Fig 3A, B). When tagged with sfGFP the major  
210 tail protein gp146 formed puncta outside the nucleus; but strikingly, when fused to  
211 GFPmut1, gp146 localized inside the nucleus (Fig 4B). GFPmut1 fusion to proteins from  
212 other phages were also transported into the  $\Phi$ KZ nucleus. PA3PhuZ tagged with  
213 mCherry formed filaments in the cytoplasm of a cell infected with  $\Phi$ KZ. However, when  
214 fused to GFPmut1, PA3PhuZ localized inside the  $\Phi$ KZ phage nucleus (Fig 4C).

215

216 **Fig 4: GFPmut1 nuclear localization is dominant in hybrid fusion proteins.** Scale  
217 bar = 1 micron.

218

219 A. GFPmut1 fused to host 50s ribosomal subunit L28 localizes inside the phage  
220 nucleus while a fusion of the same protein to mCherry localizes in the cytoplasm.

221

222 B. GFPmut1 fused to  $\Phi$ KZ tail protein gp146 is mislocalized inside the phage nucleus  
223 while a fusion of the same protein to sfGFP shows it localizes as puncta in  
224 cytoplasm.

225

226 C. GFPmut1 fused to  $\Phi$ PA3 PhuZ protein is seen inside the phage nucleus while a  
227 fusion of the same protein to sfGFP forms filaments in the cytoplasm.

228

229 D. A fusion of mCherry-GFPmut1 shows both proteins are fluorescent inside the  
230 phage nucleus.

231

232 E. A fusion of GFPmut1-mCherry shows both proteins fluoresce inside the phage  
233 nucleus after infection. The two fusions in (D) and (E) indicate that GFPmut1 can  
234 be fused at both the N and C terminus and retain nuclear targeting. The ability of  
235 mCherry to fluoresce indicates that the protein is folded and functional.

236

237 F. Timelapse of mCh-GFPmut1 shows that both proteins are diffuse in the cytoplasm  
238 before infection but move into the nucleus as infection progresses. White arrows  
239 indicate two nuclei.

240

241

242 Although the results above suggested that GFPmut1 was able to target proteins  
243 to the nucleus, we could not be sure the entire fusion was imported. It remained  
244 possible that only GFPmut1 was transported after the target protein was cleaved off. To  
245 determine if a tagged protein was imported along with GFPmut1, we created fusions of  
246 mCherry at either the N or C terminal ends of GFPmut1 (mCherry-GFPmut1 and  
247 GFPmut1-mCherry). This also allowed us to determine if the localization of the target  
248 protein was affected by the position of the GFPmut1 fluorescent tag. Both fusions were

249 transported into the nucleus, indicating that fusions to either terminus resulted in nuclear  
250 targeting (Fig 4D, 4E). In time-lapse microscopy of cells expressing mCherry-GFPmut1,  
251 both green and red fluorescent signals were visible in the cytoplasm before infection.  
252 After infection, both green and red fluorescence moved into the nucleus over time,  
253 demonstrating that both proteins are transported into the nucleus where they both  
254 remain folded (Fig 4F, white arrows). Altogether, our results demonstrate that GFPmut1  
255 can be used to target a fully folded, functional protein to the phage nucleus.

256 We next attempted to use GFPmut1 to import proteins that might be useful for  
257 gene editing. Previous attempts to circumvent the phage nucleus barrier and edit  
258 phage genomes relied upon fusing the nuclear targeted RecA-like protein KZgp152 to  
259 CRISPR-cas enzymes of interest (26). The RecA-like protein fusion successfully  
260 imported a restriction enzyme but failed to import Cas9 (26). Therefore, we tested the  
261 ability of GFPmut1 to import three proteins from different CRISPR-cas systems: Cas3,  
262 Cas9, and Cas13 (27-31). When fused to sfGFP, all three localized in the cytoplasm,  
263 indicating that all three of these proteins are normally excluded from the nucleus (Fig  
264 5A, C, E). In contrast, tagging them with GFPmut1 targeted them into the nucleus (Fig  
265 5B, D, F). These results support our previous hypothesis that the phage nucleus  
266 provides a physical barrier that protects phage DNA from endogenous host cell  
267 nucleases. In addition, we also examined the host protein SbcB, a single-stranded DNA  
268 nuclease that likely functions in host DNA recombination and repair (32, 33). Like the  
269 CRISPR-cas proteins, the SbcB-sfGFP fusion localized in the cytoplasm and was  
270 excluded from the phage nucleus (Fig 5G). When SbcB was fused to GFPmut1,  
271 fluorescence was observed inside the phage nucleus (Fig 5H). Cells expressing sbcB-

272 GFPmut1 also showed misshapen phage nucleoids compared to the smoother shape of  
273 the DNA inside infected cells expressing sbcB-sfGFP (Fig 5H). Quantitation of the  
274 DAPI intensity of the infection nucleoid in both strains showed that infected SbcB-  
275 GFPmut1 cells had a 20% lower average DAPI intensity (approximately 6000 counts,  
276 n=187) compared to SbcB-sfGFP expressing cells (average of 7500 counts, n=133),  
277 suggesting that internalization of SbcB-GFPmut1 slightly reduces DNA replication or  
278 enhances its degradation (Supplemental Fig 2C). When comparing infection of cells  
279 expressing SbcB-GFPmut1 to the SbcB-sfGFP counterpart,  $\Phi$ KZ replication was  
280 reduced approximately 10-fold (Supplemental Fig 2A, 2B). Thus, we have shown that  
281 GFPmut1 can be used as a nuclear localization tool during  $\Phi$ KZ infection.

282 **Fig 5: GFPmut1 can be used to artificially import proteins into the  $\Phi$ KZ nucleus,  
283 even those that are detrimental to phage reproduction. Scale bar = 1 micron**

284

285 A. Cas13 fused to sfGFP localizes outside the phage nucleus.  
286 B. Cas13 fused to GFPmut1 localizes inside the phage nucleus.  
287 C. Cas3 fused to sfGFP localizes outside the phage nucleus  
288 D. Cas3 fused to GFPmut1 localizes inside the phage nucleus.  
289 E. Cas12 fused to sfGFP localizes outside the phage nucleus.  
290 F. Cas12 fused to GFPmut1 localizes inside the phage nucleus  
291 G. SbcB-sfGFP localizes outside the phage nucleus  
292 H. SbcB-GFPmut1 localizes inside the phage nucleus with the phage DNA.

293

294

295 **Discussion**

296 Our major finding is that the fluorescent protein GFPmut1, and fusions to it, are  
297 transported into the  $\Phi$ KZ phage nucleus. However, this phenotype is unique to  $\Phi$ KZ, as  
298 GFPmut1 is excluded from the nucleus of the two related phages  $\Phi$ PA3 and 201 $\Phi$ 2-1.  
299 We found these results surprising given the high degree of similarity between these  
300 three related jumbo *Pseudomonas* phages (9, 10, 34) and since the cryoEM tomogram  
301 of  $\Phi$ KZ infected cells show a nucleus that is indistinguishable from that of its related  
302 phages. The GFPmut1 localization data suggests functional divergence in the selective  
303 abilities of these three phage nuclear transport systems.

304 It remains unclear why GFPmut1 is able to enter the  $\Phi$ KZ nucleus. Remarkably,  
305 a single amino acid change (F99S) completely switches GFPmut1 localization from  
306 100% nuclear to 100% cytoplasmic. One hypothesis is that a protein surface motif is  
307 required for recognition by a yet to be identified transport system. The positions of the  
308 mutations which have an effect on localization (F99S, M153T) occur on the outer  
309 surface of GFPmut1 and imply recognition of the folded structure. The fluorescence of  
310 both GFPmut1 and mCherry prior to import supports this idea as well. These results  
311 suggest the existence of transport machinery that specifically engages proteins destined  
312 for the nucleus. In this model, the surface of GFPmut1 is fortuitously recognized as a  
313 substrate and imported by the machinery and the F99S abolishes this interaction.

314 The unexpected finding of GFPmut1 nuclear targeting raised the possibility that  
315 we might be able to use this protein as a convenient way to both label and target  
316 proteins to the nucleus of  $\Phi$ KZ. Understanding which fluorescent proteins are localized  
317 outside the  $\Phi$ KZ nucleus versus which ones are imported is critical for studies of protein

318 localization and will allow us to develop valuable tools for future studies. These results  
319 suggest that we can use three different colors of fluorescent proteins (blue, CFP; red,  
320 mCherry; or green, sfGFP, GFP<sup>+</sup>, and GFPmut1) to localize proteins during  $\Phi$ KZ  
321 infection, and that we can use GFPmut1 as a tool to specifically target proteins into the  
322 nucleus.

323 Using GFPmut1 to manipulate the  $\Phi$ KZ nucleus gives us the ability to target and  
324 possibly edit phage DNA. Previous attempts to modify the DNA of these large phages  
325 have failed (26), most likely because of the physical barrier afforded by the nuclear  
326 shell. Our data support our previous hypothesis that a major function of the phage  
327 nucleus is protect phage DNA against host defenses, such as CRISPR-cas and  
328 restriction enzymes, (12, 26). We now show that GFPmut1 can be used to efficiently  
329 circumvent the phage nucleus barrier and target gene editing enzymes into the nucleus,  
330 opening up the possibility of genetically manipulating these large phages. Further  
331 studies of this targeting phenomenon will also provide insight into the methods utilized  
332 by phage  $\Phi$ KZ for protein sorting. Though the mechanisms used by  $\Phi$ KZ may differ  
333 from the other two phages, determining the specific differences will shed light on the  
334 transport systems of the phage nucleus as well as the relationships between these  
335 phages. Once we understand the molecular basis of selectivity, we may be able to  
336 manipulate it to target proteins to the nuclei in the other phages as well.

337

338 **Materials and Methods**

339

340 Strain, growth condition, and bacteriophage preparation

341 *Pseudomonas chlororaphis* strain 200-B was grown on Hard Agar (HA) containing 10 g  
342 Bacto-Tryptone, 5 g NaCl, and 10 g agar in 1L ddH<sub>2</sub>O and incubated at 30°C overnight  
343 (35). *Pseudomonas aeruginosa* strains PA01 and PA01-K2733 (pump-knockout strain)  
344 were grown on Luria-Bertani (LB) media containing 10g Bacto-Tryptone, 5g NaCl, 5g  
345 Bacto-yeast extract in 1L ddH<sub>2</sub>O and incubated at 37°C overnight. Lysates for phages  
346 201Φ2-1, ΦPA3, and ΦKZ were made by infecting 5mL of host cultures in early log  
347 stage (OD600 = 0.2-0.3) with 500μl of high titer lysate and rolling overnight at 30°C.  
348 The phage lysates were then clarified by centrifugation at 15,000 rpm for 10 minutes  
349 and syringe filtered through a 0.45 micron filter before storage at 4 °C.

350

351 Plasmid constructions and bacterial transformation

352 Fluorescent-tagged phage proteins were constructed with the pHRED30T vector as a  
353 backbone (36). Phage genes were PCR amplified from phage lysates then ligated into  
354 the pHRED30T backbone via isothermal assembly. The assemblies were  
355 electroporated into DH5 $\alpha$  *E. coli* and plated on LB supplemented with gentamycin  
356 sulfate (15μg/mL). Constructs were confirmed with sequencing and subsequently  
357 electroporated into either *P. chlororaphis* strain 200-B, *P. aeruginosa* strains PA01,  
358 and/or PA01-K2733. *P. chlororaphis* strain was grown on HA supplemented with  
359 gentamycin sulfate (25μg/mL) and *P. aeruginosa* strains PA01 and PA01-K2733 were

360 grown on LB supplemented with gentamycin sulfate at 300  $\mu$ g/mL or 15 $\mu$ g/mL,  
361 respectively. See Supplemental Table 1 for a list of plasmids and strains.

362

363 Phage titers

364 Bacterial cultures were grown in LB Gent 15 liquid broth to late log. 0.5mL culture was  
365 then mixed with 4.5mL 0.35% LB top agar and 25uL 20% arabinose (for 0.1% induction)  
366 and the mixture poured onto a LB Gent 15 plate. After the top agar lawn had solidified,  
367 5uL of 10x serial dilutions were spotted onto the lawn and the plate was incubated at  
368 30deg overnight.

369

370 Fluorescent Microscopy

371 The bacterial cells were grown on 1% agarose pads in glass well slides, containing 25%  
372 LB, 1ug/mL FM4-64, 1ug/mL DAPI, and 0.1-0.5% arabinose to induce protein  
373 expression at desired levels. These pad slides were incubated at 30°C for 3 hours in a  
374 humid chamber. For infection beginning at timepoint 0, 5-10  $\mu$ l of high-titer lysate ( $10^{10}$   
375 pfu/ml) was added to pads then incubated again at 30°C. At desired time points after  
376 phage infection, a coverslip was put on the slide and fluorescent microscopy performed.

377

378 Cells were visualized on an Applied Precision DV Elite optical sectioning microscope  
379 with a Photometrics CoolSNAP-HQ2 camera (Applied Precision/GE Healthcare) was  
380 used to visualize the cells. For static images, the cells were imaged for at least 8 stacks  
381 from the focal plane with 0.15  $\mu$ m increments in the Z-axis and, for time- lapse imaging,  
382 the cells were imaged from a single stack at the focal plane for desired length of time

383 with selected intervals with Ultimate Focusing mode. Microscopic images were  
384 deconvolved  
385 using SoftWoRx v5.5.1. Image analysis and processing were performed in Fiji.  
386

387 **Tomography Sample Preparation and Data Acquisition**

388 Infection of *P. chlororaphis* with 201Φ2-1 and *P. aeruginosa* cells with phages ΦKZ and  
389 ΦPA3 was done as indicated above. At 70 minutes post infection, cells were scraped off  
390 from the surface of the pad using ¼ LB media. 7 µl of cells were deposited on holey  
391 carbon coated QUANTIFOIL® R 2/1 copper grids that were glow discharged using  
392 Pelco easiGlow™ glow discharge cleaning system. Manual blotting from the side of the  
393 grid opposite to the cells using Whatman No. 1 filter paper removed excess liquid such  
394 that cells form a monolayer on the surface of the grid. Cells were then plunge-frozen in  
395 a mixture of ethane/propane using a custom-built vitrification device (Max Planck  
396 Institute for Biochemistry, Munich). Grids were then mounted into modified FEI  
397 Autogrids™ to avoid any mechanical damage to the delicate grids during subsequent  
398 transfer steps. Then, these clipped grids were transferred into Scios (Thermo Fisher  
399 Scientific, formerly FEI), a dual-beam (cryo-FIB/SEM) microscope equipped with a  
400 cryogenic stage. Thin sections of 100-250 nm, or lamellae, were prepared as previously  
401 described in Chaikeeratisak et al., 2017 containing 10-12 cells each. Tilt-series were  
402 collected from typically -65° to +65° with 1.5° or 2° tilt increments using SerialEM<sup>4</sup> in a  
403 300-keV Tecnai G2 Polara microscope (FEI) equipped with post-column Quantum  
404 Energy Filter (Gatan) and a K2 Summit 4k x 4k direct detector camera (Gatan). Images  
405 were recorded at a nominal magnification of 34,000 with a pixel size of 0.61 nm. The

406 dose rate was set to 10-12 e/physical pixel at the camera level. Frame exposure was  
407 set to 0.1 seconds, with a total exposure in a frame set to be determined by an  
408 algorithm targeting an average count number. The total dose in a tomogram was  
409 typically  $\sim$ 100-120 e/ $\text{A}^2$  with a defocus of -5  $\mu\text{m}$ . The dataset for this study consists of  
410 16 tomograms from 7 FIB-milled lamellas. Reconstruction of tilt-series was done in  
411 IMOD (37) using patch tracking method. Semi-automatic segmentation of the  
412 membranes was done using TomoSegMemTV (38) an open-source software based on  
413 tensor voting, followed by manual segmentation with Amira software (FEI Visualization  
414 Sciences Group).

415

416 Point mutation graph

417 PA01 cells infected by  $\Phi$ KZ were imaged 60 to 70 minutes post infection with DAPI  
418 staining. Infected cells were identified by the presence of a bright, circular DAPI stain in  
419 the center of the bacterial cells corresponding to the presence of phage DNA within the  
420 phage nucleus. ImageJ ([imagej.nih.gov/ij](http://imagej.nih.gov/ij)) was used to bisect infected cells and obtain  
421 GFP intensity profiles along their lengths. Each of these intensity profiles were  
422 normalized by the length of the cell and normalized again to the GFP intensity at the  
423 initial measured end of the cell. Intensity profiles were plotted per cell as well as  
424 averaged.

425

426 PDB structure of GFPmut1

427 The amino acid structure for GFPmut1 was used with the Phyre2 Protein Fold  
428 Recognition Server ([www.sbg.bio.ic.ac.uk/phyre2](http://www.sbg.bio.ic.ac.uk/phyre2)) to obtain an estimated structure for

429 GFPmut1 from pSG1729. This sequence differs from EGFP structure 2Y0G by  
430 substitutions V1M, L195S and L232H. The resulting structure was viewed with  
431 ChimeraX ([www.rbvi.ucsf.edu/chimerax](http://www.rbvi.ucsf.edu/chimerax)). Alignment of fluorescent proteins was made  
432 using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>)

433

434 **DAPI quantification**

435 Images of individual infected cells were cropped using ImageJ. A mask of the phage  
436 nucleus was generated using Otsu's method in Matlab 2017b and the mean DAPI  
437 fluorescence was calculated from the raw image intensity within the region of the mask.  
438 The complementary image to the mask was used to estimate background fluorescence.

439

440 **Growth Curves**

441 Bacterial cultures were grown to late log and then diluted to OD600 0.1. The diluted  
442 cultures were induced to 0.1% arabinose and 100uL was aliquoted into each well of 96  
443 well plates. 10uL of phage dilutions were added to appropriate wells. The plate was  
444 incubated in the Tecan Infinite M200, shaking, at 30degrees Celsius and OD600 was  
445 measured every 10 minutes for 360 cycles (6 hours).

446

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451 Baker.

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546

547

548 **Supplemental Fig 1: A chart showing the amino acid modifications of GFP  
549 variants over time.**

550

551 **Supplemental Fig 2: sbcb-GFPmut1 shows a small reduction in phage  
552 reproduction.**

553

554 A.  $\Phi$ KZ phage titer on a lawn of *Pseudomonas aeruginosa* expressing *sbcB-GFPmut1*. Titer, calculated at  $2 \times 10^{11}$  pfu/mL is reduced approximately 10-fold  
555 compared to (B).

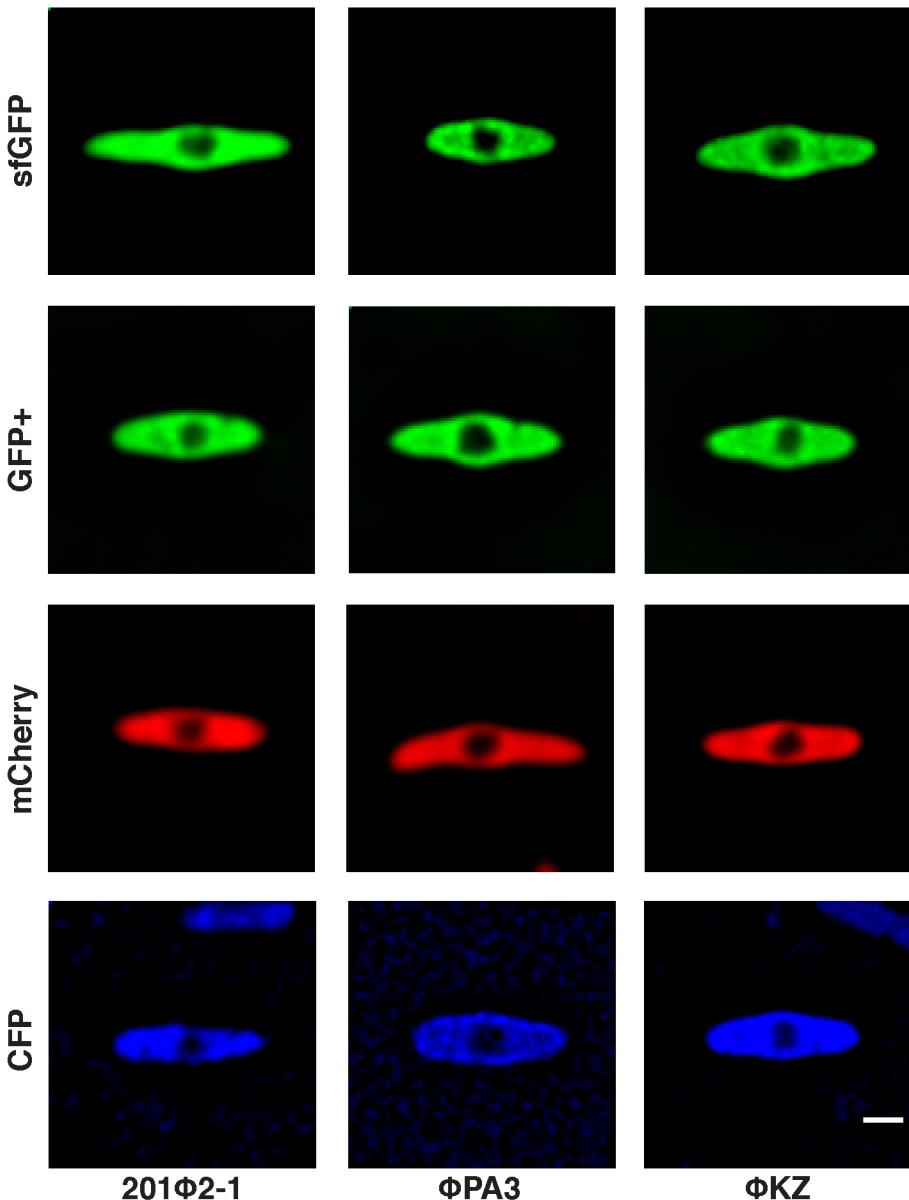
557

558 B.  $\Phi$ KZ phage titer on a lawn of *Pseudomonas aeruginosa* expressing *sbcB-sfGFP*.  
559 Titer is calculated as approximately  $2 \times 10^{12}$  pfu/mL.

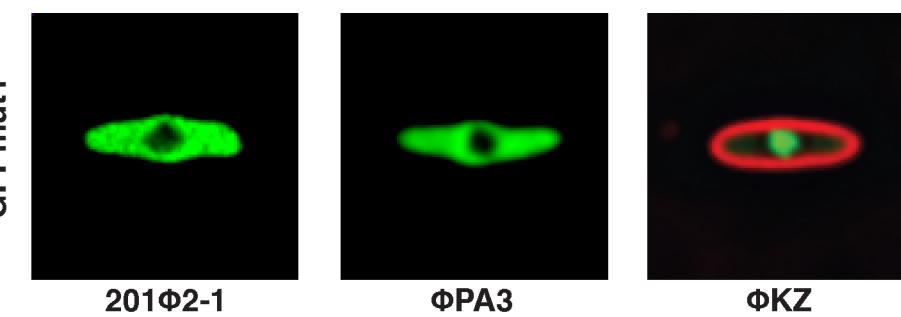
560

561 C. A histogram of DAPI (DNA stain) intensity indicates that cells expressing *sbcB*-  
562 *mut1* (blue columns, n=187) have lower intensity, compared to cells expressing  
563 *sbcB-sfGFP* (n=133). This suggests that DNA concentration is reduced by the  
564 presence of the host nuclease inside the phage nucleus.

A

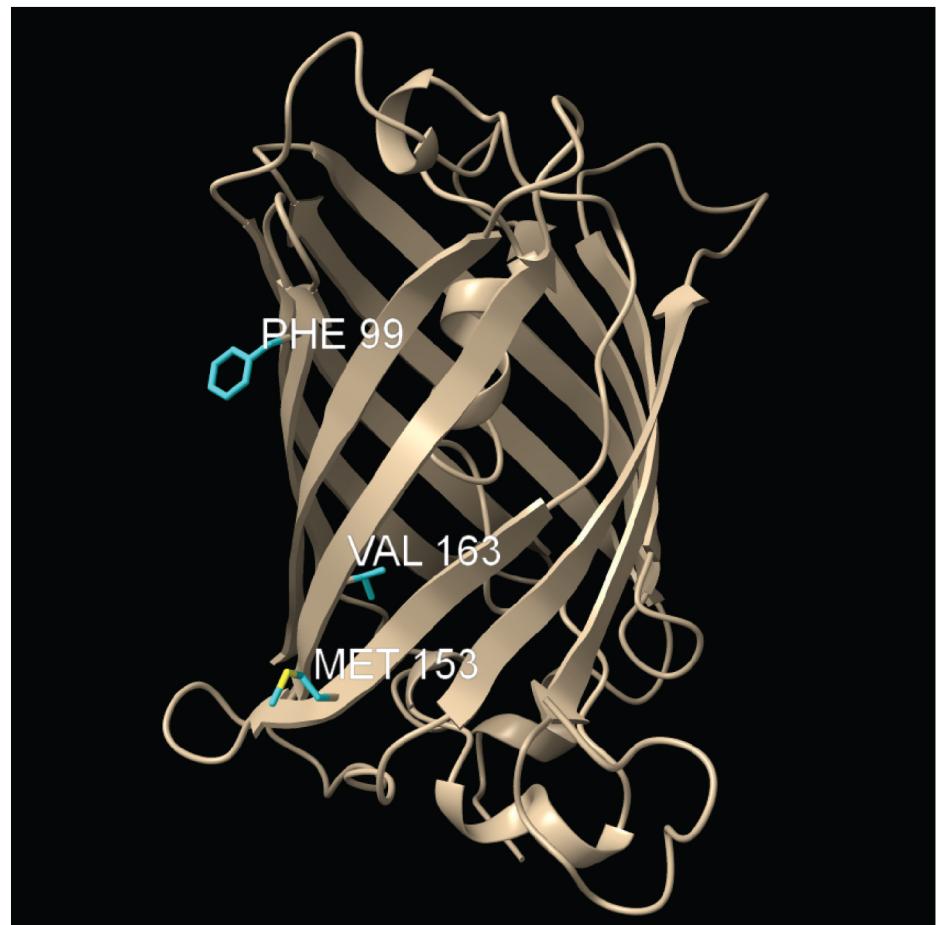


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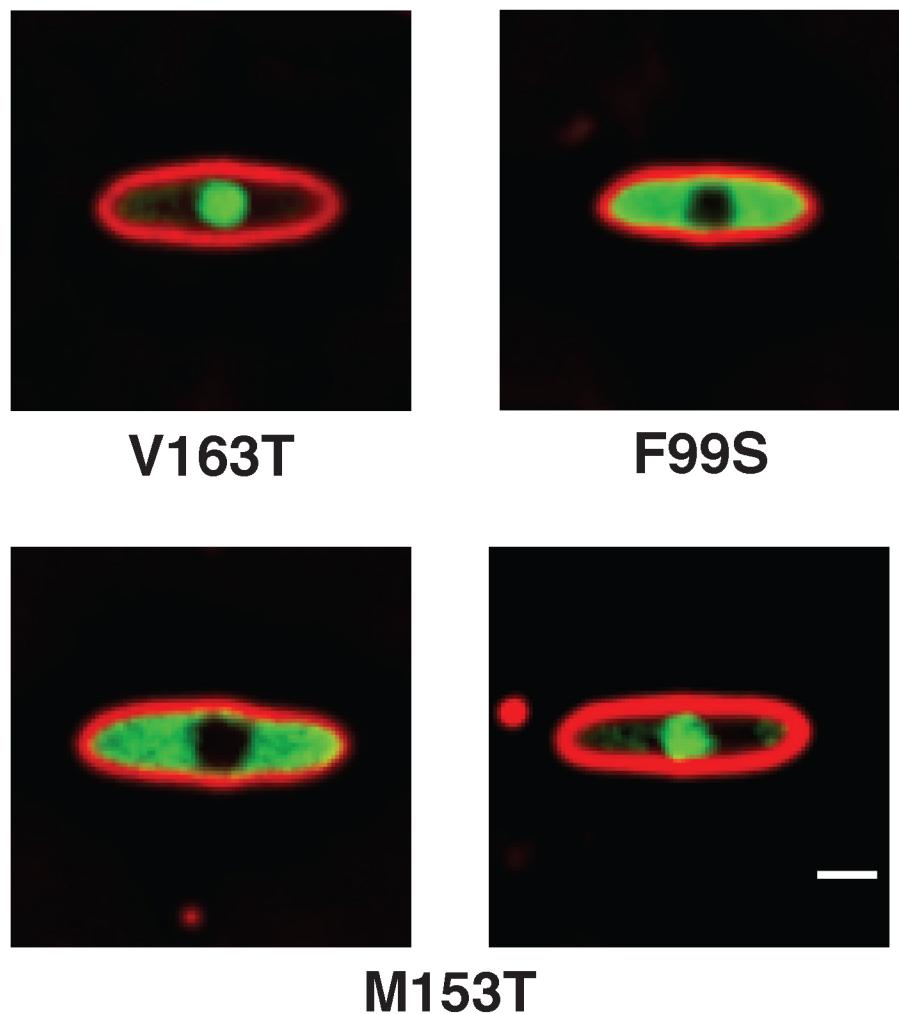


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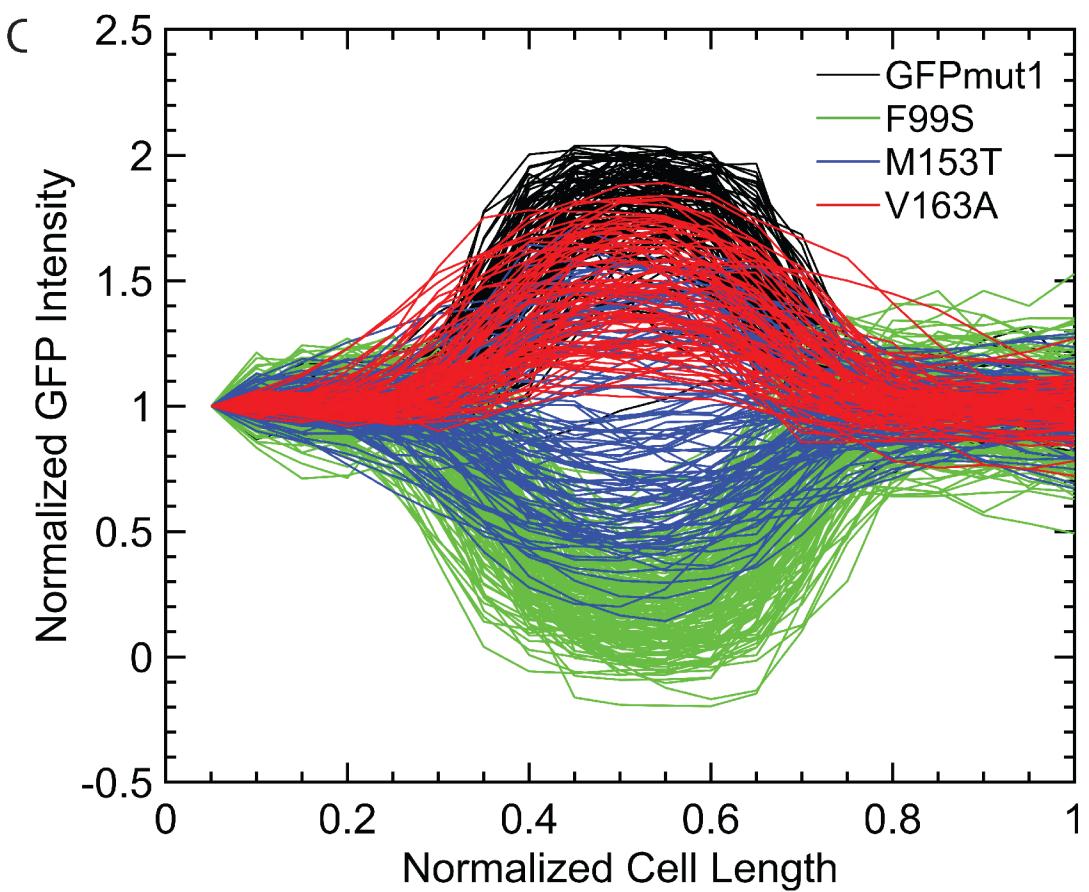
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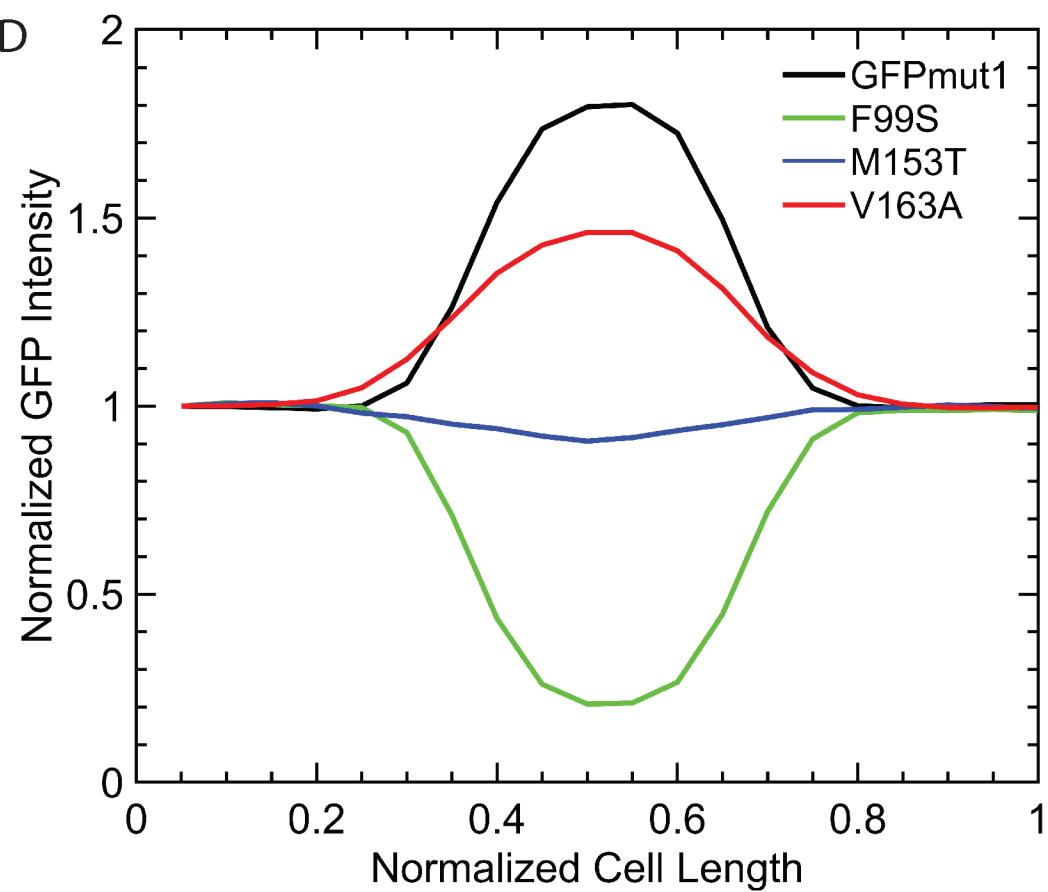
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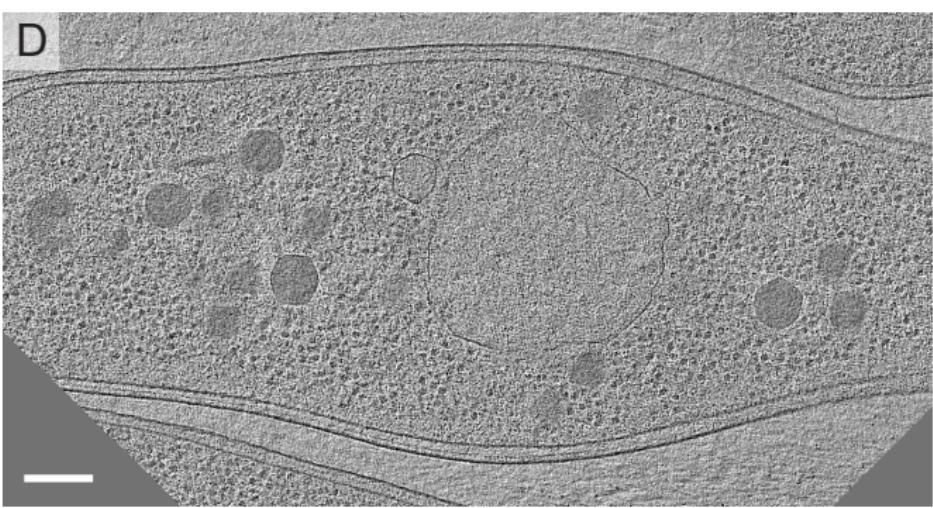
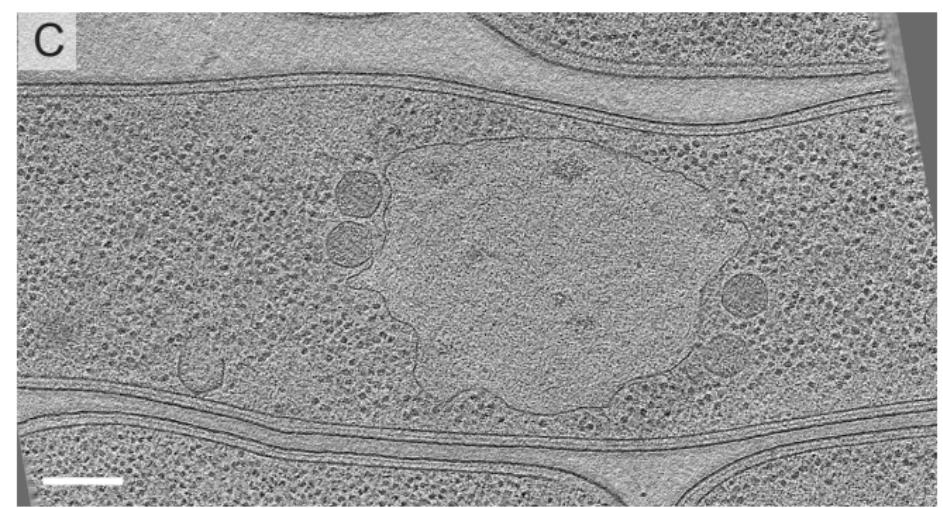
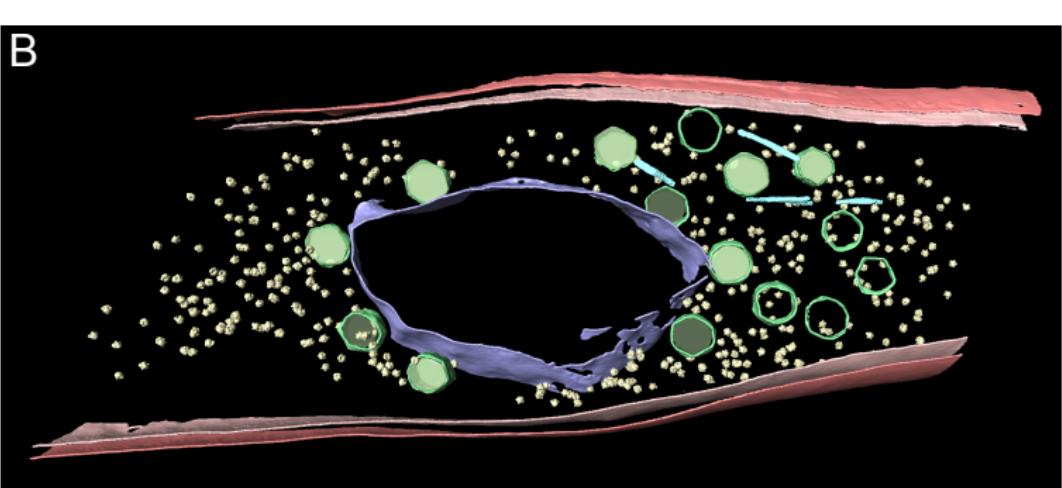
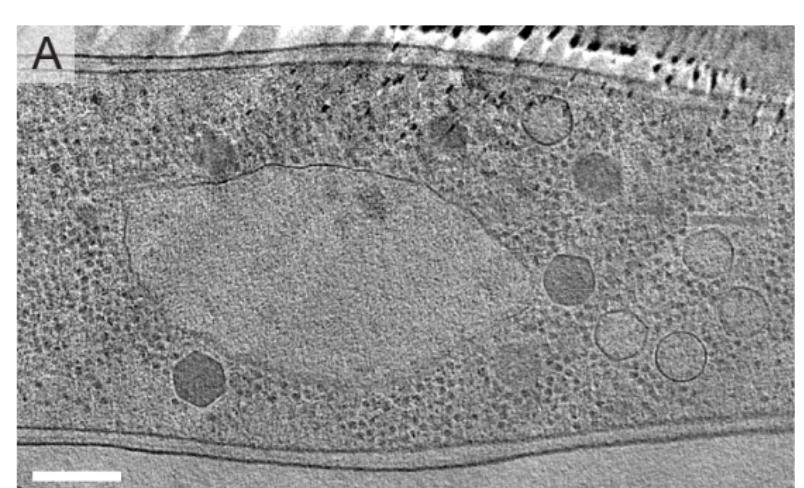


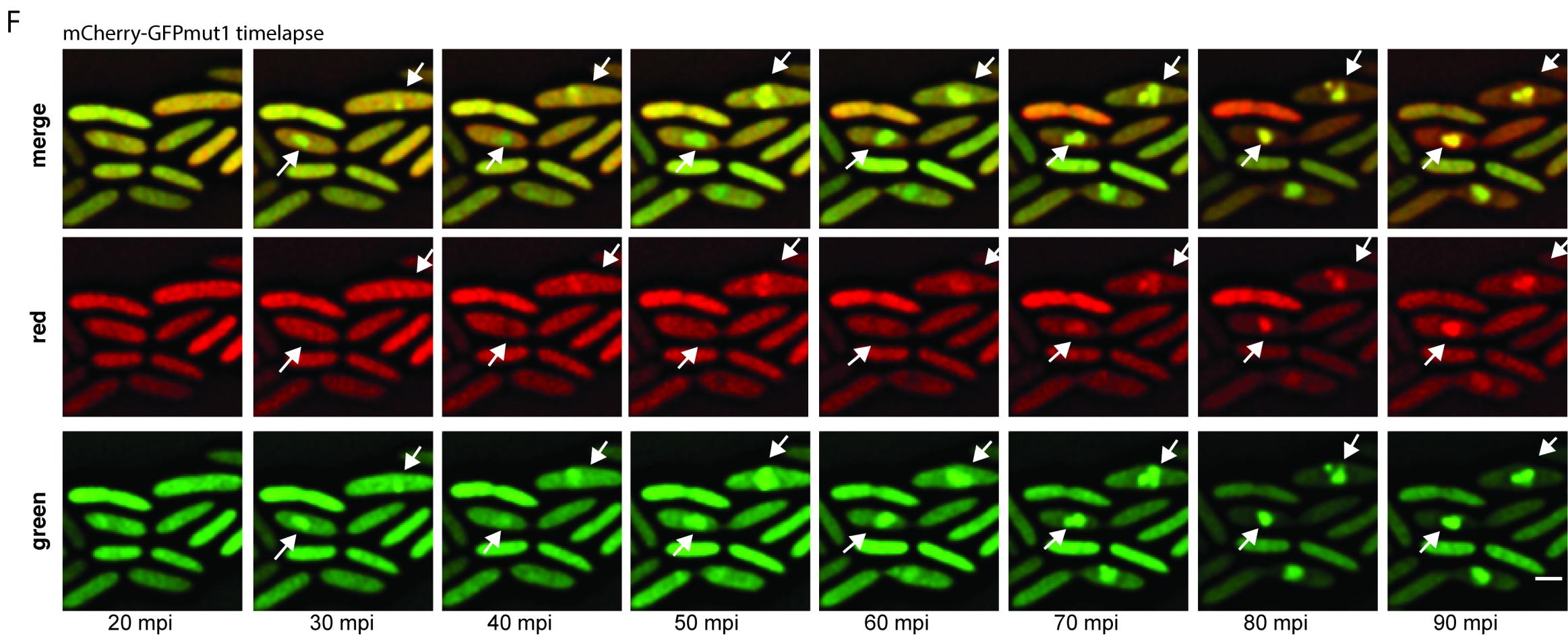
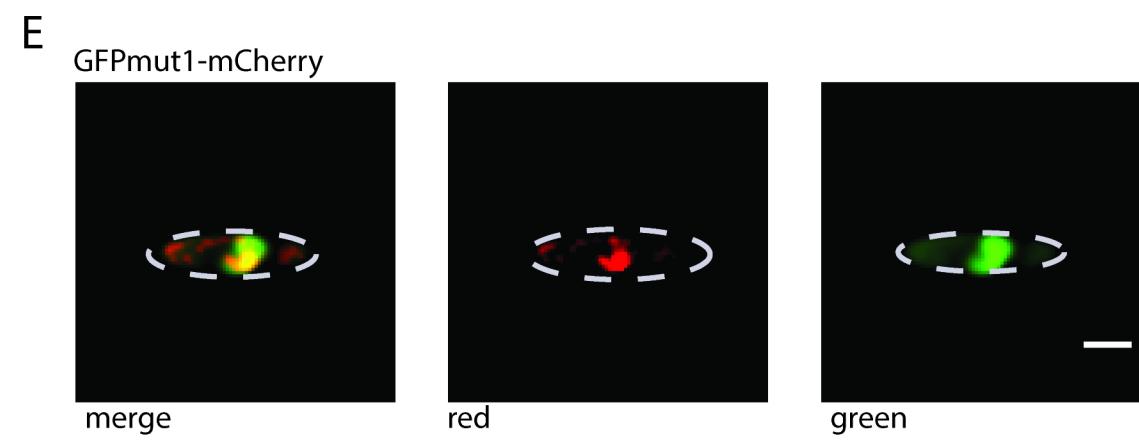
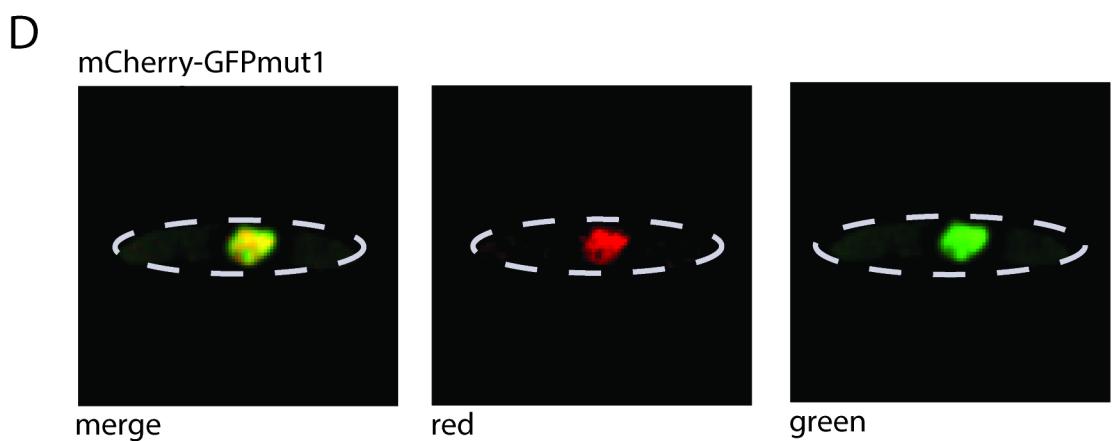
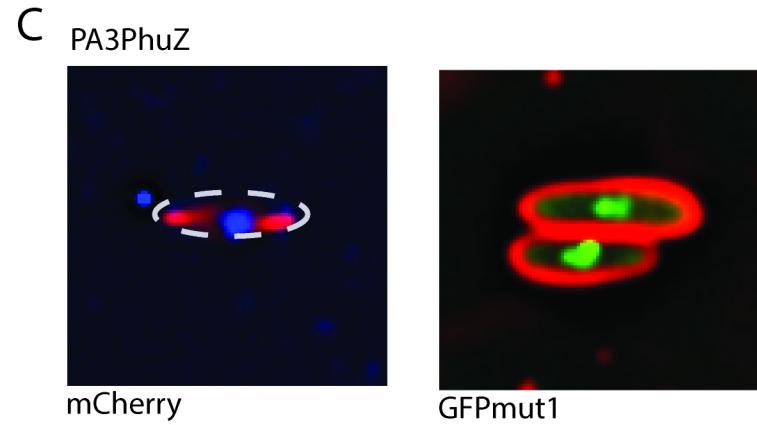
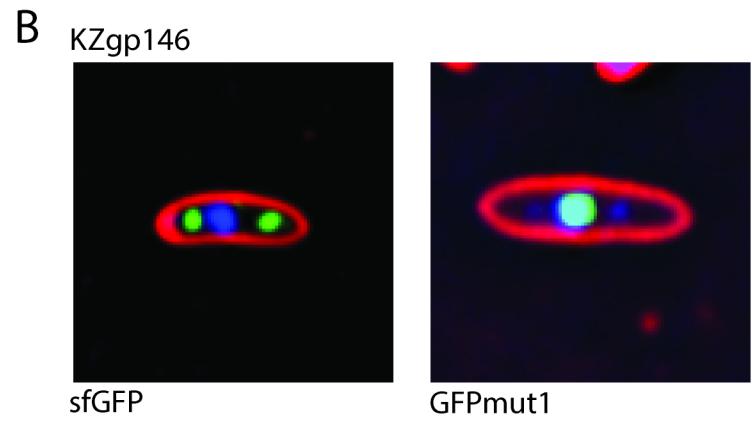
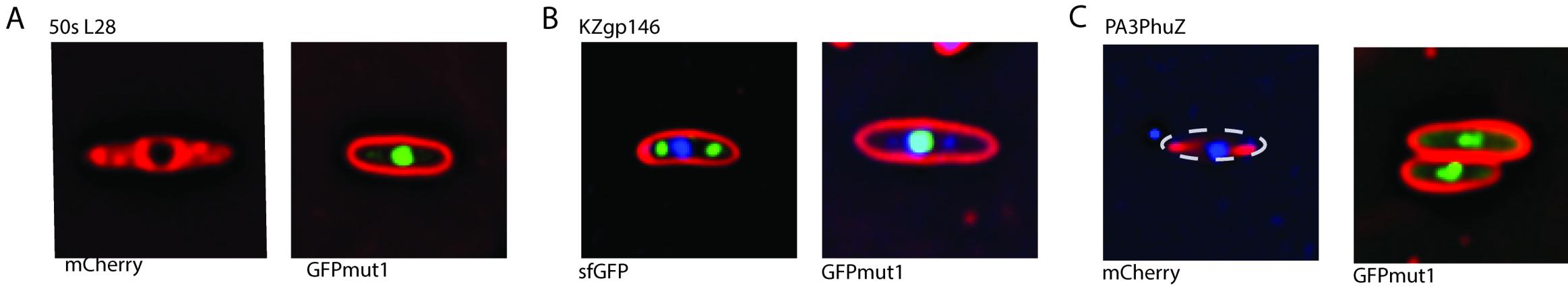
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D

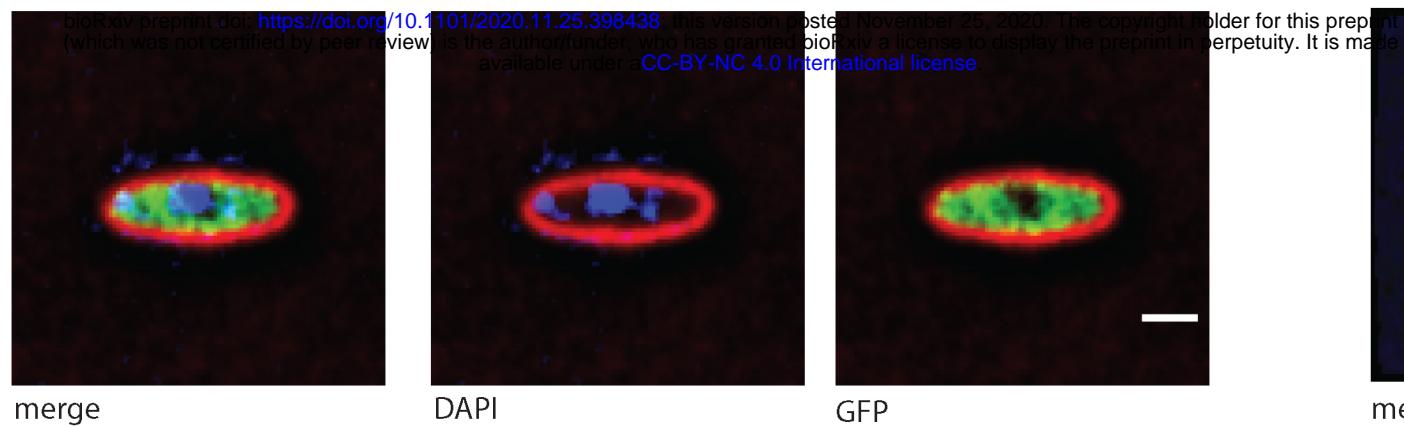






**A**

cas13-sfGFP



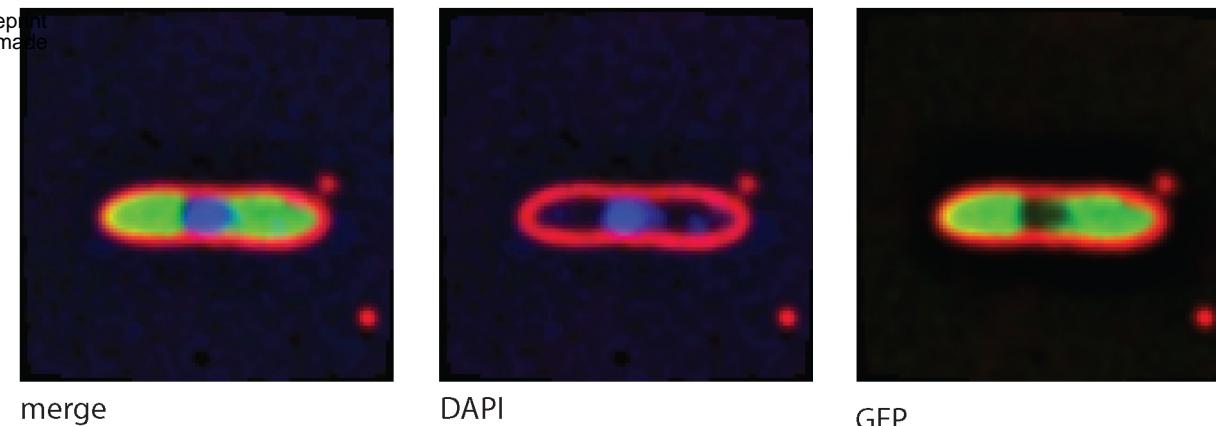
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DAPI

GFP

**E**

cas9-sfGFP



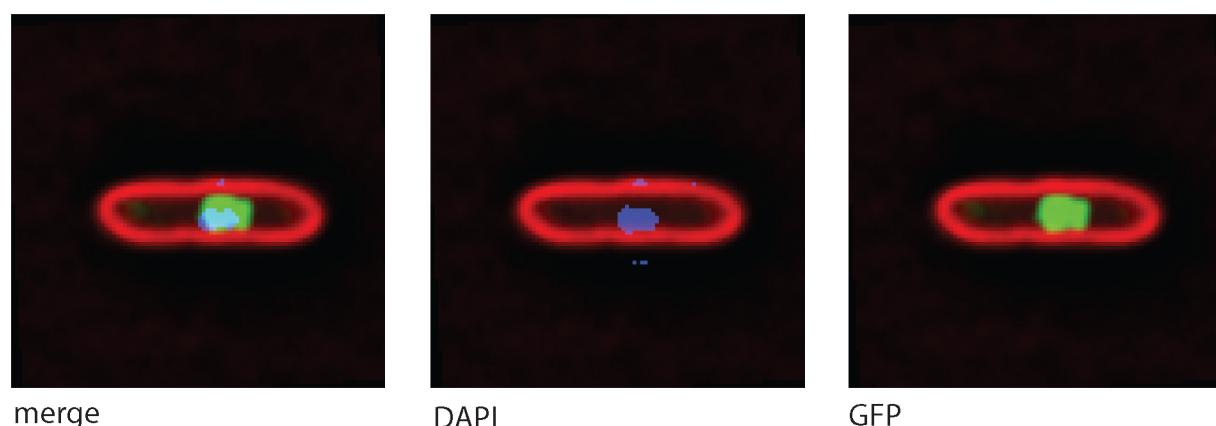
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DAPI

GFP

**B**

cas13-GFPmut1



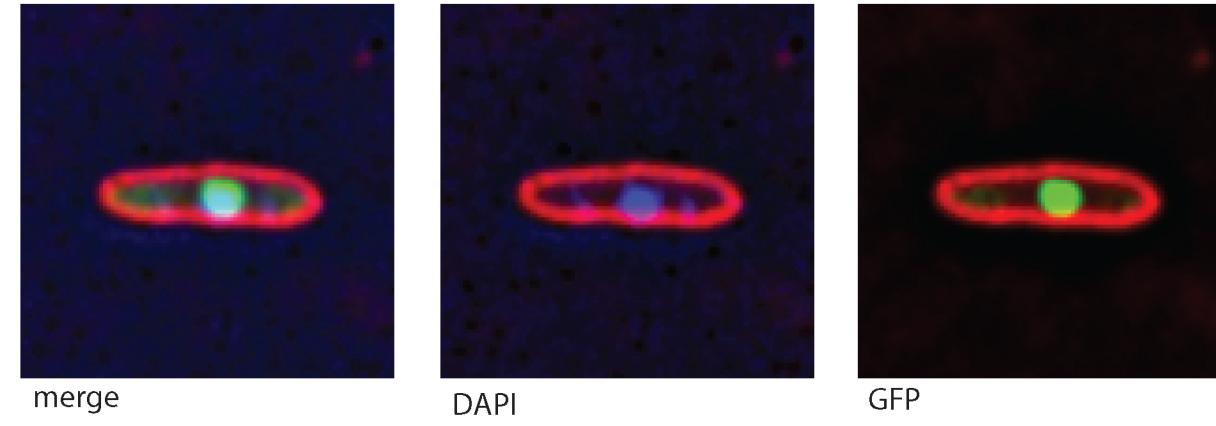
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DAPI

GFP

**F**

cas9-GFPmut1



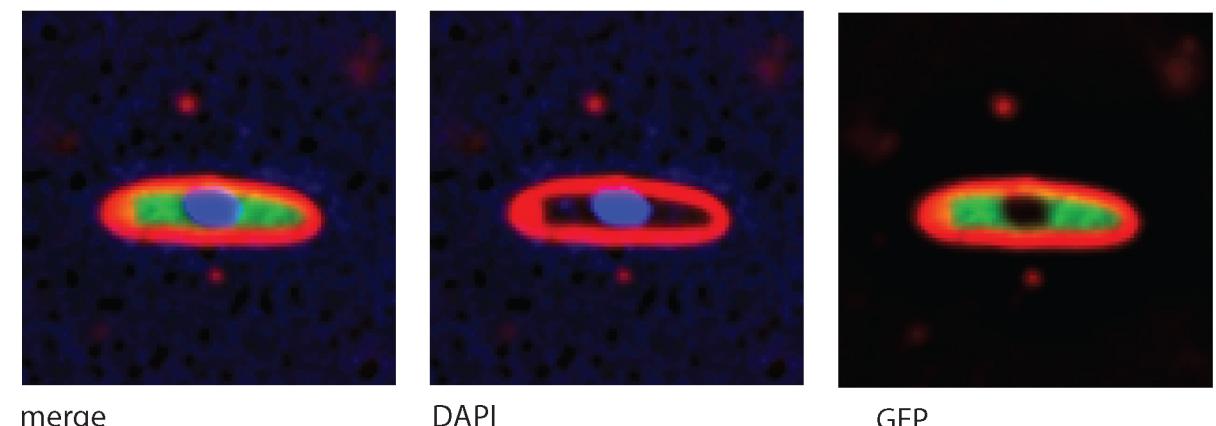
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DAPI

GFP

**C**

cas3-sfGFP



merge

DAPI

GFP

**G**

sbcB-sfGFP



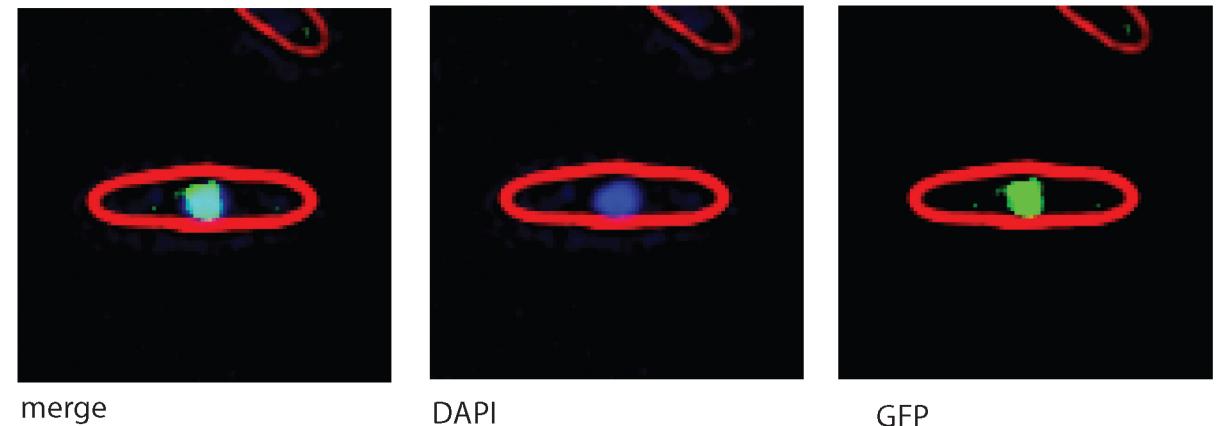
merge

DAPI

GFP

**D**

cas3-GFPmut1



merge

DAPI

GFP

**H**

sbcB-GFPmut1



merge

DAPI

GFP