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TORC2-Gad8 dependent myosin phosphorylation
modulates regulation by calcium.

Karen Baker¹, Irene A. Gyamfi¹, Gregory I. Mashanov², Justin E. Molloy²,
Michael A. Geeves¹ and Daniel P. Mulvihill^{1,3}

¹ School of Biosciences, University of Kent, Canterbury, Kent, CT2 7NJ, UK.

² The Francis Crick Institute, 1 Midland Road, London NW1 1AT, UK

³ Author for correspondence e-mail: d.p.mulvihill@kent.ac.uk
Tel: +44 (0) 1227 827239

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Running Title: Phosphorylation & calcium co-dependent myosin regulation

30 **Abstract**

31 Cells respond to changes in their environment through signalling networks
32 that modulate cytoskeleton and membrane organisation to coordinate cell
33 cycle progression, polarised cell growth and multicellular development. Here,
34 we define a novel regulatory mechanism by which the motor activity and
35 function of the fission yeast type 1 myosin, Myo1, is modulated by TORC2
36 signalling dependent phosphorylation. Phosphorylation of the conserved
37 serine at position 742 within the neck region changes both the conformation of
38 the neck region and the interactions between Myo1 and its associating
39 calmodulin light chains. S742 phosphorylation thereby couples calcium and
40 TOR signalling networks in the modulation of myosin-1 dynamics to co-
41 ordinate actin polymerisation and membrane reorganisation at sites of
42 endocytosis and polarised cell growth in response to environmental and cell
43 cycle cues.

44

45 **Introduction**

46 The actin cytoskeleton underpins cellular organisation by maintaining cell
47 shape through the transmission of mechanical signals between the cell
48 periphery and nucleus, to influence protein expression, organisation and
49 cellular architecture in response to needs of the cell. Myosins, actin-
50 associated motor-proteins, work in collaboration with an array of actin-binding
51 proteins to facilitate global cytoskeletal reorganisation and a plethora of other
52 processes including cell migration, intracellular transport, tension sensing and
53 cell division (O'Connell *et al*, 2007). Each of the many classes of myosin
54 contain three distinct domains: an actin-binding ATPase motor domain, that
55 exerts force against actin, a lever arm or neck region that contains light chain
56 binding IQ motifs, and a tail region which specifies cargo binding and other
57 molecular interactions.

58 Although the different classes of myosin perform very different cellular
59 functions, they all operate via the same basic mechanism: the motor domain
60 undergoes cyclical interactions with actin coupled to the breakdown of ATP.
61 Each molecule of ATP that is converted to ADP and inorganic phosphate can
62 generate movement along actin of between 5-25 nm and force of up to 5 pN.
63 Regulation of acto-myosin motility is multi-faceted (Heissler & Sellers, 2016a),
64 combining regulatory pathways operating via the actin track (historically called
65 thin-filament regulation), or myosin-linked regulation (historically called thick-
66 filament regulation). This latter control is often mediated via phosphorylation
67 of the heavy chain or light chain(s), or by calcium-regulation of light chain
68 binding (Heissler & Sellers, 2016b). Phosphorylation at the conserved "TEDS"
69 site motif within the myosin motor domain of class 1 myosin affects acto-
70 myosin interaction (Bement & Mooseker, 1995), while phosphorylation within
71 the tail region of class 5 myosin controls cargo binding (Rogers *et al*, 1999).
72 In contrast, phosphorylation of class 2 myosin light chains and/or heavy chain
73 can change the folded state of the heavy chain to alter actin interaction and
74 the ability to form filaments (Redowicz, 2001; Kendrick-Jones *et al*, 1987;
75 Pasapera *et al*, 2015). Thus, phosphoregulation of myosin can occur in the
76 head, neck and tail regions, as well as the light chains, and its impact vary
77 across myosin classes and between paralogues within the same class. The

78 impact of phosphorylation upon the motile function of most myosins remains
79 to be established.

80

81 The genome of the fission yeast, *Schizosaccharomyces pombe*, encodes five
82 myosin heavy chains from classes 1, 2, and 5 (Win *et al*, 2002). The single
83 class 1 myosin (UniProt Accession: Q9Y7Z8), here termed Myo1, is a 135
84 kDa protein, with motor domain, neck region (with two canonical IQ motifs)
85 and a 49 kDa tail region containing a, so-called, myosin tail-homology-2
86 domain (MYTH-2), a membrane-binding pleckstrin homology (PH) domain, an
87 SH3 domain and a carboxyl-terminal acidic region. The acidic region
88 associates with, and activates, the Arp2/3 complex to nucleate actin
89 polymerisation (Lee *et al*, 2000). The myosin motor has a conserved TEDS
90 site, which is phosphorylated to modulate the protein's ability to associate with
91 actin (Attanapola *et al*, 2009). Myo1 associates with membranes, primarily at
92 sites of cell growth, where it is required for endocytosis, actin organisation and
93 spore formation (Sirotkin *et al*, 2005; Lee *et al*, 2000; Itadani *et al*, 2007).

94

95 Calmodulin or calmodulin-like light chains associate with the IQ motifs within
96 the myosin neck to regulate both the length and stiffness of the lever arm
97 (Trybus *et al*, 2007) and behaviour of the motor domain (Adamek *et al*, 2008).
98 Calmodulins are ubiquitous calcium binding proteins that associate with and
99 regulate the cellular function of diverse proteins. Calcium associates with up
100 to four EF hand motifs within the calmodulin molecule to instigate a
101 conformational change that modulates its affinity for IQ motifs (Crivici & Ikura,
102 1995).

103

104 *S. pombe* encodes for two calmodulin like proteins, Cam1 and Cam2 (Takeda
105 & Yamamoto, 1987; Itadani *et al*, 2007). Cam1 is a typical calmodulin that
106 associates with IQ domain containing proteins in a calcium dependent
107 manner, to modulate functions as diverse as endocytosis, spore formation,
108 cell division and spindle pole body integrity (Takeda & Yamamoto, 1987;
109 Moser *et al*, 1995; 1997; Itadani *et al*, 2010). Although Cam2 shares Cam1's
110 ability to regulate Myo1, Cam2 differs from Cam1 in two important respects:

111 Cam2 is not essential for viability and is predicted to be insensitive to calcium
112 (Sammons *et al*, 2011; Itadani *et al*, 2007). Furthermore, while cells lacking
113 Cam2 show defects in spore formation following sexual differentiation they
114 have no significant growth-associated phenotypes during the vegetative
115 growth cycle.

116

117 TOR (Target of Rapamycin) signaling plays a key role in modulating cell
118 growth in response to changes in cell cycle status and environmental
119 conditions from yeast to man (Laplante & Sabatini, 2012; Hartmuth &
120 Petersen, 2009). The mTOR kinase forms two distinct protein complexes TOR
121 complex 1 (TORC1) and TOR complex 2 (TORC2), that are each defined by
122 unique components, that are highly conserved across species. TORC1
123 contains the Regulatory Associated Protein of mTOR (RAPTOR), whereas in
124 TORC2 RAPTOR is replaced with the Rapamycin-Insensitive Companion Of
125 mTOR (RICTOR). Both TORC1 and TORC2 complexes control cell migration
126 and F-actin organisation (Liu & Parent, 2011). TORC2 plays a key role in
127 regulating the actin cytoskeleton in yeasts, *Dictyostelium discoideum* and
128 mammalian cells, modulating actin organisation and growth in response to cell
129 cycle progression and the cellular environment (Jacinto *et al*, 2004; Baker *et*
130 *al*, 2016; Lee *et al*, 2005).

131

132 In *S. pombe*, TORC2 recruits and phosphorylates the fission yeast AGC
133 kinase, Gad8 (Matsuo, 2003), a homologue of human SGK1/2 kinase to
134 regulate cell proliferation, the switch of bipolar cell growth, cell fusion during
135 mating and meiosis (Du *et al*, 2016). While the basic principles of the control
136 of the calcium signalling and phosphorylation signalling pathways are
137 understood, little is known about the interplay between these parallel modes
138 of regulation.

139

140 We have used molecular cell biological, biochemical and single molecule
141 biophysical techniques to identify and characterise a novel TORC2-Gad8
142 dependent system regulating calcium-dependent switching of different
143 calmodulin light chain(s) binding to the neck region of Myo1. We define the

144 contribution that each calmodulin makes to the regulation of this conserved
145 motor protein and describe how they affect the conformation of Myo1 lever
146 arm. We propose that a concerted mode of regulation by calcium and
147 phosphorylation controls motility and function of Myo1 in response to cell
148 cycle progression.

149 **Results**

150 ***S. pombe* myosin-1 is phosphorylated within the IQ neck domain.**

151 Phospho-proteomic studies of the fission yeast, *S. pombe*, (Carpy *et al*, 2014;
152 Wilson-Grady *et al*, 2008) have revealed a conserved phosphoserine residue
153 located within the IQ motif-containing neck region of class I & V myosins
154 (Figure 1A). The location of this AGC family kinase consensus phosphoserine
155 site (Pearce *et al*, 2010) has the potential to impact myosin activity and
156 function by affecting light chain binding and conformation of the lever arm. We
157 generated polyclonal antibodies that recognised *S. pombe* myosin-1 when
158 phosphorylated at this conserved serine at position 742 (Myo1^{S742}). Myo1^{S742}
159 phosphorylation was significantly reduced in cells lacking Ste20 (the fission
160 yeast homologue of the core TORC2 component, RICTOR), and abolished in
161 cells lacking the downstream AGC kinase, Gad8. Thus, Myo1^{S742} is
162 phosphorylated in a TORC2 - Gad8 kinase dependent manner (Figure 1B).

163

164 Within cells Gad8 kinase activity is reduced through phosphorylation of a
165 conserved threonine (T6) residue (Du *et al*, 2016; Halova *et al*, 2013) (Figure
166 1C). A significantly reduction of Myo1^{S742} phosphorylation was observed in
167 cells expressing phospho-mimetic Gad8.T6D (Figure 1D), which has reduced
168 Gad8 kinase activity (Du *et al*, 2016). *S. pombe* cells lacking either TORC2 or
169 Gad8 display defects in actin organisation, regulating polarised growth and
170 control of cell cycle progression (Petersen & Nurse, 2007; Du *et al*, 2016).
171 Similarly, replacing Myo1 serine 742 with a phosphorylation resistant alanine
172 residue in *myo1.S742A* cells blocked cell division when cultured for an
173 extended period in restricted-growth media (mean length \pm SEM (μ m): wt -
174 6.67 ± 0.3 ; *myo1.S742A* - 18.50 ± 1.3 (n>300)) (Figure 1E). Therefore, while
175 Gad8 may not directly phosphorylate Myo1^{S742}, phosphorylation of this
176 residue is dependent upon the TORC2-Gad8 signalling pathway.

177 We conclude that TORC2 directed Gad8 dependent phosphorylation at S742
178 regulates Myo1 activity.

179

180 **Phosphorylation affects Myo1 lever arm structure.**

181 As serine 742 lies within the IQ motif of the Myo1 neck region, we asked
182 whether Myo1^{S742} phosphorylation alters calmodulin binding and the
183 conformation of the neck region. Isoforms of the Ca²⁺ sensitive fission yeast
184 calmodulin (wild type Cam1 and a Cam1.T6C cysteine mutant, allowing
185 conjugation to a fluorescent probe) were purified, from bacteria co-expressing
186 the fission yeast NatA amino- α -acetyl-transferase complex, in their native
187 amino-terminally (Nt) acetylated forms (Eastwood *et al*, 2017). Two methods
188 were used to measure Ca²⁺-dependent changes in Cam1 conformation. First,
189 a FRET-based sensor was generated consisting of N-terminal CyPet donor
190 and C-terminal, YPet acceptor fluorophores were fused in-frame with Cam1
191 (Nguyen & Daugherty, 2005) (Figure 2A). Second, Nt-acetylated Cam1.T6C
192 was conjugated to the cysteine-reactive synthetic fluorophore 2-(4'-
193 (iodoacetamido) anilino naphthalene-6-sulfonic acid (IAANS)). As IAANS
194 fluorescence changes in response to changes in its local environment, the
195 fluorescence emission of this fusion will change in response to calcium
196 induced changes in Cam1 conformation. Ca-binding affinity reported by the
197 Cam1-FRET sensor (Figure 2C pCa₅₀: 6.12) reflects the global change in
198 Cam1 conformation, while the Ca²⁺-dependent change in IAANS'
199 fluorescence signal (Figure 2C-inset pCa₅₀: 6.54) reflects changes in local
200 environment of the amino lobe of Cam1.

201

202 Together these probes demonstrated that Ca²⁺ binding induced a change in
203 Cam1 conformation. The rate of Ca²⁺ ion release from Cam1 was
204 independently measured by monitoring changes in fluorescence of the Ca²⁺
205 indicator Quin-2 (Tsien, 1980). The time-course of Ca²⁺ ion release exhibited
206 three phases; fast, medium and slow of approximately equal amplitude (rate
207 constants 137, 12.9 and 2.0 s⁻¹ respectively) indicating the cation has different
208 affinities for each Ca²⁺ binding lobe of Cam1 (Figure 2D).

209

210 To characterise Cam1 binding to the IQ neck region of Myo1, recombinant
211 FRET constructs were produced in which CyPet and YPet were separated by

212 either one, or both, Myo1 IQ motifs (Myo1^{IQ1}-FRET, Myo1^{IQ2}-FRET, Myo1^{IQ12}-
213 FRET) (Figure 2B & Figure 2-figure supplement 1). Cam1 binding to the IQ
214 motif(s) stabilises the α -helix and results in a drop in FRET signal in the
215 absence of calcium (Figure 2E-G). This drop in signal correlates with a Cam1
216 bound IQ12 neck region length of 4.6 nm (Wu & Brand, 1994), close to the 4.7
217 nm length predicted from the modelled structure (based upon PDB structure
218 4R8G). Analysis of interactions between Cam1 and Myo1^{IQ12}-FRET revealed
219 two distinct phases to the association of Cam1 molecules with the combined
220 Myo1^{IQ12} motifs. Each phase contributed 50% of the overall change in signal
221 (Figure 2F). The first Cam1-Myo1^{IQ12} binding event corresponded to an affinity
222 of less than 0.1 μ M (binding was too tight to calculate affinity with higher
223 precision), while the second event correlated with an approximately 10-fold
224 weaker binding affinity (0.68 μ M). This association was sensitive to calcium
225 (pCa of 5.87) (Figure 2C), indicating that Cam1 can only associate with both
226 Myo1 IQ motifs at low cellular Ca²⁺ concentrations. Interestingly while Cam1
227 bound tightly to a single, isolated, Myo1^{IQ1} alone ($K_d < 0.1 \mu$ M), no detectable
228 association was observed for the equivalent single Myo1^{IQ2} motif (Figure 2E).
229 Together these data are consistent with a sequential cooperative binding
230 mechanism by which the stable residency of Cam1 in the first IQ position is
231 required before calmodulin can bind to Myo1^{IQ2}.

232

233 Replacing serine 742 within the IQ neck region with a phosphomimetic
234 aspartic acid residue had no significant impact upon the affinity, calcium
235 sensitivity or cooperative nature of the interaction between Myo1 and Cam1
236 (Figure 2F). However, the S742D replacement resulted in a change in
237 maximum FRET signal upon Cam1 binding (F_{max} 46.05 vs 31.64) (Figure 2F),
238 indicating Myo1^{S742} phosphorylation changes the conformation of the lever
239 arm upon Cam1 binding, rather than modulating the affinity for Cam1.

240

241 **Phosphorylation regulates Myo1 dynamics and endocytosis.**

242 Immunofluorescence using Myo1^{S742} phospho-specific antibodies confirmed
243 the presence of serine-742 phosphorylated Myo1 to cortical foci (Figure 3A).

244 To explore how this phosphorylation affected Myo1 and calmodulin dynamics
245 *in vivo*, we generated prototroph *S. pombe* strains in which endogenous
246 *myo1*, *cam1*, or *cam2* genes were fused to cDNA encoding for monomeric
247 fluorescent proteins (Figure 3-figure supplement 1). Using high-speed (20 fps)
248 single molecule TIRF imaging we explored how Myo1^{S742} phosphorylation
249 impacts Myo1 and Cam1 dynamics and function *in vivo*. Myo1 and Cam1
250 associated with the cell membrane in two distinct ways: we observed both
251 rapid, transient, binding of single Myo1 molecules to the cell membrane,
252 characterised by low-intensity, single, stepwise, changes in intensity (Video
253 1), alongside longer endocytic events that were much brighter and took much
254 longer (Video 2).

255

256 The rapid, single molecule, Myo1 and Cam1 interactions with the membrane
257 had low mobility ($0.03 \mu\text{m}^2\cdot\text{s}^{-1}$), ~10-times slower than diffusion of integral
258 membrane proteins (Mashanov *et al*, 2010). The individual, diffraction-limited
259 fluorescent spots appeared and disappeared at the cell membrane in a single
260 step. The durations of these short single molecule events (defined as the
261 period over which individual objects were observed and their paths tracked)
262 were exponentially distributed with mean lifetime of $\sim 8 \text{ s}^{-1}$ ($n = 152$) (Video 1).
263 The distribution of the durations of individual Myo1 events is shown in Figure
264 3-figure supplement 1. In contrast, during endocytic events, the fluorescence
265 signal increased gradually (at a rate corresponding to $\sim 13 \text{ molecules}\cdot\text{s}^{-1}$), to a
266 peak amplitude corresponding to ~ 45 molecules of mNeogreen.Myo1, that
267 persisted for $\sim 6 \text{ s}$ (plateau phase), before decaying back to baseline level (at
268 a rate of $\sim 14 \text{ molecules}\cdot\text{s}^{-1}$) (Figure 3B, Video 2). The duration (T_{dur}) of
269 endocytic events (measured as described in the Methods) was $13.84 \text{ s} \pm 0.39$
270 (mean \pm SEM, $n=50$) (Figure 3C). While there was significant variation in the
271 maximum mNeogreen.Myo1 intensity ($2373 \pm 155 \text{ AU}$), there was no
272 correlation between maximum intensity (measured during the plateau phase)
273 and event duration (not shown).

274

275 Fluorescence intensity dynamics of Cam1.GFP during endocytic events was

276 similar to mNeogreen.Myo1, but T_{dur} was significantly shorter ($P < 0.0001$),
277 $10.99 \text{ s} \pm 0.21$ ($n=52$) while the peak (plateau) intensity was roughly double
278 that measured for mNeogreen.Myo1 and equivalent to ~ 90 GFP molecules
279 (Figure 3C) consistent with Cam1 occupying both IQ sites within the Myo1
280 neck region. The briefer event duration observed for Cam1 is best explained
281 by Cam1 dissociating from Myo1 before Myo1 leaves the endocytic patch.
282 This idea was confirmed by two-colour imaging of *mNeogreen.myo1*
283 *cam1.mCherry* cells which revealed how Myo1 and Cam1 arrived
284 simultaneously at the endocytic patch before Cam1.mCherry disassociated
285 $\sim 3 \text{ s}$ before mNeogreen.Myo1 (Figure 3D, Figure 3-figure supplement 1).

286

287 Myo1 and Cam1 dynamics in *myo1.S742A* cells during endocytosis revealed
288 how Myo1^{S742A} had average assembly/disassembly rates and peak intensity
289 measurements that were identical to wild type Myo1, yet the duration of the
290 signal (T_{dur}) was 1.5 sec shorter ($12.3 \text{ s} \pm 0.31$ $n=67$) (Figure 3E & Figure 3-
291 figure supplement 1). Consistent with the *in vitro* data, the *myo1.S742A*
292 mutation did not impact on the ability of Cam1 molecules to bind both IQ
293 motifs, as average assembly/disassembly rates, and plateau intensity for
294 Cam1 were the same in both wild type and *myo1.S742A* cells. However,
295 Myo1^{S742A} and Cam1 proteins disassociated simultaneously and somewhat
296 earlier during the endocytic event than in otherwise isogenic wild type cells.

297

298 **Myo1 S742 is phosphorylated in a cell cycle dependent manner to**
299 **regulate polarised cell growth.**

300 Upon cell division, fission yeast cells grow exclusively from the old cell end
301 that existed in the parental cell. At a point during interphase (called New End
302 Take Off -NETO) there is a transition to bipolar growth (Mitchison & Nurse,
303 1985). This cell cycle switch in growth pattern correlates precisely with a
304 parallel redistribution of endocytic actin patches (Marks & Hyams, 1985).

305

306 These TIRF imaging data were consistent with widefield, 3D, time-lapse
307 imaging that showed lifetimes of Myo1 and Cam1 foci were shorter in

308 *myo1.S742A* cells when compared to *myo1⁺* (Table 1). In contrast, while the
309 *myo1.S742A* allele did not affect accumulation of Cam2 or LifeACT to sites of
310 endocytosis (Table 1), the rate of endocytosis (measured by actin foci
311 lifetimes) differed significantly ($p < 0.01$) between the old and new ends of
312 *myo1-S742A* cells but not wild type (lifetimes at old end & new cell end: wt
313 11.96 ± 2.28 & 11.39 ± 1.07 ; *myo1.S742A* 14.17 ± 3.3 & 11.09 ± 1.29 sec (mean \pm
314 s.d.)). Therefore, while Myo1^{S742} phosphorylation does not impact the
315 assembly of Myo1-Cam1 endocytic foci, it regulates the myosin-1 to modulate
316 the activity and function of the ensemble of endocytic proteins during bipolar
317 growth.

318

319 As the *myo1.S742A* allele only affected actin dynamics at the old cell end
320 during bipolar growth we examined whether this post-translational
321 modification was subject to cell cycle dependent variance. Analysis of extracts
322 from cell division cycle mutants arrested in G1 (*cdc10.v50* cells) prior to
323 NETO (Marks *et al*, 1986) or late G2 (*cdc25.22* cells) after NETO, revealed
324 that Myo1^{S742} is phosphorylated in a cell cycle dependent manner (Figure 4A).
325 This was confirmed by monitoring Myo1^{S742} phosphorylation in cells
326 synchronised with respect to cell cycle progression (Figure 4B - D). These
327 data established that at its peak in early interphase (prior to the transition to a
328 bipolar growth pattern), approximately half of cellular Myo1 is phosphorylated
329 on S742, before dropping to undetectable levels by the end of late G2 (the
330 Cdc25 execution point), prior to entry into mitosis. *myo1.S742A* cells have a
331 longer average length compared to wild type (*myo1⁺*: 9.77 ± 1.77 μm ;
332 *myo1.S742A*: 13.2 ± 2.47 μm . t-test >99% significance $n > 500$).

333

334 In addition to the NETO phenotype, a significant proportion of *myo1.S742A*
335 cells exhibited significant issues with the ability to maintain, linear, polarised,
336 growth, as 24.7% of cells developed a bent morphology (i.e. growth deviates
337 by $>5^\circ$ from longitudinal axis) (Figure 4E - F). The *myo1.S742A* allele did not
338 have an additive effect upon the growth polarity defects associated with cells
339 lacking Tea4, a polarity determinant protein that plays an important role in

340 integrating actin cytoskeleton function with regulation of polarized cell growth
341 (Martin *et al*, 2005; Tatebe *et al*, 2005) (Figure 5A). Consistently, cell wall
342 staining revealed a significantly higher than normal proportion of *myo1.S742A*
343 cells exhibiting monopolar growth, compared to equivalent wild type,
344 indicating disruption in the switch from monopolar to bipolar growth (Figure
345 4E - F). This was confirmed by tracking the cellular distribution of the actin
346 patch marker, Sla2/End4, following cell division. Sla2 failed to redistribute to
347 the newly divided end of *myo1.S742A* cells during interphase (Figure 5B).
348 This failure to switch to bipolar growth in *myo1.S742A* cells, as well as restrict
349 growth upon nutrient depletion (Figure 1E), is consistent with the reduced
350 growth rate at the end of log phase and growth to an overall higher density
351 upon reaching stationary phase (Figure 5C).

352

353 We conclude that cell cycle dependent changes in Myo1^{S742} phosphorylation
354 modulate the ability of the myosin lever arm region to regulate endocytosis
355 and polarised growth.

356

357 **Cam2 associates with internalised endosomes and not Myo1 during**
358 **vegetative growth.**

359 Myo1 has been reported to associate with a second calmodulin like protein,
360 Cam2, via its second IQ motif (Sammons *et al*, 2011). However, our data
361 indicate Cam1 occupies both Myo1 IQ motifs during endocytosis. Widefield
362 microscopy revealed Myo1 and Cam1 dynamics (Figure 6A) at endocytic foci
363 differ significantly from Cam2 dynamics. Cam2 is recruited to sites of
364 endocytosis later than Myo1 and Cam1, but prior to vesicle scission/budding,
365 whereupon, like CAPZA^{Acp1}, Sla2 and actin, it remains associated with
366 laterally oscillating, internalised, endosomes (Figure 6B-C). Similarly,
367 simultaneous imaging of Cam1 and Cam2 in *cam1.mCherry cam2.gfp* cells
368 revealed how each protein localises to a significant proportion of foci lacking
369 the other calmodulin, to highlight the different timing of engagement of each
370 molecule with the endocytic machinery (Figure 6D). Finally, while Cam1
371 recruitment to endocytic foci is abolished when Myo1 is absent (Figure 6E),

372 the intensity, volume and number of Cam2 foci actually increases in the
373 absence of Myo1 (Figure 6F Table 1), even though, internalisation and lateral
374 “oscillating” dynamics of Cam2, and actin were dependent on Myo1 (Figure
375 6F & G). We assume that this arises from the requirement for prior action of
376 Cam1 for vesicle budding.

377

378 TIRF imaging revealed that on average a total of ~30 Cam2 molecules were
379 recruited to each endocytic focus (compared to 45 and 90 molecules
380 observed for Myo1 and Cam1 respectively), and the kinetics of its recruitment
381 to foci differed significantly to that observed for both Myo1 and Cam1. Cam2
382 signal often increased steadily, before an abrupt decline (Figure 7A), which
383 contrasts with the more gradual (sigmoidal) rise and decay in intensity
384 observed for Myo1 and Cam1 (Figure 3C & E). TIRFM confirmed that Cam2
385 remained associated with the endocytic vesicles after they were internalised
386 and their connection with the cell membrane was broken (Video 3).
387 Background corrected intensity traces of Cam2 dynamics at the membrane
388 patch before, during, and after the end of endocytosis showed the signal
389 rapidly dropped to baseline (<1s) (Figure 7A), with the Cam2 labelled vesicles
390 remaining visible close to the membrane but moved inwardly, away from the
391 location of the endocytic event. A large number of these mobile, internalised
392 Cam2 labelled vesicles were seen moving within the cytoplasm with relatively
393 low cytosolic background signal (Video 3), indicating that much of the Cam2
394 was associated with endocytic vesicles and remained bound to mature
395 endosomes. We conclude that endocytosis was inhibited, with Cam2
396 persisting on the endosome while Myo1 remained at the plasma membrane
397 during and after endosome abscission, as previously reported (Figure 6A,
398 Video 2) (Sirotkin *et al*, 2010; Berro & Pollard, 2014; Picco *et al*, 2015). Thus,
399 whilst Cam1 and Cam2 both localise to sites of endocytosis, they appear to
400 do so at different times, and each have different Myo1 dependencies.

401

402 To correlate Myo1-Cam1 dynamics at sites of endocytosis with internalisation
403 of the mature endosome into the cytoplasm, we followed Cam1 and Cam2

404 dynamics simultaneously in *cam1.mCherry cam2.gfp* cells (Video 4). An
405 average curve (Figure 7B), generated from profiles of 65 two colour individual
406 endocytic events, synchronized relative to T_{start} of Cam1 (see Fig. 3B & C),
407 shows that Cam2 moves away from the cell surface shortly after Cam1 leaves
408 but before Myo1, with the time of abscission (T_{scis}) occurring on average 11.4
409 sec after the event starts (T_{start}). Therefore endosome scission takes place
410 immediately prior to the Myo1 disassembly phase (Figure 3B), and around the
411 time Cam1 dissociates from Myo1 (Figure 3C). Intriguingly, while the overall
412 distribution of Myo1 and Cam1 appeared unaffected in *cam2 Δ* cells, the
413 number, volume and intensity of foci were significantly reduced (Figure 7C-D
414 Table 1).

415

416 **Serine 742 phosphorylation increases the affinity of a single Cam2 for** 417 **Myo1.**

418 *In vitro* analysis revealed how two Cam2 molecules can associate with the
419 unphosphorylated Myo1^{IQ12} region (Figure 2F) with 2 distinct phases. In
420 contrast to Cam1 in which the two Myo1^{IQ12} binding events contributed equally
421 to the change in FRET signal, for Cam2 70% of the signal change was
422 brought about by a single binding event, associated with an affinity of 1.10
423 μM . The smaller amplitude and tighter binding signal is not accurately
424 measurable, but the combined change in signal is consistent with 2 binding
425 events.

426

427 As predicted from sequence analysis, Cam2 failed to associate with calcium
428 (Figure 2D), and its conformation and interactions with Myo1 were insensitive
429 to the divalent cation (Figure 2G). Like Cam1, Cam2 had a higher affinity for
430 the first IQ motif (0.4 μM) than for both IQ1 and IQ2 together, and failed to
431 bind IQ2 alone (Figure 2E). Cam1 calcium binding, as measured by IAANS
432 labelling or change in Quin-2 fluorescence were unaffected by Cam2, while
433 gel filtration and fluorescence binding assays provided no evidence of a direct
434 physical interaction between the two proteins (Figure 2-figure supplement 2).
435 Interestingly a difference observed in fluorescence amplitudes between Cam1

436 and Cam2 binding to the IQ12 motif indicates an impact upon the
437 conformation of the lever arm (Figure 2G), providing a potential mechanism to
438 directly control Myo1 motor activity. However, Myo1^{S742} phosphorylation had
439 no measurable impact upon the dynamics and distribution of Cam2 within *S.*
440 *pombe* cells undergoing normal vegetative growth (Figure 8A Table 1).

441

442 **Cam1 and Cam2 associate with Myo1 during meiosis.**

443 Calcium levels within log phase yeast cells are relatively low (100-200 nM)
444 (Ma *et al*, 2011; Miseta *et al*, 1999), and so provide favourable conditions for
445 Cam1 to associate with Myo1 (pCa - 5.87). Analysis of cell fluorescence
446 indicated the relative abundance of Myo1 : Cam1 : Cam2 within the *S. pombe*
447 cell to be 0.25 : 1.56 : 1 (Table 1), which is similar to the ratios defined by
448 quantitative proteomic analysis of 0.45 : 1.56 : 1 (Marguerat *et al*, 2012).
449 Similarly, image analysis of Cam1-GFP fluorescence showed how 1.7% of
450 Cam1 associated with discrete foci within cells (Table 1), 40% of which was
451 dependent upon Myo1, with the majority associating with the SPB (Figure 6E).
452 This indicates ~0.68% of cellular Cam1 associates with Myo1 at dynamic
453 endocytic foci. These relative protein levels, binding affinities and low Ca²⁺
454 concentrations favour Cam1 binding to Myo1, over Cam2 at both IQ sites
455 (Figure 8B), consistent with *in vivo* observations.

456

457 While Ca²⁺ levels are low during vegetative growth, sporadic prolonged
458 calcium bursts occur upon pheromone release during mating (Carbó *et al*,
459 2016; Iida *et al*, 1990), and levels elevate significantly (~10 fold) during the
460 subsequent meiosis and sporulation (Suizu *et al*, 1995). Cam1 would be less
461 likely to bind to Myo1 in these conditions (pCa 5.87). We observed Myo1^{S742}
462 is phosphorylated in mating and meiotic cells (Figure 8C). Cam2 abundance
463 simultaneously increases significantly in relation to Cam1 upon starvation,
464 mating and entry into meiosis (Mata & Bähler, 2006; Mata *et al*, 2002). These
465 conditions favour interactions between Myo1-Cam2 over an association of
466 Cam1 with Myo1 (Figure 8B), which is consistent with both Myo1 and Cam2
467 playing important roles at the leading edge of forespore membrane formation

468 during meiosis (Toya *et al*, 2001; Itadani *et al*, 2007). Consistent with this
469 prediction, Myo1, Cam1, Cam2 foci lifetime and dynamics differ significantly to
470 those observed in vegetative cells ($P < 0.0001$), lasting significantly longer (>1
471 min) in meiotic and sporulating cells (Table 1). In contrast to vegetative cells,
472 during meiosis and subsequent spore formation, like Myo1 and Cam1, cortical
473 foci containing accumulations of Cam2 and actin were less dynamic, lacking
474 any oscillation and remaining in a fixed position, with significantly longer
475 lifetime than within actively growing cells (Figure 8D, Table 1, Video 5-8).
476 Consistent with this, endocytosis is significantly diminished in fusing and
477 meiotic cells (Figure 8E) containing the stable Myo1/Cam1/Cam2/actin foci,
478 indicating the the reduction in foci dynamics minimises endocytosis in meiotic
479 cells.

480 Finally, we used the *myo1.S742A* allele to monitor the impact of Myo1^{S742}
481 phosphorylation on Myo1, Cam1 and Cam2 dynamics during meiosis. In
482 contrast to wild type cells, the lifetime of Myo1 and Cam1 foci were not
483 significantly different to each other in *myo1.S742A* cells. In addition the
484 lifetime of the Myo1 and Cam1 foci in *myo1.S742A* cells were significantly
485 reduced from wild type. Cam2 dynamics did not correlate with Myo1 in
486 *myo1.S742A* cells, which is in contrast to wild type (Table 1). Myo1 and Cam1
487 foci were also seen to be shorter in *cam2Δ* cells during meiosis, when
488 compared to wild type (Table 1). These data indicate Myo1^{S742}
489 phosphorylation is required for Cam2 to interact with the Myo1 IQ motif and
490 thereby reduce Myo1 foci dynamics.

491 The majority of Cam2 foci remained present in the cell for greater than 2 mins
492 in meiotic cells lacking Myo1^{S742} phosphorylation. Such timing differs
493 significantly from the dynamics of non-phosphorylatable Myo1^{S742A}, indicating
494 normal Cam1 and Cam2 interactions with Myo1 were abolished. Consistent
495 with observations of *myo1.S742A* cells grown to stationary phase (Figure 1E),
496 heterothallic (h^{90}) nitrogen starved G₁ arrested *myo1.S742A* cells failed to
497 inhibit polar growth (Figure 8F). 27.9% of mating *myo1.S742A* cells continued
498 to grow at their mating (schmooing) tips (*myo1⁺* 1.8% vs *myo1.S742A* 27.9%
499 cells had long schmoo tips. $n > 100$), and meioses frequently produced asci
500 with an abnormal number of unequally sized spores (Figure 8F arrowheads)

501 (*myo1⁺* – 0.9%, *myo1.S742A* – 13.1%. n>100). This spore defect phenotype
502 is reminiscent of the meiotic phenotype of *cam2Δ* cells (Itadani *et al*, 2007),
503 which supports the view that an increase in cellular Ca²⁺ and Myo1^{S742}
504 phosphorylation are both key for Cam2 association with and regulation of
505 Myo1.

506

507 These data support a model by which changes in calcium levels and TORC2
508 dependent phosphorylation status provides a simple two stage mechanism for
509 modulating motor activity by modifying lever arm conformation as well as
510 switching calmodulin light chain preference in order to co-ordinate myosin
511 function with changing environmental and cell cycle dependent needs of the
512 cell (Figure 8B & F).

513 Discussion

514 Myosins are subject to diverse modes of regulation, including modulation of
515 the composition of the actin track, changes to cargo and light chain
516 interactions, alongside phosphorylation to change core physical properties of
517 the motor. Here we describe a newly discovered mechanism by which
518 phosphorylation of the myosin heavy chain (Figure 1) regulates light chain
519 specificity, lever arm conformation and flexibility, to impact upon cellular
520 function. During the vegetative life cycle, at basal levels of cellular calcium, *S.*
521 *pombe* Myo1 preferentially associates with two molecules of the calcium
522 regulated calmodulin light chain Cam1 (Figures 2 & 3). During early stages of
523 the cell cycle phosphorylation of the Myo1 neck region (Figure 8G), changes
524 the conformation of the Cam1 associated lever arm to moderate motor
525 activity, and thereby regulate the rate of endocytosis, and a switch from
526 monopolar to bipolar growth (Figure 5).

527

528 There is a significant increase in TORC2 and Gad8 activity upon starvation
529 and thereby promote the onset of the meiotic lifecycle (Halova *et al*, 2013;
530 Laboucarié *et al*, 2017; Martín *et al*, 2017). Upon starvation there is an
531 increase in Myo1 serine 472 phosphorylation (Figure 8), and *myo1.S742A*
532 cells fail to arrest growth in response to starvation (Figures 1E & 8F).
533 The IQ region phosphorylation, combined with an increase in cytosolic Ca²⁺
534 levels observed during G1, starvation and meiosis, switches light chain
535 preference to favour recruitment of a single molecule of the calcium
536 insensitive calmodulin like, Cam2. However it is the worth noting that there
537 are currently differences of opinion on the relationship between levels of Gad8
538 activity and cytosolic calcium (Cohen *et al*, 2014). The structures of the IQ
539 region of Myosin-1 and calmodulin (Lu *et al*, 2014), suggest that
540 phosphorylation of Myo1^{S742} is likely to impact Cam2 binding at the 1st IQ
541 position. Furthermore, our data reveals that CaM is unable to associate with
542 IQ2 alone, as occupancy of IQ1 is required before a second CaM can bind to
543 IQ2 (i.e. regulatory cooperative binding). This switch in light chain occupancy
544 may provide a mechanism to change the stiffness of the Myo1 neck region
545 (i.e. the “lever arm”) and thereby modulate the movement and force it

546 produces during the acto-myosin ATPase cycle and/or the load-sensitivity of
547 its actin-bound lifetime.

548

549 While observations within budding yeast indicate that motor activity from a
550 ring of myosins at the lip of the endosome (Mund *et al*, 2018) is necessary for
551 endocytic internalisation, the mechanism by which the myosin interacts with
552 actin to facilitate this localised activation is unknown (Sun *et al*, 2006). Here
553 we find that the size of the early endocytic patch determines the number of
554 Myo1 molecules necessary to generate a critical local concentration of Arp2/3
555 nucleated actin filaments (Barker *et al*, 2007). At the critical concentration
556 myosin heads are able to interact with actin filaments nucleated from either
557 adjacent Myo1 tails or WASP activated Arp2/3 complexes, tethered to the
558 membrane via molecules such as the Talin-like Sla2 (Sirotkin *et al*, 2005;
559 2010). The Myo1 is then primed to act as a tension sensor against the actin
560 filament, as it pushes against the membrane of the internalised endosome,
561 which grows against the significant 0.85 MPa (8.3 atm) turgor pressure within
562 the cell (Minc *et al*, 2009).

563

564 The number of Myo1 molecules at the plasma membrane focus remains
565 constant, as the membrane is internalised, until 2 seconds after Cam1
566 disassociates from Myo1. While the trigger for Cam1 release is unknown, the
567 speed in which the event takes places indicates that it is likely to be initiated
568 by a rapid localised spike in calcium. This could perhaps be driven by a critical
569 level of membrane deformation coupled to calcium influx - similar to
570 processes proposed for mechano-transduction and the role of mammalian
571 myosin-1 within the stereocilia of the inner ear (Adamek *et al*, 2008; Batters
572 *et al*, 2004).

573

574 Once Cam1 detaches from the Myo1 molecule, the neck loses rigidity,
575 reducing tension between the myosin motor and actin filament, to promote
576 detachment from F-actin (Lewis *et al*, 2012; Menten *et al*, 2018). Given that
577 alone, single molecules of Myo1 do not reside for long at the plasma

578 membrane (off rate is $\sim 8 \text{ sec}^{-1}$ Video 1), without an interaction with actin Myo1
579 would leave the endocytic patch a second or so after losing its Cam1 light
580 chain . Therefore, after Cam1 release, there is a 2 sec delay in the
581 disappearance of Myo1 signal, as it disassociates from the endocytic
582 machinery (Figure 3C - D).

583 The conformation and rigidity of the Myo1 lever arm would therefore play a
584 key role in modulating the tension sensing properties of the motor domain.
585 This is consistent with our data, where wild type phosphorylation competent
586 Myo1 resides at the membrane ~ 1.8 sec longer than Myo1 mutant protein that
587 cannot be phosphorylated at serine 742 (Myo1^{S742A}) (Figure 3-figure
588 supplement 1). Phosphorylation-dependent changes in the conformation of
589 the myosin neck provide a simple mechanism to modulate the rate of
590 endocytosis according to the size and needs of the cell. Similarly, in the
591 presence of Ca^{2+} and Myo1^{S742} phosphorylation, a single Cam2 resides at IQ1
592 motif of the neck (Figure 8B). While bringing about a change in the
593 conformation of the first half of the myosin lever arm (adjacent to the motor
594 domain), the vacant IQ2 motif allows flexibility within the carboxyl half of the
595 neck region. This would provide a relatively tension insensitive motor, that
596 stalls against the actin polymer, and would therefore persist significantly
597 longer at the endocytic foci, as observed to occur here in meiotic cells (Figure
598 8D, Table 1). These changes in lever arm properties change the overall rate
599 of endocytosis, as observed in differences in the time taken for endosomes to
600 internalise within the cytoplasm (Table 1 & 2).

601

602 Thus phosphorylation-dependent changes in the calcium regulated
603 conformation and rigidity of the myosin lever arm could provide a universal
604 mechanism for regulating the diverse cytoplasmic activities and functions of
605 myosin motors within all cells.

606

607 **Materials and Methods**

608 **Yeast cell culture:** Cell culture and maintenance were carried out according
609 to (Moreno *et al*, 1991) using Edinburgh minimal medium with Glutamic acid
610 nitrogen source (EMMG) unless specified otherwise. Cells were cultured at 25
611 °C unless stated otherwise and cells were maintained as early to mid-log
612 phase cultures for 48 hours before being used for analyses. Genetic crosses
613 were undertaken on MSA plates (Egel *et al*, 1994). All strains used in this
614 study were prototroph and listed in Supplementary File 1.

615 **Molecular Biology:** *cam1⁺* (SPAC3A12.14), *cam1.T6C* and *cam2⁺*
616 (SPAC29A4.05) genes were amplified as *Nde1 - BamH1* fragments from
617 genomic *S. pombe* DNA using o226/o227 and o393/o394 primers and cloned
618 into pGEM-T-Easy (*Promega*, Madison, WI, USA). After sequencing the
619 subsequent genes were cloned into pJC20 (Clos *et al.*, 1990) to generate
620 bacterial calmodulin expression constructs. DNA encoding for the FRET
621 optimized fluorophores CyPet and YPet (Nguyen and Daugherty, 2005) were
622 each amplified using primers o405 / o406 and o403 / o404 respectively. o406
623 also incorporated DNA at the 3' end of the CyPet ORF encoding for the first
624 IQ motif of the Myo1 neck region, while o404 included DNA encoding a
625 Gly3His6 tag at the 3' of the YPet ORF. The two DNA fragments were cloned
626 into pGEM-T-Easy in a three-way ligation reaction to generate pGEM-CyPet-
627 Myo1IQ1-YPet. The CyPet-Myo1^{IQ1}-YPet DNA was subsequently sequencing
628 and cloned as a *Nde1 - BamH1* fragment into pJC20 (Clos & Brandau, 1994)
629 to generate pJC20CyPet-Myo1^{IQ1}-YPet. Complementary oligonucleotides
630 o425 & o426 were annealed together and ligated into *BglIII - Xho1* cut
631 pJC20CyPet-Myo1^{IQ1}-YPet to generate pJC20CyPet-Myo1^{IQ12}-YPet. Similarly,
632 complementary oligonucleotides o429 & o430 were annealed together and
633 subsequently ligated into *Sal1-BglIII* cut pJC20CyPet-Myo1^{IQ1}-YPet and the
634 subsequent *Xho1* fragment was excised to generate pJC20CyPet-Myo1^{IQ2}-
635 YPet. Site directed mutagenesis was carried out using pJC20CyPet-Myo1^{IQ12}-
636 YPet template and o427 & o428 primers to generate pJC20CyPet-
637 Myo1^{IQ12}S742D-YPet. Complementary oligonucleotides o449 & o450 were
638 annealed together and ligated into *Nru1 - Xho1* digested pJC20CyPet-
639 Myo1^{IQ12}S742D-YPet to generate pJC20CyPet-Myo1^{IQ12}S742A-YPet. All

640 plasmids were sequenced upon construction. Strains with fluorophore tagged
641 alleles of *cam1*⁺ and *cam2*⁺ were generated as described previously using
642 appropriate template and primers (Bähler *et al*, 1998). Strains in which the
643 *myo1.S742A*, *myo1.S742D*, *mNeongreen-myo1*, *mNeongreen-myo1.S742A*,
644 or *mNeongreen-myo1.S742D* alleles replaced the endogenous *myo1*⁺ gene
645 (SPBC146.13c) were generated using a marker switching method (Maclver *et*
646 *al*, 2003). Oligonucleotides are described in Supplementary File 2.

647 **Protein expression & purification:** All recombinant proteins were expressed
648 and purified from BL21 DE3 *E. coli* cells, except Cam1 proteins where BL21
649 DE3 pNatA cells (Eastwood *et al*, 2017) were used to allow amino-terminal
650 acetylation (Figure 2-figure supplement 1). *Calmodulin purification:* Cell
651 lysates were resuspended in Buffer A (50 mM Tris, 2 mM EDTA, 1 mM DTT,
652 0.1 mM PMSF, pH 7.5) and precleared by high speed centrifugation (48,500
653 RCF; 30 min; 4 °C), before ammonium sulphate was added to the supernatant
654 at 35 % saturation, incubated for 30 minutes at 4 °C. Precipitated proteins
655 were removed by centrifugation (48,500 RCF; 30 min; 4 °C). For Cam1
656 purifications the precipitation cleared supernatant was added to a pre-
657 equilibrated 10 ml phenyl sepharose (CL-4B) column (Buffer B: 50 mM Tris, 1
658 mM DTT, 1 mM NaN₃, 5 mM CaCl₂, pH 8.0), washed in 4 volumes of Buffer B
659 before eluted as fractions in Buffer C (50 mM Tris, 1 mM DTT, 1 mM NaN₃, 5
660 mM EGTA, pH 8.0). For Cam2 purification the precipitation cleared
661 supernatant underwent a second round of ammonium sulphate precipitation
662 and clearing, and the subsequent supernatant subjected to isoelectric
663 precipitation (pH 4.3) and centrifugation (48,500 RCF: 30 minutes; 4 °C). The
664 resultant pellet was resuspended in Buffer A, heated to 80 °C for 5 minutes
665 and denatured proteins removed by centrifugation (16,000 RCF; 5 min). *His-*
666 *tagged* proteins were purified in native conditions using prepacked, pre-
667 equilibrated 5ml Ni²⁺ columns.

668 **Immunological techniques:** Standard immunological methods were used as
669 described (Harlow & Lane, 1988) . Serine 742 phosphorylation-state specific
670 anti-Myo1 antibodies were raised against phosphate-conjugated peptide
671 encompassing Myo1 serine 742 in SPF rabbits (Eurogentec, Seraing,
672 Belgium), which were subsequently affinity-purified.

673 **Analysis of yeast extracts:** Protein extracts were prepared and analysed as
674 described elsewhere (Baker *et al*, 2016). For western blot analysis anti-Myo1
675 sera was diluted 1:1000, while Myo1 serine 742 phosphorylation state specific
676 antibodies were used at a dilution of 1:50. Gel densitometry was undertaken
677 using ImageJ software.

678 **Fast reaction kinetics:** All transient kinetics were carried out using a HiTech
679 Scientific DF-61 DX2 Stopped Flow apparatus (TgK Scientific, Bradford-upon-
680 Avon, UK) at 20°C. All data was acquired as the average of 3-5 consecutive
681 shots and analysed using the KineticStudio software supplied with the
682 equipment. Quin-2 fluorescence was excited at 333 nm and used a Schott
683 GG445 cut off filter to monitor fluorescence above 445 nm. IAANS (2-(4'-
684 (iodoacetamido)anilino)-naphthalene-6-sulfonic acid) was excited at 335 nm
685 and fluorescence was monitored through a GG455 filter. For the FRET
686 measurements, CyPet was excited at 435 nm and YPet emission was
687 monitored through a combination of a Wrattan Gelatin No12 (Kodak) with a
688 Schott GG495 nm filter to monitor fluorescence at 525-530 nm.

689 **Fluorescence spectra:** Emission spectra were obtained using a Varian Cary
690 Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara,
691 CA) using a 100 µl Quartz cuvette. For FRET measurements samples were
692 excited at 435 nm (CyPet excitation) and emission was monitored from 450 –
693 600 nm with both slits set to 1 nm. Affinity experiments were carried out using
694 1 µM IQ-FRET protein with varying concentrations of Cam1 or Cam2 in a final
695 volume of 100 µl in analysis buffer of 140 mM KCl, 20 mM MOPS, pH 7.0 with
696 or without 2 mM MgCl₂ and with 2 mM of EGTA, CaCl₂ or Ca²⁺-EGTA as
697 required. Distances between FRET fluorophores were calculated as described
698 previously (Wu & Brand, 1994) using an CyPet-YPet R₀ value of 53.01.

699 **Live cell imaging:** Live cell widefield fluorescence imaging was undertaken
700 as described previously (Baker *et al*, 2016). For Total Internal Reflection
701 Fluorescence Microscopy (TIRFM) *S. pombe* cells were immobilized on №1,
702 Ø 25 mm lectin coated coverslips and placed into imaging chambers filled
703 with EMMG medium. A previously described custom TIRF Microscope
704 (Mashanov *et al*, 2003) was used to image individual cells at a rate of 20 fps
705 in either single or dual colour mode. Lasers: 488 nm/100 mW and 561 nm/150

706 mW (*Omicron*, Germany); emission filters 525/50 nm and 585/29 nm, dichroic
707 mirror 552 nm (*Semrock*, NY); all lenses and mirrors (*Thorlabs*, NJ), except
708 two Ø3 mm mirrors (*Comar Optics*, UK) which directed light in and out of the
709 100× 1.45 NA objective lens (*Olympus*, Japan). Sequences of images were
710 captured using one or two iXon897BV cameras (*Andor Technology*, UK) with
711 custom made acquisition software. 100% laser power (488 nm) was used to
712 image individual mNeongreen-Myo1 and Cam1-GFP molecules. The laser
713 intensity was reduced to 20% during endocytosis imaging experiments to
714 minimize photobleaching. All imaging was undertaken at 23 °C.

715 **Immunofluorescence:** Immunofluorescence microscopy was performed as
716 described previously (Hagan & Hyams, 1988), except gluteraldehyde was
717 omitted. Images were captured using the above widefield imaging system.
718 Anti-Myo1 sera (Attanapola *et al*, 2009) were used at a dilution of 1:100, while
719 affinity purified Myo1 serine 742 phosphorylation state specific antibodies
720 were used at a dilution of 1:10.

721 **Image analysis:** *Widefield data* was analysed using Autoquant software
722 (*MediaCybernetics*, Rockville, MD, USA). All 3d image stacks were subjected
723 to blind 3d deconvolution before analysis. Average size and number and
724 cellular distribution of foci were calculated from all foci present within ≥ 30
725 cells for each sample examined. Timing of foci events were calculated from
726 kymographs generated in *Metamorph* software (*Molecular Devices*,
727 Sunnyvale, CA, USA). The proportion of cells displaying a bent cell phenotype
728 was determined from more than >350 calcofluor (1 mg.ml⁻¹) stained cells for
729 each strain. Bent cells were defined by a deviation in the direction of growth of
730 > 5° from the longitudinal axis. *TIRF data* analyses, including single molecule
731 detection and tracking, was undertaken using GMimPro software (Mashanov
732 & Molloy, 2007). Endocytic events were identified by creating an image
733 representing the standard deviation of each pixel over the whole video
734 sequence (known as a “z-projection”). Bright spots in this image correspond to
735 regions of the yeast cell that showed large intensity fluctuations associated
736 with endocytic activity. Intensity trajectories in these regions of interest (ROIs)
737 (0.5 µm diameter, 5x5 pixels) were saved for future analysis. To correct for
738 local variation in background signal, the average intensity in a region 1.5 µm

739 diameter around the endocytosis site (but not including the central ROI) was
740 subtracted. Data from ROIs that were contaminated by other endocytosis
741 events, occurring in close proximity and close in time, were manually
742 excluded from the analysis. It was critical to identify accurately the start and
743 end of each endocytosis event so that individual traces could be averaged. To
744 facilitate this, the rising and falling phases of the intensity trace were fitted
745 with a straight line (60 data points, 3 sec duration), see Figure 3C for
746 example. The intercept of this line with the baseline intensity gave the t_{start} and
747 t_{end} values and event duration ($T_{\text{dur}} = T_{\text{end}} - T_{\text{start}}$) (see Figure 7A). The
748 amplitude (intensity) of the event A_{av} was measured at the middle of the event
749 by averaging 60 data points from 5th to 8th second from the T_{start} , grey bar on
750 Figure 3B. Intensity traces for each given condition were synchronised to the
751 starting point (T_{start}) and averaged (except Cam2-GFP traces which were
752 synchronised using t_{start} measured from simultaneously acquired Cam1-
753 mCherry signal). Similarly, traces were synchronised to their end point (T_{end})
754 and averaged. The mean duration of the events (T_{dur}) for each condition was
755 then used to reconstruct the mean intensity changes with calculated errors for
756 event amplitude and timing (Table 2). We used the results of single molecule
757 imaging experiments to calculate the number of single fluorescent molecules
758 contributing to the spot intensity at a given time. Since the falling and rising
759 phases of most events fitted well to a simple linear equation, the *slope* of the
760 fitted lines was used to estimate the rate of accumulation and dissociation of
761 the fluorescent molecules (Figure 3C). As Cam2-GFP remained bound to the
762 endocytic vesicle, when vesicle scission occurred intensity fell rapidly to zero
763 as the vesicle diffused from the TIRF evanescent field; the time of scission
764 was defined as T_{scis} (Figure 7A). Single particle tracking was performed using
765 GMimPro (Mashanov & Molloy, 2007) (ASPT module) so that the paths (or
766 trajectories) of individual Myo1 molecules bound to cell membrane could be
767 traced. Trajectories were analysed to yield mean intensities for individual
768 mNeonGreen and eGFP labelled proteins, which could be used to estimate
769 the number of fluorescently-tagged molecules associated with each
770 endocytotic event. Intensity-versus-time plots were generated from averages
771 of >30 foci for each protein in each genetic background examined.

772

773

774

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785

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1015 **Figure Legends**

1016 **Figure 1. Myo1 serine 742 phosphorylation is TORC2 dependent.** (A)
1017 Anti- (A) Sequence alignment of myosin IQ regions highlights an AGC kinase
1018 consensus sequence, conserved in class I and V myosins. Underlined
1019 residues mark residues within IQ motifs. (B) Western blots of extracts from
1020 *myo1⁺*, *myo1-S742A*, *gad8Δ* and *ste20Δ* cells probed with phospho-specific
1021 anti-Myo1^{S742} (upper panel) and anti-Myo1 (lower panel) antibodies
1022 demonstrate antigen specificity and a Myo1^{S742} phosphorylation state
1023 dependence upon the TORC2-Gad8 pathway. Ponceau staining was used to
1024 monitor equal loading. Relative Myo1^{S742} phosphorylation levels were
1025 calculated from 5 independent equivalent experiments (mean ± sd). (C) A
1026 schematic of the TORC2-Gad8 signalling pathway. (D) Myo1^{S742}
1027 phosphorylation is reduced in *gad8.T6D* cells, which have reduced Gad8
1028 kinase activity. Relative Myo1^{S742} phosphorylation levels were calculated from
1029 3 independent equivalent experiments (mean ± sd). (E) Nitrogen starved WT
1030 and *myo1.S742A* cells. In contrast to WT in which growth arrests,
1031 *myo1.S742A* cells continue to growing upon nitrogen starvation induced G1
1032 arrest. Scale – 5 μm.

1033 **Figure 2. In vitro characterisation of interactions between Myo1 and**
1034 **Cam1.** (A) Predicted models of the CyPet-Cam1-YPet FRET reporter protein
1035 (Cam1-FRET) in the absence (upper panel) and presence (lower panel) of
1036 Ca²⁺. (B) Predicted models of the CyPet-Myo1^{IQ12}-YPet FRET reporter
1037 protein (Myo1IQ12-FRET) in the absence (upper panel) or presence (lower
1038 panel) of Calmodulin binding (Cypet – cyan; Cam1 – yellow; YPet – green; IQ
1039 domain - magenta). (C) pCa curve plotting Ca²⁺ dependent changes in
1040 acceptor fluorescence (plotted as ΔYPet signal) of Cam1-FRET protein (red),
1041 Cam1 association with Myo1^{IQ12}-FRET (black) and change in fluorescence of
1042 IAANS labelled Cam1-T6C (inset). (D) Transient curves of changes in Quin2
1043 fluorescence induced by Ca²⁺ release from Cam1 (red) with 3 exponential fit
1044 best fit (black) and Cam2 (blue) illustrate only Cam1 associates with Ca²⁺. (E)
1045 Curves plotting Cam1 (black) and Cam2 (red) dependent changes of FRET
1046 donor signal of Myo1-FRET proteins containing single IQ domains (IQ1 –

1047 empty shapes; IQ2 – filled shapes) show each CaM associates with IQ1 but
1048 not to an equivalent single IQ2 motif region. (F) Curves plotting Cam1
1049 (squares and triangles) and Cam2 (circles and lozenge) dependent changes
1050 of FRET donor signal of either 0.5 μM wild type (black & red) or S742D
1051 phosphomimetic (blue) Myo1^{IQ12}-FRET proteins show while phosphorylation
1052 does not significantly impact Cam1 binding, it results in a ~50% drop in Cam2
1053 interaction. (G) Spectra of 0.5 μM Myo1^{IQ12}-FRET reporter alone (grey line), or
1054 mixed with 10 μM saturating concentrations of: Cam1 + Ca²⁺ (black dotted
1055 line), Cam1 - Ca²⁺ (black solid line), Cam2 + Ca²⁺ (red dotted line), or Cam1 -
1056 Ca²⁺ (red solid line).

1057 **Figure 3. Myo1 and Cam1 dynamics in wild type and *myo1.S742A* cells.**
1058 (A) Maximum projections from 31-z stack widefield immunofluorescence
1059 images of wild-type cells probed with anti-Myo1 (left panel) or anti-Myo1^{S742*}
1060 phosphospecific (right panels) antibodies illustrate S742 phosphorylated Myo1
1061 localises to endocytic foci (Scale - 5 μm). (B) An example relative intensity
1062 trace of a single mNeogreen.Myo1 endocytic event. Linear fitting (grey lines,
1063 60 points) was used to find the maximum gradient for both rising and falling
1064 slopes. The intercept with zero intensity level was used to calculate T_{start}, T_{end},
1065 and subsequently the duration of the event T_{dur}. See detailed description in
1066 the Methods section. Insert: An arrow highlights the analysed endocytotic
1067 event (5x5 pixels area). (C) Averaged profile from individual Myo1 (blue) and
1068 Cam1 (red) membrane association events, synchronised relative to T_{start} and
1069 T_{end}. Dotted lines show fitted rising (Myo1: 537 AU/sec, Cam1: 1073 AU/sec)
1070 and falling (Myo1: 567 AU/sec, Cam1: 1028 AU/sec) gradients. (D) An
1071 example fluorescence trace from simultaneously two-colour imaging Myo1
1072 (blue line) and Cam1 (red line) membrane association event observed in
1073 *mNeogreen.myo1 cam1.mCherry* cells is consistent with relative intensities
1074 and timings observed using single fluorophore strains. (E) Averaged intensity
1075 trajectories of individual Myo1 (blue line) and Myo1.S742A (red line)
1076 endocytosis events from TIRFM imaging of *mNeogreen.myo1* and
1077 *mNeogreen.myo1.S742A* cells respectively.

1078 **Figure 4. Myo1 S742 is phosphorylated in a cell cycle dependent manner**

1079 **to affect polarised growth.** (A) Western blots of extracts from G₁ arrested
1080 *cdc10.v50* cells, and pre-mitotic G₂ arrested *cdc25.22* cells probed with
1081 phospho-specific anti-Myo1^{S742} (upper panel) and anti-Myo1 (lower panel)
1082 antibodies demonstrate Myo1^{S742} phosphorylation occurs prior to the Cdc10
1083 execution point in monopolar G1 cells, and is not detectable by the Cdc25
1084 execution point at end of G2 (n=3). (B - D) A *cdc10.v50* culture was
1085 synchronized in G1 by shifting to 36 °C for 240 min before returning to 25 °C
1086 at time 0. Samples of cells were taken every 20 min from the release and
1087 processed for western blotting to monitor Myo1^{S742} phosphorylation. The
1088 membrane was first probed with phosphospecific anti-Myo1^{S742*} antibodies
1089 (B) and subsequently probed with anti-Myo1 antibodies (C) to monitor total
1090 Myo1. Both phosphorylated (1) and non-phosphorylated (2) Myo1 bands can
1091 be observed in B. Equal loading was monitored by Ponceau staining of the
1092 membrane. (D) Densitometry measurements of phosphorylated Myo1^{S742}
1093 (from B) and total Myo1 (both bands from C) are plotted along with the % of
1094 cells in the culture with septa. (E) Myosin-1 distribution (green), calcofluor
1095 stained regions of cell growth (magenta) and cell outline (transmitted image)
1096 of prototroph *mNeogreen.myo1⁺* and *mNeogreen.myo1.S742A* cells
1097 cultured in EMMG at 34 °C. Asterisks highlight cells with morphology defects.
1098 Scale: 10 µm. (F) Calcofluor stained WT and *myo1.S742A* cells. Asterisks
1099 highlight long bent cells displaying monopolar growth. Scale - 5 µm.

1100

1101 **Figure 5. Myo1^{S742} phosphorylation impacts polarised cell growth.** (A)
1102 Average length and frequency of growth defects in wt, *myo1.S742A*, *tea4Δ*,
1103 *cam2Δ*, *tea4Δ myo1.S742A*, and *tea4Δ cam2Δ* cells. (B) Ratio of Sla2-
1104 mCherry fluorescence at “new”: “old” cell end, averaged from >30 growing
1105 mid-log *sla2-mCherry myo1⁺* (upper panel) and *sla2-mCherry myo1.S742A*
1106 (lower panel) cells. Boxes plot median and quartile for each length measured,
1107 lines are plotted from the mean average value at each length measured. (C)
1108 Averaged growth curves from 3 independent experiments of prototroph wild
1109 type (empty circles) and *myo1.S742A* (grey filled circles) cells cultured in
1110 EMMG at 34 °C. Slower growth is apparent at the end of log phase in

1111 *myo1.S742A* cells, which go grow more until reaching stationary phase. Error
1112 bars denote s.d.

1113

1114 **Figure 6. Cam2 associates with internalised endocytic vesicles.** (A)
1115 Kymographs of GFP labelled foci from maximum projections of 13-z plane
1116 timelapse images of *mNeongreen.myo1* (upper panel), *cam1.gfp* (middle
1117 panel) and *cam2.gfp* (bottom panel) cells illustrate the static nature of Myo1
1118 and Cam1 endocytic foci when associated with the plasma membrane (black
1119 arrows). Spindle Pole Body (asterisk) associated Cam1 foci are highlighted. In
1120 contrast Cam2 foci displayed extensive lateral movements. (B) Kymographs
1121 generated from single z-plane timelapse images of single endocytic foci
1122 surface during vesicle formation and subsequent internalisation. While Myo1
1123 and Cam1 only associate with the plasma membrane, Cam2, Sla2 and actin
1124 are internalised on the vesicle after scission. These kymographs are not
1125 aligned temporally. (C) Kymographs of Cam2 and Sla2 co-internalisation in
1126 *sla2.mCherry cam2.gfp* cells. (D) Maximum projection of 31-z slice image of
1127 *cam1.mCherry cam2.gfp* cells reveals Cam1 (magenta) and Cam2 (green)
1128 colocalise in a subset of endocytic foci. (E-G) Single frames (left panels) and
1129 kymographs (right panels) from maximum projections of 13-z plane timelapse
1130 images of *cam1.gfp* (E), *cam2.gfp* (F) and *LifeACT.mCherry* (G) in either
1131 *myo1⁺* (upper panels) or *myo1 Δ* (lower panels) cells. These show while only
1132 Cam1 recruitment to endocytic foci is dependent upon Myo1, the myosin is
1133 required for internalisation of Cam2-GFP and LifeACT.mCherry foci. Scales:
1134 5 μ m.

1135 **Figure 7. Cam2 does not impact Myo1 or Cam1 dynamics in vegetative**
1136 **cells.** (A) An example fluorescence trace of a Cam2 membrane binding and
1137 vesicle internalisation event from TIRFM imaging of *cam2.gfp* cells. An abrupt
1138 drop in the fluorescence was marked as "scission time" (T_{scis} , grey vertical
1139 line). Insert: An arrow shows the location of the monitored endocytic event
1140 (5x5 pixels area). (B) Averaged profile from 65 individual Cam2 membrane
1141 association events (green line), together with averaged Cam1-mCherry profile
1142 (red) from two-colour TIRFM imaging of *cam1.mCherry cam2.gfp* cells.

1143 Events were synchronized relative to Cam1 T_{start} . Grey line denotes mean
1144 time of vesicle scission (T_{scis}). See detailed description in the Methods
1145 section. (C) Maximum projection of 31-z slice widefield image of a mixture of
1146 *yfp.myo1 sid4.tdTomato* (wt - with a red labelled spindle pole body marker)
1147 and *yfp.myo1 cam2Δ* (asterisks) cells. Red labelled SPBs allows
1148 differentiation between *cam2⁺* and *cam2Δ* cells in the same field. (D)
1149 Maximum projection of 31-z slice widefield image of a mixture of prototroph
1150 *cam1.gfp sid4.tdTomato* (wt – with a red labelled spindle pole body marker)
1151 and *cam1.gfp cam2Δ* (asterisks) cells. Red labelled SPBs allows
1152 differentiation between *cam2⁺* and *cam2Δ* cells in the same field. Scales – 5
1153 μm .

1154 **Figure 8. Myo1 S742 phosphorylation regulated Cam1 and Cam2**
1155 **dynamics during meiosis.** (A) Kymographs of Cam2.GFP foci dynamics in
1156 *myo1⁺* (upper panel) and *myo1.S742A* (lower panel) cells. (B) Scheme of
1157 consequence of phosphorylation of Myo1^{Ser742} (small empty circle) and Ca²⁺
1158 levels upon Cam1 (light grey filled circle) and Cam2 (dark grey filled circle)
1159 binding to the IQ1 (solid thick black line) and IQ2 (compound line) motifs of
1160 Myo1, and impact on relative orientation of the myosin lever arm (dashed
1161 arrow). Highlighted combination of unphosphorylated Myo1^{S742} & Ca²⁺ does
1162 not normally occur in cells. (C) Western blots of extracts from G1 arrested
1163 *cdc10.v50 myo1⁺*, *cdc10.v50 myo1-S742A*, and conjugation arrested (starved,
1164 premeiotic cells) *fus1Δ* cells, as well as meiotic spores, probed with phospho-
1165 specific anti-Myo1^{S742} antibodies (upper panel; ponceau staining lower panel)
1166 confirm Myo1S742 remains phosphorylated from the end of G1, through
1167 conjugation until the end of meiosis (n=5). Relative Myo1^{S742} phosphorylation
1168 levels were calculated from 3 independent equivalent experiments (mean \pm
1169 sd). (D) Maximum projection of 13-z slice GFP fluorescence image and
1170 transmitted light image from a timelapse of vegetative (cell 1) and meiotic (cell
1171 2) *gfp-act1* cells. Image from a GFP-act signal. Kymographs in the right
1172 panels were generated along the two dotted axes. (E) Maximum projection of
1173 mNeogreen-Myo1 fluorescence (left), FM4-64 fluorescence (middle) and
1174 transmitted light images of a mixed population of vegetative, fusing (*) and
1175 sporulating (**) *mNG-myo1⁺* cells, illustrate endocytosis is reduced in meiotic

1176 cells. Scale - 10 μm . (F) Micrographs of *myo1.S742A* cell morphology on solid
1177 starvation media. Asterisks - cells with unregulated growth and polarity
1178 defects; arrows - cells with elongated or abnormally bent shmooing
1179 (conjugation) tips; arrow heads - meiotic cells with defective spore formation.
1180 Scale - 5 μm . (G) A schematic of the TORC2 - Gad8 - Myo1^{S742} signalling
1181 pathway.

1182

1183 **Supplementary Data Legends**

1184 **Figure 2-figure supplement 1. Purified proteins used during *in vitro***
1185 **studies.** Coomassie stained SDS-PAGE gel of recombinant proteins
1186 expressed and purified during this study. From left to right lanes contain (L)
1187 protein standard; (1) Nt-acetylated Cam1; (2) Nt-acetylated Cam1-T6C; (3)
1188 Cam1-FRET; (4) Cam2; (5) IQ12 peptide (not used during this study); (6)
1189 Myo1IQ12-FRET; and (7) Myo1IQ12S742D-FRET.

1190 **Figure 2-figure supplement 2. Cam1 and Cam2 do not interact directly.**
1191 (A) Overlaid OD280 spectra were recorded of eluate from a Superdex 75 gel
1192 filtration column which had been loaded with either Cam1 (grey line), Cam2
1193 (black line) or both Cam1 and Cam2 (red line) under identical 4 mM EGTA
1194 buffer conditions. (B) Maximum IAANS fluorescence values (440 nm) of 0.5
1195 μ M Cam1-IAANS at a range of pCa values. Black symbols show values of
1196 Cam1-IAANS, red symbols show values of Cam1-IAANS with 5 μ M Cam2
1197 protein. 2 mM Ca- EGTA buffers were used to give indicated pCa values.
1198 pCa₅₀ values calculated from Origin fitting analysis - Hill equation.

1199 **Figure 3-figure supplement 1. Relative TIRF profiles.** (A) Maximum
1200 projections of 31-z stack widefield images of *mNG.myo1*, *cam1.gfp* and
1201 *cam2.gfp* cells (Scales - 5 μ m). (B) Combined profiles of averaged intensity
1202 trajectories of Myo1 (blue) and Cam1 (red) endocytosis events in wild type
1203 (solid lines) or *myo1.S742A* strains (dashed lines). TIRF imaging, see
1204 Methods section for the description. Insert: The distribution of the durations of
1205 individual wild type Myo1 events.

1206 **Supplementary File 1: Strains used during this study.**

1207 **Supplementary File 2: Oligonucleotides used during this study.**

1208 **Video 1:** Single molecule Myo1 membrane association events. TIRFM
1209 imaging of *mNeongreen.myo1* cell showing rapid single molecule interactions
1210 of Myo1 with the plasma membrane, apparent as small bright green spots of a
1211 diffraction limited size, visible over background from the camera noise
1212 (highlighted by tracking white lines). These single myosin1 molecules have

1213 limited residency time (off rate 7.8 s^{-1}) and mobility ($0.03 \mu\text{m}^2.\text{s}^{-1}$) at the
1214 plasma membrane. 63 fps @ 23°C . The plot at the end of the Video was
1215 constructed from analysis of a 50 sec full length Video.

1216 **Video 2:** Endocytic Myo1 events. TIRFM imaging of *mNeongreen.myo1* cells
1217 showing endocytosis associated interactions of Myo1 at the plasma
1218 membrane. The myosin1 quickly accumulates at the site of endocytosis and
1219 5-8 seconds of reaching a maximum, rapidly leaves the endocytic site. The
1220 accumulated myosin remains immobile on the membrane for the duration of
1221 the event. 20 fps @ 23°C .

1222 **Video 3:** Endocytic Cam1 events. TIRFM imaging of *cam2.gfp* cell showing
1223 Cam2 recruiting to endocytic vesicles, to which it remains associated after
1224 scission and internalisation of the endosome. At the start of each event each
1225 spot is immobile, but at the end of endocytic event the vesicle oscillates as it
1226 is internalised into the cytoplasm. 20 fps @ 23°C .

1227 **Video 4:** Comparison of Cam1 and Cam2 dynamics. TIRFM imaging of
1228 *cam1.mCherry cam2.gfp* cell showing early recruitment of Cam1 (red) and
1229 subsequent recruitment of Cam2 (green) to the sites of endocytosis. Cam1
1230 disassociates prior to vesicle scission, while Cam2 remains associated with
1231 the internalised endosome, so that at the beginning of the endocytic event the
1232 spots are red, but during the event they became yellow as Cam2 (green) is
1233 recruited to the vesicle. At the end of the event vesicle became green as
1234 Cam1 dissociates from the endocytotic site. 20 fps @ 23°C .

1235 **Video 5:** Myo1 dynamics in interphase and meiotic cells. Timelapse of
1236 maximum projections from 13-z slice widefield images of *mNeongreen.myo1*
1237 cells showing typical examples of Myo1 dynamics in vegetative and meiotic
1238 (highlighted with arrow) cells. Frame rate: 650 msec / frame.

1239 **Video 6:** Cam1 dynamics in interphase and meiotic cells. Timelapse of
1240 maximum projections from 13-z slice widefield images of *cam1.gfp* cells
1241 showing typical examples of Cam1 dynamics in vegetative and meiotic
1242 (highlighted with arrow) cells. Frame rate: 650 msec / frame.

1243 **Video 7:** Cam2 dynamics in interphase and meiotic cells. Timelapse of
1244 maximum projections from 13-z slice widefield images of *cam2.gfp* cells
1245 showing typical examples of Cam2 dynamics in vegetative and meiotic
1246 (highlighted with arrow) cells. Frame rate: 650 msec / frame.

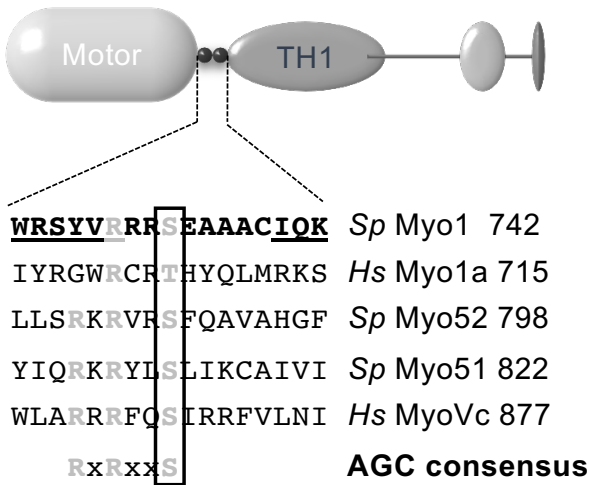
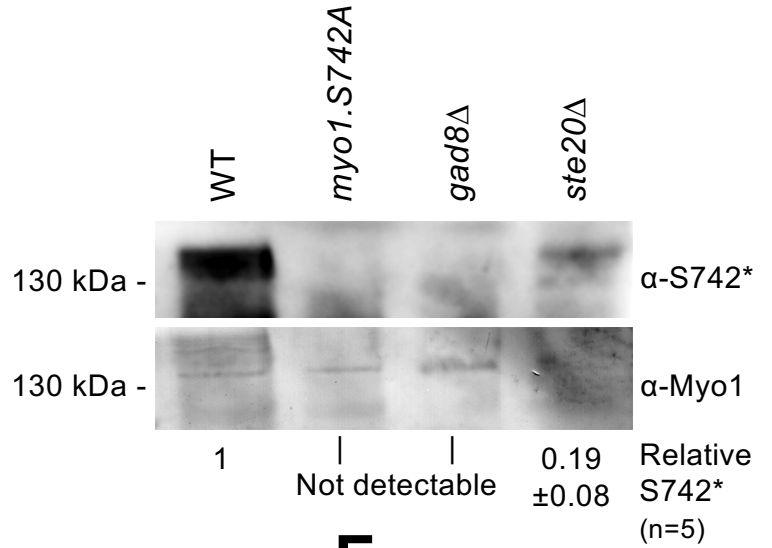
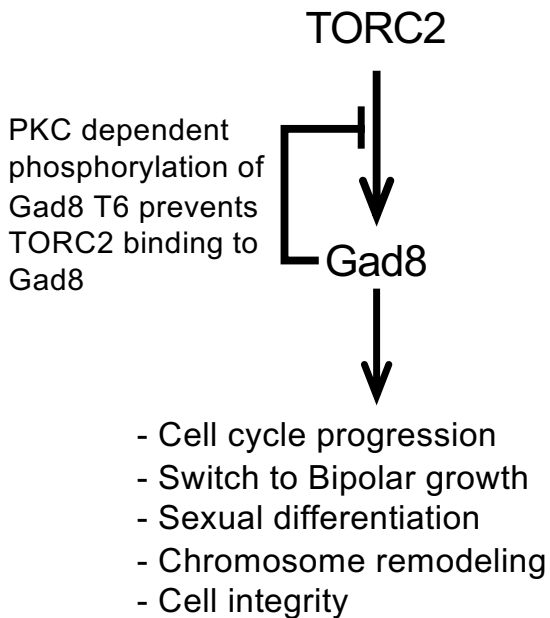
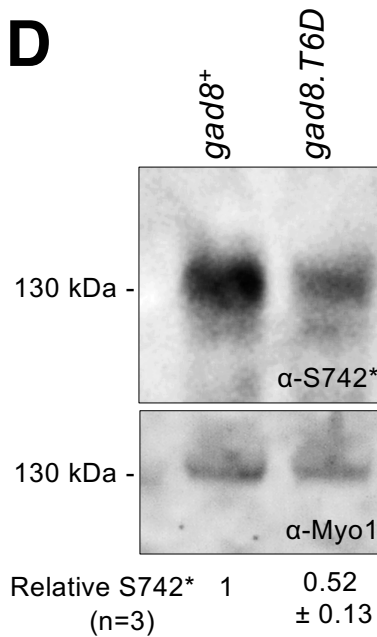
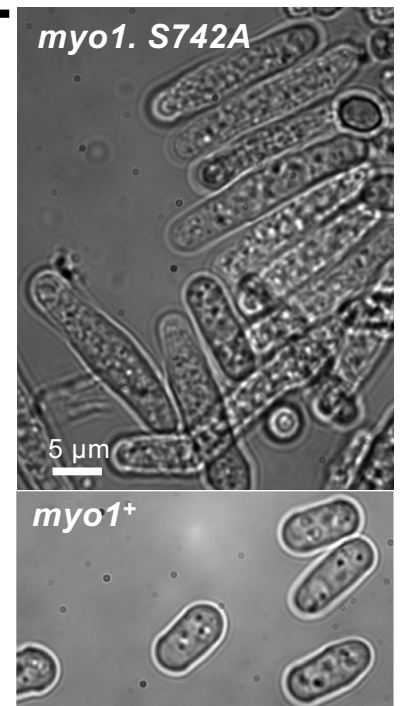
1247 **Video 8:** Act1 dynamics in interphase and meiotic cells. Timelapse of
1248 maximum projections from 13-z slice widefield images of *gfp.act1* cells
1249 showing typical examples of Act1 dynamics in vegetative and meiotic
1250 (highlighted with arrow) cells. Frame rate: 650 msec / frame.

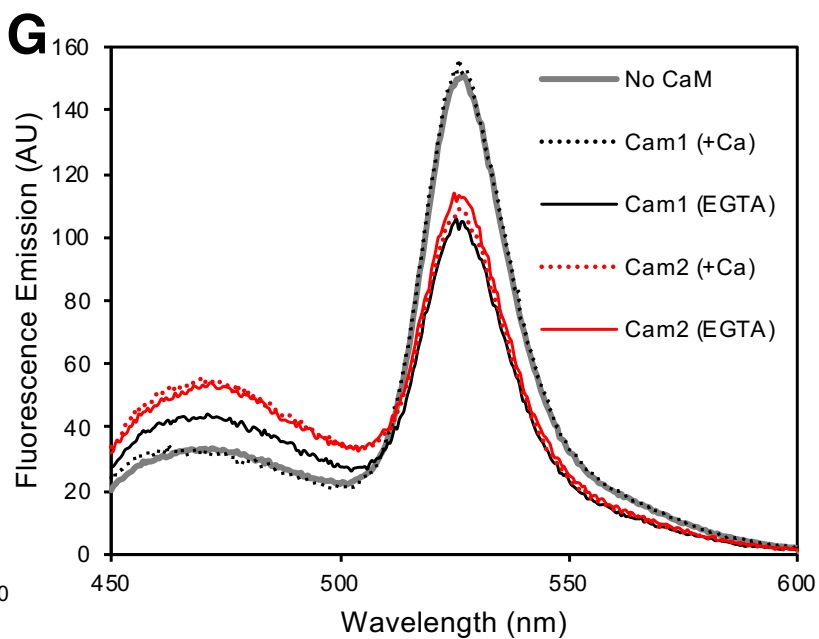
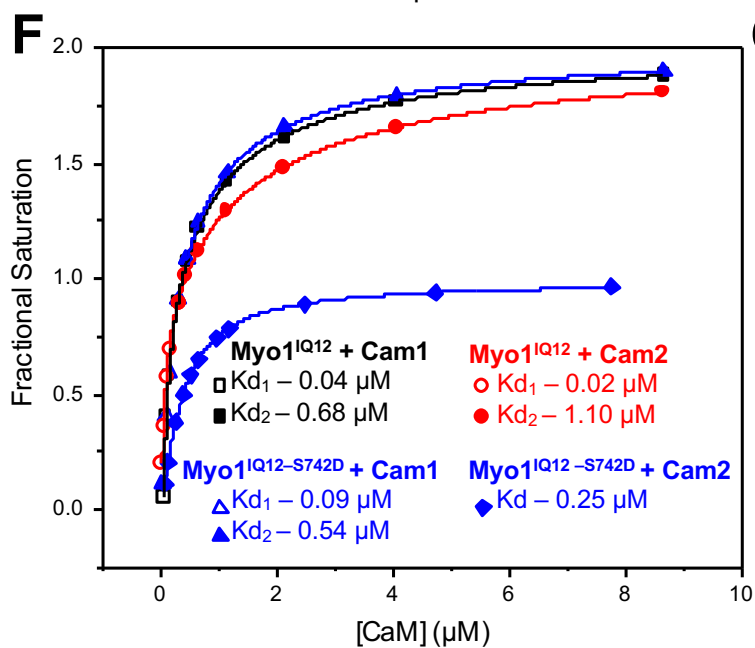
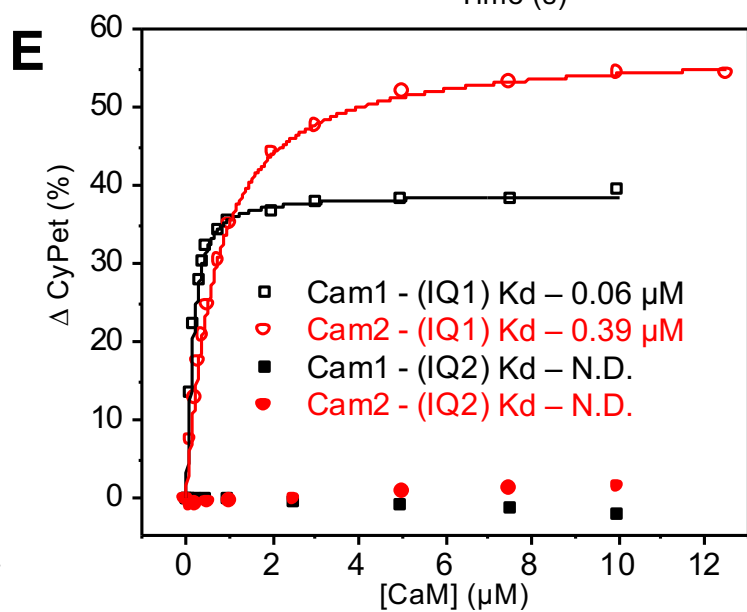
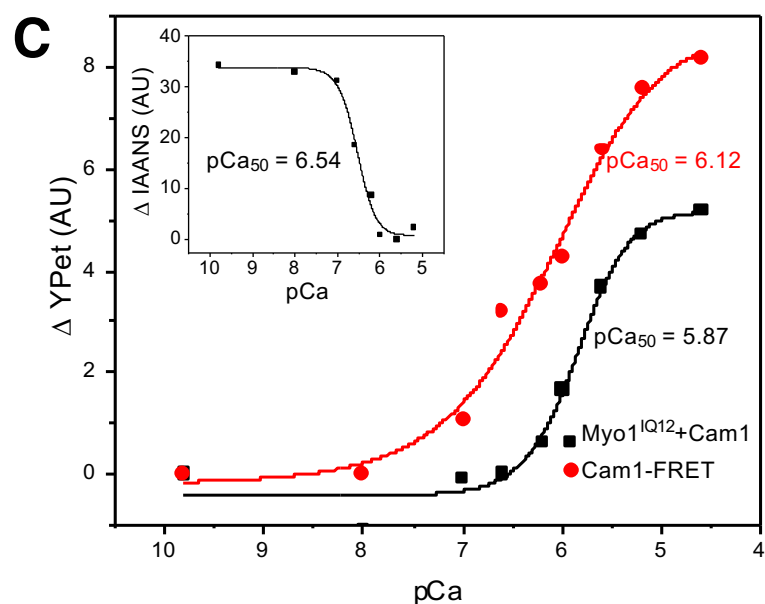
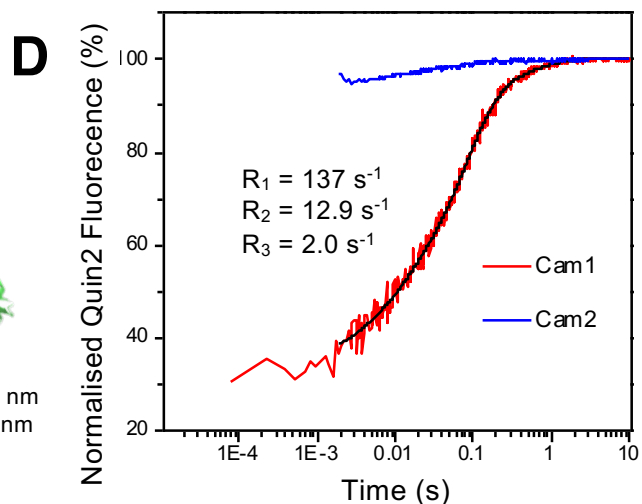
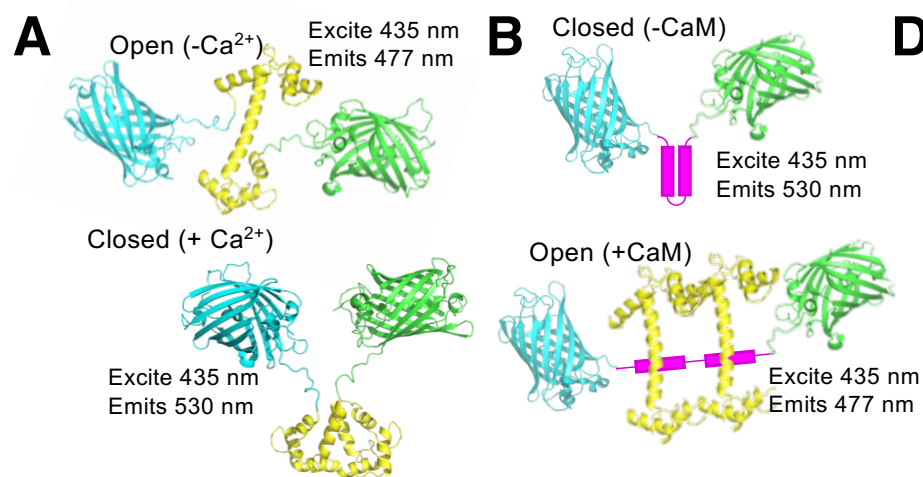
1251 **Supplementary File 1:** Strains used during this study.

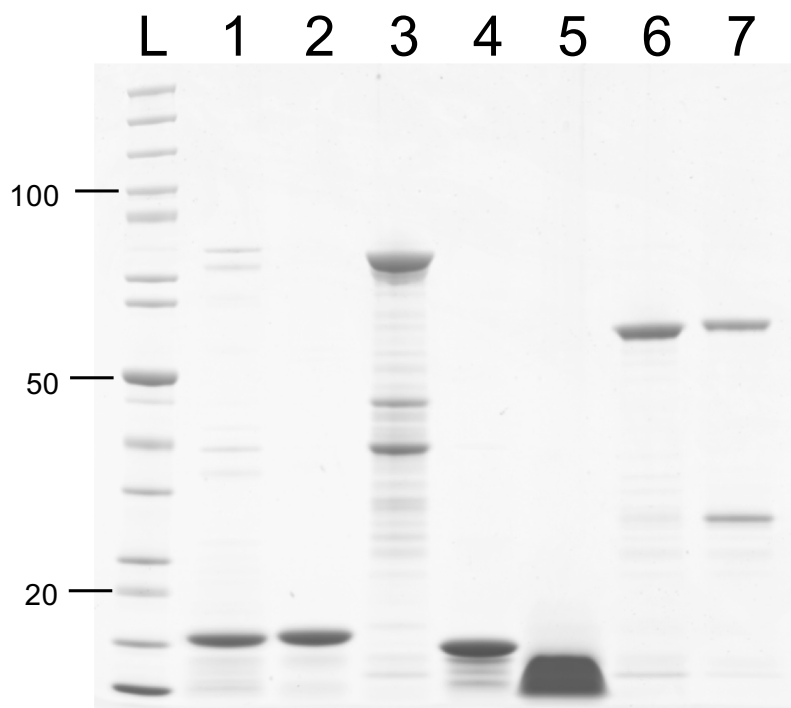
1252 **Supplementary File 2:** Oligonucleotides used during this study.

1253

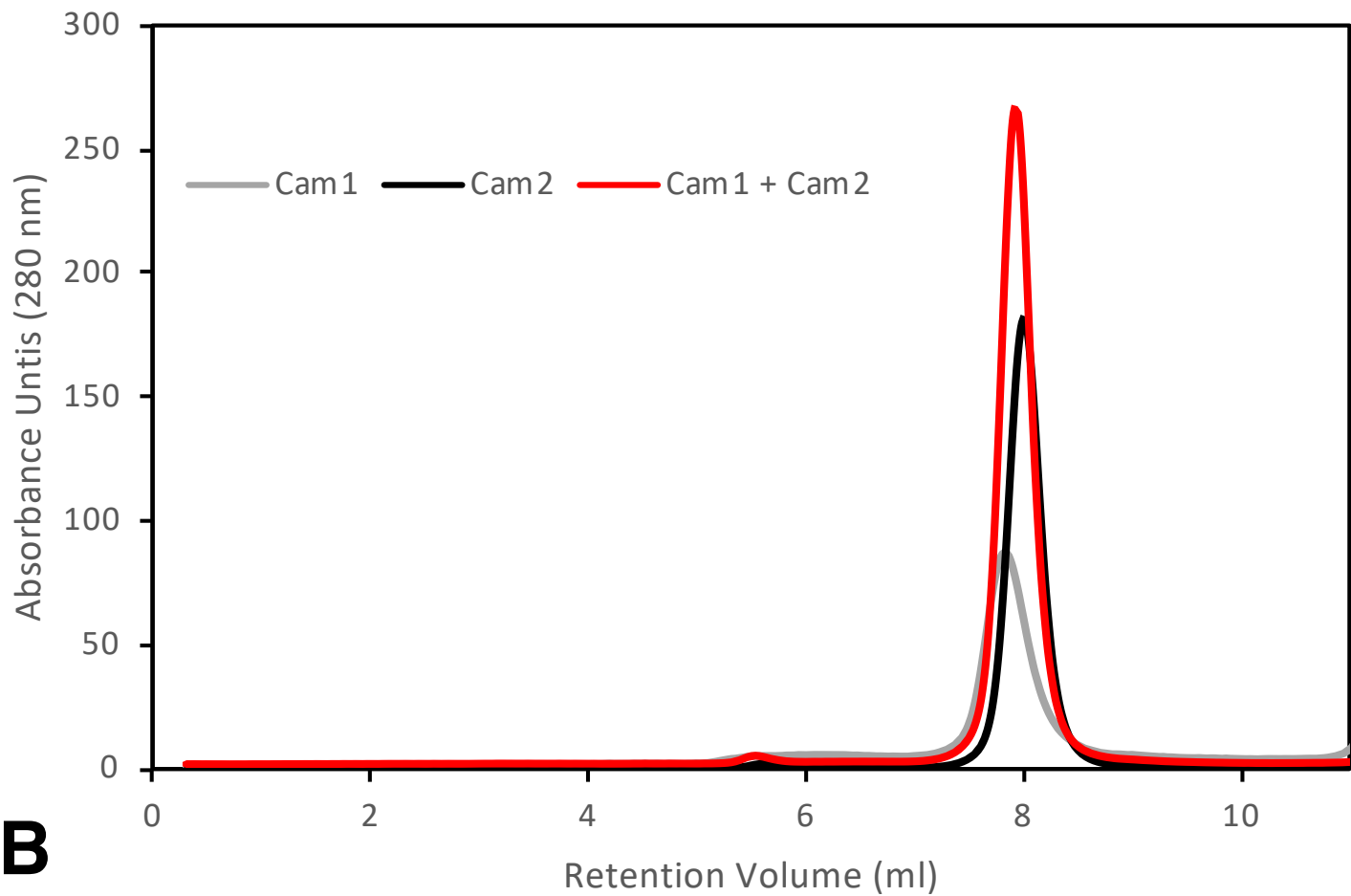
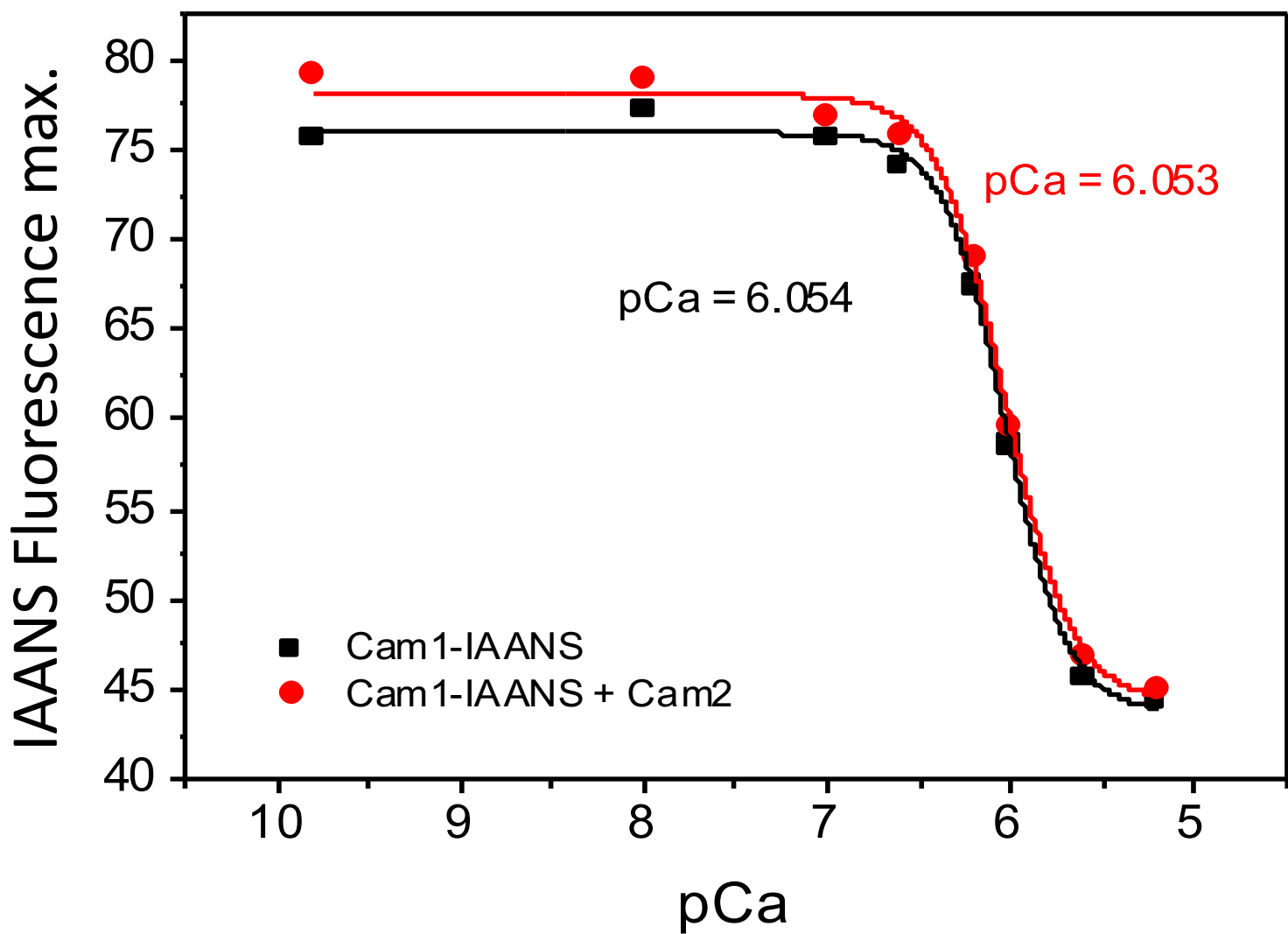
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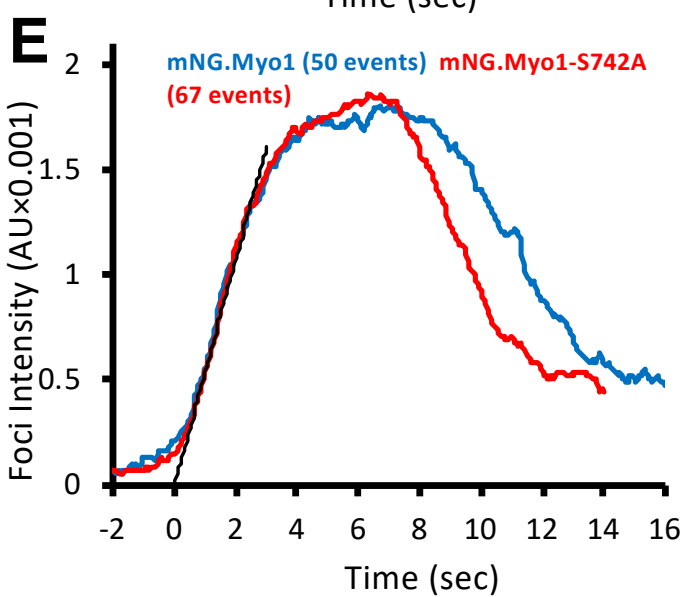
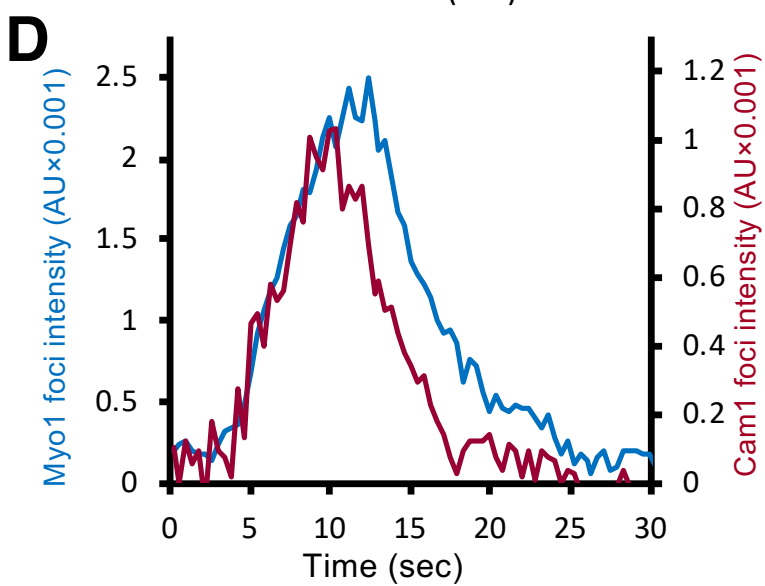
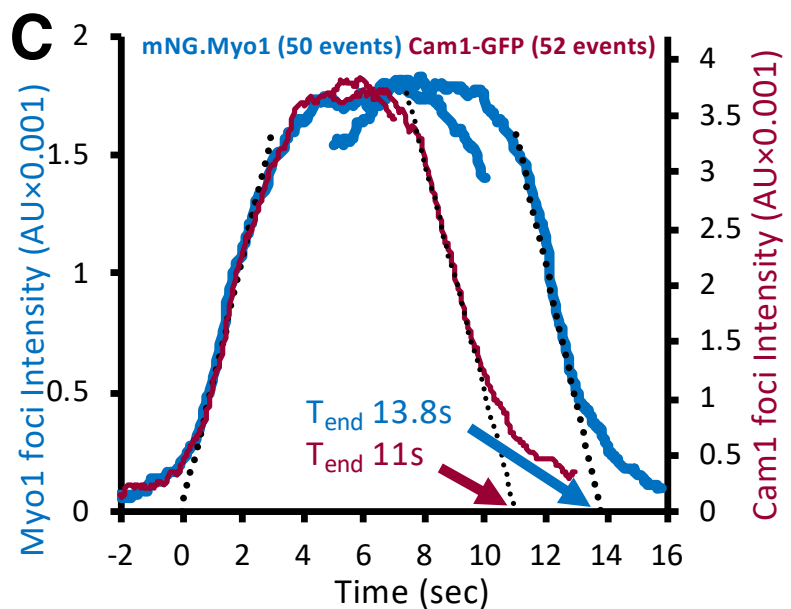
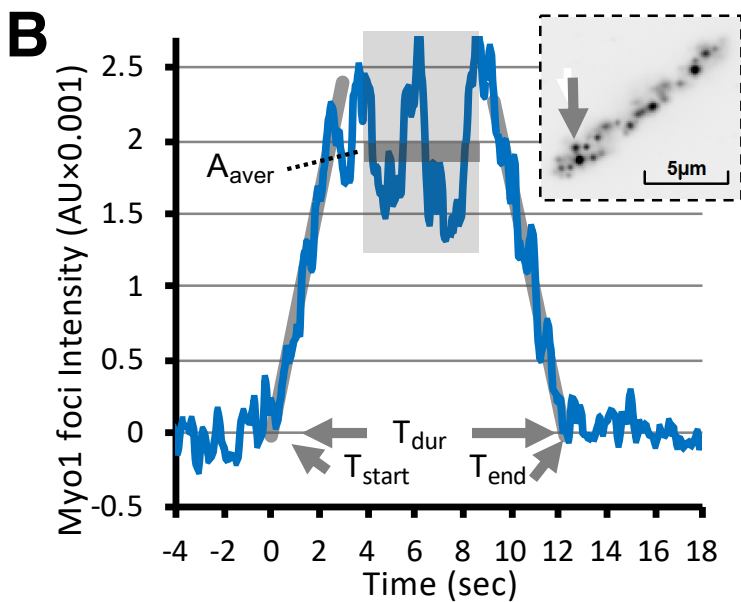
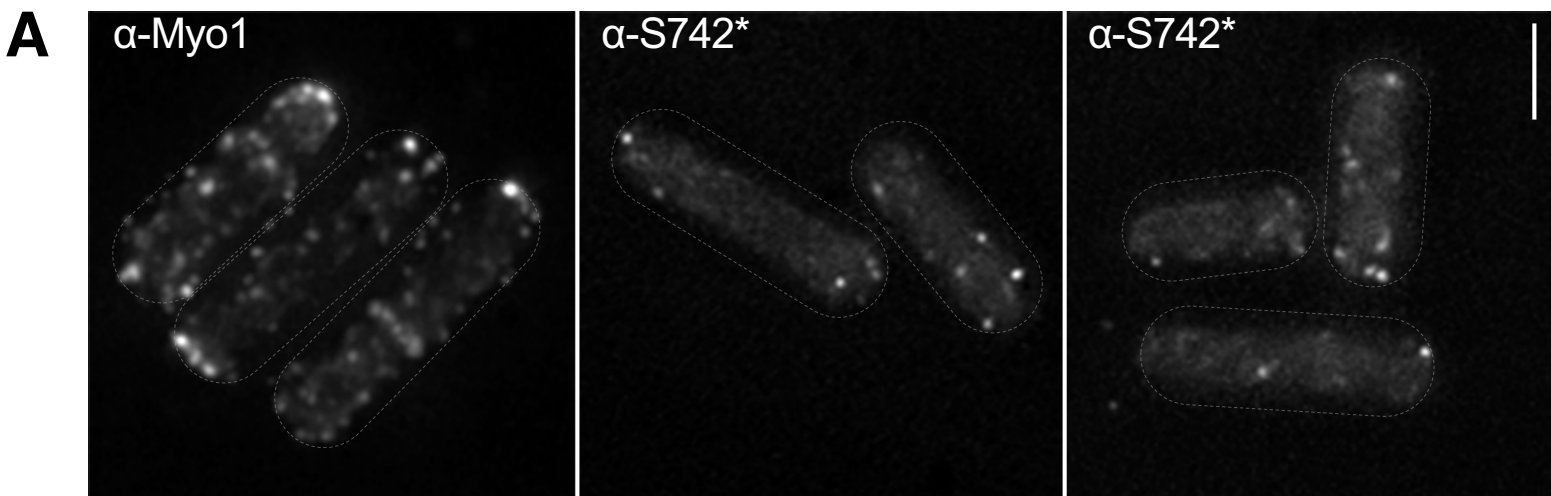
A**B****C****D****E**

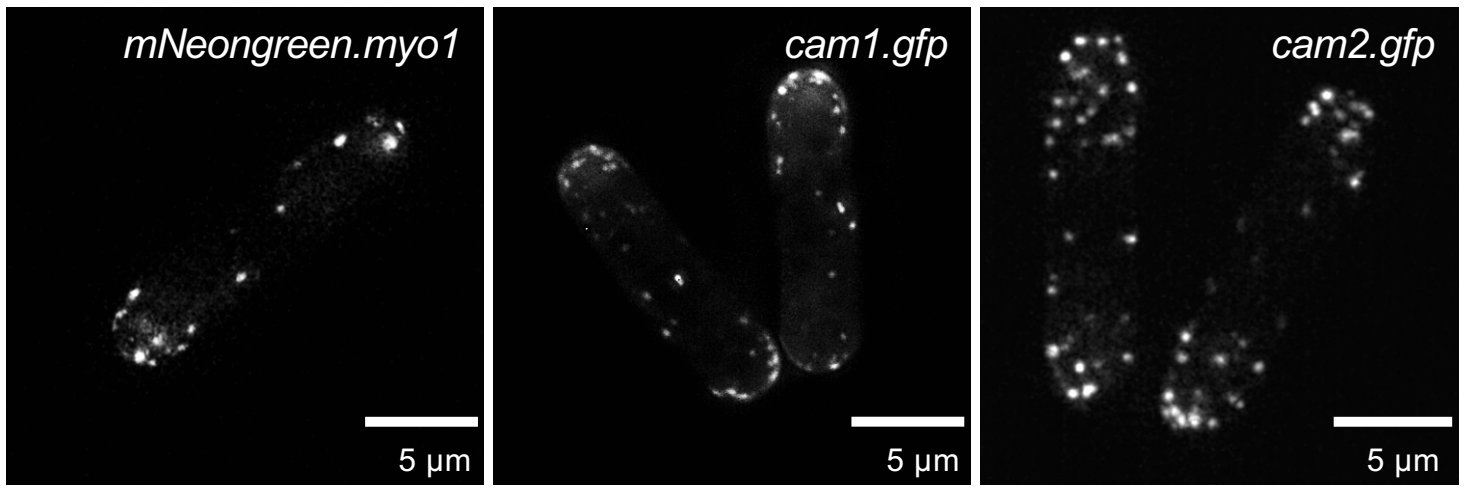
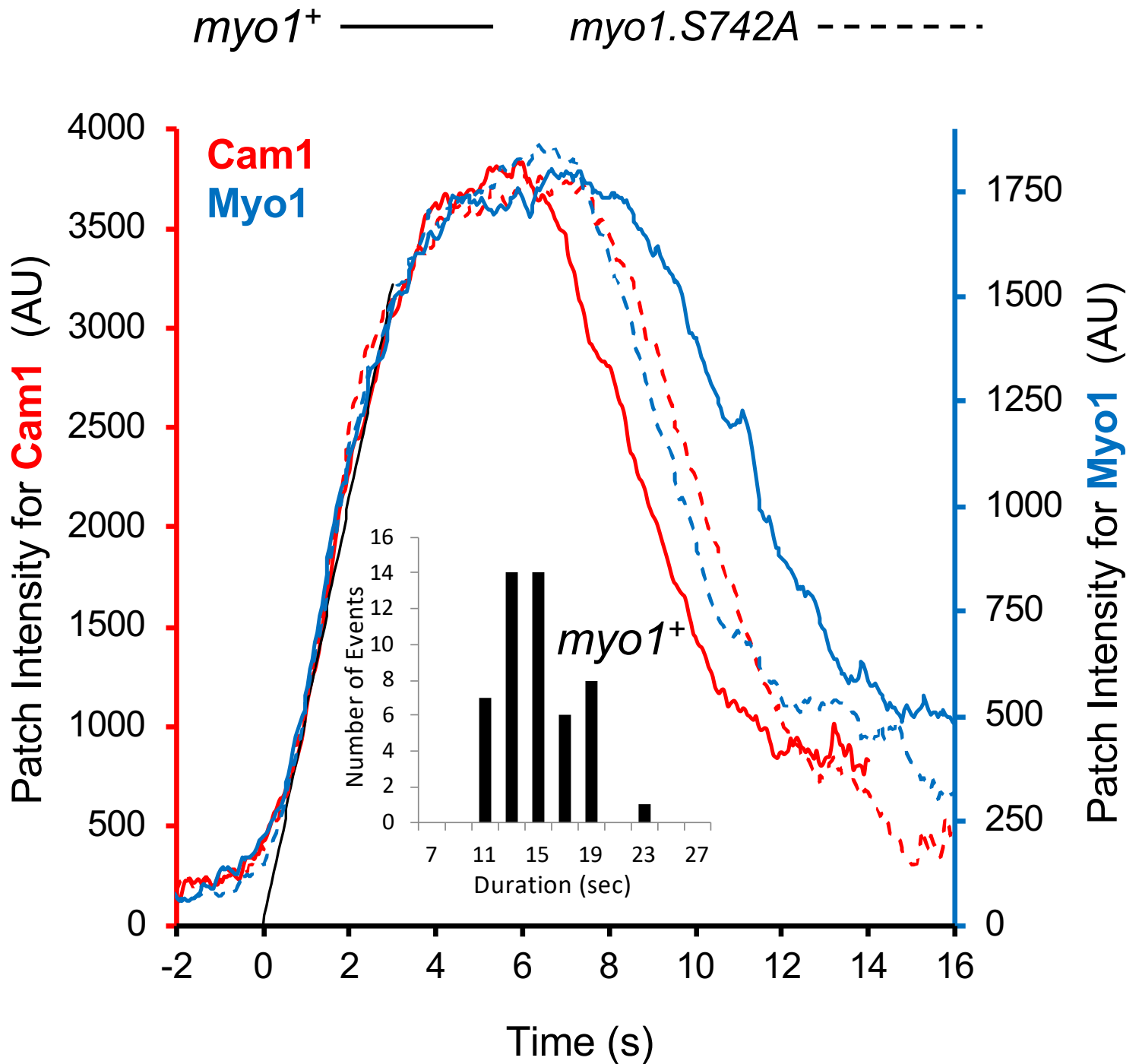


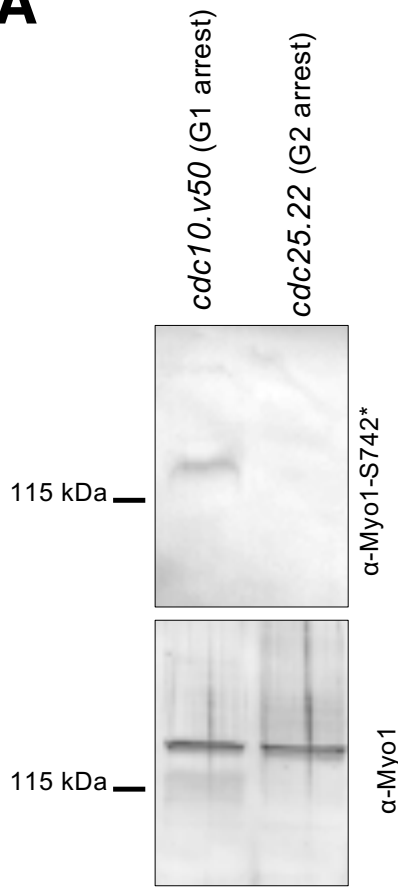
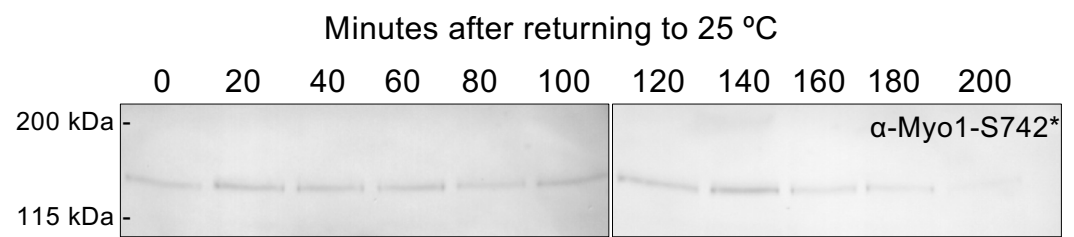
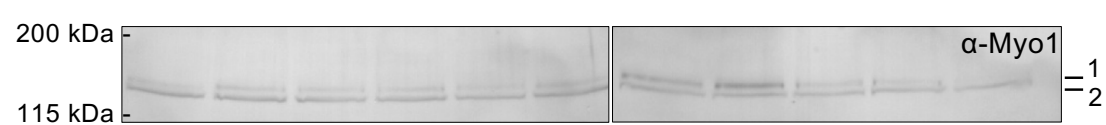
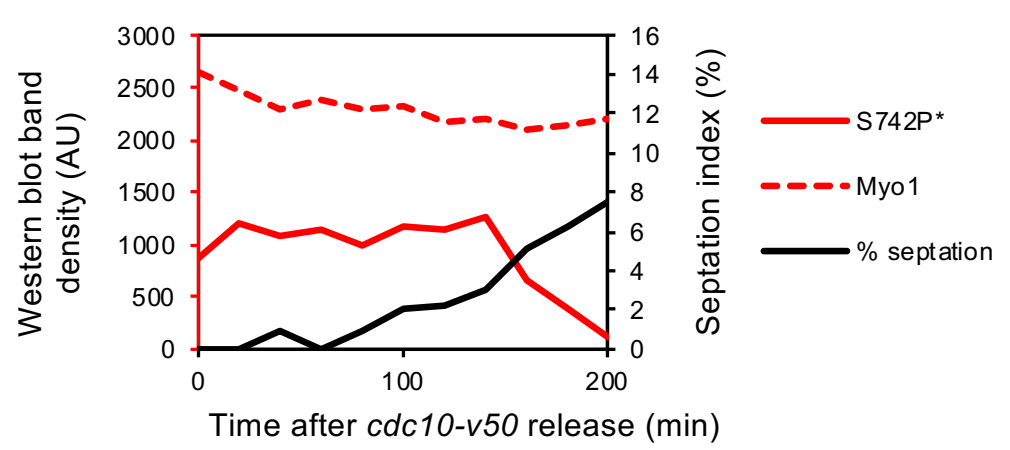
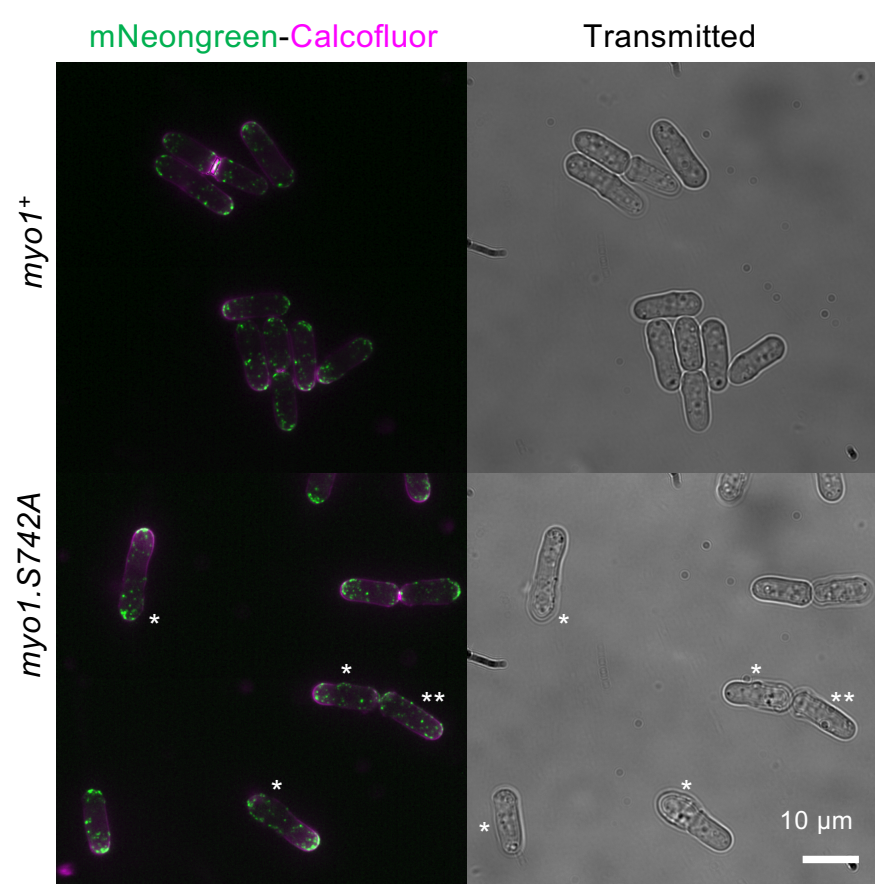
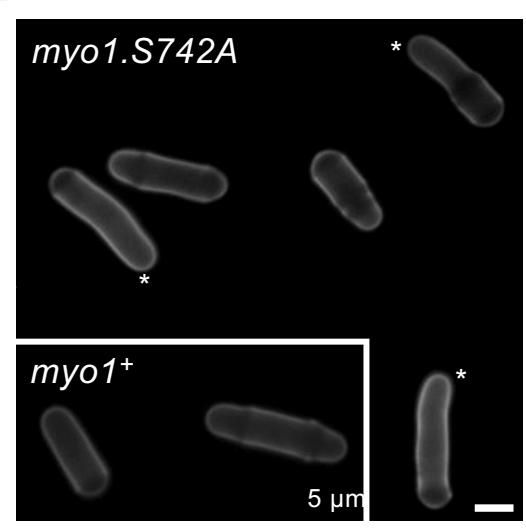


- L – Ladder
- 1 – Cam1-ACE
- 2 – Cam1-IAANS
- 3 – Cam1-FRET
- 4 – Cam2
- 5 – IQ12 peptide – not used in study
- 6 – Myo1IQ12 FRET
- 7 – Myo1IQ12-S742D FRET

A**B**

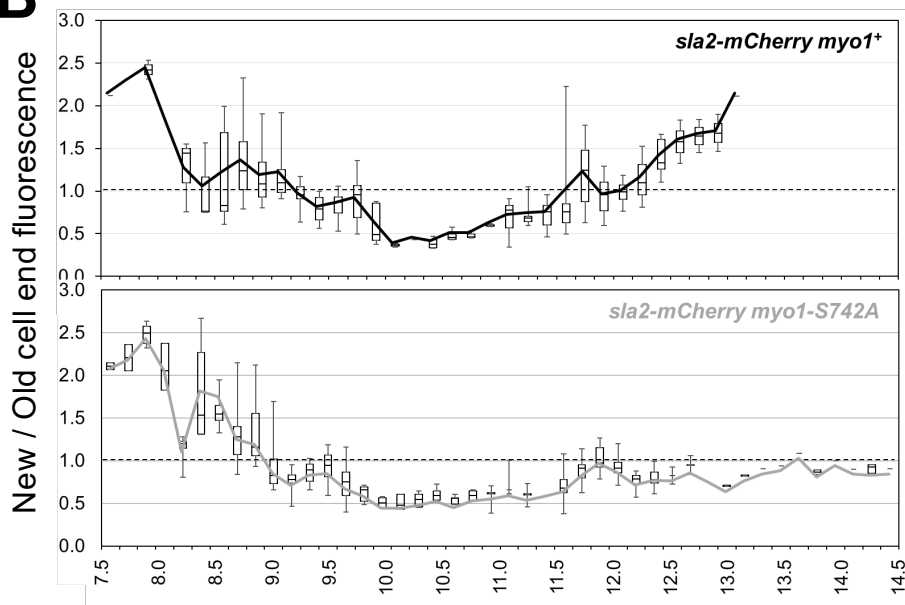
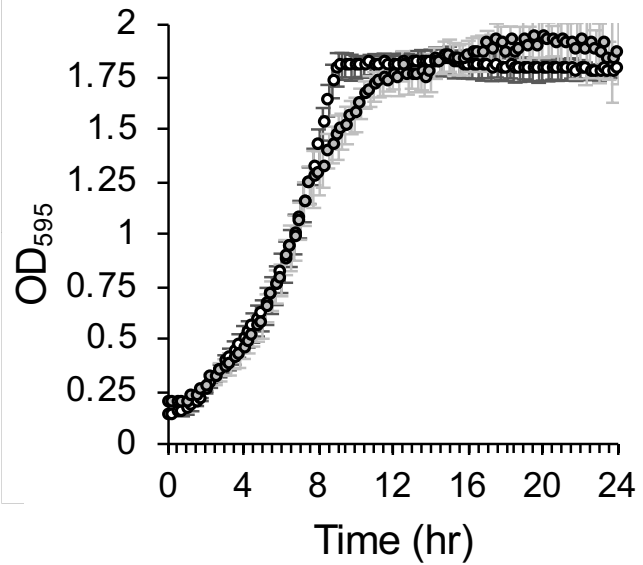


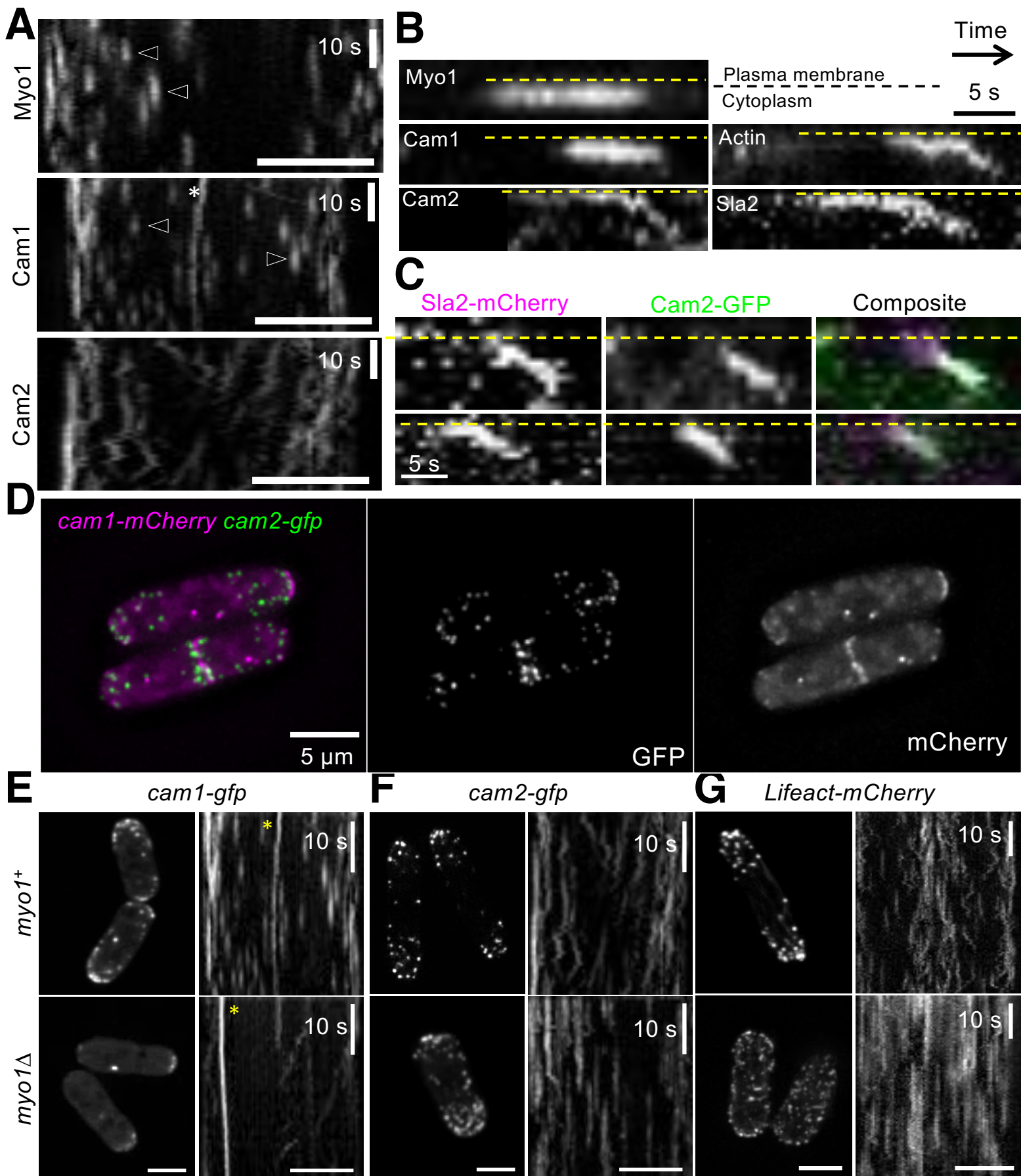
A**B**

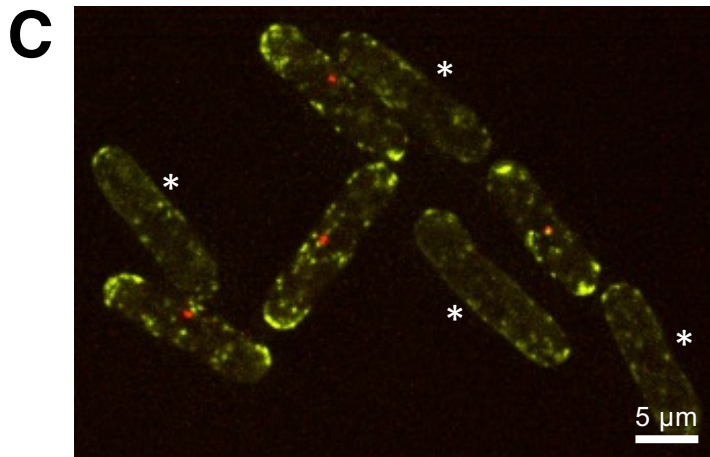
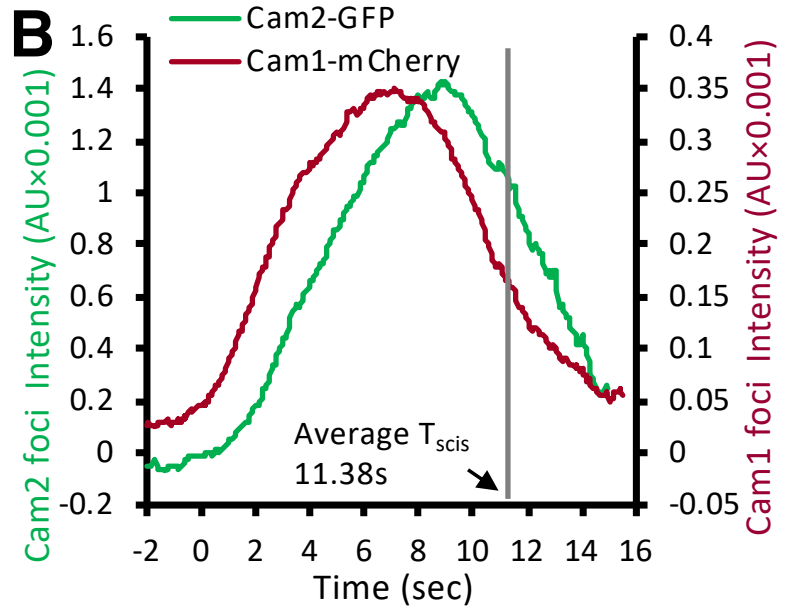
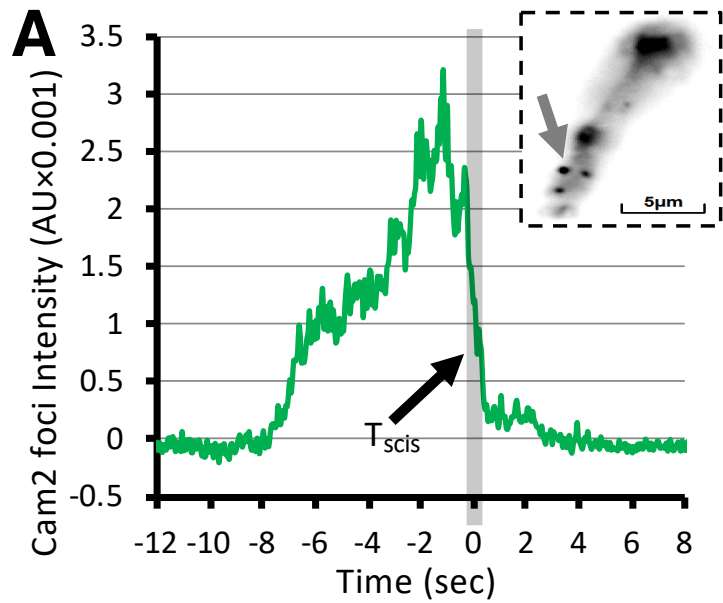
A**B****C****D****E****F**

A

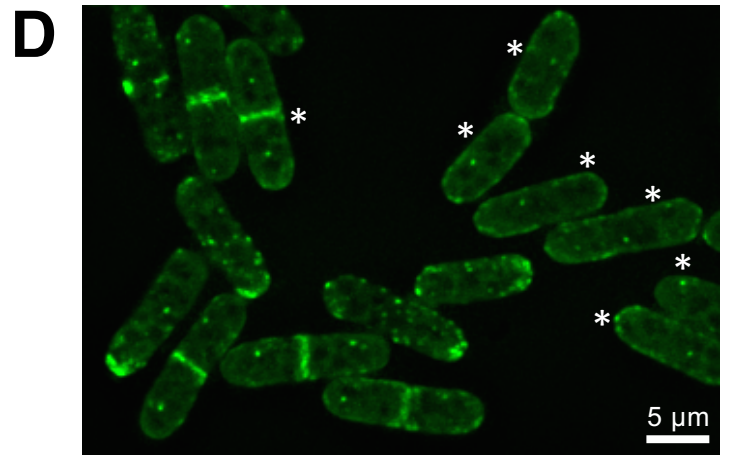
	wt	<i>myo1.S742A</i>	<i>tea4</i> Δ	<i>cam2</i> Δ	<i>tea4</i> Δ <i>myo1.S742A</i>	<i>tea4</i> Δ <i>cam2</i> Δ
Average cell length (μm)	9.77	13.2	12.5	13.2	12.5	12.7
% Bent $>5^\circ$ off long axis	0.4	24.7	51.7	18.3	49.4	66.7
% multiple growth zones	0.0	0.0	5.7	0.0	4.6	18.9
n	>500	>500	88	83	88	91

B**C**





yfp.myo1⁺ sid4.tdTomato (SPB) *cam2⁺*
and *myo1⁺ cam2 Δ* (*) cells



cam1.gfp sid4.tdTomato (SPB) *cam2⁺*
and *cam1.gfp cam2 Δ* (*) cells

