

## Monitoring *S. pombe* genome stress by visualizing end-binding protein Ku

Chance Jones & Susan L Forsburg<sup>1</sup>  
Program in Molecular & Computational Biology  
University of Southern California  
Los Angeles CA 90089

<sup>1</sup> corresponding author Forsburg@usc.edu 213-740-7342

### **Abstract**

Studies of genome stability have exploited visualization of fluorescently tagged proteins in live cells to characterize DNA damage, checkpoint, and repair responses. In this report, we describe a new tool for fission yeast, a tagged version of the end-binding protein Pku70 which is part of the KU protein complex. We compare Pku70 localization to other markers upon treatment to various genotoxins, and identify a unique pattern of distribution. Pku70 provides a new tool to define and characterize DNA lesions and the repair response.

## 1 **Introduction**

2           The response to genome stress and DNA repair can be observed in living cells in real  
3 time, by monitoring fluorescently-tagged DNA damage response proteins (Lisby, M. *et al.* 2004;  
4 Lukas, C., *et al.* 2005; Nagy, Z., & Soutoglou, E., 2009; Polo, S. E., & Jackson, S. P. 2011).  
5 This has allowed characterization of dynamic processes that respond to damage and preserve  
6 genome integrity, including cell cycle, checkpoint, repair, and recovery pathways. In the fission  
7 yeast *S. pombe*, accumulation of foci of the single-strand DNA binding protein Ssb1 (a subunit  
8 of Replication Protein A /RPA), and of the recombination protein Rad52, have been used to  
9 characterize intrinsic genome stresses as well as the response to external genotoxins (Meister, P.,  
10 *et al.* 2003; Kilkenny, M. L., *et al.* 2008; Carneiro, T., *et al.* 2010; Bass, K. L., *et al.* 2012;  
11 Sabatinos, S.A., *et al.* 2012). These proteins recognize and respond to single strand DNA  
12 accumulation, which can result from exonuclease activity, resection, processing of replication  
13 forks and recombination intermediates, or R-loop or D-loop formation (Zeman, M. K., &  
14 Cimprich, K. A., 2014)., Sabatinos, S. A., & Forsburg, S. L., 2015). Importantly, this has led to  
15 identification of distinct patterns of accumulation that can serve as fingerprints for different  
16 forms of genome stress (e.g., Sabatinos, S.A., *et al.* 2012; Sabatinos, S.A., *et al.* 2015  
17 Ranatunga).

18           The fission Pku70 protein is the orthologue of the Ku70 subunit of the conserved  
19 heterodimeric Ku complex (Baumann, P., & Cech, T. R., 2000). Ku is abundant and binds  
20 efficiently to DNA double strand breaks (DSBs) (Fell, V. L., & Schild-Poulter, C. 2015; Shibata,  
21 A., *et al.* 2018). Ku is associated with the non-homologous end-joining (NHEJ) mechanism of  
22 DNA double strand break (DSB) repair (Mahaney, B. L., *et al.* 2009) and protects telomeres  
23 (Baumann, P., & Cech, T. R., 2000; Ferreira, M. G., & Cooper, J. P. 2001). Additionally, it  
24 recognizes “one-sided” double strand breaks and ends associated with regressed replication forks  
25 (Teixeira-Silva, A., *et al.* 2017; Foster, S. S., *et al.* 2011; Langerak, P., *et al.* 2011).

26           Ku binding to DNA ends inhibits the resection and accumulation of single strand DNA  
27 that otherwise drives homologous recombination (Shibata, A., *et al.* 2018). Its activity is  
28 coordinated with the Mre11-Rad50-Nbs1 (MRN) protein complex, another early responder to  
29 DNA double strand breaks (Shibata, A., *et al.* 2018; Syed, A., & Tainer, J. A. 2018). MRN is  
30 also linked to DNA DSB end binding (Wang, Q., *et al.* 2014) and resection (Shibata, A., *et al.*  
31 2014) and contributes to DNA damage checkpoint activation (Chahwan, C., *et al.* 2003; Paull, T.

32 T. 2015). The Mre11/Rad32 subunit is able to drive endonucleolytic cleavage of DNA ends that  
33 are blocked by covalently bound proteins such as Spo11 or Top2 (Hartsuiker, E., *et al.* 2009;  
34 Milman, N., *et al.* 2009; Rothenberg, M., *et al.* 2009; Hartsuiker, E., *et al.* 2009; Garcia, V., *et al.*  
35 2011; Reginato, G., *et al.* 2017). To some degree, Ku and MRN act as mutual antagonists; Ku  
36 inhibits short-range resection driven by MRN, and MRN removes Ku to facilitate homologous  
37 recombination (HR) over NHEJ; and to prevent inappropriate repair of single-end breaks  
38 (Langerak, P., *et al.* 2011; Shao, Z., *et al.* 2012; Myler, L. R., *et al.* 2017; Shibata, A., *et al.*  
39 2018). Interestingly, loss of Ku partly suppresses the sensitivity to DNA damage and replication  
40 blocking toxins associated with mutation of MRN (Tomita, K., *et al.* 2003; Williams, R. S., *et al.*  
41 2008; Limbo, O., *et al.* 2007; Langerak, P., *et al.* 2011; Teixeira-Silva, A., *et al.* 2017), which can  
42 lead to excessive Exo1 driven resection but impaired RPA recruitment (Teixeira-Silva, A., *et al.*  
43 2017).

44 In this report, we describe the development of a new fluorescent marker for fission yeast,  
45 the Pku70 subunit of the Ku protein complex that recognizes DNA ends. We constructed a  
46 *pku70<sup>+</sup>*-citrine fusion and integrated into the genome in wild type fission yeast under the  
47 endogenous promoter. We examined its behavior and accumulation in treated and untreated wild  
48 type cells in response to different genotoxins. We compared localization of Ku to Rad52, RPA,  
49 and Mre11 markers and observe a pattern of foci that is distinct from other markers. This  
50 provides a new tool to characterize responses to different forms of genotoxic stress and a useful  
51 addition to the fission yeast tool kit for investigation of the 3-Rs of DNA replication, repair, and  
52 recombination.

53

## 54 **Results**

### 55 **Construction of strains with fluorescently tagged Pku70 and Mre11**

56 Ku (a heterodimer of Pku70/80) and MRN (Mre11/Rad50/Nbs1) protein complexes are  
57 known for high affinity for binding DNA ends (Fell, V. L., & Schild-Poulter, C. 2015; Shibata,  
58 A., *et al.* 2018). We tagged Pku70 on its C-terminal end with Citrine fluorescent protein and  
59 integrated into the endogenous locus (see methods). Using a similar strategy we also tagged  
60 Mre11 on its C terminal end with mCherry fluorescent protein. The resulting strains were  
61 compared to wild type, *pku70Δ*, *mre11Δ* and *rad51Δ* for their growth on four typical genotoxic  
62 drugs: methyl methanesulfonate (MMS), which creates alkylation damage that inhibits DNA

63 replication fork progression; camptothecin (CPT), which blocks Topoisomerase I cleavage;  
64 hydroxyurea (HU), which causes nucleotide starvation and fork pausing; and Phleomycin  
65 (phleo), a radio-mimetic that causes single- and double-strand breaks. Both the Mre11-mCherry  
66 and Pku70-Citrine tagged strains behaved the same as WT under normal growth and genotoxic  
67 stress. The  $\Delta pku70$  strain also shows no sign of genotoxin sensitivity, as reported previously  
68 (Manolis, K. G., *et al.* 2001; Sánchez, A., & Russell, P. 2015). (Fig. 1A)

69

## 70 **Pku70 and Mre11 have increased nuclear signal following genotoxic stress**

71 When we imaged the tagged strains under normal growth conditions, we observed a few  
72 scattered foci in Pku70-citrine cells and diffuse nuclear fluorescence in Mre11-mCherry cells  
73 (Fig. 1B). We next examined the distribution of signal in cells treated with MMS, CPT, Phleo,  
74 or HU at 32°C after 4 hours. There is a significant increase of cells with individual Pku70 nuclear  
75 foci in MMS, CPT, and to a lesser extent Phleo. Cells treated with HU did not show any  
76 significant difference from WT (Fig. 1C). In contrast, the Mre11-mCherry signal showed diffuse  
77 pan-nuclear staining in untreated cells (Fig 1B). Following 4 hours of treatment with the four  
78 genotoxic drugs, Mre11-mCherry did not show obvious foci. Rather, we observed generalized  
79 areas of increased fluorescence over threshold, but these typically were not well-defined discrete  
80 puncta as seen with other markers.

81

## 82 **Colocalization of Pku70 and Mre11 with other markers of DNA damage**

83 Previous studies of genome instability in fission yeast have imaged the single stranded  
84 binding protein Ssb1 (Rad11, RPA) and the homologous recombination protein Rad52 in  
85 response to different forms of replication stress (Meister, P., *et al.* 2003; Kilkenny, M. L., *et al.*  
86 2008; Carneiro, T., *et al.* 2010; Bass, K. L., *et al.*, 2012; Sabatinos, S. A., *et al.* 2012). We  
87 examined co-localization using CPT, MMS, HU and Phleo in a strain with Pku70-citrine, Rad52-  
88 mCherry, and RPA-CFP. Four hours after drug addition at 32°C, we determined frequency of  
89 colocalization among all three tagged proteins. While there was partial overlap, Ku is not  
90 completely concordant with the other markers (Fig. 2A).

91 The number of foci per nucleus was calculated and binned as either 1 or  $\geq 2$  foci using an  
92 automatic foci counter in ImageJ as described in the materials and methods (Fig. 2B). We  
93 observed that CPT 20 $\mu$ M contained the highest frequency of Pku70 foci, then MMS, Phleo, and

94 HU. The difference from prior observation likely reflects a somewhat different drug dosage:  
95 CPT levels were raised from  $10\mu\text{M}$  to  $20\mu\text{M}$  in order to produce an enhanced response and  
96 MMS was lowered from  $.9\text{mM}$  to  $.45\text{mM}$  to better resolve single foci.

97 Colocalization was determined using the objects-based method in the ImageJ plugin  
98 JACoP (see materials and methods). Fig. 2C shows the proportion of Pku70-Citrine foci that  
99 overlap with a thresholded region for Rad52-mCherry or RPA-CFP. For CPT, MMS, and Phleo  
100 these proportions vary from 60-90%. In contrast, the scattered foci in HU showed only about  
101 30% of Ku co-associating with another marker. Fig 2D shows the proportion of Rad52-mCherry  
102 foci that have a colocalizing Pku70-Citrine focus. CPT contained the highest proportion of  
103 Rad52 as well as RPA with overlapping Pku70 foci, whereas HU contained the lowest.

104 We performed a similar study with Mre11-mCherry but could not perform the same  
105 quantitation because Mre11-mCherry does not form discrete foci. We observed areas of  
106 generally increased fluorescence but never clear puncta as with Pku70, Rad52, or RPA.  
107 Observing these cells in three-dimensional reconstruction showed no obvious colocalization  
108 between Rad52-YFP and RPA-CFP in live cell video microscopy, or in static images  
109 (Supplemental Fig. 1; Fig. 3A,B).

110

### 111 **Pku dynamics in S phase specific damage**

112 The genotoxin MMS causes alkylation damage, generating lesions that block DNA  
113 polymerase (Lundin, C., *et al.* 2005). This typically results in replication template switching  
114 (Barbour and Xiao 2003; Andersen *et al.* 2008). Previous work has suggested that Ku is  
115 recruited by blocked and regressed replication forks (Teixeira-Silva, A., *et al.* 2017). Therefore,  
116 we investigated the dynamics of Ku response to MMS treatment as a model for disruptions in  
117 fork progression. We used live cell video microscopy to observe cells containing Rad52-  
118 mCherry and Pku70-Citrine over a 5hr period of MMS treatment at  $28^\circ\text{C}$ . We observe distinct  
119 dynamics for Rad52 and Pku70 recruitment during treatment. While absolute timing differs in  
120 individual cells, typically a Ku focus appears for a short time and partially co-localized with  
121 Rad52.

122 Fig 4A shows a representative newborn cell that has just entered S phase, 1h and 20m  
123 after drug treatment. The diffuse Rad52-mCherry signal distributes into smaller foci which then  
124 coalesce into two large foci. Pku70-Citrine colocalizes at the center of these large foci for about

125 20-40 minutes. The large Rad52-mCherry foci persist another 60 minutes and then begin to  
126 dissipate. Retention time of Pku-Citrine foci in MMS is  $\leq 20$  minutes with a fraction of cells  
127 maintaining it longer between 20 and 40 minutes. In contrast, Rad52 foci extend over a much  
128 longer period of time ranging from 20 all the way up to 160 minutes (Fig. 4B). Overall Rad52  
129 tends to appear slightly earlier than Pku70 in most cells and disappears much later (Fig. 4C).  
130 (Additional time-lapse images found in Supplemental Fig. 2 and 3)

131

## 132 **Discussion**

133         Localization of repair puncta in fission yeast has been a well-established means of  
134 observing DNA damage, quantified by counting foci, determining pixel intensity or size of foci,  
135 and three-dimensional position in the nucleus (Green, M. D., *et al.*, 2015). The most frequently  
136 used fluorescent tags used in *S. pombe* for observing DNA lesions are the recombination protein  
137 Rad52 and single strand DNA binding protein Rad11, a subunit of RPA (Meister, P., *et al.*, 2003;  
138 Carneiro, T., *et al.* 2010; Sabatinos, S. A., *et al.* 2012). Studies have shown that in cycling wild  
139 type cells, approximately 10-20% of cells show evidence of single RPA or Rad52 foci, likely due  
140 to sporadic S phase events. These proteins show distinct patterns in response to genotoxic  
141 stresses induced by mutations in the replication or repair pathways (Sabatinos, S. A., *et al.* 2012;  
142 Sabatinos, S. A., *et al.* 2015; Ranatunga, N. S., & Forsburg, S. L. 2016), or in response to  
143 exogenous agents such as hydroxyurea (HU), which causes replication fork stalling (Thelander,  
144 L., & Reichard, P. 1979); MMS, an alkylating agent that generates lesions that block the  
145 replication fork (Lundin, C., *et al.* 2005); camptothecin (CPT), a topoisomerase I inhibitor that  
146 leads to S-phase specific double strand breaks (Li, T. K., & Liu, L. F. 2001); and bleo- or  
147 phleomycin, radiomimetic drugs that causes single- and double-strand breaks (Povirk, L. F.  
148 1996).

149         The current study seeks to expand the library of tagged proteins, part of our strategy to  
150 develop a fingerprint for the response to different forms of genotoxic stress. We investigated  
151 fluorescently tagged Mre11 and Pku70 as markers for DNA breaks.

152         The MRN complex is one of the earliest responders to DSBs (Shibata, A., *et al.* 2018;  
153 Syed, A., & Tainer, J. A. 2018). and is essential to drive resection (Wang, Q., *et al.* 2014;  
154 Shibata, A., *et al.* 2018; Langerak, P., *et al.* 2011; Teixeira-Silva, A., *et al.* 2017). Our Mre11-  
155 mCherry construct showed a diffuse pan-nuclear signal in untreated cells. We did not see

156 obvious focus formation of Mre11-mCherry following treatment with genotoxins. Rather, it  
157 maintained a diffuse signal with regions of brightness. In other systems, MRN has been shown to  
158 be an immediate responder to double strand breaks induced by ionizing radiation (Maser, R. S.,  
159 *et al.* 1997). Our failure to see this form of localization may indicate the timing of our analysis,  
160 and diffuse distribution of lesions in drug treated cells, compared to concentrated sites of damage  
161 from of ionizing radiation.

162 Previous whole-cell localization of Pku70 in *S. pombe* was carried out using C terminal  
163 epitope tagged Pku70 and immunofluorescence on fixed cells (Manolis, K. G., *et al.* 2001). In  
164 unperturbed cells, there is a diffuse pan-nuclear localization. Association of Ku with DNA ends  
165 has been investigated using CHIP methods; in wild type cells, it is not enriched unless the MRN  
166 complex is missing (Langerak, P., *et al.* 2011; Teixeira-Silva, A., *et al.* 2017). Visualization of  
167 Pku70 in live fission yeast cells has not previously been performed.

168 We saw few Ku foci in WT cells, consistent with previous reports. Treatment for 4 hours  
169 with our panel of genotoxins showed that HU has little to no accumulation of Ku foci. Treatment  
170 with CPT causes a modest but limited increase in the fraction of cells with foci at 10 $\mu$ M and a  
171 much more drastic increase at 20 $\mu$ M. Similarly, phleomycin, a radiomimetic that causes DNA  
172 breaks throughout the cell cycle, has a modest but limited increase in foci.

173 The most dramatic increase in fraction of cells with foci was observed with MMS at  
174 0.9mM, an alkylating agent that results in error-free and error prone base excision repair during  
175 S phase, and thus leading to trans lesion synthesis. This induction in MMS is consistent with  
176 prior observations suggesting that Ku is recruited to regressed or broken replication forks in  
177 order to stabilize the free end (Langerak, P., *et al.* 2011; Teixeira-Silva, A., *et al.* 2017). This  
178 suggests that even in MRN<sup>+</sup> cells, there are situations where Ku remains associated with sites of  
179 genome stress.

180 We observed a substantial colocalization between RPA or Rad52 and Ku, in cells treated  
181 with MMS, CPT, or Phleo. This result was a surprise as many models suggest Pku should be  
182 removed by the time resection and recombination proteins are recruited. One possibility for the S  
183 phase specific toxins is that Pku could be binding to reversed forks at repair centers. Previous  
184 studies suggest that Pku plays a role at reversed forks in order to maintain genome stability,  
185 particularly in cells with defective HR repair such as *brc1 $\Delta$*  (Sánchez, A., & Russell, P. 2015;



186 Teixeira-Silva, A., *et al.* 2017). This may reflect that other mechanisms generate ssDNA besides  
187 exonuclease activity, including helicase unwinding and strand invasion.

188 To address this finding in dynamic conditions, we examined MMS-treated cells as a  
189 model for stalled replication forks. Previously, we showed that MMS induces a dramatic  
190 increase in RPA and Rad52 foci relative to other genotoxins (Ranatunga, N. S., & Forsburg, S.  
191 L. 2016). We observe substantial recruitment of Rad52-mCherry and brief, partial co-localization  
192 of Pku70. The Pku70 signal, largely in 1-2 foci, appears after Rad52 and disappears before  
193 Rad52 is resolved. Further molecular work will be required to determine what this signal  
194 represents.

195 It is likely that Ku foci will define distinct structures associated with particular forms of  
196 replication stress. For example, in a recent study, we showed that a mutant *mcm4-dg* with a  
197 defect in the MCM helicase accumulates Ku foci (Kim, S. M., & Forsburg, S. L. 2020). This  
198 accumulation can be reversed by activation of the Mus81 resolvase. Mus81 is essential for  
199 viability in *pku80Δ brc1Δ* mutants (Sánchez, A., & Russell, P. 2015), indicating a collaboration  
200 between Ku and Mus81 in response to replication stress. Our Pku70-citrine fusion will be a key  
201 reagent in dissecting this and other activities.



## 202 **Methods**

### 203 **Cell growth and physiology**

204 Fission yeast strains are described in Table 1, and were grown as in (Sabatinos *et al.*, 2012).

205

### 206 **Construction of Tagged strains**

207 All fragments were lengthened using the Expand Long Template PCR System (Roche  
208 Diagnostics, Mannheim Germany). Primers were designed using the NCBI Primer design tool  
209 and optimized to an annealing temperature of 52-54°C (Ye J, *et al.* 2012). Full length fragments  
210 were transformed using electroporation and selected using the appropriate marker (Sabatinos, S.  
211 A., & Forsburg, S. L. 2010). Upon transformation instead of plating directly onto selective  
212 minimal media the cells were first plated on YES for 24 hours then replica plated onto YES-Hph.  
213 Candidate colonies growing on Hph after 4-5 days were then restreaked onto Hph twice and  
214 visually screened for nuclear localizing foci.

215

### 216 **Pku-Citrine::Hph**

217 The Pku C-terminal Citrine fragment was formed from 5 fragments, Pku 5' overhang (FY2710 +  
218 FY2711), Citrine (FY2561 + FY2562), Hph (FY2563 + FY2564), Citrine UTR (FY2565 +  
219 FY2566), and Pku 3' UTR overhang (FY2712 + FY2713). The Citrine and Citrine UTR  
220 fragments were lengthened from addgene plasmid pKT0139 (Sheff, M. A., & Thorn, K. S.  
221 2004). The Hph fragment was lengthened from pFA6a-hphMX6 (Hentges, P., *et al.* 2005). The  
222 5' and 3' UTR overhang fragments were lengthened from phenol:chloroform extracted WT  
223 (FY527) DNA (Forsburg, S. L., & Rhind, N. 2006). The Citrine, Hph, and Citrine UTR  
224 fragments were first lengthened to form a full Citrine::HPH fragment. A single PCR reaction was  
225 then done with Pku 5' overhang, Citrine HPH, and Pku 3' UTR overhang fragments forming the  
226 full fragment. This fragment was then used for transformation.

227

### 228 **Mre11-mCherry::Hph**

229 The Mre11 C-terminal mCherry fragment was formed from 4 fragments, Mre11 5' overhang  
230 (FY2888 + 2998), mCherry (FY2890 + FY2863), Hph (FY2864 + FY2892), Mre11 3' UTR  
231 overhang (FY2891 + FY2893). The mCherry fragment was lengthened from extracted DNA,  
232 FY8381 (Yu, Y., *et al.* 2013). The Hph fragment was lengthened from the previously formed

233 Citrine::Hph fragment above. The Mre11 5' and 3' UTR overhang fragments were lengthened  
234 from extracted WT DNA (FY527). The mCherry::Hph fragment was first lengthened. The  
235 Mre11 5' overhang, mCherry::Hph, and Mre11 3' UTR overhang fragments were then combined  
236 in one PCR reaction forming the full fragment. The fragment was then used for transformation.

237

### 238 **Live Cell Imaging**

239 Cells were prepared as in (Green, M. D., *et al.* 2015) Medium for all live cell imaging was PMG-  
240 HULALA (PMG + Histidine, Uracil, Leucine, Adenine, Lysine, Arginine) (225mg/L each)  
241 (Sabatinos, S. A., & Forsburg, S. L. 2010) Unless specified all drug concentrations used for  
242 imaging were as follows, MMS .9mM, HU 15mM, CPT 20 $\mu$ M, Phleo 3 $\mu$ M. Strains in liquid  
243 cultures at 32°C were grown to mid-log phase. Cells concentrated by a brief microfuge spin were  
244 applied to 2% agarose pads made from PMG + HULA and prepared on glass slides sealed with  
245 VaLaP (1/1/1 w/w/w vasoline/lanolin/paraffin). Static images were performed at room  
246 temperature 22°C and long term timelapse images were taken at a constant temperature of 28°C.  
247 Images were acquired with a DeltaVision Core (Applied Precision, Issaquah, WA) microscope  
248 using a 60x N.A. 1.4 PlanApo objective lens and a 12-bit Photometrics CoolSnap HQII CCD.  
249 The system x-y pixel size is 0.109 $\mu$ m. softWoRx v4.1 (Applied Precision, Issaquah, WA)  
250 software was used at acquisition. Excitation illumination was from a Solid-state illuminator, CFP  
251 was excited and detected with a 438/24,470/24 filter set (excitation intensity attenuated to 10%)  
252 and a 400ms exposure; YFP was excited and detected with a 513/17,559/38 (excitation intensity  
253 attenuated to 32% for Rad52-YFP and 50% for Pku70-Citrine) filter set and a 200ms exposure.  
254 A suitable polychroic mirror was used. Sections of static timepoints were 20 .20 $\mu$ m z-sections.  
255 Long-term time-lapse videos used 8 z-steps of .35 $\mu$ m. 3-D stacks were deconvolved with  
256 manufacturer provided OTFs using a constrained iterative algorithm, images were maximum  
257 intensity projected for presentation. Images were contrast adjusted using a histogram stretch with  
258 an equivalent scale and gamma for comparability. Brightfield images were also acquired.

259

### 260 **Image processing and analysis**

261 Images were contrast adjusted using an equivalent histogram stretch on all samples.  
262 Significance was assessed with Mann Whitney tests. For publication Long-term time lapse  
263 videos were stabilized in ImageJ using the package “StackReg” by Philippe Thevanaz from the

264 Biomedical Imaging Group at the Swiss Federal Institute of Technology Lausanne. (Thevenaz, P.,  
265 *et. Al* 1998). Foci were automatically quantified using a computational algorithm based on  
266 uniform threshold per fluorescence channel as described by the light microscopy core facility at  
267 Duke University (<https://microscopy.duke.edu/guides/count-nuclear-foci-ImageJ>). Object based  
268 colocalization analysis was performed using the ImageJ plugin JACoP on the same images used  
269 for the foci quantification. However this object based colocalization analysis method still  
270 requires observer-based thresholding before analysis. In order to mitigate observer-based  
271 thresholding bias the number of observed objects after thresholding per fluorescence channel was  
272 calculated to be within 10 foci of the automatically counted foci during the previous computer-  
273 based foci quantification analysis described above.

## References

- Bass, K. L., Murray, J. M., & O'Connell, M. J. (2012). JCS ePress online publication date 24 February 2012.
- Baumann, P., & Cech, T. R. (2000). Protection of telomeres by the Ku protein in fission yeast. *Molecular biology of the cell*, 11(10), 3265-3275.
- Carneiro, T., Khair, L., Reis, C. C., Borges, V., Moser, B. A., Nakamura, T. M., & Ferreira, M. G. (2010). Telomeres avoid end detection by severing the checkpoint signal transduction pathway. *Nature*, 467(7312), 228-232.
- Chahwan, C., Nakamura, T. M., Sivakumar, S., Russell, P., & Rhind, N. (2003). The fission yeast Rad32 (Mre11)-Rad50-Nbs1 complex is required for the S-phase DNA damage checkpoint. *Molecular and cellular biology*, 23(18), 6564-6573.
- Fell, V. L., & Schild-Poulter, C. (2015). The Ku heterodimer: function in DNA repair and beyond. *Mutation Research/Reviews in Mutation Research*, 763, 15-29.
- Ferreira, M. G., & Cooper, J. P. (2001). The fission yeast Taz1 protein protects chromosomes from Ku-dependent end-to-end fusions. *Molecular cell*, 7(1), 55-63.
- Forsburg, S. L., & Rhind, N. (2006). Basic methods for fission yeast. *Yeast*, 23(3), 173-183.
- Foster, S. S., Balestrini, A., & Petrini, J. H. (2011). Functional interplay of the Mre11 nuclease and Ku in the response to replication-associated DNA damage. *Molecular and cellular biology*, 31(21), 4379-4389.
- Garcia, V., Phelps, S. E., Gray, S., & Neale, M. J. (2011). Bidirectional resection of DNA double-strand breaks by Mre11 and Exo1. *Nature*, 479(7372), 241-244.
- Green, M. D., Sabatinos, S. A., & Forsburg, S. L. (2015). Microscopy techniques to examine DNA replication in fission yeast. In *DNA Replication* (pp. 13-41). Humana Press, New York, NY.
- Gould, K. L., Burns, C. G., Feoktistova, A., Hu, C. P., Pasion, S. G., & Forsburg, S. L. (1998). Fission yeast *cdc24+* encodes a novel replication factor required for chromosome integrity. *Genetics*, 149(3), 1221-1233.
- Hartsuiker, E., Mizuno, K., Molnar, M., Kohli, J., Ohta, K., & Carr, A. M. (2009). Ctp1CtIP and Rad32Mre11 nuclease activity are required for Rec12Spo11 removal, but Rec12Spo11 removal is dispensable for other MRN-dependent meiotic functions. *Molecular and cellular biology*, 29(7), 1671-1681.
- Hentges, P., Van Driessche, B., Tafforeau, L., Vandenhoute, J., & Carr, A. M. (2005). Three novel antibiotic marker cassettes for gene disruption and marker switching in *Schizosaccharomyces pombe*. *Yeast*, 22(13), 1013-1019.
- Kilkenny, M. L., Doré, A. S., Roe, S. M., Nestoras, K., Ho, J. C., Watts, F. Z., & Pearl, L. H. (2008). Structural and functional analysis of the Crb2-BRCT2 domain reveals distinct roles in checkpoint signaling and DNA damage repair. *Genes & development*, 22(15), 2034-2047.
- Kim, S. M., & Forsburg, S. L. (2020). Active replication checkpoint drives genome instability in fission yeast *mcm4* mutant. *Molecular and Cellular Biology*.
- Langerak, P., Mejia-Ramirez, E., Limbo, O., & Russell, P. (2011). Release of Ku and MRN from DNA ends by Mre11 nuclease activity and Ctp1 is required for homologous recombination repair of double-strand breaks. *PLoS genetics*, 7(9).

- Liang, D. T., Hodson, J. A., & Forsburg, S. L. (1999). Reduced dosage of a single fission yeast MCM protein causes genetic instability and S phase delay. *Journal of Cell Science*, *112*(4), 559-567.
- Limbo, O., Chahwan, C., Yamada, Y., de Bruin, R. A., Wittenberg, C., & Russell, P. (2007). Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination. *Molecular cell*, *28*(1), 134-146.
- Li, T. K., & Liu, L. F. (2001). Tumor cell death induced by topoisomerase-targeting drugs. *Annual review of pharmacology and toxicology*, *41*(1), 53-77.
- Lisby, M., Barlow, J. H., Burgess, R. C., & Rothstein, R. (2004). Choreography of the DNA damage response: spatiotemporal relationships among checkpoint and repair proteins. *Cell*, *118*(6), 699-713.
- Lundin, C., North, M., Erixon, K., Walters, K., Jenssen, D., Goldman, A. S., & Helleday, T. (2005). Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable in vivo DNA double-strand breaks. *Nucleic acids research*, *33*(12), 3799-3811.
- Lukas, C., Bartek, J., & Lukas, J. (2005). Imaging of protein movement induced by chromosomal breakage: tiny 'local' lesions pose great 'global' challenges. *Chromosoma*, *114*(3), 146-154.
- Mahaney, B. L., Meek, K., & Lees-Miller, S. P. (2009). Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. *Biochemical Journal*, *417*(3), 639-650.
- Manolis, K. G., Nimmo, E. R., Hartsuiker, E., Carr, A. M., Jeggo, P. A., & Allshire, R. C. (2001). Novel functional requirements for non-homologous DNA end joining in *Schizosaccharomyces pombe*. *The EMBO journal*, *20*(1-2), 210-221.
- Maser, R. S., Monsen, K. J., Nelms, B. E., & Petrini, J. H. (1997). hMre11 and hRad50 nuclear foci are induced during the normal cellular response to DNA double-strand breaks. *Molecular and cellular biology*, *17*(10), 6087-6096.
- Meister, P., Poidevin, M., Francesconi, S., Tratner, I., Zarzov, P., & Baldacci, G. (2003). Nuclear factories for signalling and repairing DNA double strand breaks in living fission yeast. *Nucleic acids research*, *31*(17), 5064-5073.
- Milman, N., Higuchi, E., & Smith, G. R. (2009). Meiotic DNA double-strand break repair requires two nucleases, MRN and Ctp1, to produce a single size class of Rec12 (Spo11)-oligonucleotide complexes. *Molecular and cellular biology*, *29*(22), 5998-6005.
- Myler, L. R., Gallardo, I. F., Soniat, M. M., Deshpande, R. A., Gonzalez, X. B., Kim, Y., ... & Finkelstein, I. J. (2017). Single-molecule imaging reveals how Mre11-Rad50-Nbs1 initiates DNA break repair. *Molecular cell*, *67*(5), 891-898.
- Nagy, Z., & Soutoglou, E. (2009). DNA repair: easy to visualize, difficult to elucidate. *Trends in cell biology*, *19*(11), 617-629.
- Paull, T. T. (2015). Mechanisms of ATM activation. *Annual review of biochemistry*, *84*, 711-738.
- Polo, S. E., & Jackson, S. P. (2011). Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. *Genes & development*, *25*(5), 409-433.
- Povirk, L. F. (1996). DNA damage and mutagenesis by radiomimetic DNA-cleaving agents: bleomycin, neocarzinostatin and other enediynes. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *355*(1-2), 71-89.

- Ranatunga, N. S., & Forsburg, S. L. (2016). Characterization of a Novel MMS-Sensitive Allele of *Schizosaccharomyces pombe* mcm4+. *G3: Genes, Genomes, Genetics*, 6(10), 3049-3063.
- Reginato, G., Cannavo, E., & Cejka, P. (2017). Physiological protein blocks direct the Mre11–Rad50–Xrs2 and Sae2 nuclease complex to initiate DNA end resection. *Genes & development*, 31(23-24), 2325-2330.
- Rothenberg, M., Kohli, J., & Ludin, K. (2009). Ctp1 and the MRN-complex are required for endonucleolytic Rec12 removal with release of a single class of oligonucleotides in fission yeast. *PLoS genetics*, 5(11).
- Sabatinos, S. A., & Forsburg, S. L. (2015). Managing single-stranded DNA during replication stress in fission yeast. *Biomolecules*, 5(3), 2123-2139.
- Sabatinos, S. A., & Forsburg, S. L. (2010). Molecular genetics of *Schizosaccharomyces pombe*. In *Methods in enzymology* (Vol. 470, pp. 759-795). Academic Press.
- Sabatinos, S. A., Green, M. D., & Forsburg, S. L. (2012). Continued DNA synthesis in replication checkpoint mutants leads to fork collapse. *Molecular and cellular biology*, 32(24), 4986-4997.
- Sánchez, A., & Russell, P. (2015). Ku stabilizes replication forks in the absence of Brc1. *PLoS one*, 10(5).
- Schindelin, J.; Arganda-Carreras, I. & Frise, E. et al. (2012), "[Fiji: an open-source platform for biological-image analysis](https://doi.org/10.1038/nmeth.2019)", *Nature methods* 9(7): 676-682, PMID 22743772, doi:[10.1038/nmeth.2019](https://doi.org/10.1038/nmeth.2019)
- Shao, Z., Davis, A. J., Fattah, K. R., So, S., Sun, J., Lee, K. J., ... & Chen, D. J. (2012). Persistently bound Ku at DNA ends attenuates DNA end resection and homologous recombination. *DNA repair*, 11(3), 310-316.
- Sheff, M. A., & Thorn, K. S. (2004). Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae*. *Yeast*, 21(8), 661-670.
- Shibata, A., Jeggo, P., & Löbrich, M. (2018). The pendulum of the Ku-Ku clock. *DNA repair*, 71, 164-171.
- Shibata, A., Moiani, D., Arvai, A. S., Perry, J., Harding, S. M., Genois, M. M., ... & Ismail, A. (2014). DNA double-strand break repair pathway choice is directed by distinct MRE11 nuclease activities. *Molecular cell*, 53(1), 7-18.
- Syed, A., & Tainer, J. A. (2018). The MRE11–RAD50–NBS1 complex conducts the orchestration of damage signaling and outcomes to stress in DNA replication and repair. *Annual review of biochemistry*, 87, 263-294.
- Teixeira-Silva, A., Saada, A. A., Hardy, J., Iraqui, I., Nocente, M. C., Fréon, K., & Lambert, S. A. (2017). The end-joining factor Ku acts in the end-resection of double strand break-free arrested replication forks. *Nature communications*, 8(1), 1-14.
- Thelander, L., & Reichard, P. (1979). Reduction of ribonucleotides. *Annual review of biochemistry*, 48(1), 133-158.
- Thevenaz, P., Ruttimann, U. E., & Unser, M. (1998). A pyramid approach to subpixel registration based on intensity. *IEEE transactions on image processing*, 7(1), 27-41.
- Tomita, K., Matsuura, A., Caspari, T., Carr, A. M., Akamatsu, Y., Iwasaki, H., ... & Yoshinaga, K. (2003). Competition between the Rad50 complex and the Ku heterodimer reveals a role for Exo1 in processing double-strand breaks but not telomeres. *Molecular and cellular biology*, 23(15), 5186-5197.

- Wang, Q., Goldstein, M., Alexander, P., Wakeman, T. P., Sun, T., Feng, J., ... & Wang, X. F. (2014). Rad17 recruits the MRE11-RAD50-NBS1 complex to regulate the cellular response to DNA double-strand breaks. *The EMBO journal*, *33*(8), 862-877.
- Williams, R. S., Moncalian, G., Williams, J. S., Yamada, Y., Limbo, O., Shin, D. S., ... & Moiani, D. (2008). Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. *Cell*, *135*(1), 97-109.
- Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden T (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 13:134.
- Yu, Y., Ren, J. Y., Zhang, J. M., Suo, F., Fang, X. F., Wu, F., & Du, L. L. (2013). A proteome-wide visual screen identifies fission yeast proteins localizing to DNA double-strand breaks. *DNA repair*, *12*(6), 433-443.
- Zeman, M. K., & Cimprich, K. A. (2014). Causes and consequences of replication stress. *Nature cell biology*, *16*(1), 2-9.

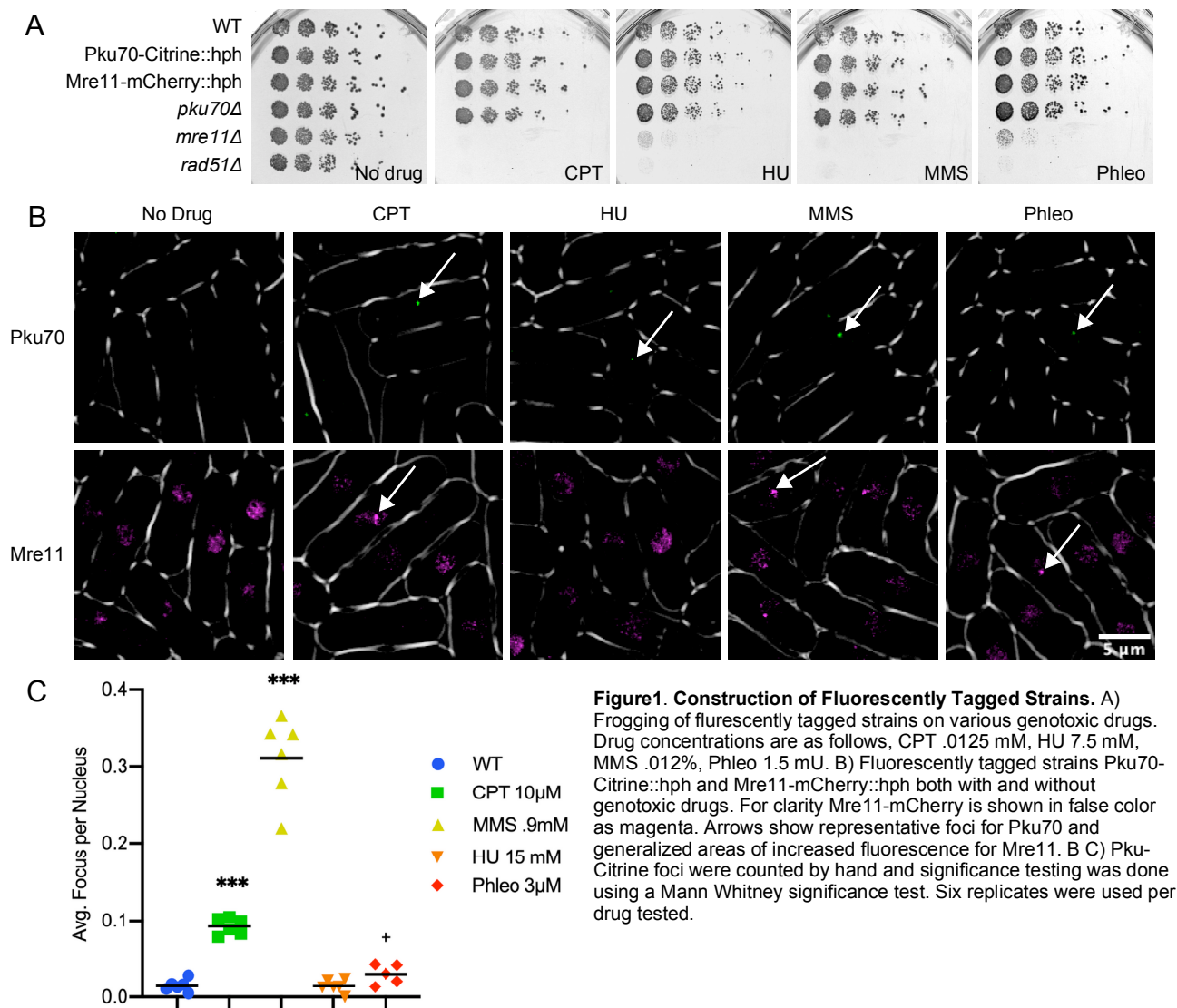


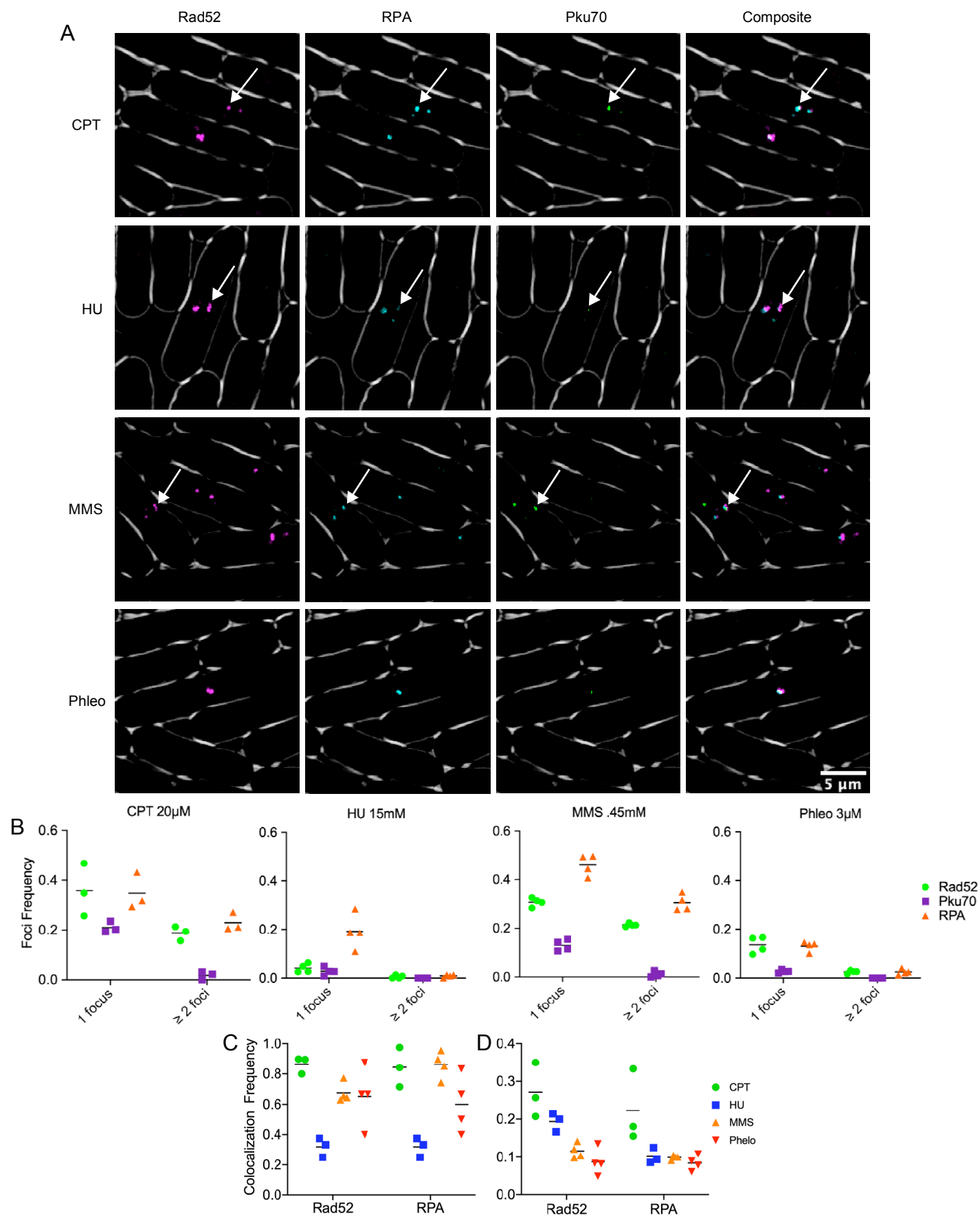
**Table 1: Strains**

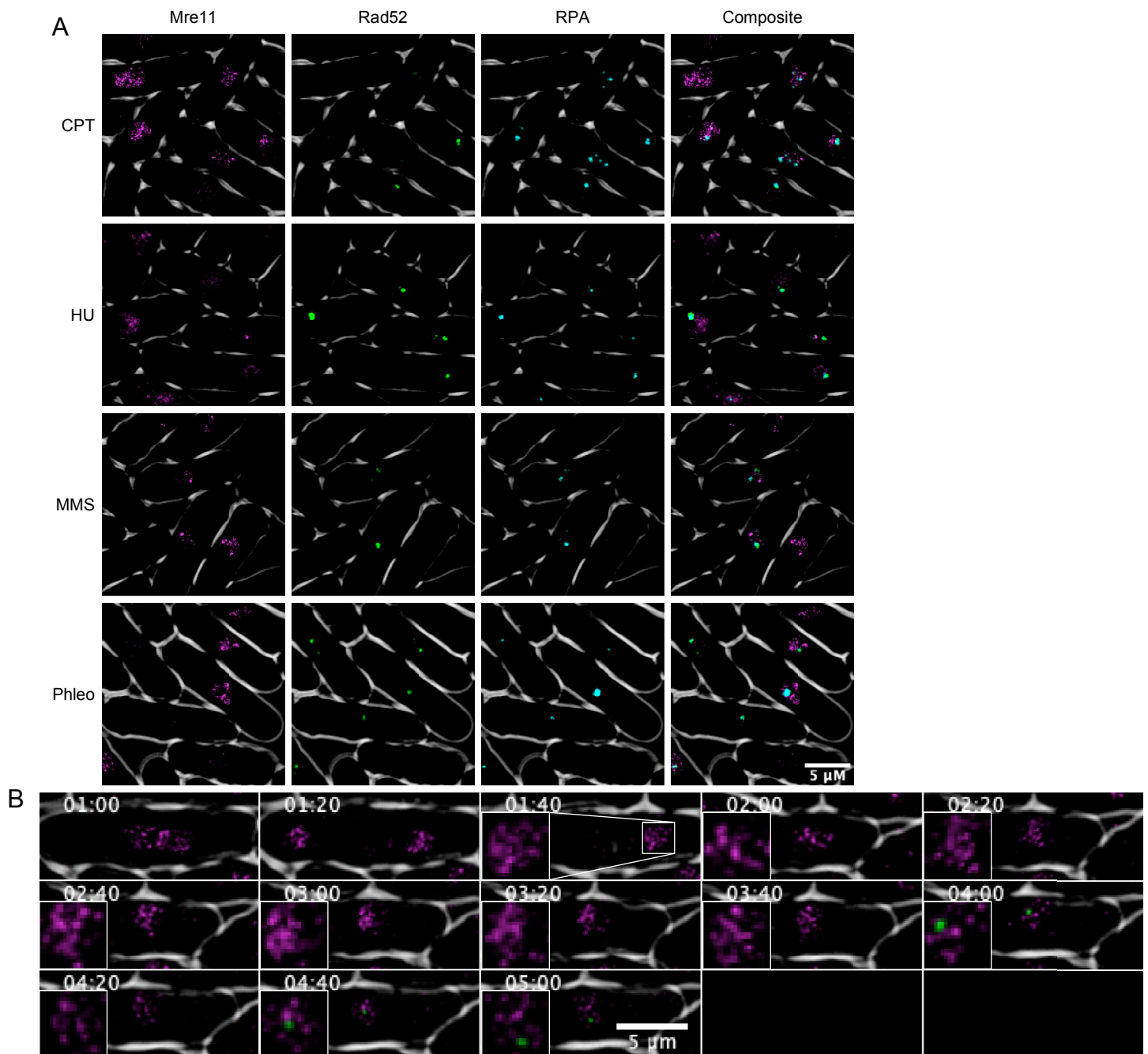
FY527	h- his3-D1 ade6-M216 ura4-D18 leu1-32	Gould, K. L., 1998
FY528	h+ his3-D1 ade6-M210 ura4-D18 leu1-32	Liang, D. T., 1999
FY8488	h+ Pku70-citrine::hph his3-D1 ade6-M210 ura4-D18 leu1-32	This study
FY8558	h- Pku70-citrine::hph his3-D1 ade6? ura4-D18 leu1-32	This study
FY8661	h+ Mre11-mCherry::hph his3-D1 ade6-M210 ura4-D18 leu1-32	This study
FY8662	h- Mre11-mCherry::hph his3-D1 ade6? ura4-D18 leu1-32	This study
FY8381	h- Rad52-mCherry::kan ura4-D18 leu1-32	Yu, Y., 2013
FY8625	h- Pku70-citrine::hph Rad52-mCherry::kan his3-D1? ade6? ura4-D18 leu1-32	This study
FY8698	h90 Mre11-mCherry::hph, Pku70-citrine::hph, his3-D1 ade6? ura4-D18 leu1-32	This study
FY4743	h- rad11-Cerulean::hphMX rad22-YFP-natMX leu1-32 ade6-M210 ura4-D18	Sabatinos, S. A., 2012
FY8687	h90 Mre11-mCherry::hph, RPA-Cerulean::hphMX, Rad52-YFP-natMX his-D1? ade6-M210 ura4-D18 leu1-32	This study
FY9381	h- Rad11-Cerulean::hphMX pKu70-citrine::hph rad52-mCherry::natMX6 ura4-D18 leu1-32 his? ade?	This study

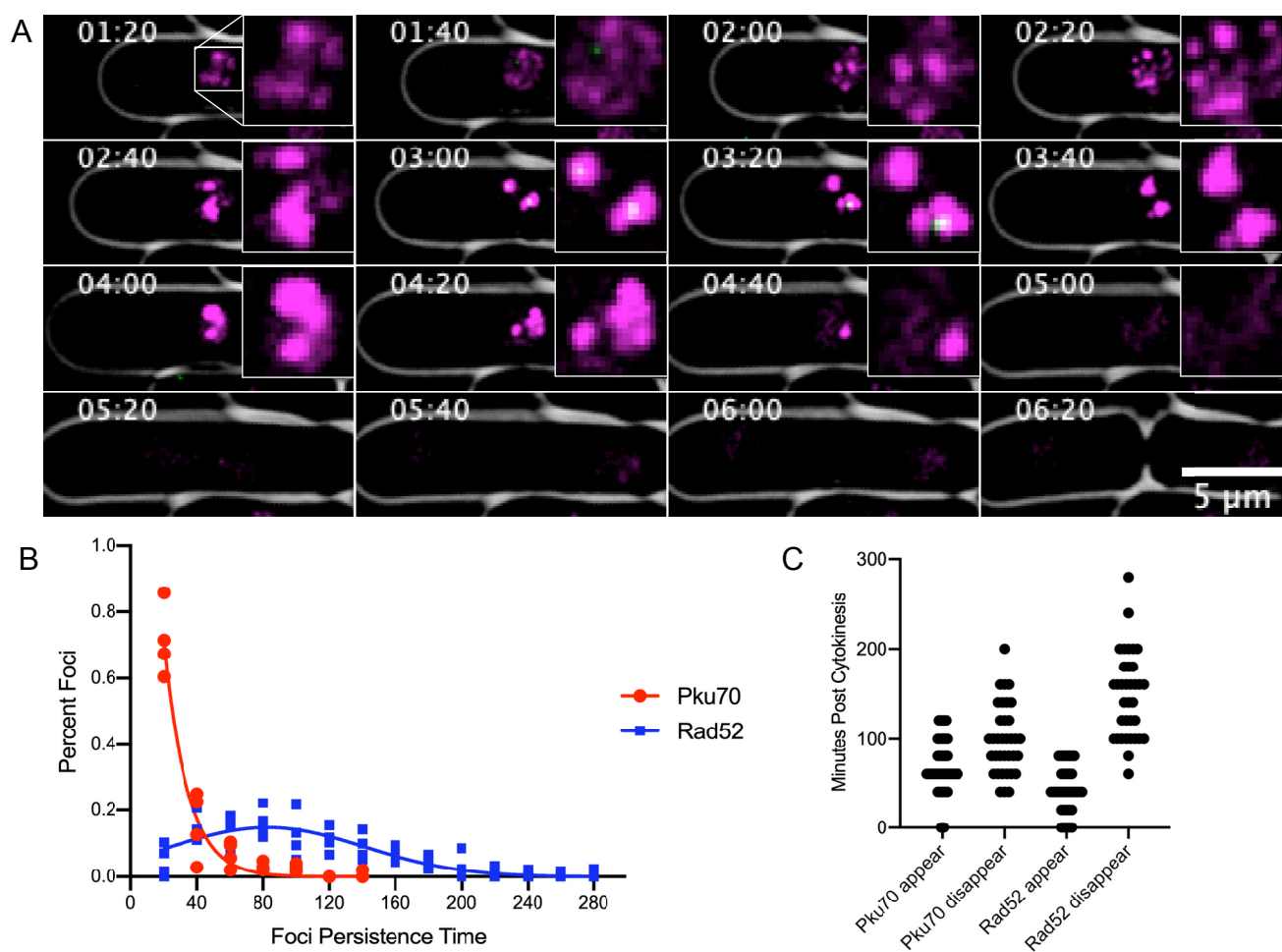
**Table 2: Primer List**

FY2561	atgtctaaaggtgaagaattattcac	Citrine Fwd	This study
FY2562	gtattctggcctccatgtcttattgtacaattcatcca	Citrine Rev	This study
FY2563	gacatggaggccagattac	Hph Fwd	This study
FY2564	agtatagcgaccagcattc	Hph Rev	This study
FY2565	gaatgctggtcgctactactggcgccacttctaataa	3' Citrine UTR Fwd	This study
FY2566	ccctgttatccctagcggatct	3' Citrine UTR Rev	This study
FY2710	tgtaacattttagcgcgtc	5' Pku Fwd	This study
FY2711	aattctcaccttagacattaattttgacatagttcg	5' Pku Rev	This study
FY2712	gaatgctggtcgctactactgacaagaaaatattaagat	3' UTR Pku Fwd	This study
FY2713	agcatacgttagtgaaggttga	3' UTR Pku Rev	This study
FY2888	tacgaagctcaaggaaccgt	5' Mre11 Fwd	This study
FY2889	gccctgctaccatcatctaaaatttcg	5' Mre11 Rev	This study
FY2890	cgaattttgatgatggtgagcaagggc	mCherry Fwd	This study
FY2863	ctgctcgacatgttcatctgtacctccgggtctta	mCherry Rev w/TEF	This study
FY2864	gacgagctgtacaagtaggacatggaggccagaat	Hph Fwd	This study
FY2892	attgataagatcaacagtatagcgaccagcattcacatacg	Hph Rev	This study
FY2891	ctggtcgctactactgtgatcttataaaattttgttaagtgtacct	3' UTR Mre11 Fwd	This study
FY2893	cgcactatcgctttgtgtgc	3' UTR Mre11 Rev	This study

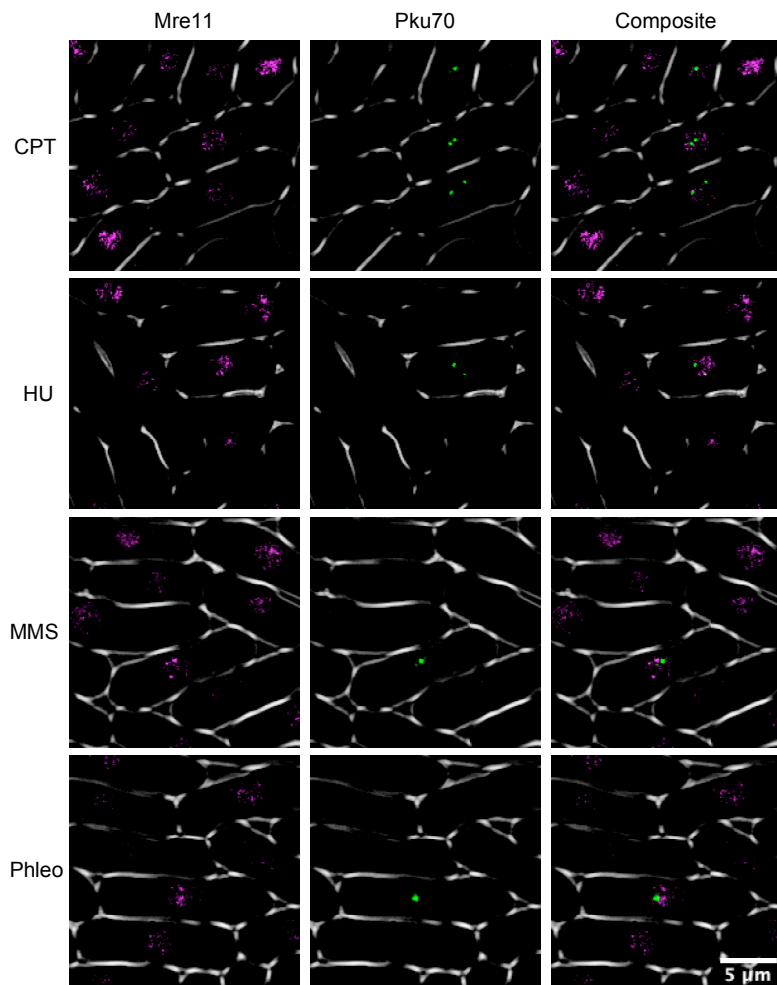




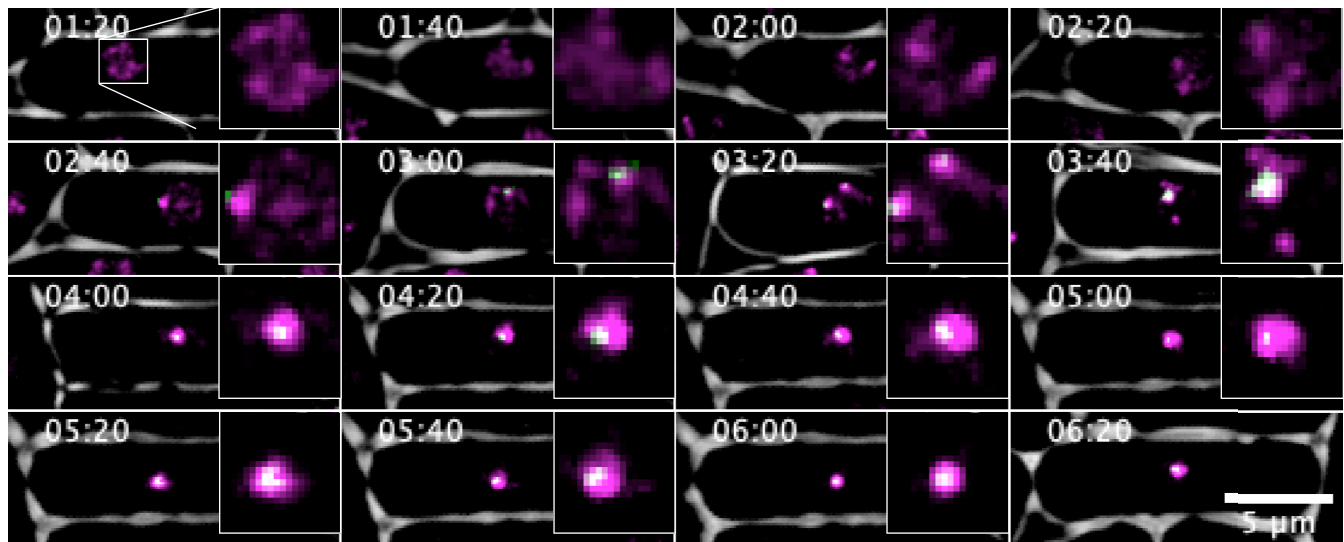




**Figure 4. Pku localization in a dynamic timecourse of MMS treatment.** A) Fluorescent time lapse images of Pku70-Citrine colocalizing with Rad52-mCherry. For clarity Rad52-mCherry is shown in magenta and Pku-Citrine is shown in green. Imaging was started at 80 minutes post addition of .45mM MMS and cells were imaged at 28°C. Timecourse images were taken every 20 min. B) Persistence time of Rad52-mCherry (n=205) and Pku70-Citrine (n=195). C) Appearance and disappearance times for Pku70-Citrine and Rad52-YFP in individual mononucleate cells. T=0 first time point after completion of cytokinesis. Horizontal density shows higher quantity of foci appearing or disappearing at that time point. (n=35 cells).

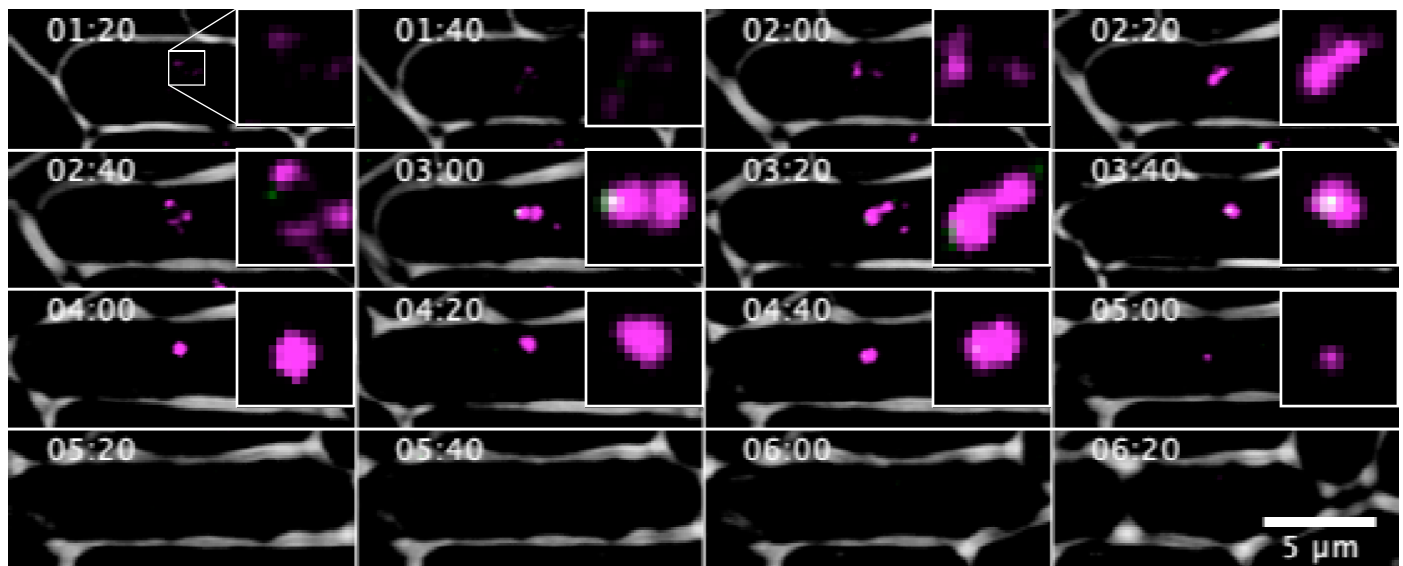


**Supplemental Figure 3. Colocalization of Pku70 and Mre11.** Cells were treated in .45mM MMS for 4 hours at 32°C. Mre11-mCherry is shown in false color as magenta and Pku70 is shown in green for clarity.



**Supplemental Figure 2. Timelapse of Pku70 and Rad52.** Fluorescent time lapse images of Pku70-Citrine colocalizing with Rad52-mcherry. For clarity Rad52-mCherry is shown in magenta as well as Pku-Citrine being shown in green. Imaging was started at 80 minutes post addition of .45mM MMS and cells were grown at 28°C. Timecourse images were taken every 20 min.





**Supplemental Figure 3. Timelapse of Pku70 and Rad52.** Fluorescent time lapse images of Pku70-Citrine colocalizing with Rad52-mCherry. For clarity Rad52-mCherry is shown in magenta as well as Pku-Citrine being shown in green. Imaging was started at 80 minutes post addition of .45mM MMS and cells were grown at 28°C. Timecourse images were taken every 20 min.