Functional organization of protein determinants of meiotic DNA break hotspots

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During *Schizosaccharomyces pombe* meiotic prophase, homologous chromosomes are co-aligned by linear elements (LinEs) analogous to the axial elements of the synaptonemal complex (SC) in other organisms. LinE proteins also promote the formation of meiotic DNA double-strand breaks (DSBs), the precursors of cross-overs. Rec10 is required for essentially all DSBs and recombination, and three others (Rec25, Rec27, and Mug20) are protein determinants of DSB hotspots – they bind DSB hotspots with high specificity and are required for DSB formation there. These four LinE proteins co-localize in the nucleus in an interdependent way, suggesting they form a complex. We used random mutagenesis to uncover recombination-deficient missense mutants with novel properties. Some missense mutations changed essential residues conserved among *Schizosaccharomyces* species. DSB formation, gene conversion, and crossing-over were coordinately reduced in the mutants tested. Based on our mutant analysis, we revised the rec27 open reading frame: the new start codon is in the previously annotated first intron. Genetic and fluorescence-microscopy assays indicated that the Rec10 N- and C-terminal regions have complex interactions with Rec25. These mutants are a valuable resource to elucidate further how LinE proteins and the related SCs of other species regulate meiotic DSB formation to form crossovers crucial for meiosis.

Meiosis is a special process in which diploid cells undergo one round of DNA replication followed by two consecutive rounds of chromosome segregation to generate haploid gametes. In the first meiotic division, homologous chromosomes physically associate with each other via crossing-over during prophase, resulting in the reciprocal exchange of chromosome portions, and then segregate. This unique feature of meiosis – high-levels of homologous recombination – is critical for sexually reproducing species and is regulated in multiple ways. Homologous recombination is initiated by DNA double-strand breaks (DSBs) formed by the conserved protein Spo11 (called Rec12 in fission yeast, studied here)1. These programmed DSBs are not evenly distributed throughout the genome: the frequency of breakage is much higher than average at widely-spaced loci called DSB hotspots. Here, we describe novel mutants of proteins critical for determining DSB hotspots. These proteins also comprise a structure important for the pairing of homologs described below.

During meiotic prophase in most eukaryotes studied, a tripartite chromosomal structure called the synaptonemal complex (SC) is observed under the electron microscope [reviewed in ref. 2]. The SC consists of a central element and two parallel lateral elements to which chromatin loops are attached, thereby aligning and holding together the two homologous chromosomes. Axial elements, formed shortly after replication, become the lateral elements of the SC later in meiosis. The ultra-structure of the SC is highly conserved, but the proteins composing it are highly diverged among species.

In meiotic prophase of the fission yeast *Schizosaccharomyces pombe*, linear elements (LinEs), but not full-length SC, are detected by electron microscopy (EM) of spread-out nuclear contents; LinEs appear similar to lateral elements of the SC of other species but do not extend end-to-end on the chromosomes in such EM analyses2,3. Like the SC, LinE formation requires a meiosis-specific sister chromatid cohesion complex containing Rec8 and Rec11 subunits, and phosphorylation of one of them (Rec11) by a casein kinase 1 ortholog4,5. Rec8 forms filamentous structures with the chromosomes aligned in parallel from one end to the other during the horsetail stage of meiosis as observed in live cells by super-resolution structured illumination microscopy (SIM)6.

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This observation reveals a structural similarity of S. pombe meiotic chromosones and those of other species. The time-course of LinE development, like that of the SC, is coordinated with other meiotic prophase events – LinEs form after replication but before recombination is complete \(^{8,9}\). Although LinEs differ somewhat from the SC in structure or stability, both SC and LinE proteins are required for high levels of homolog pairing and meiotic recombination \(^{10-14}\).

Four *S. pombe* proteins have been identified as LinE components – Rec10, Rec25, Rec27 and Mug20. The morphology of immunostained Rec10 is similar to that of LinEs observed by EM, suggesting Rec10 is a primary structural component \(^1\). In the C-terminal truncation mutant rec10-155, LinEs are not detectable by EM \(^{13,15}\). Rec25, Rec27 and Mug20 are small proteins (125–151 amino acids) predicted to form coiled-coil domains, which are also found in SC proteins. In rec25Δ or rec27Δ cells, no Rec10 immunostaining signal, and therefore no LinEs, can be detected by fluorescence microscopy \(^1\). In the absence of Mug20, LinEs fail to develop to full-length \(^{12}\) or to form distinct nuclear foci \(^{16}\). Furthermore, these four LinE components interdependently co-localize in meiotic nuclei of live cells; i.e., focus-formation of any one depends on each of the others tested \(^{11,16}\). Rec10 physically interacts with the other three proteins, as detected by mass spectrometry of immuno-precipitates, and directly with Rec25, as detected in yeast two-hybrid assays \(^{16,17}\). Thus, these four proteins appear to function as a physical complex (designated the LinE complex), although there is no direct biochemical evidence for such a complex. Two other proteins, Hop1 and Mek1, also localize to LinEs and their localization depends on Rec10 \(^{16}\).

LinE proteins are crucial for meiotic recombination. Like elimination of Rec12 or any of its partner proteins, deletion of *rec10* abolishes meiotically induced homologous recombination in all intervals tested and DSB-formation genome-wide \(^{16,18,19}\). Rec10 interacts with Rec15, a Rec12 partner protein, in two-hybrid assays \(^{20}\) and appears to activate Rec12 for DSB formation. Rec25, Rec27 and Mug20 are meiotic DSB hotspot determinants – they bind specifically to nearly all hotspots and are essential for most hotspot DSBs \(^{16}\). In contrast to deletion of *rec10*, deletion of *rec25*, *rec27* or *mug20* reduces recombination frequencies strongly in some intervals (100-fold or more) but only modestly in others (as little as 2-fold) \(^{11,12,21}\). Thus, Rec10 acts in DSB formation genome-wide, whereas Rec25, Rec27, and Mug20 see to function primarily or exclusively at DSB hotspots.

Although LinE proteins play a fundamental role in DSB formation, their mechanistic role is still unclear. Reduced recombination frequency could be a consequence of reduced DSB formation, but the available data do not exclude an additional function of LinE proteins in DSB repair after break formation. Some *rec10* non-deletion mutants (*rec10-109, rec10-144 and rec10-155*) were used to study LinE development and function in recombination and homologous chromosome pairing \(^{4,13,15,22}\), but no *rec25*, *rec27* and *mug20* alleles other than deletions have been reported, to our knowledge. Non-deletion mutants are especially useful to study multi-functional proteins, such as LinE proteins involved in DSB formation, homolog pairing and recombination. Here, we identified multiple missense and nonsense alleles of *rec25, rec27, mug20* and *rec10* and investigated recombination and DSB formation in these mutants. We revisited the *rec27* open reading frame and the deduced sequence of the *Rec27* protein. We also investigated *Rec25*-GFP focus-formation in multiple *rec10* missense mutants. Our results demonstrate the importance of certain conserved amino acids, lend further insight into LinE formation and function, and provide material useful for further investigations. Our study of LinE proteins provides a new perspective on the still-unclear relationship between the SC (axial elements), DSB formation, and recombination.

### Results

**A screen for novel LinE protein mutants deficient in meiotic recombination.** We designed a genetic screen to isolate new *rec25*, *rec27*, and *mug20* mutations affecting meiotic recombination by modifying a screen for recombination-deficient (Rec−) mutants described previously \(^{23}\) (Fig. 1). We used a diploid strain heteroallelic for *mat* (*h/ι*) and for two non-complementing *ade6* mutations (*ade6-3049/ade6-M26*). Upon starvation, these cells undergo meiosis and recombine to ade− at high frequency due to the *ade6-3049* and *ade6-M26* recombination hotspots \(^{19}\). *ade6-3049* and *ade6-M26* are also DSB hotspots; each is a single base-pair change mutation of ade6− that creates a binding site for the transcription factor Atf1-Pcr1 \(^{24,25}\). Binding of Atf1-Pcr1, as well as LinE proteins, is required for DSB formation there \(^{16,26}\). Chromatin structural changes accompanying this binding appear to facilitate, by an unknown mechanism, DSB formation by Rec12 and its putative partner proteins \(^{27-29}\). A LinE gene, such as *rec25*, was deleted on each chromosome but was present on a derivative of the low copy plasmid pFY20 \(^{30}\) as a mutagenized library. After sporulation, spores were spotted on YEAg plates without supplementary adenine, on which non-recombinant (Ada−) spores form red colonies, while recombinant (Ada+) spores form white colonies; this produces a red lawn with some white (Ada−) papillae. When the diploid cells express a recombination-deficient (Rec−) LinE mutant protein from the plasmid, the frequency of white papillae is reduced.

We used mutagenic PCR to form a library of plasmids with mutations restricted to the LinE protein gene. We sequenced the LinE protein gene from randomly chosen plasmids (9 for *rec25*, 10 for *rec27*, and 8 for *mug20*) and found 0.27 base-pair changes per *rec25* gene (0.12/kb), 2.5 base-pair changes per *rec27* gene (1.2/kb), and 0.65 base-pair changes per *mug20* gene (0.36/kb). We transformed an appropriate diploid strain with these libraries and screened ~400 transformants for *rec25* or *rec27* and ~200 transformants for *mug20*. We identified 43, 32, and 15 Rec− candidates (i.e., with reduced frequency of white papillae) of *rec25, rec27*, and *mug20*, respectively. Among these candidates, 24, 17, and 6 were sequenced (Suppl. Figs S1–S3). Missense mutants, especially those changed in conserved amino acids, were chosen for further study. For all subsequent experiments reported here, the mutations were put onto the chromosome at the endogenous location, to ensure stability and appropriate gene expression.

**Four amino acids essential for Rec25 activity.** We analyzed four missense mutations of *rec25* – *rec25-234* (N36D, L137P), *rec25-235* (N63I), *rec25-236* (K124I), and *rec25-237* (L90P) – with substitutions of amino acids conserved in at least four of the five examined *Schizosaccharomyces* species (N36 is not conserved; Suppl.
Fig. S4A). Each of these mutations reduced recombinant frequencies, both ade6 intragenic (gene conversion) and ade6–arg1 intergenic (crossing-over), by factors not significantly different from that of the rec25 deletion (null) mutation (Fig. 2A; Suppl. Table S3; by unpaired t-test p > 0.07, except for rec25-235 gene conversion in the ade6-M26 x ade6-52 cross with p = 0.04). ade6 gene conversion was reduced by factors of 30–50, and ade6–arg1 crossing-over by factors of 16–28, indicating the importance of Rec25, and these residues specifically, for recombination.

Revised rec27 ORF starts within the annotated first intron. The rec27 open reading frame (ORF) in the public database (http://www.pombase.org/) has ten sequential As (adenine) in the first exon close to the designated ATG start codon. We sequenced the rec27+ ORF of two not-closely-related wild-type strains (GP13 and GP20) and found that each had ten sequential As, as expected. We found, however, that two isolates from our screen had only nine As (rec27-248) or eleven As (rec27-249) but were as recombination-proficient (Rec+ +) as another isolate with ten As, which we designate wild type (rec27+ +) (Fig. 3A). These potential frameshift mutations would result in truncated Rec27 proteins only 20 and 22 amino acids long, yet they had wild-type recombination phenotype.

This unexpected observation raises the question, where does rec27 translation start? Recent RNA-seq data show abundant transcripts covering only part of the former first intron, followed by a short exon and then the rest of the gene as designated in the database.11. We fortunately recovered a mutation in the first annotated intron [rec27-246 (M1L, N50I)] which exhibited the mutant null phenotype and changed a Met codon to a Leu codon. This Met codon, but not another Met codon toward the 5′ end of this intron, is covered by the RNA-seq data. We conclude, based on these data, that rec27-246 changed the true start codon ATG (Met) to TTG (Leu) in what we conclude is the wild-type first exon (Table 1 and Fig. 3). Using these data, we revised the rec27 ORF such that rec27 translation starts within the former first intron at nucleotide (nt) 156 (relative to the previously designated ATG). Thus, the newly designated first exon extends from nt 156 to 196 and the new first intron extends from nt 197 to 247 (Fig. 3B). As a result, the predicted Rec27 protein has nine amino acids less than the annotated Rec27 protein and 12 amino acids different at the N-terminus. As expected, the revised Rec27 protein aligned better with other Schizosaccharomyces species Rec27 and C. elegans SYP-2 proteins, which were previously shown to share sequence similarity (Fowler et al.16; Suppl. Fig. S4B). The nucleotide changes and amino acid changes of the rec27 mutants listed in Table 1 and Suppl. Fig. S2 were defined based on the revised ORF.

Four rec27 missense mutations with recombination-reduced or null phenotype. Three rec27 missense mutants – rec27-228 (K28E), rec27-240 (K36E), and rec27-241 (Q119K) – were modestly or strongly Rec− in meiosis. The meiotic gene conversion at ade6 was reduced only 2- to 7-fold in rec27-228 and rec27-240 compared to that in wild-type cells. Crossing-over in the ade6–arg1 interval was reduced by a factor of 1.4 for rec27-238 and by a factor of 3 for rec27-240 (Fig. 2B; Suppl. Table S3). rec27-241, however, was strongly Rec−: it
reduced both gene conversion and crossover frequencies to rec27Δ levels (Fig. 2B; Suppl. Table S3). rec27-238 and rec27-240 mutations changed K (lysine) to E (glutamic acid) at conserved sites. Interestingly, the mutation with the greatest defect (Q119) is not among the conserved sites of Schizosaccharomyces species; it is the seventh amino acid from the C-terminus and is apparently missing in two other related species (Table 1; Suppl. Fig. S4B).

Since the Rec− mutants rec27-238 (K28E) and rec27-240 (K36E) altered conserved K (lysine) to E (glutamic acid), we used site-directed mutagenesis to similarly alter five other conserved K or R (arginine) sites to E (R32E, K52E, K57E, K65E and K108E) (Table 1). rec27-239 (R32E), but not the other mutations – rec27-242 (K52E), rec27-243 (K57E), rec27-244 (K65E) and rec27-245 (K108E) – modestly reduced meiotic recombination (Fig. 2B; Suppl. Fig. S5; Suppl. Table S3). The amino acids altered in the three Rec− mutations, K28E, R32E and K36E, are conserved and clustered, suggesting this region is part of a functional domain.

Figure 2. Meiotic recombination of newly isolated LinE mutants. Meiotic gene conversion at ade6 (two allele pairs) and crossing-over between ade6 and arg1 in LinE mutants were measured as described in Methods. Data (mean ± SEM) are from Supplementary Table S3; n ≥ 3 for each mutant. NS (not significant; P > 0.05), **(P < 0.01), and ****(P < 0.001) indicate significance of the difference from wild type by unpaired t-test.
Figure 3. Revised Rec27 ORF starts within the first formerly designated intron. (A) Meiotic recombination between ade6-M26 and ade6-52 in rec27 mutants. Data (mean ± SEM) are from Supplementary Table S3; n = 4 for each strain. NS (non-significant) and * (P < 0.05) indicate significance of the difference from rec27+ by unpaired t-test. (B) Map of revised rec27 ORF (lower line) compared to the previous annotation (upper line) (http://www.pombase.org/spombe/result/SPBC577.05c). Pink boxes are exons.

### Table 1. Nucleotide and amino acid changes in LinE mutants.

| Allele number | Nucleotide change | Amino acid change |
|---------------|------------------|------------------|
| rec25-234     | A106G, T410C     | N36D, L137P      |
| rec25-235     | A188T            | N63I             |
| rec25-236     | A371T, C421A      | K124I            |
| rec25-237     | T269C            | L90P             |
| rec25-238     | A132G            | K28E             |
| rec27-239     | A144G, G145A     | R32E             |
| rec27-240     | A156G            | K36E             |
| rec27-241     | C454A            | Q119K            |
| rec27-242     | A204G            | K52E             |
| rec27-243     | A219G            | K57E             |
| rec27-244     | A243G            | K65E             |
| rec27-245     | A421G            | K108E            |
| rec27-246     | A1T, A199T       | M1Le, N50I       |
| rec27-248     | Aα, at −104 from ATG | None |
| rec27-249     | Aβ, at −104 from ATG | None |
| mug20-250     | G70A, T198C      | L52P             |
| mug20-251     | T276A            | V78E             |
| rec10-109     | G526A, G533A     | V176I, G178D     |
| rec10-116     | G712A            | W275*            |
| rec10-133     | C2185T           | Q729*            |
| rec10-134     | G811A, G1301A    | E271K, R434H     |
| rec10-136     | G925A, G1809A    | E309K, W663*     |
| rec10-144     | G2180A          | G727E            |
| rec10-155     | LEU2 insertion after G2079 | E691I and frame-shift |
| rec10-216     | AGGGAT (550–555) TTCACA | R184F, D185T |

*Relative to ATG (A = bp 1 of the revised rec27 ATG; see Fig. 3B).Nucleotide change does not change protein coding. With additional mutation Aα at −104 from ATG. Site-directed mutation. The nucleotide change at the translational start, from ATG to TTG, is unlikely to allow translation to begin. From22. Isolated by random mutagenesis of two codons (R184 and D185). Non-sense mutation.
but −Δ. Crossovers, however, were decreased substantially less:
level of rec10-116 (W575 and L52) are not conserved in species other than the closest related species Schizosaccharomyces rec10-109 rec25 rec10-155 and/109 (less than 20 Ade cross were tested for recombinants. Frequencies were converted to cM using Haldane’s equation. cCollective C-terminal 101 amino acids with ten additional amino acids encoded by the insertion 11 (Suppl. Fig. S6). Two rec10Δ (Table 2). reduced gene conversion and rec10-216 11*, (W575 but not sequenced (except for rec10-155 rec10-144 rec10-144 mutations. *Ade+ spores/million viable spores, as mean of two independent experiments or mean ± SEM of four independent experiments. >200 total colonies and >20 Ade+ colonies were counted for each determination except for rec10Δ, rec10-116, rec10-109/rec10-155 and rec10-109 rec25Δ (less than 20 Ade+ colonies were counted). 120 spore colonies from each cross were tested for recombinants. Frequencies were converted to cM using Haldane’s equation. ‘Collective data for rec25*, rec27* and mug20* (Supplementary Table S3). rec10-175::kanMX6. ‘From11; wild-type genetic distances were 15 cM (lys3 – ural1 and 31 cM (ural1 – met5). ‘From*; wild-type genetic distance was 73 cM (ade6 – arg1). ND, not determined. ‘From9; Ade+ recombinants were from homothallic h+ ade6-M26 ura4-294 rec10-133 (or rec10-136) crossed with heterothallic h+ ade6-52 rec10-109; wild-type frequency was 1600. ‘rec25-180:kanMX6. About 280 spore colonies from 4 independent crosses were tested. Frequencies were converted to cM using Haldane’s equation.

| Mutant         | Gene conversion ade6-M26 × 52  | Crossing-over (cM)  |
|----------------|--------------------------------|---------------------|
| rec10Δ         | 1200 ± 41 (4)                 | 14 ± 28             |
| rec10Δ         | 2                              | 0.2 ± 0.5           |
| rec10-109      | 27                             | 13 ± 35             |
| rec10-116      | 4.5                            | 9.2 ± 1.3           |
| rec10-133      | 6                              | 13 ± 1.5            |
| rec10-134      | 8.1                            | 13 ± 1.5            |
| rec10-136      | 21                             | 13 ± 1.5            |
| rec10-144      | 85                             | 6.3 ± 0.3           |
| rec10-155      | 3.8                            | 16 ± 5              |
| rec10-216      | 8.3 ± 0.5 (4)                  | 1.2 ± 0.3 (4)       |
| rec10Δ         | 41                             | 9.6 ± 0.6           |
| rec10-109/rec10-144 | 650 ± 20 (4)            | 8 ± 15              |
| rec10-109/rec10-155 | 11 ± 0.8 (4)         | 15 ± 0.8            |
| rec10-216/rec10-144 | 200 ± 21 (4)              | ND                  |
| rec10-216/rec10-155 | 2 ± 0.25 (4)               | ND                  |
| rec10-109 rec25Δ | 2                              | 5.2 ± 0.8           |
| rec10-155 rec25Δ | 8.7 ± 0.6 (4)             | 11 ± 0.8            |
| rec10-144 rec25Δ | 24 ± 2.4 (4)                | ND                  |

Table 2. Locus-specific reduction of meiotic recombination in rec10 mutants. *Ade+ spores/million viable spores, as mean of two independent experiments or mean ± SEM of four independent experiments. >200 total colonies and >20 Ade+ colonies were counted for each determination except for rec10Δ, rec10-116, rec10-109/rec10-155 and rec10-109 rec25Δ (less than 20 Ade+ colonies were counted). 120 spore colonies from each cross were tested for recombinants. Frequencies were converted to cM using Haldane’s equation. ‘Collective data for rec25*, rec27* and mug20* (Supplementary Table S3). rec10-175::kanMX6. ‘From11; wild-type genetic distances were 15 cM (lys3 – ural1 and 31 cM (ural1 – met5). ‘From*; wild-type genetic distance was 73 cM (ade6 – arg1). ND, not determined. ‘From9; Ade+ recombinants were from homothallic h+ ade6-M26 ura4-294 rec10-133 (or rec10-136) crossed with heterothallic h+ ade6-52 rec10-109; wild-type frequency was 1600. ‘rec25-180:kanMX6. About 280 spore colonies from 4 independent crosses were tested. Frequencies were converted to cM using Haldane’s equation.

Two mug20 missense mutations with recombination-reduced or null phenotype. In two mug20 missense mutants – mug20-250 (L52P) and mug20-251 (V78E) – gene conversion and crossing-over were reduced. Both types of recombination were modestly reduced in mug20-251 (1.5 to 3-fold) but were reduced to the level of mug20Δ in mug20-250 (Fig. 2C; Suppl. Table S3). The amino acids changed in these two mutants (V78 and L52) are not conserved in Schizosaccharomyces species other than the closest related species S. kambucha (Suppl. Fig. S4C).

Analysis of eight new and previously isolated rec10 mutations. Rec10 is essential for meiotic recombination: deletion of rec10 decreases recombinant frequencies about 1000-fold, to the levels in mutants lacking Rec12, the protein with the active site for DSB formation (Table 2)19. Here we analyzed seven rec10 mutants (rec10-109, rec10-116, rec10-133, rec10-134, rec10-136, rec10-144 and rec10-155) previously described but not sequenced (except for rec10-144 and rec10-155)11,19,22,23,32,33 and one newly created mutant (rec10-216).

The first rec10 mutant isolated, rec10-109, had two nearby amino acids changed in its conserved N-terminal quarter – V176I and G178D (Suppl. Fig. S4D; Table 1). Gene conversion at ade6 was reduced by a factor of 100, but, remarkably, crossing-over in the lys3 – ural1 or ural1 – met5 intervals on another chromosome was not affected, compared to wild-type cells (Table 2). This observation is consistent with the previous results with rec10-109 which indicated that Rec10 is a region-specific activator of meiotic recombination15,23,33.

rec10-216 (R184F, D185T) was newly generated by random mutagenesis of two N-terminal codons for amino acids conserved in all Schizosaccharomyces species (Suppl. Fig. S4D) and close to the sites mutated in rec10-109, rec10-216 reduced gene conversion and ade6 – arg1 crossing-over nearly to the level of rec10Δ (Table 2). rec10-116 (W575*), rec10-133 (Q729*), rec10-134 (E271K, R434H), and rec10-136 (E309K, W603*) were previously isolated as nitroguanidine-induced Rec+ mutants32, and three contain non-sense mutations (designated by *). rec10-155 is an insertion of S. cerevisiae LEU2 into the coding sequence44 and results in Rec10 lacking the C-terminal 101 amino acids with ten additional amino acids encoded by the insertion11 (Suppl. Fig. S6). Two mutations – rec10-116 (W575*) and rec10-155 (E691I and frame-shift) – decreased ade6 gene conversion to the level of rec10Δ. Crossovers, however, were decreased substantially less: < 11-fold reduction in these mutants but > 50-fold reduction in rec10Δ11,19 (Table 2). rec10-144 (G727E) changes an amino acid in a conserved region that is removed by the rec10-155 truncation (E691I and frame-shift). Both rec10-144 and rec10-155 reduced recombination similarly for crossing-over, but rec10-144 had less effect on gene conversion at ade6 (Table 2).

We conducted complementation analyses by testing N-terminal mutations rec10-109 (V176I, G178D) and rec10-216 (R184F, D185T) with the C-terminal truncation mutation rec10-155 (E691I and frame-shift) and the
missense mutation rec10-144 (G727E). Both rec10-109 and rec10-216 partially complemented the missense mutation rec10-144, consistent with previous reports of rec10-109 and rec10-144 complementation\(^{22,32}\), but failed to complement the truncation mutation rec10-155 (Table 2). Deletion of rec25 in the N-terminal mutant rec10-109 reduced the recombinant frequency to the rec10Δ level at ade6 and also significantly reduced crossing-over (\(p < 0.01\) by chi-square test). But deletion of rec25 in the C-terminal rec10-155 truncation or even in the missense mutation rec10-144 did not have this strong additive effect (Table 2). These results suggest that Rec25 interacts with the C-terminal region of Rec10 and agrees with the absence of LinEs in the rec10-155 C-terminal truncation mutant\(^{15}\).

**DSBs are reduced or eliminated in parallel with recombination reduction in the newly isolated LinE mutants.** The deficiency of recombination observed in LinE mutants could result from failure either to form or to repair DSBs. DSBs are abolished genome-wide in rec10Δ mutants and strongly reduced at DSB hotspots in rec27Δ; at the few hotspots tested, DSBs are also strongly reduced in rec25Δ\(^{16,19,21}\). We therefore assayed DSB formation at the ade6-3049 hotspot in representative novel LinE mutants. Cells were induced to enter meiosis synchronously using the pat1-114 temperature-sensitive repressor of meiosis in the rad50S background to accumulate unrepaired broken DNA\(^{18}\). There were no detectable DSBs in strong Rec− mutants – rec27-241 (Q119K), rec25-235 (N63I) and mug20-250 (L52P) (Figs 4 and S7). In weaker Rec− mutants – rec27-238 (K28E), rec27-240 (K36E) and rec27-241 (Q119K),
Figure 5. Rec25-GFP localization in rec10 missense mutants. Cells containing Rec25-GFP and the indicated rec10 mutations were induced for meiosis and fixed for fluorescence microscopy. The upper panels show Rec25-GFP, while the lower panels show DAPI-stained DNA. The figures show only the time points when GFP signal was detected (from 1.5 hr to 4 hr) after meiotic induction.
rec27-240 (K36E) and mug20-251 (V78E) – DSBs at ade6-3049 accumulated to 22%, 10% and 16%, respectively, of total DNA, compared to 30% in wild-type cells (mean percentage of broken DNA at 5 hr and 6 hr). Overall, DSBs were reduced in parallel with the reduction of recombinant frequencies (Figs 2 and 4).

**Rec25-GFP forms nuclear foci in a rec10 C-terminus mutant but not in an N-terminus mutant.** The formation of nuclear foci by Rec25-GFP, Rec27-GFP, Mug20-GFP and immunostained-Rec10 in nuclear spreads depends on the presence of each of the other tested LinE proteins, suggesting that these four proteins form an active complex11, 12, 16. To test whether mutations in the largest protein, Rec10, interrupt formation of this putative complex, we investigated Rec25-GFP nuclear focus-formation in three rec10 mutants. rec10-109 (V176I, G178D) and rec10-216 (R184E, D185T) alter the N-terminal region, and rec10-144 (G727E) alters the C-terminal region. In rec10-109 cells, Rec25-GFP fluorescence became visible in the nucleus about 1.5 hr after meiotic induction, similarly as in wild-type cells, but the Rec25-GFP foci were scarce, faint or not distinct, even at 4 hr after meiotic induction (Fig. 5). In rec10-216 cells, Rec25-GFP entered the nucleus with similar kinetics, but the signal was less concentrated (weaker, and without distinct foci) than in wild-type cells, especially at early time points, and nuclear accumulation was not clear until later in meiosis (Fig. 5). This suggests that in rec10-216 mutant cells Rec25-GFP protein does not enter the nucleus efficiently. Flow cytometry showed that DNA replication in rec10-109 and rec10-216 cells was normal, indicating that the retarded Rec25-GFP accumulation and focus formation in the nucleus was not caused by delayed meiotic progression (data not shown); indeed, both mutants entered meiosis 1 with normal timing compared to control cells (Fig. 5). In rec10-144 cells, Rec25-GFP appeared in the nucleus at the same time as in wild-type cells, and with similar intensity, but only a few foci were formed on top of a pan-nuclear signal not observed in the control at times when discrete Rec25-GFP foci were present (Fig. 5).

**Discussion**

Missense mutations are especially useful to study a protein's function, particularly that of multi-functional proteins such as the *S. pombe* LinE proteins. We report here eleven newly isolated missense mutations of LinE proteins – four rec25 mutants, four rec27 mutants, two mug20 mutants and one rec10 mutant – and their levels of meiotic recombination, DSB formation and putative complex formation to understand further how these proteins act in meiosis. Additionally, we revised the rec27 ORF.

LinE components share some similarities with SC proteins found in other species. Rec10 has limited amino-acid sequence similarity to Red1, an *S. cerevisiae* axial element protein; the similarity is limited to ~10% of the protein near the C-terminus that in both proteins is predicted to form a coiled-coil8 (Suppl. Fig. S4D). Red1 appears to play a role in the bias toward DSB repair with the homolog rather than with the sister chromatid35, 36. Rec27 and Mug20 share amino-acid sequence similarity with *Caenorhabditis* SC proteins SYP-2 and DDL-1, respectively36. Rec25, Rec27, and Mug20 are also predicted to be coiled-coil proteins, a feature of many SC proteins from numerous organisms. In addition, LinE components are highly conserved among *Schizosaccharomyces* species. For example, the *S. pombe* and *S. kambucha* genomes share 99.5% nucleotide sequence identity37, and their Rec25, Rec27, and Mug20 proteins are 100% identical, and Rec10 98% identical (Suppl. Fig. S4). Among all annotated orthologs in the genomes of three other *Schizosaccharomyces* species, the mean amino-acid sequence identities, compared to those of *S. pombe*, range from 66% for *S. octosporus* to 55% for *S. japonicus*37. Among the four individual LinE proteins, amino-acid sequence identities, compared to those of *S. pombe*, range from 40% for Mug20 of *S. cryophilus* to 25% for Rec10 of *S. japonicus*. Although these LinE proteins are somewhat less conserved than proteins in general, as is typical for meiotic proteins, the conclusions with *S. cerevisiae* proteins, such as the *S. pombe* Rec25, Rec27, and Mug20 are also predicted to be coiled-coil proteins, a feature of many SC proteins from numerous organisms. In addition, LinE components are highly conserved among *Schizosaccharomyces* species. For example, the *S. pombe* and *S. kambucha* genomes share 99.5% nucleotide sequence identity37, and their Rec25, Rec27, and Mug20 proteins are 100% identical, and Rec10 98% identical (Suppl. Fig. S4). Among all annotated orthologs in the genomes of three other *Schizosaccharomyces* species, the mean amino-acid sequence identities, compared to those of *S. pombe*, range from 66% for *S. octosporus* to 55% for *S. japonicus*37. Among the four individual LinE proteins, amino-acid sequence identities, compared to those of *S. pombe*, range from 40% for Mug20 of *S. cryophilus* to 25% for Rec10 of *S. japonicus*. Although these LinE proteins are somewhat less conserved than proteins in general, as is typical for meiotic proteins, the conclusions with *S. pombe* mutants, described here, are likely to extend to the other *Schizosaccharomyces* species and perhaps to more distant species such as *C. elegans* as well.

In the rec25, rec27 and mug20 mutants studied here, there is co-ordinate reduction or loss of DSBs and recombination frequency. Meiotic recombination is initiated by DSBs, whose formation requires multiple proteins. LinE proteins Rec10, Rec25, Rec27 and Mug20 form a putative LinE complex and are required for most DSBs at hotspots, to which they bind with high specificity16, 19, 21. Since the viable spore yields of the LinE mutants studied here were not strongly reduced (unpublished data), the observed recombination-deficiency of these mutants is likely a consequence of subdued complex formation or impaired DSB formation rather than inefficient DSB repair. Missense LinE mutants are a useful resource to address these aspects of DSB regulation and to identify protein domains involved in these processes.

The LinE mutants we identified reduced recombination to different levels: four rec25 mutants – rec25-234 (N36D, L137P), rec25-235 (N63I), rec25-236 (K124I), and rec25-237 (L90P), one rec27 mutant – rec27-241 (Q119K), and one mug20 mutant – mug20-250 (L52P) had the null phenotype, indicating that these amino acids are critical for each protein's function, although some of these amino acids are not conserved among *Schizosaccharomyces* species (Suppl. Fig. S4). As noted above, three rec27 mutants – rec27-238, rec27-239 and rec27-240 – clustered in a small N-terminal region of Rec27 and reduced recombination frequencies moderately; similar reductions were observed in mug20-251.

Since the recombination-deficiencies in these LinE mutants spanned a range from null to nearly wild-type, we measured DSBs at the *ade6-3049* hotspot in these mutants. DSBs in mutants with strongly reduced recombination – rec25-235 (N63I), rec27-241 (Q119K) and mug20-250 (L52P) – were reduced to background level. In other mutants with reduced but not abolished recombination – rec27-238 (K36E) and mug20-251 (V78E) – DSBs were correspondingly reduced but not abolished (Figs 4 and S7). The detectable DSBs in these mutants suggest that the putative Rec10-Rec25-Rec27-Mug20 complex formed and accumulated in the nucleus, but the complex's binding-efficiency to hotspots may be inefficient and thereby reduce but not eliminate DSB formation. At the DSB hotspots *ade6-3049* and *mbs1*, DNA breaks are repaired 3- or 4-times more frequently
with the sister chromatid than with the homolog\textsuperscript{38–40}. We infer that in the rec27-238, rec27-240 and mug20-251 mutants DSBs are repaired with the same bias as in wild-type cells, since DSB and recombinant frequencies are reduced in parallel.

A KR cluster near the N-terminus of Rec27 is important for its function. Three rec27 mutants changed K (lysine) or R (arginine) to E (glutamic acid): rec27-238 (K28E), rec27-239 (R33E), and rec27-240 (K36E) are each separated by three amino acids and clustered near the N-terminus of Rec27. They partially reduced recombination (both gene conversion and crossing-over) (Fig. 2B; Suppl. Table S3) and partially reduced DSBs at ade6-3049 (Figs 4 and S7). These three amino acids are identical in the Rec27 proteins of five Schizosaccharomyces species and may be part of a functional domain to perform the protein’s function.

Our data indicate complex interactions of the N- and C-terminal domains of Rec10. Rec25-GFP, Rec27-GFP and Mug20-GFP interdependently co-localize at about 15 distinct foci in meiotic nuclei; nuclear accumulation of the proteins and formation of foci depends on Rec10\textsuperscript{11,16}. In the absence of one or another LinE protein, there is no observed GFP accumulation in the nucleus, even though where tested the proteins’ abundance in the cells is similar to that in wild-type cells\textsuperscript{11}. The nuclear foci of LinE proteins are unlikely to be single protein molecules visible in the microscope we used. Instead, these foci may represent multiple distant regions on a chromosome bound by LinE proteins and forming a cluster that locally regulates DSB formation (Fowler et al., unpublished data).

Rec10, but not the other LinE proteins, has a predicted nuclear localization signal (NLS) in the middle of the protein (K494-S503) (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). In mitotic cells, ectopically expressed Rec10 strongly localizes in the nucleus, while Rec25 and Mug20 are distributed evenly over the cytoplasm and nucleus\textsuperscript{41}. Rec25-Rec27-Mug20-Rec10 may form a complex and then be brought into the nucleus by Rec10. This view is consistent with Rec10, but not the other LinE proteins, having an obvious NLS. Alternatively, the different components may independently enter the nucleus and LinE complex formation may cause nuclear retention. Since Rec25, Rec27, and Mug20 are small (125–151 amino acids), they may freely diffuse into the nucleus but not accumulate there without association with Rec10.

The rec10 mutations we used to investigate Rec25-GFP focus-formation did not occur in this predicted NLS. In these rec25 mutants Rec25-GFP accumulated in the nucleus, although in the case of the N-terminal mutant rec10-216 (R184F, D185T) less efficiently and with some delay (Fig. 5). This suggests that LinE complex formation or nuclear import is defective in this mutant, which affects two amino acids located in a region of Rec10 (H183-L191) identical in five Schizosaccharomyces species (Fig. S4D). The deficiency in localization of the LinE complex agrees with the recombination deficiency of the rec10-216 mutant, which shows a strong reduction in recombination (gene conversion and crossing-over) nearly to the levels of rec10Δ (Table 2).

In another N-terminal mutant rec10-109 (V176I, G178D), LinE formation assayed in chromosome spreads is considerably reduced\textsuperscript{4}. In intact rec10-109 cells, we observed that Rec25-GFP signal was concentrated in the nucleus with normal kinetics, suggesting that LinE complex formation and nuclear import or retention are normal but the signal did not evolve into dot-like foci as distinct as those in rec10Δ cells (Fig. 5). Gene conversion at the hotspot ade6-M26 is reduced in rec10-109 by a factor > 40, but, remarkably, crossing-over is not significantly reduced in two other intervals on another chromosome (Table 2)\textsuperscript{21,22,24}. Genome-wide analysis showed that most of the DSBs at most hotspots, including ade6-3049, are strongly reduced but not eliminated in rec10-109; at some hotspots, however, DSBs are still prominent\textsuperscript{46}. The defect in LinE focus formation agrees with the genome-wide analysis of DSBs in this mutant. The LinE complex’s binding to hotspots may be defective in the rec10-109 mutant, and the recombination still observed in rec10-109 mutants may originate mostly from DSBs outside of hotspots\textsuperscript{46}.

A different Rec25-GFP signal was observed in intact cells of the C-terminal rec10-144 (G727E) mutant. A few distinct foci formed in the nucleus although later than, and not as efficiently as, in wild-type cells. The Rec25-GFP nuclear foci in rec10-144 may be limited to a set of hotspot clusters noted above; alternatively, binding of the LinE complex to hotspots may be unstable, hampering normal levels of DSB formation at the hotspot.

In the rec10-109 (V176I, G178D) mutant, the residual gene conversion at the hotspot ade6-M26 and crossing-over in the intervals tested (lys3 – ura1 and ura1 – met5) was Rec25-dependent: deletion of rec25 in rec10-109 reduced gene conversion to the rec10Δ level and crossing over to the rec25Δ level (Table 2). This result indicates that there is still some LinE-dependent recombination activity in rec10-109. Perhaps LinE complexes still load onto some hotspots but at such a low level that they cannot be as readily detected by Rec25-GFP fluorescence as in rec10Δ cells; alternatively, hotspot clustering may be defective (see above). Unlike the N-terminal mutant rec10-109, the C-terminal mutant rec10-155 (E691I and frame-shift) did not manifest this genetic interaction with rec25Δ: the recombinant frequencies in the double mutant rec10-155 rec25Δ were the same as that in each single mutant, which are similar (Table 2). This suggests that Rec25 interacts with the Rec10 C-terminus, and that this interaction is lost in the truncated Rec10-155 protein. The similar recombination phenotypes of the C-terminal rec10-144 (G727E) and rec25Δ mutants and the mild effect of rec25Δ in rec10-144 background are also consistent with Rec25 interacting with this region of Rec10.

Our study shows that rec10 mutations altering either the N-terminal region (rec10-109; V176I, G178D) or the C-terminal region (rec10-144; G727E) are defective in LinE focus formation. This result and the intragenic complementation of N-terminal and C-terminal missense rec10 mutations (Table 2)\textsuperscript{22,32} suggest that Rec10 protein is self-interacting. There may be domains provided from different Rec10 molecules in a dimeric or higher order complex. In addition, the C-terminal region appears to interact with Rec25 for DSB formation. Two adjacent residues (R184, D185) in the N-terminal region may have a role in LinE complex formation or nuclear import. In summary, the rec10 mutants studied here differ from rec10Δ with respect to their interactions with Rec25, assayed both genetically and microscopically, but no clear pattern has yet emerged to explain these alterations.

The eleven novel missense mutations of LinE proteins reported here will help shed more light on LinE complex organization, regulation, and functions of the SC in DSB formation to form meiotic crossovers.
Materials and Methods

**S. pombe** strains, growth media, and genetic methods. Suppl. Table S1 lists the *S. pombe* strains used in this study. Media for cell growth and methods for meiotic crosses and random spore analysis were described.

**S. pombe** plasmids, oligonucleotides, and site-directed mutant construction. rec25-256::ura4+, rec27-257::ura4+ and mug20-258::ura4+ were constructed using the method described. Briefly, the ura4+ ORF (open reading frame) was amplified from plasmid pFY20 using primers OL3266 and OL3267 (Suppl. Table S2) with 80 bp of homology to DNA flanking the rec25 ORF, or OL3268 and OL3269 with 80 bp of homology to DNA flanking the mug20 ORF. For rec27-257::ura4+ construction, a 1.8 kb DNA fragment containing ura4+ with ~250 bp of homology to DNA flanking each side of the rec27 ORF, as formerly annotated, was generated in two steps by DNA polymerase chain-reactions (PCRs); first, ~250 bp DNA fragments upstream or downstream of the former rec27 ORF were amplified in PCRs using OL3441 with OL3448, or OL3439 with OL3440, respectively, and wild-type (strain GP13) genomic DNA. Then, the purified PCR products together with pFY20 as template were used to amplify the 1.8 kb ura4+ by a secondary PCR using primers OL3442 and OL3443. Since the PCR products from the first step overlap with the 5′ and 3′ ends of ura4+ on plasmid pFY20, the second step PCR generated ura4+ with ~250 bp of homology to regions flanking rec27. The PCR products containing ura4+ and homology to regions flanking rec25, rec27 and mug20 were used to transform strain GP4915 (ura4-D18) to uracil prototrophy. Integration at the rec25 locus was verified by a PCR using primers OL326 and OL3258; OL326 and OL3260 for mug20; and OL3255 and OL3256 for rec27.

Plasmids pLM06, pLM08 and pLM09 with wild-type rec25+, rec27+ and mug20+, respectively, were constructed as follows. The rec25+ gene with 297 bp of 5′ and 1118 bp of 3′ flanking DNA was amplified from wild-type (strain GP13) genomic DNA using OL3250 and OL3251 (primers), and the PCR fragment was cloned into pFY20 at the SalI site to produce plasmid pLM06. The rec27+ gene with 292 bp of 5′ and 314 bp of 3′ flanking the rec27 ORF, as formerly annotated, was PCR-amplified using OL3248 and OL3249 and cloned into pFY20 at the KpnI site to produce pLM07. The mug20+ gene with 291 bp of 5′ and 597 bp of 3′ flanking DNA was PCR-amplified using OL3253 and OL3254 as primers and cloned into pFY20 at the KpnI site to produce plasmid pLM09.

The mutations rec27-239, rec27-242, rec27-243, rec27-244, and rec27-245 were introduced into plasmid pLM08 by mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent catalog # 210518). Primers used to introduce point mutations were: OL3399 and OL3400 for rec27-239; OL3401 and OL3402 for rec27-242; OL3403 and OL3404 for rec27-243; OL3405 and OL3406 for rec27-244; and OL3407 and OL3408 for rec27-245. After sequencing to confirm the mutations, the mutated rec27 ORFs with flanking homologous sequences were released from the plasmids by KpnI digestion and integrated into the chromosome at the rec27 locus by transformation of strain GP8513 (rec27-257::ura4+) to 5-fluorouracil (FOA)-resistance. Integration at the rec27 locus was identified by a PCR using primers OL3255 and OL3256.

rec10-216 was generated as follows: primers OL2502 and OL2503, each of which contains six random nucleotides in place of the wild-type codons for R184 and D185, were used to generate randomly mutagenized R184, D185 mutations in rec10. Plasmid pFY20 (PvuII plus AflII digestion to remove the template plasmid DNA, the mutant plasmids were introduced into ultracompetent *E. coli* XL10-Gold cells (Agilent) by transformation to ampicillin-resistance. Plasmids were isolated from several isolated colonies and sequenced using OL1192; three were mutant: R184V, D185*, R184F, D185T, and R184I, D185S. (Indicates a non-sense mutation.) Strain GP6993 (rec10-175::kanMX6) was transformed with the mutated plasmids and crossed with GP6994 (rec10-175::kanMX6) to assay intergenic and intragenic recombination. The missense mutation R184F, D185T (rec10-216) showed strong recombination deficiency, equal to that of the nonsense mutation R184V, D185T (rec10-261) (data not shown). The rec10-216 ORF with flanking homologous sequences was released from the plasmid by PvuII and NsiI digestion and integrated into the chromosome at the rec10 locus by transformation of strain GP7301 (rec10-260::ura4+) to 5-fluorouracil (FOA)-resistance. Integration at the rec10 locus was verified by a PCR using primers OL1778 and OL1779. GP7301 (rec10-260::ura4+) was constructed by transformation of strain GP6994 to Ura+ using a 1.8 kb ura4+ PCR fragment with 80 bp of homology to DNA flanking the rec10 ORF [amplified from pFY20 which contains ura4+ using OL2722 and OL2723 as primers]. Integration at the rec10 locus was verified by PCR using primers OL1778 and OL1779.

**rec25**, **rec27** and **mug20** mutant library construction. Random mutations were introduced into each Line ORF using the Genemorph II EZClone Domain Mutagenesis Kit (Agilent catalog # 200552). Oligos OL3257 and OL3258 for rec25, OL3255 and OL3256 for rec27, and OL3259 and OL3260 for mug20 were used to synthesize gene fragments containing random mutations using plasmid pLM06, pLM08 or pLM09, respectively, as templates. After denaturation and annealing to pLM06, pLM08 or pLM09, the mutated PCR products served as “mega-primers” for a PCR with a specialized high-fidelity enzyme mix in the kit. After DpnI digestion to remove the template plasmid DNA, the mutant plasmids were introduced into ultracompetent *E. coli* XL10-Gold cells by transformation to ampicillin-resistance. DNA from separate pools of ~6500 transformants for rec25, ~4000 transformants for rec27, and ~2800 transformants for mug20 generated the rec25, rec27 and mug20 mutant libraries. This DNA was used to transform diploid strains GP8370 (rec25Δ), GP8367 (rec27Δ), and GP8484 (mug20Δ) to uracil prototrophy by selection on EMM2 + Ade plates incubated at 32°C for 5–6 days to allow growth into visible colonies, mating, and sporulation. Single colonies were picked into 75 μl of sterile water in a sterile 96-well plate, and 4 μl of the cell suspension was spotted onto NBA + Ade “keeper” plates. Then, 25 μl of glusulase (1:50 dilution in water) was added to each well. After overnight incubation at 32°C 100 μl of 60% ethanol was added to each well and held...
at room temperature for 30 min. The plates were centrifuged at 4000 rpm (2880 × g) for 4 min in an Eppendorf centrifuge model 5810; the cells were washed twice with 125 μl of sterile water and suspended in 125 μl of sterile water. Aliquots of 4 μl of the spore suspensions were spotted on YEAs plates and incubated for 4 days at 32 °C to assay the Ade+ recombinant frequencies (Fig. 1).

The rec27 mutant genes were put into the chromosome as for the site-directed rec27 mutant genes described above. The rec25 mutant genes with flanking DNA were released by Kpn1 and XmaI digestion and used to transform GP8210 (rec25-256::ura4+) to FOA-resistance; a PCR using OL3257 and OL3258 as primers identified homologous replacements. The mug20 mutant genes were released by Ksal digestion and similarly transferred to strain GP8211 (mug20-258::ura4+) and identified by a PCR using OL3259 and OL3260 as primers.

**Assay for meiotic DSBs.** Meiotic induction and DNA analysis were described by45,46. DNA in agarose plugs was digested with BsrG1 overnight at 37 °C, subjected to electrophoresis through 0.8% agarose, and transferred to a nylon membrane (Zeta-probe GT membrane, Biorad). DNA broken at the ade6-3049 DSB hotspot was detected by hybridization to a 1 kb probe from the right end of the 10.1 kb BsrG1 fragment. The probe was a PCR product amplified from wild-type genomic DNA using OL3693 and OL3694 as primers and labeled with [α-32P]dCTP (3000 Ci/mmol). Signals were detected using a Typhoon storage PhosphorImaging system (GE Healthcare).

**Fluorescence microscopy.** Diploid pat1-114 rec10 mutant cells producing Rec25 with GFP fused to its C-terminus (rec25-204::GFP) were induced for meiosis, and the GFP signals were examined as described48. Cells were fixed sequentially with 100% methanol and 100% acetone before and at different times after induction of meiosis. The cell suspension was spread on a poly-L-lysine coated slide, acetonate allowed to evaporate, and mounting solution (Vectorshield; Vector Laboratories, Inc.) supplemented with DAPI (2 μg/ml) added. Images are maximal projections of 9–11 sections, step size of 0.4 μm, to cover the whole cell (approx. 4 μm total). DNA images are a single focal plane, because out-of-focus DAPI fluorescence obscures the projection. Images were obtained with a Nikon Eclipse 90i microscope equipped with a 1/4 Oil Plan APO VC lens, a Hamamatsu ORCAER camera, and MetaMorph software (Molecular Devices).

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Author Contributions

All authors participated in the design of the study, analyzed the data, and wrote and reviewed the manuscript text. L.M., K.R.F., and C.M.C. performed the experiments and prepared the figures.

Additional Information

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