

## A spontaneous mutation in MutL-Homolog 3 (HvMLH3) affects synapsis and crossover resolution in the barley desynaptic mutant des10

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1 **Title:** A spontaneous mutation in MutL-Homolog 3 (HvMLH3) affects synapsis and  
2 crossover resolution in the barley desynaptic mutant *des10*.

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22

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24

25 **Summary**

26 ) Although meiosis is evolutionarily conserved, many of the underlying mechanisms  
27 show species specific differences. These are poorly understood in large genome plant  
28 species such as barley (*Hordeum vulgare* L.) where meiotic recombination is very  
29 heavily skewed to the ends of chromosomes.

30 ) The characterisation of mutant lines can help elucidate how recombination is  
31 controlled. We used a combination of genetic segregation analysis, cytogenetics,  
32 immunocytology and 3D imaging to genetically map and characterize the barley  
33 meiotic mutant *DESYNAPTIC 10* (*des10*).

34 ) We identified a natural exonic deletion in the ortholog of *MutL-Homolog 3* (*HvMlh3*)  
35 as the causal lesion. Compared to wild-type, *des10* mutants exhibit reduced  
36 recombination and fewer chiasmata, resulting in the loss of obligate crossovers and  
37 leading to chromosome mis-segregation. Using 3D-SIM, we observed that normal  
38 synapsis progression was also disrupted in *des10*, a phenotype that was not evident  
39 with standard confocal microscopy and that has not been reported with *Mlh3* knock-  
40 out mutants in Arabidopsis.

41 ) Our data provide new insights on the interplay between synapsis and recombination in  
42 barley and highlight the need for detailed studies of meiosis in non-model species.  
43 This study also confirms the importance of early stages of prophase I for the control  
44 of recombination in large genome cereals.

45

## 46 INTRODUCTION

47 Meiotic recombination is one of the principal forces underlying genetic diversity and a driver  
48 for evolution as well as progress in crop breeding programmes (Riley et al, 1981). A deeper  
49 understanding of this process offers the opportunity to manipulate recombination and  
50 improve the speed and accuracy of plant breeding in order to address the needs of food  
51 security within a period of increased environmental constraints (Able et al, 2009; Martinez-  
52 Perez, 2009). This is particularly true in cereals such as wheat, barley, oats, and rye as well as  
53 in many forage grasses that show a highly skewed distribution of meiotic crossovers (CO)  
54 relative to gene content, with large portions of the chromosomes around the centromeric  
55 regions rarely recombining (Higgins et al, 2012; IBGSC et al, 2012; Kunzel et al, 2000;  
56 Kunzel and Waugh, 2002; Ramsay et al, 2014). Interestingly this CO distribution phenotype  
57 is not found in *Arabidopsis* nor in either rice or *Brachypodium*, grass species with much  
58 smaller genomes (Chen et al, 2002; Huo et al, 2011, Salomé et al, 2012). The control of  
59 recombination and the interlinked processes of early meiotic progression have been  
60 intensively studied in model eukaryotic organisms with comparative studies being undertaken  
61 in mammalian species and the standard model plants *Arabidopsis* and rice (Baudat et al,  
62 2013; Gerton and Hawley, 2005; Luo et al, 2014; Mercier et al, 2014), but they have yet to be  
63 deciphered in large genome cereals.

64

65 During meiosis, homologous recombination starts with the formation of programmed DNA  
66 double-stranded breaks (DSB) by the protein SPO11 that is found in all eukaryotes (Keeney,  
67 2008; Metzler-Guillemain and de Massy, 2000, Stacey et al, 2006). The DSB ends are  
68 resected by the MRE11 complex (MRE11-Rad50-Xrs2 in yeast, MRE11-Rad50-NSB1 in  
69 plants) to generate 3' ssDNA tails (Daoudal-Cotterell et al, 2002; Nicolette, 2010, Raynard et  
70 al, 2008) which are then coated by the recombinases RAD51 and DMC1 to mediate strand  
71 invasion resulting in a joint molecule (D-Loop) (Shinohara et al, 1997, Da Ines et al, 2012;  
72 Kathiresan et al, 2002; Kurzbauer et al, 2012). The subsequent repair occurs either by  
73 synthesis-dependent strand annealing (SDSA) resulting in non-crossovers (NCO) or via a  
74 double Holliday junction (dHj) (Hunter, 2007; Bzymek et al, 2010, Matos and West, 2014;  
75 Bzymek et al 2010). Protein complexes (MSH4-MSH5, MER3) stabilize the dHjs (Nakagawa  
76 and Kolodner, 2002; Snowden et al, 2004;) that are mostly resolved into crossovers (CO) by  
77 the MutL homologs MLH1-MLH3 (Ranjha et al, 2014; Rogacheva et al, 2014) with a certain  
78 fraction resolved into NCO by a helicase-dependent mechanism in *Arabidopsis* (Knoll and

79 Puchta, 2011). Orthologues for many of these proteins have been identified in plants,  
80 suggesting a broadly conserved mechanism for crossover formation (Higgins et al, 2014; Luo  
81 et al, 2014; Mercier et al, 2014). It has been postulated that in Arabidopsis 85% of crossovers  
82 arise from a pathway under the control of the ZMM (ZYP, MSH, MER) group of proteins  
83 (Higgins et al, 2004; Higgins et al, 2005; Mercier et al, 2005). This pathway produces Class I  
84 COs which exhibit interference, the phenomenon where the presence of a CO reduces the  
85 probability of an additional CO in an adjacent interval with the remaining COs being Class II  
86 that do not exhibit interference (Higgins et al, 2008).

87

88 Homologous pairing, recombination and synapsis have been extensively studied, but the  
89 interdependence between these processes remains to be fully resolved and may differ  
90 between species (Santos, 1999; Zickler, 2006). In cereals, telomeres cluster during early  
91 meiosis to bring homologous chromosomes together and initiate synapsis (Colas et al, 2008;  
92 Higgins et al, 2012). During zygotene the two homologues progressively synapse along their  
93 entire length and the process is completed at pachytene (Santos, 1999; Zickler, 2006). The  
94 synaptonemal complex then disassembles but the chromosomes remain held together by  
95 chiasmata (the cytogenetic manifestation of the COs). At metaphase I the bivalents align at  
96 the equatorial plate and each of the homologous chromosomes then separates at anaphase I. A  
97 second round of cell division then follows, resulting in sister chromatid separation and the  
98 formation of haploid cells (Stack and Anderson, 2001).

99

100 While much of our current understanding has been developed in small genome models, it is  
101 now being extended to large and complex genome non-model crops such as barley, where  
102 recent cytogenetic studies have described meiotic progression and the chronology of meiotic  
103 events (Barakate et al, 2014; Higgins et al, 2012; Phillips et al, 2012). Although largely  
104 conforming to expectations, specific observations such as the clustering of the telomeres and  
105 the spatiotemporal organization of the recombination machinery differs from Arabidopsis  
106 (Armstrong et al, 2001, Barakate et al, 2014; Higgins et al, 2012; Phillips et al, 2012). Even  
107 in related grasses (e.g. barley vs. rice) there are conflicting reports of the direction of change  
108 in the number of chiasmata formed after disrupting the amount of the synaptonemal complex  
109 protein ZIPPER1 (HvZYP1/OsZEP1) (Barakate et al, 2014; Wang et al, 2010) hinting at  
110 significant functional differences between related components of the overall meiotic  
111 machinery.

112

113 To explore meiosis in a large genome crop we have been using a collection of barley  
114 *DESYNAPTIC* mutants that were determined cytologically in 1970s to have an aberrant  
115 meiotic phenotype with the presence of univalents being ascribed to premature desynapsis  
116 (Lundqvist et al, 1997). Here we have taken a classical forward genetics approach to map the  
117 spontaneous semi-sterile *DESYNAPTIC 10* (*des10*) mutant (Lundqvist et al, 1997) and  
118 identify the causal mutation as a deleted exon in the mismatch repair gene *HvMlh3*. Using a  
119 combination of genetic segregation analysis and super-resolution immuno-cytology we show  
120 that the mutation has a deleterious effect on recombination and crossing over. The unique  
121 form of the *des10* mutant allele results in the coding sequence being maintained in frame  
122 allowing immuno-fluorescent visualisation of the protein in both mutant and wild-type,  
123 providing novel insights into its importance in the very early stages of meiosis.

124

## 125 **MATERIALS AND METHODS:**

### 126 **Plant material**

127 Plants were grown under 16h of light at 18-20°C and 8h of dark at 16°C. For cytology, the  
128 cultivar (*cv.*) Bowman (wild-type) and its nearly-isogenic line BW230 (*des10*) were grown in  
129 a growth cabinet until meiosis. Anthers were checked for meiosis stage and fixed in  
130 formaldehyde. To assess the effect of *des10* on recombination, F<sub>2</sub> and F<sub>3</sub> populations derived  
131 from BW230 x *cv.* Morex were grown in a glasshouse and young leaf tissue were collected in  
132 96 well plates for DNA extraction and genotypic analysis. Plants were grown to maturity to  
133 assess fertility.

### 134 **Mapping and sequencing**

135 Frozen plant material was disrupted in a lysis buffer using a Qiagen grinder and DNA  
136 extracted with Qiagen DNA extraction kit using an automated station QIAxtractor®  
137 (Qiagen). Initial genetic mapping utilised a custom 384 SNP genotyping array using the  
138 Illumina beadXpress platform. For mapping we used the segregation of the semi-sterile  
139 phenotype of *des10* as a Mendelian trait. Using JoinMap 4.0 (Kyazma) software, loci were  
140 assigned to linkage groups and two rounds of regression mapping used to order the loci  
141 within groups. The iterative development of custom KASPar© SNP assays (KBioscience)  
142 derived from alignments of genic sequences, known to map in this interval, were mined for  
143 polymorphism between *cvs.* Bowman and Morex, and these used to delineate the interval

144 containing *des10* to a single 1.02Mb BAC contig (contig\_38558) containing six annotated  
145 genes (<http://mips.helmholtz-muenchen.de/plant/barley/fpc/index.jsp>). Primers were  
146 designed to amplify the genomic sequences of the six genes within the BAC contig and all  
147 other possible syntenic genes and the PCR products sequenced using big dye V3.1 reaction  
148 kit and analysed on an ABI Prism 3730. For cDNA sequencing mRNA from young  
149 inflorescences and anthers from BW230(*des10*) and Bowman was extracted using an RNA  
150 extraction kit (Qiagen) in presence of DNaseI. cDNA was made using the standard protocol  
151 of the Superscript III kit (Life Technologies) and sequenced using specific primers  
152 encompassing the deleted region.

### 153 **Recombination frequency**

154 F<sub>3</sub> individuals derived from selfed seed from F<sub>2</sub> individuals homozygous for *des10* or wild-  
155 type alleles at *HvMlh3* were used for the recombination assay. The genome wide genetic  
156 mapping utilised the custom 384 SNP genotyping array. Three independent ~20cM intervals  
157 on 4H (centromeric), short arm of 6H (distal) and long arm of 7H (distal) were studied in  
158 more depth using KASP© assays.

### 159 **Immunocytology**

160 Anthers were fixed in 4% formaldehyde (1X PBS/0.5% Triton™ X-100) for 20 to 30  
161 minutes, rinsed twice in 1XPBS/0.5% Triton™ X-100 and tapped to release the meiocytes.  
162 Meiocytes suspension (30µl) were transferred onto a *Polysine® slide (Poly-L-Lysine coated*  
163 *slides)* and left to air dry (room temperature) and without squashing to preserve the 3D  
164 conformation. Slides were first blocked 30 minutes in 3% BSA in 1XPBS, 0.1% Triton™ X-  
165 100 and then incubated in the primary antibody solution which consisted of one or multiple  
166 antibodies (raised in rabbit or rat) diluted in blocking solution in a wet chamber for 1 hour at  
167 room temperature followed by 24-48h at 4°C. The antibodies that have been previously  
168 described were; anti-AtASY1, -AtZYP1, -HvMLH3, -AtRAD51, -AtMHS4, -AtDMC1  
169 (Barakate et al, 2014; Higgins et al, 2012; Phillips et al, 2012; Phillips et al, 2013). We also  
170 prepared a new barley antibody, anti-HvZYP1 (Rat), from an immunization with two  
171 individual peptides (Dundee Cell Product) to confirm the ZYP1 phenotype. Slides were  
172 warmed for 30 minutes to 1 hour at room temperature before washing for 15 minutes in  
173 1XPBS and incubating for up to 2 hours at room temperature in a secondary antibody  
174 solution consisting of a mixture of anti-rabbit Alexa Fluor® (488 or 568) and/or anti-rat  
175 Alexa Fluor® (568 or 488) (Invitrogen) diluted in 1XPBS. Slides were washed 15min in

176 1XPBS, counterstained with Hoechst 33342 (Life Technologies) for 15 minutes, and  
177 mounted in Vectashield® (H-1000, Vectorlabs).

### 178 **DNA *in situ* hybridization**

179 For chiasmata counts, anthers were fixed in Ethanol/Acetic acid (3:1) for 24 hours and stored  
180 in 70% Ethanol at 4°C until use. Slide preparation and DNA *in situ* hybridizations were  
181 performed as previously described (Higgins et al, 2012) using rDNA 5s-digoxigenin and  
182 rDNA 45s-biotin probes to identify the individual chromosomes.

### 183 **Time course**

184 Stems were injected with 0.5ml to 1ml of 10µM 5-ethynyl-2'-deoxyuridine (EdU) in the  
185 region of the inflorescence (under the base of the spike) and also two thirds of the way up  
186 along the length of the stem. The EdU solution was left in the stems for 2 h to allow for its  
187 incorporation into S-phase nuclei as previously described (Higgins et al, 2012). Spikes were  
188 collected and fixed in fresh 4% formaldehyde/PBS fixative for 30 minutes to 1 hour at  
189 various time-points (6, 18, 24, 48, and 68 hours after the 2 hours of EdU pulse). Fixed anthers  
190 were prepared for immuno-detection with anti-ASY1 (primary and secondary incubation) as  
191 described above, immediately followed by EdU detection as per the suppliers protocol. EdU  
192 was detected with Click-iT® EdU Alexa Fluor® 488 HCS assay kit (Life Technologies) with  
193 45 minutes incubation instead of 30 minutes in the supplied protocol. Slides were  
194 counterstained with Hoechst 33342 (2µg/ml, Life Technologies), mounted in Vectashield®  
195 (H-1000, Vectolabs) and sealed.

### 196 **Microscopy**

197 For confocal microscopy, 3D Confocal stack images (512x512, 12bits) were acquired on a  
198 LSM-Zeiss 710 fitted C-Apochromat 63x/1.20 W Korr M27 oil objective. Laser light (405,  
199 488, 561 and or 594nm) were used at 2-4%, sequentially with 2 (up to 4) lines averages. 3D  
200 stack slices were taken at 0.25 to 0.44 µm interval at pixel dwell 1.58 µs. For SC spreads,  
201 imaging was performed using a Nikon Eclipse 90i microscope as previously described  
202 (Higgins et al., 2012; Barakate et al, 2014). For structured illumination microscopy, 3D-SIM  
203 images were acquired on a DeltaVision OMX Blaze (GE Healthcare) fitted with an Olympus  
204 PlanApo N 60x 1.42 NA oil objective. Laser light from solid state lasers (405, 488 and  
205 564nm), shuttered by high speed tilt mirrors and coupled into a broadband single mode  
206 optical fibre was split into three beams. 3D interference pattern in the sample plane are



207 generated by focusing of the beams onto the back focal plane of the objective lens. Striped  
208 illumination patterns are shifted by five phase steps and rotated by 3 angles ( $-60^\circ$ ,  $0^\circ$  and  
209  $+60^\circ$ ), providing a set of 15 images per unprocessed z-section. Interference patterns were  
210 phase shifted by directing the outer two beams through a separate pair of windows with  
211 individual tilt control. Phase of the interference pattern at the sample plane was shifted due to  
212 the change in the path length for the respective outer beam, while lateral refractive beam  
213 translation was canceled by tilting a given window pair in complementary directions. Angles  
214 of pattern orientation were shifted by a tilt mirror, directing the three beams pattern to one of  
215 three mirror clusters; the beam pattern from each of the three rotation paths was redirected  
216 back to a common exit path by reflecting a second time from the tilt mirror. Exposure times  
217 were typically between 100 and 200 ms, and the power of each laser was adjusted to achieve  
218 optimal intensities of between 1,000 and 3,000 counts in a raw image of 15-bit dynamic  
219 range of Edge sCMOS camera (PCO AG, Germany). The lowest possible laser power was  
220 chosen for each channel to minimize photo bleaching. Unprocessed image stacks were  
221 composed of 15 images per z-section (five phase-shifted images per each of three  
222 interference pattern angles). The microscope was routinely calibrated by measuring channel  
223 specific optical transfer functions (OTFs) to optimize lateral and axial image resolution  
224 (channel dependent and typically  $\sim 120$  and  $\sim 300$ nm, resp.). Super-resolution three-  
225 dimensional image stacks were reconstructed with SoftWoRx 6.0 (GE) using channel specific  
226 OTFs and Wiener filter setting of 0.002 (0.005 for the DAPI channel) to generate a super-  
227 resolution three-dimensional image stack. Images from the different colour channels,  
228 recorded on separate cameras, were registered with SoftWorx 6.0 alignment tool (GE), based  
229 on alignment parameters obtained from calibration measurements with 100nm-diameter  
230 TetraSpeck beads (Life Technologies).

### 231 **Imaging and modelling**

232 Images were processed with the respective microscope software package, or with external  
233 imaging tools like Fiji (ImageJ 1.49m) for deconvolution (Schindelin et al, 2012; Vonesch  
234 and Unser, 2008) and Imaris 8.1.2 (Bitplane) for 3D projection and MLH3 counting. Barley  
235 MLH3 protein modelling was obtained by submitting the protein sequence of the intact  
236 protein and the truncated version to the SWISS-MODEL workspace (Bordoli et al, 2009).

237

238

239 **RESULTS**

240 ***des10* is the result of a mutation in the mismatch repair gene *HvMlh3*.**

241 *des10* is a spontaneous semi-sterile mutant of the barley cultivar (*cv.*) Betzes (Lundqvist et al,  
242 1997). The original mutation was backcrossed repeatedly to *cv.* Bowman then selfed to  
243 produce the Bc<sub>5</sub>F<sub>3</sub> near-isogenic line BW230 (*des10*) (Fig. 1a) (Druka et al, 2010). To  
244 identify the lesion causing the observed phenotype, we genetically mapped the *des10*  
245 mutation using an F<sub>2</sub> population (n=168) derived from a cross between BW230 (*des10*) and  
246 the *cv.* Morex to the long arm of chromosome 5H (Fig. 1b) using a standard SNP marker set  
247 (Close et al, 2009; Druka et al, 2011). By extending the population to 1102 F<sub>2</sub> plants and  
248 using additional KASP<sup>TM</sup> SNP markers developed using published genome sequence data  
249 from *cvs.* Morex and Bowman (IBGSC et al, 2012), we located *des10* to a 0.2 cM interval  
250 encompassed entirely within a 1.02 Mb BAC contig (contig\_38558) containing six annotated  
251 genes (Fig. 1b). Sequencing all six genes revealed a single polymorphism between BW230  
252 (*des10*) and Betzes in MLOC\_52425 (Fig. 1b) consisting of a 159 bp deletion that removes  
253 the entire seventeenth exon of a putative gene model encoding HvMutL-homolog 3  
254 (HvMLH3 - GenBank accession no. JQ855501, Fig. S1a), but maintaining the open reading  
255 frame of the downstream exons (Fig. S1b and Fig. 2a). The deleted exon encodes the majority  
256 of the conserved DQHAX<sub>2</sub>EX<sub>4</sub>E metal binding motif essential for the endonuclease activity  
257 of HvMLH3 (Fig. 2b), a mismatch repair protein that has a role in the resolution of double  
258 Holliday junctions (dHj) arising from the ZMM dependent CO pathway (Jackson et al, 2006;  
259 Lipkin et al, 2000; Nishant et al, 2008; Phillips et al, 2013). Deletion of this domain is  
260 predicted to affect protein conformation (Fig. 2c-d), potentially destabilizing the MutL  
261 protein complex (MLH1-MLH3) required for resolution of dHJs (Guarne et al, 2004; Ranjha  
262 et al, 2014).

263 ***des10* has fewer chiasmata than observed in wild-type.**

264 To confirm and further characterise the meiotic phenotype of *des10* mutants we used  
265 fluorescence *in situ* hybridization (FISH) with probes against 45S and 5S rDNA to determine  
266 chiasma frequencies and CO at metaphase I in wild-type and *des10*. While homologues are  
267 normally paired at pachytene in both genotypes (Fig. 3a,e), *des10* exhibits fewer chiasmata.  
268 In the wild-type metaphase I, the number of chiasmata ranged from 16 to 20 per nucleus with  
269 the mean frequency of 18.4±1.3 (n=21) (Fig. 3b,i,j) slightly lower than CO numbers

270 (mean=21.8) estimated from genetic maps (Close et al, 2009; IBGSC et al, 2012) but closer  
271 than previous estimates (Nilsson et al, 1993). In *des10* we observed significantly fewer  
272 chiasmata ranging from 5 to 13 per nucleus with a mean of  $9.2 \pm 2.1$  (n=57) (Fig. 3f,i,j) and we  
273 also observed the presence of univalents ( $1.7 \pm 2.0$ , n = 57) (Fig. 3f,i) leading to occasional  
274 aberrant chromosome segregation at anaphase I (Fig. 3c,g), genetically unbalanced tetrads  
275 (Fig. 3d,h) and a subsequent semi-fertile phenotype. Given the similar size of the seven  
276 chromosome pairs, the distribution of chiasmata per nucleus in *des10* can be compared to that  
277 expected assuming a Poisson distribution of the number of chiasmata observed (Jones, 1967).  
278 The observed distribution was significantly different from that expected from a random  
279 distribution (p=0.034), which indicated that although the presence of univalents is indicative  
280 of a substantial disruption, some control of CO distribution remained (Jones, 1967, Jackson et  
281 al, 2006).

#### 282 ***des10* shows reduced genetic recombination frequency.**

283 Given the recessive nature of the mutation we investigated the effect of *des10* on genetic  
284 recombination using segregating F<sub>3</sub> families derived from specific F<sub>2</sub> individuals from the  
285 BW230 (*des10*) x Morex cross that were homozygous for either the wild-type (n=188 across  
286 15 F<sub>2</sub> families) or *des10* mutant (n=183 across 16 F<sub>2</sub> families) allele at *HvMlh3*. The  
287 reconstituted chromosome linkage maps generated from the segregation data within the F<sub>3</sub>  
288 families derived from wild-type F<sub>2</sub> individuals were comparable to the barley consensus map  
289 (Close et al, 2009; IBGSC et al, 2012). However the maps derived from the segregation data  
290 within the F<sub>3</sub> families derived from *des10* F<sub>2</sub> individuals showed considerably less  
291 recombination, being only 45.9% of the length of the maps derived from wild-type families  
292 (excluding chromosome 5H due to the selection at the *HvMlh3* locus) (Fig. 4, Fig. S2). There  
293 was little evidence to suggest that the reduction in recombination varied across the genome  
294 with similar reductions observed in subtelomeric (44.2%) or centromere-proximal regions  
295 (51.4%) (Fig. S2) with the estimates of genetic to physical distance ratios in wild-type and  
296 *des10* changing from 1.16 to 0.46 cM/Mb in distal subtelomeric regions and from 0.06 to  
297 0.03 cM/Mb in proximal regions. The reduction in recombination frequency was confirmed  
298 by comparisons at three specific intervals delineated by KASP SNP markers on a larger  
299 number of individuals from F<sub>3</sub> families (wild-type, n=695 across 22 F<sub>2</sub> families; *des10*, n=556  
300 across 24 F<sub>2</sub> families) that all showed a significant differences in recombination with *des10*  
301 lines showing an mean reduction to 39% wild-type recombination frequency (26.0-54.6%

302 ( $p=8.4^{e-8}-0.01$ ) (Fig. S3). The reduction in recombination in these F<sub>3</sub> families paralleled the  
303 reduction of chiasmata observed cytogenetically in the mutant *des10* compared to wild-type.

304 **Chromosome pairing is normal but the normal progression of synapsis appears**  
305 **compromised in *des10*.**

306 Given the importance of the interplay between synapsis and recombination in CO formation  
307 (Santos, 1999, Zickler, 2006), we compared synapsis in *des10* and wild-type using antibodies  
308 raised against AtZYP1 and the axial element associated protein AtASY1 (Barakate et al,  
309 2014; Higgins et al, 2012; Phillips et al, 2012) using Structured Illumination Microscopy  
310 (SIM). Axis formation and the initiation of synapsis during leptotene were comparable in  
311 wild-type (Fig. 5a-b and Fig. S4) and *des10* (Fig. 5g-h and Fig. S4). By mid-zygotene in  
312 wild-type most of the chromosomes were paired (Fig. 5c,d) and the typical tri-partite  
313 structure of the SC was visible (Fig. 5d, white arrow) with the ZYP1 signal suggesting new  
314 synapsis initiation sites as shown previously (Phillips et al, 2012). The tri-partite structure is  
315 also clearly visible at pachytene in wild-type with complete synapsis evident (Fig. 5e,f).  
316 Using confocal-microscopy, synapsis appeared to progress normally with the linearization of  
317 the ZYP1 signal during zygotene-pachytene (Fig. S5). However with SIM there appeared to  
318 be a difference in the relative positioning of ZYP1 compared to wild-type with a highly  
319 punctate ZYP1 signal observed in *des10* at mid zygotene (Fig. 5i,j) or later (Fig. 5k,l) that  
320 precluded the discernment of the SC tri-partite structure at mid-zygotene (Fig. 5j) or late  
321 zygotene/pachytene (Fig. 5l). The problems of homologue pairing were also indicated by  
322 unsynapsed ASY1 regions resembling the previously described “peg and coalescent” process  
323 (Colas et al, 2008) at early zygotene in *des10* (Fig. 5j, arrows). However the punctuated  
324 appearance of ZYP1 seen with SIM was not obvious when using confocal images, where the  
325 ZYP1 signal appeared linear in *des10* (Fig. S5). This suggests that the homologous  
326 chromosomes are aligned but that either the SC is not fully mature in the mutant or that in  
327 *des10* the chromatin structure is altered precluding binding of the ZYP1 antibody. Using  
328 Imaris we were able to track the individual bivalents of the later zygotene/pachytene cells in  
329 *des10* (Figure 5k) and show that the distance between the ASY1 labelled homologues were  
330 maintained at 0.1 $\mu$ m (Figure 6) as previously reported at pachytene (Phillips et al, 2012)  
331 suggesting that despite the non-linear ZYP1 these cells are fully synapsed.

332

333 ***des10* displays delayed synapsis.**

334 The observations of a perturbed synaptic progression were unexpected given that synapsis  
335 has been reported as normal in both Arabidopsis and mouse knock-out *mlh3* mutants  
336 (Jackson et al, 2006; Lipkin et al, 2002) albeit that these have not been analysed using 3D-  
337 SIM. In order to better understand how and when the mutation in *des10* was having this  
338 effect, we conducted a time course analysis using 5-ethynyl-2'-deoxyuridine (EdU) labelling.  
339 After collecting spikes of the same size in both wild-type and *des10* for each time point,  
340 meiocytes were spread from the central spikelets (numbers 3 to 10). This enabled several  
341 stages of meiosis to be studied for each spike with the EdU intensity/distribution and ASY1  
342 linearity/intensity under confocal microscopy being used to classify the cells. A total of 27,  
343 163, 98 and 141 cells were counted at 18h, 24h, 48h and 68h respectively in the wild type and  
344 26, 172, 167 and 46 cells were counted at 18h, 24h, 48h and 68h respectively in *des10*. We  
345 observed that early meiotic events in *des10* were comparable to wild-type with the presence  
346 of the telomere bouquet, which produces a concentrated ASY1 signal at one side of the  
347 nucleus (Higgins et al, 2012), at 6h (Fig. 7a-c) and 18h (Fig. 7d-f). However by 48 hours  
348 (Fig. 7j-l) while in wild-type there were roughly equal numbers of cells in zygotene and  
349 pachytene with 8% in later stages, in *des10*, 87% of the total cells were in zygotene with no  
350 cells found at pachytene, although 5% were at later stages. This result corresponds to the  
351 apparent defect in synapsis described above suggesting that in *des10*, cells appear suspended  
352 at zygotene with very few exhibiting a mature pachytene (with a strong linear ZYP1 signal  
353 relating to chromosome condensation). At 68h (Fig. 7m-o), similar levels of metaphase I  
354 were found in wild-type and *des10*, but while 100% of them are labelled in wild-type, 29% of  
355 the total metaphase I cells were not labelled in *des10*, indicating that in *des10* they have lost  
356 synchronicity, potentially due to the delay in synapsis. Although this lack of synchronicity  
357 made estimates difficult for the majority of the cells, the total length of prophase does not  
358 appear generally different between wild-type and *des10*. Thus, unlike the 25 hours delay in  
359 reaching metaphase I in knock-out *AtMlh3* mutants (Jackson et al, 2006), *des10* cells exhibit  
360 no overall (or little) time delay compared to wild-type. Moreover, a comparison of the stages  
361 of meiotic progression relative to changes in meiocyte size based on DNA staining (Fig. S6)  
362 revealed that the expected chromosomal changes were delayed in *des10* relative to wild-type  
363 (Higgins et al, 2012; Jackson et al, 2006; Kleckner et al, 2004).

364

365 **HvMLH3 foci detectable in wild-type and *des10***

366 Using high resolution immuno-cytology, we observed that the HvMLH3 antibody (Phillips et  
367 al, 2013) produced a punctate signal associated with the nucleus at zygotene in wild-type  
368 barley with some MLH3 signal associated with chromatin and the forming SC (Fig. 8a-d). At  
369 late zygotene/early pachytene, synapsis of the chromosomes in wild-type progressed via  
370 ZYP1 polymerization and although the MLH3 signals are detectable in the nucleus, a subset  
371 of more distinct MLH3 foci become evident on the SC (Fig. 8e-h, triangles). At late  
372 pachytene, (Fig. 8i-l), polymerization of ZYP1 is complete and distinct MLH3 foci are  
373 evident as previously described (Phillips et al, 2013). Using 3D stacks, the final MLH3 foci  
374 count (Fig. 8j, triangles and Fig. S7) averaged 20.8 ( $\pm$  3.4, n=19) per cell (Table S1) for the  
375 wild-type, which closely corresponds to the average chiasma count of 18.4 at metaphase I.

376 The exonic deletion in *des10* almost entirely removes the functional HvMLH3 metal binding  
377 motif but as the mutation left *HvMlh3* in frame, it potentially produces detectable protein that  
378 is endonuclease deficient and under the control of its native promoter. This was confirmed  
379 with immuno-cytology with the HvMLH3 antibody in conjunction with HvZYP1 allowing  
380 the observation of the mutant protein in relation to the synaptonemal complex formation. As  
381 the problems of synapsis in *des10* that are evident when using 3D-SIM could complicate  
382 accurate staging when using the ZYP1 antibody without ASY1, the staging was also carried  
383 out using confocal images where the ZYP1 signal appears linear in *des10* (Fig. S5).

384 At zygotene, we observed a similar MLH3 signal in the nucleus in *des10* (Fig. 8m-p) as in the  
385 wild-type (Fig. 8a-d). However at late zygotene/pachytene, judged by the stage of ZYP1  
386 polymerization, distinct foci are much less apparent in *des10* (Fig. 8q-t) with a higher  
387 background MLH3 signal present in the nucleus (Fig. 8t). At the pachytene-like stage in  
388 *des10*, distinct foci do form and the final number could be estimated using 3D image stacks  
389 (Fig. 8v, triangles and Fig. S8) with the mean being 7.7 foci/cell ( $\pm$  1.6, n=30) (Table S1)  
390 which is close to the observed average of 9.2 chiasmata per nucleus. The distribution of the  
391 number of MLH3 foci per nucleus in *des10* was significantly different from a Poisson  
392 distribution (p=0.011), confirming our earlier conclusion from chiasmata counts that the COs  
393 are not random (Jackson et al, 2006). Interestingly although the number of cells was limited  
394 and the count subject to experimental error, the MLH3 foci distribution in *des10* did however  
395 just fit a binomial distribution expected given the number of foci found in the mutant and  
396 wild-type.

397 In addition, we found that DSB formation was not disturbed in *des10* and progressively  
398 formed in both wild-type and *des10* from the distal regions and localized to the axial  
399 elements as previously described in barley (Fig. S9 and S10) (Barakate et al, 2014; Higgins et  
400 al, 2012; Phillips et al, 2012). However, higher numbers of RAD51, DMC1 and MSH4 foci  
401 were found in *des10*, compared to the wild-type, (Table 1, Fig. S9 and S10) suggesting that  
402 the mutation is either affecting DSB numbers as previously reported in ZMM mutants  
403 (Thacker et al, 2014), or the dynamics of DSB repair.

404

## 405 **DISCUSSION**

### 406 ***des10* is a spontaneous mutation in *HvMlh3***

407 Using classical forward genetics we show that the spontaneous semi-sterile barley *des10*  
408 mutant is the consequence of a deletion of exon 17 of *MutL-homolog 3* (*HvMlh3*) that  
409 contains most of the conserved C-terminal metal binding endonuclease domain. The *des10*  
410 mutant showed a clear meiotic phenotype with a reduction in chiasmata number relative to  
411 wild-type that mirrors the reduction seen in the knock-out mutants in Arabidopsis, the only  
412 other plant for which *mlh3* mutants have been characterised (Jackson et al, 2006). As in  
413 Arabidopsis, the presence of some univalents indicates that the remaining COs are  
414 insufficient in number in some cells to ensure accurate chromosome segregation.

415 This similar level of reduction in chiasmata in the MLH3 mutants indicates that, as expected,  
416 the deletion of the majority of the conserved metal binding motif essential for the  
417 endonuclease activity (Nishant et al, 2008) in *des10* mimics the complete knock-out of the  
418 gene. The effects observed were however less severe than those found in classical ZMM  
419 mutants in Arabidopsis and *Zyp1* knockdowns in barley (Higgins et al, 2004; Barakate et al  
420 2014) which also corresponds with the phenotypes observed in Arabidopsis MLH1 and  
421 MLH3 mutants (Dion et al, 2007; Jackson et al, 2006). Importantly given the nature of the  
422 mutation we were able to count the MLH3 foci directly in both wild-type and in *des10* unlike  
423 in the Arabidopsis and mouse knockout studies. These MLH3 foci counts confirmed the  
424 reduction observed with chiasmata counts, showing a reduction to 37% (7.7/20.8) compared  
425 to wild-type that mirrored the estimates of chiasmata counts (50%: 9.2/18.4) and interestingly  
426 close to the ratio found with chiasmata counts in Arabidopsis (39%) (Jackson et al, 2006).

427 This mutant phenotype in both species is consistent with a post-ZMM role for MLH3 in the  
428 resolution of predetermined CO sites (Jackson et al, 2006, Zakharyevich et al, 2010). This  
429 interpretation was supported in Arabidopsis by the fit of the mutant cell chiasmata  
430 frequencies to a binomial distribution that modelled the probability (p) of the independent  
431 resolution of dHJs as COs at each of a preselected set of (k) recombination intermediates.  
432 However in Arabidopsis, the chiasmata frequencies also fitted a simpler discrete Poisson  
433 distribution about the mean, potentially indicative of the random nature of the remaining  
434 COs. Importantly this simpler random distribution was not supported in this study with both  
435 the counts of chiasmata and MLH3 foci in *des10* being significantly different from the  
436 expected Poisson distributions while the MLH3 foci distribution in *des10* did only just fit a  
437 binomial distribution expected given the number of foci found in the mutant and wild-type.

438 The effect of *des10* was observed genetically on recombination frequency with the F<sub>3</sub> map  
439 length of families derived from F<sub>2</sub> individuals homozygous for the *des10* allele at *HvMlh3*  
440 being 45.9% the map length of wild-type. Interestingly there was little evidence to suggest  
441 that the reduction in recombination varied across the genome despite the known temporal-  
442 spatial control of recombination in barley (Higgins et al 2012). This observation corresponds  
443 well with the assumption that MLH3 is involved in the resolution of predefined CO  
444 intermediates derived from ZMM pathway and thus *des10* should not affect the distribution  
445 of designated CO events but will affect the proportion of these that are resolved as CO, i.e.  
446 will affect recombination frequency but not recombination distribution.

447 Intriguingly a similar proportion of wild-type CO was observed in *des10* (37%) as in  
448 AtMLH3 knockouts (39%). While the mechanism by which the dHJs are resolved in the  
449 absence of a functional MLH3 is unclear (Jackson et al 2003), the involvement of other  
450 complexes such as MLH1-PMS2 have been suggested (Lipkin et al, 2002). Considering the  
451 interaction between the MLH1-MLH3 complex and MMS4-MUS81 in yeast (de los Santos et  
452 al, 2003; Fabre et al, 2003; Wang and Kung, 2002), and the known involvement of MUS81 in  
453 mammalian (Holloway et al, 2008) and plant CO resolution (Higgins et al, 2008a), it is  
454 possible that the resolution of the Class I COs in MLH3 mutants is mediated via the Class II  
455 machinery while maintaining the ZMM CO designations and interference (Zakharyevich et  
456 al, 2010).

457 **HvMLH3 foci evident at zygotene**



458 The development of MLH3 foci with the developing SC at zygotene is earlier than the  
459 classical expectation, where SC associated foci are generally observed at pachytene on  
460 completion of synapsis (Lipkin et al, 2002). However this early development of MLH3 foci  
461 during zygotene is supported by observations in mouse and Arabidopsis (Kolas et al, 2005;  
462 Jackson et al 2006) and of other MutL homologs in other species (Baker et al, 1995;;  
463 Storlazzi et al, 2010). The earlier association of MLH3 signal with the nucleus at zygotene  
464 before the appearance of clear foci was surprising but showed a punctate but regular  
465 organisation of stretches of ZYP1 signal separated by MLH3 foci (Fig. 7d). This would  
466 suggest that our observations are unlike the association with heterochromatic repeats found in  
467 mouse (Baker et al, 1995) or with chromatin organization suggested during chromosome  
468 segregation in humans (Roesner et al, 2014). This would therefore indicate that MLH3 is  
469 recruited earlier to the newly formed axes potentially during synapsis, rather than on mature  
470 chromosomes axes, as suggested by animal studies reporting the presence of MLH3 at  
471 pachynema.

472 In *des10*, the mutation affects the dimerization domain of HvMLH3 that would potentially  
473 cause a change in the conformation of the C-terminal domain and thus possible difficulties in  
474 forming the heterodimer with MLH1 that is required for the resolution of dHJs (Guarne et al,  
475 2004; Ranjha et al, 2014; Rogacheva et al, 2014). However the capacity of the complex to  
476 bind to chromatin would likely to be unaffected given the intact DNA binding domain, and as  
477 HvMLH3 is still recruited to the axis, its DNA binding activity appears to remain effective.  
478 This would parallel the behaviour of the yeast mutant MLH3 7 that also lacks the  
479 endonuclease motif, but is normally recruited to the DNA (Roesner et al, 2013). Interestingly  
480 the MLH3 7 studies also showed a higher turnover of the protein in the mutant that could  
481 tally with the higher background and staining of the nucleolus in this study. The early  
482 meiotic effects seen in *des10* may therefore be a manifestation of the timing of the binding of  
483 MLH3 with the continued presence of the defective protein on the axis generating a  
484 phenotype not detected in a knockout (Jackson et al, 2006; Lipkin et al, 2002)

#### 485 ***des10* displays altered synapsis progression**

486 The barley *des10* phenotype revealed a perturbation in the progression of synapsis compared  
487 to the wild-type that became evident at zygotene. This unexpected effect on synapsis and the  
488 associated delay in meiotic progression is broadly similar to phenotypes observed for ZMM  
489 mutants (Barakate et al, 2014; Higgins et al, 2004; Novak et al, 2001) although not as severe.

490 Assembled ZYP1 appears to be fairly linear under confocal microscopy and subsequent  
491 meiotic progression indicates that the chromosome are sufficiently aligned to allow some  
492 crossover resolution. However with structured illumination microscopy ZYP1 did show a less  
493 continuous signal than wild-type and was associated with a longer zygotene/pachytene  
494 transition in *des10*. While the observed differences in synapsis could have been exacerbated  
495 by an increased sensitivity of the mutant protein containing complexes to the cytological  
496 procedures, such effects would *de facto* imply a change in structure. The observed  
497 perturbation of synapsis was consistent with the timing of the appearance of MLH3 signal  
498 and potentially relates to the changed binding dynamics of the mutant protein. Similarly the  
499 delay at zygotene observed in *des10* cells would be concomitant with the observed changes in  
500 structure associated with ZYP1 signal and the difficulty in observing cells with a classic  
501 pachytene appearance given the apparent problems of synapsis. It is thus unclear whether  
502 full synapsis is achieved in this desynaptic mutant or how many cells achieve full synapsis  
503 although cells clearly do progress through to diplotene.

504

505 The interplay between recombination and synapsis is a standard feature of meiotic mutant  
506 studies and is inherent in the grouping of ZIP1 (ZYP1) and mismatch repair genes in the  
507 ZMM pathway (Mercier et al, 2014; Osman et al, 2011). However the processes of  
508 recombination and synapsis are not inseparable, with DSB formation and CO imposition  
509 known to occur prior to synapsis in some species (Fung et al, 2004; Santos, 1999; Thacker et  
510 al, 2014). While our data show that CO imposition is retained in *des10*, it also suggests that  
511 SC progression is dependent on accurate CO resolution. Synapsis would therefore appear to  
512 involve different stages; with chromosome engagement, alignment and initiation being ZMM  
513 dependent (Thacker et al, 2014) but progression and maturation also being dependent on  
514 subsequent CO resolution by MLH3. We noted that RAD51/DMC1 counts were higher in  
515 *des10* and attribute this to the concomitant change in the observed timing of meiotic  
516 progression rather than a direct effect on DSB formation, as seen in ZMM mutants (Thacker  
517 et al, 2014). The observed difference in timing of MLH3 action compared to Arabidopsis  
518 could reflect the specific nature of the *des10* mutation combined with the advantages of  
519 visualising in a large genome with high resolution microscopy. Whether our observations  
520 reflect an earlier role for MLH3 in plants in general or specifically in barley, they are  
521 consistent with the known spatio-temporal difference between barley and Arabidopsis in  
522 early meiosis and the considerable variation in genome size and organisation of  
523 heterochromatin between these species (Higgins et al, 2012). There are differences in

524 chromosome dynamics of the two species in early prophase (Armstrong et al, 2001) with  
525 barley exhibiting a temporal differentiation in recombination initiation between distal and interstitial  
526 chromosomal regions that correspond to the relative timing of replication and the differentiation of the  
527 genome by chromatin modifications (Higgins et al, 2012; Baker et al, 2015).

528

529 In summary we have taken advantage of genetic and genomic resources in barley to identify  
530 an exonic deletion in the orthologue of *MutL-Homolog 3 (Mlh3)* as the causal lesion in a  
531 natural semi-sterile *DESYNAPTIC 10 (des10)* mutant. *des10* exhibits reduced recombination  
532 and fewer chiasmata than the wild-type, congruent with our expectations for the post-ZMM  
533 role of HvMLH3 in the resolution of predetermined CO sites. The reduction in chiasmata  
534 resulted in the loss of obligate crossing-over leading to chromosome mis-segregation and the  
535 semi-sterile phenotype. This study thus confirms the conserved role of MLH3 in barley  
536 previously assumed in earlier studies (Phillips et al, 2013) and the non-random nature of the  
537 CO distribution in the mutant as postulated but not demonstrated in Arabidopsis (Jackson et  
538 al, 2003). Importantly, in barley MLH3 foci are evident at zygotene, earlier than expected,  
539 although this has been observed in other systems (Kolas et al, 2005) and that using 3D-SIM  
540 super-resolution microscopy we were able to observe that *des10* also exhibited aberrant  
541 synaptonemal complex progression at this stage, associated with a meiotic delay. We  
542 interpret this as meaning that the resolution of CO is initiated early in barley and that its  
543 disruption in *des10* compromises synapsis progression with the associated change in the  
544 dynamics of the mutant MLH3 protein. Thus, in barley both crossover imposition and  
545 crossover resolution occur prior to full synapsis, affirming the importance of the early stages  
546 of prophase I for the control of recombination. The integration of genetic and cytological  
547 approaches to dissect the mutant phenotype of *Hvmlh3* establishes the tractability of studying  
548 meiosis in large genome cereals. The size of the genome facilitates cytological discrimination  
549 of the profound changes in chromosome structure during prophase I and is potentially  
550 associated with specific changes in timing of meiotic processes when compared to physically  
551 smaller model systems.

552

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563

#### 564 **AUTHORS CONTRIBUTIONS:**

565 I.C., L.R., and R.W designed the study. I.C., M.M., J.D.H, D.P., and L.R. and carried out  
566 experiments and analysis. A.B. cloned and sequenced the full-length HvMLH3 cDNA and  
567 provided resources and supervision for cDNA sequencing. M.P. carried out 3D-SIM imaging  
568 and reconstruction. M.P., A.B., C.H., S.J.A. and F.C.H.F. advised on experiment and/or  
569 manuscript revisions. I.C., R.W., A.B., F.C.H.F, S.J.A, C.H. and L.R. wrote the paper.

570

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788 **FIGURE LEGENDS**

789 **Figure 1: *des10* mapping.**

790 *des10* exhibits a semi-sterile phenotype **(a)** producing fewer seeds per ear ( $7.0\pm 2.7$ ) in *des10*  
791 than wild-type ( $13.7\pm 3.7$ ). The *des10* region **(b)** was initially delineated between two SNP  
792 markers (11\_11273 and 11\_21203) on the long arm of chromosome 5H and then fine mapped  
793 on an extended F<sub>2</sub> population to a 0.2cM region between two markers (MLOC\_17896 and  
794 MLOC\_34818) located on the same BAC contig (contig\_38588). The only exonic  
795 polymorphism for the genes within this BAC contig was a deletion in MLOC\_52425  
796 encoding the barley ortholog of *HvMLH3*.

797 **Figure 2: *des10* mutation.**

798 **(a)** Alignment of *HvMlh3* gene sequences in wt and *des10*, with exons are highlighted in  
799 grey. The 159bp deletion removes the entire 17th exon coding for the peptide sequence  
800 HAADERIRLEELRSK without affecting reading frame. **(b)** The Histidine kinase-like  
801 ATPases and the MutL\_Trans domains are unaffected but the *des10* Mutl-C domain is  
802 missing the majority of the metal binding motif QHAADERIRLEE (red box). This 15 amino  
803 acid deletion potentially affects the Mutl-C conformation in *des10* **(c)** as compared to the  
804 wild-type **(d)**.

805 **Figure 3: Reduced chiasmata and abnormal chromosome segregation in *des10*.**

806 3D confocal optical section of wild-type **(a-d)** and *des10* **(e-h)** meiocytes (Scale bars 10  $\mu$ m).  
807 At pachytene, homologous chromosomes are paired in both wild-type **(a)** and *des10* **(e)** as  
808 shown with the 45s (red) and 5s (green) probes. wild-type metaphase I **(b)** has seven ring  
809 bivalents that can be identified with 45S (red) and 5S (green) probes, whereas *des10*  
810 metaphase I **(f)** averages 9.2 chiasmata per nucleus with occasional univalents. During  
811 anaphase I, chromosomes segregate to each pole in wild-type **(c)** while chromosome mis-  
812 segregation is evident in *des10* **(g)**. Tetrad are normal and genetically balanced in wt **(d)** but  
813 not in *des10* **(h)** showing **(i)** Histogram of the distribution of chiasmata per cell for *des10* and  
814 wild-type and **(j)** a table of the number of chiasmata per chromosome in wild-type and *des10*.

815

816 **Figure 4: Genetic mapping in F<sub>3</sub> families.**

817 Alignment of the physical sequence (in Mbp) with consensus genetic map (in cM) for  
818 chromosome 1H with comparisons to the genetic maps calculated from BW230 (*des10*) x  
819 Morex F<sub>3</sub> families derived from F<sub>2</sub> individuals homozygous for either the wild-type or *des10*  
820 mutant allele at *HvMlh3*. Estimated centromere position on genetic map marked in red.

821 **Figure 5: Comparison of synapsis in wild-type and *des10*.**

822 Progression of synapsis in wild-type and *des10* demonstrated by the immuno-localization of  
823 AtASY1 (green) and AtZYP1 (magenta) on formaldehyde fixed meiocytes. Cells visualised  
824 by 3D-SIM show the progression of synapsis in wild-type (**a-f**) and *des10* (**g-l**) at leptotene  
825 (**a,b,g,h**), zygotene (**c,d,i,j**) and pachytene (**e,f,k,l**) together with detailed views of white  
826 squared regions (squares in **a,c,e,g,i,k**) shown in (**b,d,f,h,j,l**). Scale bars 5µm

827 **Figure 6: Imaris modelling of *des10* pachytene like cell.**

828 **a)** 3D view from Imaris of *des10* cell from Figure 5k with ASY1 labelling. **b)** Individual  
829 bivalent labelling in different colours using Imaris tracking. **c)** the distance between the two  
830 homologous chromosomes is 0.1µm corresponding to the wt SC distance.

831 **Figure 7: EDU time course in wt and *des10*.**

832 Percentage of cells in each meiotic stage category and 3D confocal optical sections of wild-  
833 type and *des10* meiocytes at 6h (**a-c**), 18h (**d,f**), 24h (**g-i**), 48h (**j-l**) and 68h (**m-o**). Scale bars  
834 5µm. PM, TB, Lept, Zyg, Pach, Dip, MI, AI signifying Pre-meiotic, Telomere bouquet,  
835 Leptotene, Zygotene, Pachytene, Diplotene, Metaphase I and Anaphase I respectively.

836 **Figure 8: Distribution of ZYP1 and HvMLH3 during prophase.**

837 Wild-type (**a-l**) and *des10* (**m-x**) meiotic progression monitored using antibodies raised  
838 against HvZYP1 (magenta) and HvMLH3 (green) using 3D-SIM with detailed views of white  
839 squared regions (squares in **c,g,k,o,s,w** shown in **d,h,l,p,t,x**). At early zygotene in both wild-  
840 type (**a-d**) and *des10* (**m-p**) MLH3 signal is abundant (**b,n**) in the nucleus including  
841 associations with the chromosomes axes (**d,p**). This continues into early pachytene, in both  
842 wild-type (**e-h**) and *des10* (**q-t**). However, in the wild-type (**g,h**) a few foci with a stronger  
843 signal potentially marking the finalized COs become evident (triangles) while it is difficult to  
844 differentiate foci in *des10* (**s,t**). At late pachytene (**i-l, u-x**), CO foci (triangles) are seen

845 clearly in wild-type as compared to weaker un-associated signals (circle) (**k,l**). Weaker SC  
 846 associated foci (**w-x**) are discernible in *des10* (triangles) though considerable MLH3 signal  
 847 remains in the nucleus and on the axes. Scale bars 5 $\mu$ m

848 **Table 1: Recombination foci in wild-type and *des10*.**

849 Table showing the number of AtDMC1, AtRAD51 and AtMSH4 foci in wild-type and *des10*  
 850 at the telomere bouquet, the stage of de-clustering of the telomere and the linear ASY1 stage.

Stage	Protein	WT	<i>des10</i>	TTEST static
Telomere bouquet	RAD51	127.27 $\pm$ 55.38	142.2 $\pm$ 49.3	2.46E-01
De-clustering	RAD51	164.96 $\pm$ 63.12	240.8 $\pm$ 80.6	6.48E-04
Linear ASY1	RAD51	349.3 $\pm$ 79.5	700.3 $\pm$ 128.2	1.721E-05
	DMC1	361.3 $\pm$ 62.9	766.8 $\pm$ 147.3	4.4873E-06
	MSH4	323.2 $\pm$ 33.4	639.5 $\pm$ 79.5	3.53682E-06

851

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853

854 **SUPPORTING INFORMATION**

855 **Figure S1: *des10* cDNA and polymorphism.**

856 PCR-sequencing of MLOC\_52425 (*HvMLH3*) revealed a single polymorphism between  
857 *des10* and wild-type lines including Bowman and the original mutant background cultivar  
858 Betzes (**a**), due to a 159bp deletion potentially removing the 17th exon. Sequencing amplified  
859 cDNA from anthers and young inflorescence in both wild-type and *des10* confirmed that the  
860 mutant cDNA was missing the 17th exon, leaving the sequence in frame (**b**).

861 **Figure S2: Recombination in F<sub>3</sub> families for chromosomes 2H-7H.**

862 Comparison of the consensus genetic maps (a) for chromosomes 2H-7H with those  
863 calculated from BW230 (*des10*) x Morex F<sub>3</sub> families derived from F<sub>2</sub> individuals  
864 homozygous for either the wild-type (b) or *des10* mutant allele at *HvMlh3* (c). The position of  
865 the centromere is marked in red on all consensus maps and the position of *des10* marked in  
866 red on the 5H consensus map.

867 **Figure S3: Recombination in F<sub>3</sub> families at three intervals.**

868 KASP markers were designed to SNPs delineating intervals (box) in three contrasting  
869 genomic regions (centromeric 4H, distal 6HS and distal 7HL) (**a**). Recombination in the three  
870 unlinked genetic intervals is reduced by 61% in individuals in F<sub>3</sub> families derived from F<sub>2</sub>  
871 individuals homozygous for the *des10 HvMlh3* allele compared to those derived from  
872 individuals homozygous for the wild-type allele (**b**).

873

874 **Figure S4: Synapsis details in wild-type and *des10*.**

875 Detailed progression of synapsis in wild-type (**a-l**) and *des10* (**m-x**) cells demonstrated by the  
876 immuno-localization of AtASY1 (green) and AtZYP1 (magenta) on formaldehyde fixed  
877 meiocytes. Cells visualised by 3D-SIM show the progression of synapsis in wild-type at  
878 leptotene (**a-d**), zygotene (**e-h**), and pachytene (**i-l**) together with detailed views of white  
879 squared regions compare to the progression of synapsis in *des10* at leptotene (**m-p**), zygotene  
880 (**q-t**), and pachytene (u-x) together with detailed views of white squared regions. Scale bars  
881 5µm.

882 **Figure S5: Comparison of Confocal and Structured Illumination Microscopy images.**

883 Confocal images of (a) wild-type and (b) *des10* show a linear HvZYP1 (magenta) signal at  
884 pachytene and it is possible to count the number of HvMLH3 (green) foci in wild-type on the  
885 newly formed SC. The same cells imaged by 3D-SIM show that in wild-type (c), ZYP1 is  
886 indeed linear along the chromosome. In *des10* (d), ZYP1 signal is seen along the entire  
887 length of the chromosome suggesting that pachytene is achieved but the signal remains non-  
888 continuous, suggesting that ZYP1 loading is not complete.

889 **Figure S6: Meiocyte size.**

890 The sizes of the nucleus were estimated using chromatin stain diameter ( $\mu\text{m}$ ) at different  
891 stages of meiosis (a) gauged by ASY1 and ZYP1 labelling. Results show a lack of  
892 synchronicity between cell size and meiotic stage in *des10* relative to wild-type (b).

893 **Figure S7: HvMLH3 foci count in wild-type late pachytene.**

894 (a) 3D confocal image of wild-type pachytene labelled with HvZYP1 (magenta) and  
895 HvMLH3 (green) with the nucleus showing 21 MLH3 foci on the ZYP1 axes. (b) 3D SIM  
896 image gallery of wild-type pachytene labelled with HvZYP1 (magenta) and HvMLH3  
897 (green) with numbering highlighting the foci presumably marking crossovers. This nucleus  
898 shows 18 MLH3 foci on the ZYP1 axes.

899 **Figure S8: HvMLH3 foci in *des10*.**

900 (a) 3D confocal image of a *des10* cell labelled with HvZYP1 (magenta) and HvMLH3  
901 (green). This cell appears to be at pachytene as the ZYP1 signal is quite linear. Despite a high  
902 MLH3 background, 5 MLH3 foci can be seen associated with the ZYP1 axes. (b) 3D SIM  
903 image gallery of a *des10* cell labelled with HvZYP1 (magenta) and HvMLH3 (green) with  
904 the numbering highlighting the foci presumably marking crossovers. This nucleus shows 10  
905 MLH3 foci on the ZYP1 axes although with 3D SIM the ZYP1 appears non-linear.

906 **Figure S9: 3D localisation of RAD51.**

907 AtRAD51 (green) protein initially loads onto the chromosome from the telomere region in  
908 both wild-type (a) and *des10* (b). As RAD51 protein signal moves from telomere to more  
909 proximal regions it becomes possible to count individual foci in wild-type (c) and *des10* (d).  
910 Scale bars  $5\mu\text{m}$ .

911

912 **Figure S10: RAD51, DMC1 and MSH4 foci on SC spreads (squash).**

913 Co- immunolocalization was also performed for (a) AtASY1 (green) and AtRAD51  
914 (magenta), (b) AtASY1(green) and AtDMC1 (magenta), and (c) AtASY1 (green) and  
915 AtMSH4 (magenta), to monitor the localization of DSBs on the axial elements. In both wild-  
916 type and *des10* early (RAD51 and DMC1) and intermediate (MSH4) recombination proteins  
917 load on the chromosome axis (ASY1) revealing that crossing over intermediates are formed  
918 in both wild-type and *des10*. Scale bars 5 $\mu$ m.

919

920 **Table S1: MLH3 foci counts.**

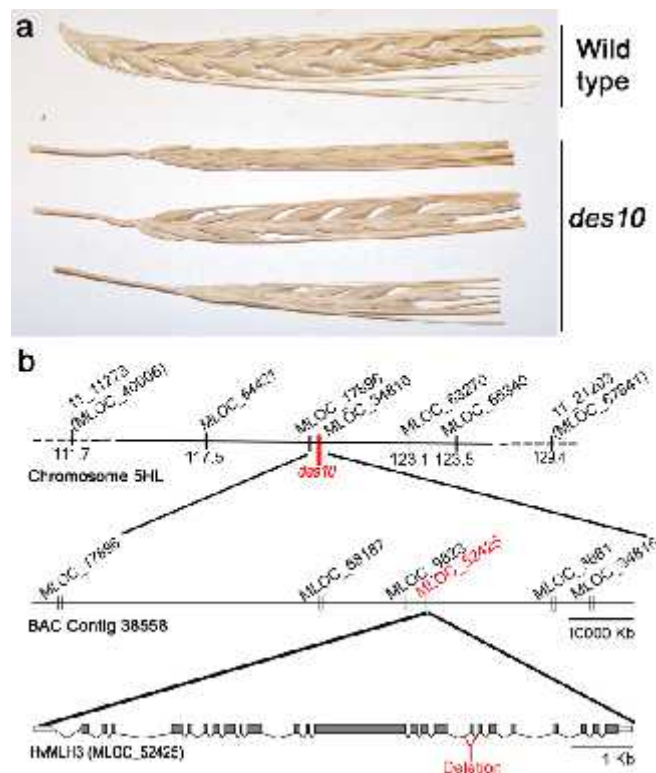
921 Table showing the number of MLH3 foci at late pachytene in 3D stack images of wild-type  
922 and *des10* cells.

923



**Figure 1: *des10* mapping.**

*des10* exhibits a semi-sterile phenotype (a) producing fewer seeds per ear ( $7.0 \pm 2.7$ ) in *des10* than wild-type ( $13.7 \pm 3.7$ ). The *des10* region (b) was initially delineated between two SNP markers (11\_11273 and 11\_21203) on the long arm of chromosome 5H and then fine mapped on an extended F<sub>2</sub> population to a 0.2cM region between two markers (MLOC\_17896 and MLOC\_34818) located on the same BAC contig (contig\_38588). The only exonic polymorphism for the genes within this BAC contig was a deletion in MLOC\_52425 encoding the barley ortholog of *HvMLH3*.



**Figure 2: *des10* mutation.**

**(a)** Alignment of *HvMlh3* gene sequences in wt and *des10*, with exons are highlighted in grey. The 159bp deletion removes the entire 17th exon coding for the peptide sequence HAADERIRLEELRSK without affecting reading frame. **(b)** The Histidine kinase-like ATPases and the MutL\_Trans domains are unaffected but the *des10* MutL-C domain is missing the majority of the metal binding motif QHAADERIRLEE (red box). This 15 amino acid deletion potentially affects the MutL-C conformation in *des10* **(c)** as compared to the wild-type **(d)**.

**a**

```

Wild type  AAGCAGATTCATTCGCGCCGTCATATCGCCGAAATGCTACTGCTGCTTCATCGAGCTTCTAGCTTACTTCATTCACGCGCE
des10      AAGCAGATTCATTCGCGCCGTCATATCGCCGAAATGCTACTGCTGCTTCATCGAGCTTCTAGCTTACTTCATTCACGCGCA
           R K E T P V T S G E M I L E V D Q

Wild type  CTCCIGGCAGATGAAATTAITTTTACGCGTAATCTGTTTATCGTTTCCTCCAGCGAATAAATAGCGAAATCCCAAGAA
des10      CTCCIGGCAGATGAAATTAITTTTACGCGTAATCTGTTTATCGTTTCCTCCAGCGAATAAATAGCGAAATCCCAAGAA

Wild type  GTCGACCTTTGACTTCGAACTCCTCCAGCCCTTCGAAATATAATCTGTAATATAATTAACCAATAATTTCTTTCCAGCTTTTCTT
des10      GTCGACCTTTGACTTCGAACTCCTCCAGCCCTTCGAAATATAATCTGTAATATAATTAACCAATAATTTCTTTCCAGCTTTTCTT

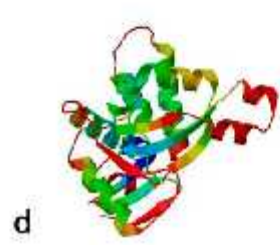
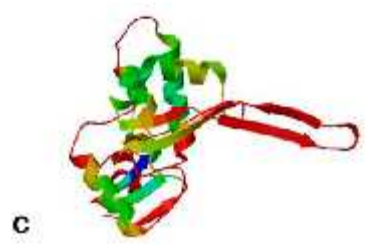
Wild type  GTTGTTCAGGATTTTTCGATATATCTCCAGATACAGAAATATTCGCGTTATATCCGACACTGAAATTCGAAAGCATCCAI
des10      GTTGTTCAGGATTTTTCGATATATCTCCAGATACAGAAATATTCGCGTTATATCCGACACTGAAATTCGAAAGCATCCAI

Wild type  TTCVLAASTVSTTAAITTCGTAAGLAAKATMTECAATFSGATGACAAALACATTTTCGCGGATTTTSSAAAGLCAI
des10      TTCVLAASTVSTTAAITTCGTAAGLAAKATMTECAATFSGATGACAAALACATTTTCGCGGATTTTSSAAAGLCAI

Wild type  GTCGIAINDTACCTCTGCGCGCTGCGTAAAGGATGCGTTTGGAGGAGCTCCGTAACGAAATGTTGTAACGCTA
des10      -----
           H A A D E R I R L E E L R S K

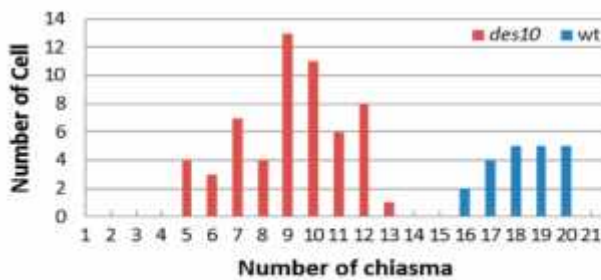
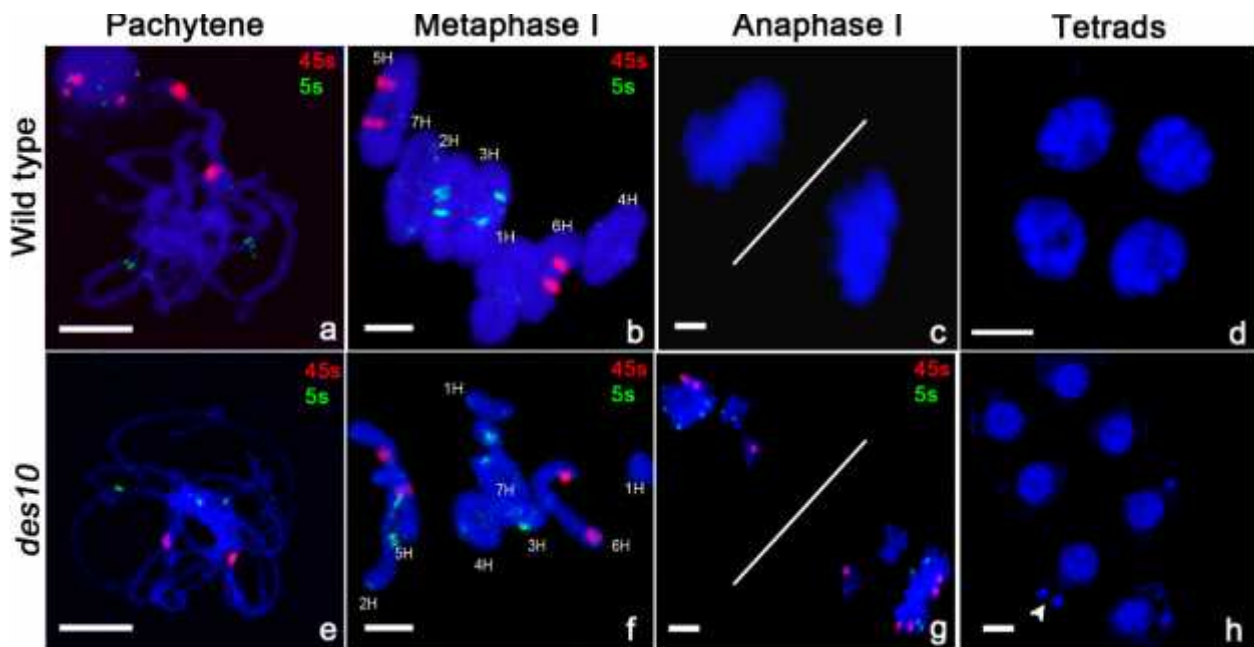
Wild type  AACTTACCAAGGAACTACTTTTAAATAGGGGTTTTCATTTAACRTSTTAACTATGTTGGATTTTCAGGTTTTCAGGAAI
des10      -----
           V I S D

Wild type  GATGCGGAGATTTTCGATATATCTCCAGATACAGAAATATTCGCGTTATATCCGACACTGAAATTCGAAAGCATCCAI
des10      GATGCGGAGATTTTCGATATATCTCCAGATACAGAAATATTCGCGTTATATCCGACACTGAAATTCGAAAGCATCCAI
           D D R G I T Y L D S E X E X
    
```



**Figure 3: Reduced chiasmata and abnormal chromosome segregation in *des10*.**

3D confocal optical section of wild-type (a-d) and *des10* (e-h) meocytes (Scale bars 10  $\mu$ m). At pachytene, homologous chromosomes are paired in both wild-type (a) and *des10* (e) as shown with the 45s (red) and 5s (green) probes. wild-type metaphase I (b) has seven ring bivalents that can be identified with 45S (red) and 5S (green) probes, whereas *des10* metaphase I (f) averages 9.2 chiasmata per nucleus with occasional univalents. During anaphase I, chromosomes segregate to each pole in wild-type (c) while chromosome mis-segregation is evident in *des10* (g). Tetrads are normal and genetically balanced in wt (d) but not in *des10* (h) showing (i) Histogram of the distribution of chiasmata per cell for *des10* and wild-type and (j) a table of the number of chiasmata per chromosome in wild-type and *des10*.



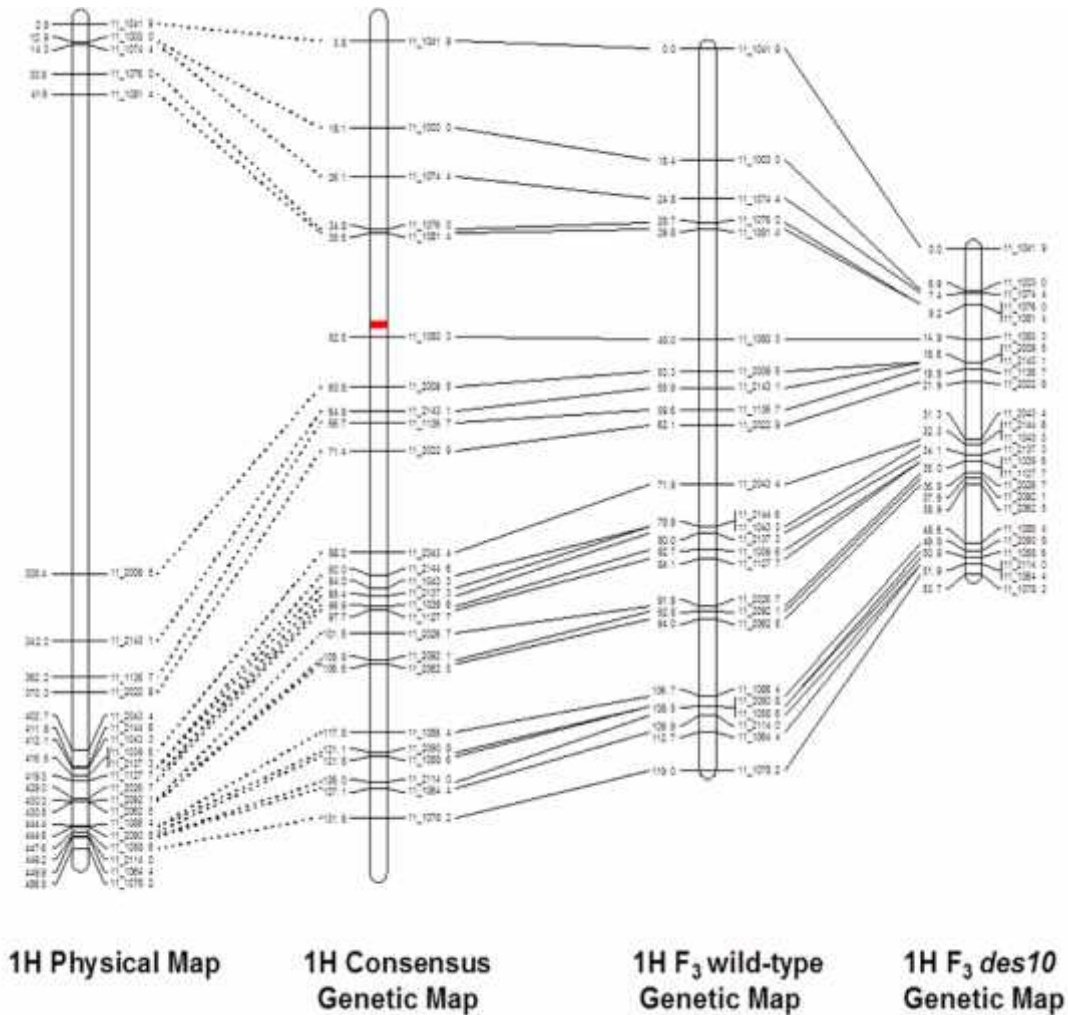
i- Distribution of chiasma number per cell

	1H	2H	3H	4H	5H	6H	7H	Total
wt	2.4	2.9	3.0	2.5	2.5	2.6	2.4	18.4
<i>des10</i>	1.1	1.3	1.3	1.4	0.9	1.0	1.2	9.2

j. Chiasma number per bivalent

**Figure 4: Genetic mapping in F<sub>3</sub> families.**

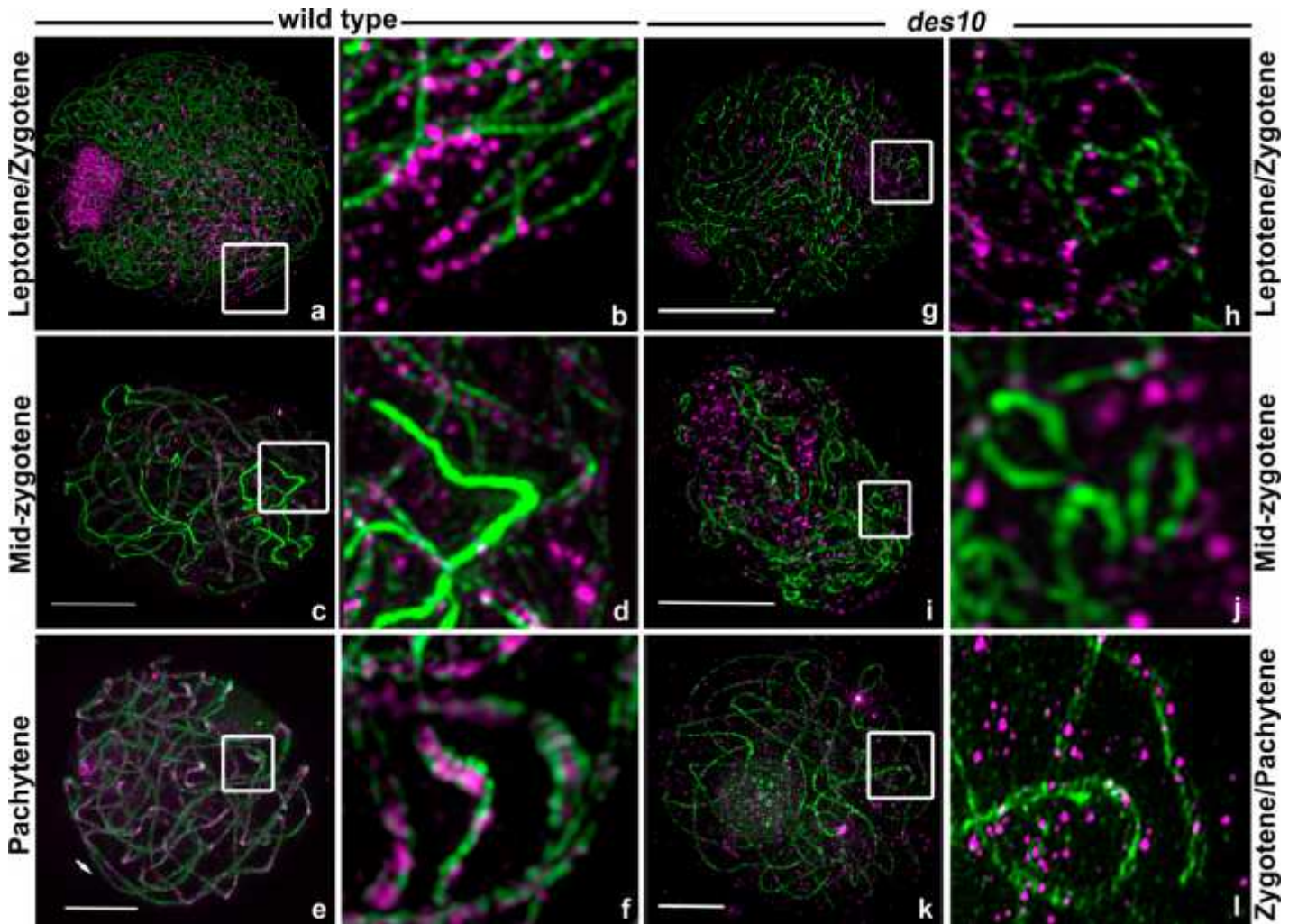
Alignment of the physical sequence (in Mbp) with consensus genetic map (in cM) for chromosome 1H with comparisons to the genetic maps calculated from BW230 (*des10*) x Morex F<sub>3</sub> families derived from F<sub>2</sub> individuals homozygous for either the wild-type or *des10* mutant allele at *HvMlh3*. Estimated centromere position on genetic map marked in red.





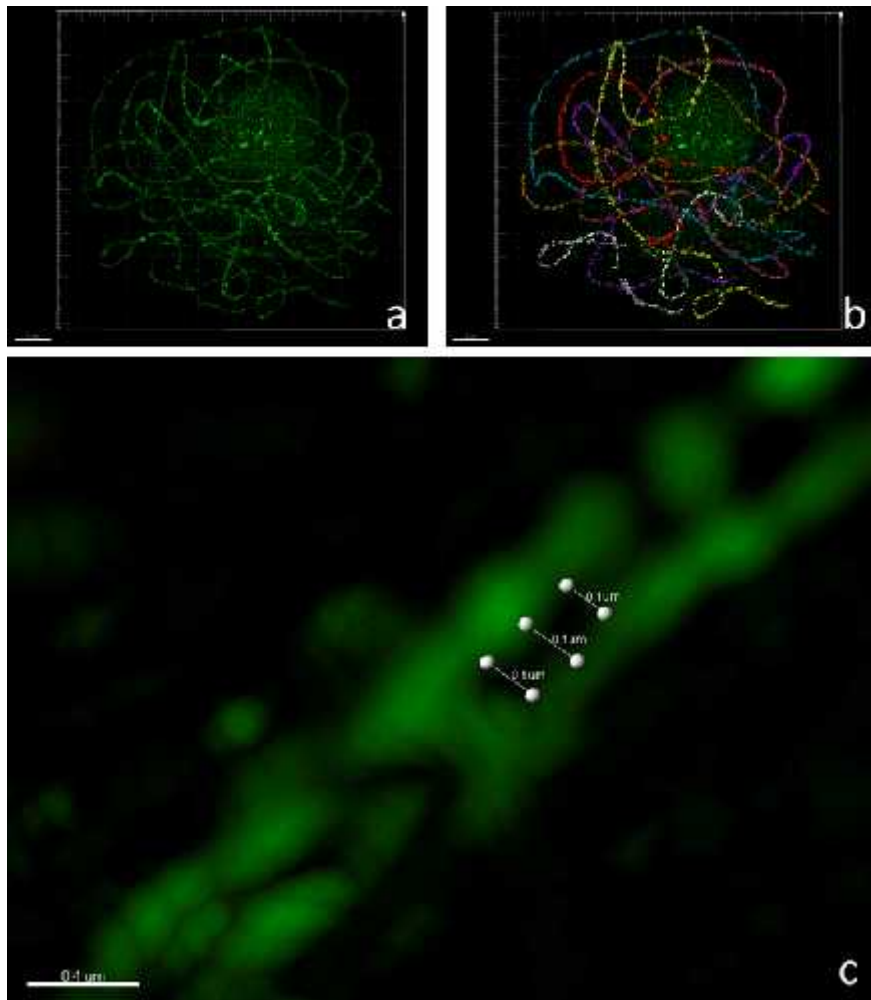
**Figure 5: Comparison of synapsis in wild-type and *des10*.**

Progression of synapsis in wild-type and *des10* demonstrated by the immuno-localization of AtASY1 (green) and AtZYP1 (magenta) on formaldehyde fixed meiocytes. Cells visualised by 3D-SIM show the progression of synapsis in wild-type (**a-f**) and *des10* (**g-l**) at leptotene (**a,b,g,h**), zygotene (**c,d,i,j**) and pachytene (**e,f,k,l**) together with detailed views of white squared regions (squares in **a,c,e,g,i,k**) shown in (**b,d,f,h,j,l**). Scale bars 5 $\mu$ m



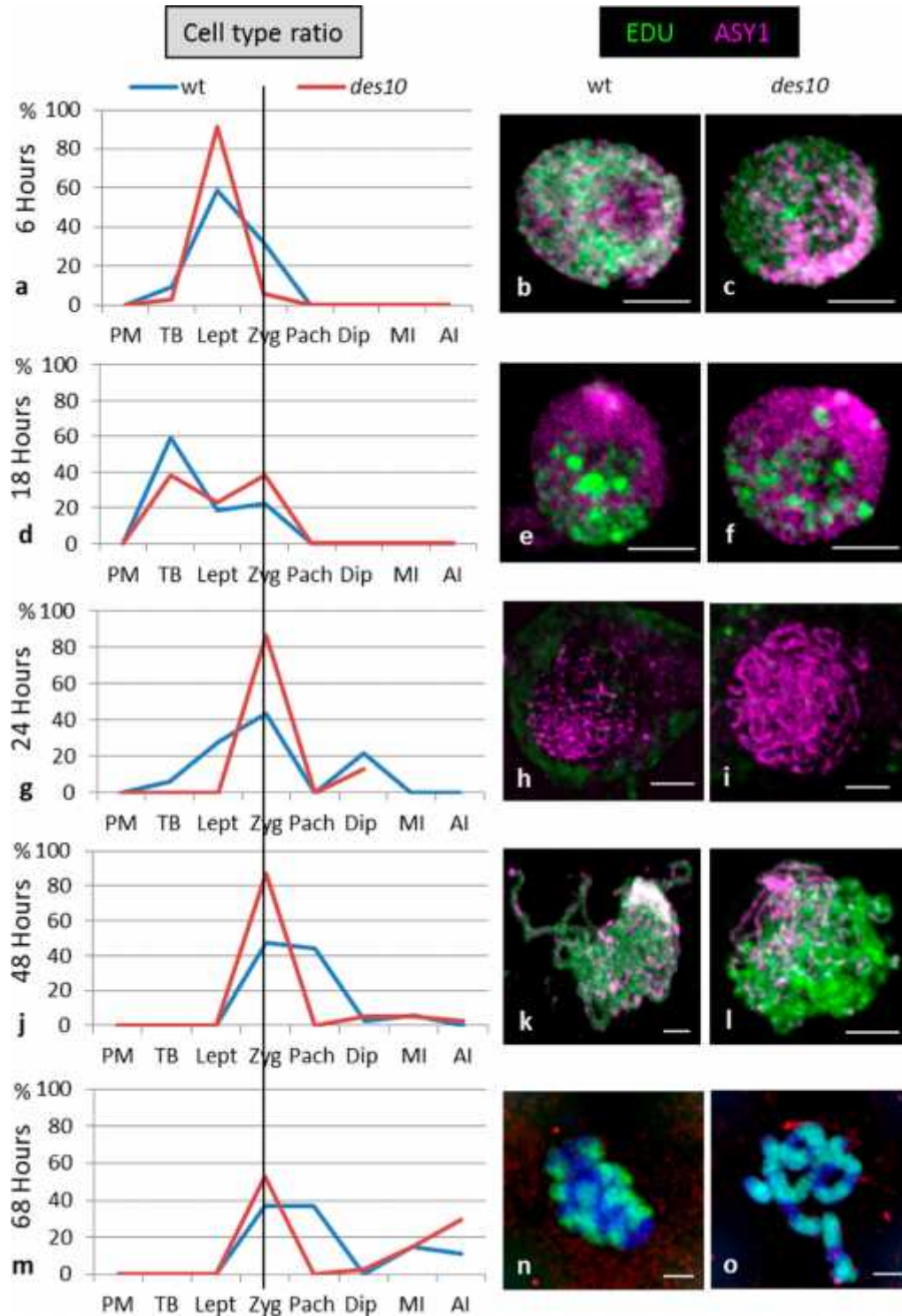
**Figure 6: Imaris modelling of *des10* pachytene like cell.**

**a)** 3D view from Imaris of *des10* cell from Figure 5k with ASY1 labelling. **b)** Individual bivalent labelling in different colours using Imaris tracking. **c)** the distance between the two homologous chromosomes is  $0.1\mu\text{m}$  corresponding to the wt SC distance.



**Figure 7: EDU time course in wt and *des10*.**

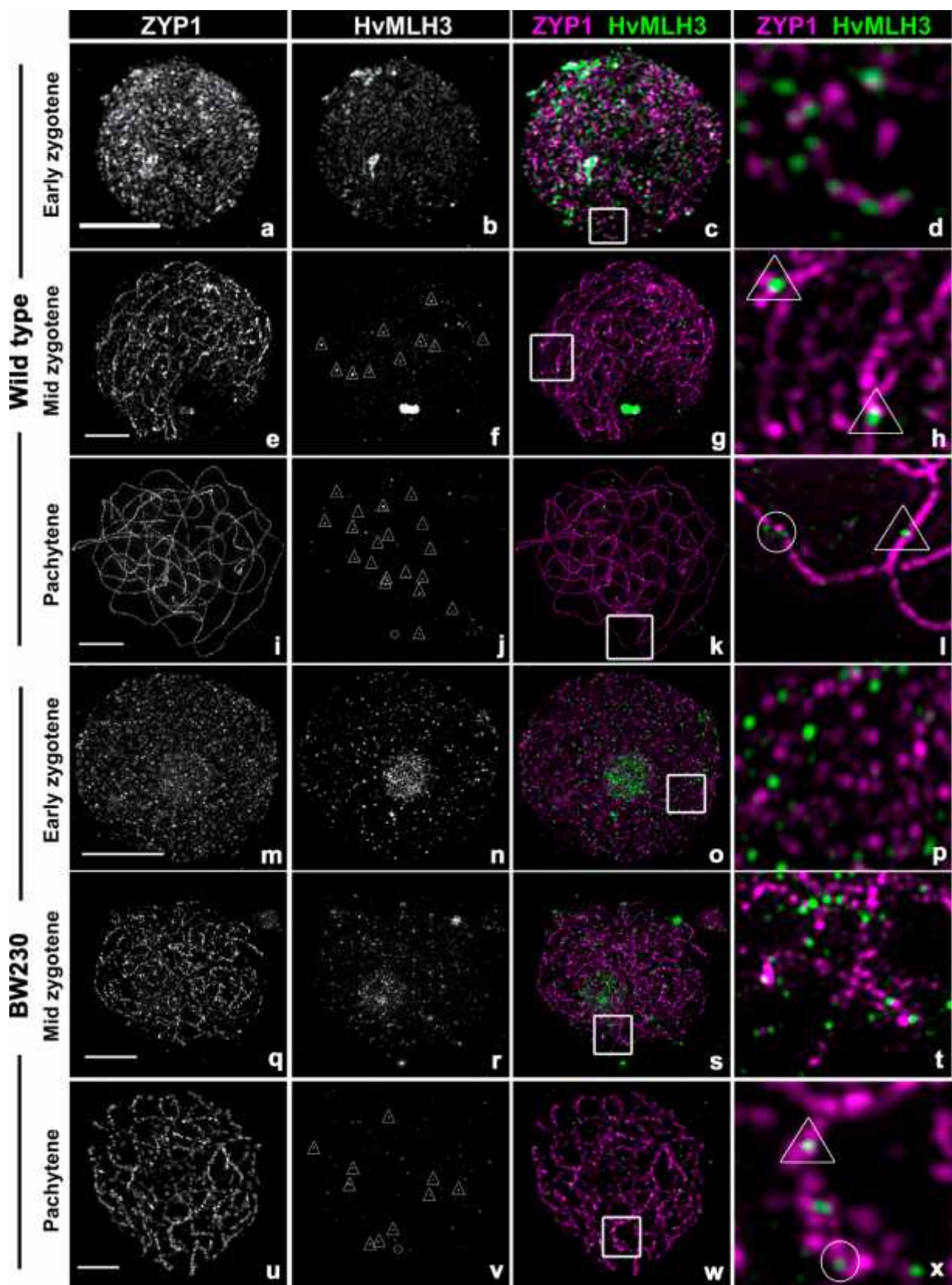
Percentage of cells in each meiotic stage category and 3D confocal optical sections of wild-type and *des10* meiocytes at 6h (a-c), 18h (d,f), 24h (g-i), 48h (j-l) and 68h (m-o). Scale bars 5µm. PM, TB, Lept, Zyg, Pach, Dip, MI, AI signifying Pre-meiotic, Telomere bouquet, Leptotene, Zygotene, Pachytene, Diplotene, Metaphase I and Anaphase I respectively.



**Figure 8: Distribution of ZYP1 and HvMLH3 during prophase.**

Wild-type (**a-l**) and *des10* (**m-x**) meiotic progression monitored using antibodies raised against HvZYP1 (magenta) and HvMLH3 (green) using 3D-SIM with detailed views of white squared regions (squares in **c,g,k,o,s,w** shown in **d,h,l,p,t,x**). At early zygotene in both wild-type (**a-d**) and *des10* (**m-p**) MLH3 signal is abundant (**b,n**) in the nucleus including associations with the chromosomes axes (**d,p**). This continues into early pachytene, in both wild-type (**e-h**) and *des10* (**q-t**). However, in the wild-type (**g,h**) a few foci with a stronger signal potentially marking the finalized COs become evident (triangles) while it is difficult to differentiate foci in *des10* (**s,t**). At late pachytene (**i-l, u-x**), CO foci (triangles) are seen clearly in wild-type as compared to weaker un-associated signals (circle) (**k,l**). Weaker SC associated foci (**w-x**) are discernible in *des10* (triangles) though considerable MLH3 signal remains in the nucleus and on the axes. Scale bars 5 $\mu$ m





**Table S1: MLH3 foci counts.**

Table showing the number of MLH3 foci at late pachytene in 3D stack images of wild-type and *des10* cells.

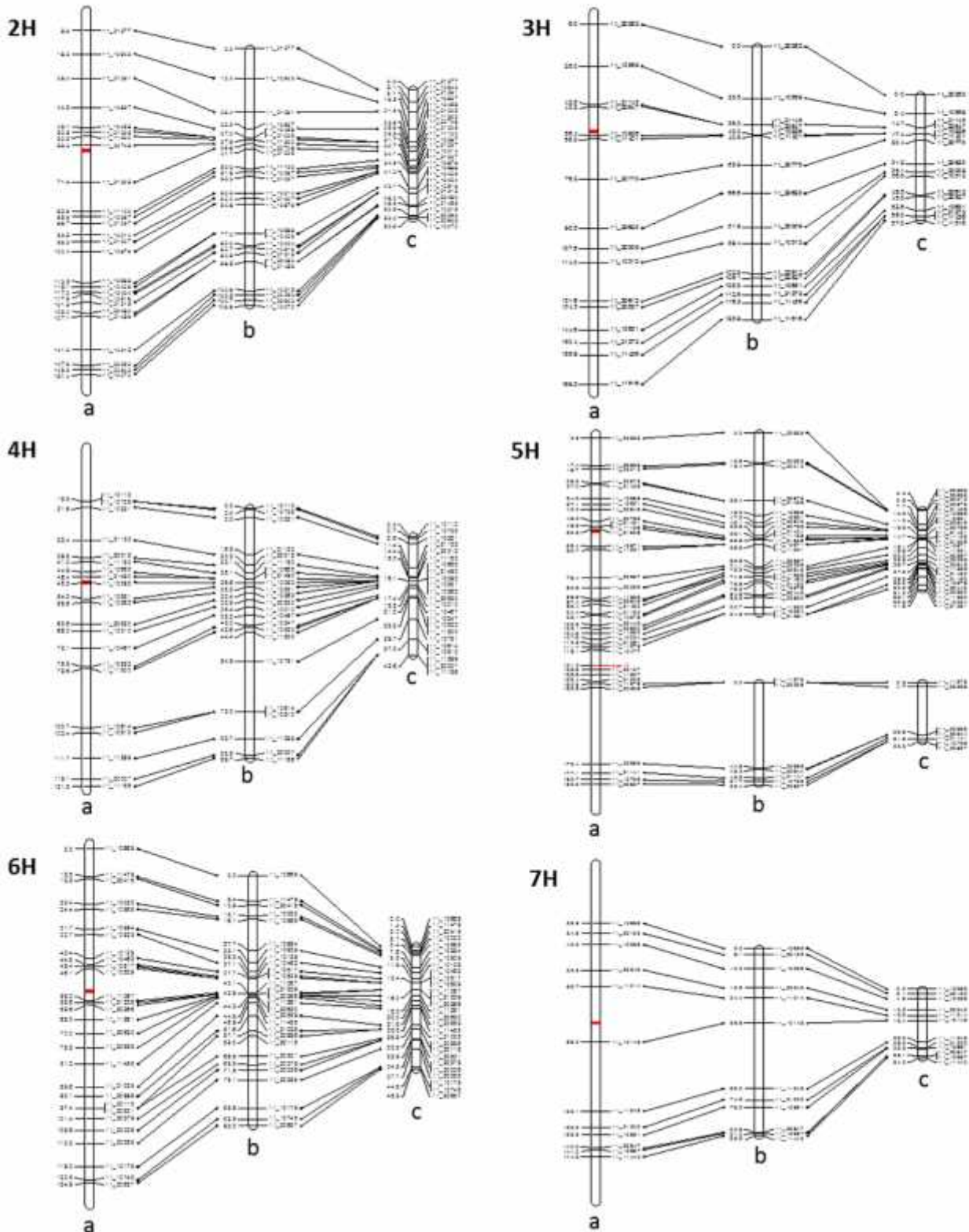
wild-type Image no.	wild-type MLH3 foci	<i>des10</i> Image no.	<i>des10</i> MLH3 foci
Image 1	23	Image 1	7
Image 2	22	Image 2	7
Image 3	22	Image 3	6
Image 4	26	Image 4	7
Image 5	26	Image 5	9
Image 6	20	Image 6	7
Image 7	21	Image 7	6
Image 8	26	Image 8	9
Image 9	20	Image 9	6
Image 10	20	Image 10	7
Image 11	24	Image 11	8
Image 12	15	Image 12	6
Image 13	16	Image 13	10
Image 14	23	Image 14	4
Image 15	18	Image 15	6
Image 16	21	Image 16	8
Image 17	20	Image 17	12
Image 18	17	Image 18	8
Image 19	16	Image 19	9
		Image 20	9
		Image 21	6
		Image 22	8
		Image 23	8
		Image 24	9
		Image 25	8
		Image 26	7
		Image 27	8
		Image 28	8
		Image 29	9
		Image 30	10
<b>Foci Total</b>	<b>396</b>		<b>232</b>
<b>Number of Cell</b>	<b>19</b>		<b>30</b>
<b>mean No./nucleus</b>	<b>20.84</b>		<b>7.73</b>
<b>SD</b>	<b>3.40</b>		<b>1.60</b>





**Figure S2: Recombination in F<sub>3</sub> families for chromosomes 2H-7H.**

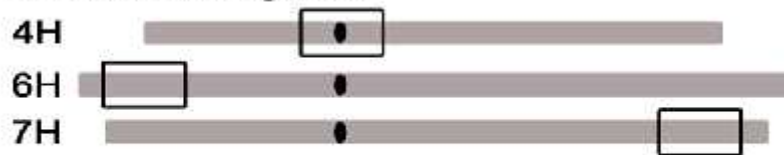
Comparison of the consensus genetic maps (a) for chromosomes 2H-7H with those calculated from BW230 (*des10*) x Morex F<sub>3</sub> families derived from F<sub>2</sub> individuals homozygous for either the wild-type (b) or *des10* mutant allele at *HvMlh3* (c). The position of the centromere is marked in red on all consensus maps and the position of *des10* marked in red on the 5H consensus map.



**Figure S3: Effect on recombination in F<sub>3</sub> families.**

KASP markers were designed to SNPs delineating intervals (box) in three contrasting genomic regions (centromeric 4H, distal 6HS and distal 7HL) (a). Recombination in the three unlinked genetic intervals is reduced by 61% in individuals in F<sub>3</sub> families derived from F<sub>2</sub> individuals homozygous for the *des10 HvMih3* allele compared to those derived from individuals homozygous for the wild-type allele (b).

**a Intervals targeted**



●=Centromere

4H interval = 11\_21122 - 11\_10262

6H interval = 11\_10669 - 11\_10868

7H interval = 11\_11243 - 11\_11440

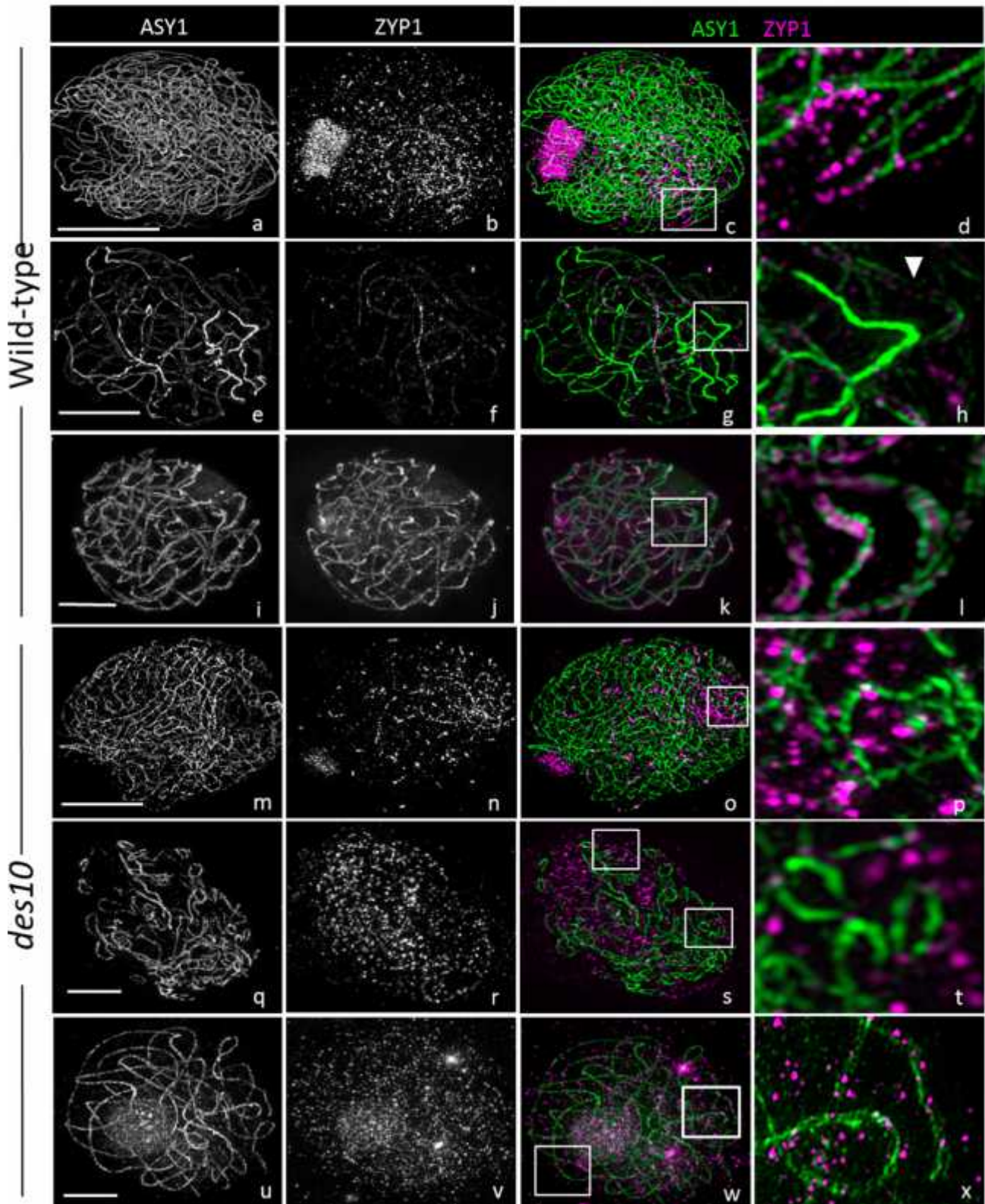
**b**

Interval	wt	<i>des10</i>	%
4H	0.184	0.100	55%
6H	0.380	0.099	26%
7H	0.412	0.150	36%
mean			39%



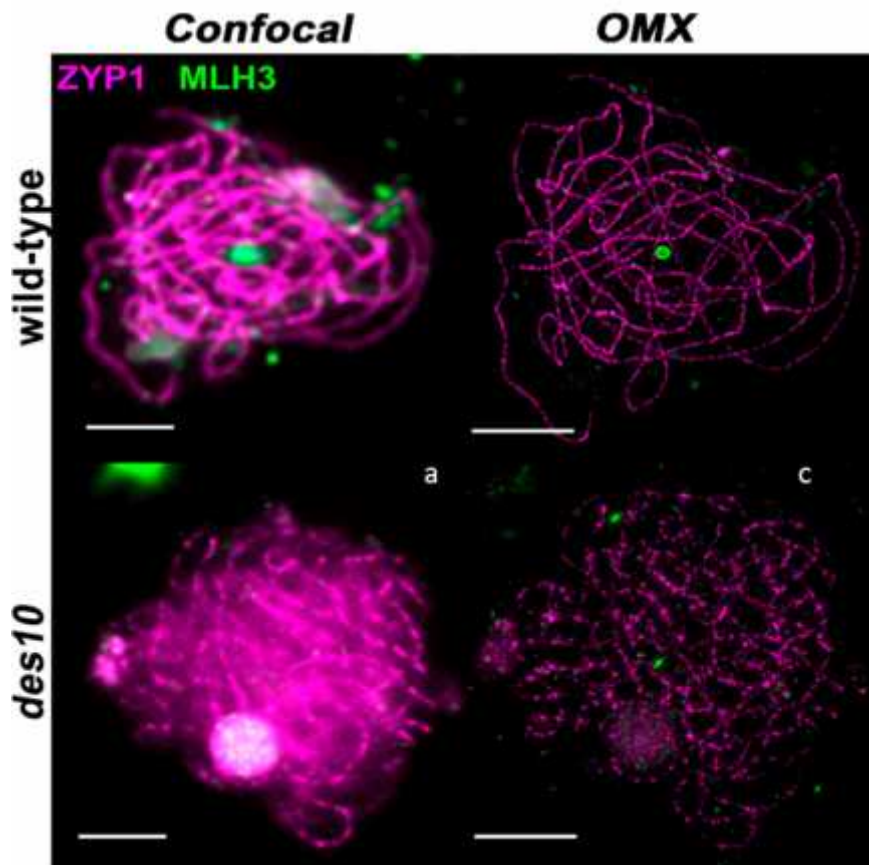
**Figure S4: Synapsis details in wild-type and *des10*.**

Detailed progression of synapsis in wild-type (a-l) and *des10* (m-x) cells demonstrated by the immuno-localization of AtASY1 (green) and AtZYP1 (magenta) on formaldehyde fixed meiocytes. Cells visualised by 3D-SIM show the progression of synapsis in wild-type at leptotene (a-d), zygotene (e-h), and pachytene (i-l) together with detailed views of white squared regions compare to the progression of synapsis in *des10* at leptotene (m-p), zygotene (q-t), and pachytene (u-x) together with detailed views of white squared regions. Scale bars 5µm.



**Figure S5: Comparison of Confocal and Structured Illumination Microscopy images.**

Confocal images of (a) wild-type and (b) *des10* show a linear HvZYP1 (magenta) signal at pachytene and it is possible to count the number of HvMLH3 (green) foci in wild-type on the newly formed SC. The same cells imaged by 3D-SIM show that in wild-type (c), ZYP1 is indeed linear along the chromosome. In *des10* (d), ZYP1 signal is seen along the entire length of the chromosome suggesting that pachytene is achieved but the signal remains non-continuous, suggesting that ZYP1 loading is not complete.

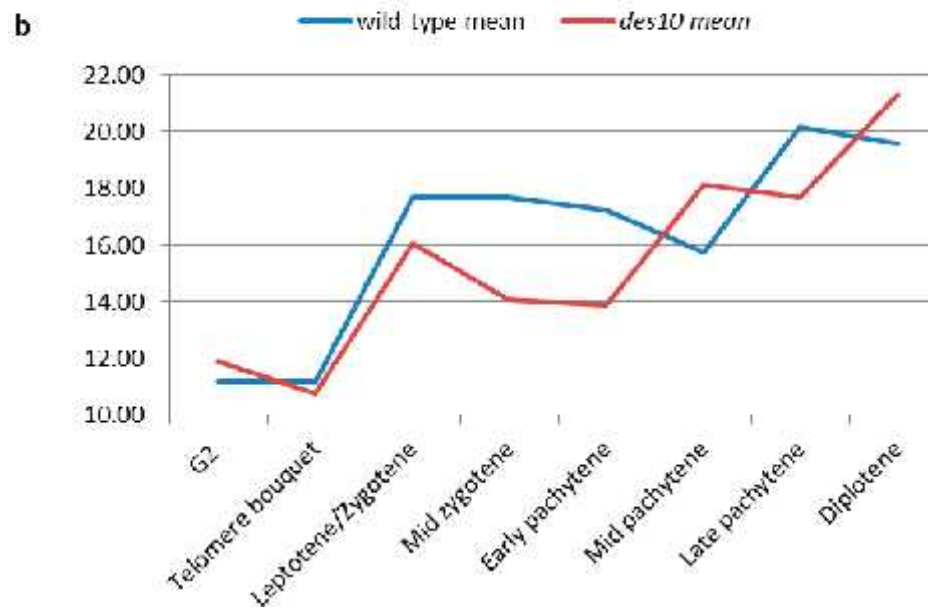


**Figure S6: Meiocyte size.**

The sizes of the nucleus were estimated using chromatin stain diameter ( $\mu\text{m}$ ) at different stages of meiosis **(a)** gauged by ASY1 and ZYP1 labelling. Results show a lack of synchronicity between cell size and meiotic stage in *des10* relative to wild-type **(b)**.

**a**

	wild-type mean	<i>des10</i> mean
G2	11.19	11.91
Telomere bouquet	11.18	10.77
Leptotene/Zygotene	17.68	16.05
Mid zygotene	17.66	14.06
Early pachytene	17.23	13.90
Mid pachytene	15.73	18.14
Late pachytene	20.15	17.69
Diplotene	19.56	21.31

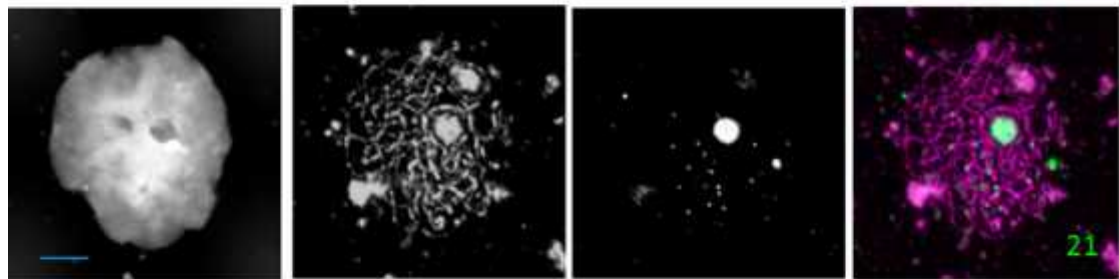




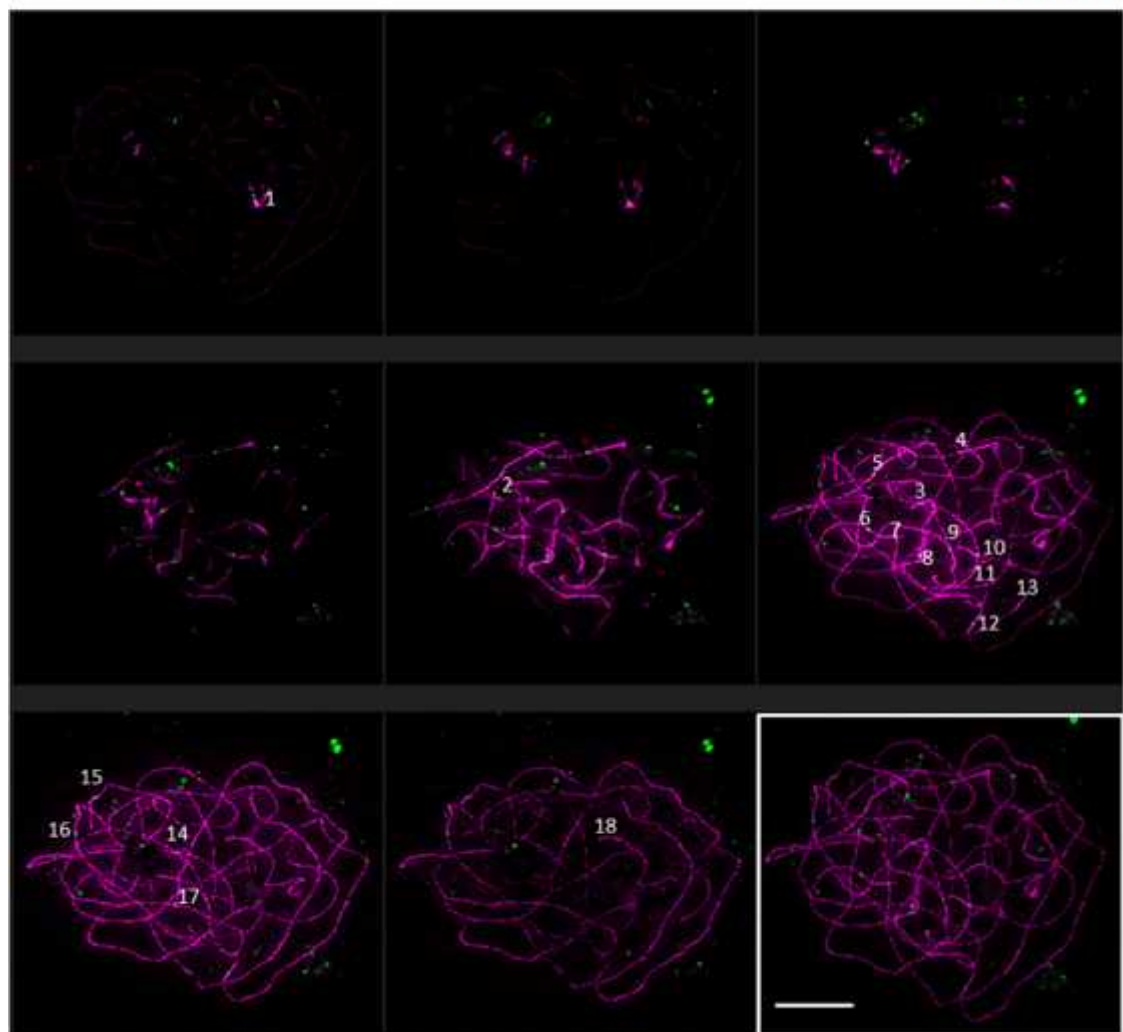
**Figure S7: HvMLH3 foci count in wild-type late pachytene.**

(a) 3D confocal image of wild-type pachytene labelled with HvZYP1 (magenta) and HvMLH3 (green) with the nucleus showing 21 MLH3 foci on the ZYP1 axes. (b) 3D SIM image gallery of wild-type pachytene labelled with HvZYP1 (magenta) and HvMLH3 (green) with numbering highlighting the foci presumably marking crossovers. This nucleus shows 18 MLH3 foci on the ZYP1 axes.

**A: 3D Confocal**



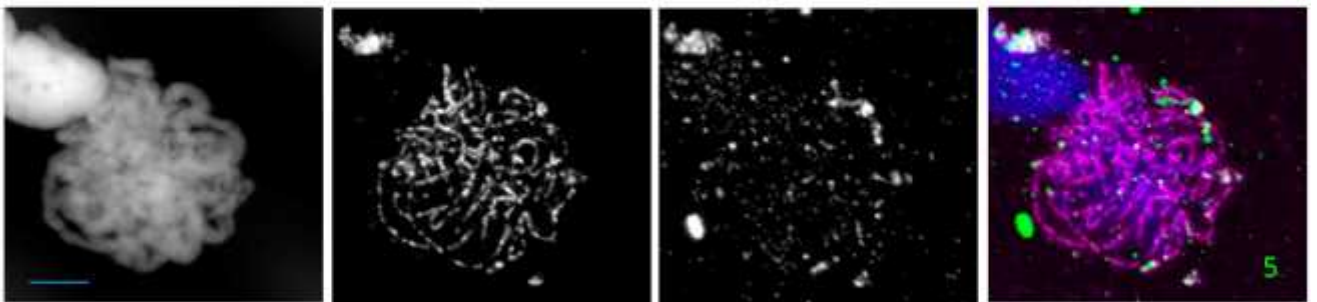
**B: 3D SIM**



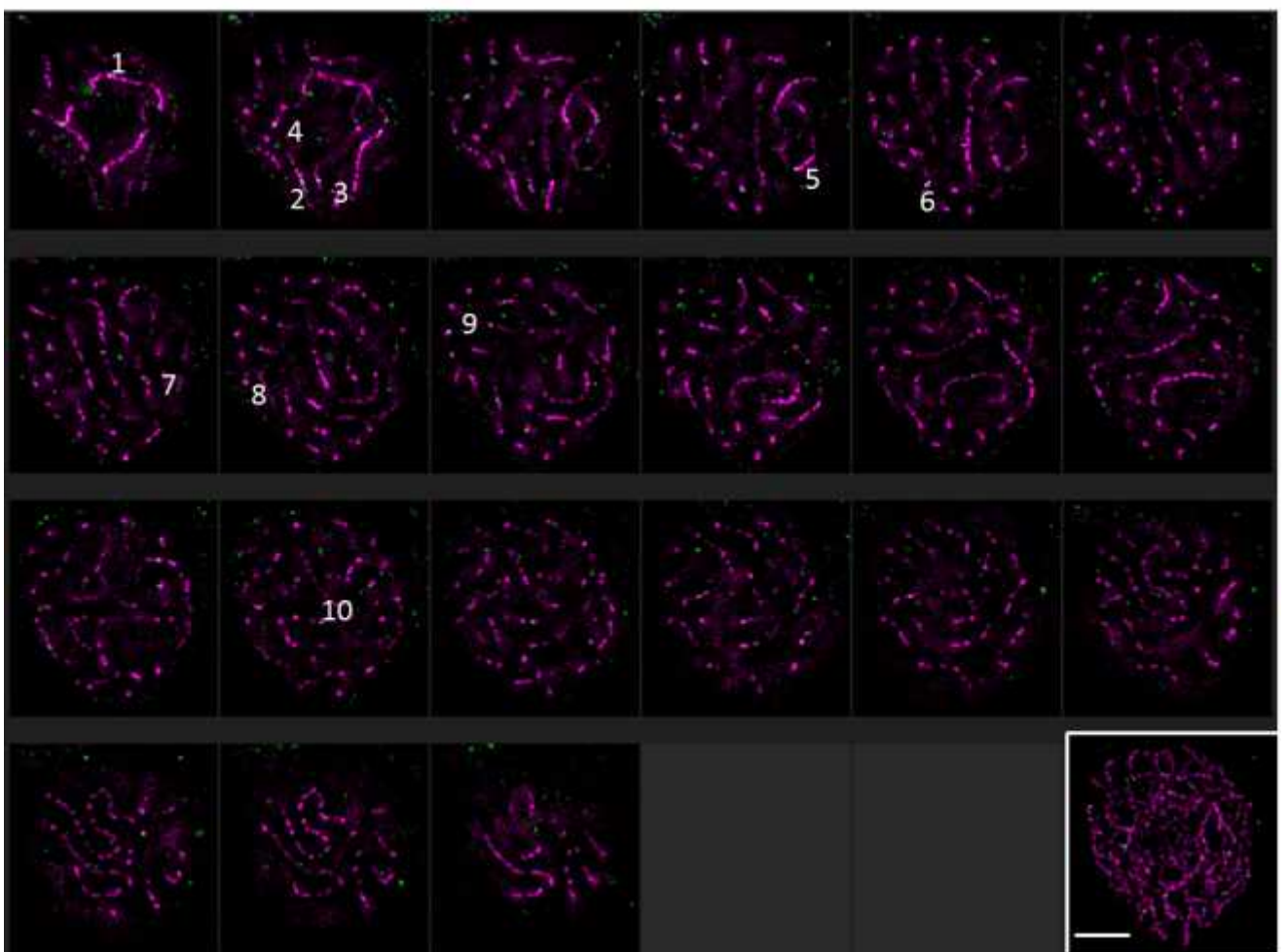
**Figure S8: HvMLH3 foci in *des10*.**

(a) 3D confocal image of a *des10* cell labelled with HvZYP1 (magenta) and HvMLH3 (green). This cell appears to be at pachytene as the ZYP1 signal is quite linear. Despite a high MLH3 background, 5 MLH3 foci can be seen associated with the ZYP1 axes. (b) 3D SIM image gallery of a *des10* cell labelled with HvZYP1 (magenta) and HvMLH3 (green) with the numbering highlighting the foci presumably marking crossovers. This nucleus shows 10 MLH3 foci on the ZYP1 axes although with 3D SIM the ZYP1 appears non-linear

**A: Confocal**

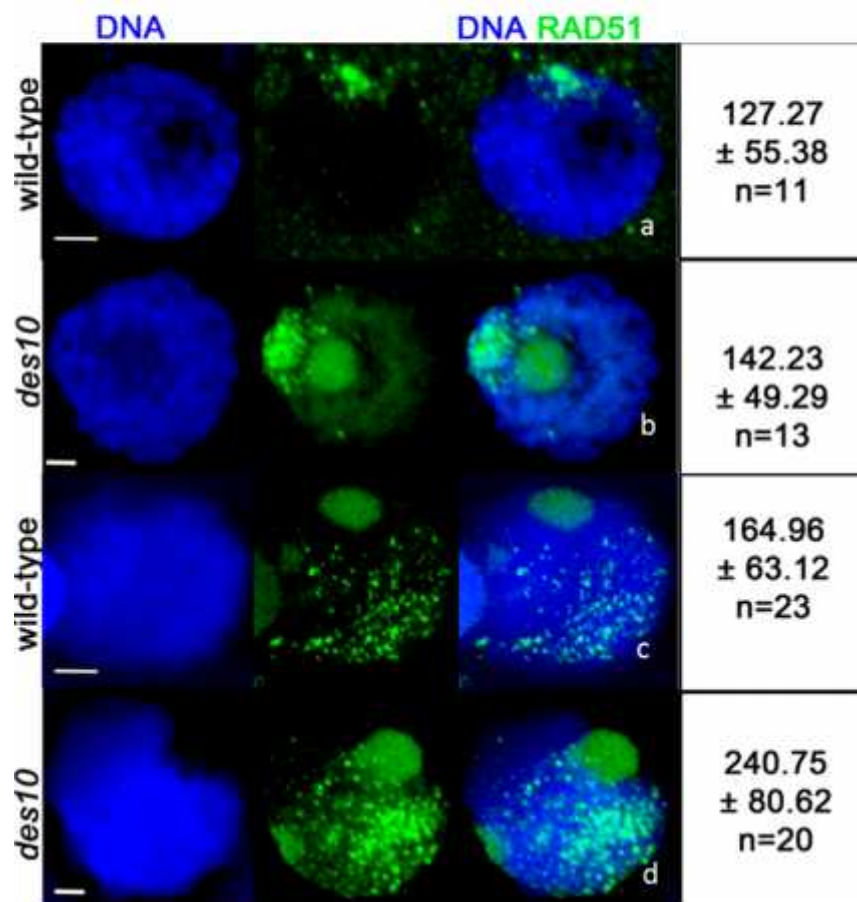


**B: 3D SIM**



**Figure S9: 3D localisation of RAD51.**

AtRAD51 (green) protein initially loads onto the chromosome from the telomere region in both wild-type (a) and *des10* (b). As RAD51 protein signal moves from telomere to more proximal regions it becomes possible to count individual foci in wild-type (c) and *des10* (d). Scale bars 5µm.





**Figure S10: RAD51, DMC1 and MSH4 foci on SC spreads (squash).**

Co-immunolocalization was also performed for (a) AtASY1 (green) and AtRAD51 (magenta), (b) AtASY1 (green) and AtDMC1 (magenta), and (c) AtASY1 (green) and AtMSH4 (magenta), to monitor the localization of DSBs on the axial elements. In both wild-type and *des10* early (RAD51 and DMC1) and intermediate (MSH4) recombination proteins load on the chromosome axis (ASY1) revealing that crossing over intermediates are formed in both wild-type and *des10*. Scale bars 5µm.

