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RESEARCH ARTICLE

Eveline Doll · Monika Molnar · Yasushi Hiraoka
Jürg Kohli

Characterization of *rec15*, an early meiotic recombination gene in *Schizosaccharomyces pombe*

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Abstract In *S. pombe* strains mutant for *rec15* aberrant ascus morphology, reduced spore viability and severe reduction of meiotic recombination was detected. Genetic and cytological analysis identified frequent interruption of meiosis after the first division, and nondisjunction I, as the main segregation errors in the mutant. Chromosome segregation at meiosis I was not random in *rec15*, suggesting the presence of a backup system for correct segregation of achiasmate chromosomes. The analysis of meiotic progression in time-course experiments revealed that the major meiotic events, such as the onset of premeiotic DNA synthesis, of horse-tail nuclear movement, and of the first meiotic division occurred earlier in *rec15* than in wild-type. The early onset of meiotic events is a novel observation for an early recombination mutant and implies a function of *rec15* protein already at or before DNA synthesis.

Keywords Fission yeast · Meiosis · *rec15* · recombination

Introduction

During meiosis two nuclear divisions follow a single round of DNA replication thus halving the chromosome number of the meiocyte. The first (reductional) division is longer than the second (equational) division and has some special features. Chromosomes emerge from the meiotic DNA replication having two tightly associated sister chromatids. In meiosis I homologous chromosomes pair and undergo high levels of meiotic recombination. In most organisms the pairing process is accompanied by the formation of a meiosis specific structure, the synaptonemal complex (SC) (Page and Hawley 2004). Meiotic double strand breaks (DSB) are processed to crossovers, which in turn leads to the formation of chiasmata. These physical connections between the homologous chromosomes help to balance the tension which is imposed on them by their bipolar attachment to the opposite spindle poles. At the end of meiosis I, sister chromatid cohesion distal to the chiasmata is dissolved and homologous chromosomes segregate to opposite poles. Lack of chiasmata or precocious resolution of sister chromatid cohesion causes segregation errors and leads to the generation of offspring with irregular chromosome number.

The fission yeast *Schizosaccharomyces pombe* has a haploid life cycle. Upon nutritional starvation haploid cells of opposite mating type conjugate and the diploid zygotes undergo meiosis immediately (zygotic meiosis). By interruption of the mating process before commitment to meiosis, diploid vegetative strains can be obtained. When starved for nutrients these diploid cultures undergo meiosis with relatively high synchrony (azygotic meiosis) (Egel 1973; Egel and Egel-Mitani 1974). Meiosis in fission yeast has some unusual features. *S. pombe* does not form SCs, thus, the classical meiotic stages defined by the development of the SC cannot be applied to fission yeast meiosis. Instead of SCs, it has linear elements (LE), which appear in meiotic prophase and resemble the axial cores of other eukaryotes (Bähler et al. 1993; Lorenz et al. 2004; Olson 1978). Some of the early recombination mutants in *S. pombe* show defects in LE formation, especially those affecting cohesion and linear element proteins (Kitajima et al. 2003; Lorenz et al. 2004; Molnar et al. 1995; Molnar et al. 2003;

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E. Doll · J. Kohli (✉)
Institute of Cell Biology, University of Bern,
Baltzerstrasse 4, CH-3012 Bern, Switzerland
E-mail: juerg.kohli@izb.unibe.ch
Tel.: +41-31-6314654
Fax: +41-31-6314616

M. Molnar
Department of Microbiology and Biotechnology,
Faculty of Sciences, University of Debrecen,
4032 Debrecen, Hungary

Y. Hiraoka
Kansai Advanced Research Center, National Institute
of Information and Communications Technology,
Nishi-ku, Kobe 651-24, Japan

Parisi et al. 1999) A striking feature of *S. pombe* meiosis is the oscillatory movement of the prophase nuclei led by the SPB and the attached telomere cluster (Chikashige et al. 1994). This movement confers a special shape (horse-tail) to the prophase nuclei and lasts throughout the meiotic prophase (Hiraoka et al. 2000). The movement contributes to the pairing of homologs, and is required for wild-type levels of recombination (Yamamoto et al. 1999). *S. pombe* has only three chromosomes (Kohli et al. 1977). This low chromosome number is advantageous for segregation studies, because it allows the tracing of individual chromosomes through meiosis, and thus facilitates the detection of different missegregation types (Molnar et al. 2001a; Molnar et al. 1995; Molnar et al. 2001b). Furthermore, meiotic recombination deficient mutants can be isolated in fission yeast easily because they produce enough viable spores even when chromosomes segregate at random (De Veaux et al. 1992; Ponticelli and Smith 1989).

Mutations in the *rec15* gene caused severe defects both in meiotic intragenic and intergenic recombination (De Veaux et al. 1992; Lin and Smith 1995). Sequencing of *rec15* revealed a 49 bp intron-containing gene with a coding capacity for a protein of 180 amino acids. No sequence similarity could be detected to proteins in data bases (Davis and Smith 2001; Lin and Smith 1995). In *pat1-114* background, *rec15* is expressed two hours after induction of meiosis (Lin and Smith 1995) and in h^+/h^- azygotic meiosis the expression of *rec15* peaks four hours after meiosis induction (Mata et al. 2002), http://www.sanger.ac.uk/perl/SPGE/geexview). The present study aims at the examination of chromosome segregation in *rec15* mutant strains by genetic and cytological means. In addition, meiotic progression in *rec15* mutants was examined in individual living cells, and in meiotic time-course experiments. We show that *rec15* mutants have phenotypes in common with other early meiotic recombination mutants of fission yeast.

Materials and methods

Strains, media and standard genetic methods

S. pombe strains used in this study are listed in Table 1. *rec15-154::LEU2* strains were derived from GP1688 or GP1825 and *rec15::kanMX* strains from ED58 by standard genetic crosses. The standard media yeast extract agar (YEA), yeast extract liquid (YEL) and malt extract agar (MEA) were as described by Gutz et al. (1974). Minimal medium (MMA) contained 0.67% Difco Nitrogen Base without amino acids, 1% glucose and 1.8% agar. YEA + 5 and MEA + 5 were supplemented with100 mg/liter adenine, uracil, leucine, lysine and histidine. MMA was supplemented with nutrients according to the experimental requirements at 100 mg/liter. Crosses were carried out on MEA + 5 at 25°C. YEA + 5 was used as a standard growth medium at 30°C, except for *tps13–24* (25°). For microscopic observations EMM2-N liquid medium (EMM2 minimal medium without nitrogen source) was applied (Moreno et al. 1991). For meiotic time course experiments *S. pombe* synthetic minimal medium (PM) and PM-N (PM without NH_4Cl) were used (Beach et al. 1985; Watanabe et al. 1988). The classical genetic methods (random spore analysis, dissection of asci, and interrupted mating for construction of diploid strains) were performed as described (Gutz et al. 1974). In random spore experiments for determination of spore viability and meiotic recombination frequencies approximately 200 spores were analyzed.

Gene disruption and sequencing of *rec15* mutations

To disrupt *rec15*, the method of Bähler et al. (1998) was followed. First a PCR reaction was carried out on pFA6a-kanMX6 with primers carrying sequences directly upstream (forward primer: GTCGCGTGTC TCGACTTTTT TCTAGTGAAA ACGAACATTC CCTTTAACCA AACCTGCAAT TAAGCGTTAC GGGAATTTTA ACGGAAACGA TCATTAAATA CGTACGCTGC AGGTCGACGG) and downstream (reverse primer: TGAATATATT GCATCGCTCA CTATGAAAAA ACGGTTTGAT TGGTGTTTAA TTTTAATTGA TATCATAGAA AAGCTTTTAA ATGAGGCTGA TCTAAGTATT CATCGATGAA TTCGAGCTCG) of the *rec15* coding region. The resulting fragment was used to transform strain 84–3330 by the lithium acetate method (Ito et al. 1983). Proper integration of the fragment into the genome was verified by PCR and tetrad analysis. PCR with the control primers (forward: TCGTCTTGCT GAGTTGGAGG; reverse: CTGAGAGTTC TGCTCGGTGA) yielded the expected 2 kb and 1.2 kb fragments for disruption and control strain, respectively. After protoplast fusion (Sipiczki and Ferenczy 1977) between strains 84–330 and 1–24 the resulting diploid was sporulated and the regular segregation (2:2 segregation in tetrad analysis) of G418 resistant clones (Bahler et al. 1998) verified.

To determine the sequences of the *rec15-124* and *rec15-127* mutations, PCR fragments were generated with the control primers described above. The resulting template was then sequenced with the following primers (forward: AATGGGCATC CAATATACTG; reverse: ATATTGCATC GCTCACTATG).

Segregation analysis in classical genetic system

A detailed protocol for studying chromosome segregation in random spores and dyads was described earlier (Molnar et al. 2001b). Strains were crossed as described above, the crossing material was digested with snail digestive juice (1:500 dilution of Helix Pomatia Juice, BioSepra, France), and the liberated spores were plated and grown on YEA + 5 plates at 25°C. After recording colony morphology and microscopic inspection of cell

Table 1 Strains

Strain	Genotype	Origin[a]
972	*h*$^-$	Berne collection
975	*h*$^+$	Berne collection
1–21	*h*$^+$ *ade6-M210*	Berne collection
1–24	*h*$^-$ *ade6-M216*	Berne collection
3–86	*h*$^+$ *leu1-32*	Berne collection
3–85	*h*$^-$ *leu1-32*	Berne collection
5–179	*h*$^-$ *aro5-110*	Berne collection
16–632	*h*$^-$ *leu1-32 ura4-D18*	Berne collection
41–1628	*h*$^-$ *lys1-131 leu1-32*	Berne collection
82–3249	*h*$^-$ *ade6-M210 tps13-24 ura4-D18*	Berne collection
82–3244	*h*$^+$ *ade6-M216 lys1-131 ura4-D18*	Berne collection
84–3330	*h*^{90} *leu1-32 ura4-D18*	Berne collection
ED1	*h*$^-$ *ade6-M216 leu1-32*	3–86×1–24
ED3	*h*$^+$ *ade6-M210 leu1-32*	3–85×1–21
ED8	*h*$^+$ *ade6-M210 leu1-32 tps13-24*	82 3249×ED3
ED9	*h*$^-$ *ade6-M216 leu1-32 lys1-131*	82 3244×ED1
GP569	*h*^{90} *rec15-124*	DeVeaux et al. 1992
GP631	*h*^{90} *rec15-127*	DeVeaux et al. 1992
GP1688	*h*$^-$ *ade6-52 ura4-294 leu1-32 rec15-154::LEU2*	Lin et al. 1995
GP1825	*h*$^+$ *ade6-M26 ura4-294 leu1-32 rec15-154::LEU2*	Lin et al. 1995
MM1	*h*^{90} *ade6-M26 ura4-294 leu1-32 rec15-154::LEU2*	isolated from GP1825
ED10	*h*$^-$ *ade6-M210 leu1-32 rec15-154::LEU2*	ED3×GP1688
ED11	*h*$^+$ *ade6-M216 leu1-32 rec15-154::LEU2*	ED1×GP1825
ED18	*h*$^-$ *rec15-154::LEU2 leu1-32*	3–86×GP1688
111–4424	*h*$^+$ *rec15-154::LEU2 leu1-32*	3–85×GP1825
ED19	*h*$^-$ *ade6-M210 leu1-32 tps13-24 rec15-154::LEU2*	ED8×ED10
ED5	*h*$^+$ *ade6-M216 leu1-32 lys1-131 rec15-154::LEU2*	ED9×ED11
ED22	*h*$^+$ *aro5-110 leu1-32*	3–86×5–179
ED23	*h*$^-$ *aro5-110 leu1-32 rec15-154::LEU2*	ED18×ED22
ED13	*h*$^+$ *lys1-131 leu1-32 rec15-154::LEU2*	42–1628×111–4424
JB6	*h*$^+$ */h*$^-$ *ade6-M210/ade6-M216*	Berne collection
ED58	*h*^{90} *ura4-D18 leu1-32 rec15::kanMX*	This study
ED59	*h*$^-$ *rec15::kanMX*	ED58×L972
ED60	*h*$^+$ *rec15::kanMX*	ED58×L975
ED64	*h*$^+$ *ade6-M210 rec15::kanMX*	ED58×1–21
ED65	*h*$^-$ *ade6-M216 rec15::kanMX*	ED58×1–24
ED67	*h*$^-$ *leu1-32 ura4-D18 rec15::kanMX*	ED58×16–632
rec15dip	*h*$^+$ */h*$^-$ *ade6-M210/ade6-M216 rec15::kanMX/rec15::kanMX*	ED64×ED65
MKY7A-4	*h*$^+$ *leu1-32 ura4-D18 his7*$^+$ *::lacI-GFP lys1*$^+$ *::lacOp*	Yanagida M.
EDhet	*h*$^-$ *leu1-32 ura4-D18 his7*$^+$ *::lacI-GFP lys1*$^+$ *::lacOp rec15::kanMX*	MKY7A-4×ED67
MKY7der	*h*^{90} *leu1-32 ura4-D18 his7*$^+$ *::lacI-GFP lys1*$^+$ *::lacOp*	isolated from MKY7A-4
ED74	*h*^{90} *leu1-32 ura4-D18 his7*$^+$ *::lacI-GFP lys1*$^+$ *::lacOp rec15::kanMX*	MKY7der×ED58

[a]When strains resulted from crosses of other strains presented in the table, the respective crosses are indicated

size (ploidy), the colonies were analyzed further for centromere-linked markers. Lysine auxotrophy (*lys1*, marker of chromosome I) was tested on appropriately supplemented MMA plates. Temperature sensitivity (*tps13*, marker of chromosome II) was checked on YEA + 5 plates at 37°C. YEA was used to check the *ade6* marker on chromosome III. The *ade6-M210* mutation confers dark red pigment, the *ade6-M216* mutation light red colour, and the heterozygous *ade6-M210/ade6-M216* configuration white colour due to complementation. The full genotype of clones classified as diploid or aneuploid by colony morphology and microscopic examination was determined after self-sporulation (*h*$^+$ */h*$^-$ diploids and aneuploids) or after crossing with *h*$^+$ and *h*$^-$ tester strains (*h*$^+$ */h*$^+$ or *h*$^-$ */h*$^-$ diploids and aneuploids). After dissection of two-spored asci, germination and division of the spores was examined by microscopy. The dyad analysis was completed as described above.

Segregation analysis and observation of meiotic stages in living cells

Microscopic observation of living cells took place at 26°C, with the DeltaVision microscope system (Applied Precision, Inc., Seattle, WA). Details of the microscope system were described previously (Haraguchi et al. 1999). To follow chromosome segregation in living cells, strains carrying tandem repeats of LacO DNA sequences at the centromere-linked *lys1* locus, and a GFP-LacI-NLS fusion construct at the *his7* locus were used (Nabeshima et al. 1998). These strains are designated as *his7*$^+$ *::lacI-GFP lys1*$^+$ *::lacO* in Table 1. Meiosis was induced by overnight incubation of cells on MEA plates at 26°C. Zygotes were stained with 5 μg/ml Hoechst 33342 dye in distilled water for 15 min at room temperature, then resuspended in EMM2-N and mounted on a cover slip for microscopic examination. Firstly, zygotes with two nuclei (after meiosis I) were identified

using an excitation filter with a narrow peak at 380 nm (Haraguchi et al. 1999). Then images were taken with a FITC filter through the whole nuclei (10 sections, 0.3 μm distances).

To follow the meiotic divisions in living cells, zygotes were stained with Hoechst 33342 dye and mounted on cover slips as described above. Images were taken at 2 min intervals with an exposure time of 0.2–0.5 seconds. To observe karyogamy and the length of horse-tail movement, strain ED58 was transformed with pYC551 as described previously (Molnar et al. 2001a). This pREP1 expression vector-based plasmid (Maundrell 1990) carries the sequences for the NLS-GFP protein. Images were taken with FITC filters with an exposure time of 0.2 seconds, at 10 min intervals. Image processing and analysis were carried out using the Delta Vision program (Applied Precision, Seattle, WA). For a more detailed description of the microscope system, preparation of specimens and fluorescence imaging of living cells see Molnar et al. (2001a).

Meiotic time-course experiments were carried out according to Bähler et al. (1993). Strains ED7 and JB6 were streaked on YEA plates and incubated for 4 days at 30°. Four single colonies were picked for the first precultures in YEL and incubated for 24 h at 30°. 50 to 100 μl from each were dropped on MEA + 5 plates and incubated for 24 h at 30°. The second precultures were inoculated from YEL to YEL and grown at 30° for 24 h. The sporulation capacity of the cells on MEA + 5 was checked microscopically and by iodine staining. The best sporulating clone was chosen to inoculate PM (1:50 and 1:100). The precultures were grown at 30° for 16–18 h. The cell titer of the PM cultures were determined, and one with $1 - 2\times10^7$ cells per ml was chosen for the time-course experiment. Cells were centrifuged, the pellet was washed with distilled water, and centrifuged again. The pellet was then resuspended in PM-N and 0 h samples for FACS and DAPI analysis were taken. Samples (1 ml) were taken every hour up to 10 hours, and a final one after 24 hours. Samples were spun and the pellet was resuspended in ice cold 70% ethanol to fix the cells for DAPI and FACS analysis.

DAPI staining and FACS analysis

To analyze the nuclei, cells were washed with water, and aliquots mixed with an equal amount of DAPI (4',6-diamino-2-phenylindol) dye (2.5 μg/ml). At every time point at least 200 cells were examined.

Samples were washed twice in 50 mM Na-Citrate (pH 7.0). For time points 0, 1 and 2 hours the cell titer was determined by a counting chamber. Cells were diluted to a titer of 4×10^6 cells per ml. To 1 ml sample 25 μl RNAse A (10 mg/ml) were added and samples were incubated at 37°C for 1 hour. The cells were then transferred to 5 ml Falcon tubes and stained with 1 ml propidium iodide solution (2.5 μg/ml in 50 mM Na-Citrate). Samples were stored on ice and in the dark for three days for diffusion of propidium iodide. Before measuring on a FACS Scan, samples were sonicated at 10% with a Branson sonifier for 8×0.5 sec with 0.5 sec break after each cycle. Data were analyzed with the CellQuest program.

Results and discussion

Sequences of *rec15* point mutations and construction of a full deletion

rec15 mutant strains GP569 (*rec15-124*) and GP631 (*rec15-127*) were isolated in the original screen for recombination deficient mutants by DeVeaux et al. (1992). To determine the nature of these two mutations, DNA sequencing was carried out. The *rec15-124* mutation turned out to be a C to T transition at nucleotide 199 (relative to the first coding nucleotide). It results in a stop codon and severe truncation of the protein. The *rec15-127* sequence has a G to A transition at nucleotide 206. It affects the first nucleotide of the consensus sequence at the 5' intron splicing site (Kupfer et al. 2004). Therefore, it is likely to interfere with splicing of the 49 bp long intron of *rec15*. Both mutations are likely to confer a null phenotype which is in accordance with the reported strong deficiency in meiotic recombination (De Veaux et al. 1992; Lin and Smith 1995). To construct a full deletion of *rec15*, the whole ORF was replaced by the *kanMX* cassette (Materials and Methods). The *rec15-154::LEU2* mutant isolated by Lin and Smith (1995) was also used for analysis of ascus morphology, spore viability, and chromosome segregation studies by the genetic assay. The *rec15::kanMX* mutant was mainly examined in the cytological studies.

Aberrant ascus morphology, reduced spore viability, and meiotic recombination deficiency in *rec15* mutants

Microscopic examination of zygotic asci of *rec15* mutant strains revealed highly irregular ascus morphology (Fig. 1a, b). *rec15* mutants produced asci of variable spore number and size in contrast to the uniform asci of the control strain. *rec15-154::LEU2* showed 50% asci with four spores, 18% asci contained three spores, 27% two spores and 5% one spore. The *rec15::kanMX* deletion showed almost identical results. DAPI-staining showed not only the size and placement of spores in the asci that were abnormal, but also that they contained DNA in variable amounts (Fig. 1a). The predominant irregularity was the high abundance of asci with two and three spores (Fig. 1b). The two deletion constructs (*rec15-154::LEU2* and *rec15::kanMX*) showed almost identical phenotypes. Similar ascus morphology has been observed in other early recombination-deficient mutants: *rec6*, *rec7*, *rec12* and *rec14* (Davis and Smith 2001; Molnar et al. 2001a; Sharif et al. 2002). In fission yeast, the spindle pole body (SPB) plays a crucial role in

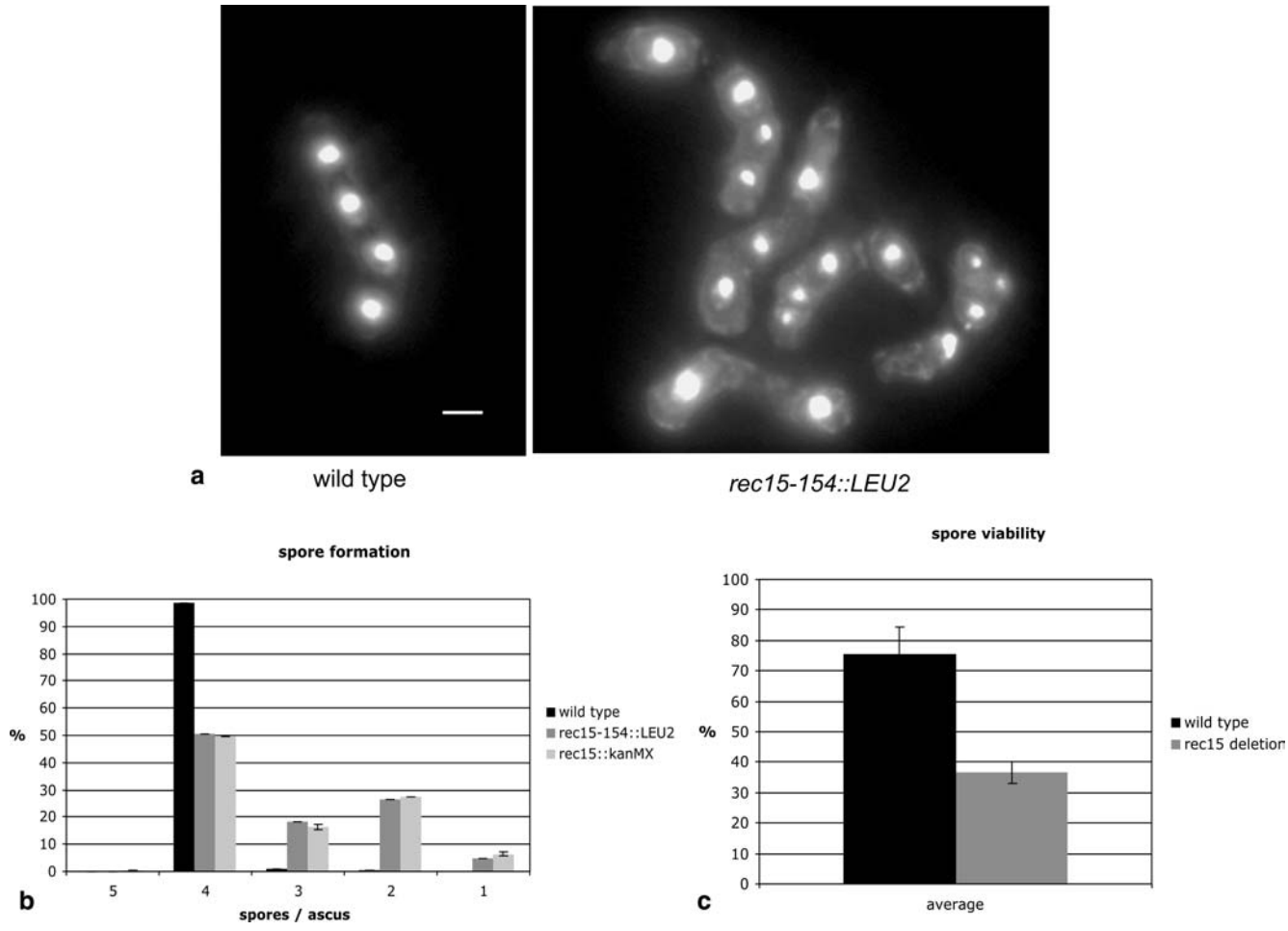

Fig. 1 Ascus formation and spore viability. **a** An ascus from a wild type $h^{+} \times h^{-}$ cross showing four regular spores and asci from a cross of strains GP1688 and GP1825 (homozygous for *rec15-154::LEU2*) were stained with DAPI. Bar, 5 µm **b** The numbers of spores in asci of a wild type cross, and of crosses homozygous for two different *rec15* disruptions. The cross for *rec15-154::LEU2* was GP1688×GP1825, that for *rec15::kanMX* a sporulation of the homothallic strain ED58 (Tab 1). Averages and standard errors of three experiments are presented **c** The viability of spores was measured in the same crosses as described in b. Averages and standard errors of five measurements are given

sporulation: it determines the timing of forespore membrane assembly which occurs only after completion of meiosis I in wild-type cells (Shimoda 2004). In a temperature sensitive *cdc2* mutant forming two-spored asci, SPB modification occurs at metaphase I and the forespore membrane is formed during meiosis I (Shimoda 2004). Perturbation of SPB function may also happen in the recombination-deficient mutants.

Mutants deficient in meiotic recombination show reduced spore viability. Most other mutants tested showed a spore viability of 20 – 30%, higher than the expected 12.5% from random segregation (Molnar et al. 1995; Ponticelli and Smith 1989). For *rec15*, an even higher spore viability of 37% was determined in random spore analysis (Fig. 1c). Spore viability also was very high in the dyad analysis (41%, see Table 3). To test meiotic recombination in *rec15*, Lin and Smith (1995) measured recombination frequencies in several regions on the chromosome arms and close to the telomeres. To complement their study, intergenic meiotic recombination was measured in the *rec15-154::LEU2* mutant in two centromere proximal regions. Each experiment was carried out three times. For the *lys1-aro5* interval on chromosome I $< 0.5\%$ recombination frequency was obtained in the cross of two *rec15* strains (ED23×ED13) compared to 29% in the control (ED22×41-1628). In the *tps13-mat1* interval on chromosome II 1.5% and 43.6% recombination frequencies were determined in the *rec15* (ED19×ED5) and the control cross (ED8×ED9), respectively. The severe, genome-wide decrease in meiotic recombination in the *rec15* mutant is explained by the lack of meiotic double-strand breaks (Young et al. 2004).

rec15-deficient meiosis leads to the formation of aneuploid and diploid spores

The unusual ascus formation and reduced spore viability indicated irregular meiosis. To get insights into chromosome segregation, first an unselected population of

Table 2 Random spore analysis of chromosome segregation in *rec15-154::LEU2* and *rec*$^+$ crosses

Segregation of markers [a,b]			Cell size	Deduced ploidy of spore	No. of colonies	
lys1	*tps13*	*ade6*			*rec15*	*rec*$^+$
+ or –	+ or –	*M210* or *M216*	Haploid	Haploid	131	207
+ or –	+ or –	*M210/M216*	Haploid	Disomic chromosome III[c]	5	
+ + or –	+ + or –	*M210/M210* or *M216/M216*	Diploid	Diploid, homozygous for centromere markers	26	
+ / –	+ / –	*M210/M216*	Diploid	Diploid, heterozygous for centromere markers	1	
+ + or –	+ + or –	*M210/M210* and *M216/M216*	Diploid	Aneuploid, (2n + 1 or 2) chromosome III[c]	32	
+ / –	+ + or –	*M210/M210* or *M216/M216*	Diploid	Aneuploid, (2n + 1 or 2) chromosome I[c]	5	
+ + or –	+ / –	*M210/M210* or *M216/M216*	Diploid	Aneuploid, (2n + 1 or 2) chromosome II[c]	0	
+ / –	+ + or –	*M210/M216*	Diploid	Aneuploid, (2n + 1 or 2) chromosome I and III[c]	6	
+ / –	+ / –	*M210/M210* or *M216/M216*	Diploid	Aneuploid, (2n + 1 or 2) chromosome I and II[c]	2	
Total					208	207

[a]Strains used for this study: *h*$^+$ *ade6-M216 leu1-32 rec15-154::LEU2 lys1-131*×h$^-$ *ade6-M210 leu1-32 rec15-154::LEU2 tps13-24* and *h*$^+$ *ade6-M216 lys1-131*×h$^-$ *ade6-M210 tps13-24*

[b]Location of markers: *lys1*,*tps13* and *ade6* are centromere-linked markers on chromosome I, II and III, respectively. The genotypes of the cells in the colonies that resulted from the spores are shown

[c]The colonies in these classes were mostly small and of irregular shape. Colonies of the other classes were mostly large and of regular shape

spores was examined. In fission yeast aneuploid genomes are unstable. Only disomics for chromosome III can be maintained over many generations by selection, and form colonies, but are continuously producing haploid segregants (Molnar et al. 1995; Niwa and Yanagida 1985). When strains to be crossed are marked with two different alleles of *ade6* on chromosome III (*ade6-M210* and *ade6-M216*), disomic spores can be detected by the mixed color (dark and light red, respectively) of the segregating haploid clones (see also Materials and Methods). Colonies derived from disomic spores are usually small and contain cells of small (haploid) size. In segregation studies, centromere-linked markers (*lys1*, *tps13* and *ade6*) serve to distinguish first and second division segregation patterns. Truly diploid spores produce large colonies with large diploid cells, and the cells show apparent first or second division segregation patterns for the centromere markers. By the study of the recombination deficient mutant *rec7*, a novel type of aneuploid spores (2n + 1 or 2n + 2, called hyper-diploids in this text) was detected (Molnar et al. 2001b). These hyper-diploids form small colonies, but the cells in the colonies are of large (diploid) size. Mixed segregation patterns of the centromere markers, and the segregation of diploid clones with both colony colors, are diagnostic for this type of aneuploid spores.

For *rec15-154::LEU2* chromosome segregation was analyzed by crossing ED19 with ED5, in comparison to the cross ED8 with ED9 (*rec*$^+$). Colonies were classified according to their genotype (centromere marker pattern), cell size and the deduced ploidy of the spores giving rise to the colonies (Table 2). The most striking feature of the mutant was the frequent formation of 2n + 1 or 2n + 2 aneuploid spores. In random spore analysis it could not be decided, whether the original spores carried one or two extra copies of the chromosome(s) in question. Hyper-diploids represented 21.6% of all the viable meiotic products. Among them, aneuploids for chromosome III were the most frequent (15% or 18% when the hyper-diploids for chromosome I and III are included). Hyper-diploid segregants could be explained by inclusion of two nuclei into one spore. But this was ruled out as the major cause by microscopic observation of DAPI-stained asci and spores: only 2–3% of the spores contained two separately stained bodies. The second remarkable observation was the high number of colonies with diploid cells homozygous for all the centromere markers (13%). Disomics for chromosome III represented only a minor fraction among the irregular meiotic products of the *rec15* mutant (2%).

Table 3 Classification of *rec15-154::LEU2* dyads

Deduced genotypes of colony-forming spores [a]	Number[b]
Dyads with two colony-forming spores	30
2n/2n, both homozygous for centromere markers	10 (3)
2n/2n, one homo-, other heterozygous for centromere markers	4
2n/2n, both heterozygous for centromere markers	10 (5)
n/2n, diploid homozygous for centromere markers	2
n/n, 1 with same centromere markers, 3 with different centromere markers	4
Dyads with one colony-forming spore	25
2n + 2 or 2n + 1 chromosome III	5 (3)
2n + 2 or 2n + 1 chromosome I	5 (3)
2n + gene conversion chromosome II	1
2n + 2 or 2n + 1 chromosome I and gene conversion chromosome II	1
2n + 1, chromosomes I and III	4 (1)
2n, homozygous for centromere markers	4 (3)
n	2
Dyads with no colony-forming spore	49
Total	104

[a]Strains used for dyad analysis: *h*$^+$ *ade6-M216 leu1-32 rec15-154::LEU2 lys1-131*×h$^-$ *ade6-M210 leu1-32 rec15-154::LEU2 tps13-24*

[b]2n spores usually produced colonies with uniformly large cells (diploid size). Some of the 2n spores gave rise to colonies with mixed (diploid and haploid) cell size. The number of these dyads is indicated in parenthesis

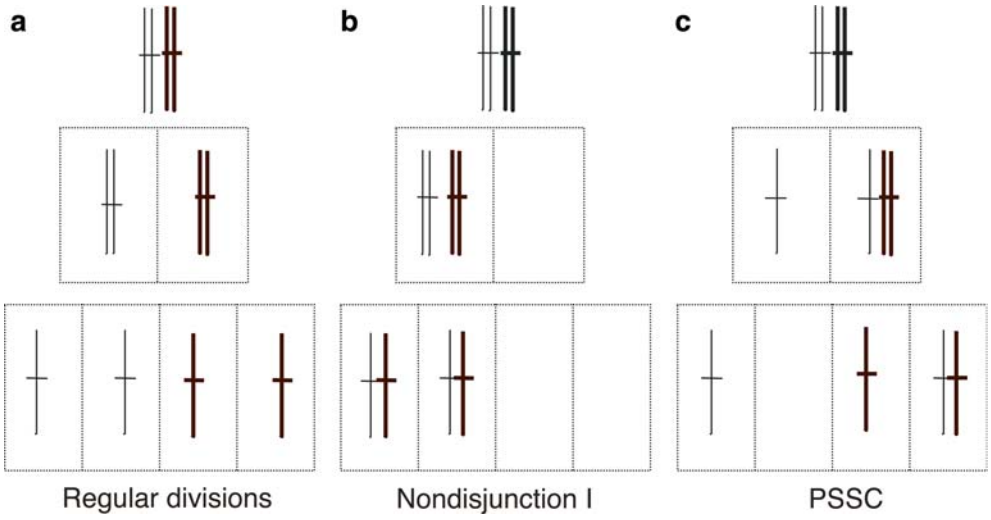

Fig. 2 Meiotic chromosome segregation errors. **a** Regular meiotic chromosome segregation **b** Nondisjunction at meiosis I **c** Precocious separation of sister chromatids

Also, when *rec15* would be partially defective in karyogamy, omission of meiosis altogether could result in dyads with apparent first division segregation. This was ruled out, since microscopic observation of live karyogamy showed no difference from wild-type (see below).

rec15-154::LEU2 meiosis is frequently interrupted after the first division

The frequent occurrence of two-spored asci (Fig. 1b) and the many diploid and hyperdiploid meiotic products (Table 2) suggested that dyad analysis could reveal the type of missegregation in *rec15*. The applied genetic assay allowed the detection of nondisjunction at meiosis I (Fig. 2b), precocious separation of sister chromatids (PSSC, Fig. 2c), and the omission of meiosis II. The combination of nondisjunction I with omission of meiosis II could also be scored. When meiosis II is skipped the middle row of Fig. 2 shows the relevant chromosome configurations. For this study, 104 dyads of the cross ED19×ED5 were dissected (Table 3). From 30 dyads two colonies were obtained. In 10 dyads out of these 30, both colony-forming spores showed homozygosity for all centromere markers, indicating regular disjunction I. These asci evidently stopped meiosis after the first division (Fig. 2a, middle row). In 10 dyads both spores showed heterozygosity for all centromere markers. Although second division segregation patterns may arise, when meiocytes skip the first division, another origin of these dyads is more likely. Precocious separation of sister chromatids occurring for all three chromosomes

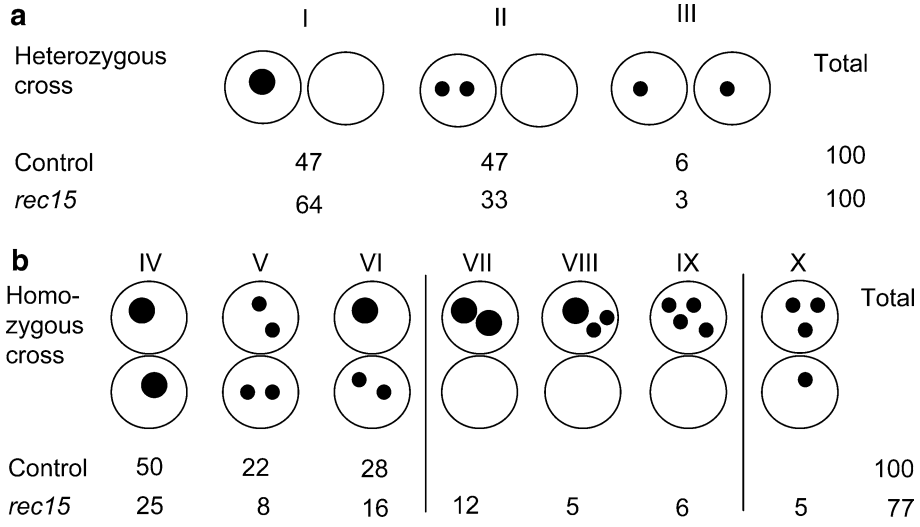

Fig. 3 The assay of chromosome segregation in living cells. **a** Segregation patterns observed in crosses heterozygous for the GFP construct at the centromere of chromosome I. Strain EDhet was crossed with ED60 (*rec15::kanMX* strains) and MKY7A-4 was crossed with the wild type strain 972 (control). Classes I and II correspond to the expected segregation patterns in meiosis I. The class III pattern derives either from crossover or from precocious separation of sister chromatids (PSSC) **b** Segregation patterns observed in crosses homozygous for the GFP construct. The homothallic strains ED74 (*rec15::kanMX*) and MKY7der (control) were induced to undergo meiosis. Classes IV to VI correspond to normal meiosis I segregation, classes VII to IX to nondisjunction I, and class X to PSSC. Large circles indicate chromosomes with joint sister chromatids, small circles separated sister chromatids

caused second division segregation patterns in the *rec8* mutant (Molnar et al. 1995; Watanabe and Nurse 1999). This type of mis-segregation was detected in the *rec15* mutant as well (Fig. 3, see below). Since precocious separation of sister chromatids for all three chromosomes is formally equivalent to omission of meiosis I and a normal meiosis II, the occasional occurrence of omission of meiosis I as origin of dyads cannot be excluded. Finally, in four dyads one spore showed heterozygosity, the other one homozygosity, which is hard to explain by the known recombination and segregation mechanisms.

In the dyads carrying haploid spore(s), chromosome loss and interruption of the meiotic divisions might have occurred. In 25 dyads only one spore formed a colony; the other spore did not germinate, or stopped after two or three divisions. Among these dyads we searched for evidence for nondisjunction I (Fig. 2b, middle row). When nondisjunction I occurs for chromosome III only, the surviving spore segregates three colony colors: dark red (*ade6-M210*), light red (*ade6-M216*), and white (*ade6-M210*/*ade6-M216* complementation). The majority of the colony-forming spores proved to be hyperdiploid, but their origin (nondisjunction I or precocious separation of sister chromatids) could not be decided unambiguously. Because this genetic study of chromosome segregation did not fully reveal the segregation error(s) occurring in the mutant, we decided to examine meiosis in living *rec15* mutant cells.

Live observation of *rec15* mutant meiosis: nondisjunction I and achiasmate chromosome segregation

To follow chromosome segregation in living cells, the *lacO*/*lacI-GFP* construct was introduced into *rec15::kanMX* mutant and control strains. These strains carry tandem repeats of the *lac* operon integrated at the *lys1* locus (Nabeshima et al. 1998) which maps 4 cM from the centromere of chromosome I (Kohli et al. 1977). Binding the lacI-GFP protein allows the detection of the segregation pattern of chromosome I. Firstly, zygotes with two nuclei were identified, and then the number of GFP signals in the daughter nuclei was examined as described in Materials and Methods. To detect precocious separation of sister chromatids (PSSC), crosses heterozygous for the GFP construct were performed (Fig. 3a). Class III represents PSSC, classes I and II indicate regular segregation. Two signals were visible in class II nuclei, because sister chromatids separated already for the second meiotic division. In the control cross 6% of the zygotes fell into class III. They may have resulted from crossover between the centromere and the *lys1* locus or from PSSC. The class III meiocytes of the *rec15* mutant most likely were due to PSSC, because the strong reduction in meiotic recombination in *rec15* tightens the linkage of *lys1* to its centromere.

To test the fidelity of chromosome disjunction at meiosis I, crosses homozygous for the GFP construct were analyzed (Fig. 3b). Classes IV, V and VI indicate regular disjunction of homologous chromosomes. In some nuclei sister chromatids were separated already for the second division (classes V and VI). Classes VII, VIII and IX were diagnostic for nondisjunction at meiosis I. Class X showed a pattern typical of precocious separation of sister chromatids. In the control cross, irregular segregation was not detected. In the *rec15* mutant 30% of the examined meiocytes fell into classes VII to IX identifying nondisjunction I as the main segregation error. In the absence of meiotic recombination (chiasmata) homologous chromosomes were expected to segregate randomly at meiosis I (50% regular segregation, 50% nondisjunction I). In *rec15*, homologs segregated regularly in 64% of the meiosis I divisions. This was significantly more frequent than expected from random segregation (χ^2 test, $p < 0.005$). Nonrandom homolog segregation was described before for *rec7*, another early meiotic recombination mutant (62%, Molnar et al. 2001a), and also for *rec12*, which was defective for the DSB-forming enzyme (63%, Davis and Smith 2001; Sharif et al. 2002). It was proposed that fission yeast possesses a system promoting homolog segregation independent of meiotic recombination (Davis and Smith 2001; Molnar et al. 2001a; Sharif et al. 2002). The present study is in accordance with the previous observations and suggests that an enhanced homolog disjunction in achiasmate meiosis is a general feature of the early recombination-deficient mutants. Some precocious separation of sister chromatids also occurred in *rec15* (6.5%, Fig. 3b). In the previously tested *rec7* mutants (Molnar et al. 2001b) only 2.5% of PSSC were detected. The significance of low level PSSC in these early recombination mutants is unclear.

Besides the segregation analysis, living *rec15* mutant cells were examined microscopically for all stages of meiosis. Karyogamy and horse-tail nuclear movements took place regularly in the mutant. The horse-tail stage lasted 147 ± 9.5 minutes (four experiments), which corresponds to the length of horse-tail movement in wild-type cells 146 ± 14 minutes (Hiraoka et al. 2000). Meiotic divisions were monitored in strain MM1 carrying the *rec15-154::LEU2* mutant. The first meiotic division took considerably more time in the *rec15* mutant than in wild-type cells. 35 ± 10 minutes were determined as the mean of twelve experiments, compared to 22 ± 3 minutes in wild type (Molnar et al. 2001a). During the lengthened first division, chromosomes wandered back and forth between the spindle poles. This phenotype was observed in 9 out of 12 experiments. Half of the monitored divisions ended with apparently even, the other half with obviously uneven distribution of the nuclear material between the two resulting nuclei. Wandering of chromosomes has been observed before in the *rec7* and *rec14* mutants. It was proposed to be part of the mechanism that improves chromosome segregation in achiasmate meiosis (Molnar et al. 2001a). Some obser-

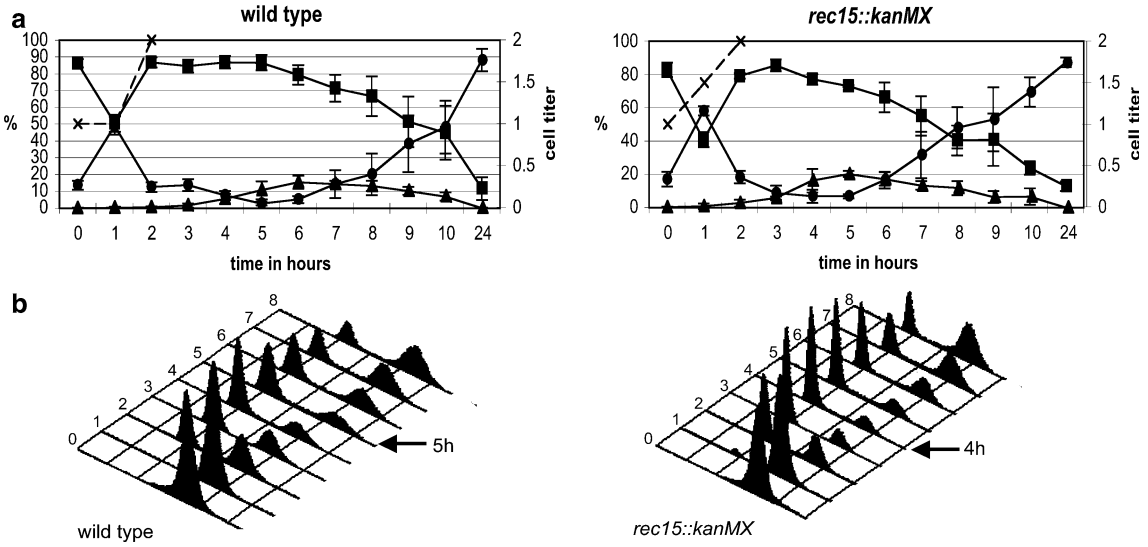

Fig. 4 Meiosis progression assayed by DAPI-staining and FACS analysis. **a** Samples of cells taken at the indicated times after induction of meiosis were stained with DAPI from the wild type strain JB6 (five independent experiments) and the *rec15*Δ strain rec15dip (three independent experiments). The cells were classified with respect to the number (one: squares, two or more: circles) and shape of nuclei (horse-tails: triangles). In addition the cell titer is indicated for time points 0–3 hours (crosses). The peak of cells with more then one nucleus at 1 hour is due to the last mitotic division that the cells undergo before entering meiosis. A comparison of the two panels documents the earlier onset of cytokinesis, horse-tail formation, and meiosis I in the *rec15* mutant. This earlier onset was also observed using diploids carrying the *rec15-154::LEU2* mutation (data not shown) **b** FACS profiles of wild type (JB6) and *rec15* deficient meiosis (rec15dip). The cells enter with a 4C DNA content (G2). By performing the last mitosis and cytokinesis, the cells enter G1 (2C DNA content). The 4C peak increases again when premeiotic DNA synthesis starts. The mutant showed an earlier 4C to 2C transition, a consequence of the early cytokinesis in *rec15*Δ . DNA synthesis starts in *rec15*Δ an hour earlier than in wild type. The experiment was carried out three times and representative panels are shown

vations were consistent with occasional omission of meiosis II in the *rec15* mutant, but reliable results could not be obtained with this method. Also, cases of unequal distribution of the nuclear material in meiosis II were observed, which were likely the consequence of impaired first divisions.

Timing of meiotic events reveals an additional early function of *rec15*

Meiotic progression in time-course experiments was analyzed in the *rec15::kanMX* and *rec15-154::LEU2* mutants with identical results. The experiments were

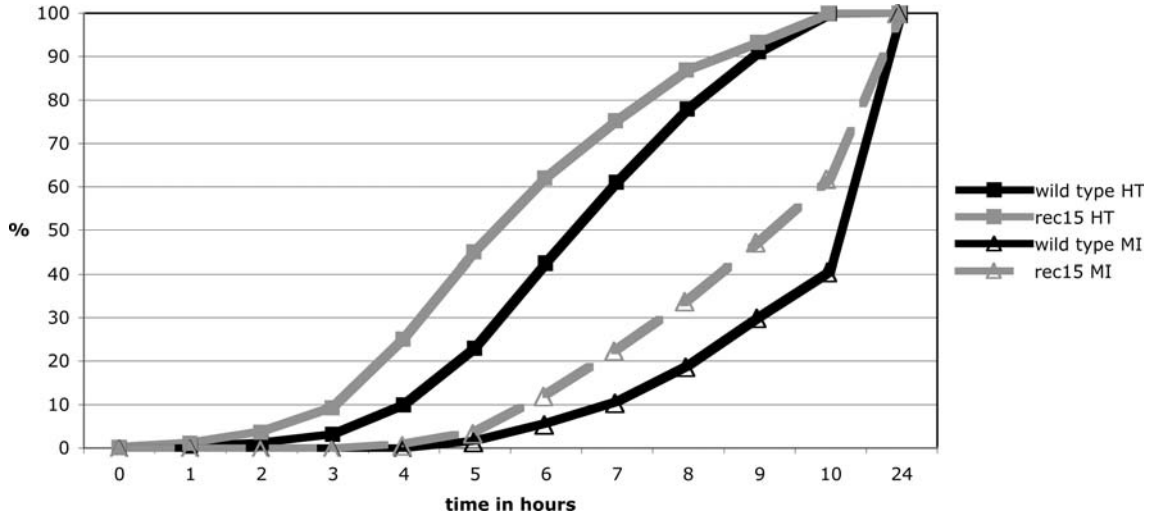

Fig. 5 Onset of the horse-tail (HT) nucleus and meiosis I stages The percentage of HT nuclei observed at all time points were summed up and the total set as 100%. The sum of HT nuclei up to each time point was then standardized to this total. This way of standardization was chosen because of the different amount of HT nuclei observed in wild type and in the mutant strain. To standardize the sum of MI meiocytes at each time point the amount of asci observed at 24 hours was used. At least 200 cells were scored at each time point

always carried out with fresh clones. Care was taken not to overgrow the precultures to avoid nutritional stress before induction of meiosis by nitrogen starvation (see Material and Methods). Meiosis was followed by DAPI staining of the nuclei and by phase contrast microscopy to check sporulation (Fig. 4a). One hour after meiotic induction, the final mitotic division took place as indicated by a peak of cells having two nuclei both in the *rec15* and wild-type cultures. The first difference between mutant and wild type appeared at this final mitotic division. The wild-type culture doubled its cell titer between one and two hours after meiotic induction. No difference was detected between the zero and one hour time-points. Contrastingly, the *rec15* culture already had at one hour 50% more cells than at time-point zero. As *rec15* mutants did not show higher growth rate in PM medium (data not shown), this difference was likely due to faster cytokinesis in the PM-N medium. Although the peak of transcription of *rec15* in wild-type background is four hours after shifting the cells to nitrogen-free medium (Mata et al. 2002), http://www.sanger.ac.uk/perl/SPGE/geexview, our finding suggests that early low expression of *rec15* may have a role in slowing the last mitotic division. Such a change in the timing of the final mitotic division in meiotic time-courses has not been reported so far.

For the *S. pombe* mitotic cell cycle, information on the length of DNA synthesis cannot be gained directly by FACS analysis, because the cells start DNA synthesis before they undergo cytokinesis (Gomez and Forsburg 2004). In meiotic time-courses, h^+ /h^- cells spend about three hours in G1 after the last mitotic division, and then enter premeiotic S phase (Fig. 4b). FACS analysis revealed that *rec15* premeiotic DNA synthesis started one hour before that of wild type (Fig. 4b). The beginning of horse-tail nuclear movement was also advanced for an hour in the *rec15* mutant (Fig. 4a). The primary data on horse-tail movement and occurrence of meiosis I presented in Fig. 4 were used for compilation of the summation curves in Fig. 5. For each time point the number of cells in the respective stage was added to the corresponding numbers of all earlier time points. For the horse-tail curve the total at 24 hours was set as 100% and used to standardize the summation values for each time point. The same was done for meiosis I. A clear difference between *rec15* and wild-type was observed (Fig. 5). The first meiotic division occurred earlier in *rec15* than in the wild type. The difference was more pronounced for the horse-tail stage. When present, the Rec15 protein seems to delay premeiotic DNA synthesis (Fig. 4b) and subsequent meiotic events such as horse-tail movement and meiosis I (Fig. 5). This phenotype was also observed for other early meiotic recombination mutants (Doll et al., unpublished). The surprising finding of altered timing of meiotic events in *rec15* deserves further investigation and may lead to a better understanding of the regulation of the meiotic cell cycle.

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