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RESEARCH ARTICLE

Functional interactions among members of the meiotic initiation complex in fission yeast

Silvia Steiner · Jürg Kohli · Katja Ludin

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Abstract DNA double-strand breaks (DSBs) initiate meiotic recombination in *Schizosaccharomyces pombe* and in other organisms. The Rec12 protein catalyzes the formation of these DSBs in concert with a multitude of accessory proteins the role of which in this process remains to be discovered. In an all-to-all yeast two-hybrid matrix analysis, we discovered new interactions among putative members of the meiotic recombination initiation complex. We found that Rec7, an axial-element associated protein with homologies to *Saccharomyces cerevisiae* Rec114, is interacting with Rec24. Rec7 and Rec24 also co-immunoprecipitate in *S. pombe* during meiosis. An amino acid change in a conserved, C-terminal phenylalanine in Rec7, F325A interrupts the interaction with Rec24. Moreover, *rec7*F325A shows a recombination deficiency comparable to *rec7*Δ. Another interaction was detected between Rec12 and Rec14, the orthologs of which in *S. cerevisiae* Spo11 and Ski8 interact accordingly. Amino acid changes Rec12Q308A and Rec12R309A disrupt the interaction with Rec14, like the according amino acid changes Spo11Q376A and Spo11RE377AA loose the interaction with Ski8. Both amino acid changes in Rec12 reveal a recombination deficient *rec12*$^{-}$ phenotype. We propose that both Rec7–Rec24 and Rec12–Rec14 form subcomplexes of the meiotic recombination initiation complex.

Keywords Meiosis · Recombination · Spo11 complex · *Schizosaccharomyces pombe*

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S. Steiner · J. Kohli · K. Ludin (✉)
Institute of Cell Biology, University of Bern,
3012 Bern, Switzerland
e-mail: katja.ludin@izb.unibe.ch

Introduction

Meiosis is essential for all sexually reproducing eukaryotes to form haploid gametes or spores from diploid cells. One round of chromosome duplication is followed by two rounds of chromosome segregation. In the first meiotic division (meiosis I) homologous chromosomes separate, whereas in the second meiotic division (meiosis II) sister chromatids move apart. Faithful chromosome segregation is crucial for the production of viable gametes and is enabled by physical connections between the two homologs (chiasmata) caused by recombination events. In addition, meiotic recombination contributes profoundly to genetic diversity. After obligatory DNA replication, recombination is initiated by the formation of DNA double-strand breaks (DSBs) catalyzed by the Spo11 protein (reviewed in Keeney 2001). Spo11 shares homology with subunit A of archaeal topoisomerase Top6 (Bergerat et al. 1997). It is ubiquitously found in organisms of the *Eukarya* and *Archaea* kingdom, however its function was mainly studied in the ascomycete *Saccharomyces cerevisiae*. The DNA DSB is formed by two trans-esterification reactions involving active site tyrosines of a Spo11 dimer. Spo11 remains transient covalently attached to the 5'-end of the broken DNA (Keeney et al. 1997). Later, Spo11 gets released from the DNA by endonucleolytic cleavage (Neale et al. 2005). The liberated 5'-ends allow further strand resection, probably by exonucleolytic activities. Exposed

single-stranded DNA strands are substrates to recombinases (e.g. Rad51 and Dmc1, among others), the strand invasion ability of which eventually leads to the formation of crossovers or non-crossovers.

Next to Spo11, nine proteins are known to be indispensable for DSB formation and thus meiotic recombination in *S. cerevisiae*: Mre11, Rad50, Xrs2, Ski8, Rec102, Rec104, Mer2, Mei4, and Rec114 (reviewed in Keeney 2001). Based on physical and genetical interactions among these 10 proteins, three subgroups have been defined: Mre11, Rad50, and Xrs2 (MRX complex); Spo11, Ski8, Rec102, and Rec104; and Rec114, Mer2, and Mei4 (Arora et al. 2004).

The evolutionary conserved MRX complex is not only required for meiotic DSB formation, but also holds numerous functions in meiotic and mitotic DNA repair (reviewed in Borde 2007). However, its dependency on DSB formation is not conserved in *Arabidopsis thaliana* (Puizina et al. 2004) or in fission yeast (Young et al. 2004).

Rec102 and Rec104 seem to form a functional unit; they interact genetically (Salem et al. 1999; Kee and Keeney 2002; Jiao et al. 2003) and physically (Arora et al. 2004; Kee et al. 2004; Maleki et al. 2007; Kee and Keeney 2002; Jiao et al. 2003). Both localize to chromatin at the time of DSB formation. Rec102 is distributed uniformly across the chromosomal DNA during meiosis, with preference for chromatin loops, but not for hotspot regions (Kee et al. 2004). Nuclear localization and chromatin association of Rec102 and Rec104 is inter-dependent and depends on Spo11 and Ski8 as well (Kee et al. 2004). In addition, Spo11 association with chromatin requires Rec102/Rec104 (Prieler et al. 2005; Sasanuma et al. 2007).

Ski8 and Spo11 interact physically (Uetz et al. 2000; Arora et al. 2004). Ski8 re-localizes during meiotic prophase from the cytoplasm to the nucleus in a Spo11 dependent manner (Arora et al. 2004). Spo11 and Ski8 need to interact for DSBs to be formed and it is presumed that Ski8 stabilizes the association of Spo11 with meiotic chromosomes (Arora et al. 2004). Furthermore, Ski8 contains the classical seven-bladed propeller structure of WD repeat proteins (Matsumoto et al. 1993; Madrona and Wilson 2004), which is a widely employed protein interaction motif, serving as a scaffold for the assembly of other proteins (reviewed in Smith et al. 1999), e.g. it promotes the interaction between Spo11 and Rec102/Rec104 (Arora et al. 2004). The function of Ski8 as a loading platform might also explain its other function in RNA metabolism in vegetatively growing cells (Ridley et al. 1984). This role, however, is genetically separable from its role in meiosis (Arora et al. 2004).

The third subgroup consists of Mer2, Mei4, and Rec114. The three proteins co-localize on meiotic chromosomes and co-immunoprecipitate (Maleki et al. 2007; Li et al. 2006). Mer2 interacts with itself, Rec114, Mei4, and Xrs2 (Arora et al. 2004). Mer2 forms foci on meiotic chromosomes and gets phosphorylated by Cdc28-Clb5/Clb6 and Cdc7-Clb4, which is essential for its interaction with other DSB proteins and their loading to chromatin (Henderson et al. 2006; Sasanuma et al. 2008; Wan et al. 2008). Rec114 localizes independently of other DSB proteins to the DNA and is needed for Spo11 association with hot-spot DNA (Prieler et al. 2005; Sasanuma et al. 2007; Maleki et al. 2007). Furthermore, its over-expression prevents DSB formation (Bishop et al. 1999). Rec114 as well as Mei4 interact with Rec102 and Rec104 in a yeast two-hybrid assay (Arora et al. 2004; Maleki et al. 2007). It might well be that Rec102/Rec104 bridge the interaction between Rec114/Mei4/Mer2 and Spo11/Ski8.

Other proteins, which are not directly engaged in the formation of DSBs but might be needed for proper loading or activation of the recombination initiation complex might affect DSB formation. As DSBs occur mainly in already open chromatin regions, e.g. promotor regions with transcription factor mediated chromatin remodeling, any protein responsible for opening the chromatin might be involved in recruiting the recombination initiation complex (reviewed in Lichten 2008). Mentionable are also the meiosis-specific proteins Red1, Mek1, and Hop1, which associate to form the axial elements of the synaptonemal complex (SC), a tripartite structure present in many organisms to mediate pairing of homologous chromosomes (reviewed in Zickler and Kleckner 1999). As such, these three proteins form the basis for the chromatin loops of the sister chromatids. *red1*$^{-}$, *mek1*$^{-}$, or *hop1*$^{-}$ mutants lead to DSB reduction from 5 to 15% of the wild type (Carballo et al. 2008; Mao-Draayer et al. 1996; Xu et al. 1997). This reduction implies a more supporting than essential role in DSB formation.

In the distantly related ascomycete *Schizosaccharomyces pombe*, 10 proteins are assumed to be required for meiotic DSB formation: Rec6, Rec7, Rec8, Rec10, Rec11, Rec12, Rec14, Rec15, Rec24, Rec25, Rec27, and Mde2 (Ponticelli and Smith 1989; De Veaux et al. 1992; Cervantes et al. 2000; Martin-Castellanos et al. 2005; Gregan et al. 2005). *S. pombe* does not possess a SC but structures similar to the axial elements, the linear elements (LinEs) are observed (Bähler et al. 1993; Lorenz et al. 2004). The proper assembly of the LinEs requires inter-dependably Rec10, Rec25, and Rec27 (Davis et al. 2008), as well as Rec8 and Rec11, the meiotic cohesins responsible for sister chromatid cohesion during meiosis I (Molnar et al. 1995; Parisi et al. 1999; Kitajima et al. 2003). LinE-associated proteins, Hop1 and Mek1, are not absolutely required but are needed for wild-type levels of DSB formation (Latypov et al. 2010).

Besides Rec12, two more *S. pombe* DSB-proteins have homologs in *S. cerevisiae*: Rec14 is the homolog of Ski8

(Evans et al. 1997) and Rec7 is the homolog of Rec114 (Fox and Smith 1998). A *rec14*$^{-}$ mutant reflects, as *ski8*$^{-}$, a mitotic slow-growth phenotype (Evans et al. 1997; Deutschbauer et al. 2005), which might be related to its function in RNA metabolism. Rec12's association with hot-spot DNA depends on Rec14 as well as on Rec6 (Ludin et al. 2008).

Before karyogamy, up to three Rec7 foci are formed per nucleus, as detected in cytological experiments with living cells (Molnar et al. 2001b). Later during meiosis, Rec7 foci reach a maximum in prophase I and decreases afterwards, but some foci persist until meiosis I (Molnar et al. 2001b). The formation of the prophase I foci depends on Rec10 (Lorenz et al. 2006). As *rec15*$^{-}$, *rec7*$^{-}$ shows a prolonged meiosis I stage, probably due to chromosome segregation difficulties, seen in a lagging chromosome phenotype (Molnar et al. 2001a) which is also a characteristic of *mde2*$^{-}$ (Gregan et al. 2005). In addition, *rec7*$^{-}$ crosses produce homozygous diploid spores with a high frequency arising by omission of meiosis II (Molnar et al. 2001b). This phenomenon is found in *rec12*$^{-}$ (Davis and Smith. 2003), *rec14*$^{-}$ (Molnar et al. 2001a), *rec15*$^{-}$ (Doll et al. 2005), and *rec24*$^{-}$ mutants as well (Martin-Castellanos et al. 2005). Whether this phenotype is caused by the same mechanism in all these mutants remains to be investigated.

There must be candidates for the meiotic recombination initiation complex among the remaining proteins in the above list. To further characterize the interplay among potential candidates of the *S. pombe* meiotic initiation complex, we employed a yeast two-hybrid all-against-all matrix of the proteins mentioned above. Here, we present three new interactions, Rec7 with Rec24, Rec12 with Rec14, and Rec15 with Mde2, respectively.

Materials and methods

Culture methods and yeast strains

Media and general methods for *S.cerevisiae* were used as described in (Ausubel et al. 2009). Media and general methods for *S. pombe* are described in (Gutz et al. 1974; Moreno et al, 1991). Synthetic medium, EMM with 2% glucose, and EMM without nitrogen source (EMM-N) with 1% glucose were described in (Nurse et al. 1976). Supplements were added to a final concentration of 0.1%, where appropriate. Construction of *rec7HA3*::*kan*^{R} was done by the PCR-based method after Bähler with primers #SIL9 and #SIL10 (Table S3) and plasmid pFA6a-HA3 (Bähler et al. 1998). Similarly, *rec24TAP4*::*kan*^{R} was constructed with primers #SIL1 and #SIL2 and pFA6a-TAP4 (P2021, K. Gould). After sequencing of the constructs, both Rec7HA3 and Rec24TAP4 were tested for functionality. In crosses homozygous for *rec7HA*::*kan*^{R} and *rec24TAP4*::*kan*^{R} spore viability was tested on YEA medium under the tetrad microscope by visually counting non-germinating spores versus appearing micro colonies.

Construction of diploid strains

A diploid h^{-}/h^{-} strain expressing epitope-tagged Rec7HA3 and/or Rec24TAP4 was constructed by scoring for a mitotic crossover between the mating-type region and the centromere on chromosome II of a h^{+}/h^{-} diploid strain. This strain was procured by mating a haploid *pat1-114 ade6-M210 lys7-2* strain with a haploid *pat1-114 ade6-M216 leu2-120* strain for 12 h on a SPO plate. The *ade6-M210* and *ade6-M216* alleles on chromosome III complement each other, whereas the *lys7-2* and *leu2-120* markers on chromosome I are closely linked, reducing the possibility of a mitotic crossover event between them. Cell material was collected, resuspended in water, and plated on MMA plates. After 4 days at 25°C, large colonies were picked and re-isolated on MMA plates. The absence of sporulation was investigated by iodine staining after 3 days growth on MEA plates. The mating-type of the non-sporulating diploids was verified with back-crossings to tester strains 975 h^{+} or 972 h^{-}.

Yeast two-hybrid system

The PCR-based recombinational cloning method after Hudson et al. (1997) was used to integrate the intronless *gene-of-interest* into the yeast two-hybrid (Y2H) vectors pOAD and pOBD, respectively, resulting in a fusion between either sequences of the activation- or the binding domain of the GAL4 gene with sequences of *gene-of-interest* (free *gene-of-interest* C-terminus). Primers and templates used for PCR-cloning are listed in Table S1 and primer sequences in Table S3. The *rec7* point mutation (*rec7F325A*, TTC to GCC) was introduced by PCR with a long primer containing the base substitution and the resulting PCR fragment was cloned as described above (Table S1, S3). The *rec12* point mutations (*rec12Q308A*, CAG to GCG, and *rec12R309A*, GGA to GCA, respectively) were introduced by fusion PCR using primers with homologous sequence over the base substitutions, and the resulting PCR fragment was cloned as described above (Table S1, S3). After recombinational transformation into *S. cerevisiae* strains PJ694A or PJ694α (Table 1; James et al. 1996), plasmids were isolated (QIAprep Spin Miniprep Kit) and sequence-confirmed. Yeast two-hybrid analysis was performed in diploid cells which were derived from mated *S. cerevisiae* strains PJ694α and PJ694A (Table 1), transformed with sequence-checked pOBD or pOAD derivatives. Expression of three reporter genes

Table 1 Strain list

Yeast strain	Genotype	Reference
S. cerevisiae		
PJ69-4a	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δgal80ΔLYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et al. (1996)
PH69-4α	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δgal80ΔLYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et a.l (1996)
S. pombe		
153-6133	h^+ *lys1-131 ade6-M26*	Berne collection
153-6136	h^- *lys1-131 ade6-469*	Berne collection
KLY399	h^- *lys1-131 ade6-469 rec12Δ::hyg*^{R}	This study
KLY402	h^+ *lys1-131 ade6-M26 rec12Δ::hyg*^{R}	This study
KLY410	h^- *lys1-131 ade6-469 ura4-D18 rec7Δ::ura4*	This study
KLY414	h^+ *lys1-131 ade6-M26 ura4-D18 rec7Δ::ura4*	This study
SILY117	h^-/h^- *rec7HA3::kan*^{R}*/rec7HA3::kan*^{R} *rec24-TAP4::kan*^{R}*/rec24TAP4::kan*^{R} *lys7-2/leu2-120 ade6-M210/ade6-M216 pat1-114/pat1-114*	This study
SILY123	h^-/h^- *rec7HA3::kan*^{R}*/rec7HA3::kan*^{R} *lys7-2/leu2-120 ade6-M210/ade6-M216 pat1-114/pat1-114*	This study

(P_{GAL2}-*ADE*2, P_{GAL2}-*HIS*3, and P_{GAL2}-*lacZ*) was assayed: growth on drop-out minimal media missing leucine, tryptophane, and adenine; growth on drop-out minimal media missing leucine, tryptophane, and histidine, but replenished with 25 mM 3-aminotriazole (3-AT); and β-galactosidase activity assayed in permeabilized cells (Ludin et al. 1998).

S. pombe plasmid constructions

pSIL2 was constructed in several steps: first, pMR3, a pREP41 (Basi et al. 1993) derivate containing a thiamine-repressible, myc epitope-tagged *rec12* in the *Sal* I site, was used to amplify the *nmt1*-promotor sequence with primers #SIL81 and #SIL82, producing *Pst*I and *Xho*I restriction site overhangs. A second PCR amplification with primers #SIL83 and #SIL84 and pMR3 produced a fragment containing 13 myc epitopes followed by *nmt1*-terminator sequences with *Xho*I and *Kpn*I restriction site overhangs. The two fragments were fused by PCR amplification using primers #SIL81 and #SIL84 and sequence homology over the *Xho*I restriction site. The resulting PCR fragment was ligated into the *Kpn*I and *Pst*I restriction sites of pYC36 (Chikashige et al. 2004) to form pSIL2. *S. pombe* genes were PCR amplified using a forward primer with a *Xho*I restriction site overhang and a reverse primer with a *Sal*I and a *Xho*I restriction site overhang (Table S2, S3) with genomic DNA (*rec7*, *rec12*) or Y2H plasmid DNA (*rec7*F325A, *rec12*Q308A, and *rec12*R309A) as target sequence. *Xho*I digested PCR fragments were ligated into *Xho*I digested pSIL2 to create myc epitope-tagged constructs (e.g. pSIL2-Rec7$_{myc}$) or *Xho*I, *Sal*I digested PCR fragments were ligated into *Xho*I digested pSIL2 to create untagged constructs with a stop codon 21 bp after the last gene-specific codon (e.g. pSIL2-Rec7). All constructs were sequenced before transformation.

Intragenic recombination assay

pSIL2 or pSIL2 derivatives were transformed into h^+ *ade6-M26 lys1-131* strains (with or without deletion of *rec7* or *rec12*, respectively) and integrated at the *lys1* locus by homologous recombination between the C-terminal truncated *lys1* gene on pSIL2 and *lys1-131*. Transformants, which were able to grow on GMA+ade media were crossed with strains of the background h^- *ade6-469 lys1*$^+$*::pSIL2* with or without deletion of *rec7* or *rec12* by the following procedure: parental strains were grown to stationary phase in EMM+ade medium for at least 48 h. As much as 500 µl of each culture was mixed with the respective partner culture, briefly centrifuged, and the cell pellet washed by vortexing extensively with 500 µl 50 mM Na-phosphate at pH 7.0. Cells were again centrifuged and resuspended totally in 50 µl 50 mM Na-phosphate at pH 7.0. As much as 5 µl of this cell suspension was spotted on a SPO plate or on SPO+thiamine (2 µM) plate and incubated for 48 h at 25°C. Crossing material was then scratched from the plate and incubated for 4 h in 500 µl water containing 0.2% glusulase (*Helix pomatia* Juice. PALL Life Sciences) and 0.05% lysing enzyme (Sigma L-1412) at 30°C. The spore suspension was centrifuged and the pellet was resuspended in 500 µl 50 mM Na-phosphate at pH 7.0. A 5 µl of this spore suspension, or

ten-fold dilutions, was spotted on GMA and GMA+adenine plates and growth was scored after 4 days at 30°C. Recombination analysis was repeated at least three times with individual *lys*$^+$ transformants. Homozygous wild-type crosses, heterozygous mutant crosses, and homozygous mutant crosses were always performed in parallel. Spore viability analysis (Rothenberg et al. 2009) of all crosses listed in Figs. 3 and 7 is given in Table S4.

Western blot

Protein extracts (Caspari et al. 2000) were separated on 10% SDS polyacrylamide gels and blotted on PVDF membranes (Immobilon, Millipore) in a wet blot chamber (BioRad) for 1 h 15 min at 400 mAmp. After over night blocking at 4°C with 5% milk in PBS-T (20 mM phosphate, 150 mM NaCl, and 0.05% Tween), the membrane was probed with one of the following antibodies: monoclonal mouse anti-HA HRP coupled (12CA5, Roche) at a dilution of 1/400 or monoclonal mouse anti-HA (12CA5, Roche) at a dilution of 1/1,000 and polyclonal rabbit anti-mouse HRP coupled (DAKO) for detection of Rec7HA, polyclonal rabbit anti-HRP antibody in combination with HRP (PAP, DAKO) at a dilution of 1/15,000 for detection of Rec24TAP4.

Co-immunoprecipitation

Diploid strains (Table 1) were grown to an OD_{595} of 0.4 in EMM at 25°C. The cells were washed once with water and resuspended in EMM-N. After 16 h of nitrogen starvation cells were arrested in G1. Meiosis was induced by a temperature shift to 34°C after the addition of NH_4Cl to a final concentration of 0.5 g/l. At each timepoint from 0 to 8 h, 1 ml of cell culture was taken washed once with H_2O then resuspended in 1 ml 70% EtOH for DAPI and FACS analysis. At time points 0 h (T0) and 3.5 h (T3.5) 175 ml cell culture was centrifuged for 3 min at 3,000 rpm (Eppendorf 5417C), washed with 175 ml PBS (20 mM phosphate, 150 mM NaCl) with freshly added phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM, and centrifuged again. The cell pellet was resuspended in 300 µl PBS with freshly added PMSF, protease inhibitors (Complete Protease Inhibitor Cocktail, EDTA-free, Roche) and phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail, Roche). A ceramic mortar and pestle were cooled with liquid N_2. Thick cell suspension was pipetted into the mortar filled with liquid N_2 and then pestled for at least 5 min under constant addition of N_2. The cell powder was stored at −80°C until further use. The cell powder was resuspended in 600 µl lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 8.7% Glycerol, 1 mM PMSF, protease, and phosphatase inhibitors) and centrifuged for 5 min at 4°C in a micro centrifuge at maximal speed. The supernatant was transferred into a new eppendorf tube and a centrifugation of 15 min at maximal speed followed. The supernatant was transferred into a new eppendorf tube and if needed was adjusted to 1,025 µl with lysis buffer (whole cell extract, WCE). As much as 25 µl WCE was mixed with 25 µl 2x Lämmli buffer (125 mM Tris–HCl, 20% glycerol, 4% SDS, 0.02% Bromphenol blue, pH 6.8), boiled and stored at −20°C (Input). IgG sepharose (IgG Sepharose^{TM} 6 Fast Flow, GE Healthcare) was pre-washed at 4°C by resuspension and centrifugation (1,000 rpm in a micro centrifuge) five times with 50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween, and twice with lysis buffer. A 30 µl of the pre-washed sepharose matrix was incubated with 1 ml of WCE for 2 h at 4°C on a rotating wheel. PMSF was added to a final concentration of 1 mM every 30 min. The sepharose matrix was washed 6 times with 1 ml lysis buffer, resuspended in 50 µl 2x Lämmli buffer, and boiled and stored at −20°C.

Results and discussion

Yeast two-hybrid analysis among putative members of the meiotic recombination initiation complex

To test for physical interactions between the fission yeast DSB proteins, we carried out a yeast two-hybrid (Y2H) assay in pair-wise combinations. We cloned cDNA sequences of *rec6*, *rec7*, *rec10*, *rec12*, *rec14*, *rec15*, *mde2*, *rec24*, and *rec25* behind the *GAL4* DNA-binding domain or the *GAL4* activation domain in the vectors pOBD or pOAD, respectively (see Materials and methods). We failed after many attempts to clone *rec27* cDNA and left it out for the analysis. We also included in the Y2H assay *rec23* and *rec26*, the gene products of which were reported to co-immunoprecipitate in *S. pombe*. They served us as an interaction control (Chikashige et al. 2006). *rec23* and *rec26* mutants also showed decreased DSB formation, but were found to be involved in bouquet formation, thus their synonyms *bqt2* and *bqt1*, respectively.

The diploid Y2H strain PJ69-4 contains three reporter genes under the control of different *GAL* promotors: P_{GAL2}-*ADE2*, P_{GAL1}-*HIS3*, and P_{GAL7}-*lacZ*. The auxotrophic reporter genes were used for an all-against-all Y2H analysis. If an interaction is apparent, growth on media lacking adenine, or on media lacking histidine but replenished with 3-aminotriazole as an inhibitor of the sparsely expressed *HIS3* gene product, is feasible. We confirmed the interaction between Rec23/Bqt2 and Rec26/Bqt1 in both construct combinations, Gal4BD-Rec23 with Gal4AD-Rec26 and Gal4AD-Rec23 with Gal4BD-Rec26, respectively (Fig. 1). Besides this control interaction, three novel interactions

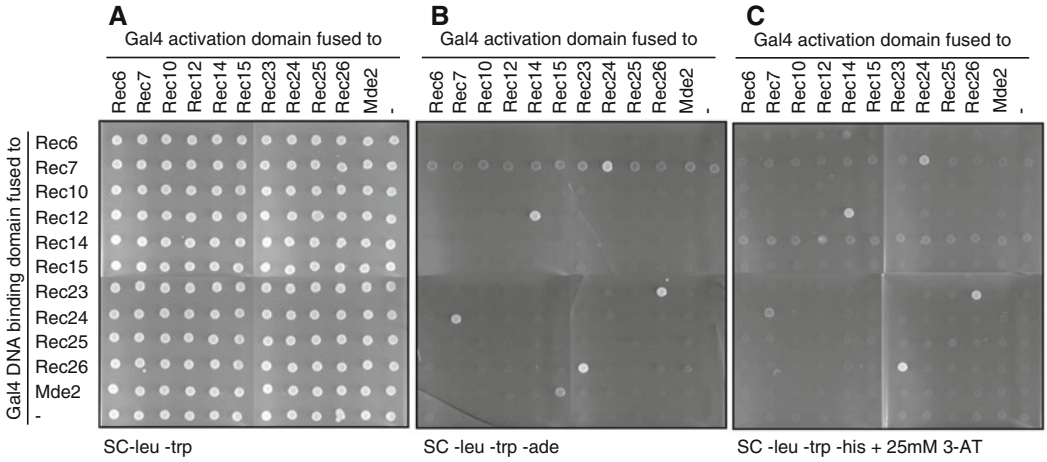

Fig. 1 Matrix of yeast two-hybrid interaction analysis of early meiotic recombination proteins. Gal4 activation domain or DNA binding domain fusions to *S. pombe* proteins involved in early meiosis were assayed in the *S. cerevisiae* yeast two-hybrid strain PJ69-4 in pairwise combinations for growth of synthetic complete media lacking leucine and tryptophan (SC leu–trp) for the presence of both fusion constructs (**a**), on synthetic complete media lacking adenine, leucine, and tryptophan (SC ade–leu–trp) for expression of the P_{GAL2}-*ADE2* reporter (**b**), and on synthetic complete media lacking histidine, leucine, and tryptophan (SC his–leu–trp) for expression of the P_{GAL2}-*HIS3* reporter (C). "–" stands for empty vector

were found; we detected an interaction between Rec7 and Rec24: the expression of both combinations stimulated P_{GAL2}-*ADE2* and P_{GAL1}-*HIS3* expression. Gal4BD-Rec7 was showing a mild self-activation phenotype when tested for expression of P_{GAL2}-*ADE2*. Similarly, the Rec7 homolog in *S. cerevisiae*, Rec114 activated a *lacZ* reporter when fused to a DNA-binding domain (Arora et al. 2004). Another interaction was detected between Rec12 and Rec14, although only the combination Gal4BD-Rec12 with Gal4AD-Rec14 stimulated expression of P_{GAL2}-*ADE2* and P_{GAL1}-*HIS3*. Finally, an interaction between Mde2 and Rec15 was found, but only the combination Gal4BD-Mde2 with Gal4AD-Rec15 stimulated transcription of the P_{GAL2}-*ADE2* reporter gene. The interactions between Rec7 and Rec24 and between Rec12 and Rec14 were studied in more detail. Although the interaction was seen only in one construct combination, it was interesting to see whether similar interaction parameters applied to Rec12–Rec14 as it was shown for *S. cerevisiae* Spo11–Ski8 (Arora et al, 2004). The interaction of Mde2 with Rec15 was not further characterized because the weak activation of the P_{GAL2}-*ADE2* reporter gene could neither be confirmed with the P_{GAL1}-*HIS3* (Fig. 1c) nor the P_{GAL7}-*lacZ* reporter gene (data not shown). Nevertheless, sterical hindrances of Gal4 fusion constructs as well as fission yeast, meiosis-specific changes in the post-translational protein modification might limit the score in this Y2H interaction analysis.

Rec12/Rec14 interaction resembles Spo11/Ski8 interaction

It was previously shown that amino acid changes in the C-terminus of Spo11, Q376A, or RE377AA reduce the Y2H interaction with Ski8 vigorously (Arora et al. 2004). In addition, these amino acid changes trigger a recombination defect similar to *spo*11Δ. Both amino acids, glutamine at position 308 and arginine at position 309 are conserved in Rec12. In a Y2H analysis with Gal4BD-Rec12Q308A and Gal4AD-Rec14, a reduced interaction was detected with the P_{GAL2}-*ADE2* reporter gene; whereas no interaction was found with the P_{GAL1}-*HIS3* and the P_{GAL7}-*lacZ* reporter genes (Fig. 2; Table 2). The interaction between Gal4BD-Rec12R309A and Gal4AD-Rec14 was completely abolished with all three reporters (Fig. 2; Table 2). A hydrophobic amino acid patch in Ski8 is involved in the interaction with Spo11 and especially phenylalanine at position 59 seems to be crucial, as determined by crystal structure (Cheng et al. 2004).

At the equivalent position 57 in Rec14 a proline is located instead of a phenylalanine. Nevertheless, we wondered if the N-terminus of Rec14 is involved in the interaction with Rec12. A Y2H analysis with Gal4BD-Rec12 and Gal4AD-Rec14ΔN, missing the N-terminal 61 amino acids, revealed a loss of interaction based on the lack of activation with all three reporter genes (Fig. 2; Table 2).

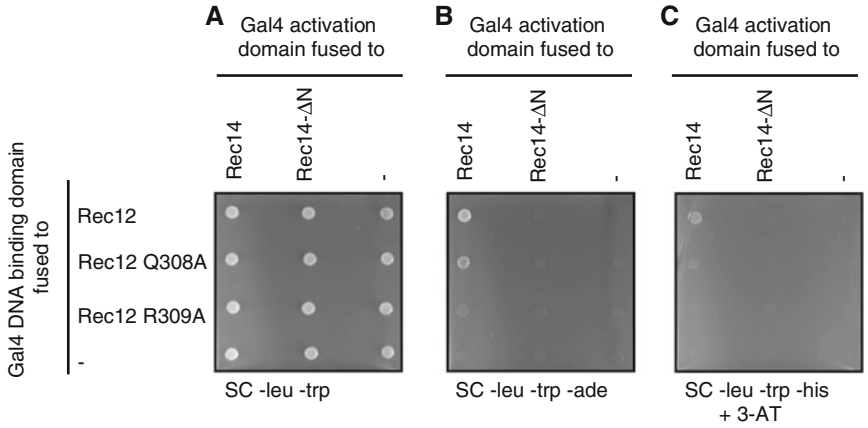

Fig. 2 Yeast two-hybrid interaction analysis of Rec12 and Rec14. Gal4 DNA-binding domain fusions to Rec12, Rec12Q308A, or Rec12R309A in combination with Gal4 activation domain fusions to Rec14 or Rec14ΔN were assayed for growth on synthetic complete media lacking leucine and tryptophan (SC leu–trp) for the presence of both fusion constructs (**a**), synthetic complete media lacking adenine, leucine, and tryptophan (SC ade–leu–trp) for the expression of the P_{GAL2}-*ADE2* reporter (**b**), and on synthetic complete media lacking histidine, leucine, and tryptophan (SC his–leu–trp) for expression of the P_{GAL2}-*HIS3* reporter (C). "–" stands for empty vector

Table 2 Y2H interaction of Rec12 and Rec14 domains analyzed with the P_{GAL7}-*lacZ* reporter

pOAD	pOBD	β-gal activity[a]	SEM[b]
Rec14	Rec12	6.6	1
Rec14	Rec12Q308A	0.2	0.2
Rec14	Rec12R309A	<0.1	
Rec14	–	1.0	0.4
Rec14ΔN	Rec12	<0.1	
Rec14ΔN	Rec12Q308A	<0.1	
Rec14ΔN	Rec12R309A	<0.1	
Rec14ΔN	–	0.9	0.3
–	Rec12	<0.1	
–	Rec12Q308A	<0.1	
–	Rec12R309A	<0.1	

[a] The mean β-galactosidase activity from 3 to 10 independent transformants is given in Miller units

[b] SEM stands for standard error of the mean

Interaction loss between Rec12Q308A or Rec12R309A and Rec14 leads to recombination deficiency in *S. pombe*

We wondered whether the amino acid substitutions Q308A and R309A in Rec12, which were responsible for the loss of interaction with Rec14, lead to meiotic recombination deficiency in *S. pombe*. We integrated *rec12*, *rec12Q308A*, and *rec12R309A*, controlled from a thiamine-repressible promotor, into a *rec12Δ* strain and asked whether they can complement the *rec12*Δ meiotic recombination defect. In a qualitative recombination assay, we measured intragenic recombination between *ade6-M26* and *ade6-469* in crosses heterogenous for the integrated *rec12* construct ("Materials and methods", Fig. 3). The same recombination proficiency as in a heterozygous *rec12*Δ cross was seen in crosses with integrated *rec12*, independently of the thiamine condition. Obviously, Rec12 protein is not a limiting factor for proper recombination initiation as also suggested from Western blot analysis by the high amount of free Rec12 protein in a meiotic time course when probed for bound oligonucleotides (Rothenberg et al. 2009). In crosses with *rec12Q308A* or *rec12R309A* integrations, few adenine prototrophs were detected. Spore viability of these crosses was not significantly different from the spore viability of the homozygous *rec12*Δ cross (Table S4). Western blot analysis confirmed that myc epitope tagged Rec12Q308A and Rec12R309A were expressed (Figure S1).

From these results we conclude that amino acid Q308 and R309 in Rec12 and the N-terminus of Rec14 are required for mutual interaction. This interaction is essential for proper recombination levels. The fact that homologous amino acids/domain in the distantly related yeasts are required for this interaction shows not only phylogenetical, but also a structural conservation of part of the DSB machine.

The C-terminus of Rec7 interacts with the N-terminus of Rec24

To find out which part of Rec7 participates in the physical interaction with Rec24, Y2H plasmids were constructed with one out of four Rec7 sequence parts, Rec7A to Rec7D (Table S1, S3). A Y2H interaction analysis was performed between these partial Rec7 proteins and full length Rec24 in both combinations, e.g. Gal4BD-Rec7A with Gal4AD-Rec24

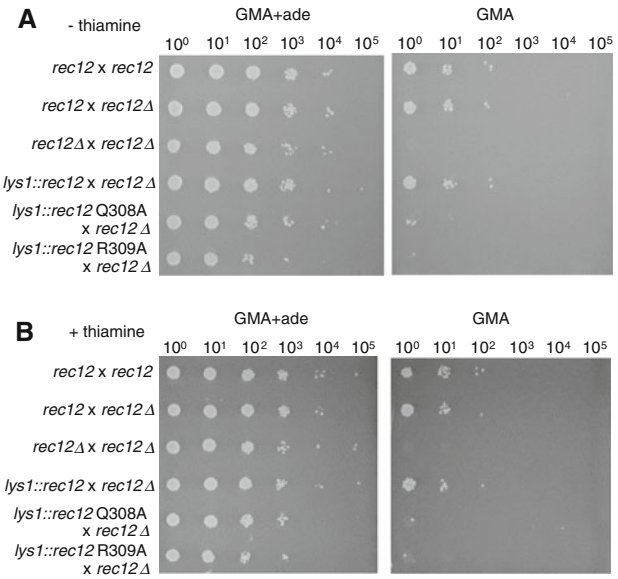

Fig. 3 Amino acid changes in Rec12, Q308A and R309A, lead to a recombination defect. Undiluted and tenfold dilutions of spore suspensions from derepressed (**a**, SPO media) or repressed conditions (**b**, SPO media with thiamine) of homozygous *rec12*, heterozygous *rec12*Δ, homozygous *rec12*Δ, heterozygous *lys1::*P_{nmt1}*-rec12*, heterozygous *lys1*::P_{nmt1}*-rec12Q308A*, and heterozygous *lys1*::P_{nmt1}*-rec12R309A* crosses, grown on minimal media containing adenine (GMA+ade, *left panel*) or on minimal media without adenine (GMA, *right panel*)

and Gal4BD-Rec24 with Gal4AD-Rec7A. P_{GAL2}*-ADE2*, P_{GAL1}*-HIS3* (Fig. 4), and P_{GAL7}*-lacZ* expressions (Table 3) were stimulated only when Rec24 was combined with Rec7D. When Gal4BD- or Gal4AD-construct fused to different parts of Rec24 (A to D) is expressed together with Gal4AD-Rec7 or Gal4BD-Rec7, only Rec24A showed a Y2H interaction with Rec7 (Fig. 4; Table 3).

So far, no Rec24 orthologs were found. However, a multiple sequence alignment (ClustalX; Thompson et al. 1994) of Rec7 with orthologs found among sequenced ascomycete genomes pointed toward a conserved phenylalanine at position 325 in part D of Rec7 (Fig. 5). Changing this phenylalanine to alanine impaired the interaction of Gal4BD-Rec7F325A with Gal4ADRec24 and Gal4AD-Rec7F325A with Gal4BD-Rec24, as seen with all three reporter genes (Fig. 4; Table 3).

From these results we conclude that the C-terminus of Rec7, especially the phenylalanine at position 325, and the N-terminus of Rec24 are crucial for the interaction between these proteins.

Rec7 interacts with Rec24 in *S. pombe*

To demonstrate that Rec7 and Rec24 interact during *S. pombe* meiosis, we performed co-immunoprecipitation

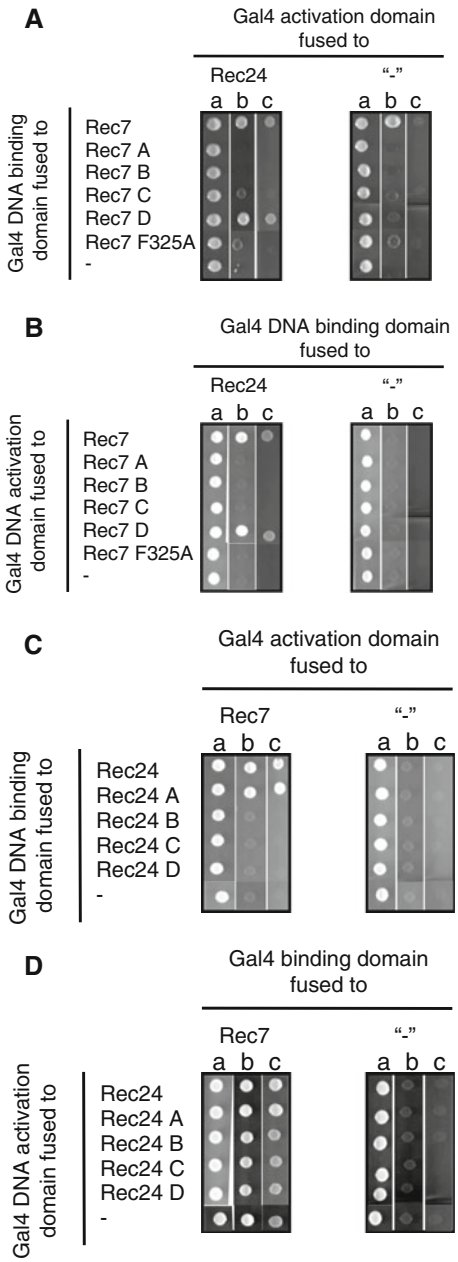

Fig. 4 Yeast two-hybrid interaction analysis of Rec7 and Rec24 domains. Gal4 DNA binding domain (**a**) or activation domain fusions (**b**) to Rec7, Rec7A (amino acids: 1-87), Rec7B (80–175), Rec7C (168–263), Rec7D (256–340), or Rec7F325A were assayed with Gal4 activation domain or Gal4 DNA binding domain fusions to Rec24 on synthetic complete media lacking leucine and tryptophan (*a*, SC leu–trp), synthetic complete media lacking adenine, leucine, and tryptophan (*b*, SC ade–leu–trp), and synthetic complete media lacking histidine, leucine, and tryptophan (*c*, SC -his -leu -trp) for expression of the P_{GAL2}*-ADE2* and P_{GAL2}*-HIS3* reporters. Similarly, Gal4 DNA binding domain (**c**) or activation domain fusions (**d**) to Rec24, Rec24A (amino acids: 1–112), Rec24B (105–180), Rec24C (174–264), and Rec24D (257–357) were assayed with Gal4 activation domain or Gal4 DNA binding domain fusions to Rec7 for growth on the same media (*a–c*).“–” stands for empty vector

Table 3 Y2H interaction of Rec7 and Rec24 domains analyzed with the P_{GAL7}-*lacZ* reporter

pOAD-	pOBD-	β-gal activity[a]	SEM[b]
Rec24	Rec7	24.4	3.5
Rec24	Rec7A	<0.1	
Rec24	Rec7B	<0.1	
Rec24	Rec7C	4.3	0.8
Rec24	Rec7D	15.5	1.4
Rec24	Rec7F325A	0.2	0.1
Rec24	–	0.2	0.1
–	Rec7	2.2	0.6
–	Rec7A	<0.1	
–	Rec7B	<0.1	
–	Rec7C	3.1	0.8
–	Rec7D	0.3	0.2
–	Rec7F325A	0.1	0.1
Rec7	Rec24A	61.5	11.3
Rec7	Rec24B	<0.1	
Rec7	Rec24C	0.2	0.2
Rec7	Rec24D	0.1	0.1
–	Rec24A	0.1	0.1
–	Rec24B	<0.1	
–	Rec24C	<0.1	
–	Rec24D	0.5	0.3
Rec7	Rec24	10.9	2.5
Rec7A	Rec24	<0.1	
Rec7B	Rec24	<0.1	
Rec7C	Rec24	<0.1	
Rec7D	Rec24	60.2	14.2
Rec7F325A	Rec24	<0.1	
–	Rec24	<0.1	
Rec7	–	0.5	0.2
Rec7A	–	0.2	0.1
Rec7B	–	0.5	0.3
Rec7C	–	0.5	0.4
Rec7D	–	0.2	0.1
Rec7F325A	–	0.1	0.1
Rec24A	Rec7	40.4	14.9
Rec24B	Rec7	1.4	0.4
Rec24C	Rec7	1.1	0.2
Rec24D	Rec7	1.9	0.6
Rec24A	–	0.6	0.3
Rec24B	–	0.4	0.3
Rec24C	–	0.2	0.1
Rec24D	–	0.1	0.1

[a] The mean β-galactosidase activity from 3 to 10 independent transformants is given in Miller units

[b] SEM stands for standard error of the mean

experiments with tagged proteins from *pat1-114* meiotic cell samples. Inducing meiosis in a *pat1-114* mutant strain has the advantage of high synchronicity (Beach 1985), but the disadvantage of a chromosome segregation defect in the first meiotic division (Yamamoto and Hiraoka 2003). However, it has been used systematically for the timing of early meiotic events including DSB formation (Cervantes et al. 2000; Young et al. 2002). Functional Rec7HA3 and Rec24TAP4 were expressed in a h^-/h^-*pat1-114/pat1-114* diploid strain (Table 1, "Materials and methods") and Rec24TAP4 was immuno-precipitated at time points 0 or 3 h after meiotic start (for FACS analysis, see Figure S2), utilizing the affinity of the Protein A epitope to human immunoglobulin. As shown in Fig. 6, Rec7HA3 co-precipitated only when Rec24TAP4 was present in the protein extracts. This result confirms the interaction of Rec7 and Rec24. The occurrence of this interaction in both experimental systems, the Y2H analysis and the co-immunoprecipitation experiment, suggests that the mutual interaction is not dependent on fission yeast post-translational modifications.

Loss of interaction between Rec7 and Rec24 leads to recombination deficiency

To test whether the lost interaction of Rec7F325A with Rec24 in the Y2H analysis is relevant for a functional *S. pombe* meiotic recombination initiation complex and thus for wild-type recombination levels, we integrated *rec7F325A* as well as *rec7* wild-type sequences into the *lys1* locus in a *rec7*Δ strain and checked for complementation of recombination deficiency by measuring intragenic recombination between *ade6-M26* and *ade6-469*. Both constructs were under the control of a thiamine-repressible promotor. Heterozygous *lys1::rec7* x *rec7*Δ crosses showed similar recombination proficiency under derepressing conditions (no thiamine) than heterozygous *rec7* x *rec7*Δ crosses (Fig. 7a). The addition of thiamine to the sporulation media decreases the recombination level (Fig. 7b) indicating a need for high amounts of Rec7 protein. Heterozygous crosses *lys1::rec7*F325A × *rec7*Δ, however, were indistinguishable from homozygous *rec7*Δ x *rec7*Δ crosses. Spore viability of these crosses was not significantly different from the spore viability of the homozygous *rec7*Δ cross (Table S4). Western blot analysis confirmed that a myc epitope tagged Rec7F325A was expressed (Figure S1).

In conclusion we can say, that the consequence of interaction loss between Rec7F325A and Rec24 in the Y2H analysis converts into a meiotic recombination deficiency of

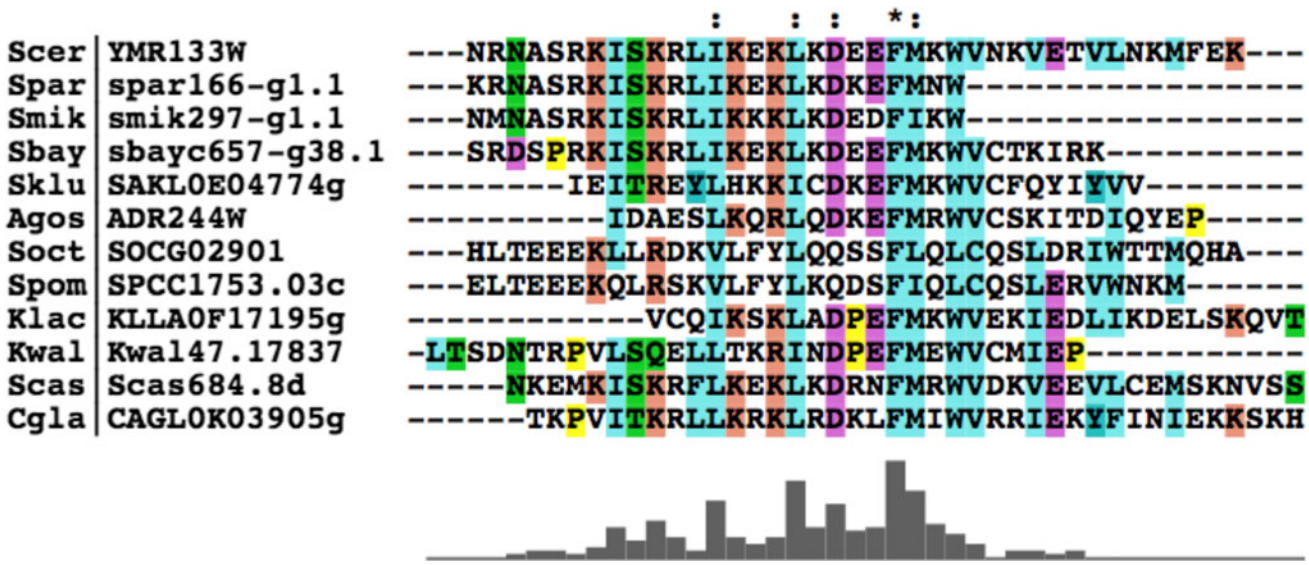

Fig. 5 Multiple sequence alignment of the C terminus of Rec7 homologs. Sequences homologs from various ascomycetes of Rec7 were aligned with the CLUSTAL-X program. The very C-terminal patch of homology is shown, containing the C terminal ends of all homologs. Positions with similar amino acids are indicated with vertical double dots above the aligned sequences, whereas identical amino acids are indicated with a star. The *bar diagram* below the aligned sequences indicates the amount of homology in an arbitrary scale

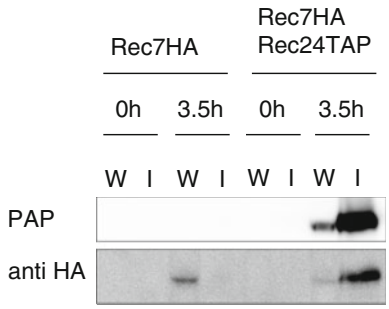

Fig. 6 Rec7 physically interacts with Rec24 in *S. pombe* during meiosis. Co-immunoprecipitation was performed with extracts from diploid strains at the start of meiosis (0 h) and after 3.5 h. Rec24TAP was immunoprecipitated by the affinitiy of its protein A moiety to IgG sepharose. The strains carried either Rec7HA (SILY123) or Rec7HA and Rec24TAP (SILY117). *I* stands for co-immunoprecipitate and corresponds to 40 times the amount of loaded whole cell extract (W). The *upper lane* shows immuno-detection with PAP antibody, the *lower panel* shows the re-probing with a mouse anti-HA antibody

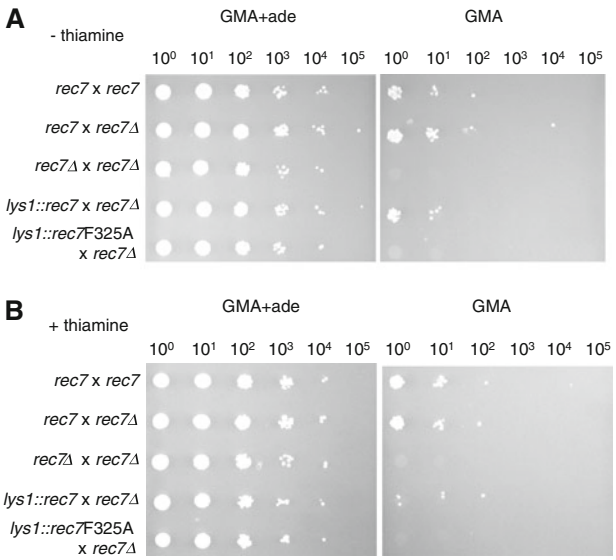

Fig. 7 Recombination is abolished in *rec7F325A*. Undiluted and tenfold dilutions of spore suspensions from derepressed (**a**, SPO media) or repressed conditions (**b**, SPO media with thiamine) of homozygous *rec7*, heterozygous *rec7*Δ, homozygous *rec7*Δ, heterozygous *lys1::P*$_{nmt1}$*-rec7*, and heterozygous *lys1::P*$_{nmt1}$*-rec7F325A* crosses, grown on minimal media containing adenine (GMA+ade, *left panel*) or on minimal media without adenine (GMA, *right panel*)

rec7F325A in *S. pombe*. The conservation of this phenylalanine in Rec7 homologs might suggest an important contact point with other, although not conserved DSB-proteins.

Preliminary TAP purification experiments indicate that Rec15 purifies with Rec24, and Rec6 with Rec14 (own unpublished observations) suggesting that post-translational protein modifications might be needed for these interactions and thus were not detected in the Y2H system. These putative interactions and the here presented Rec7/Rec24 and Rec12/Rec14 interaction, together with the observed Y2H interaction of Rec15 with Mde2 let us propose the following tentative sub-complexes: Rec7/Rec24/Rec15/Mde2 and Rec12/Rec14/Rec6. The decreased, but not erased recombination level of Rec12Q308A and Rec12 R309A might suggest that another contact point between Rec12 and Rec14 exists, maybe through Rec6.

Assembly of the meiotic recombination initiation complex must happen in several, distinguishable steps. First, meiotic cohesins Rec8 and Rec11 load on chromosomes. Next, proteins of the LinEs (Rec10, Rec25, and Rec27) accumulate in a cohesin-dependent manner. As suggested by Lorenz et al. (2006), the LinEs might then serve as a loading platform for proteins or sub-complexes that are more directly involved in the actual DSB formation process. Their results indicate that Rec7, and probably the other members of its sub-complex Rec24, Rec15, and Mde2, locate in a Rec10-dependent way on chromatin. Our results with the thiamine-repressible promotor also suggest that the amount of Rec7 is crucial for the initiation process. Rec7 loading is not dependent on Rec12, which must mean that Rec12 and its probable sub-complex is loaded afterwards.

It remains to be investigated which factors influence the interplay between these sub-complexes. Further experiments are needed to elucidate the true composition and functional relevance of the proposed sub-complexes in the time frame of meiosis.

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