The *Saccharomyces cerevisiae* gene ECM11 is a positive effector of meiosis

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Abstract

Ecm11 is classified as a protein involved in yeast cell wall biogenesis and organization, but in this paper, we provide evidence that it is involved in meiosis as well. Mutants with deleted *ECM11* exhibit complex defects in meiosis: replication, recombination and chromosome segregation are affected. The *ecm11Δ* diploid strains sporulate more slowly and less efficiently than parental strains with wild type copies of *ECM11*. Fluorescence activated cell sorter scans of DNA content during sporulation showed that meiotic DNA synthesis is initiated at the same time in parental and *ecm11Δ* strains, but is less efficient in the knockout strain. By recombination tests, we demonstrated that *ECM11* is required for crossing-over, but not for gene conversion. In the absence of *ECM11* gene product, viability of spores is reduced to 50% and predominantly two viable spores per tetrad are formed. Our results suggest that *ECM11* is required in early stages of meiosis where its function is related to DNA replication and crossing-over.

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1. Introduction

*ECM11* corresponds to the open reading frame YDR446W. The predicted *ECM11* gene product has 302 amino acids and shows no significant similarity to any other protein in the database. In the large-scale screen of gene inactivation by transposon mutagenesis, identifying Calcofluor white sensitive mutants, *ECM11* was found among many other genes [1]. Accordingly, Ecm11 is classified as a protein involved in yeast cell wall biogenesis and organization. Calcofluor white binds to chitin and glucan and interferes directly with the supramolecular organisation of the cell wall [2]. Cells with inactivated *ECM11* are also hypersensitive to the lytic enzyme zymolase, indicating defects in the cell wall structure. Additionally, they are hypersensitive to inhibitor of β-1,3-glucan synthetase, Papulacandin B, and to antibiotic Hygromycin B, indicating defects in glucan biosynthesis [1]. Although many of the genes identified by this screen have their main role in the cell wall biogenesis, some genes coding for proteins whose main function is involved in DNA metabolism were also identified, e.g. DNA repair protein Mre11, involved in meiotic recombination [3], ubiquitin like protein Rad23, involved in DNA damage recognition and repair [4], DNA damage check-point protein Mec3 [5] and tran-
scription factor Swi6, responsible for cell cycle dependent gene activation and initiation of meiosis [6].

Results from our previous work suggest that Ecm11 is a nuclear protein [7]. We also detected two-hybrid interaction between Ecm11 and Cdc6, essential protein involved in the initiation of DNA replication [8]. We postulated that ECM11 gene has additional, not yet known function in the yeast cell.

From the micro-array hybridisation data, available at the Stanford Genome Database (SGD), it is apparent that ECM11 transcript does not oscillate during the cell cycle. Yet, transcription of the ECM11 gene is elevated significantly during meiosis, showing its possible role in this process. Several genes are involved in highly coordinated process of meiosis. It was shown that transcription of about 1000 genes is altered during meiosis; half of them are up-regulated and the rest are down-regulated. The majority of meiotic genes are also involved in vegetative growth, while some of them are meiosis specific or have a different function in meiosis than in mitosis. From the micro-array hybridisation data, available at the Stanford Genome Database (SGD), it is apparent that transcription of the ECM11 gene is elevated significantly during meiosis. Its transcript level relative to the control was elevated for 7.7-fold after 2 h, 16.7-fold after 5 and 7 h, 20.0-fold after 9 h and 11.1-fold after 11 h of cultivation in sporulation media (http://db.yeastgenome.org/cgi-bin/SGD/expression/expressionConnection.pl).

Obviously, ECM11 showed an early to middle pattern of induction in meiosis, as do about 95 other yeast genes. They are initially induced in the first 2 h after transfer to the sporulation medium and still have high transcript levels at 5 or 7 h after induction. There are three groups of meiotic genes with almost identical expression patterns as ECM11: the meiotic recombination genes such as MND1 and REC114 [9,10], the synaptonemal complex genes such as RED1, MSH4 [11,12] and the meiotic specific cohesion gene REC8 [13]. Using those data, we hypothesized that ECM11 has a role in early stages of meiosis.

Meiosis is a complex developmental program in which diploid cells reduce their chromosome number by half providing the offspring cells to get one copy of each chromosome. After receiving a signal from the environment, yeast cells reorganise the transcriptional program of genes and duplicate their DNA. Meiotic replication program is generally quite similar to that of vegetative cell cycle, but meiotic S phase is universally several times longer than mitotic in the same organisms. It has been proposed that this difference is related to the need for laying down of specialized chromosome features utilized at later stages for interhomolog interactions. Chromosome pairing and meiotic recombination are essential for proper chromosome segregation in meiosis I. The pairing of homologous chromosomes is mediated by a synaptonemal complex protein network. Crossover and non-crossover, two types of recombination, are generated between homologs during meiotic prophase. But only crossovers are associated with reciprocal exchanges of chromosome arms and are crucial for the accurate segregation of homologs to opposite poles. Meiotic recombination is initiated by double-strand breaks. The ends of the double-strand breaks are resected on their 5’-strands to produce 3’-single-strand tails that interact with a homologous chromosome to produce a single-end invasion and then double-Holliday junctions. The double-Holliday junctions are resolved by structure-specific endonucleases [14]. After chromosome pairing and recombination, two successive segmentations and spore wall formation occur. Finally, yeast cells end up with four haploid spores per ascus. The central, unique features of meiosis are homologue pairing, interhomologue recombination and synaptonemal complex formation. These regulated events are expected to use meiosis-specific regulators and there we suppose to be the role of ECM11.

In this work, we demonstrate that Ecm11, previously identified as a protein involved in yeast cell wall biogenesis, is indeed required in meiosis. We show that ecm11Δ phenotype includes diminished meiotic DNA synthesis, reduced crossing-over, increased gene conversion events, reduced sporulation efficiency and reduced spore viability.

2. Materials and methods

2.1. Strains

Saccharomyces cerevisiae strains used are listed in the Table 1. In haploid strains, the ECM11 locus was disrupted and strains were mated to result in diploid strains. Control strains were made by transformation diploid strains with YCp33-ECM11.

To generate YCp33-ECM11, ECM11 ORF with 500 bp promoter region and 230 bp terminal region was isolated by PCR amplification using primers PE1, 5’-CGG GGT ACC CTT AAA CGT TAG ACT GGC TGG GACA-3’, and PE2, 5’-CCC GAA TTC TTC CGC TCA AAG AGA CAG ACG TTA-3’. The PCR product was digested with Kpn1 and EcoRI and the resulting 1.6 kb product was inserted into YCplac33 (YCplac33 was a gift from RD Gietz) [15].

Cloning experiments were performed with the E. coli strain DH5α, using standard media [16].

2.2. Media and growth conditions

For vegetative growth, cells were grown in standard YPD medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) at 30 °C and 160 rpm. To induce meiosis,
cells were grown to $10^7$ cells/ml, washed and transferred to the half volume of SPM (0.3% potassium acetate, 0.02% raffinose); cells were shaken at $30 \degree C$ and 180 rpm [17]. Alternatively cells were grown to $3 \times 10^7$ cells/ml in YPA (1% yeast extract, 2% Bacto-Peptone, 2% potassium acetate), washed and transferred to the half volume of SM (0.82% sodium acetate, 0.19% potassium chloride, 0.035% magnesium sulphate, 0.12% sodium chloride); cells were shaken at $30 \degree C$ and 180 rpm [18].

2.3. Gene disruption

ECM11 gene was deleted and replaced by kanMX4 gene as described by Wach et al. [19]. The deletion strains were generated by transforming the yeast strain with linear PCR fragments containing kanMX4 gene flanked by terminal sequences homologous to ECM11 gene. Replacement of the genes was verified by PCR analysis using specific oligonucleotides (sequences of oligonucleotides are available upon request).

2.4. DAPI staining of spores in asci

Samples were collected at specified time points after cells were transferred to the sporulation medium and fixed in 70% ethanol. After ethanol was removed by centrifugation, cells and asci were re-suspended in distilled water and stained for 15 min with DAPI (1 $\mu$g/ml). Cells were washed with water and visualized using Carl Zeiss Axiovert 135 microscope (Germany) with DAPI filter (Chroma, Sweden).

2.5. FACS analysis

One-millilitre samples of meiotic cultures were withdrawn every hour. Cells were fixed in 70% ethanol, washed with water, treated with RNAase A and protease as described [20] and stained with propidium iodide (10 $\mu$g/ml). Fifteen thousand cells of each sample were analysed by Becton–Dickinson FACS analyser.

2.6. Return-to-growth experiment

Meiotic gene conversion was measured by return-to-growth experiment as described [21] using isogenic wild type (yC) and ecm11D (yCecm11-) strains. At specified time points cells were withdrawn from sporulation medium and plated onto YPD and synthetic media lacking the appropriate amino acid (LEU). The frequency of Leu+ recombinants was calculated as the ratio of Leu+ colonies to the total number of colonies on YPD, at each time point.

2.7. Spore dissection

Spores were dissected using a Narishige micromanipulator with Carl Singers’ dissection needles, following standard protocols [22].
3. Results

3.1. The ecm11Δ strains exhibit reduced sporulation efficiency

To determine the influence of Ecm11 on progression of meiotic process, ecm11Δ strains were constructed. The ECM11 gene was deleted in various haploid strains and, by the crossbreeding, homozygous and heterozygous strains were constructed.

By monitoring the sporulation process of ecm11Δ strains, we observed a delay of sporulation and decrease in the percentage of asci obtained, compared to the isogenic wild type strain. In the homozygous ecm11Δ strains about 20% less cells formed ascis than in the wild types. Analysis of sporulation of heterozygous strains yielded slightly higher sporulation efficiency than in homozygous ecm11Δ strains. Almost the same sporulation efficiency as in heterozygous strains was observed in ecm11Δ strains carrying a copy of ECM11 gene on the centromeric plasmid. Heterozygous strains with additional copy of ECM11 on the plasmid yielded nearly the same sporulation efficiency as the isogenic wild type. These results show that the reduced sporulation efficiency is a consequence of ecm11Δ mutation. Several strains and sporulation media were tested and similar results were obtained in all cases (Table 2).

Surprisingly, wild type strains carrying an extra copy of ECM11 gene on the centromeric plasmid also showed reduced sporulation efficiency comparing to the wild types (Table 2), indicating sensitivity of the cells to the copy number of the gene.

Following the kinetics of sporulation, we found that the first spores appeared about 4 h later in ecm11Δ strain than in the wild type. Both strains completed sporulation at the same time, but ecm11Δ strain had a reduced percentage of asci.

Since deletion of ECM11 resulted in reduced meiotic process progression with reduced sporulation efficiency, Ecm11 is defined as a positive effector of meiosis.

3.2. Deletion of ECM11 affects meiotic replication

To determine if deletion of ECM11 influences meiotic replication, DNA content of yeast cells was analysed by flow cytometry. After 2 h of incubation in the sporulation medium, meiotic DNA synthesis was initiated in both strains simultaneously. However, the number of cells replicating DNA was significantly lower in the mutated strain. The replication process was the most intense between the second and the fourth hour of sporulation. In this period, the percentage of 4N cells in the wild type cell population increased for 36%, while in the mutant cells this increase was only 18%. After this period, both strains showed similar kinetics. The ecm11Δ mutation had no effect on the time at which cells enter S phase, occurring about 2 h after transfer to the sporulation medium. The ecm11Δ mutation also did not influence the length of S phase, but the fraction of cells entering S phase was reduced in comparison to the wild type (Fig. 1). However, in the heterozygous strain and in the strain with a functional copy of ECM11 gene on the plasmid, DNA replication is indistinguishable from the wild type strain.

Table 2
Sporulation efficiency (% asci) of strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wild type</th>
<th>ecm11Δ</th>
<th>Heterozygous</th>
<th>Wild type + YCp33-ECM11</th>
<th>ecm11Δ + YCp33-ECM11</th>
<th>Heterozygous + YCp33-ECM11</th>
</tr>
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<tbody>
<tr>
<td>MAS</td>
<td>96 ± 1</td>
<td>73 ± 5</td>
<td>85 ± 5</td>
<td>80 ± 6</td>
<td>79 ± 4</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>CG</td>
<td>85 ± 5</td>
<td>65 ± 6</td>
<td>74 ± 5</td>
<td>71 ± 6</td>
<td>73 ± 6</td>
<td>81 ± 7</td>
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<tr>
<td>yC</td>
<td>58 ± 3</td>
<td>35 ± 4</td>
<td>55 ± 3</td>
<td>52 ± 5</td>
<td>53 ± 5</td>
<td>56 ± 6</td>
</tr>
</tbody>
</table>

Fig. 1. Analysis of DNA content during sporulation. (a) Progression of DNA replication was monitored by FACS analysis of samples collected at specified time points during meiotic time courses. Cells progress from unreplicated state (2C) to a state in which bulk DNA replication is complete (4C). (b) The fractions of cells with 4C DNA content, plotted as a function of time, were evaluated from the corresponding FACS profiles. Time courses were conducted three times and average values were calculated.
In all strains tested (MAS, CG, yC), ecm11Δ mutants showed the same generation time as wild type strains during vegetative growth in rich media.

3.3. ECM11 gene affects spore viability

Asci of the parental MAS and homozygous ecm11Δ strains were dissected and compared. As ecm11 cells are hypersensitive to the lytic enzyme zymolase, the ecm11Δ ascis are hypersensitive as well. Ascis were prepared for dissection by degrading 10 µl ascis suspension in $1 \times 10^{-2}$ U lyticase and 17 min incubation for the wild type MAS strain, but only in $5 \times 10^{-3}$ U lyticase and 5 min incubation for the MASecm11Δ strain.

In the parental strain, 98% of spores germinated after dissection, while only 51% spores of the ecm11Δ strain survived. The majority of ecm11Δ ascis (56%) produced only two viable spores, while only 1% of such ascis were observed in the parental strain. The quantity of ascis with three viable spores was similar in both strains (Fig. 2).

Thus, our results show that ECM11 is required for efficient segregation of chromosomes in meiosis I.

As 56% of MASecm11Δ ascis contained two viable spores, they were stained with DAPI and the presence of DNA was determined. Over a 104 sporated ascis were inspected and all of them had DNA in all four nuclei.

3.4. The ecm11Δ mutation reduces the frequency of meiotic crossing-over, but not the frequency of gene conversion

Since the majority of ecm11Δ ascis produced only two viable spores, we wander the property of those spores. MAS strains have non-functional ura3 gene on its original location on both chromosomes V, but they carry functional UR3 gene in CYC1 locus on one of the two chromosomes X. Dissected ascis of MASecm11Δ strain with two viable spores were replica plated on URA drop-out plates and analyzed. In 84% of ascis both spores were either URAΔ or uraΔ and only in 16% of ascis both viable spores have different URA phenotype. Since CYC1 is about 35 cM from the centromere [23], we assumed that crossing-over in ecm11Δ strain is significantly reduced.

To determine whether gene conversion is affected in ecm11Δ mutant cells, the return-to-growth experiment was done. Strains yC and yCecm11Δ carry two different LEU1 alleles that upon intragenic recombination give rise to the LEU1 prototrophs. As shown in Fig. 3, there was consistent difference between wild type and ecm11Δ cells in the kinetics of meiotic recombination. The ecm1Δ cells showed increased gene conversion events. Considering that ecm1Δ cells initiated meiotic DNA replication at lower extent than the wild type cells, the difference is even bigger. The heterozygous strain and the strain with an additional copy of ECM11 gene on the plasmid expressed almost the same frequency of gene conversion as the wild type strain.

4. Discussion

4.1. The ecm11Δ mutation affects sporulation efficiency

In the homozygous ecm11Δ strains we observed a delay of sporulation process and a decrease in the percentage of ascis, compared to the isogenic wild type strains. Heterozygous strains yielded slightly higher sporulation efficiency than ecm11Δ strains. Wild type strains carrying ECM11 on the centromeric plasmid also showed reduced sporulation efficiency in comparison to the isogenic wild types. Obviously, sporulation efficiency depended on the copy number of ECM11 gene in the cell during meiosis. Since sporulation process depended on exact copy number of the ECM11 in the cell, it is most probably that its gene product participates in a larger complex determining the proper assembly of its components.

On the other hand, the heterozygous strains and the wild type strains with additional copy of ECM11 on the plasmid, replicated DNA and expressed gene conversion as wild type strains. In addition, despite significant delay of spore appearance in the homozygous ecm11Δ strains, we did not observe a delay in meiotic
DNA replication or gene conversion processes in those strains. Obviously, ECM11 gene affects replication- and gene conversion-related processes, but get additional role in the downstream meiotic processes, which require highly coordinated ECM11 gene regulation.

4.2. ECM11 is required for meiotic DNA replication

To determine if deletion of ECM11 influences meiotic replication, the DNA content of yeast cells was analysed by flow cytometry. FACS analysis was done in MAS strains (isogenic derivative of SK-1), but it was recapitulated in CG strains because of the clumping property of MAS strain.

FACS analysis showed that the number of ecm11Δ cells entering S phase was reduced in comparison to the wild type. The ecm11Δ cells entered S phase at the same time as wild type cells. The ecm11Δ mutation also did not influence the length of S phase, but the fraction of cells entering S phase was reduced.

Since in all strains tested, deletion of the ECM11 gene had no impact on generation time during vegetative growth in rich media, we assumed that the effect of the ecm11Δ mutation on S phase is limited to meiosis. Switching meiotic DNA replication was similar to mitotic DNA replication although there were some important differences. Recent results suggest that meiotic cells tolerate lower activity of certain replication initiation proteins than mitotic cells [24]. It is obvious that specific proteins are present in meiosis, which are able to partially suppress mutant phenotype of some replication initiation proteins.

The hindrance of DNA replication caused by deletion of the ECM11 gene may be due to many reasons: the lower efficiency of origin firing, the lower progression of DNA replication or the consequence of modifications in some other meiotic process. Based on our FACS data we cannot exclude any of these possibilities.

We found in two-hybrid screen, that Ecm11 interacts strongly with essential protein Cdc6 having a pivotal role in the initiation of DNA replication. We also observed genetic interactions between Cdc6 and Ecm11. Moderate suppression of cdc6-1 mutation by over-expression of ECM11 was detected [8] and deletion of ECM11 in cdc6-1 genetic background enhanced thermo-sensitivity of cdc6-1 mutation (unpublished result). Beside that, ECM11 showed synthetic interaction with CDC8, a gene coding thymidylate kinase that is required for DNA synthesis [25]. Those data suggest direct involvement of ECM11 in DNA replication.

4.3. ECM11 is required for crossing-over

In the present investigation, we found that spore viability of the ecm11Δ mutants was reduced to 50%. We also observed that the majority of ecm11Δ asci produced two viable spores, confine the effect of Ecm11 in fidelity of chromosome segregation to meiosis I. Because of meiosis I non-disjunction of homologous chromosomes, the two survival spores carry chromosomes that result from the sister chromatides of original diploid. As meiotic map distance between CEN and CYC1 is approximately 35 cm, the expected recombination would result in at least 50% of the sister chromatides containing different markers [23,26]. As there were only 16% of asci with both viable spores having different URA phenotype, we assumed that crossing-over in ecm11Δ strain is significantly reduced.

Surprisingly, the results of return-to-growth experiment showed slightly increased gene conversion events in ecm11 cells. Similar outcome was observed in mer3Δ strain and two possible explanations were proposed: the mutation impaired the crossover control that was imposed at an early step of recombination, before the differentiation of intermediates into crossovers or non-crossovers, or there was a default pathway in the mutant that gives non-crossovers only [27].

Ecm11 belongs to the specific set of proteins that are required for crossing-over but not gene conversion, as genes required for synaptonemal complex formation (ZIP1, ZIP2, ZIP3), mutS homologues MSH4, MSH5, some mismatch repair genes (MLH1, MLH3, EXO1) and meiosis specific DNA helicase MER3. Mutants of some of those genes (msh5, mer3, zip1, zip2, zip3) exhibit normal level of double-strand breaks and non-crossover products but strong coordinate defects in single-end invasions, double Holliday junctions and crossover products implying that those mutants are specifically defective in converting double-strand breaks to single-end invasions [28].

As proper segregation of homologous chromosomes at the reductional division of meiosis does not require gene conversion, but requires crossing-over to establish chiasmata, which physically connect homologues after disassembly of the synaptonemal complex [29], chromosome segregation defects in ecm11Δ cells could be due to reduced crossing-over in those cells.

Summarising our data, we can conclude that the absence of the functional ECM11 gene product affects meiotic DNA synthesis and crossing-over resulting in abnormal chromosome segregation and poor spore viability. Taken together, our results show that ECM11 is a positive effector of meiosis.

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