A surprising role for the Sch9 protein kinase in chromosome segregation in *Candida albicans*

Neha Varshney*,†, Alida Schaekel*,†,§, Rima Singha*, Tanmoy Chakraborty*,†, Lasse van Wijlick§,†, Joachim F. Ernst§,†,‡† and Kaustuv Sanyal*,††

* Molecular Mycology Laboratory, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064, India, † Department Biologie, Molekulare Mykologie and § Manchot Graduate School Molecules of Infection, Heinrich-Heine-Universität, Düsseldorf, Germany

† These authors contributed equally to this work

‡ Present address: Department of Microbiology, University of Szeged, Középfasor, Szeged, Hungary 672


**Running title:** Role of Sch9 in chromosome segregation

**Keywords:** kinase, Sch9, centromere, chromosome segregation, kinetochore

†† Corresponding authors:

Joachim F. Ernst
Department Biologie, Molekulare Mykologie;
Heinrich-Heine-Universität Düsseldorf;
40225 Düsseldorf, Germany
Tel/Fax: +49 (211) 811-5176
e-mail: joachim.ernst@uni-duesseldorf.de

Kaustuv Sanyal
Molecular Mycology Laboratory,
Molecular Biology and Genetics Unit,
Jawaharlal Nehru Centre for Advanced Scientific Research,
Jakkur, Bangalore 560064,
India
Tel : + 91 (80) 2208 2878
Fax: + 91 (80) 2208 2766
e-mail: sanyal@jncasr.ac.in

Copyright 2015.
ABSTRACT The AGC kinase Sch9 regulates filamentation in Candida albicans. Here, we show that Sch9 binding is most enriched at the centromeres in C. albicans, but not in Saccharomyces cerevisiae. Deletion of CaSch9 leads to a 150-750-fold increase in chromosome loss. Thus, we report a previously unknown role of Sch9 in chromosome segregation.

Target of rapamycin complex 1 (TORC1) is a major regulator of cell growth and nutrient sensor in all eukaryotic cells. In a pathogenic yeast C. albicans, the AGC kinase Sch9, one of the direct downstream targets of TORC1, represses filamentation in hypoxia and high CO₂ conditions. Sch9 performs distinct functions in growth and morphogenesis depending on the availability of O₂ and CO₂ (STICHTERNOTH et al. 2011). Earlier studies indicated that absence of Sch9 increases chronological life span of S.cerevisiae (FABRIZIO et al. 2001). However, sch9 mutant cells of C. albicans have a reduced longevity only in normoxic conditions but not in hypoxic condition (STICHTERNOTH et al. 2011).

In this study, we sought to determine the genomic binding sites of the HA-tagged Sch9 protein by ChIP on chip (ChIP-chip) experiments under normoxia as well as hypoxia with and without elevated CO₂ levels in C. albicans. Remarkably, the major binding peaks of Sch9 coincided with the centromere (CEN) regions. Centromeric Sch9 binding was observed under normoxia (Figure 1A) as well as under hypoxia with or without 6% CO₂ (Figure 1B). Under all conditions, a few reproducible Sch9 binding peaks occurred outside the CEN regions as well (not shown). The ChIP-chip data was validated by semi-quantitative (data not shown) and quantitative PCR analysis using CEN5- and CEN7-specific primers (Figure 1C).

Enrichment of Sch9 binding at CEN regions led us to examine its possible role in the stability of the kinetochore, a multiprotein complex forms on the CEN DNA. The centromere-kinetochore complex plays a central role in the microtubule-kinetochore mediated process of chromosome segregation. First, we analysed the nuclear morphology in wild-type (CAI4) and mutant cells (CAS1 and CCS3) (strain construction, Southern confirmation and genotype of strains are described in the Supporting Text,
Figure S1 and Table S1 respectively). Except for a marginal increase in proportion of large-budded cells (at G2/M stage) with unsegregated nucleus in the \textit{sch9} mutant cells (CAS1 and CCS3) as compared to the wild-type (CAI4), no significant difference was evident (Figure S2). A marginal increase observed in the proportion of large-budded mutant cells having unsegregated nuclear mass as compared to wild-type is insignificant, since the wild-type cells also showed unsegregated DNA mass, as expected, during pre-anaphase stage of the cell cycle. Moreover, a significant delay in G1 in \textit{sch9} mutant added to the complexity of analysis. Like \textit{S. cerevisiae}, centromeres are clustered throughout the cell cycle in \textit{C. albicans} (ROY et al. 2011; SANYAL and CARBON 2002; THAKUR and SANYAL 2012). Depletion of an essential kinetochore protein leads to centromere declustering and delocalization of the centromere-specific histone Cse4 in \textit{C. albicans} (THAKUR and SANYAL 2012). However, we neither found centromere declustering nor any significant change in the centromeric histone Cse4 levels at the kinetochore (Cse4-GFP intensity) in wild-type (strain 8675) and \textit{sch9} mutant (strain 8675T) strains (Figure 2A). To further investigate the role of Sch9 in Cse4 localization at the centromeres, we performed Cse4-ChIP assays with wild-type (J200) and mutant cells (J200T). We analyzed enrichment of Cse4 at \textit{CEN5} and \textit{CEN7} regions both by semi-quantitative (Figure S3) and qPCR (Figure 2B) (Primer sequences are listed in Table S2). Cse4 binding was found to be similar at the centromeres in presence or absence of Sch9. Thus, Sch9 does not seem to play a direct role in Cse4-mediated kinetochore integrity in \textit{C. albicans}.

Many kinetochore proteins play crucial but non-essential roles in the process of chromosome segregation (GHOSH et al. 2001; MEASDAY et al. 2002; ORTIZ et al. 1999; PODDAR et al. 1999; SANYAL et al. 1998). The centromeric localization of Sch9 prompted us to examine whether Sch9 plays a role in high fidelity chromosome segregation. One or both the alleles of \textit{SCH9} were deleted from the diploid genome of the \textit{C. albicans} wild-type strain RM1000AH which was previously used to study chromosome loss (SANYAL et al. 2004). Each homolog of chromosome 7 is marked by an auxotrophic marker \textit{HIS1} or \textit{ARG4} in RM1000AH (Figure 3A). The strains were confirmed by
Southern blot analysis (Figure S1). We previously reported that the natural rate of loss of a chromosome in wild-type *C. albicans* (SN148) is $<5 \times 10^{-4}$/cell/generation (MITRA et al. 2014). Two independent null (*sch9/sch9*) mutant strains (RMKS2A and RMKS2B) exhibited 150-750-fold increase in chromosome loss as compared to the spontaneous rate of loss of a chromosome (Figure 3B). Even heterozygous (*SCH9/sch9*) mutants of *SCH9* (RMKS1A and RMKS1B) exhibited chromosome loss at a rate higher than the wild-type (Figure 3B). This high rate of chromosome loss is comparable to the loss rate exhibited by several *S. cerevisiae* kinetochore mutants. The role of Sch9 in chromosome segregation was further validated by re-integrating the *SCH9* ORF including its native promoter and terminator sequences at the *RPS10* locus. While the chromosome loss was completely suppressed in re-integrants (RMKS1AR and RMKS1BR) generated in *SCH9/sch9* mutant background (RMKS1A and RMKS1B), a reduced rate of loss was observed in re-integrants (RMKS2AR and RMKS2BR) in *sch9/sch9* null mutant background (RMKS2A and RMKS2B) (Figure 3C). While this assay measures the loss of heterozygosity, the loss of an unlinked single nucleotide polymorphism (SNP) on chromosome 7 along with the loss of a marker gene on the same chromosome could determine the loss of the entire chromosome. We observed that SNPs on the chromosome 7 in the strains used in this study are absent, as compared to another *C. albicans* strain reported previously (FORCHE et al. 2009). However, binding of Sch9 to all centromeres combined with a higher rate of loss of the marker gene by the homozygous and heterozygous mutants led us to conclude that absence of Sch9 indeed increases the rate of chromosome loss. This confirms the critical role of Sch9 in the high fidelity process of chromosome segregation.

To verify, if centromere DNA binding of the Sch9 kinase also occurs in other yeast species outside the *Candida*-specific CTG clade, we carried out a genome-wide ChIP-chip experiment to localize HA-tagged ScSch9 (PASCUAL-AHUJR and PROFT 2007) in *S. cerevisiae*. No detectable binding of ScSch9 to any centromere region was found. However, as in *C. albicans*, the rDNA locus showed significant ScSch9 binding in *S. cerevisiae* (data not shown). We conclude that Sch9
binding to centromeres is not a general feature among hemiascomycetes fungi and may have arisen specifically in *C. albicans*, a member of the CTG clade, while binding to rDNA remained conserved. It would be tempting to speculate that the association of Sch9 with centromeres might have arisen specifically in the CTG clade. Nevertheless, Sch9 is important for growth in both *S. cerevisiae* and *C. albicans* (Pascual-Ahuir and Proft 2007; Stichternoth et al. 2011).

Rapid fungal growth requires effective biosynthetic, metabolic and regulatory activities of cells. Nutrient abundance is signaled by the Tor1 pathway via the Sch9 AGC kinase. Thus, the remarkable strong binding of Sch9 to centromeres could be related to effective chromosomal replication. Incidentally, deletion of a centromere-proximal replication origin leads to a moderate increase in chromosome loss (Mitra et al. 2014). In addition, phospho-regulation of kinetochore proteins by kinases (such as Aurora B kinase / polo-like kinase 1) has been shown to be critical for proper chromosome segregation (McKinley and Cheeseman 2014; Shang et al. 2003). Unlike short 125 bp genetically determined sequence-specific point centromeres of *S. cerevisiae*, *C. albicans* chromosomes contain unique sequence-independent epigenetically specified regional centromeres (Baum et al. 2006; Sanyal et al. 2004; Thakur and Sanyal 2013). While the targets of this AGC kinase Sch9 are largely unknown, centromere binding of this protein selectively in *C. albicans* but not in *S. cerevisiae* provides new insights of functional evolution of a protein in organisms having different types of centromeres.

**ACKNOWLEDGEMENTS**

This work was supported by a grant from Department of Biotechnology, Govt. of India and intramural funding by JNCASR to KS. We gratefully acknowledge the support by grants of the Jürgen Manchot Stiftung Düsseldorf (AS, LvW) and the ERA-NET PathoGenoMics project OXYstress (JFE). NV and RS are supported by fellowships from CSIR and UGC (Govt. of India), respectively.
**FIGURES**

**Figure 1**

**Figure 1** Genomic localization of the Sch9 kinase. The ChIP-chip procedure was carried out essentially as described previously (Lassak et al. 2011; Schaeckel et al. 2013). *C. albicans* genomic tiling microarrays (NimbleGen) were probed pair-wise by immune-precipitated chromatin of a strain expressing HA-tagged Sch9 (AF1006) and the corresponding control strain (CAS1). Two independent cultures were assayed for each combination of strains. (A) An overview of Sch9 binding. Significant binding peaks were calculated by the NimbleScan software (NimbleGen) and colour-coded according to their FDR values in red (FDR ≤ 0.05), orange (FDR ≤ 0.1), yellow (FDR 0.1-0.2) and grey (FDR > 0.2). Significant Sch9 binding peaks were detected at centromeres by genomic ChIP-chip on all *C. albicans* chromosomes. In addition, a significant peak occurred at the rDNA locus (open arrow). (B) Examples for centromeric binding of Sch9 at *CEN5* and *CENR*. Scaled log₂...
ratios of cells grown in normoxia and hypoxia with or without 6 % CO₂ are shown. Note that Sch9 enrichment was obtained for cells grown under normoxia or hypoxia. (C) Enrichment at CEN7, CEN5 and non-centromeric region was analyzed using quantitative PCR. qPCR analysis reveals the mean fold enrichment of Sch9 at the centromeres obtained in two independent ChIP experiments (+/- SD) relative to the no-tag control and normalized to the input samples. The calculation was done with two biological replicates (two ChIP samples) and each measurement was performed in triplicates. Significant difference was observed in Sch9 recruitment at the CEN5 and CEN7 region (p<0.05) and (p<0.01) respectively (shown by asterick).

**Figure 2**

*Figure 2* Sch9 is not required for Cse4-mediated kinetochore stability. (A) Microscopy images showing Cse4-GFP signal intensities in wild-type 8675 (CSE4-GFP/CSE4;SCH9/SCH9) and mutant 8675T (CSE4-GFP/CSE4;sch9/sch9). *C. albicans* wild-type and mutant strains where CSE4 is GFP-tagged were grown overnight at 30° in normoxic conditions in YPDU, washed with water and images were taken using a confocal laser scanning microscope (LSM 510 META, Carl Zeiss). The brightest GFP signal in each cell was determined using the Image J software as described before (ROY *et al.* 2011). Briefly, an equal area from each cell was selected. The average pixel intensity was measured and corrected for the background by subtracting the lowest pixel intensity value in the field from the average. Then the mean GFP intensity was measured using the Image J software and graph was plotted using Graph Pad Prism. Measurement was taken from 45 cells in each case. The experiment was performed twice. Standard error of mean (t test) was used to calculate statistical significance (p<0.05). For strain construction (See Supplementary text). (B) Cse4 localisation at the centromere is not affected by absence of Sch9. Standard ChIP assays were performed on strains CAKS102 (CSE4-TAP/CSE4;SCH9/SCH9) and J200T (CSE4-TAP/Cse4; sch9/sch9) (grown at 30° in normoxic conditions) using anti-Protein A antibodies. Enrichment at CEN7, CEN5 and non-centromeric region was analysed by quantitative PCR. PCR using total DNA (T) or ChIP DNA fractions with (+) or without (-) antibodies was performed. qPCR analysis reveals the enrichment of Cse4 at the centromere as a percentage of the total chromatin input and values
were plotted as mean of triplicates ± SD. No significant difference was observed in CaCse4 recruitment at the CEN5 and CEN7 region (p>0.05). Percent Input was calculated as 100*2^(adjusted input-Ct(IP)) (MUKHOPADHYAY et al. 2008).

**Figure 3**

![Figure 3](image)

**Figure 3** Chromosome loss assay. (A) Schematic of chromosome loss assay. (B and C) The chromosome loss assay was performed with two independent transformants of both mutants and revertants, as described before (SANYAL et al. 2004). The numbers indicate the summation of colonies patched in independent experiments. Briefly, the strains were grown approximately for 20 generations on YPDU medium at 30° in normoxic conditions. Subsequently, approximately 1000 cells
were plated on YPDU agar plates for each transformant and incubated at 30° for 2 days. The single colonies were patched on SD minimal medium (SD) without arginine (CM-arg), SD without histidine (CM-his) and YPDU. The chromosome loss rate was calculated by the number of colonies that were unable to grow on selective media divided by the total number of colonies grown on non-selective media.

REFERENCES


ORTIZ, J., O. STEMMANN, S. RANK and J. LECHNER, 1999 A putative protein complex consisting of Ctf19, Mcm21, and Okp1 represents a missing link in the budding yeast kinetochore. Genes Dev 13: 1140-1155.


