Loss of Function of Sth1, The Catalytic Component of RSC (Remodel the Structure of Chromatin) Complex Grossly Alter the Chromatin Architecture

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Abstract. Chromatin architecture has a profound effect on the gene expression in eukaryotes. It is constantly modulated in the cells in response to different stress condition and during the normal physiological process in the cell. The chromatin is also modulated during the cell growth and division, where several proteins involved during the cell cycle are synthesized, and hence the gene expression profile and chromatin state of an actively dividing cell differ from that of a resting cell in G0 state. *Candida albicans*, which is a harmless commensal in human host and an opportunistic fungal pathogen also show dynamic chromatin architecture, and this is facilitated by the several epigenetic determinants, which modulate the chromatin architecture. In this context, RSC (Remodel the structure of chromatin) complex in *C. albicans* is previously shown to be crucial for cell viability and to carry out several DNA templated events, like kinetochore function and cohesion enrichment. To correlate the role of RSC in kinetochore function with the chromatin architecture at centromeric and non-centromeric region, here we have shown that the chromatin at non-*CEN7* regions shows lesser occupancy of nucleosomes in absence of Sth1 protein (catalytic component of RSC complex), which is due to the reduced binding but not due to the reduced expression of the histones.

Keywords: Chromatin · Nucleosomes · Candida albicans · RSC

1 Introduction

C. albicans infection is widespread across the globe. *C. albicans* exist in different morphogenic forms which provide an adaptive advantage to the organism to thrive in adverse conditions tolerating the stresses and hence evade the host immune attack. Thus, morphogenesis in *C. albicans* is closely linked to stress tolerance and pathogenesis. The switching between the different morphogenic forms and stress tolerance in *C. albicans* is controlled epigenetically where alteration of the chromatin organization with the help of various epigenetic factors play a pivotal role. Histone modifiers, histone variants and ATP dependent chromatin remodelers are the different epigenetic factors in *C. albicans*

V. Agarwal et al. (Eds.): BIOSANGAM 2022, ABSR 24, pp. 168–175, 2023.
https://doi.org/10.2991/978-94-6463-020-6_17

which have profound impact on the biology of *C. albicans*. Among these, the different chromatin remodeler have important roles in the biology of the fungi. SWI/SNF complex (a member of the SWI/SNF family of chromatin remodeler) has crucial role in the morphogenesis, and transcriptional control in the fungi. We have shown previously that RSC complex (another member of SWI/SNF family) is crucial for cell viability, kinetochore clustering, cohesion function, faithful chromosome segregation and cell cycle control by spindle assembly check point by the functional characterization of the catalytic component Sth1 of the RSC complex [1]. Recently through the transcriptomic and proteomic profiling, it was found that the genes targeted by the transcription factors involved in the cell wall damage response (Cas5, Sko1), and several stress responses (Cta8, Cwt1, Skn7, Sko1) are up regulated in cells depleted of Sth1 [2], suggesting RSC function with these TFs in the regulation of gene expression. We have also reported that the global chromatin architecture is altered in cells depleted of Sth1 [1]. To further explore the chromatin architecture at the centromeric and non-centromeric region, and correlating with the observed role in kinetochore clustering, cohesion function and hence faithful chromosome segregation [1], we analysed the centromeric (*CEN*) and non-centromeric (non-*CEN*) regions of the chromatin. We have found a gross reduction in the level of nucleosomes near *CEN7* and *non-CEN7* regions. Further we found that the histone 3 enrichment was significantly decreased at the non-*CEN7* regions, but not *CEN7*, though there was no change in the level of protein expressed.

2 Material and Methods

2.1 Strains, Media and Growth Condition

Candida albicans strains used in this study are listed in the Table 1. *C. albicans* cells were grown in YPD media (1% Yeast extract, 2% peptone, 2% dextrose and 0.01% uridine) or SCD (Synthetic Complete Dextrose, 0.67% YNB and 2% dextrose). PCK1 promoter overexpresses the protein when the cells are grown in 2% succinate as carbon source. All *C. albicans* strains were grown at 30 °C unless otherwise stated.

2.2 Chromain Imunoprecipitation (ChIP)

Chromatin Immunoprecipitation Assay and qPCR was performed as described before with modifications [3]. Formaldehyde crosslinking of Hht21-V5 protein was done for 15 min at room temperature. Sth1 protein was depleted by following the growth regime as described. Overnight grown culture from permissive medium (YPSU) was transferred into repressive (YPDU) medium, grown for 12–14 h, repassaged into fresh repressive medium, grown for additional 4–5 h, till the final OD_{600} reaches ~1. Same growth regime was followed for the wild type cells. Chromatin was sheared by sonicating the spheroplasts for 21 s ON and 1 min OFF on ice for 13 rounds generating the DNA fragments in the range of 300 bp–500 bp. 5 μg/ml of Anti V5 (Sigma Cat. No. R96025) antibody was used for the pull down of crosslinked DNA. Protein-DNA were de-crosslinked at 65 °C for 14–16 h and DNA was purified using SureExtract PCR purification kit (Nucleo-pore, Genetix, India) as per the manufacturer's instructions and eluted in 50 μl

of elution buffer. qPCR was performed on the Bio-Rad CFX96 Real Time System with iTaq Universal Sybr Green Supermix (Bio-Rad, United States). Calculations for Enrichment/Input values were made as described elsewhere (Fernius and Marston, 2009) using the given equation: $\Delta Ct = Ct(ChIP)\text{-}\{Ct(Input) - \log E\ (Input\ dilution\ factor)\}$, where E is the value of specific primer efficiency; Enrichment/Input $= E^{\wedge -1Ct}$. Error bars were calculated from the standard deviation from two technical replicates from at least two independently grown cultures.

2.3 MNase Digestion of Chromatin and Southern Blotting

Around 4×10^9 cells were harvested and treated with 20T Zymolyase (MP Biomedicals) to make spheroplast. The spheroplasts were resuspended in MNase digestion buffer and equal units (0.375 units) of MNase enzyme (Sigma, Cat. No. 3755) were added. Digestion was carried out at 37 °C for 0, 10, 20, and 40 min and 0.25 mM EDTA supplemented with 5% SDS was added to stop the reaction. DNA was extracted, purified and quantified. 3 μg of purified DNA was separated by electrophoresis on 1.4% agarose gel and was stained using ethidium bromide (EtBr). The *sth1* mutant cells have pseudohyphal phenotype and are difficult to make spheroplast, so twice the volume of cell culture of the mutant than the wild type cells were taken (both grown for 12 h) to obtain equal amount of chromatin to start the MNase digestion reaction, as shown by the presence of equal amount of purified undigested DNA at 0 min time point for each sample (Undigested, UD in Fig. 1).

Southern blotting was performed as described previously [4].

2.4 Protein Extraction and Western Blot

Dry cell pellets were boiled for 10 min in water bath and were resuspended in 80 μl of ice-cold ESB buffer (1.5 mM TRIS-HCl, pH6.8, 2 M DTT, 20% SDS, 0.3% bromophenol blue and 50% glycerol), supplemented with 1X protease inhibitor cocktail (Roche, 11873580001). 100 μl of acid-washed glass beads (0.5 mm) were added to each tube. This mix was vortexed five times, with 1 min on vortexer and 1 min on ice. 50 μl of ESB buffer was again added, followed by 1 min vortexing. The lysate was cleared by centrifugation at 6,800 g for 30 s, supernatant collected, boiled in the presence of SDS loading dye, and subjected to electrophoresis on 8%–10% SDS PAGE. Protein samples were then transferred to nitrocellulose membrane (Pall Lifesciences, BSP0161), membrane blocking was done with 5% skimmed milk in TBST solution and subjected to epitope-specific primary antibodies (anti V5 (Sigma Cat. No. R96025) or anti-tubulin (YOL1/34, Abcam, Cat. No. ab64332)) and HRP-conjugated secondary antibodies. Development of blots was done using ECL substrate (Bio-Rad, 1705060).

3 Result

3.1 Loss of Sth1 Function Generates Possible Long Regions of Nucleosome Free Regions (NFR) in *C. Albicans*

As a function of remodeling activity, it has been demonstrated that RSC has the ability to alter nucleosome structure in vitro [5, 6]. In *S. cerevisiae* it has been shown that RSC is

recruited to the centromeric loci and it is believed that RSC mediated chromatin remodeling is crucial to establish a proper centromeric chromatin that supports kinetochore function and a global chromatin architecture that facilitates sister chromatid cohesion [7, 8]. In our previous study, we too observed that Sth1 could be targeted to the centromeres and loss of this protein can cause declustered centromeres and cohesin loading defect [1]. Concomitant to these, we have previously shown that the gross chromatin architecture is altered in the cells depleted of Sth1[1]. We further propose that the loss of Sth1 function generates possible long regions of chromatin devoid of any nucleosomes in these cells. This proposal arises by the reported findings in *S. cerevisiae*, where was reported that nucleosome depletion causes the alteration of centromeric chromatin architecture [9]. Since we have found RSC components localized at the centromere in *C. albicans* [1] similar to what has been observed in *S. cerevisiae* [6], we argue that the centromeric chromatin in *C. albicans* may be altered in the absence of Sth1 as shown for *sth1* in *S. cerevisiae* [5, 6]. The nucleosome phasing of Cse4 containing centromeric chromatin is different from that of the bulk of the chromatin in *C. albicans* [10]. This difference can be easily visualized as a relatively smeared DNA from the centromeric chromatin versus a distinct nucleosomal ladder from the bulk chromatin when the DNA from the MNase digested chromatin is probed using the DNA sequences from different regions of the chromosomes [10]. To investigate if the absence of Sth1 can cause any alteration in this pattern, we analyzed the MNase digested chromatin by Southern blot hybridization using the radiolabelled DNA probes from the centromere (*CEN7*) and the region flanking the centromere (pericentromere). In the wild type and in the cells overexpressing Sth1, we indeed observed a trailing smear (Fig. 1A, lanes 14–16 and 18–20, regions highlighted by the bracket) when the probe from the CEN7 was used while such a smear was absent when the probe from the non-centromeric region was used (Fig. 1A, lanes 2–4 and 6–8). Notably, chromatin from the Sth1 depleted cells also yielded a trailing smear when probed by *CEN* probe, and lacked such smear when probed by the non-*CEN* probe, but the length of such smear was greatly reduced (Fig. 1, comparing lane 14 or 18 vs. 22 and 15 or 19 vs. 23). Southern blots from both the centromeric and the non-centromeric regions of *sth1* cells showed similar profiles which were observed previously [1] in the corresponding EtBr stained gels depicting the global chromatin. These results suggest that there is an overall reduction in the level of nucleosomes in the *sth1* cells, at least at the probed regions, either because of a gross reduction in the association of nucleosomes with the DNA or because of a change in the relative positions of nucleosomes such that they are more sparsely distributed. Moreover, in the Sth1 depleted chromatin, the presence of fewer di-nucleosomes at the centromeric region (Fig. 1, lanes 22,23) compared to no di-nucleosomes at the non-centromeric regions (Fig. 1, lanes 10,11) suggests that the extent of depletion of nucleosomes at the non-centromeric region is perhaps more than that at the centromeric region in the mutant.

Centromeric (using CEN probe) and pericentromeric (using non-CEN probe) regions are probed using Southern blot hybridization. A trailing smear along with the nucleosome bands (mono, di, tri, etc.) are seen when CEN probe is used while only distinct bands of nucleosomes are seen when non-CEN probe is used. The length of the smear generated by the CEN probe in the sth1 is smaller than that in the wild type or over-expressed cells. Wild type: *STH1/STH1-TAP*, Overexpression: *sth1/PCK1-STH1-TAP* grown in YPSU

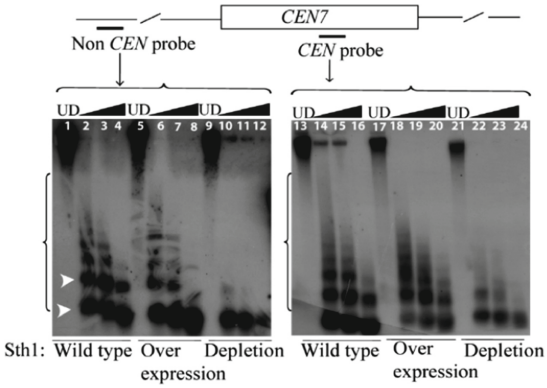

Fig. 1. Non-centromeric (non-CEN7) region of chromatin shows lesser occupancy of nucleosome

(Yeast extract, Peptone, Succinate, Uridine), Depletion: *sth1/PCK1-STH1-TAP* grown in YPDU (Yeast extract, Peptone, Dextrose, Uridine). UD: Undigested or 0 min. Wells 2,3,4; 6,7,8; 10,11,12; 14,15,16; 18,19,20; 22,23,24: 10, 20, 40 min of digestion with MNase enzyme.

3.2 Relative Enrichments of Histone H3 at Non-centromeric But Not Centromeric Regions are Reduced in Cells Depleted of Sth1

To address whether the association of nucleosomes with the DNA changes in the *sth1*, we examined the level of histone 3 (H3), coded by *HHT21* gene in *C. albicans*, on the chromatin. By Chromatin immunoprecipitation (ChIP) assay, we investigated the relative occupancy of Hht21 tagged with V5 at the centromeric and non-centromeric regions. We indeed observed that when Sth1 was depleted, the overall occupancy of Hht21 on the chromatin, but not its expression, was reduced by almost 1.5 fold at the non-centromeric regions (Fig. 2A, 2B). However, we failed to detect any such effect at the centromeric region perhaps because Cse4 replaces most of the H3 at this region and previously we did not detect any change in the enrichment of Cse4 at the centromere in the Sth1 depleted cells with respect to wild type cells [1]. From the above results, we conclude that in the absence of Sth1, the global chromatin architecture is grossly altered. This alteration may cause an overall open chromatin structure that may lead to centromere malfunction, improper recruitment of cohesins, DNA damage and loss of cell viability.

A. ChIP assay showing the association of Hht21 with the chromosomal loci in the wild type, the cells overexpressing Sth1 and depleted of Sth1. The statistical significance between the compared values are indicate by the P values for the two-tailed paired t-test.
B. Expression of Hht21 remains same with the altered level of expression of Sth1, as shown by immunoblot of Hht21-V5 using antibodies against V5.

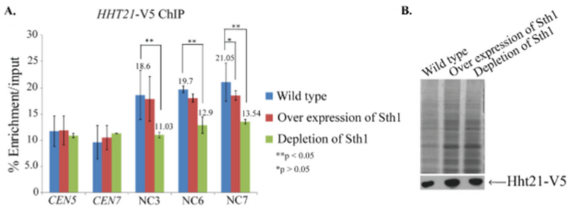

Fig. 2. *HHT21* binding to non-CEN7 regions but not its expression decreases when Sth1 is depleted in the cells

Table 1 .

S. No.	Strain name	Genotype	Reference
1.	SGC78	*STH1/STH1-TAP*	This study
2.	SGC79	*sth1/PCK1-STH1-TAP*	This study
3.	SGC339	*ura3::imm434/ura3::imm 434 iro1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2,HHT21-V5(URA3)/HHT21*	[13]
4.	SCG341	*ura3::imm434/ura3::imm 434 iro1/iro1::imm434 his1::hisG/his1::hisG leu2/leu2,HHT21-V5(URA3)/HHT21,sth1::FRT/PCK1pr(ARG4)-STH1*	This study

4 Discussion and Conclusion

From our results, it can be envisaged that RSC function is required for proper chromatin architecture that helps in chromatin driven events such as centromere function and cohesin recruitment as reported earlier [1]. For these processes, RSC is believed to maintain a dynamic chromatin state by moving, ejecting or restructuring the nucleosome using the energy of ATP hydrolysis. Therefore phasing of the nucleosome is expected to change in *rsc* mutant which has been shown in *S. cerevisiae* [5, 6]. To investigate such a change in rsc mutant in *C. albicans*, we challenged the chromatin from the *sth1* mutant and the wild type cells with MNase and found a global reduction in nucleosome occupancy across the chromatin as visualized by the disappearance of nucleosome ladder in the mutant [1]. Using locus specific probes, we found reduced nucleosome occupancy both at the centromere and non-centromere regions which was confirmed by ChIP assay by showing a decreased occupancy of Hht21 at the non-centromeric loci in the *sth1* mutants. These are the clear indications of an altered chromatin structure in *rsc* mutant in *C. albicans* which may lead to observed phenotypes in the mutant. It is to be noted that RSC is known for changing the position of the nucleosomes in such a way that NFR (Nucleosome Free Region) can be generated/maintained that can "open up" chromatin for certain gene promoters facilitating transcription [11, 12]. However, in contrast to this notion, from our results, it appears that in *C. albicans*, it is the loss of RSC function that can lead to an overall opening of the chromatin by decreasing the nucleosome density along the chromatin. This indicates a somewhat contrasting epigenetic regulation

of chromatin by RSC complex in *C. albicans* which open up new avenue for further investigation.

Acknowledgment. I am thankful to Prof. Kaustuv Sanyal, JNCASR, Bangalore for providing strain SGC339 using in this study. I am thankful to Prof. Santanu Kumar Ghosh, IITB for providing me the required laboratory space and other infrastructure facilities. This work was funded by BRNS (37(1)/14/30/2015/BRNS), DBT (BT/PR13909/BRB/10/1432/2015) Govt. of India and Research seed money (RSM), code 1129, NIT Warangal. PP was supported by DBT fellowship (DBT-JRF/2012-13/209), Govt. of India. at IITB.

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