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Original Paper

Unravelling the role of Set2 protein domains in H3K36 methylation in *Saccharomyces cerevisiae*

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Abstract

Histone methylation plays a crucial role in gene expression and chromatin structure regulation. In *Saccharomyces cerevisiae*, the Set2 protein is responsible for the methylation of histone H3 at lysine 36 (H3K36), which is associated with transcriptional regulation, RNA processing, and DNA repair. This study investigates the specific functions of individual domains within the yeast Set2 protein by utilizing PCR-based domain deletions and subsequent western blot analysis to assess their impact on H3K36 methylation status. The results demonstrate that the SET domain alone is sufficient for H3K36 dimethylation, while optimal trimethylation necessitates the presence of additional domains, including the central autoinhibitory domain. Furthermore, the SRI domain is found to be essential for both di- and trimethylation when considering the full-length Set2 protein. These findings underscore the critical role of Set2 domains in modulating Set2 enzymatic activity.

Key words: central autoinhibitory domain, chromatin, genome, histone, histone methylation, H3K36, H3K36me2, H3K36me3, Set2, SET domain, SRI domain, transcription.
Abbreviations: HB, histone binding domain; SRI, Set2-Rpb1 interaction domain; TCA, trichloracetic acid.

Introduction

In eukaryotic cells, DNA serves as the principal repository of genetic information and is hierarchically compacted in the form of chromatin within the nucleus. The fundamental unit of chromatin structure is the nucleosome, comprising an octameric histone core with two copies each of histones H2A, H2B, H3, and H4, around which approximately 146 – 147 base pairs of DNA are tightly wound (Luger et al. 1997; Davey et al. 2002). Chromatin, a nucleoprotein complex, presents a significant barrier to essential DNA-templated processes such as replication, transcription, and repair and thereby intricately regulates the eukaryotic genome (van Steensel 2011). To navigate through this barrier, cells employ a sophisticated regulatory network involving histone chaperones, ATP-dependent chromatin remodelers, and histone-modifying enzymes (Paranjape et al. 1994; Kingston et al. 1996; Tsukiyama, Wu 1997; Carey et al. 2006; Workman 2006; Kulaeva et al. 2009; Kulaeva et al. 2010; Bannister, Kouzarides 2011).

Histone chaperones mediate the proper assembly and disassembly of histone octamers around DNA (Avvakumov et al. 2011; Gurard-Levin et al. 2014; Hammond et al. 2017), while ATP-dependent remodelers reposition nucleosomes to modulate chromatin compaction (Flaus, Owen-Hughes 2004; Smith, Peterson 2005; Saha et al. 2006; Clapier, Cairns 2009). Histone-modifying enzymes introduce post-translational modifications, such as methylation, to regulate chromatin accessibility and gene expression (Kouzarides 2007; Ruthenburg et al. 2007). Among these modifications, trimethylation of lysine 36 on histone H3 (H3K36me3) is critical for transcriptional regulation, RNA processing, and DNA repair (Wagner, Carpenter 2012; Pfister et al. 2014; Lim et al. 2018; Lam et al. 2022; Lam, Chen 2022; Sharda, Humphrey 2022). Dysregulation of H3K36me3 is linked to various diseases, notably cancer (Xiao et al. 2021).

In yeast, Set2 is solely responsible for all methylation states of H3K36 (Lee, Shilatifard 2007). In contrast, in mammals, the homologous SETD2 enzyme specifically catalyzes the trimethylation of H3K36, with other enzymes handling di- and monomethylation (Edmunds et al. 2008; Yuan et al. 2009). Set2-mediated H3K36 methylation in yeast facilitates proper chromatin refolding post RNAPII passage by targeting histone deacetylation via the Rpd3S HDAC complex, preventing cryptic transcription initiation (Carrozza et al. 2005). The evolutionary conservation

of H3K36 methylation underscores its importance in DSB repair and genomic stability (Jha, Strahl 2014). In *Schizosaccharomyces pombe*, Set2 deletion disrupts the balance between NHEJ and HR (Pai et al. 2014), while in *Saccharomyces cerevisiae*, Set2 interacts with genes suppressing chromosomal rearrangements, indicating its role in maintaining chromosomal integrity (Putnam et al. 2012). H3K36 methylation is crucial for organismal development, influencing gene activation and regulation (Venkatesh, Workman 2013). It also regulates mRNA and ncRNA expression dynamics (Kim et al. 2016), and controls replication timing in *S. cerevisiae* (Pryde et al. 2009). Furthermore, H3K36 methylation by Set2 enhances transcriptional responses to genotoxic stress and supports effective DNA replication (Pai et al. 2017). During nutrient stress, H3K36 methylation ensures transcriptional fidelity by preventing cryptic transcription within gene bodies (McDaniel et al. 2017).

Set2's regulatory mechanisms are complex and interdependent, involving interactions with elongating RNAPII via Set2-Rpb1 interaction domain (SRI) and histone H4 through its histone binding domain (HB) (Krogan et al. 2003; Li et al. 2003; Xiao et al. 2003; Kizer et al. 2005; Du et al. 2008). Despite extensive research, the precise regulation of Set2 activity remains incompletely understood, necessitating further investigation. This study elucidated the roles of specific Set2 domains in mediating H3K36 methylation in *S. cerevisiae*, advancing the understanding of its regulatory mechanisms.

Materials and methods

Construction of yeast strains

All yeast strains used in this study are isogenic to BY4741 (Table 1). Standard genetic methods involving PCR-based homologous recombination, as described by Winston et al. (1995) were employed for strain construction and are described in the following sections.

Table 1. Yeast strains used in the present study

Strain	Genotype	Source
BY4741	Mat a his3D1 leu2D0 met15D0 ura3D0	Open Biosystems
Set2-TAP	Mat a his3D1 leu2D0 met15D0 ura3D0 set2-TAP	This study
set2ΔC-TAP	Mat a his3D1 leu2D0 met15D0 ura3D0 set2ΔC-TAP	This study
set2ΔAID-TAP	Mat a his3D1 leu2D0 met15D0 ura3D0 set2ΔAID-TAP	This study
set2ΔSRI-TAP	Mat a his3D1 leu2D0 met15D0 ura3D0 set2ΔSRI-TAP	This study
Δset2	Mat a his3D1 leu2D0 met15D0 ura3D0 Δset2 (KAN)	This study

PCR amplification

PCR amplification of the cassette was performed using the primers with overhangs corresponding to the target site and used for yeast transformation. The primers used in this study are listed in Table 2. For complete gene deletions, the *kanMX6* cassette from plasmid pFA6a-kanMX6 was amplified. The forward primer included a 40 bp overhang upstream of the start codon, and the reverse primer included a 40 bp overhang downstream of the stop codon. For domain deletions and simultaneous protein tagging, the TAP-*URA* cassette was amplified from plasmid pBS1539 with primers containing overhangs corresponding to the target site.

Preparation of competent cells

Competent yeast cells were prepared through a multi-step protocol. Initially, a single yeast colony was inoculated into 3 mL of yeast-peptone-dextrose medium (1% yeast extract, 2% peptone, 2% dextrose) and incubated overnight at 30 °C with shaking at 220 rpm. The next day, 50 mL of 2X yeast-peptone-dextrose-adenine medium (2% yeast extract, 4% peptone, 4% dextrose, 200 mg L^{-1} adenine hemisulfate) was inoculated with 1 mL of the starter culture and grown to an OD_{600} of 1.5 at 30 °C. Cells were harvested by centrifugation at 4000 rpm for 3 min, washed once with 25 mL of sterile water, followed by 1 mL of 0.1 M lithium acetate. The cells were then centrifuged at 14000 rpm for 30 s, and the pellet was resuspended in 500 μL of 0.1 M lithium acetate, followed by a 5 min incubation at room temperature to create a competent cell suspension suitable for transformation.

Yeast transformation

To perform the transformation, 100 μL of competent yeast cells were used. First, 7 μL of salmon sperm DNA (10 mg mL^{-1}) was added, followed by 2 μg of precipitated PCR product. The mixture was then incubated at room temperature for 5 min. Next, 240 μL of 50% polyethylene glycol, 36 μL of 1 M lithium acetate at pH 7.5, and sterile water were sequentially added to make a final volume of 360 μL. The mixture was gently mixed and incubated at 30 °C for 30 min, followed by a heat shock at 42 °C for 20 min. After that, the cells were harvested by centrifugation at 13000 rpm, washed with sterile water, and resuspended in 100 μL of autoclaved deionized H_2O for subsequent plating on selective media.

Western blotting of total protein

The total protein was extracted using the trichloroacetic acid (TCA) precipitation method. Briefly, cells from 5mL of culture in the log phase were harvested by centrifugation at 5000 rpm for 5 min. The cell pellet was washed with autoclaved deionized water and resuspended in 100 μL of

Table 2. Oligonucleotides used in the present study

Primer	Sequence	Description
P1	5'GAAAACGTGAAACAAGCCCCAAATATGCATG TCTGGTTAATACGACTCACTATAGGGCGA 3'	Reverse primer for replacement of SET2 stop codon by TAP-URA
P2	5'ATCAACAAGGATGTCTTCTCCTCCACCTTCAA CATCATCATCCATGGAAAAGAGAAGATG 3'	Forward primer for TAP tagging SET2 at the genomic locus
P3	5'CTACTGTGAGGAGCCAAATTGTATTGGGTTTC TCGGTGGTTCCATGGAAAAGAGAAGATG 3'	Forward primer for deletion of C-terminal region of SET2 gene
P4	5'CTACTGTGAGGAGCCAAATTGTATTGGGTTTC TCGGTGGTGGTTTACCTCCAGGCTGGGAGAT3'	Forward primer for deletion of AID domain
P5	5'CAAAAAACTAGTGGAAGCAAAAGAGGCTAAG CGGTTGAAATCCATGGAAAAGAGAAGATG3'	Forward primer for deletion of SRI domain
P6	5'TCAAACCTTTCTCCTTTCCTGGTTGTTGTTTT ACGTGATCCGGATCCCCGGGTTAATTAA3'	Forward primer for deletion of SET2 by kanMX6
P7	5'GAAAACGTGAAACAAGCCCCAAATATGCATGT CTGGTTAAGAATTCGAGCTCGTTTAAAC3'	Reverse primer for deletion of SET2 by kanMX6

20% TCA followed by the addition of 100 μL of TCA buffer (20 mM Tris-HCl pH 8.0, 50 mM ammonium acetate, 2 mM EDTA pH 8.0, and 0.2 mM PMSF) and 200 μL of acid-washed glass beads. Cells were lysed by bead beating and the cell lysate was centrifuged at 14000 rpm, 4 °C for 20 min to precipitate the total protein. Precipitated proteins were separated by SDS-PAGE and visualized by western blotting using antibodies against TAP-Tag (Thermo Fisher Scientific), H3K36me2 (Abcam), H3K36me3 (Abcam), and H4 (Abcam).

Results

Expression of different Set2 deletion versions

PCR-based methods were used to delete and simultaneously tag different domains of histone methyltransferase with TAP-tag as discussed above. The deleted versions of Set2 are represented in Fig. 1A. The expression of different deleted versions of Set2 was analyzed by western blotting using anti-TAP antibody (Fig. 1B) and all the deleted versions of Set2 showed normal expression.

Specific domains within the Set2 protein contribute to the efficiency and specificity of methylation

The purpose of our study was to investigate the role of specific domains within the Set2 protein in mediating H3K36 methylation. To achieve this, yeast strains that had different domains of Set2 deleted were used. Total protein from these strains was extracted and analyzed by western blotting with antibodies against H3K36 methylation. The findings provided detailed insights into the functional significance of Set2 protein domains in mediating H3K36 methylation. Robust levels of H3K36 dimethylation and

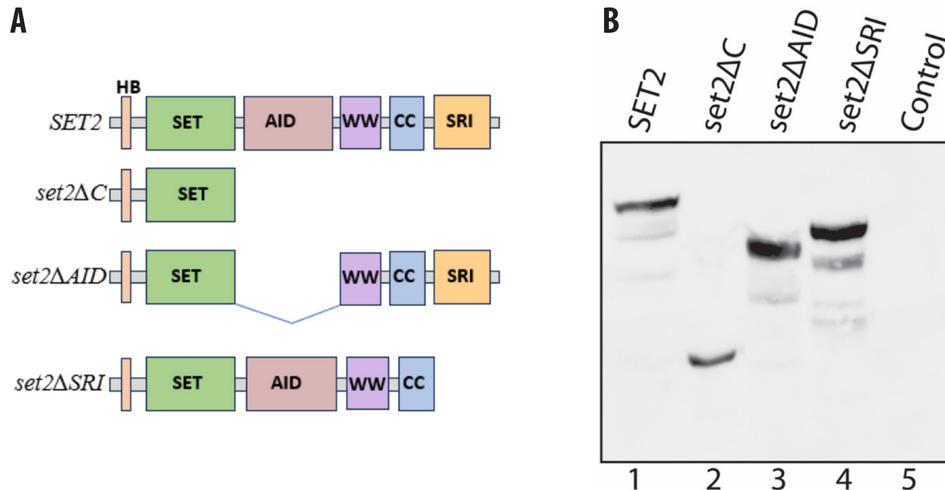

Fig. 1. Expression of Set2 domains. A, a schematic representation illustrates Set2 and various deletion versions lacking the designated domains. B, different domains of Set2 were deleted and simultaneously tagged with the TAP tag. Expression of tagged versions of proteins was analyzed by western blotting probed with anti-TAP antibody. Lane 1: full-length Set2 protein; Lane 2: set2ΔC having only catalytic domain intact; Lane 3: set2ΔAID, Lane 4: set2ΔSRI; Lane 5: wild type no tag control.

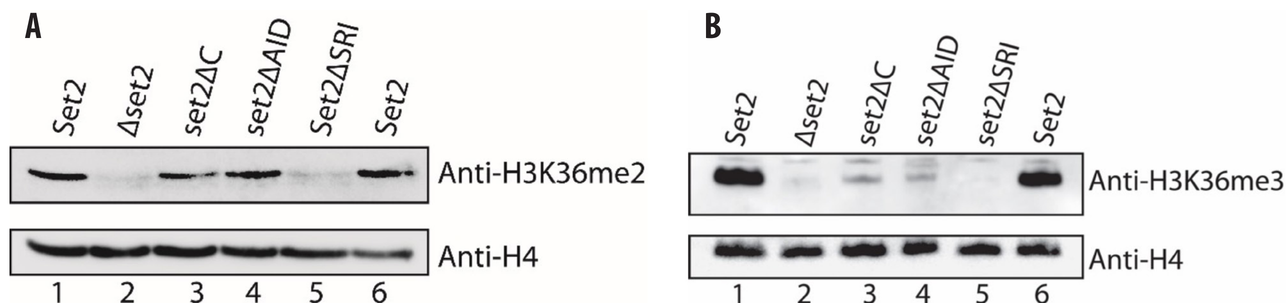

Fig. 2. Functional analysis of Set2 reveals domain-specific control over histone methylation patterns. Total cell lysates from strains expressing either Set2 or indicated deletion versions were resolved by SDS-PAGE and analyzed by western blot. A, Western blot probed with anti-H3K36me2. Lane 1 and Lane 6: full-length Set2 protein; Lane 2: Δset2; Lane 3: set2ΔC; Lane 4: set2ΔAID; Lane 5: set2ΔSRI. An anti-H4 antibody serves as a loading control in all lanes. B, Western blot probed with anti-H3K36me3. Lane 1 and Lane 6: full-length Set2 protein; Lane 2: Δset2, Lane 3: set2ΔC; Lane 4: set2ΔAID; Lane 5: set2ΔSRI. An anti-H4 antibody serves as a loading control in all lanes.

trimethylation in wild-type yeast were observed (Full-length Set2). However, keeping only the catalytic SET domain intact and deleting all other domains resulted in decreased trimethylation levels (Fig. 2B), but dimethylation levels persisted (Fig. 2A). This indicates that the SET domain alone is sufficient for dimethylation, which is consistent with previous studies. Interestingly, we found that deletion of the central region containing the autoinhibitory domain did not alter dimethylation levels (Fig. 2A), but resulted in a drop in trimethylation levels (Fig. 2B). This suggests that the autoinhibitory domain plays a specific role in mediating the trimethylation of H3K36. In contrast, when only the SRI domain was deleted, and all other domains were intact, a loss of both di- and trimethylation of H3K36 was observed (Fig. 2A & B). This suggests that the SRI domain is essential for both di- and trimethylation of H3K36 in the context of full-length Set2 protein. Overall, these observations highlight the importance of domain-domain interactions in governing histone modification by the Set2 protein. Specifically, the SET domain alone is sufficient for dimethylation, while other domains contribute to the efficiency and specificity of methylation in the context of the full-length protein.

Discussion

Histone post-translational modifications are crucial for the regulation of chromatin architecture and gene expression in *S. cerevisiae*. Despite its relatively simplistic genome compared to higher eukaryotes, *S. cerevisiae* shares numerous conserved epigenetic mechanisms with more complex organisms (Marino-Ramirez et al. 2006; Dahiya et al. 2020; Frigerio et al. 2023). Histone methylation at lysine residues is particularly a significant modification, modulating transcriptional activation, repression, and DNA repair pathways. Set2, a key histone methyltransferase in yeast, is primarily responsible for catalyzing H3K36 methylation (Strahl et al. 2002). This modification is indispensable for regulating transcription elongation, mRNA processing, and genomic stability (Wagner, Carpenter 2012; Pfister et al. 2014; Lim et al. 2018; Lam, Chen 2022; Lam et al. 2022; Sharda, Humphrey 2022).

While the role of Set2 in chromatin dynamics is well-documented, its regulatory mechanisms are not fully elucidated. The present findings shed light on the intricate functional interplay between different domains within the Set2 protein that govern H3K36 methylation. Although the SET domain is essential for its catalytic activity, our

Fig. 3. Comparison of the protein domains of budding yeast Set2 and human SETD2. The architectural organization of Set2 in budding yeast and its human homolog SETD2 exhibits remarkable conservation of domains: Both proteins feature a highly conserved domain arrangement, including a catalytic SET domain responsible for methyltransferase activity. Adjacent to the SET domain lies the Autoinhibitory Domain (AID), whose precise function remains largely elusive. Following the AID, a tandem arrangement of the WW, coiled-coil (CC) domains, and Set2-Rpb1 Interaction (SRI) domain are located at the C-terminal end.

data reveal that other domains within Set2 are critical for achieving efficient and specific methylation. This functional synergy among Set2 domains underscores the complexity of histone modification dynamics and highlights the importance of domain interactions in modulating enzymatic activity.

The present experiments with yeast strains having different Set2 domains deleted strongly suggest the importance of these domains in Set2 activity. It was demonstrated that the presence of the catalytic domain alone is sufficient for the di-methylation of H3K36 in yeast; however, trimethylation necessitates the presence of additional domains. In *S. cerevisiae*, Set2 uniquely mediates all three methylation states of H3K36 (Lee, Shilatifard 2007). However, previous studies have shown that chromatin factors such as Ctk1 and Spt6 are specifically required for H3K36me3 deposition by Set2, suggesting a specialized function for H3K36me3 distinct from H3K36me1/2 (Youdell et al. 2008). The precise mechanisms by which Ctk1 and Spt6 facilitate H3K36me3 deposition remain unclear. Given the involvement of non-catalytic domains of Set2 in H3K36 trimethylation, it is plausible that factors like Ctk1 and Spt6 modulate Set2 activity through direct or indirect interactions with these domains. Further investigation is warranted to delineate these interactions and their regulatory implications for Set2's enzymatic function.

The implications of the present findings extend beyond yeast biology and have relevance for understanding the regulation of H3K36 methylation in human cells. SETD2, the human homologue of Set2, shares structural and functional similarities with its yeast counterpart and is responsible for trimethylating histone H3 at lysine 36 in humans (Fig. 3). The role of SETD2 and its associated H3K36me3 in tumour suppression is well known. SETD2 mutations have been implicated in various cancer types, including renal cell carcinoma, glioblastoma, and leukaemia, highlighting its tumour-suppressive role (Duns et al. 2010; Wen et al. 2014; Li et al. 2016; Niu et al. 2020; Zhou et al. 2020; Tsang et al. 2021; Yang et al. 2022; Ma et al. 2023). Loss of SETD2 function often leads to global reductions in H3K36me3 levels, which are associated with genomic instability and tumour progression (Pfister et al. 2014; Sharda, Humphrey 2022). Many of the mutations in SETD2 linked with cancer are in domains outside the SET domain (Fahey, Davis 2017). Given the conservation of Set2/SETD2 function, our findings regarding the role of specific protein domains in mediating H3K36 methylation states especially trimethylation of H3K36 in *S. cerevisiae* provide valuable context for future studies aimed at understanding the role of SETD2 domains in cancer development and progression. Future investigations intended at elucidating the molecular mechanisms governing SETD2-mediated histone methylation may uncover novel therapeutic targets for cancer treatment. It may be possible to identify specific protein domains that could serve as targets for therapeutic intervention. Targeting these domains could potentially restore normal histone modification dynamics, inhibit tumour progression, and improve clinical outcomes for cancer patients.

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