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Sus1p facilitates pre-initiation complex formation at the SAGA-regulated genes independently of histone H2B de-ubiquitylation

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Abstract

Sus1p is a common component of transcriptional co-activator, SAGA (Spt-Ada-Gcn5-Acetyltransferase), and mRNA export complex, TREX-2 (Transcription-export 2), and is involved in promoting transcription as well as mRNA export. However, it is not clearly understood how Sus1p promotes transcription. Here, we show that Sus1p is predominantly recruited to the upstream activating sequence of a SAGA-dependent gene, *GALI*, under transcriptionally active conditions as a component of SAGA to promote the formation of pre-initiation complex (PIC) at the core promoter, and consequently, transcriptional initiation. Likewise, Sus1p promotes the PIC formation at other SAGA-dependent genes, and hence transcriptional initiation. Such function of Sus1p in promoting PIC formation and transcriptional initiation is not mediated via its role in regulation of SAGA's histone H2B de-ubiquitylation activity. However, Sus1p's function in regulation of histone H2B ubiquitylation is associated with transcriptional elongation, DNA repair and replication. Collectively, our results support that Sus1p promotes PIC formation (and hence transcriptional initiation) at the SAGA-regulated genes independently of histone H2B de-ubiquitylation, and further controls transcriptional elongation, DNA repair and replication via orchestration of histone H2B ubiquitylation, thus providing distinct functional insights of Sus1p in regulation of DNA transacting processes.

Keywords

Transcription; Sus1p; RNA polymerase II; DNA repair and replication

Introduction

Transcriptional initiation involves a coordinated assembly of various general transcription factors (GTFs) and RNA polymerase II at the core promoter to form the preinitiation

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complex (PIC). Such an assembly of transcription factors at the promoter is promoted by activator (1, 2). An activator binds to DNA upstream of TATA-box or core promoter via its DNA binding domain, and recruits a co-activator through its activation domain (1, 2). Co-activator then aids in the formation of PIC (1, 2). SAGA is a well-characterized co-activator (1, 3-6). It is a large multi-protein complex, and is involved in transcription of ~10% of genes in *Saccharomyces cerevisiae* (1). Although the SAGA complex was initially discovered in yeast, it is evolutionarily conserved from yeast to humans (1, 3, 4, 6). SAGA possesses two enzymatic activities: histone H3 acetyltransferase (HAT) and histone H2B ubiquitin protease or de-ubiquitylation (DUB) activity. These activities are crucial in regulating post-translational modifications of histones H3 and H2B, thereby causing an alteration of an active or inactive state of the chromatin. In yeast, SAGA is composed of at least 20 components which are organized into several modules such as DUB, HAT, TAF (TBP-associated factor) and SPT (Suppressor of Ty) (1, 3-9). The DUB module possesses histone H2B lysine 123 (K123) de-ubiquitylation or ubiquitin protease activity, and has four subunits, namely Ubp8p, Sgf11p, Sgf73p and Sus1p. The HAT module has histone H3 K9/14 HAT activity, and consists of Gcn5p, Ada2p, Ada3p and Sgf29p. A set of TAFs constitutes the TAF module that has been implicated in regulation of SAGA's HAT activity and interaction with activator. The SPT module contains Tra1p, Spt3p, Spt7p, Spt8p, Ada1p and Spt20p, and contacts with activator and PIC to regulate transcriptional initiation in addition to its role in maintaining SAGA's overall structural integrity. Thus, different modules of SAGA perform distinct functions in regulation of histone covalent modifications and transcriptional initiation.

Sus1p regulates SAGA's histone H2B de-ubiquitylation activity by maintaining the association of Ubp8p (that has histone H2B de-ubiquitylation or ubiquitin protease activity) with SAGA (10-12). It is a small 11 kDa protein, and is evolutionarily conserved among eukaryotes. Its human homologue is ENY2 (13). Preliminary results suggest the links of ENY2 to different pathological states (10). In *Drosophila*, ENY2/E(Y)2 is involved in the barrier activity of Su(Hw)-dependent insulators (14). It also functions as a cofactor to regulate transcriptional activity of nuclear receptors (15). In yeast, transcription of ~9% genes is affected by Sus1p (12). Likewise, it is involved in gene expression in higher eukaryotes (13). Thus, Sus1p/ENY2 is an important regulator of gene expression for cellular functions.

Sus1p was initially identified by synthetic lethality screening with mRNA export adaptor Yra1 mutant allele (12). It also interacts physically with mRNA export factors, Thp1p and Sac3p, which are the integral components of the TREX-2 complex (10, 12). The TREX-2 complex binds to various nucleoporins of the nuclear pore complex, and mediates the export of mRNA from nucleus to cytoplasm (10-12). Thus, Sus1p is likely to affect mRNA export. Indeed, Sus1p has been shown to regulate mRNA export (10-12). Therefore, through its interaction with SAGA and TREX-2, Sus1p bridges transcription with mRNA export. However, it is not clearly understood how Sus1p regulates transcription. Here, we demonstrate that Sus1p stimulates the PIC formation (and hence transcriptional initiation) at the SAGA-regulated genes independently of its function in controlling SAGA's histone H2B de-ubiquitylation activity. Further, Sus1p is involved in transcriptional elongation, DNA

repair and replication via modulation of histone H2B deubiquitylation. These results provide significant mechanistic and functional insights of Sus1p as presented below.

Results

Sus1p is recruited to the UAS of a SAGA-dependent gene, *GAL1*, and promotes PIC formation at the core promoter for enhanced transcriptional initiation

As mentioned above, Sus1p is a common component of SAGA and TREX-2, and thus, has been shown to be involved in transcription and mRNA export. Further, Sus1p has been implicated in translocation of *GAL1* during active transcription (10, 11, 16, 17). Therefore, Sus1p functions at the interface of SAGA and TREX-2 in gene translocation, transcription and mRNA export. Hence, Sus1p is expected to be recruited to *GAL1* during active transcription. In fact, we find that Sus1p is predominantly associated with the upstream activating sequence (UAS) of *GAL1*, but not with the core promoter or coding sequence in galactose-containing growth medium (Figures 1A and 1B). Sus1p is not found to be associated with *GAL1* UAS in raffinose-containing growth medium (Figures 1A and 1B). Thus, Sus1p associates with the *GAL1* UAS under transcriptionally active (galactose-containing growth medium), but not inactive (raffinose-containing growth medium), conditions. Like Sus1p, SAGA-associated Sgf73p and Ubp8p are recruited to the *GAL1* UAS in a SAGA-dependent manner (18, 19). Similarly, the other components of SAGA are recruited to the *GAL1* UAS under transcriptionally active conditions (18-22). However, Sac3p, a specific component of TREX-2, is not found to be associated with *GAL1* UAS under transcriptionally active conditions (Figure 1C). These results support that Sus1p is recruited to the *GAL1* UAS as a component of SAGA.

Next, we analyzed the function of Sus1p in formation of the PIC at the core promoter of *GAL1*. In this direction, we determined the recruitment of TBP and RNA polymerase II to the *GAL1* core promoter in the presence and absence of Sus1p. TBP nucleates the assembly of GTFs to form the PIC. Thus, the formation of PIC and hence RNA polymerase II recruitment to the *GAL1* core promoter would be impaired in the absence of TBP. We find that the recruitment of TBP to the *GAL1* core is significantly decreased in the absence of Sus1p (Figure 2A). Likewise, the recruitment of the largest subunit (Rpb1p) of RNA polymerase II to the *GAL1* core promoter is reduced in the *sus1* strain (Figure 2B). Rpb1p maintains the structural integrity of RNA polymerase II (23). Thus, reduced recruitment of Rpb1p (Figure 2B) implies decreased association of RNA polymerase II with the *GAL1* core promoter in the absence of Sus1p. As a non-specific DNA control, we analyzed the ChIP signal at the transcriptionally inactive region of chromosome V as done previously (24). As expected, a background level of ChIP signal was observed at the inactive region in chromosome V (Figures 2A and 2B). Together, these results support that Sus1p promotes the formation of PIC at the *GAL1* core promoter.

As mentioned above, transcriptional initiation involves the PIC formation at the core promoter. Since Sus1p facilitates the PIC formation at the *GAL1* core promoter, transcription of *GAL1* would likely to be decreased in the absence of Sus1p. To test this, we carried out primer extension analysis in the *sus1* and wild type strains. Our primer extension analysis reveals that transcription of *GAL1* is impaired in the *sus1* strain (Figure

2C; Supplementary Figure S1). As a control, we also analyzed transcription of a SAGA-independent gene, *RPS5*, in the presence and absence of Sus1p. Since *RPS5* is a SAGA-independent gene (20), we would not observe reduced *RPS5* transcription in the *sus1* strain. Indeed, we do not find an alteration of *RPS5* transcription in the *sus1* strain in comparison to the wild type equivalent (Figure 2C). Therefore, our results support that Sus1p promotes transcription of the SAGA-dependent *GAL1* gene, but not SAGA-independent *RPS5* gene. Such transcriptional stimulatory role of Sus1p is mediated via facilitated formation of the PIC at the *GAL1* core promoter (Figures 2A and 2B). Consistently, the *sus1* strain grows slowly in comparison to the wild type equivalent in galactose-containing growth medium that requires expression of *GAL* genes including *GAL1* (Figure 2D).

Sus1p facilitates the PIC formation by enhancing recruitment of Sgf73p to the GAL1 UAS

Next, we analyzed how Sus1p promotes the PIC formation and hence transcriptional initiation. Sus1p has been biochemically shown to reside within DUB module of SAGA that contains Sgf73p, Upb8p, Sus1p and Sgf11p (7-9, 25, 26). Sgf73p plays a crucial function in maintaining SAGA's global structural integrity, and hence PIC formation and transcriptional initiation (19, 27). Further, Sgf73p links DUB module with the rest of SAGA (5, 7-9, 25). Sus1p may regulate the association of Sgf73p with SAGA, and control the PIC formation via Sgf73p. To test this, we analyzed the association of Sgf73p with the *GAL1* UAS in the presence and absence of Sus1p. In this direction, we tagged Sgf73p by HA epitope at its chromosomal locus in the wild type and *sus1* strains, and then performed the ChIP assay. We find that Sgf73p is predominantly associated with the *GAL1* UAS (Figure 3A). An anti-Myc was used as a non-specific antibody, and generated background signal (Figure 3A). Interestingly, we find that the association of Sgf73p with SAGA is decreased in the absence of Sus1p in comparison to its wild type equivalent (Figure 3B). However, the association of activator Gal4p with the *GAL1* UAS is not altered in the *sus1* strain (Figure 3B). Thus, Sus1p facilitates the recruitment of Sgf73p to the *GAL1* UAS. Such facilitated recruitment of Sgf73p to the *GAL1* UAS in the presence of Sus1p is likely to enhance the PIC formation at the core promoter and hence transcriptional initiation, as our previous studies (19) demonstrated the stimulatory role of Sgf73p in PIC formation and transcriptional initiation.

The stimulatory function of Sus1p in PIC formation at GAL1 is not mediated via histone H2B de-ubiquitylation

We find that Sus1p promotes Sgf73p recruitment and hence PIC formation at *GAL1*. In addition, Sus1p may play an additional function in the PIC formation via an impaired recruitment of Upb8p (or concurrent reduction of histone H2B deubiquitylation), as previous studies demonstrated the role of Sus1p in recruitment of Upb8p and hence histone H2B deubiquitylation (25, 28, 29). Intriguingly, we find that the loss of Upb8p does not alter the recruitment of TBP and the the largest subunit (Rpb1p) of RNA polymerase II to the *GAL1* core promoter following 90 min transcriptional induction (Figure 3C). Likewise, previous studies (30) demonstrated the dispensability of Upb8p for recruitment of the Rpb3p component of RNA polymerase II to *GAL1*. Thus, the PIC formation at *GAL1* is not regulated by histone H2B deubiquitylation. Therefore, Sus1p does not regulate the PIC formation at *GAL1* via Upb8p (or histone H2B de-ubiquitylation).

Sus1p facilitates the PIC formation (and hence transcriptional initiation) at the SAGA-dependent ADH1 and PHO84 genes independently of histone H2B de-ubiquitylation

Our results demonstrate that Sus1p promotes the PIC formation (and hence transcriptional initiation) at a SAGA-dependent gene, *GALI*. To determine whether Sus1p plays similar functions at other SAGA-dependent genes, we analyzed the formation of PIC at the core promoters of two other SAGA-dependent genes such as *ADH1* and *PHO84* in the presence and absence of Sus1p. We find that the recruitment of TBP and RNA polymerase II to the core promoters of these genes is significantly impaired in the absence of Sus1p (Figures 4A and 4B). Thus, like the results at *GALI*, Sus1p facilitates the formation of PIC at the core promoters of the SAGA-dependent *ADH1* and *PHO84* genes. Therefore, transcription of these genes is likely to be impaired in the absence of Sus1p. Indeed, we find that transcription of these genes is reduced in the *sus1* strain in comparison to the wild type equivalent (Figure 4C; Supplementary Figure S1). As a control, we show that transcription of a SAGA-independent gene, *RPS5*, is not altered in the absence of Sus1p (Figure 4C). The stimulatory role of Sus1p in transcriptional initiation of *ADH1* and *PHO84* is not mediated via histone H2B deubiquitylation, as our previous studies (18) demonstrated that PIC formation (and transcriptional initiation) at the promoters of these genes is not altered in the absence of Ubp8p. Thus, similar to the results at *GALI*, Sus1p promotes the PIC formation (and hence transcriptional initiation) independently of histone H2B de-ubiquitylation at other SAGA-regulated genes that are constitutively active in dextrose-containing growth medium. Consistently, the growth of the *sus1* strain is decreased under similar dextrose-containing growth medium (Figure 4D).

Sus1p promotes transcriptional elongation, DNA repair and replication

SAGA, Ubp8p or Sus1p has been previously shown to be associated with the coding sequence of certain genes, and regulates histone H2B ubiquitylation (30-32). The loss of histone H2B ubiquitylation in the absence of histone H2B ubiquitylation enzymes (or in histone H2B K123R point mutant) impairs transcriptional elongation (33-39). Thus, an enhanced level of histone H2B ubiquitylation at the coding sequence in the absence of Sus1p or Ubp8p would affect transcriptional elongation. Indeed, it has been demonstrated previously that the loss of Ubp8p or increased histone H2B ubiquitylation lowers the association of Ctk1p (Serine-2 kinase for carboxy terminal domain of the largest subunit of RNA polymerase II), Set2p (histone H3 K36 methyltransferase) and serine-2-phosphorylated RNA polymerase II with the active coding sequence (30). Consequently, the null mutation of Ubp8p (or enhanced histone H2B ubiquitylation; 18, 26, 30, 40) impairs the cellular growth in solid SC-uracil medium containing 6-AU (6-Azaauracil) (30). 6-AU decreases nucleotide pools for transcriptional elongation by RNA polymerase II, and thus, results in slow (or impaired) growth phenotype upon deletion or mutation of the factors involved in transcriptional elongation (30). Thus, impaired growth of the *ubp8* strain in the presence of 6-AU (30) implicates the role of Ubp8p or histone H2B de-ubiquitylation in transcriptional elongation. Since the loss of Sus1p enhances histone H2B ubiquitylation (25, 28, 29) similar to the *ubp8* strain, transcriptional elongation is thus likely to be impaired in the *sus1* strain. Indeed, the growth of the *sus1* strain is impaired in SC-uracil medium containing 6-AU (Figure 4E), indicating the role of Sus1p in facilitation of transcriptional

elongation. Thus, the loss as well as gain (or excess) of histone H2B ubiquitylation impairs transcriptional elongation, implicating an appropriate balance of histone H2B ubiquitylation and de-ubiquitylation in the maintenance of normal transcriptional elongation.

In addition to its stimulatory function in transcriptional elongation, histone H2B ubiquitylation also promotes DNA repair (34, 41, 42). Since Sus1p is involved in modulation of histone H2B ubiquitylation via maintaining the association of Ubp8p with SAGA (25, 28, 29), it is likely to be involved in DNA repair. To test this, we analyzed the growth of the wild type and *sus1* strains in solid YPD growth medium with or without 0.026% MMS (Methyl methanesulfonate; DNA damaging agent). We find that the *sus1* strain does not grow on solid YPD medium containing 0.026% MMS (Figure 5A), while it grows normally in the absence of MMS (Figure 5A). These results indicate that Sus1p promotes DNA repair. Such stimulatory function of Sus1p in DNA repair may be mediated via its role in maintaining SAGA's histone H2B de-ubiquitylation activity. If yes, Ubp8p would enhance DNA repair. To test this, we analyzed the growth of the wild type and *ubp8* strains in solid growth medium with or without 0.026% MMS. We find that, similar to the *sus1* strain, the *ubp8* strain does not grow on solid YPD medium with 0.026% MMS, but rather grows fine in the absence of MMS (Figure 5A). Similarly, the deletion of *SGF73* (that impairs recruitment of Ubp8p, and hence histone H2B deubiquitylation; 43) shows MMS-sensitivity (Figure 5B). However, the deletion of *SPT3*, *SPT8* and *SGF29* (that do not alter SAGA's global structural integrity, and hence histone H2B de-ubiquitylation; 5, 20, 22, 44-48) do not affect the growth of yeast cells in the presence of 0.026% MMS (Figure 5B). Thus, the loss of Sus1p, Ubp8p, Sgf73p or enhanced histone H2B ubiquitylation impairs DNA repair. Likewise, the *rad6* strain does not grow on solid YPD medium containing 0.026% MMS (Figure 5A), consistent with previous studies (34, 41) that demonstrated the role of Rad6p (histone H2B ubiquitin conjugase) or histone H2B ubiquitylation in facilitation of DNA repair. Thus, the loss as well as gain (or excess) of histone H2B ubiquitylation have adverse effects on DNA repair. Similar results are also found following UV-induced DNA damage (Figures 5C and 5D).

A recent study (49) has implicated the role of histone H2B ubiquitylation in DNA replication. Consistently, the absence of Rad6 impairs cellular growth in response to replication stress by HU (Hydroxyurea) (Figure 6A). However, it remains unknown whether an excess of histone H2B ubiquitylation or impairment of histone H2B deubiquitylation in the absence of Ubp8p or Sus1p has any effect on DNA replication. To test this, we analyzed the growth of the *ubp8* and *sus1* strains in solid YPD growth medium containing HU. We find that the growth of the *ubp8* cells is dramatically impaired in the presence of HU, similar to the growth defect of the *rad6* strain in HU (Figure 6A). However, the growth of the *spt3*, *spt8* and *sgf29* strains (that do not regulate histone H2B de-ubiquitylation) is not altered in solid growth medium containing HU (Figure 6B). These results support that Ubp8p is essential for DNA replication. Similar results are also found in the absence of Sus1 (Figure 6A) that is essential to maintain DUB activity of Ubp8 (25, 28, 29). Likewise, the growth of the *sgf73* strain (that impairs histone H2B de-ubiquitylation; 43) is decreased in the presence of HU (Figure 6B). Thus, histone H2B de-ubiquitylation affects DNA replication.

Discussion

Although previous studies (12) implicated Sus1p in transcription, it was not clear how Sus1p affects transcription. Here, we show that Sus1p is recruited to the UAS of a SAGA-dependent gene, *GALI*, as a component of SAGA. Subsequently, it promotes the formation of PIC at the core promoter. Enhanced formation of PIC by Sus1p consequently increases transcriptional initiation of *GALI*. Likewise, Sus1p promotes the formation of PIC (and hence transcriptional initiation) at other SAGA-dependent genes such as *ADH1* and *PHO84*. These results show how Sus1p controls transcriptional initiation.

The absence of Sus1p impairs the recruitment of Ubp8p (25), and hence histone H2B de-ubiquitylation (25, 28, 29). However, an impaired histone H2B de-ubiquitylation in the absence of Ubp8p does not alter PIC formation (Figure 3C; 18, 30). Thus, Sus1p promotes the PIC formation independently of histone H2B de-ubiquitylation (Figure 7). Further, histone H2B ubiquitylation facilitates histone H3 K4 methylation via histone trans-tail cross-talk (50, 51, 52). However, similar to histone H2B de-ubiquitylation, histone H3 K4 methylation is dispensable for PIC formation (18, 33). Therefore, Sus1p facilitates PIC formation at the SAGA-regulated genes independently of histone H2B deubiquitylation or histone H3 K4 methylation.

Sus1p facilitates the recruitment of Sgf73p (Figure 3B) that is essential for SAGA's overall structural integrity, PIC formation and transcriptional initiation (19, 27). Thus, Sus1p appears to facilitate the PIC formation via Sgf73p (Figure 7). Further, a compromised overall structural integrity of SAGA in the absence of Sus1p (via decreased recruitment of Sgf73p) would reduce SAGA's HAT activity. Hence, decreased PIC formation at *GALI* in the *sus1* strain might be mediated via impaired histone H3 K9/14 acetylation. However, we rule out this possibility, as our previous studies (19, 20) demonstrated that SAGA's HAT activity or Gcn5p (that has HAT activity) is dispensable for the PIC formation at *GALI*. Thus, Sus1p's function in promoting the PIC formation is not mediated via SAGA's HAT activity.

Like Sus1p, Sgf73p promotes the PIC formation independently of histone H2B de-ubiquitylation, but rather via the maintenance of overall structural integrity of SAGA (Figure 7). Sgf73p may also play an additional direct role in regulation of the PIC formation and transcriptional initiation. In support of this possibility, a very recent study (53) has implicated the interaction of Sgf73p with proteasome in regulation of transcription. Further, Sgf73p has been implicated to interact with Mediator (54) that promotes transcription (21). Thus, Sgf73p is likely to play a direct role in PIC formation in addition to its role in maintaining SAGA's overall structural integrity. Likewise, Sus1p may play a direct function in addition to its role in stimulation of PIC formation via Sgf73p. In support of this possibility, Sus1p is found to be a peripheral component of SAGA (5), and has been implicated to interact with Mediator (5) that facilitates *GALI* transcription (21).

Although both the Sgf73p and Sus1p components of SAGA's DUB module are involved in the PIC formation independently of histone H2B de-ubiquitylation, it is not clear whether Sgf73p functions through Sus1p (as the loss of Sgf73p impairs recruitment of Sus1p; 31),

Sus1p functions through Sgf73p or independently. We find that the loss of Sus1p impairs the recruitment of Sgf73p and PIC formation (Figures 2A, 2B and 3B). Similarly, the loss of Sgf73p impairs the recruitment of Sus1p (31), and reduces the PIC formation (19). Thus, it is likely that the functions of Sus1p and Sgf73p are mutually dependent in stimulating the formation of PIC, and subsequently transcriptional initiation. Consistently, Sus1p and Sgf73p are found to interact with each other (5, 12, 31, 43).

In addition to promoting transcriptional initiation, Sus1p also facilitates transcriptional elongation (Figure 4E). Such function appears to be mediated via its role in regulation of histone H2B de-ubiquitylation (Figure 7). Recent studies (30) have implicated that enhanced histone H2B ubiquitylation in the absence of Ubp8p decreases transcription elongation. An excess histone H2B ubiquitylation decreases the recruitment of Ctk1p (that phosphorylates RNA polymerase II for recruitment of Set2p and mRNA 3'-end processing factors to control transcriptional elongation and mRNA processing; 55-59) to the coding sequence, thus lowering transcriptional elongation (30). On the other hand, the loss of histone H2B ubiquitylation also impairs transcriptional elongation (33-39). Therefore, an appropriate balance of histone H2B ubiquitylation and deubiquitylation is essential for optimal transcriptional elongation. Ctk1p is not involved in transcriptional initiation. Thus, while histone H2B de-ubiquitylation is dispensable for PIC formation (18), it plays an important role in maintaining proper level of histone H2B ubiquitylation for optimal transcriptional elongation.

Further, we show here that Sus1p or histone H2B de-ubiquitylation is essential for DNA repair and replication (Figures 5, 6 and 7). We find that an excess of histone H2B ubiquitylation in the absence of Sus1p or Ubp8p (25, 28, 29) impairs DNA repair and replication (Figures 5A, 5C and 6A). On the other hand, the loss of histone H2B ubiquitylation in the absence of Rad6p decreases DNA repair and replication (Figures 5A, 5C and 6A). Thus, the loss or gain of histone H2B ubiquitylation reduces DNA repair and replication. An appropriate balance of this modification is essential for optimal DNA repair and replication. Sus1p or Ubp8p play important roles in maintaining such balance. Likewise, Sgf73p and Sgf11p (that are required for recruitment of Ubp8p, and hence histone H2B de-ubiquitylation; 26, 40, 43) would also regulate the balance of histone H2B ubiquitylation/de-ubiquitylation, and thus DNA repair and replication. Indeed, we show here that, like Sus1p and Ubp8p, Sgf73p is required for DNA repair and replication (Figures 5B, 5D and 6B). However, the Spt3p, Spt8p and Sgf29p components of SAGA (that are dispensable for SAGA's overall structural integrity, and hence histone H2B de ubiquitylation activity; 5, 20, 22, 44-48) are not required for DNA repair and replication (Figures 5B, 5D and 6B). Thus, histone H2B de-ubiquitylation activity of SAGA regulates DNA repair and replication.

In summary, Sus1p promotes the PIC formation independently of histone H2B deubiquitylation, while it favors transcriptional elongation, DNA repair and replication via modulation of histone H2B de-ubiquitylation. These results provide significant functional insights of Sus1p in regulation of DNA transacting processes. Since Sus1p is conserved among eukaryotes, similar regulatory mechanisms/functions are likely to exist in humans.

Materials and Methods

Plasmids

The plasmids, pFA6a-13Myc-KanMX6 and pFA6a-3HA-His3MX6 (60), were used for genomic tagging of the proteins of interest by Myc and HA epitopes, respectively. Plasmids, pRS416 and pRS413, were used for PCR-based knock-out of the genes of interest such as *SGF29*, *SGF73* and *UBP8*.

Yeast strains

The yeast (*S. cerevisiae*) strain bearing *SUS1* knockout (*sus1*) and its isogenic wild-type equivalent were obtained from the Rodríguez-Navarro laboratory (Centro de Investigación Príncipe Felipe, Spain; 12). The yeast strain harboring the null mutation of *RAD6* (STY2; *rad6* in FM392) and the wild-type equivalent (STY1; FM392) were obtained from the Shilatifard laboratory (Ali Shilatifard, Stowers Institute for Medical Research; purchased from Research Genetics). The wild type yeast strain, YKH045, was obtained from the Osley laboratory (Mary Ann Osley, University of New Mexico Health Science Center) (61). Yeast strains harboring null mutation in *SPT3* (FY294) and *SPT8* (FY462), and their isogenic wild-type equivalent, FY631, were obtained from the Winston laboratory (Fred Winston, Harvard Medical School, Boston, MA) (44). Multiple HA epitope tags were added at the original chromosomal locus of *SGF73* in the *sus1* and wild type strains to generate GDY76 (*sus1* , Sgf73p-HA) and GDY77 (Sgf73p-HA), respectively. Likewise, multiple Myc epitope tags were added at the chromosomal loci of *SUS1* and *SAC3* in W303a to generate GDY24 (Sus1p-Myc) and GDY27 (Sac3p-Myc), respectively. The endogenous *UBP8* gene of YKH045 was disrupted, using a PCR-based gene knockout method to generate the ASY19 (*ubp8*) strain (18). Likewise, the *SGF29* and *SGF73* genes of W303a strain were deleted to generate ASY8 (*sgf29*) and ASY9 (*sgf73*), respectively (19, 22).

Growth media

Yeast strains were grown in YPD (yeast extract, peptone plus 2% dextrose) up to an OD₆₀₀ of 1.0 at 30 °C for the studies at the *ADH1*, *PHO84* and *RPS5* genes. For studies at *GALI*, yeast cells were grown in YPR (yeast extract, peptone plus 2% raffinose) up to an OD₆₀₀ of 0.9 at 30 °C, and then switched to YPG (yeast extract, peptone plus 2% galactose) for 90 min. For growth curve analysis in YPG, yeast cells were grown in YPR at 30 °C up to an OD₆₀₀ of 0.1, and then switched to YPG. Subsequently, OD₆₀₀ was measured at different time points. The growth analysis was also performed for yeast cells continuously grown in YPD.

ChIP assay

The ChIP assay was performed as described previously (18, 20, 21, 45, 62, 63). Briefly, yeast cells were treated with 1% formaldehyde, collected and resuspended in lysis buffer. Following sonication, cell lysate (400 µl lysate from 50 ml of yeast culture) was precleared by centrifugation, and then 100 µl lysate was used for each immunoprecipitation. Immunoprecipitated protein–DNA complexes were treated with proteinase K, the cross-links were reversed, and DNA was purified. Immunoprecipitated DNA was dissolved in 20

μl TE 8.0 (10 mM Tris HCl pH 8.0 and 1 mM EDTA), and 1 μl of immunoprecipitated DNA was analyzed by PCR. The PCR reactions (a total of 23 cycles) contained [α - 32 P]dATP (2.5 μCi for each 25-μl reaction), and the PCR products were detected by autoradiography after separation on a 6% polyacrylamide gel. As a control, “input” DNA was isolated from 5 μl of lysate without going through the immunoprecipitation step and suspended in 100 μl of TE 8.0. To compare the PCR signal arising from the IP DNA with that from the input DNA, 1 μl of input DNA was used for PCR analysis. Serial dilutions of input and IP DNA samples were used to assess the linear range of PCR amplification as described previously (45, 63; Supplementary Figure S2). The PCR data presented here are within the linear range of PCR analysis.

For analysis of Sus1p, Sac3p and Sgf73p recruitment, the above ChIP protocol was modified as described previously (18, 19, 22, 24, 64, 65). Briefly, a total of 800 μl lysate was prepared from 100 ml of yeast culture. Following sonication, 400 μl lysate was used for each immunoprecipitation (using 10 μl of anti-HA or anti-Myc antibody and 100 μl of protein A/G plus agarose beads from Santa Cruz Biotechnology, Inc.), and immunoprecipitated DNA sample was dissolved in 10 μl TE 8.0 of which 1 μl was used for PCR analysis (a total of 23 cycles). In parallel, PCR analysis for input DNA was performed using 1 μl DNA that was prepared by dissolving purified DNA from 5 μl lysate in 100 μl TE 8.0. The primer pairs used for PCR analysis were as follows:

<i>ADH1</i> (Core):	5'-GGTATACGGCCTTCCTCCAGTTAC-3' 5'-GAACGAGAACAATGACGAGGAAACAAAAG-3'
<i>PHO84</i> (Core):	5'-GATCCACTTACTATTGTGGCTCGT-3' 5'-GTTTGTGTGTGCCCTGGTGATCT-3'
<i>RPS5</i> (Core):	5'-GGCCAACTT CTACGCTCACGTTAG-3' 5'-CGGTGTCAGACATCTT TGAATGGTC-3'
<i>GAL1</i> (UAS):	5'-CGCTTAACTGCTCATTGCTATATTG-3' 5'-TTGTTCGGAGCAGTGC GGCGC-3'
<i>GAL1</i> (Core):	5'-ATAGGATGATAATGCGATTAGTTTTTAGCCTT-3' 5'-GAAAATGTTGAAAGTATTAGTTAAAGTGTTATGCA-3'
<i>GAL1</i> (ORF):	5'-CAGAGGGCTAAGCATGTGTATTCT-3' 5'-GTCAATCTCTGGACAAGAACATTTC-3'
Chromosome-V:	5'-GGCTGTCAGAATATGGGGCCGTAGTA-3' 5'-CACCCGAAGCTGCTTTCACAATAC-3'

Autoradiograms were scanned and quantitated by the National Institutes of Health image 1.62 program. Immunoprecipitated DNAs were quantitated as the ratio of immunoprecipitate to input, and represented as ChIP signal. The maximum ChIP signal was set to 100, and other ChIP signals were normalized with respect to 100. The normalized ChIP signal (represented as normalized occupancy) is plotted in the form of a histogram with standard deviation (S.D.; Microsoft Excel 2003). ORF, open reading frame; UAS, upstream activating sequence; and Core, core promoter.

Total RNA preparation

The total RNA was prepared from yeast cell culture following the standard protocol. Briefly, 10 ml yeast culture of a total OD₆₀₀ of 1.0 in YPD (or 90 min induced yeast culture in YPG) was harvested, and then suspended in 100 μl RNA preparation buffer (500 mM NaCl, 200 mM Tris-HCl, 100 mM Na₂EDTA, and 1% SDS) along with 100 μl phenol/chloroform/

isoamyl alcohol and 100 µl volume equivalent of glass beads (acid washed; Sigma). Subsequently, yeast cell suspension was vortexed with a maximum speed (10 in a VWR mini-vortexer; cat. no. 58816-121) five times (30 s each). The cell suspension was placed in ice for 30 s between pulses. After vortexing, 150 µl RNA preparation buffer and 150 µl phenol/chloroform/isoamyl alcohol were added to the yeast cell suspension followed by vortexing for 15 s with maximum speed on a VWR mini-vortexer. The aqueous phase was collected following 5 min of centrifugation at maximum speed in a microcentrifuge machine. The total RNA was isolated from the aqueous phase by precipitation with ethanol.

Primer extension analysis

Primer extension analysis was performed as described previously (18-21). Briefly, total RNA was prepared from 10 ml of yeast culture as described above. Fifteen micrograms of total RNA was used in the primer extension analysis using ³²P-labeled primer and AMV reverse transcriptase (Promega). The average primer extension signal of the biologically independent experiments is reported with S.D. (Microsoft Excel 2003). The primers used for analysis of *PHO84*, *ADH1*, *RPS5*, and *GAL1* mRNAs were as follows:

ADH1: 5'-TATCCTTGTTTCCAATTTACCGTGG-3'
RPS5: 5'-GACTGGGGTGAATTCTTCAACAACCTTC-3'
PHO84: 5'-GAAGACTTCTTTCAGCAACATG-3'
GAL1: 5'-CCTTGACGTAAAGTATAGAGG-3'

Analysis of transcriptional elongation on solid growth medium

The growth of the *sus1* and wild type cells was analyzed on plates containing solid SC-uracil (plus 2% dextrose) with or without 100 µg/ml 6-AU. Both wild type and *sus1* strains were transformed with a low copy number plasmid expressing the *URA3* gene, inoculated in liquid SC-uracil medium (with 2% dextrose), and grown up to an OD₆₀₀ of 0.2 at 30 °C. Subsequently, yeast cells were suspended in fresh liquid SC-uracil medium (with 2% dextrose), and grown up to an OD₆₀₀ of 0.4 prior to spotting (3 µl) on solid SC-uracil medium (plus 2% dextrose) with or without 100 µg/ml 6-AU. Yeast cells were spotted with serial dilutions. Yeast cells were grown at 30 °C, and photographed after 2 or 3 days. Growth analysis was carried out in biological triplicates, and consistent results were obtained. One representative set is included in Figure 4E.

Analysis of DNA repair on solid growth medium

The growth of the *rad6*, *sus1*, *ubp8*, *spt3*, *spt8*, *spt29*, *sgf73* and wild type cells was analyzed on plates containing solid YPD with or without 0.026% MMS (129925-5G, Sigma). Yeast cells were inoculated in YPD, and grown up to an OD₆₀₀ of 0.2 at 30 °C. Subsequently, yeast cells were suspended in fresh YPD medium, and grown up to an OD₆₀₀ of 0.4 at 30 °C prior to spotting (3 µl) on solid medium with serial dilutions. Yeast cells were grown at 30 °C, and photographed after 2, 3 or 4 days. Similar experiments were also performed for UV-induced DNA damage. Briefly, yeast cells were spotted on solid YPD, and then exposed to UV (254 nm) light (29 J/m²). Following exposure to UV light, YPD plates were wrapped by aluminum foil to avoid light, and incubated at 30 °C. Cells were photographed after 2, 3 or 4 days. Growth analysis was carried out in biological duplicates

or triplicates, and consistent results were obtained. One representative set is included in Figure 5.

Analysis of DNA replication on solid growth medium

The growth of the *rad6*, *sus1*, *ubp8*, *spt3*, *spt8*, *spt29*, *sgf73* and wild type cells was analyzed on plates containing solid YPD with or without 100 mM HU. Yeast cells were inoculated in YPD, and grown up to an OD₆₀₀ of 0.2 at 30 °C. Subsequently, yeast cells were suspended in fresh YPD medium, and grown up to an OD₆₀₀ of 0.4 at 30 °C prior to spotting (3 µl) on solid medium with serial dilutions. Yeast cells were grown at 30 °C, and photographed after 2, 3 or 4 days. Growth analysis was carried out in biological duplicates, and consistent results were obtained. One representative set is included in Figure 6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Sus1p enhances Sgf73p recruitment, PIC formation and transcription initiation.
- Sus1p promotes PIC formation independently of histone H2B de-ubiquitylation.
- Sus1p also facilitates transcriptional elongation, DNA repair and replication.
- Such functions of Sus1p are mediated via modulation of H2B de-ubiquitylation
- Sus1p differentially regulates DNA transacting processes via H2B deubiquitylation

Figure 1.

Recruitment of Sus1p to the *GAL1* UAS. (A) Schematic diagram showing the locations of different primer pairs at *GAL1* for the ChIP analysis. The numbers are presented with respect to the position of the first nucleotide of the initiation codon (+1). (B) Recruitment of Sus1p to the UAS, but not core promoter or coding sequence, of the *GAL1* gene. Yeast strain expressing Myc-epitope tagged Sus1p was grown at 30°C in yeast extract containing peptone plus 2% raffinose (YPR) up to an OD₆₀₀ of 0.9 prior to formaldehyde-based *in vivo* crosslinking or induced for 90 min in YPG (yeast extract containing peptone plus 2% galactose) before crosslinking. Chromatin immunoprecipitation was performed as described in the Materials and Methods section. Immunoprecipitation was performed using a mouse monoclonal antibody against the c-Myc epitope-tag (9E10; Santa Cruz Biotechnology, Inc.). Primer pair located at the UAS, core promoter or coding sequence of the *GAL1* gene (see Materials and Methods section) was used for PCR analysis of the immunoprecipitated DNA samples. The ratio of immunoprecipitate over the input in the autoradiogram was measured, and represented as ChIP signal. The maximum ChIP signal was set to 100. Other ChIP signals were normalized with respect to 100. The normalized ChIP signal (represented as normalized occupancy) is plotted in the form of a histogram. (C) Analysis of recruitment of Sus1p and Sac3p to the *GAL1* UAS. Yeast strains expressing Myc-tagged Sus1p and Sac3p were grown, crosslinked and immunoprecipitated as in panel B. The ratio of immunoprecipitate over the input in the autoradiogram was measured in raffinose (YPR) and galactose (YPR). The fold change in galactose relative to raffinose is plotted in the form of a histogram.

Figure 2.

Requirement of Sus1p for recruitment of TBP and RNA polymerase II to the *GAL1* core promoter. **(A and B)** Sus1p is required for recruitment of TBP and RNA polymerase II to the *GAL1* core promoter. Yeast strains were grown and crosslinked as in Figure 1B. Immunoprecipitation was carried using an anti-TBP antibody (obtained from Michael R. Green; University of Massachusetts Medical School) against TBP or 8WG16 antibody (Covance, Inc.) against the carboxy terminal domain of Rpb1p. Immunoprecipitated DNA was amplified by PCR using primer pair targeted to the *GAL1* core promoter or an inactive region within chromosome V. The maximum ChIP signal was set to 100, and other ChIP signals were normalized with respect to 100. The normalized ChIP signal (represented as normalized occupancy) is plotted in the form of a histogram. **(C)** Transcription. Total cellular RNA was prepared from the wild type and $\Delta sus1$ strains, and mRNA levels from the *GAL1* and *RPS5* genes were quantitated by primer extension analysis. Yeast cells were grown as in Figure 1B. The mRNA level of wild type strain was set to 100, and mRNA level of the $\Delta sus1$ strain was normalized with respect to 100. Normalized levels of mRNA are plotted in the form of a histogram. **(D)** Growth analysis. Both the wild type and $\Delta sus1$ strains were initially grown in YPR up to an OD₆₀₀ of 0.1 and then switched to YPG at 30 °C. Subsequently, OD₆₀₀ was measured for next 5 hr, and plotted as a function of time.

Figure 3.

Recruitment of Sgf73p to the *GAL1* UAS. (A) Recruitment of Sgf73p to the UAS, but not core promoter, of the *GAL1* gene. The wild type strain expressing HA-epitope tagged Sgf73p was grown and crosslinked as in Figure 1B. Immunoprecipitation was performed using a mouse monoclonal antibody (F-7; Santa Cruz Biotechnology, Inc.) against the HA epitope-tag. An anti-Myc was used as a non-specific antibody. The ratio of immunoprecipitate over the input in the autoradiogram was measured, and represented as ChIP signal. The maximum ChIP signal was set to 100, and other ChIP signals were normalized with respect to 100. The normalized ChIP signal (represented as normalized occupancy) is plotted in the form of a histogram. (B) Sus1p facilitates the recruitment of Sgf73p to the *GAL1* UAS. Wild type and $\Delta sus1$ strains expressing HA-epitope tagged Sgf73p were grown, crosslinked and immunoprecipitated as in Figure 1B. The ChIP signal of the wild type strain was set to 100, and the ChIP signal of the $\Delta sus1$ strain was normalized with respect to 100. The normalized ChIP signal (represented as normalized occupancy) is plotted in the form of a histogram. The Student's *t* test of Microsoft Excel 2003 (with tail = 2 and types = 3) was used to determine the p values for statistical significance of the change in the ChIP signals from three biologically independent experiments. The changes were considered to be statistically significant at $p < 0.05$. (C) Ubp8p does not regulate the recruitment of TBP and RNA polymerase II (Rpb1p) to the *GAL1* core promoter. Yeast cells were grown and crosslinked as in Figure 1B. Immunoprecipitation was carried out as in Figures 2A and 2B.

Figure 4.

Sus1p promotes the PIC formation at the *ADH1* and *PHO84* core promoters. **(A and B)** Sus1p is required for recruitment of TBP and RNA polymerase II to the *ADH1* and *PHO84* core promoters. Yeast strains were grown in YPD (yeast extract containing peptone plus 2% dextrose) up to an OD₆₀₀ of 1.0 prior to crosslinking. Immunoprecipitation was carried as in Figures 2A and 2B. The ChIP signal of the wild type strain was set to 100, and the ChIP signal of the *sus1* strain was normalized with respect to 100. The normalized ChIP signal (represented as normalized occupancy) is plotted in the form of a histogram. **(C)** Transcription. Total cellular RNA was prepared from the wild type and *sus1* strains, and mRNA levels from the *ADH1*, *PHO84* and *RPS5* genes were quantitated by primer extension analysis. Yeast cells were grown as in panels A and B. **(D)** Growth analysis. Both wild type and *sus1* strains were initially grown in YPD up to an OD₆₀₀ of 0.1 at 30 °C. Subsequently, OD₆₀₀ was measured for next 5 hr in YPD, and plotted as a function of time. **(E)** Growth analysis of the *sus1* and wild type strains in the solid SC-uracil medium containing 2% dextrose with or without 6-AU (100 µg/ml) at 30 °C.

Figure 5.

Sus1p facilitates DNA repair. **(A and B)** Growth analysis of the *sus1*, *rad6*, *ubp8*, *spt3*, *spt8*, *spt29*, *sgf73* and wild type strains in the solid YPD medium with or without 0.026% MMS at 30 °C. **(C and D)** Growth analysis of the *sus1*, *rad6*, *ubp8*, *spt3*, *spt8*, *spt29*, *sgf73* and wild type strains in the solid YPD medium with or without UV (254 nm) exposure (29 J/m²).

Figure 6. Sus1p facilitates DNA replication. **(A and B)** Growth analysis of the *sus1*, *rad6*, *ubp8*, *spt3*, *spt8*, *spt29*, *sgf73* and wild type strains in the solid YPD medium with or without 100 mM HU.

Figure 7.

Schematic diagram showing the roles of Sus1p in different DNA transacting processes. Sus1p is essential for association of Ubp8p with SAGA (25). Ubp8p is dispensable for PIC formation (Figure 3C; 18), but favors transcriptional elongation (Figure 4E; 30, 31), DNA repair (Figure 5), and DNA replication (Figure 6). Sus1p also facilitates the association of Sgf73p with SAGA (Figure 3B). Sgf73p is essential for overall structural integrity of SAGA and hence PIC formation (19, 27). Thus, Sus1p appears to promote the PIC formation via Sgf73p. Sus1p and Sgf73p may also have additional direct roles in stimulation of PIC as described in the discussion section. “+”, stimulatory effect; PIC, pre-initiation complex; and ORF, open reading frame.