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Roles of H2A.z

in Fission Yeast

Chromatin

PhD Thesis of

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My God, teach me the truth of things.

Prophet Mohammed (s.a.v.)

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SUMMARY:

In the nuclei of all eukaryotic cells, genomic DNA is highly folded, constrained and compacted by histone and non-histone proteins in a dynamic polymer called chromatin.[1] The basic unit of chromatin is the nucleosome, which is formed by histone octamer formed by H2A, H2B, H3 and H4. The nucleosome can have variations in its content via addition of N-terminal modifications of histones and insertion of histone variants instead of canonical histones. These variations in nucleosomes can be used as a mark for gene expression states such as active or repressed, chromatin compartments such as centromere, telomere or mating type locus, and different stages of cell cycle. Methylation of Histone H3 lysine residues acts as a mark for active and repressed genes. For example, methylation of Histone H3 lysine 4 is a mark for active transcription.

In addition to the growing number of histone post-translational modifications, cells can exchange canonical histones with different variants that can directly or indirectly change chromatin structure. Moreover, enzymatic complexes that can exchange specific histone variants within the nucleosome have now been identified. One such variant, H2A.Z, has recently been the focus of many studies. H2A.Z is highly conserved in evolution even more than H2A and has many different functions, while defining both active and inactive chromatin in different contexts.[2] Swr1 Complex can specifically exchange H2A for H2A.Z in yeast chromatin.[3]

The JmjC domain was first defined based on aminoacid similarities in the Jarid2 (Jumonji), Jarid1C (SmcX) and Jarid1A (RBP2) proteins.[4] JmjC domain was found to be Histone lysine demethylase.[5] JmjC domain containing

demethylases (JHDMs) can remove all three histone lysine methylation states. Some of the JmjC domains are enzymatically inactive. In S. pombe, there are seven JmjC domain containing proteins. Msc1 is the highest molecular weight JmjC domain containing protein in S. pombe.

Five of seven JmjC domain containing proteins (JDPs) in S. pombe were immunoprecipitated using TAP (Tandem Affinity Purification) method, and only Msc1 was found to be in a protein complex. This complex was homolog of Swr1 Complex (Swr1C) in S. cerevisae, although in S. cerevisae Swr1C does not contain a JDP. In S. pombe, Swr1C has 14 members. It's known that H2A.z interacts with Swr1C in S.cerevisae. However, Msc1 did not purify detectable amounts of H2A.z. To answer the question of whether H2A.z interacts with Swr1C, H2A.z was purified using TAP. An immunoprecipitation (IP) with histone H2A variant H2A.z resulted Swr1C, H2B, nucleosome assembly proteins and Kap114, an importin. Effect of Swr1 or Msc1 loss on Swr1C structure and H2A.z interaction with Swr1C was investigated. Swr1C falls apart and H2A.z interaction with Swr1C is lost when Swr1 is absent. Msc1 loss does not affect Swr1C integrity and H2A.z interaction with Swr1C.

Deletion strains of msc1, h2A.z and swr1 were constructed. These deletion strains were shown to be sensitive to UV (Ultraviolet) and Camptothecin (CMT), as h2A.z deletion being the most sensitive. Global levels of several histone modifications were screened in msc1 and h2A.z deletions. Among them, only Histone H4 lysine 20 trimethylation (H4 K20 Me3) has been shown to be lost. Histone modifications associated with H2A.z nucleosome were screened and Histone H4 lysine 16 acetylation (H4 K16 Ac) has been shown to have much

lower level compared to average level. H4 K20 Me3, Histone H3 K4 Me3 and H3 K9 Ac levels were at comparable to average level. Histones associated with Msc1 IP had lower levels of H4 K16 acetylation and H3 K36 Me3. Low level of H3 K36 Me3 suggests that histones are located at somewhere else than coding regions of genes maybe promoters. Deletion strains of msc1, h2A.z and swr1 were shown to be sensitive to TSA, a histone deacetylase (HDAC) inhibitor. Deletion strains were sensitive to TSA and had low levels of H4 K20 Me3. The relationship between H4 K20 Me3 and TSA was revealed with the finding that TSA treated wild type cells have lost H4 K20 Me3. Gene expression analysis of deletion strains revealed a significant overlap of genes between each other. Genes upregulated in deletion strains have been shown to cluster at subtelomeric region of chromosomes and to be enriched of stress- and meiosis-induced genes .

TSA sensitivity, low level of H4 acetylation in H2A.z nucleosome, loss of H4 K20 Me3 and clustering of upreguleted genes at subtelomeric region in deletion strains indicate a cooperation with HDACs. It's known that a significant portion of genes upregulated in HDAC deletions in S. pombe is induced in stress conditions or meiosis.[6] It's also known that H2A.z poises repressed promoters for activation through histone loss in S. cerevisae.[7] As a conclusion, our data with the support of just quoted findings, indicate that H2A.z and Msc1 cooperate with HDACs in the maintenance of a specific chromatin environment of temporally repressed inducible genes. We suggest that this chromatin environment keeps inducible genes silent and at the same time, genes can be activated when it's necessary such as in meiosis or stress.

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1. INTRODUCTION:

1.1 Chromatin, Epigenetics and Histone Modifications:

In the nuclei of all eukaryotic cells, genomic DNA is highly folded, constrained and compacted by histone and non-histone proteins in a dynamic polymer called chromatin.[2] A cell's identity is defined by its characteristic pattern of gene expression and silencing, so remembering who it is, consists of maintaining that pattern of gene expression through the traumas of DNA replication, chromatin assembly and the radical DNA packaging that accompanies mitosis. The mechanisms by which around 2 m of DNA is packaged into the cell nucleus while remaining functional, border on miraculous and are still poorly understood. The first stage in packaging process is the nucleosome core. The reduction in DNA length produced by this histone induced supercoiling is modest, but is an essential first step in the formation of higher order chromatin structures.[8]

Is DNA alone responsible for generating the full range of information that ultimately results in a complex eukaryotic organism such as ourselves? [2]
The view is favored that epigenetics, imposed at the level of DNA-packaging proteins (histones), is a critical feature of a genome wide mechanism of information storage and retrieval.[2] It's now widely recognized that heritable, but reversible changes in gene expression can occur without alterations in DNA

sequence.[2]

Epigenetic "on-off" transcriptional states are largely dependent on the position of a gene within an accessible(euchromatic) or an inaccessible (heterochromatic) chromatin environment.[2] For S. pombe, which appears to contain a higher order chromatin structure more closely resembling that of multicellular eukaryotes, inheritance of silenced chromatin domains has been shown to be remarkably stable during both mitosis and meiosis.[2,3] Epigenetics imparts a fundamental regulatory system beyond the sequence information of our genetic code and emphasizes that "Mendel's gene is more than just a DNA moiety."[2,7]

A nucleosome can be defined as a histone octamer made up of each of H2A, H2B, H3 and H4 with DNA wound on the outside.[1] The nucleosome is fundemental to DNA coiling and gene regulation. It serves as a basis for chromatin condensation, whose modulation controls transcription.[1] A chain of nucleosomes is coiled in a chromatin fiber through interactions of the histone tails with adjacent nucleosomes and additional proteins, these interactions may be modulated by acetylation of tails.[1] The nucleosome has an additional role namely regulation of gene expression. Particularly exciting is the growing probability that the nucleosome can transmit epigenetic information from one cell generation to the next and has the potential to act, in effect, as the cell's memory bank. This information storage function resides primarily in the

amino-terminal tails of the four core histones. The tails are exposed on the nucleosome surface and are subject to a variety of enzyme catalyzed posttranslational modifications of selected aminoacids, including lysine acetylation, lysine and arginine methylation, serine phosphorylation and attachment of the small peptide ubiquitin (Figure 1.1).[8,9]

Histones are small basic proteins consisting of a globular domain and a more flexible and charged N-terminal tail that protrudes from nucleosome.[2] Exquisite variation is provided by covalent modifications (acetylation, phosphorilation, methylation...) of the histone tail domains which allow regulatable contacts with the underlying DNA.[2]

All of these modifications can be found both in active or silenced chromatin regions, which raises the question of how combinatorial specificity is used in defining an imprint for euchromatin or heterochromatin. There may be a positive or negative crosstalk between modifications.[2] The histone code hypothesis predicts that the modification marks on the histone tails should provide binding sites for effector proteins. In agreement with this notion, the bromodomain has been the first protein module to be shown to selectively interact with a covalent mark (acetylated lysine) in the histone NH2-terminal tail. [2,4,5,6]

Histone Lysine methylation occurs on histones H3 and H4. So far, six lysine residues located on these two histones have been reported to be sites of methylation. Each of these lysine residues can be mono-, di- or trimethylated.[10]

In its extended form, chromatin appears as an array of nucleosomes, but in the nucleus, the chromatin fibres that form chromosomes undergo several levels of folding, resulting in increasing degrees of condensation.[10,11] It is known that the histone tails have an important role in this folding process.[10,12] Considering this and the fact that histone methylation primarily occurs in histone tails, it would be reasonable to propose that methylation might function to regulate chromatin structure directly by affecting the higher-order folding of the chromatin fibre. This could have important implications for chromatin-templated processes such as transcription and DNA repair, assuming that folding alters the accessibility of DNA to the proteins that mediate these processes.[10]

1.1.1 Histone H3 Lysine 4 (K4) Methylation:

Analyses of the different H3-K4 methylation states and their distribution in various organisms indicate that both the dimethyl- and trimethyl-H3-K4 modifications are enriched at actively transcribed genes.[10,13,14,15,16] While the dimethyl modification seems to be generally distributed across the body of active genes, the trimethyl modification is localized specifically to the 5 prime

end of these genes. This general methylation pattern was confirmed by a high resolution genome-wide study.[10,17]

It's proposed that specific histone modifications, including lysine methylation, are binding sites for different proteins that mediate downstream effects.[2] Consistent with this hypothesis, it has been shown that BROMO DOMAINS can recognize acetylated lysines.[10,18,19] Recent studies on histone methylation identified at least three protein motifs— the CHROMODOMAIN[20], the TUDOR DOMAIN[21,22] and the PHD Finger — that are capable of specific interactions with methylated lysine residues.[10] Recently, a new domain PHD (Plant Homeodomain) was found to bind trimethylated Histone H3 K4.[24,25]

1.1.2 Histone H4 Lysine 20 (K20) Methylation:

H4-K20 methylation is a marker of mammalian heterochromatin.[26] It has been shown that SUV4–20h1 and SUV4–20h2, two SET-domain-containing proteins that localize to pericentric regions, are responsible for this modification. Furthermore, mutations in the *D. melanogaster* homologue Su(var)4–20 impair heterochromatin formation as evidenced by the suppression of PEV(Position Effect Variagetion).[26]

The retinoblastoma protein regulates pericentric heterochromatin. While

the LXCXE binding site mutation does not disrupt pRb's interaction with the Suv4-20h histone methyltransferases, it dramatically reduces H4-K20 trimethylation in pericentric heterochromatin.[30] Interestingly, there is a link between pRb and H4-K20 trimethylation in pericentric heterochromatin.

In fission yeast, H4-K20 methylation has no apparent role in regulating gene expression or heterochromatin function. Rather, the modification has a role in DNA damage response. Loss of Set9, the enzyme that catalyzes this modification, function or the H4-K20 residue impairs cell survival after genotoxic challenge and compromises the ability of cells to maintain checkpoint-mediated cell cycle arrest. Set9 is functionally linked to the checkpoint protein Crb2 and is required for its localization to double-strand breaks (DSBs).[27]

Checkpoint Rad protein and Crb2-dependent phosphorylation of Chk1 serves as a specific marker of DNA damage checkpoint activation.[28] Phosphorylation of Chk1 (Chk1-Pi) is compromised at high doses of IR by deletion of *set9*+. Set9 and H4-K20 methylation are important elements of the fission yeast DNA damage checkpoint.[27] Set9 functions in a checkpoint Rad protein-dependent pathway but not in the replication checkpoint defined by Mrc1 and Cds1. Because *set9* is epistatic with *crb2* but not *chk1*, these data argue that the role of Set9 in the DNA damage checkpoint is to contribute to Crb2 function prior to Chk1 activation.[27]

Histone H2A C-terminal phosphorylation and H4-K20 methylation cooperate in the same Crb2 recruitment pathway, which also requires the Tudor and BRCT motifs in Crb2.[29]

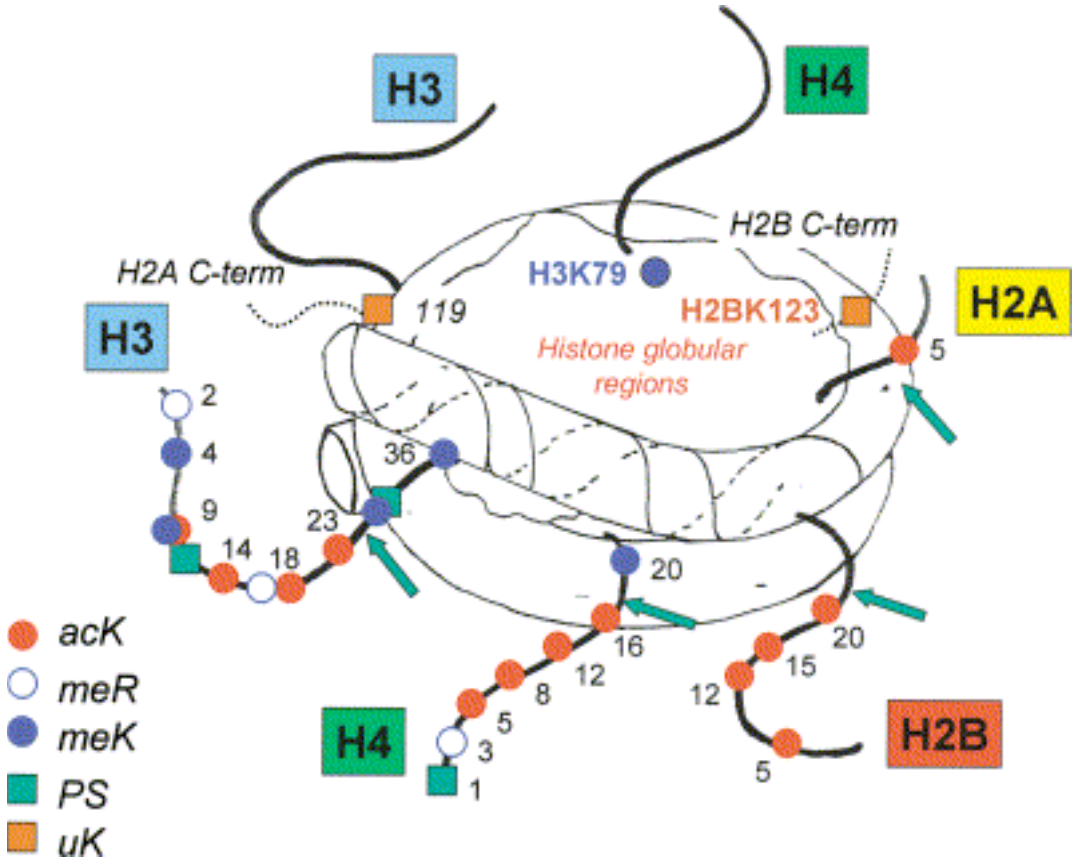

Figure 1.1 Schematic view of Nucleosome, Histone N-terminal tails and posttranslational modification sites (The figure is reproduced from Turner, 2002, Cell (ref no: 8))

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1.2 Histone H2A Variant H2A.z:

In addition to the growing number of histone post-translational modifications, cells can exchange canonical histones with different variants that can directly or indirectly change chromatin structure. Moreover, enzymatic complexes that can exchange specific histone variants within the nucleosome have now been identified. One such variant, H2A.Z, has recently been the focus of many studies. H2A.Z is highly conserved in evolution and has many different functions, while defining both active and inactive chromatin in different contexts.[1]

H2A.Z was first characterized as an H2A variant that is expressed independently of DNA replication, unlike major or S-phase histones.[1,2] It has been established as essential for viability in many organisms, H2A.Z was first characterized as an H2A variant that is highly conserved during evolution, even more than the major H2A, suggesting an important role for this variant in basic cellular mechanisms.[1,3]

H2A.Z reconstituted chromatin arrays fold more readily than H2A reconstituted arrays, though they are more resistant to interfiber condensation.[1,4] H2A.Z arrays have the ability to bind HP1a more efficiently in vitro, compared with H2A.[1,5] Furthermore, in vitro enzymatic assays revealed that H2A.Z containing nucleosomes are refractory to certain

posttranslational modifications.[1,6] Crystal structure analysis revealed that vertebrate H2A.Z binds to a metal ion at the nucleosomal surface and bears a negatively charged region that extends to H2B, which could constitute a potential interaction surface.[1,7] This interaction surface lies within an H2A.Z C-terminal region that has been characterized as essential for the survival of *Drosophila* and *Xenopus*.[1,8,9] In vitro protein–protein interaction assays revealed that H2A.Z can bind directly to INCENP, a heterochromatin binding protein.[1,10]

1.2.1 H2A.z Role in Transcription:

Many clues indicate that H2A.Z plays a positive role in gene transcription. Allis et al. (1980) discovered that H2A.Z was found exclusively in the transcriptionally active macronucleus of the protozoan *T. thermophila*, suggesting a global positive role for this histone. Later studies in yeast demonstrated this positive role for H2A.Z in transcription, as *htz1-* cells showed a defect in the activation of inducible genes for example *GAL1*.[1,12,13] Genomic expression analysis of the deletion of *HTZ1* in yeast revealed that approximately half of these downregulated genes were located less than 35 000 bp from telomeres heterochromatin, and were found in small clusters called HZADs (H2A.Z/Htz1 activated domains). Deletion of the heterochromatin component silent information regulator 2 (Sir2) can suppress the negative effect on transcription of the HZADs caused by the deletion of *HTZ1*.[1,14] In an

interesting study, H2A.Z was not only found to be upregulated during cardiac hypertrophy in mice, but essential to this function.[1,15] H2A.Z is globally bound to a large proportion of promoters. These H2A.Z containing genes (~63%) seem stochastically distributed in the genome with no particular preference in proximity to centromeres or telomeres.[1,16] Importantly, H2A.Z is preferentially localized to transcriptionally inactive promoters, as highly transcribed genes are somewhat depleted in H2A.Z.[1,16]

A functional significance of H2A.Z acetylation was demonstrated first in *T. thermophila*, as this organism cannot survive if 6 lysines in the N-terminal tail are mutated into nonacetylable arginine residues.[1,17] One study in chicken cells found that H2A.Z was acetylated in nucleosomes on the promoters of active genes and at insulators.[1,18] In another study, Babiarz et al. (2006) found that mutations of N-terminal H2A.Z lysines are lethal in combination with mutations of NuA4 components or H4 acetylable lysines. Moreover, mutations of all N-terminal acetylable lysines lead to heterochromatin spreading at telomere proximal regions, causing silencing.[1,19] In combination, these results suggest that the antisilencing function of H2A.Z could be regulated by the total charge in its N-terminal tail.[1] H2A.Z appears to be acetylated only when it is assembled into chromatin, suggesting a downstream role for its acetylation.[1,19,20]

1.2.2 Swr1 Complex:

Using different approaches, 3 independent laboratories discovered a protein complex that can specifically exchange H2A for H2A.Z in yeast chromatin.[1,21,22,23] This complex, called SWR1, was named after its catalytic subunit, which contains an ATPase/helicase domain homologous to Swi2/Snf2. Krogan et al. (2003) identified this complex in a genome-wide genetic interaction screen with *SET2*, *CDC73*, and *DST1*, 3 genes involved in transcriptional elongation and (or) chromatin metabolism.[1] Kobor et al. (2004) isolated this complex while purifying H2A.Z to identify possible interaction partners.[1] Purification of the SWR1 complex revealed that it contained 12 or 13 proteins, including histone H2A.Z, and that it could catalyze the exchange of H2A.Z in chromatin reconstituted in vitro.[1,23] The SWR1 complex in yeast has many shared subunits with the histone acetyltransferase (HAT) complex NuA4, bearing the catalytic subunit Esa1.[1,21,22,24] In higher eukaryotes, these 2 complexes seem to be fused together as a single complex, Tip60.[1,25] Among other proteins, Bdf1 has been identified as a member of this complex. Via its 2 bromodomains, Bdf1 can bind to acetylated histone tails, namely those of acetylated histone H4.[1,26] One attractive hypothesis for H2A.Z deposition is that Bdf1 can read promoter specific histone acetylation patterns and dictate localization. Deletion of *BDF1* and point mutations in lysines of H3 and H4 all diminish the presence of H2A.Z in promoters.[1,27,16]

1.2.3 H2A.z and Heterochromatin:

In contrast with the role described above in gene activation, H2A.Z has also been shown to be important in transcriptional silencing. In fact, H2A.Z becomes enriched in pericentric heterochromatin during the initial stages of mouse development.[1,10] Moreover, H2A.Z colocalizes with HP1a and INCENP, 2 heterochromatic factors important for chromosome segregation.[1,10] RNAi-mediated depletion of H2A.Z causes HP1a mislocalization and defects in chromosome segregation.[1,28] The *Drosophila* homolog of H2A.Z (H2Av) is also essential for HP1 recruitment during centromeric heterochromatin assembly.[1,29] One recent finding about H2A.z is that H2A.Z-mediated localization of genes at the nuclear periphery confers epigenetic memory of previous transcriptional state.[30]

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1.3 JmjC Domain Proteins and Msc1:

The JmjC domain was first defined based on aminoacid similarities in the Jarid2 (Jumonji), Jarid1C (SmcX) and Jarid1A (RBP2) proteins.[1-4] Homology between the JmjC domain and Cupin Metalloenzyme domains led to the identification of JmjC domain-containing factor inhibiting hypoxia (FIH) as an active protein oxygenase that can hydroxylate asparagine residues.[1,2,5,6] Given that a related group of oxygenases, the AlkB family of proteins, were previously shown to remove methylation from amine groups in modified DNA, it was predicted that chromatin associated JmjC-domain containing proteins might be involved in demethylation of modified arginine or lysine amine groups within histones.[1,7,8] This was confirmed by an activity based biochemical purification, which identified a JmjC-domain containing protein, JHDM1, as a histone H3K36-specific demethylase.[7]

JmjC domain containing demethylases (JHDMs) can remove all three histone lysine methylation states.[1] JmjC domain containing proteins were divided into seven subgroups with respect to their domain architecture and JmjC domain homology (See table 1.1).[1]

One of these groups is JARID1/JARID2 family: This family of proteins contain JmjN, AT-rich interactive, C5HC2-zinc-finger and PHD-finger domains. Jarid1A(RBP2), Jarid1B(PLU1), Jarid1C(SmcX), Jarid1D(SmcY) and Jarid2(Jumonji) are members of this family.[1] JARID1A was initially discovered as a retinoblastoma (RB)-binding protein in a yeast two-hybrid screen.[1,9] Subsequent functional analysis revealed a role for JARID1A in the activation of RB-mediated

transcription and, paradoxically, as a factor that antagonizes RB function under certain conditions.[1,10,11] JARID1A also physically associates with several nuclear hormone receptors (NRs) to facilitate NR-mediated gene expression.[1,12]

Members of the JARID2 subgroup are found in *D. melanogaster* and have orthologues in higher organisms. Mouse *Jarid2* was first identified in a gene-trap screen as an important factor in neural tube formation, and was named *Jumonji* (in reference to the Japanese character) because of the abnormal cruciform shape that the neural groove formed in the homozygous null mouse.[1,13,14]

Recently, Jarid1 group of proteins were identified as histone H3 K(Lysine)4 demethylases.[15-18] In Drosophila, The Trithorax group protein Lid is a timethyl H3K4 demethylase.[15] RBP2 is a histone demethylase in vitro and in vivo.[16] The function of the JmjC domain seems to be different within the Jarid1 and Jarid2 subgroups, as the amino acids required for enzymatic function are intact in most members of the JARID1 subgroup but completely lacking in the JARID2 subgroup.[1]

In S. pombe, there are seven JmjC domain containing proteins (Figure 1.2). Two of them, Lid2 and Ecm5 are found in a protein complex, together. This complex has a proteomic hyperlink to Set1 Complex which methylates histone H3 K4.[19] Another JmjC domain protein, Epe1 counteracts transcriptional silencing by negatively affecting heterochromatin stability.[20] Msc1 is the highest molecular weight JmjC domain containing protein in S. pombe. It has one JmjN, one C5HC2-zinc-finger and three PHD-finger domains in addition to JmjC domain. It is a multicopy supressor of a defect in the DNA damage checkpoint pathway in fission yeast.[21] Overexpression of Msc1 compensates for the loss of Chk1, a DNA damage

checkpoint protein.[21] Cells lacking Msc1 are hypersensitive to the HDAC inhibitor TSA.[21] Deletion of PHD fingers or JmjC domain also causes TSA sensitivity,[21] indicating the importance of these domains in HDAC(histone deacetylase) related function. Msc1 is in Jarid1/Jarid2 group.[1] Especially domain architecture resembles this group very well, although homology in terms of aminoacid identity is relatively low. Domain architecture of Msc1 is more similar to Jarid1 group proteins such as RBP2, because Jarid2 group proteins don't have PHD fingers but Msc1 has. On the other hand, when JmjC domains are compared, Msc1 resembles Jarid2 group proteins. As Jarid2 group JmjC domains, Msc1's JmjC domain does not contain critical residues necessary for enzymatic activity. Msc1 is probably enzymatically inactive. It may function as a specific histone methylation binding and targeting factor or it may protect a specific methylation from being demethylated.

Msc1

Lid2

Epe1

Ecm5

Spac1002.05

Spcc622.19

Spac25H1.02

jmjC **PHD** **jmjN** **BRIGHT** **C5HC2 Zinc Finger**

Figure 1.2: Domain configuration of JmjC domain proteins in S. pombe (The figure is reproduced from SMART database (smart.embl-heidelberg.de))

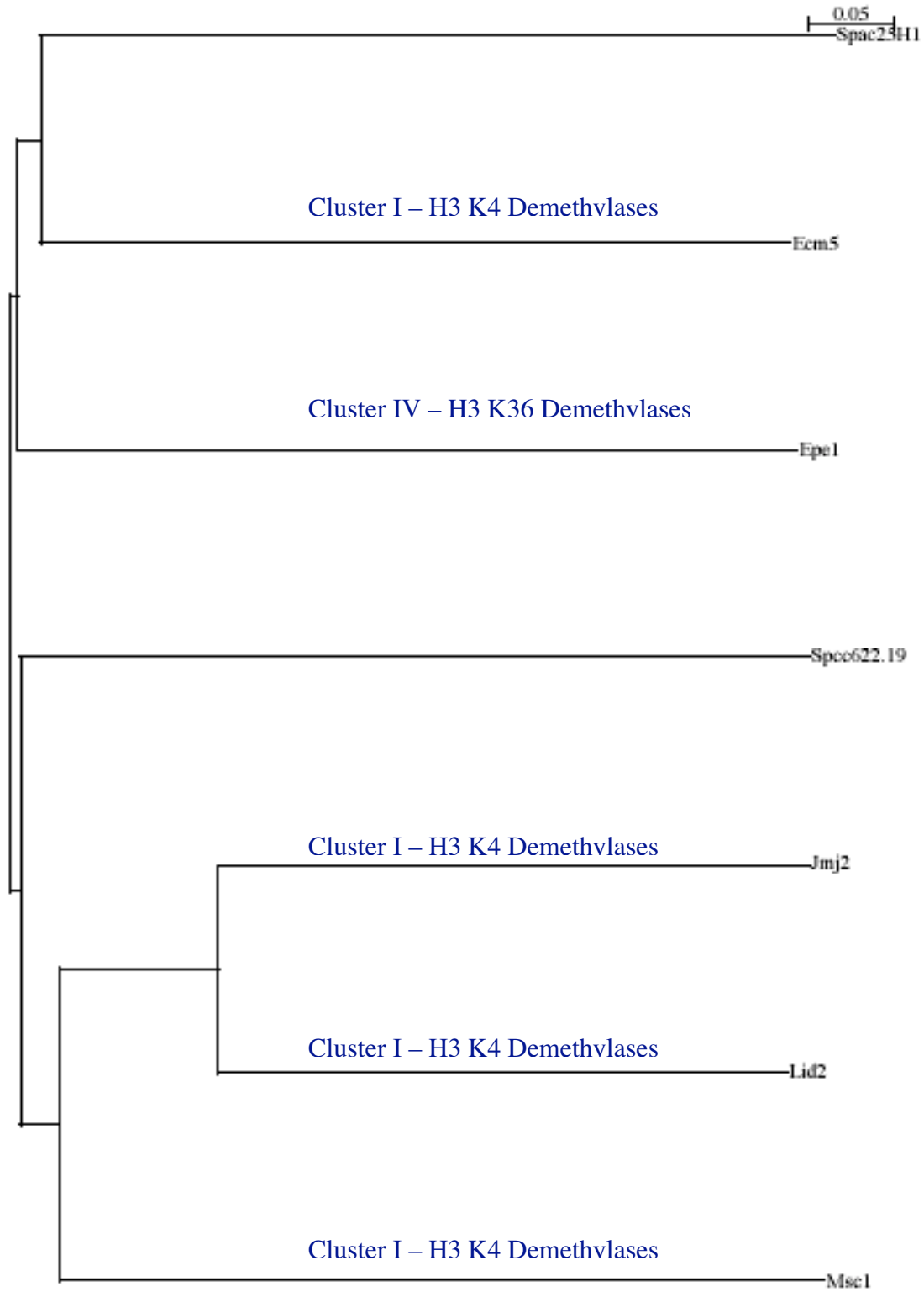

Figure 1.3: Cluster tree of JmjC domains of proteins in S. pombe indicating their degree of similarity. Some of them belonging to the clusters indicated in Table 1.1, are labeled.

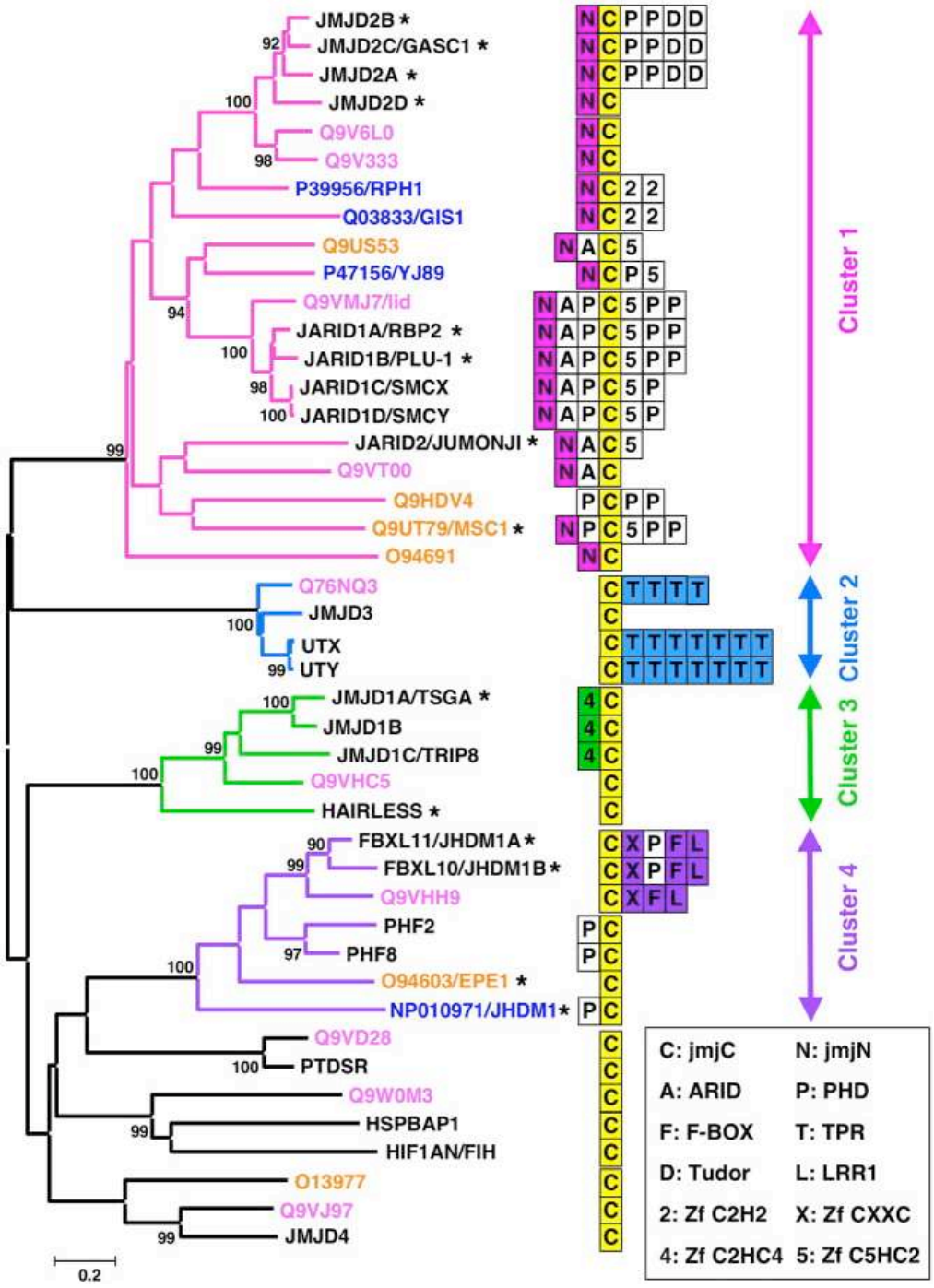
JMJD2B *
JMJD2C/GASC1 *
JMJD2A *
JMJD2D *
Q9V6L0
Q9V333
P39956/RPH1
Q03833/GIS1
Q9US53
P47156/YJ89
Q9VMJ7/lid
JARID1A/RBP2 *
JARID1B/PLU-1 *
JARID1C/SMCX
JARID1D/SMCY
JARID2/JUMONJI *
Q9VT00
Q9HDV4
Q9UT79/MSC1 *
O94691
Q76NQ3
JMJD3
UTX
UTY
JMJD1A/TSGA *
JMJD1B
JMJD1C/TRIP8
Q9VHC5
HAIRLESS *
FBXL11/JHDM1A *
FBXL10/JHDM1B *
Q9VHH9
PHF2
PHF8
O94603/EPE1 *
NP010971/JHDM1 *
Q9VD28
PTDSR
Q9W0M3
HSPBAP1
HIF1AN/FIH
O13977
Q9VJ97
JMJD4
0.2
Cluster 1
Cluster 2
Cluster 3
Cluster 4
C: jmjC
N: jmjN
A: ARID
P: PHD
F: F-BOX
T: TPR
D: Tudor
L: LRR1
2: Zf C2H2
X: Zf CXXC
4: Zf C2HC4
5: Zf C5HC2

Table 1.1: JmjC domain containing proteins were divided into seven subgroups with respect to their domain architecture and JmjC domain homology. The table depicts clustering and domain structure of the proteins from different organisms. The ones with the stars are members that have been shown to have demethylase activity or experimental data about their function (The figure is reproduced from Klose et al. 2006 Nat Rev Genet (Ref no: 1))

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1.4 Protein Complexes and Tandem Affinity Purification:

A protein complex is a group of two or more associated proteins formed by protein-protein interactions that is stable over time. In a protein complex, a number of proteins come together to form a stable assembly or machinery that can achieve multiple functions to complete a job such as DNA replication. To identify a protein complex gives important clues about the function of proteins in the complex and functional relationships between protein members of even different complexes when combined with genetic interaction data and biochemical assays. Immunoprecipitation (IP) is a favored method to identify interaction partners of the target protein. Then, the result of IP is visualized on a SDS-PAGE gel and characterized by mass spectrometry.

A generic purification protocol is available to allow routine protein complex purification for proteome analysis. TAP tag is a generic module that is used in the tandem affinity purification (TAP) method. TAP method is a tool that allows rapid purification under native conditions of complexes, even when expressed at their natural level. Prior knowledge of complex composition or function is not required. The TAP method requires fusion of the TAP tag, either N- or C-terminally, to the target protein of interest. Starting from a relatively small number of cells, active macromolecular complexes can be isolated and used for multiple applications. Its simplicity, high yield, and wide applicability make the TAP method a very useful procedure for protein purification and proteome exploration (Puig et. al. , Pijnappel et al.).

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Pijnappel WW, Schaft D, Roguev A, Shevchenko A, Tekotte H, Wilm M, Rigaut G, Seraphin B, Aasland R, Stewart AF. The S. cerevisiae SET3 complex includes two histone deacetylases, Hos2 and Hst1, and is a meiotic-specific repressor of the sporulation gene program.
Genes Dev. 2001 Nov 15;15(22):2991-3004.

2: RESULTS:

2.1: Msc1, H2A.z, Swr1 Complex and Tandem Affinity Purification: a Proteomic Perspective

2.1.1: Five of seven JDP (JmjC domain proteins) in S. pombe were TAP-tagged and affinity purification was performed:

There are seven JmjC domain proteins in S. pombe. Two of them, Lid2 and Ecm5, were previously identified in Lid2 complex (Roguev et al. 2003). The remaining five proteins were immunoprecipitated using TAP (Tandem Affinity Purification) method. Epe1, Spac1002.05, Spcc622.19 and Spac25H1.02 purifications resulted the proteins itself and it was not possible to identify these proteins in a complex under our experimental conditions. Msc1, the highest molecular weight JDP in S. pombe, purification resulted a protein complex.

2.1.2: Msc1 was copurified with proteins having homology with members of Swr1 complex in S. cerevisae.

Msc1 was purified using TAP method and the resulting material was run on the SDS-PAGE gel and protein bands were analysed using mass spectrometry (Figure 2.1A). The mass spectrometry analysis revealed that Msc1 is a member of Swr1 Complex. Proteins copurifying with Msc1 were homologous to the Swr1 Complex members in S. cerevisae (Figure 2.1A). The composition of Swr1 Complex in S. pombe and S. cerevisae are quite similar, however an important difference is that Swr1 Complex in S. cerevisae does not include a JDP. In S. pombe Swr1 Complex (Swr1C), there are 14 members. Domain configuration of these proteins was shown in figure 2.2. Several other Swr1C members, Swc2, Yaf9 and Swc4, were purified using TAP and almost the same Swr1C composition as Msc1 TAP was obtained (Figure 2.1A, Figure 2.2). In all purifications, Msc1 was a stoichiometric member of Swr1 complex.

2.1.3: Histone H2A variant H2A.z copurifies with Swr1C:

Swr1C in S. cerevisae was shown to load histone H2A variant H2A.z into nucleosomes. It's known that H2A.z interacts with Swr1C in S.cerevisae. However, both Msc1 and Swc2 TAP did not purify detectable amounts of H2A.z. To answer the question of whether H2A.z interacts with Swr1C, H2A.z was purified using TAP (H2A.z-TAP). The resulting protein bands on an SDS-

PAGE gel was shown in Figure 1B. This purification resulted Swr1C, H2B, nucleosome assembly proteins and Kap114, an importin (Figure 2.2). H2A.z interacts with two sets of proteins: one is Swr1C and the other is H2A.z specific proteins independent of Swr1C. It is a point to note that H2A.z TAP results Swr1 Complex although Msc1 and Swc2 TAP do not bring down detectable H2A.z in their Swr1C.

Figure 2.1:

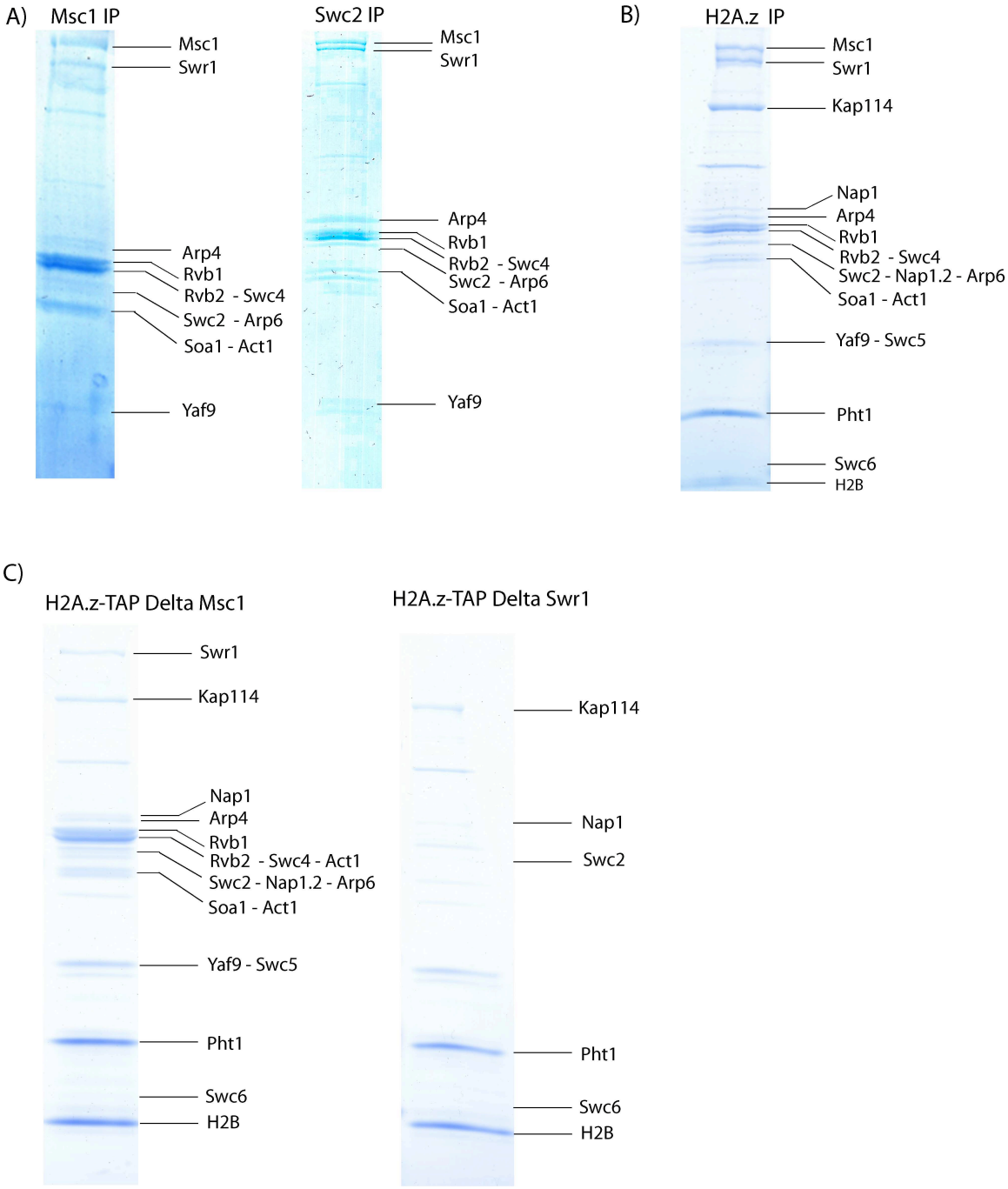

A)
Msc1 IP
Msc1
Swr1
Arp4
Rvb1
Rvb2 - Swc4
Swc2 - Arp6
Soa1 - Act1
Yaf9
Swc2 IP
Msc1
Swr1
Arp4
Rvb1
Rvb2 - Swc4
Swc2 - Arp6
Soa1 - Act1
Yaf9
B)
H2A.z IP
Msc1
Swr1
Kap114
Nap1
Arp4
Rvb1
Rvb2 - Swc4
Swc2 - Nap1.2 - Arp6
Soa1 - Act1
Yaf9 - Swc5
Pht1
Swc6
H2B
C)
H2A.z-TAP Delta Msc1
Swr1
Kap114
Nap1
Arp4
Rvb1
Rvb2 - Swc4 - Act1
Swc2 - Nap1.2 - Arp6
Soa1 - Act1
Yaf9 - Swc5
Pht1
Swc6
H2B
H2A.z-TAP Delta Swr1
Kap114
Nap1
Swc2
Pht1
Swc6
H2B

Figure 2.1: Target proteins were purified using Tandem Affinity Purification (TAP) method and run on the SDS-PAGE gel. Each band on the gel was identified by mass spectrometry. On top of each gel, the protein used for immunoprecipitation (IP) was written, and when purification is performed in the presence of a gene deletion, it's denoted by delta. The protein bands specific for affinity purification were labeled using lines and background contamination was left unlabeled. A) JmjC domain protein Msc1 was purified by TAP method and copurifying proteins, Swr1 complex members, were labeled on the gel. Another Swr1 complex member, Swc2, was purified, Swr1 complex was obtained and bands were labeled. B) Histone H2A variant H2A.z, Pht1 in S. pombe, was purified, Swr1 Complex members and some H2A.z associated proteins were obtained. C) H2A.z affinity purification was performed when Msc1 or Swr1 is missing. H2A.z didn't copurify with Swr1 Complex when Swr1 is missing.

2.1.4: Swr1 is necessary for complex integrity:

To see the effect of loss of Swr1, the motor helicase protein in Swr1C, the gene swr1 was deleted (dswr1) and immunoprecipitations (IPs) were performed. Msc1 TAP brought down only Msc1 and not other members of Swr1C when swr1 is deleted (Figure 2.2, Msc1-TAP dswr1). Msc1 interactions with other Swr1C members are all dependent on Swr1. When H2A.z TAP was performed in swr1 deletion background (H2A.z-TAP dswr1), most of the Swr1C members were missing, only Swc2 and Swc6 were present as part of Swr1C. However, H2A.z associated cluster of proteins including H2B, NAPs and importin were purified in H2A.z TAP (Figure 2.1C, Figure 2.2). In these two IPs, Msc1-TAP dswr1 and H2A.z-TAP dswr1, Swr1C members were mostly lost in TAP purifications suggesting that Swr1 is important for the structural integrity of Swr1C.

2.1.5: Loss of Msc1 does not impair H2A.z interaction with Swr1C:

H2A.z TAP in the presence of msc1 deletion (H2A.z TAP dmsc1) did not show any difference than H2A.z TAP in wild type background (Figure 2.1C). Swr1C integrity and H2A.z interactions were all preserved when msc1 deleted (Figure 2.2).

Figure 2.2:

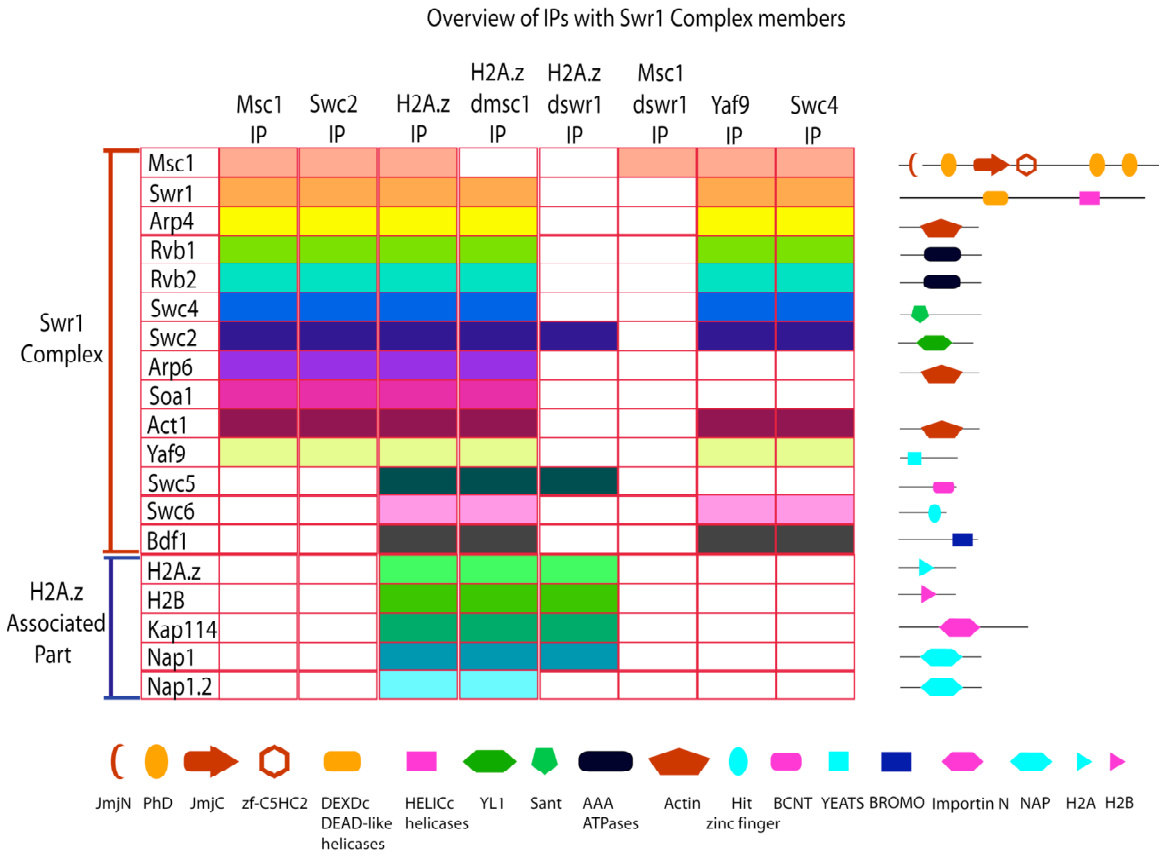


Figure 2.2: Table as an overview of immunoprecipitations (IPs) performed using various Swr1 complex members as baits. Each column represents a different IP and on top of each column the bait for the IP is written. Each row represents a protein obtained as a result of at least one IP. When an IP includes a protein represented by the row, it's colored by the specific color of this row or it's left blank. On the right side of each row, domain structure of the specified protein for that row was represented. Columns have two main parts, one is Swr1 Complex and the other is H2A.z specific part, which is only obtained in H2A.z IPs.

2.2: Msc1, H2A.z and Histone Modifications:

2.2.1: Histone modifications in deletion strains: Loss of H4 K20 Me3

Several deletion strains were constructed: msc1, h2A.z, swr1, lsd1. Lsd1 is a non-JPD histone demethylase. A strain that overxpresses Msc1 was also constructed. In these strains, whole cell extracts were blotted and antibodies against several histone modifications were used. Histone H3 lysine 4 di- and trimethyl (H3 K4 Me2 and Me3), Histone H3 lysine 36 di- and trimethyl (H3 K36 Me2 and Me3), Histone H3 lysine 9 di- and trimethyl (H3 K9 Me2 and Me3), Histone H3 lysine 9 acetylation (H3 K9 Ac), Histone H4 lysine 12 and 16 acetylation (H4 K12 and K16 Ac), Histone H4 lysine 20 mono- and trimethyl (H4 K20 Me1 and Me3) are the histone modifications screened in deletion strains (Figure 2.3). Among these modifications, only H4 K20 Me3 showed a difference. In msc1 and h2A.z deletions, this modification is decreased dramatically (Figure 2.3). This result has been repeated at least in three different experiments. H4 K20 Me1 showed no difference in deletion strains. H4 K20 Me3 is related with heterochromatin in higher eukaryotes and in S. pombe it's shown that this modification has a role double strand break repair to recruit some DNA damage checkpoint proteins (Sanders et al. 2004). It's worth to note that here global levels of histone modifications have been monitored, any changes at local compartments would be missed by this method.

Figure 2.3:

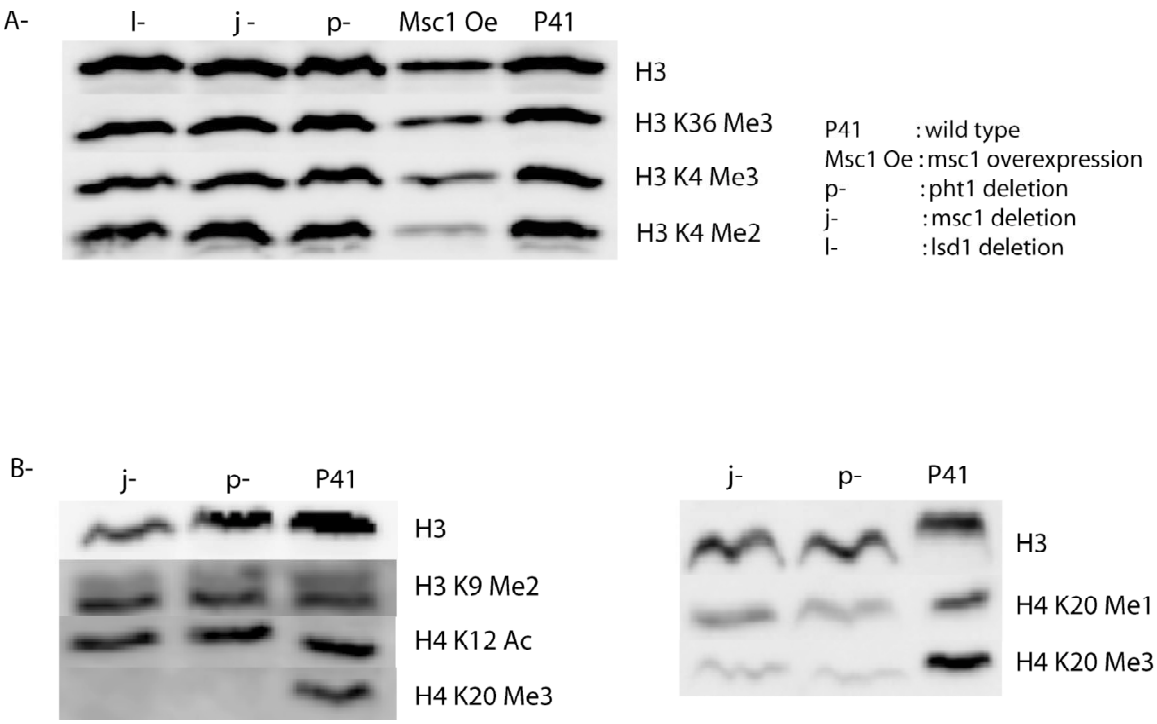


Figure 2.3: Whole cell extracts of different deletion strains were blotted and levels of various histone modifications were detected. H3 and H4: Histone H3 and H4, K: Lysine, Ac: Acetylation, Me1,2,3: Mono-, di- and trimetylation. In pht1(h2A.z) and msc1 deletion Histone H4 Lysine 20 trimethylation is decreased significantly.

2.2.2: Modifications of Histones in H2A.z TAP and Msc1 TAP:

2.2.2.1: Histone Modifications in H2A.z TAP: Underacetylated Histone H4

H2A.z TAP resulted Swr1C and H2A.z associated proteins as previously noted. However, Histone H3 and H4 were detected at very low amounts in mass spectrometry. To be able to screen histone modifications that are brought by H2A.z TAP, histones from H2A.z TAP were blotted and specific antibodies were used. The question was: what were the modifications of histones associated with H2A.z ? To answer this question, H2A.z was purified using TAP method but only first step of the method (IgG binding) was done to increase the yield of histones. IgG beads were then boiled and released proteins were blotted. As a control, whole cell extract (WCE) was used. When histone modification levels were compared between WCE and H2A.z TAP, some modifications have been found to show difference (Figure 2.4A). Histone H3 was used as loading control. H3 K36 Me3, H3 K4 Me3, H3 K9 Ac and H4 K20 Me3 showed no difference in histones from WCE and H2A.z TAP. H4 K16 Ac showed a dramatic decrease in H2A.z TAP suggesting that H2A.z associated histones have a low level of histone H4 acetylation. The next question was: Does loss of Msc1 cause change of any modifications in H2A.z TAP? To answer this question histones from H2A.z TAP and H2A.z TAP dmsc1 (msc1 is deleted) were blotted and levels of modifications were compared (Figure 2.4A). Histone H3 was used as loading control. H3 K36

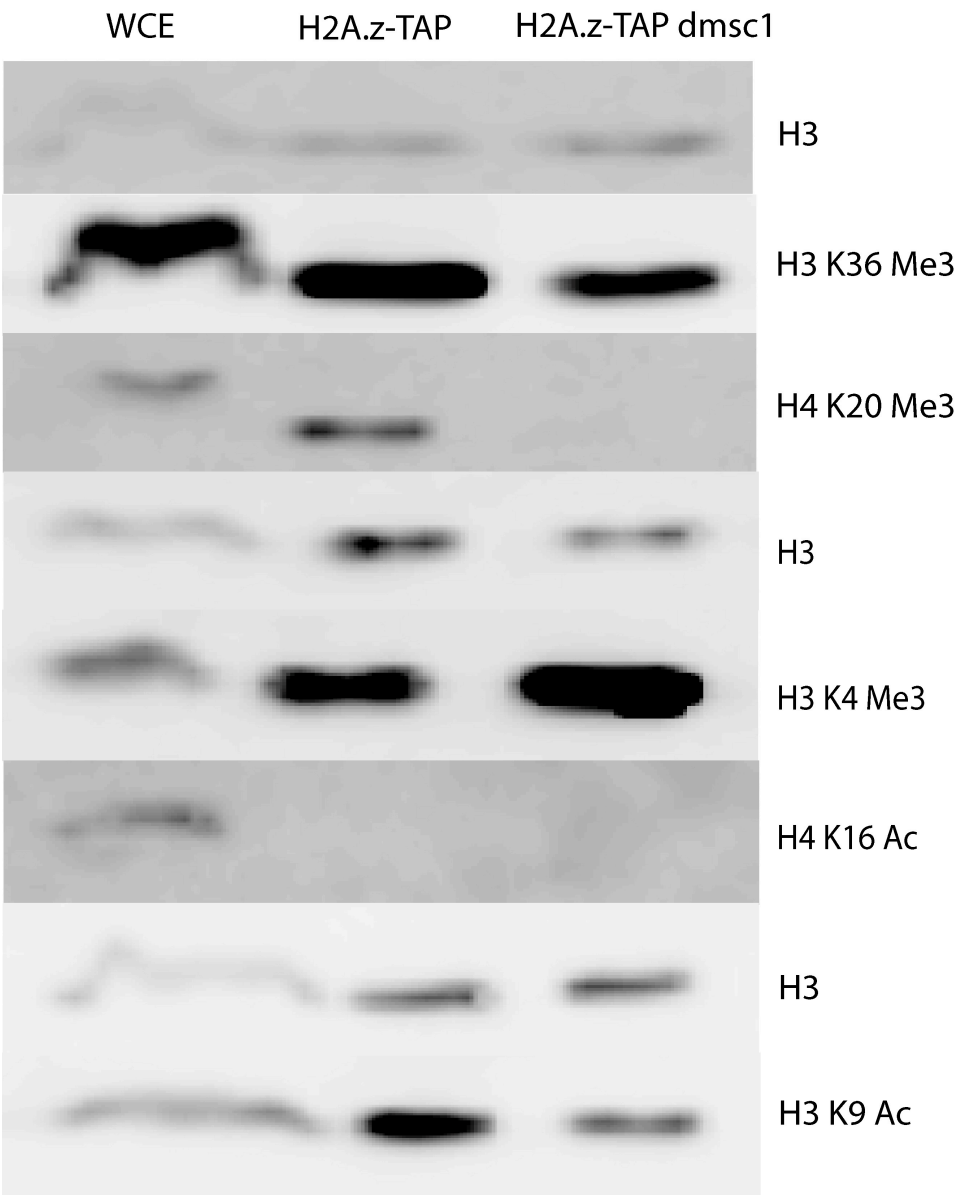
Me3, H3 K4 Me3, H3 K9 Ac and H4 K16 Ac showed no difference between H2A.z TAP and H2A.z TAP dmsc1. H4 K20 Me3 was lost, however this was due to global loss of this modification in msc1 deletion.

2.2.2.2: Histone Modifications in Msc1 TAP: Low H3 K36 Trimethylation

Histones from Msc1 TAP and WCE were blotted and monitored for different modifications (Figure 2.4B). Histones from Msc1-TAP showed a similar pattern as histones from H2A.z TAP. H3 K4 Me3, H3 K9 Me3 and H3 K9 Ac levels did not show a significant difference. H4 K16 Ac and H3 K36 Me3 were significantly decreased in Msc1-TAP when compared to WCE. It is an interesting question to ask whether histones from H2A.z TAP and Msc1 TAP come from exactly the same source? H3 K36 Me3 have been shown to decrease in Msc1 TAP although it seems not to show a dramatic change in H2A.z TAP.

Figure 2.4:

A)


WCE
H2A.z-TAP
H2A.z-TAP dmsc1
H3
H3 K36 Me3
H4 K20 Me3
H3
H3 K4 Me3
H4 K16 Ac
H3
H3 K9 Ac

B)

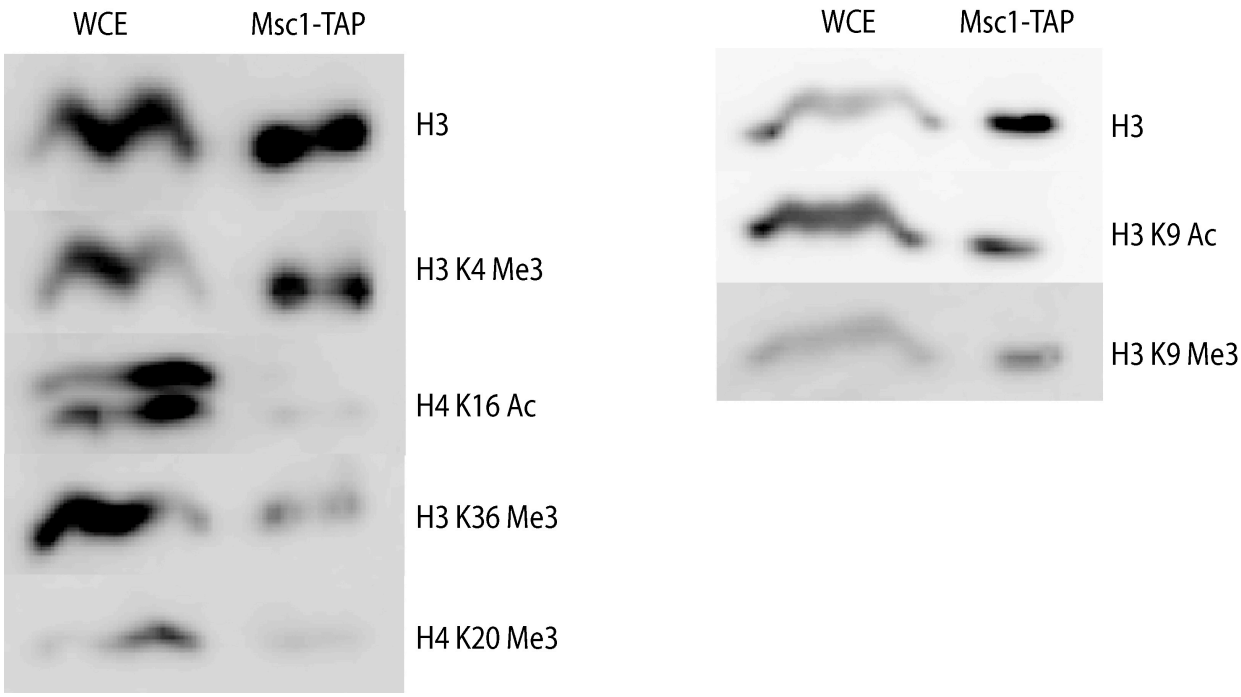


Figure 2.4: Only first step, IgG binding step, of TAP method was used for IPs. The beads were boiled in loading buffer and IPs were blotted. WCE (Whole Cell Extract) was used as a control. H3 and H4: Histone H3 and H4, K: Lysine, Ac: Acetylation, Me1,2,3: Mono-, di- and trimetylation. A) Immunoprecipitations (IPs) using H2A.z as a bait in the presence or absence of Msc1. H2A.z-TAP: H2A.z IP, H2A.z-TAP dmsc1: H2A.z IP when msc1 is deleted. Antibodies used were indicated at right hand side of each row. B) Msc1-TAP: Msc1 IP. Msc1 was used in IPs as a Swr1 complex member and histones copurifiying were blotted.

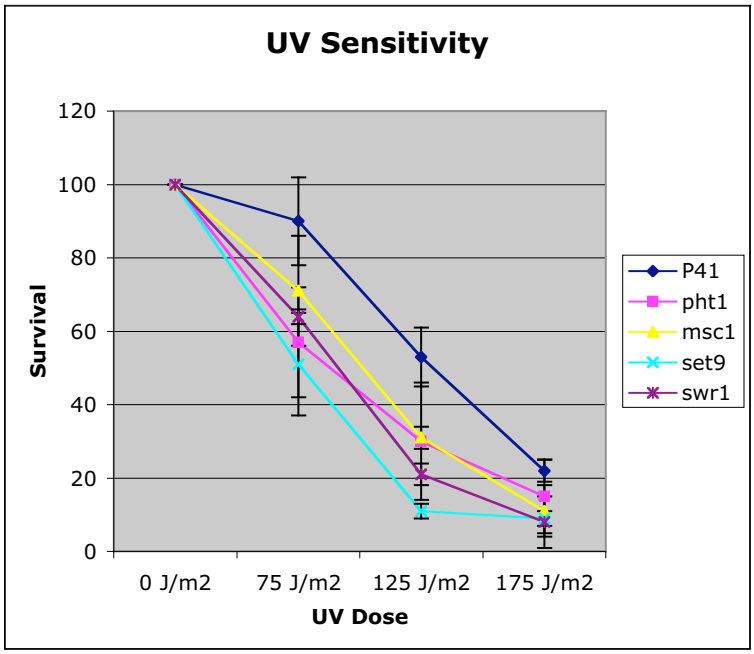
2.3: Deletion Strains and DNA Damage: UV and CMT Sensitivity

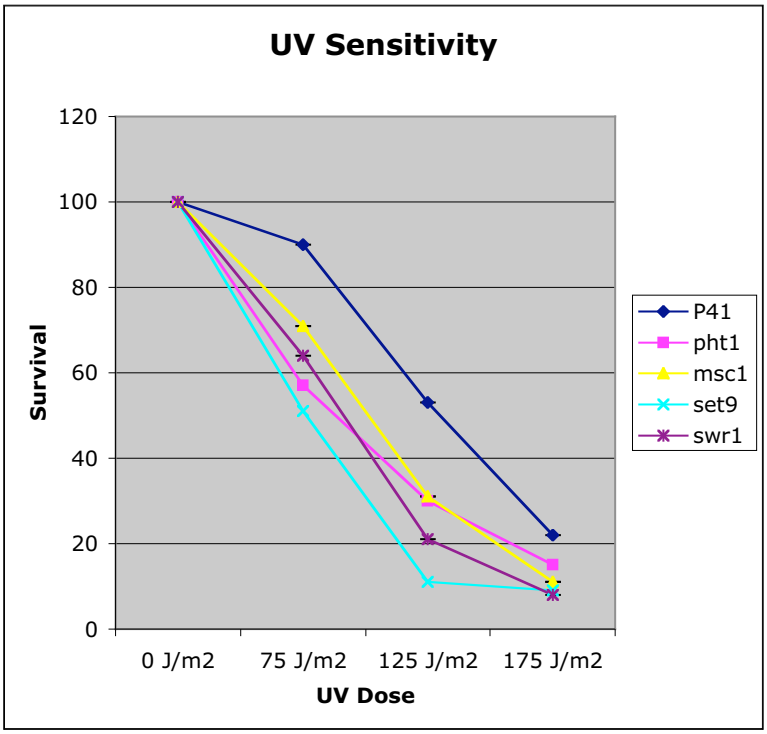
Msc1 overexpression has been shown to compensate the loss of chk1, which has a role in DNA damage checkpoint (Ahmed et al. 2004). It's also known H4 K20 methylation has a role in double strand break repair (Sanders et al. 2004) and we showed that H4 K20 Me3 is lost in msc1 and h2A.z deletions. These findings prompted us to find out the sensitivity of deletion strains to different DNA damage reagents. First, sensitivity to UV was checked in deletion strains msc1, h2A.z, swr1 and set9 (catalyzes methylation of H4 K20 Me3). Number of surviving colonies was counted after UV treatment at different doses 75, 125, 175 J/m2 (Figure 2.5A). All deletion strains have been shown to be sensitive to UV. set9 is the most sensitive. At lower doses swr1 and h2A.z are more sensitive than msc1. Sensitivity to camptothecin (CMT) was checked. Camptothecin binds Topoisomerase I, inhibits its religating activity and causes single strand breaks. Serial dilutions of deletion strains were grown on YE5S plates with different concentrations of CMT: 50, 75 and 100 uM. Deletion strains used were msc1, h2A.z, swr1, set9 and rad6 (Figure 2.5B). h2A.z was the most sensitive to CMT. set9, msc1 and swr1 showed moderate sensitivity. Another reagent, hydroxyurea (HU), is a DNA replication inhibitor and induces double strand breaks near replication forks. Serial dilutions of deletion strains were grown on YE5S plates with different concentrations of HU: 5, 7.5 and 10 mM. msc1, swr1, h2A.z and set9 showed no sensitivity to HU (Figure 2.5C). At higher concentrations, all strains

including wild type, showed same reduced growth (data not shown). MMS (Methyl methanesulfanate) is another reagent inducing double strand breaks. Serial dilutions of deletion strains were grown on YPD plates with different concentrations of MMS: 0.005, 0,0075, 0.01, 0.02, 0.03, 0,04 %. At 0,0075 and 0.01 % MMS, H2A.z showed sensitivity to MMS, however other strains did not show a significant difference than wild type (Figure 2.5C).

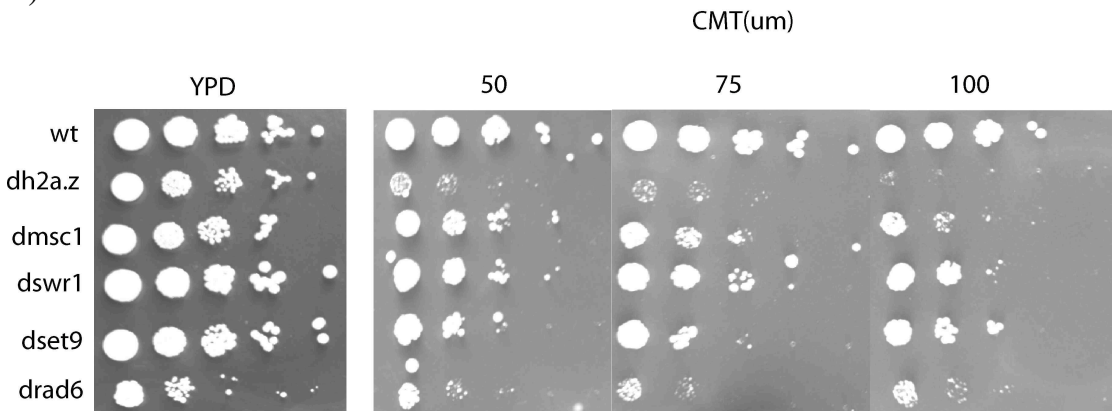
Figure 2.5:

A)


UV Sensitivity
120
100
80
60
40
20
0
Survival
0 J/m2
75 J/m2
125 J/m2
175 J/m2
UV Dose
P41
pht1
msc1
set9
swr1


UV Sensitivity
120
100
80
60
40
20
0
Survival
0 J/m2
75 J/m2
125 J/m2
175 J/m2
UV Dose
P41
pht1
msc1
set9
swr1

B)



C)

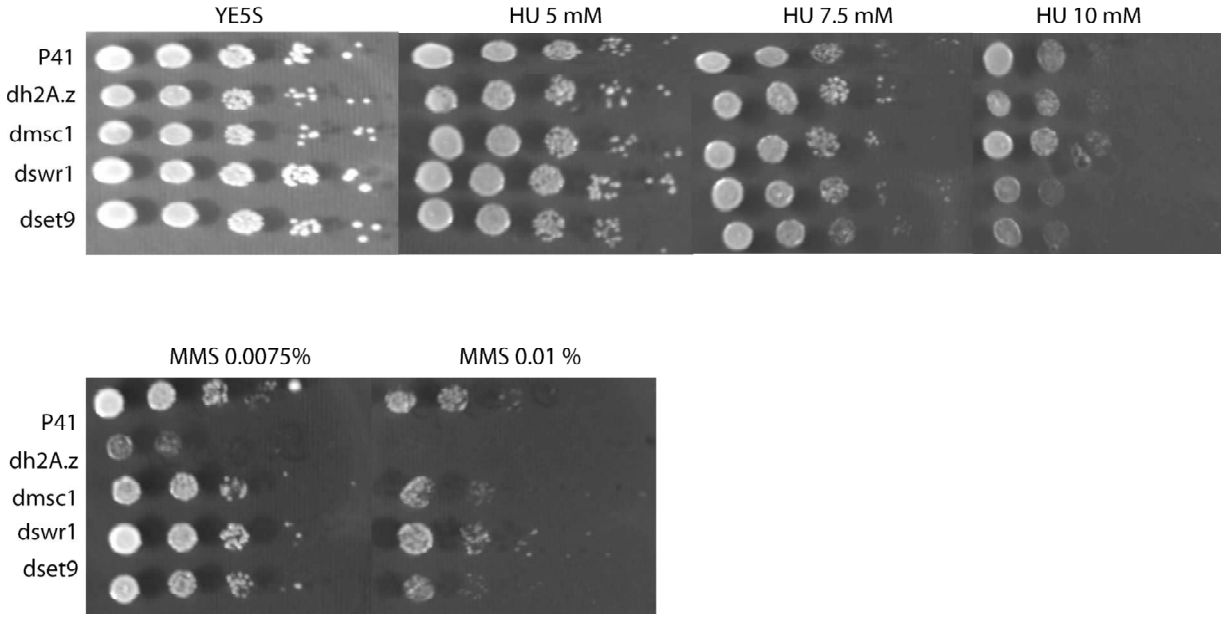


Figure 2.5: Deletion strains of h2a.z (pht1), msc1, swr1, set9, rad6 and wild type strain P41 were used in these sensitivity experiments. A) UV sensitivity of deletion strains were monitored by counting surviving colonies after certain dose of UV. Two graphs represent the same data, however second one is without error bars to have a clear graph for the reader. B) Sensitivity to different concentrations of Camptothecin (CMT) of deletion strains was monitored. C) Sensitivity to Hydroxyurea (HU) and MMS (Methyl methanesulfanate) was monitored. For UV, CMT and HU sensitivity YE5S plates were used, for MMS YPD plates were used.

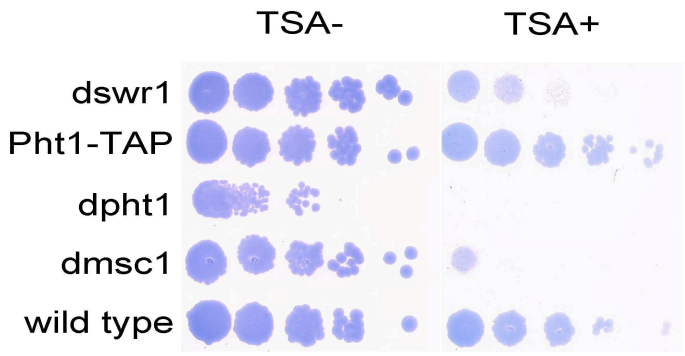
2.4: Deletion Strains, TSA sensitivity and H4 K20 Trimethylation: Network of Interactions

TSA (Trichostatin A) is a histone deacetylase inhibitor. It's reported that msc1 is sensitive to TSA (Ahmed et al.). Serial dilutions of h2A.z (pht1), swr1, msc1 and h2A.z-TAP (pht1-TAP) strains were grown on TSA (25 ug/ml) plates (Figure 2.6A). h2A.z, swr1 and msc1 have been shown to be sensitive to TSA. H2A.z-TAP was not sensitive to TSA, suggesting that TAP tag does not interfere with H2A.z function. TSA sensitivity of deletion strains suggests that Msc1 and H2A.z function in a parallel pathway with histone deacetylases. We also have shown that msc1 and h2a.z have decreased levels of H4 K20 Me3. Then we asked the question whether TSA treatment has any effect on H4 K20 Me3. Wild type cells, which have been treated with TSA, have significantly lower levels of H4 K20 Me3 (Figure 2.6B and 2.6C). Histones from H2A.z TAP were shown to be devoid of H4 K16 acetylation. And we have shown that H4 K16 Ac is increased by TSA treatment (Figure 2.6B).

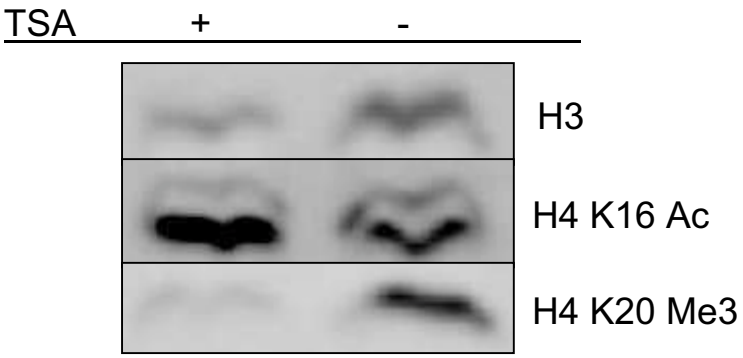
Figure 2.6:

A)

deletion strains are sensitive to TSA, a histone deacetylase inhibitor



B)



C)

TSA effect on Histone H4 K20 Me3

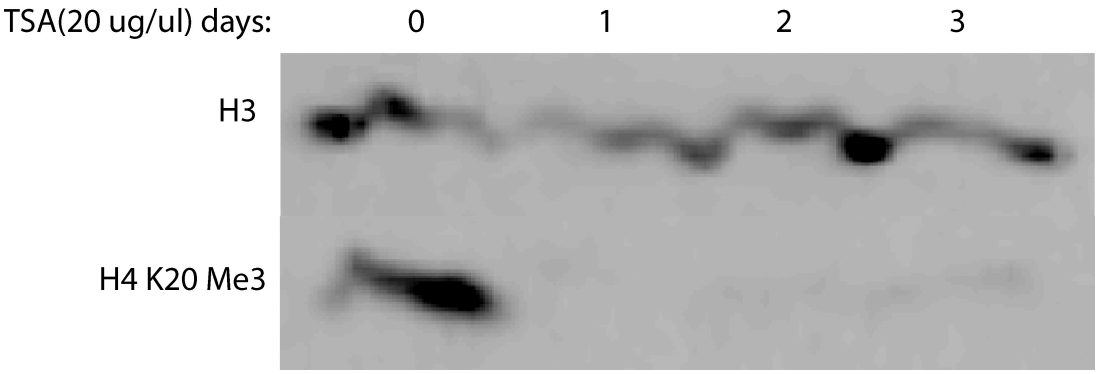


Figure 2.6: A) Serial dilutions of deletion strains of msc1, swr1 and h2a.z (pht1) and Pht1-TAP were grown on YPD plates with or without TSA(25 ug/ml). msc1, swr1 and h2a.z deletions are sensitive to TSA. B) Wild type cells were grown in YPD liquid media with or without TSA (25 ug/ml) for 24 hours and whole cell extracts were blotted. Antibodies used for the blot were indicated at the right hand side. C) Wild type cells were grown in YPD liquid media with or without TSA (20 ug/ml) for 1,2,3 days. Antibodies used for the blot were indicated at the left hand side. H3 and H4: Histone H3 and H4, K: Lysine, Ac: Acetylation, Me1,2,3: Mono-, di- and trimetylation.

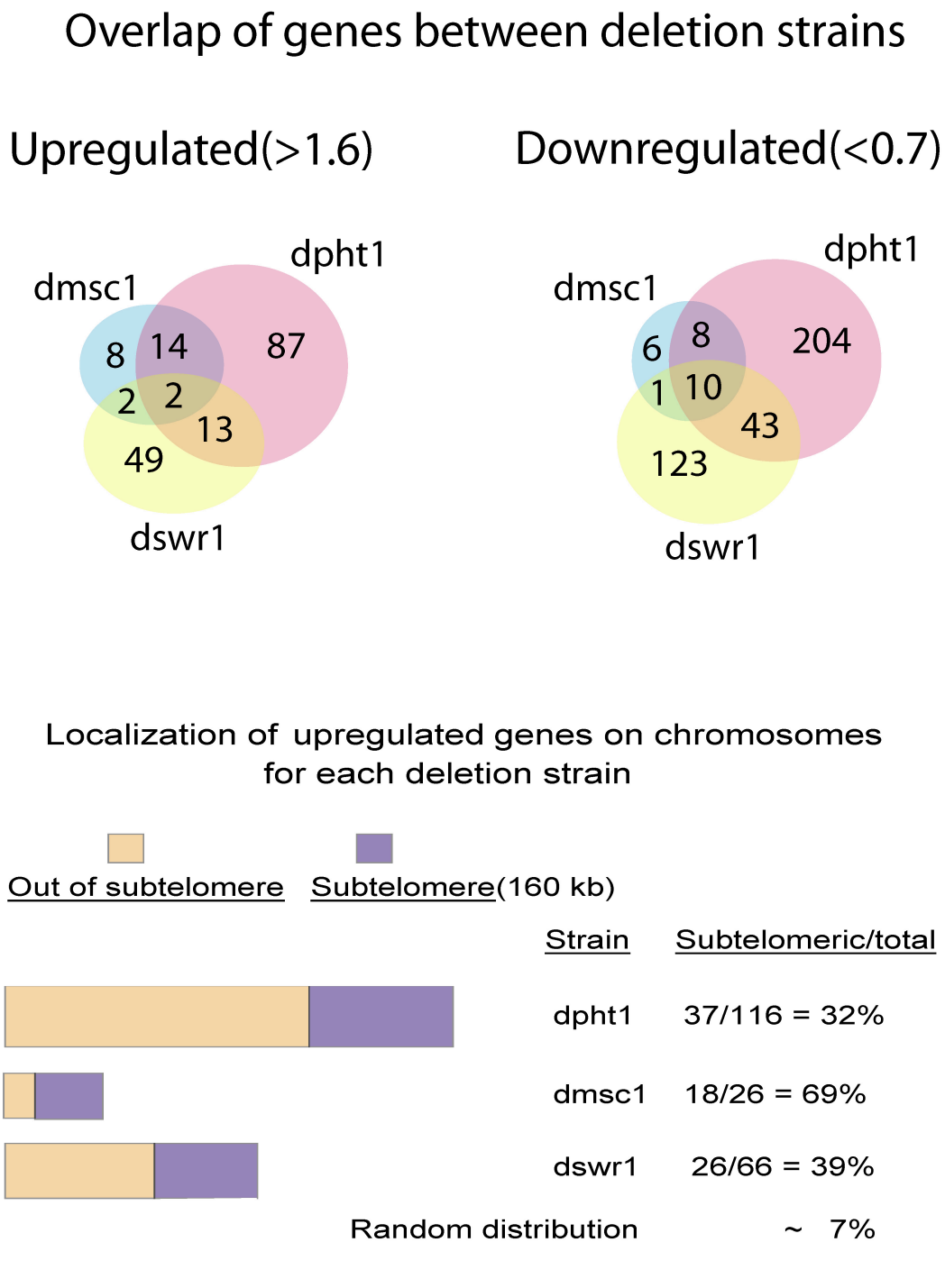
2.5: Gene Expression Profile of Deletion Strains: Subtelomeric Clustering

We've shown that H2A.z, Msc1 and Swr1 are found together in Swr1C. Then, we wanted to analyze gene expression profile of deletion strains h2A.z, msc1 and swr1. Microarray data revealed that there is a significant overlap between expression profiles of deletion strains (Figure 2.7, upper panel). In deletion strains, genes upregulated more than 1.6 fold than wild type and genes downregulated less than 0.7 fold than wild type, were analyzed and the numbers were depicted in venn diagrams for each deletion. The degree of overlap in terms of gene numbers was depicted at intersections of venn diagrams. Upregulated genes in deletion strains show an interesting localization when their position on chromosomes were analyzed for each gene deletion. A significant percent of upregulated genes localize at the end of chromosomes (Figure 2.7, lower panel). We define this end, as 160 kb from the end of chromosome and we name it as subtelomeric region. For h2A.z deletion, 37 out of 116 upregulated genes (32%) were at subtelomeric region. For swr1 deletion, 26 out of 66 upregulated genes (39%) were at subtelomeric region. For msc1 deletion, 18 out of 26 upregulated genes (69%) were at subtelomeric region. Random localization of these genes would result only 7% of genes to reside at subtelomeric region. Upregulated genes for each deletion can be divided into two groups. One group is genes residing at subtelomeric region and the other group is genes residing out of subtelomeric region. When we compare overlap between deletion strains for these two groups seperately,

overlap between genes residing at subtelomeric region is always significantly higher than genes out of subtelomeric region. This difference is more pronounced when the comparison is between h2A.z and msc1 deletions or swr1 and msc1 deletions. The overlap between msc1 and h2A.z deletions or msc1 and swr1 deletions is bigger at subtelomeric region suggesting that Msc1 and H2A.z functions, and Msc1 and Swr1 funtions cooperate at subtelomeric region stronger than other regions.

Another interesting point about upregulated genes are the ratios of stress-induced and meiosis-induced genes. The percentage of stress-induced genes in S. pombe genes is nearly 19%. The ratio of stress-induced genes for upregulated genes in deletion strains is 63%, 59%, 41% respectively for msc1, h2A.z and swr1 deletions. Especially for h2A.z and msc1, there is a significant enrichment compared to background ratio. The percentage of meiosis-induced genes in S. pombe genes is nearly 30%. The ratio of meiosis-induced genes for upregulated genes in deletion strains is 37%, 56%, 39% respectively for msc1, h2A.z and swr1 deletions. In this case, h2A.z has a significant enrichment. The ratio of meiosis-induced genes for upregulated genes for msc1 increases to 44% at subtelomeric region, showing enrichment at this region for msc1.

Figure 2.7:


Overlap of genes between deletion strains
Upregulated(>1.6)
dpht1
dmsc1
8
14
87
2
2
13
49
dswr1
Downregulated(<0.7)
dpht1
dmsc1
6
8
204
1
10
43
123
dswr1
Localization of upregulated genes on chromosomes for each deletion strain
Out of subtelomere
Subtelomere(160 kb)
Strain
Subtelomeric/total
dpht1
37/116 = 32%
dmsc1
18/26 = 69%
dswr1
26/66 = 39%
Random distribution
~ 7%

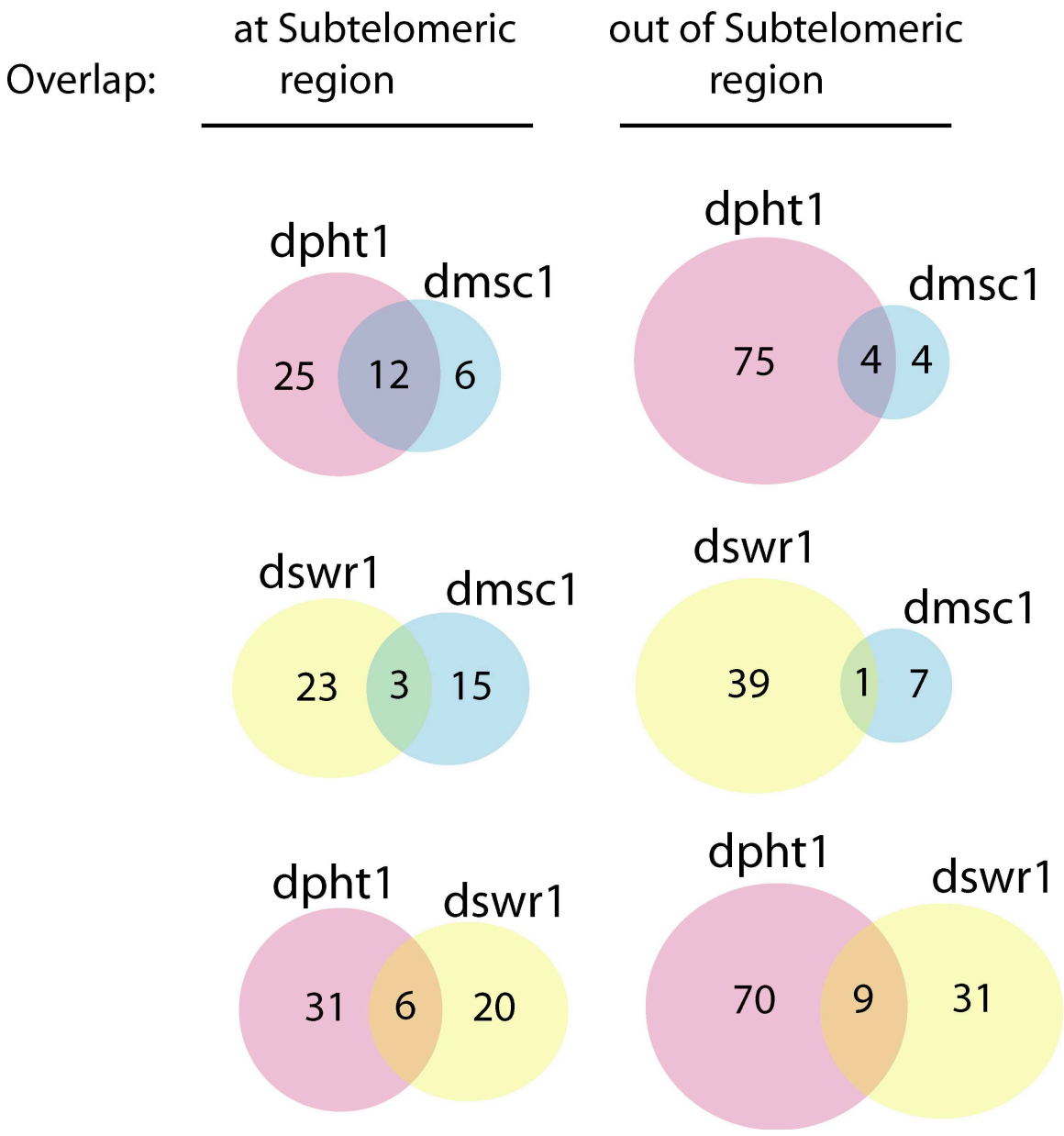


Figure 2.7: Gene expression profiles of deletion strains of pht1 (h2a.z), msc1 and swr1 were analyzed and number of common genes in the profile for upregulated and downregulated genes were indicated in venn diagrams. Each diagram represents a set of differentially expressed genes in a deletion strain. In the second part of figure, the graph represents the percentage of genes localized at subtelomeric region for each deletion. In the last part of the figure, overlaps between deletions at subtelomeric region and out of subtelomeric region for upregulated genes, were depicted using venn diagrams (Primary data is produced by Brain Wilhelm and Jurg Bahler in Wellcome Trust Sanger Institute, Cambridge, UK)

2.6: H2A.z incorporation into chromatin is impaired in the absence Swr1 or Msc1:

H2A.z as an histone variant, is found in some nucleosomes in chromatin. H2A.z-TAP resulted Swr1 Complex. The next question was whether Swr1 and Msc1 function in incorporation of H2A.z into chromatin in S. pombe. To answer this question, ChIP (chromatin immunoprecipitation) method was used. It's possible to amplify H2A.z bound DNA fragment and to monitor H2A.z localization at certain regions of chromatin using ChIP. Using H2A.z-TAP, H2A.z-TAP dswr1 and H2A.z-TAP dmsc1 strains, H2A.z incorporation into chromatin was analyzed. First, DNA immunoprecipitated from H2A.z-TAP, H2A.z-TAP dswr1 and H2A.z-TAP dmsc1, were amplified using random primers to compare levels of total immunoprecipitated DNA. A significant decrease in resulting ChIP DNA was observed in the absence of Swr1 and Msc1 (Figure 2.8). This indicates H2A.z incorporation is impaired at global level. Then several gene regions were selected and H2A.z incorporation to these regions were checked using specific primers. H2A.z incorporation is impaired in both swr1 and msc1 deletions. The impairment is more profound in swr1 deletion (Figure 2.8).

Figure 2.8:

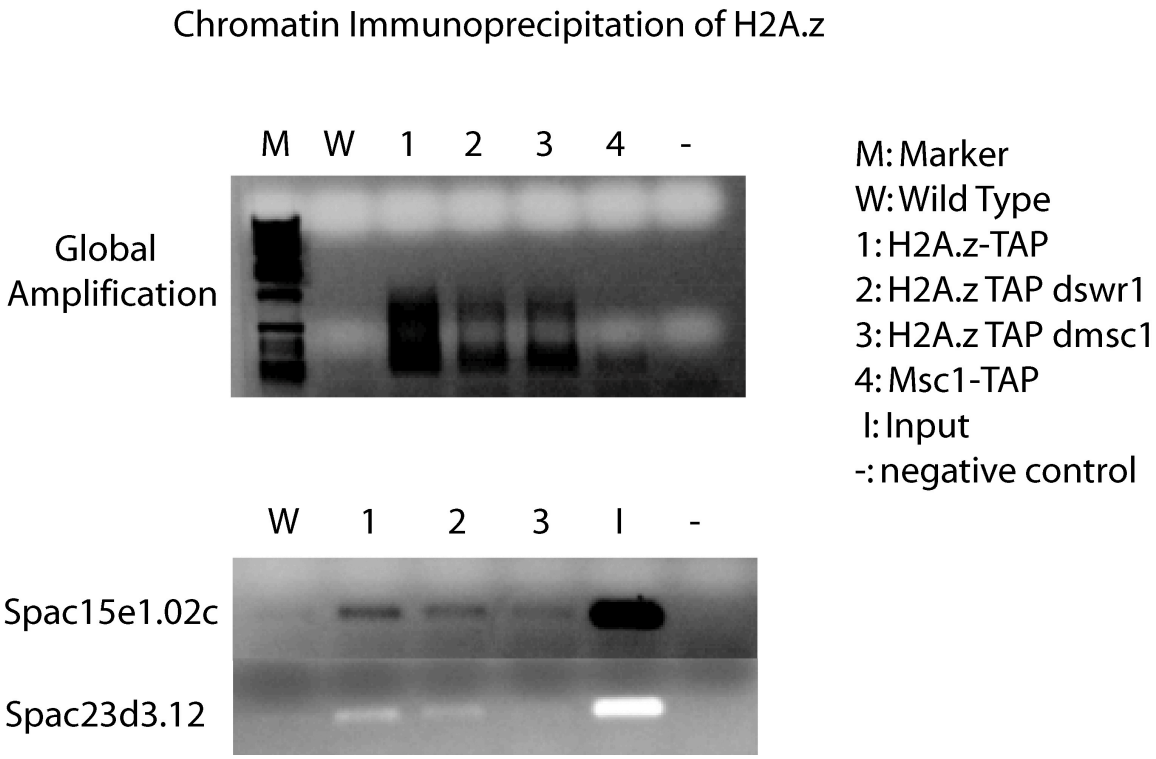


Figure 2.8: ChIP of H2A.z was performed. Resulting DNA was amplified using random primers (global amplification) or primers specific for certain genes. Names of genes were indicated. Amplified ChIP DNA from different strains was run on agarose gel. DNA source for each lane was indicated.

REFERENCES:

1. Sanders SL, Portoso M, Mata J, Bahler J, Allshire RC, Kouzarides T. Methylation of histone H4 lysine 20 controls recruitment of Crb2 to sites of DNA damage. Cell. 2004 Nov 24;119(5):603-14.

2. Ahmed S, Palermo C, Wan S, Walworth NC. A novel protein with similarities to Rb binding protein 2 compensates for loss of Chk1 function and affects histone modification in fission yeast. Mol Cell Biol. 2004 May;24(9):3660-9.

3. DISCUSSION:

3.1 Msc1 is a member of Swr1 Complex:

Msc1 was immunuprecipitated using TAP (Tandem Affinity Purification) method in S. pombe and Swr1 Complex (Swr1C) was purified as a result (Figure 2.1). The same complex was identified in S. cerevisae and Swr1C composition in two species, S. pombe and S. cerevisae, is quite similar, interestingly there is not a homolog of Msc1 in S. cerevisae Swr1C. When it's thought in terms of chromatin, there are several differences between S. pombe and S. cerevisae. Chromatin structure in S. pombe resembles chromatin of higher eukaryotes more than S. cerevisae does. S. pombe has three chromosomes with centromeric and telomeric compartments as heterochromatic regions and there is one silent mating type locus in S. pombe. Histone H3 Lysine 9 and Lysine 20 methylations (H3 K9 and 20 Me) are specific for heterochromatin in higher eukaryotes. These two modifications are missing in S. cerevisae, however S. pombe has these modifications. Msc1 may have something to do with more higher eukaryote-like chromatin structure of S. pombe, when it's considered that there is no homolog of Msc1 S. cerevisae. In S. pombe, there are 14 members of Swr1C. Three other members, Swc2, Yaf9 and Swc4 brought down Swr1C as a result of TAP method. In all these purifications, Msc1 was identified as a stochiometric member of Swr1C.

3.2 H2A.z TAP brings down Swr1C and a seperate group of H2A.z specific interaction partners:

In S. cerevisae, Swr1C is known to load Histone H2A variant H2A.z into chromatin. When H2A.z in S. pombe was purified, Swr1C and H2A.z specific proteins were obtained (Figure 2.1). Purifications with other Swr1C members did not result H2A.z, but H2A.z TAP resulted Swr1C. This may indicate that only a small portion of Swr1C is occupied with H2A.z or for a short time period, Swr1C is occupied with H2A.z. Additionally, H2A.z TAP brought down H2B, nucleosome assembly proteins and an importin. This second group of interactions of H2A.z seems to be independent of Swr1C and H2A.z interaction.

3.3 Swr1 is an important member for complex integrity and Msc1 is a peripheral member of Swr1 Complex:

Swr1 is a helicase and acts as the motor of the complex. It's central to the complex. When Swr1 is deleted, Msc1 purification only resulted Msc1 but not any other member of Swr1C. When Swr1 is deleted, H2A.z purification also did not result Swr1C (Figure 2.1, 2.2). These two findings suggest that Swr1 is central to the complex. On the other hand, it is possible that Msc1 and H2A.z directly bind Swr1 and not any other member in Swr1C. Another important point is that H2A.z keeps interactions with H2B, nucleosome

assembly proteins and importin in the absence Swr1, indicating that these interactions are independent of Swr1C. In the case of H2A.z TAP, there are still two members of Swr1C, Swc2 and Swc5, in the absence of Swr1. When H2A.z TAP was performed in the absence of Msc1, all the interactions of H2A.z is preserved, indicating that Msc1 is not essential for H2A.z interaction with Swr1C. Msc1 seems to be a peripheral member of Swr1C because in the absence of Msc1, H2A.z TAP brings down the whole Swr1C and Msc1 TAP brings down only Msc1 in the absence of Swr1.

3.4 Msc1 and H2A.z is necessary for H4 K20 trimethylation and Msc1 overexpression may stimulate a Crb2 dependent DNA damage pathway:

Deletion strains of msc1 and h2a.z showed a dramatic decrease in Histone H4 K20 trimethylation levels (Figure 2.3B). Other modifications including H3 K4 Me2, Me3, H3 K36 Me3, H3 K9 Me3, H3 K9 Ac, H4 K12 and K16 Ac did not show a difference at global level for total histones. H4 K20 Me3 is related with heterochromatin in higher eucaryotes and in S. pombe it's shown that this modification has a role in double strand break (DSB) repair and recruits Crb2, a DNA damage checkpoint protein, to DNA damage regions. Crb2 is necessary for the activation of Chk1, which in turn inhibits mitotic entry. Deletion of *crb2+* results in cells moderately more sensitive to IR than *chk1_* mutants (Willson et al., 1997). However, double *crb2_-chk1_* mutants are no more sensitive to IR than *crb2_* cells, and Chk1 phosphorylation after

DNA damage is strictly Crb2 dependent (Saka et al. 1997 and Willson et al. 1997). These findings mean that Crb2 is upstream of Chk1, Chk1 function is dependent on Crb2 and Crb2 plays an additional role in DNA damage independent of Chk1. Interestingly, Msc1 was first identified in a screen searching for suppressors of a checkpoint protein chk1. Expression of Msc1 in a multicopy vector can bypass the need of cells for chk1 under damaging conditions. When we consider all these findings together: Msc1 is necessary for H4 K20 trimethylation, H4 K20 methylation is necessary for Crb2 recruitment, Crb2 involves in DNA damage checkpoint, Crb2 is upstream of Chk1 which inhibits mitotic entry and chk1 deletion is suppressed by Msc1 overexpression, these data suggest that overexpression of Msc1 stimulates a pathway related to Crb2 and Histone H4 K20 trimethylation independently from Chk1, as a result, phenotype of deletion of chk1 is suppressed by Msc1 overexpression. Another important point here is the loss of H4 K20 Me3 in h2A.z deletion. This suggests that Msc1 involvement with this modification is directly related with H2A.z and it's not an independent function of Msc1. Recently PHD fingers of Msc1 was found to exhibit E3 ubiquitin ligase activity and Msc1 was found to interact with Rad6 homolog. (Dul and Walworth, 2007) One can speculate that Msc1 induces a modification on H2A.z (such as ubiquitylation) and this modification then acts as an upstream signal for H4 K20 Me3 as in the case of H2B ubiquitylation that is upstream for H3 K4 Me3.

3.5 The chromatin environment of H2A.z: Underacetylated at Histone H4 and normal levels of Histone H3 K4 Trimethylation and K9 Acetylation:

Histones from H2A.z TAP were analysed using antibodies specific for different histone modifications. Whole cell extract (WCE) was used as a control. The aim of this experiment was to find out a specific pattern for posttranslational modifications in H2A.z associated histones. H2A.z associated histones show difference in terms of posttranslational modifications when histone modifications from two different sources of histones: WCE and H2A.z associated histones, were compared. Several histone modifications including H3 K4 Me2 and Me3, H3 K36 Me3, H3 K9 Ac, H4 K20 Me3 and H4 K16 Ac, were screened. H4 K16 Ac showed a dramatic decrease in H2A.z associated histones (Figure 2.4A). This suggests that H2A.z is located in a chromatin environment which is underacetylated at histone H4 but not at histone H3 (Figure 3.1). It was proposed that histone H4 N-terminal tails play role in higher order chromatin structure, thus H2A.z may have some implications in higher order chromatin organization. Another interesting finding is that H4 K20 Me3 levels in H2A.z TAP and WCE. One would expect enrichment of H4 K20 Me3 in H2A.z associated histones because h2A.z deletion causes a dramatic decrease in H4 K20 Me3 levels. However, levels of H4 K20 Me3 in H2A.z TAP and WCE histones are comparable and this suggests that H4 K20 Me3 is not specific for H2A.z associated histones. Levels of H3 K4 Me3 and H3 K9 Ac are comparable in H2A.z TAP and WCE histones. These

modifications are marks for active chromatin and this suggests that H2A.z is located at euchromatic regions although we can not exclude that H2A.z may as well be located at heterochromatic regions. The modifications of H2A.z associated histones define an interesting chromatin environment. This chromatin environment possess euchromatic modifications such as H3 K4 Me3 and H3 K9 Ac, on the other hand, this environment is dramatically underacetylated at histone H4 K12 and K16.

The source of histones obtained from H2A.z TAP is questionable. Do these histones come from nucleosomes or do they come from Swr1C? It's not possible to answer this question certainly, however Swr1C holds H2A.z and H2B as a dimer and the amount of H2A.z-H2B dimer is much more than Histones H3 and H4 in H2A.z TAP. This observation strengthens the possibility that the histones are from nucleosomes that are in contact with Swr1C.

Msc1 TAP resulted histones H3 and H4 as H2A.z TAP does. Posttranslational modification patterns of histones H3 and H4 in Msc1-TAP is quite similar to ones in H2A.z TAP. Acetylation of histone H4 K16 is dramatically decreased compared to WCE and levels of H3 K4 Me3 and H3 K9 Ac are comparable to WCE (Figure 2.4B). One additional difference at Msc1-TAP histones is dramatic decrease of H3 K36 trimethylation when compared to levels at WCE histones. Such a difference was not observed at H2A.z TAP

histones. This may indicate that histones associated with Msc1 TAP represents a specific subgroup of H2A.z associated histones. Histone H3 K36 (H3 K36 Me3) trimethylation is associated with ongoing elongation by RNA pol II (Kizer et al.), thus decrease of H3 K36 Me3 in Msc1 TAP histones suggests that these histones do not localize at coding regions of genes but maybe at promoters.

3.6 The role of H2A.z and Msc1 in DNA damage pathways:

DNA damage is induced by several factors including UV, campthotecin (CMT), hydroxyurea (HU), methyl methanesulfanate (MMS). Deletion strains msc1, h2a.z, swr1 and set9 were tested for their sensitivity to these DNA damage inducing factors (Figure 2.5). h2a.z was found to be sensitive to UV, CMT and MMS. set9 was dramatically sensitive to UV and sensitive to CMT. msc1 and swr1 were sensitive to UV and CMT. Sensitivity of h2a.z deletion strain to CMT and MMS, was significantly higher than other strains. This indicates that H2A.z involves in DNA damage pathways independent of Msc1 and Swr1.

Msc1 was first identified in a screen searching for suppressors of a checkpoint protein chk1. Expression of Msc1 in a multicopy vector, can bypass the need of cells for chk1 under damaging conditions. In one of the upper panels of discussion, it was suggested that overexpression of Msc1 stimulates a

pathway related to Crb2 and Histone H4 K20 trimethylation independently from Chk1; as a result, phenotype of deletion of chk1 is suppressed by Msc1 overexpression. Moderate sensitivity of msc1 deletion strain to DNA damage (CMT and UV) and loss of H4 K20 Me3 in msc1 deletion, strengthen the suggestion that Msc1 involves in a pathway, which is related to Crb2 and Histone H4 K20 trimethylation. We do not know the pathway Msc1 involves in, but this pathway seems to involve DNA damage repair directly or indirectly. Another important point to note here is differential sensitivity of h2A.z and msc1 deletions to CMT and UV. Although both strains have decreased levels of H4 K20 Me3, h2A.z deletion is more sensitive to DNA damage than msc1 deletion. This indicates that loss of H4 K20 Me3 has a moderate and partial effect on DNA damage sensitivity of h2A.z deletion.

3.7 TSA (Trichostatin A) sensitivity of deletion strains: Cooperation with HDACs and low histone H4 acetylation of H2A.z nucleosome

TSA is an HDAC (histone deacetylase) inhibitor. In the presence of TSA, global histone acetylation levels are increased in chromatin. Deletion strains of msc1, h2a.z and swr1 were shown to be sensitive to TSA (Figure 2.6A). It's worth noting that all three strains show TSA sensitivity. Msc1, Swr1 and H2A.z are found in the same complex and TSA sensitivity points out a function that is performed by Swr1C. Sensitivity of deletion strains suggests that triple of Msc1, Swr1 and H2A.z, involves in a parallel pathway with HDACs.

HDACs are best known in gene silencing. However, cooperation of H2A.z with HDACs seems to be controversial when the data from S. cerevisae is considered. Because, in S. cerevisae, H2A.z was shown to function in gene activation and to function as an antogonist of heterochromatin. In this situation, one would not expect H2A.z to be sensitive to TSA. On the other hand, H2A.z was shown to localize at centromeric region and interact with one type of heterochromatic protein in higher eucaryotes. So in higher eucaryotes, H2A.z seems to be more related with heterochromatin. Chromatin organisation of S. pombe resembles higher eucaryotes more than chromatin of S. cerevisae does. It's likely that H2A.z in S. pombe is more similar to H2A.z of higher eucaryotes than H2A.z of S. cerevisae.

In figure 2.4A, it was shown that H2A.z associated histone H4 has low levels of acetylation although level of histone H3 acetylation is normal. This suggests that chromatin environment of H2A.z is devoid of H4 acetylation. One can speculate that H2A.z containing nucleosome is also deacetylated at histone H4 and these two factors on a nucleosome, presence of H2A.z and absence of histone H4 acetylation, play a role in the establishment of a yet unknown function. This function may be related to gene expression or higher chromatin structure. We also know that h2A.z and msc1 deletion strains are sensitive to TSA. Sensitivity of h2A.z deletion to TSA, suggests that at least one of the two properties of H2A.z nucleosomes is essential for cell viability. These two properties are the presence of H2A.z and absence of histone H4 acetylation. And sensitivity of h2A.z deletion to TSA can be explained with the requirement of H2A.z presence or absence of histone H4 acetylation in an

H2A.z containing nucleosome for cell survival.

Msc1 is a member of Swr1C and it's thought to be a peripheral member of the complex with respect to the previous data. Its function may be targeting of the complex, binding a specific modification or directing an enzymatic activity to H2A.z nucleosome. Msc1 TAP resulted Swr1C and small amounts of histones. These histones were detected by antibodies specific for different posttranslational modifications. Histones from Msc1 TAP also had low levels of histone H4 acetylation concordant with H2A.z associated histones. These two different purifications, one from a histone variant and another from a member of Swr1C, resulted histones and both resulted histones with low level of histone H4 acetylation. This strengthens the idea that H2A.z nucleosomes have low level of histone H4 acetylation and also suggests that Swr1C and Msc1 participate in loading H2A.z into a nucleosome lowly acetylated at histone H4 (Figure 3.1). Deletion of msc1 causes TSA sensitivity as h2A.z deletion. Low level of H4 acetylation with Msc1 associated histones makes sense when one considers sensitivity to TSA. Because TSA is a HDAC inhibitor and sensitivity to TSA suggests cooperation with HDACs. Low level of H4 acetylation with Msc1 associated histones, suggest HDAC activity on these histones supporting a cooperation with HDACs. One important point about the TSA sensitivity of msc1 and h2A.z deletions is their comparable degree of sensitivity. At tested concentration, both strains show similar sensitivity to TSA. On the other hand, when one considers sensitivity to CMT and UV, h2A.z deletion was more sensitive than msc1 deletion. This suggests that common and main action, which brings H2A.z and Msc1 together, is demonstrated by TSA sensitivity.

msc1 and h2A.z deletions meet at two outputs: TSA sensitivity and loss of H4 K20 Me3. It was also known that both Msc1 TAP and H2A.z TAP resulted histones with low levels of histone H4 acetylation. All these phenotypes were related to histone H4 N-terminal tail. Then the next question was whether there is a connection between TSA sensitivity and loss of H4 K20 Me3. To answer this question, two experiments were performed. First one was to check TSA sensitivity of set9 deletion and second one was to check whether TSA treatment affect levels of histone H4 K20 Me3 levels. set9 deletion was not sensitive to TSA. On the other hand, TSA treated cells had lower level of H4 K20 Me3. This indicates that trimethylation of H4 K20 is dependent on HDACs, although loss of histone H4 K20 trimethylation does not affect cells' sensitivity to an HDAC inhibitor. These data suggest that HDACs are upsteam of histone H4 K20 trimethylation. Both HDACs and H2A.z, Msc1 doublet are necessary for the maintenance of histone H4 K20 trimethylation.

3.8 Gene expression analysis of deletion strains and subtelomeric localization of upregulated genes

Gene expression profile of deletion strains h2A.z, msc1 and swr1 was analysed. Microarray data revealed that there is a significant overlap between expression profiles of deletion strains (Figure 2.7, upper panel). The number of downregulated genes in h2a.z (pht1) and swr1 deletions was more than the number of upregulated genes. In msc1 deletion, the number of up- and downregulated genes was similar. This suggests that H2A.z and Swr1 function more in gene activation than in gene silencing although there is quite a number of genes upregulated in both h2A.z and swr1 deletions. The number genes,

which have altered expression, are much lower in msc1 deletion than in h2A.z and swr1 deletions. This suggests that function of Msc1 is narrower than of Swr1 and H2A.z or it does not directly affect gene expression.

Analysis of gene expression profile was done in terms of localization of genes on chromosomes. It was checked whether genes, with altered expression in deletion strains, cluster at certain regions on chromosomes. For downregulated genes, any preference for any region on the chromosome could not be observed. For upregulated genes, a significant portion of genes was found to cluster at subtelomeric region. Subtelomeric region is defined as 160 kb from the end of the chromosome. For h2A.z deletion, 37 out of 116 upregulated genes (32%) were at subtelomeric region. For swr1 deletion, 26 out of 66 upregulated genes (39%) were at subtelomeric region. For msc1 deletion, 18 out of 26 upregulated genes (69%) were at subtelomeric region. Random localization of these genes would result only 7% of genes to reside at subtelomeric region. There is a quite significant clustering at subtelomeric region, when one compares ratios for deletion strains and random localization. Same phenomenon was observed also for some HDACs, they affect expression of subtelomeric genes (Wiren et al. 2005). This may be an explanation for TSA sensitivity of deletion strains. Another interesting finding about HDACs, is that Hos2 has an unexpected role in promoting high expression of growth-related genes by deacetylating H4 K16 Ac in their open reading frames (Wiren et al. 2005). In this study, it was shown that H2A.z associated histone H4 has low

levels of lysine 16 (K16) acetylation. Hos2 may maintain low H4 K16 Ac levels in H2A.z associated histones. It's possible that Hos2 and H2A.z cooperate in promoting high expression of growth-related genes. This may be another explanation for TSA sensitivity of deletion strains. Clr6 is the primary target of TSA in S. pombe. Mutant of clr6 has high acetylation at histone H3 K9, H3 K14, H4 K5, H4 K12 and H4 K16 at intergenic regions (IGRs) but not at open reading frames (ORFs). This indicates that primary effect of Clr6 is on promoter regions. Clr6 is the principal enzyme that functions in promoter-localized repression (Wiren et al. 2005). H2A.z in S. cerevisae has been found to localize at repressed promoters (Zhang et al. 2005). This is a common point between Clr6 and H2A.z, both function at repressed promoters. An interesting point about Clr6 is the ratio of induced genes in 72 Clr6 repressed genes with high IGR acetylation in clr6-1 mutant. 71% of these 72 repressed genes are induced during meiosis and 46% induced by environmental stress (Wiren et al. 2005). For H2A.z, 56% of 116 H2A.z repressed genes are induced during meiosis and 59% induced by stress (results section 2.5). We know that H2A.z at repressed promoters makes genes potentially ready for activation or inducible when a signal for activation comes (Zhang et al. 2005). This is the second common point between Clr6 and H2A.z, that both function at repressed promoters that are later induced at certain conditions.

Upregulated genes for each deletion can be divided into two groups. One group contains genes residing at subtelomeric region and the other contains

genes residing out of subtelomeric region. When we compare overlap between deletion strains for these two groups seperately, overlap between genes residing at subtelomeric region is always significantly higher than genes out of subtelomeric region. This difference is more pronounced when the comparison is between h2A.z and msc1 deletions. The overlap between msc1 and h2a.z deletions is more stronger at subtelomeric region (Figure 2.7). This indicates Msc1 function in Swr1C is focused at subtelomeric region and Msc1 involves in or cooperates with H2A.z function at subtelomeric region. It's tempting to speculate that Msc1-H2A.z doublet and HDACs maintain a temporary and facultative repressed state at subtelomeric region and when these genes at subtelomeric region are in need, they are activated (Figure 3.2). It was found out that H2A.z poises repressed promoters for activation through histone loss in S. cerevisae (Zhang et al. 2005). A similar mechanism may be in process for subtelomeric genes in S. pombe through a cooperation of H2A.z-Msc1 doublet and HDACs. Some subtelomeric genes, including stress induced and meiotic genes, may be poised at repressed state and presence of H2A.z may be a mark for activation at proper time in these genes (Figure 3.2).

3.9 Possible roles of Msc1 in Swr1C

I made an analysis of the experimental data so far and in the light of these analyses, one can speculate about the specific functions of Msc1. Histones from Msc1 TAP have lower level of histone H3 K36 trimethylation and an

average level of histone H3 K4 trimehylation. H3 K36 trimethylation is enriched at ORFs and is not abundant at promoters. One can speculate that Msc1 in Swr1C complex targets H2A.z to some region other than ORFs, possibly promoters of genes at certain regions (Figure 3.1). The best way to answer this question is ChIP-on-chip method with H2A.z TAP and H2A.z TAP dmsc1 strains. By using this method, one can both find out global localization of H2A.z and also effect of loss of Msc1 on this localization. If there is such a targeting to promoters, this would be to subtelomeric region primarily or only an effect at subtelomeric region was observed because of redundancy. TSA sensitivity of Msc1 suggests cooperation with HDACs. Low level of H4 K16 Ac in histones from Msc1 TAP strengthens the reliability of Msc1 and HDAC cooperation. One can speculate that Msc1 involves in H2A.z mediated repression and poising of inducible genes at subtelomeric region. There are intriguing possibilities about the biochemical activities of Msc1. Recently PHD fingers of Msc1 was found to exhibit E3 ubiquitin ligase activity and Msc1 was found to interact with Rad6 homolog (Dul and Walworth, 2007). It's tempting to speculate that Msc1 ubiquitylates H2A.z or H2B during incorporation of H2A.z into chromatin. Such kind of ubiquitylation would have some functional implications. Msc1 has JmjC domain and this domain is known to have histone lysine demethylase activity. Although JmjC domain of Msc1 is missing some of essential residues for enzymatic activity, it may bind to a specific methylated lysine and protect it from demethylation by active demethylases. One such a candidate is histone H4 K20 trimethylation. This modification is lost in the

absence of Msc1. Maintenance of H4 K20 trimethylation may depend on ubiquitylation of H2A.z or H2B by a mechanism dependent on Msc1. It's known that H3 K4 methylation depends on H2B ubiquitylation by Rad6 in S. cerevisae, a similar mechanism may take part here.

3.10 Synthesis: H2A.z and Msc1 cooperate with HDACs for temporal repression of inducible genes by forming a poised chromatin at promoters

Htz1, the yeast H2A.Z homologue, is preferentially present in promoter regions of euchromatic genes, and that this presence is inversely proportional to transcription rate and occupancy of RNA polymerase II (PolII). Htz1 is enriched at inactive genes and that high transcription is associated with less occupancy of Htz1. Htz1 preferentially resides at inactive promoters and may be involved in optimal transcription activation (Li et al. 2005). It was shown that Htz1 is enriched at the GAL1 and PHO5 promoters under repressive conditions and disappears upon gene activation (Santisteban et al. 2000). Incorporation of Htz1 during the inactive state may serve as a stable epigenetic mark but may not play a dominant role in transcription per se. Perhaps it keeps promoters in a repressed state until the appropriate activation signal is received (Li et al. 2005). Specific incorporation of the histone variant H2A.Z into the recently repressed promoter mediates retention at the nuclear periphery and rapid reactivation (Guillemette et al. 2005). These findings show that H2A.z resides at promoters when they are repressed or not being fired by transcription machinery and is necessary for optimal transcription rate.

Clr6, an HDAC in fission yeast, is the principal enzyme that functions in promoter-localized repression (Wiren et al. 2005). An interesting point about Clr6 is the ratio of induced genes in 72 Clr6 repressed genes with high IGR acetylation in clr6-1 mutant. 71% of these 72 repressed genes are induced during meiosis and 46% induced by environmental stress (Wiren et al. 2005). In deletion strains, we've shown a enrichment of stress-induced and meiosis-induced genes for upregulated genes in h2A.z and msc1 deletions (section 2.5). As H2A.z resides repressed inducible promoters, Clr6 functions in repression of inducible genes.

In our study, TSA sensitivity of h2A.z and msc1 deletions and low level H4 acetylation in H2A.z and Msc1 associated histones, suggest cooperation of H2A.z with HDACs and formation of deacetylated and H2A.z containing nucleosomes. In addition to that, subtelomeric localization of upregulated genes and high percentage of inducible genes in upregulated genes in h2A.z and msc1 deletion strains, point out a role in the repression of inducible genes in cooperation with HDACs. One can propose that H2A.z resides at inducible promoters of repressed state and these promoters are deacetylated by HDACs. Presence of H2A.z and absence of acetylation maintains a promoter that is paused and silent, on the other hand ready for activation. In this model, H2A.z poises promoters in off state. however, H2A.z still keeps promoter's ability to positively response to transcriptional activation (Figure 3.2A). It's also proposed that chromatin environment maintained by H2A.z and HDACs, is

necessary for histone H4 K20 Me3 (Figure 3.2B). This modification is erased when H2A.z or Msc1 is absent or it's treated with TSA (Figure 2.3 and Figure 2.6c). H4 K20 Me3 may involve in nucleosome stability, promoter poising or formation of an inaccessible chromatin. Presence of H2A.z seems to be a mark for a gene to be inducible although it's repressed at the moment. This role of H2A.z spreads genome wide with significant enrichment at subtelomeric region. Msc1 seems to have a role more concentrated at subtelomeric region in this scenario. It's known that in clr3 deletion, increase of histone H3 K14 acetylation significantly overlap with 0-25 kb subtelomeric region (Wiren et al. 2005). One of the best ways to reveal the details of cooperation between H2A.z, Msc1 and HDACs is to compare and find out the degree of correlation of gene expression and ChIP on chip data from the following experiments: 1. Localization of H2A.z genomewide with and without Msc1, 2. Genes upregulated in msc1 and h2A.z deletions, 3. Genes upregulated in deletion of HDACs such as clr6 and clr3, 4. Genomewide distribution of histone H4 K16 Ac (and additionally H3 K14 Ac) in wild type and clr6, clr3 deletions. Especially comparison of experiments 2 and 4, will give an idea about the relationship between H2A.z and Msc1 function and site specific histone acetylation. Also, a ChIP experiment on an inducible promoter to reveal the levels of H2A.z and histone modifications such as H4 K16 Ac, H4 K20 Me3 before and after induction, would be quite useful.

H2A.z functions in activation of many genes, in fact more than two times of genes repressed by H2A.z (Figure 2.7). Interestingly a mechanism, which is very similar to the one for inducible repressed promoters in figure 3.2, can be used to explain the role of H2A.z in transcriptional activation. After a promoter is on, it's fired by transcriptional machinery repetitively.

Transcriptional initiation includes chromatin opening, histone acetylation and histone eviction (reviewed in Li et al. 2007.). The proposed model by our study, suggests that the promoter must be restored after each firing (Figure 3.3). It was suggested that deacetylation by Rpd3 Complex and Htz1 incorporation by Swr1 Complex functions in restoration of chromatin after H2A phosphorylation and H4 acetylation in DNA repair (Utley et al. 2005, van Attikum et al. 2005). A similar restoring mechanism might take role after firing of promoter of genes. Restoring includes formation of a closed, stable or even poised chromatin. In our model, H2A.z functions in formation of such a restored promoter after transcriptional firing in cooperation with HDACs. This promoter would be reassembled and ready for refiring by transcription machinery. This restoration would provide efficent and repetitive transcription firing. When H2A.z is absent, such a restoration of promoter nucleosomes after firing would be inefficient and rapid refiring of promoter would be defective causing downregulation of gene expression (Figure 3.3).

Figure 3.1: Model: Special Chromatin Environment Associated with H2A.z and Msc1

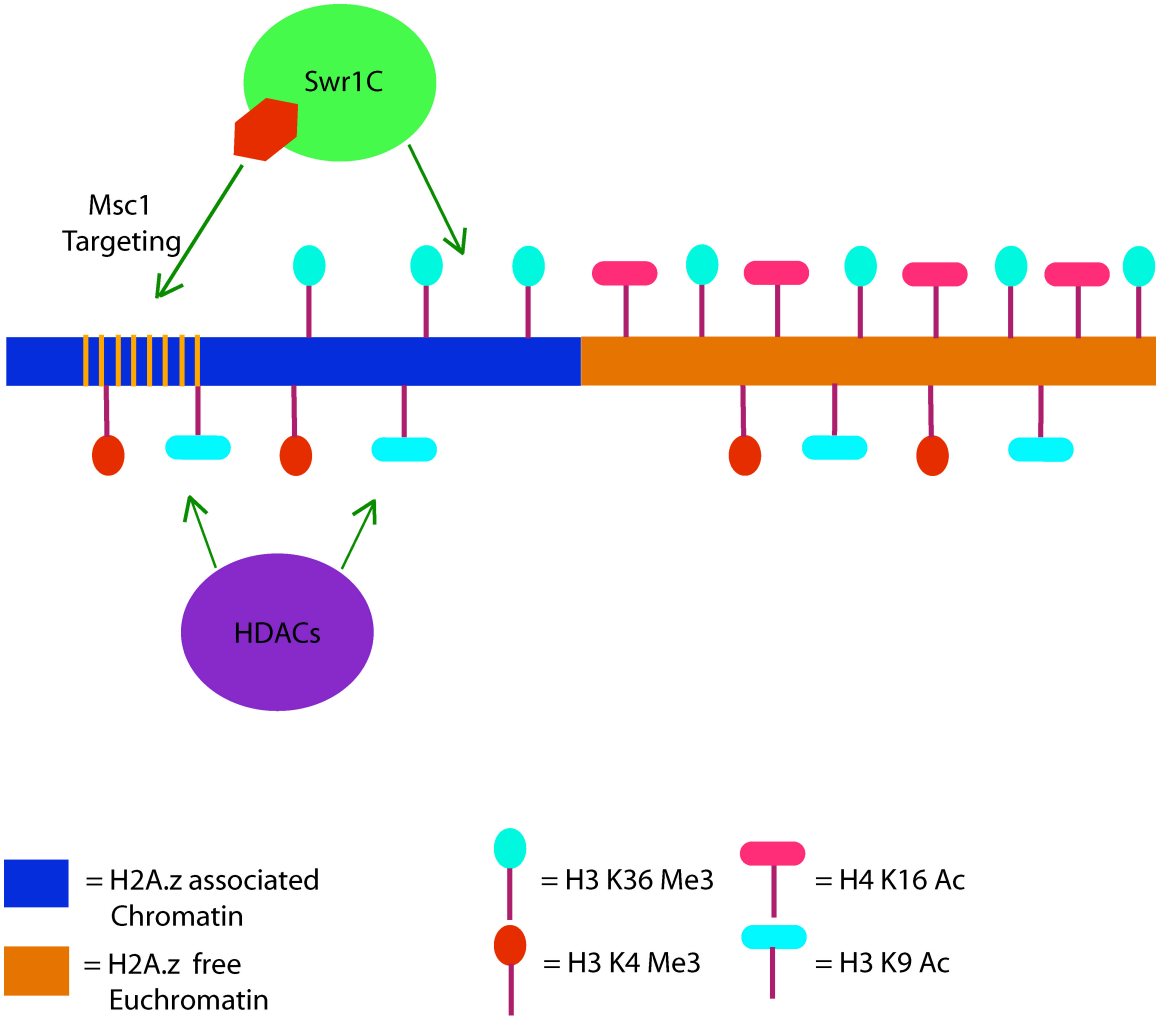
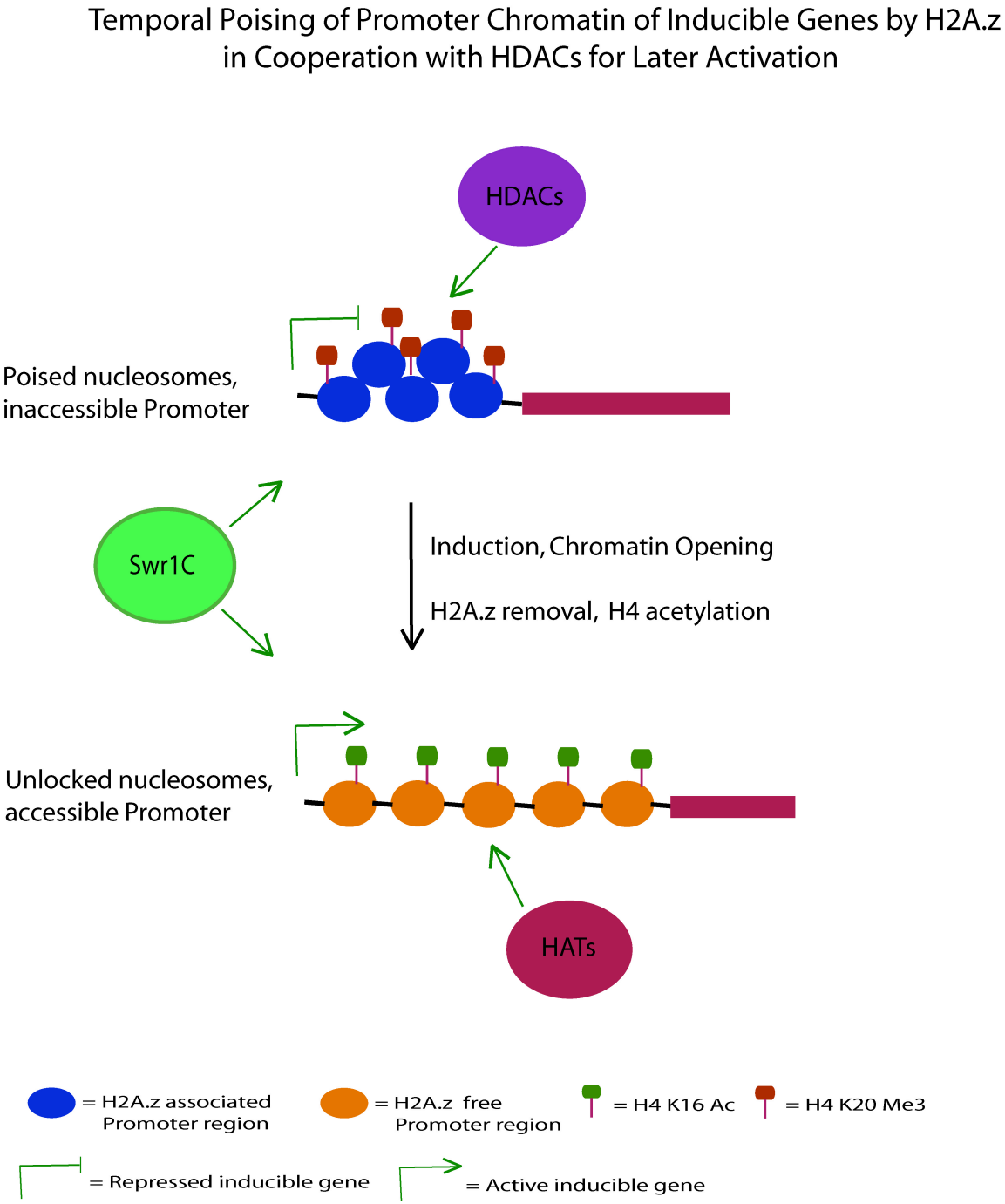


Figure 3.1: In the model proposed: H2A.z associated chromatin has low level of H4 K16 Ac, and overall levels of histone H3 K9 acetylation, histone H3 K4 and K36 trimethylation had are not different from average levels although at local regions different levels and combinations of these modifications are possible. Swr1C and HDACs cooperate in the maintenance of such a chromatin environment. Msc1 has a targeting role of H2A.z to a chromatin region devoid of H3 K36 Me3.

Figure 3.2: Model for the role of H2A.z in gene repression:

A)



B)

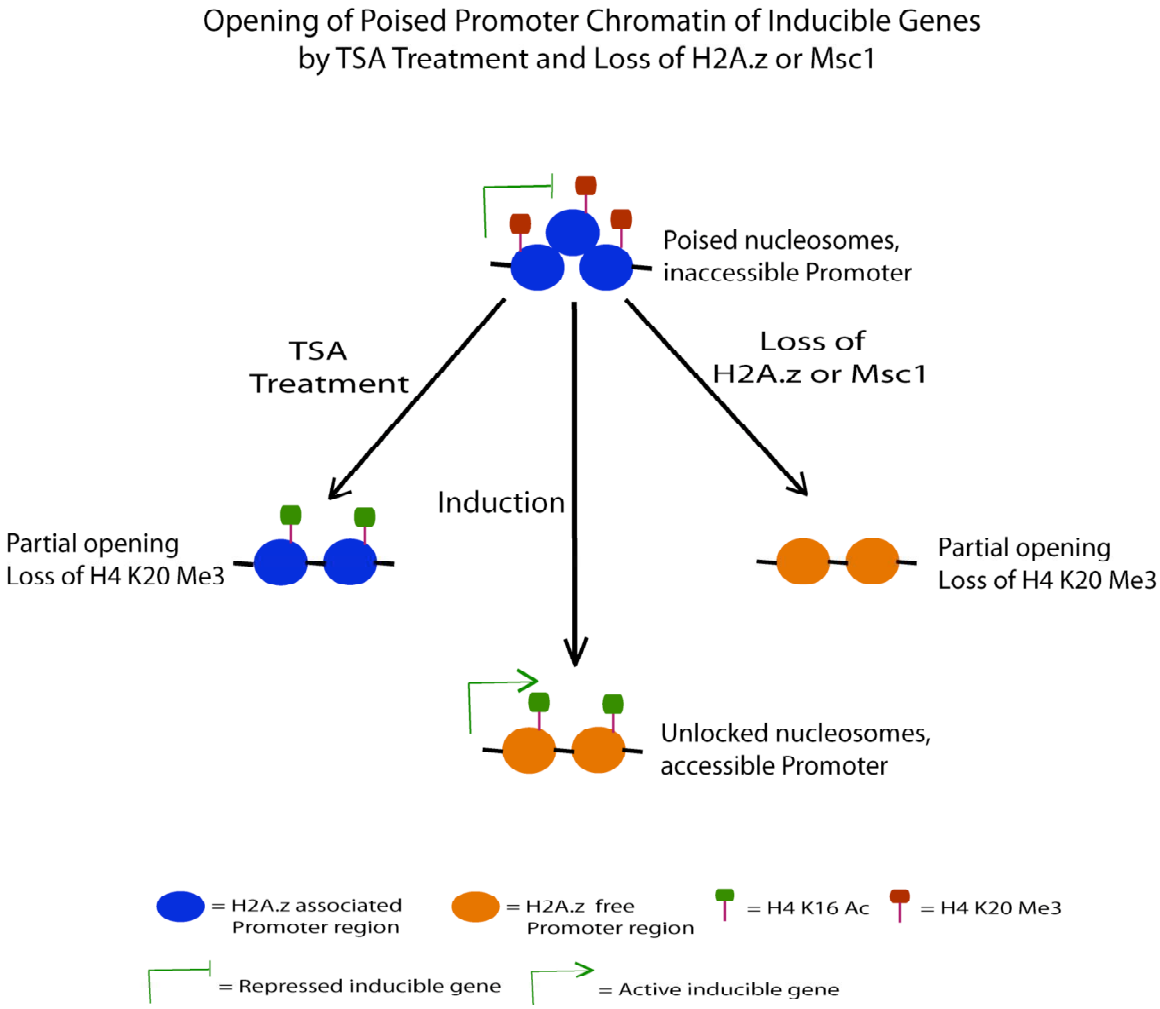


Figure 3.2: A) In the proposed model: H2A.z are in a chromatin environment devoid of H4 K16 acetylation. Presence of H2A.z and absence of H4 K16 Ac at promoters of inducible genes pause the chromatin of inducible genes at repressed state and at the same time, these genes are able to be activated when needed at times such as meiosis or stress. **B)** Treatment with TSA, causes acetylation and loss of H4 K20 Me3. Similarly, loss of H2A.z or Msc1, causes loss of H4 K20 Me3. It's thought that both TSA treatment and loss of H2A.z or Msc1, result partial opening of repressed inducible promoters.

Figure 3.3: Model for the role of H2A.z in transcriptional efficiency:

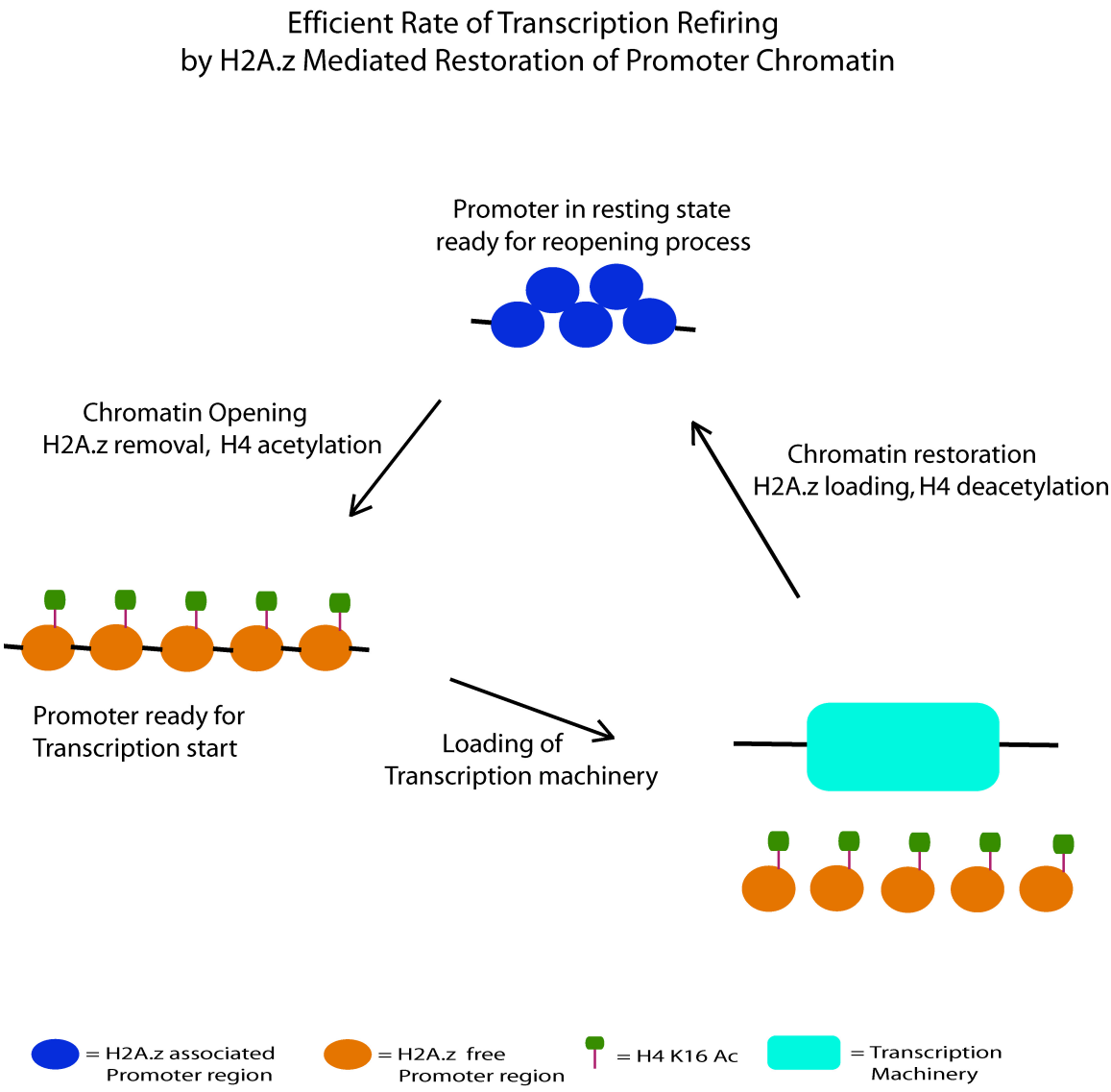


Figure 3.3: H2A.z resides in promoters and evicted when transcription is on. At the same time, it is necessary for full power transcription of many genes. In this study, a model was proposed in figure 3.2. Combining this model and recent findings from other studies, it's proposed that H2A.z functions in restoring promoter after each transcriptional firing by the machinery. It's made possible to refire a new transcriptional initiation by such sort of promoter restoration with respect to the model. Promoter is paused in restored state. Promoter is deacetylated and includes H2A.z. Promoter is unlocked, made accessible and open by loss of H2A.z and histone H4 acetylation. Then, transcription machinery is loaded and transcription starts. After transcriptional start, H2A.z is loaded and deacetylation occurs to restore the promoter.

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4. Materials and Methods:

4.1 Polymerase Chain Reaction (PCR):

PCRs were done following standard protocols. Final concentrations of materials used are: dNTPs 0.2 mM, oligonucleotides 1 uM, Taq polymerase 1 U per 50 ul, DNA template less than 1 ng/ul. The PCR machine used was Robocycler Gradient Temperature Cycler (Stratagene). PCR steps were as following: 1. Initial denaturation at 95 C for 3 min, then multiple cycles of 1 min initial denaturation at 95 C, 1 min annealing at precalculated temperature for primers, 1 min extension for each 1 kb at 72 C, and final extension for 5 min at 72 C.

4.2 Oligonucleotides:

Oligonucleotides were stored in ddH2O at –20 C to a final concentration of 100 ul. The list of oligonucleotides used in this study were listed in the following table.

Oligonucleotides:

Msc1-TAP up	gtctctaatactccttcccatttttccaaaatggactacgtattggaagaccgtaaacctgatttgtttactgaaacatac ctcagcatgTCCATGGAAAAGAGAAG
Msc1-TAP dw	aattaaagcagtttgcagatatgaaaatgttaagtgggcaaacgactaatgtaaagtatttcaattcacattatcattcc ACATACGATTTAGGTGAC
Msc1-TAP chk up	tacctcgccaaactact
Msc1-TAP chk dw	gcagaccgataccaggat
Pht1-TAP up	ggcggtggtgtattgcctcatatcaataaacagctcttgattcgtacaaaggaaaaatatcccgaggaggaagaaatta ttTCCATGGAAAAGAGAAG
Pht1-TAP dw	atgctttaatcatagaaaaaggtaaccaattaaataaaataaatccttagattgtaaaaagaaacgaaaacacaaaaatg aACATACGATTTAGGTGAC
Pht1-TAP chk up	tttgaccgctgaagtcttgga
Pht1-TAP chk dw	ggttggctgctgagacggcta
pht1 KO up	aatattctataaatttatcctctgttgttagaaattcgaaaacttgatgtttaaaggtacagcccctctgtacgcggctt tCACTCAACCCTATCTCGG
pht1 KO dw	atgctttaatcatagaaaaaggtaaccaattaaataaaataaatccttagattgtaaaaagaaacgaaaacacaaaaatg aCTCGAAATTAACCCTCAC
pht1 KO chk up	ccgtcagccagtttagtc
pht1 KO chk dw	gaatgaaaagggagcaaa
msc1 KO up	cttcattatagagacacaattttgggatttaccagggagataaaattaccaacgctgtaaattgaatcaccttggtaaag cCACTCAACCCTATCTCGG
msc1 KO dw	aaattaaagcagtttgcagatatgaaaatgttaagtgggcaaacgactaatgtaaagtatttcaattcacattatcattcc CTCGAAATTAACCCTCAC
msc1 KO chk up	ccgtcagccagtttagtc
msc1 KO chk dw	tgggttatggaaagtctt
msc1-ura ko up	cttcattatagagacacaattttgggatttaccagggagataaaattaccaacgctgtaaattgaatcaccttggtaaagc CACTCAACCCTATCTCG
msc1-ura ko dw	aaattaaagcagtttgcagatatgaaaatgttaagtgggcaaacgactaatgtaaagtatttcaattcacattatcattcc CTCGAAATTAACCCTCAC
msc1-ura ko chk up	ggctgattatctttttcacca
msc1-ura ko chk dw	gacccctctcatttttgtatt

swr1 KO up	tctgccccttacactgtccttcaacgaatggaaagaccaacgtccttccctcttatgcctcaacaccttcattatctc ctCACTCAACCCTATCTCGG
swr1 KO dw	cttattctcaaaaaacaacgaagttagagaaataaacgtttcccggttacaaccaacaaaaaagccatgtcaaaatt tgacCTCGAAATTAACCCTCAC
swr1 KO chk up	ccgtcagccagtttagtc
swr1 KO chk dw	cctactaatgcgaaactttat
swr1-ura ko up	tctgccccttacactgtccttcaacgaatggaaagaccaacgtccttccctcttatgcctcaacaccttcattatctcc tCACTCAACCCTATCTCGG
swr1-ura ko dw	cttattctcaaaaaacaacgaagttagagaaataaacgtttcccggttacaaccaacaaaaaagccatgtcaaaatttg acCTCGAAATTAACCCTCAC
swr1-ura chk up	ggctgattatctttttcacca
swr1-ura chk dw	cctactaatgcgaaactttat
Msc1 cond up	cttcattatagagacacaattttgggatttaccagggagataaaattaccaacgctgtaaattgaatcaccttggtaaag cGCGGATCTGCCGGTAGAGGTG
Msc1 cond dw	acgtttaattcctcaaagtcaacatacggaaattcaataatattttccgaggactgattttcatgagatgaatttttccgca tTTTAGCTTCCTTAGCTCCTGAAAATCTCG
Msc1 cond chk up	ATTCGTTATTGCCCCCCGATA
Msc1 cond chk dw	GTGAGGAGGCTTTTTTGGAGG
Arp6-TAP up	gagcactggaatgcgaataaaataactcgttctgagtacgaacatggagcaaacataatgacacgaaaaaggagaa tat ccatggaaaagagaag
Arp6-TAP dw	atgcgaattatcagctcgccttgagaattttgtgaagatagaaatttaacccacattattgagttttaaaatggtgatctat acga ctcactataggg
Arp6-TAP chk up	ttattattttccccatctgat
Arp6-TAP chk dw	cgtttcgttgttcttcgttta
Vps72-TAP up	tcaaatgtgcaagcatttcagcaagttcgtgaagtttacaaccaacgttattcatggagcgcaatgttaaatctttttcat TCCATGGAAAAGAGAAG
Vps72-TAP dw	agttcattgcgtacataactgaaaagtaatcaaaagaaaagaagaaataccagttattaagttcaccattaccatacat gc ACATACGATTTAGGTGAC
Vps72-TAP chk up	gcgttattacagggaagccag
Vps72-TAP chk dw	gatgaaggcgtttcgttgttc

msc1 ko chk+ up	aggaagtctggtgccgtc
msc1 ko chk+ dw	atgaagtttctgcggtgg
pht1 ko chk+ up	tgccttctccctaacaca
pht1 ko chk+ dw	ttttgagtttttgccttt
swr1 ko chk+ up	attcggaagcgtttgacactt
swr1 ko chk+ dw	ggaaaagcaatagccaatcta
Spac1002.05 TAP up	tatctttgcaagtgcgatttaagcaataagaccttacgcctaaaagtcgacgataatgagttacagaagtt gctaagttatagtggatctggttcaggatccatggaaaagagaag
Spac1002.05 TAP dw	ccaacgatgtttgtaaatgaccttgaaaatataggtttataaattgcaaaataattgttttagaaaagaata aatgtaacttacgactcactataggg
Spac1002.05 TAP chk up	ttgctcctgatttggtaa
Spac1002.05 TAP chk dw	ggctgctgagacggctat
Epe1-TAP up	gaaaataacatttatgattttgaagatcactctcctgttagggaaaaatgggggcacaggcttcggtcc agaggtgctagtagtggatctggttcaggaTCCATGGAAAAGAGAAG
Epe1-TAP dw	atgtgaactactcaagaatcataagcacgtggggataaatattcaatggtagccgaaggaaataaaaag tgccgaggtactACATACGATTTAGGTGAC
Epe1-TAP chk up	tgaagaaatgaaagcgta
Epe1-TAP chk dw	tatcataatcaagtgccc
Spac25h1.02-TAP up	gagcccggcagcaccttcgattctggttctcctccttcatcgattgtcacgatctttaaatctctcggtgattt taaagaaTCCATGGAAAAGAGAAG
Spac25h1.02-TAP dw	tacaaagacgtaagcaaaattgaagaaagcagacgcagaatgaaaaaaacatcattttaaaaggagttta gatgagtagtaACATACGATTTAGGTGAC
Spac25h1.02-TAP chk up	
Spac25h1.02-TAP chk dw	gcagaccgataccaggat
Spcc622.19-TAP up	gatcaattgtatgctactaacaagaaaaacgaaaaacgaccggcagaagacgattcaccttctcaaaag aaaacttgtcaaTCCATGGAAAAGAGAAG
Spcc622.19-TAP dw	tcattagtacaagggattttcaagggtttgtattaataaatttaaaattattcaattctaaatgttttataaataagt gttACATACGATTTAGGTGAC
Spcc622.19-TAP chk up	ttgcaggatacaaaaccc
Spcc622.19-TAP chk dw	tgaatttgttgtccacgg

4.3 Plasmids:

Plasmids were used as templates to amplify targeting cassettes for yeast transformations.

The following plasmids were usede in this study:

pDS1 c-TAP (KanMX6), pKS-KanMX6, pKS-URA4, pEVP11-TetR, pEVP11-BSD

4.4 Yeast Strain Construction and Media:

All media formulations were standard unless difference is indicated. All S. pombe strains were grown in rich medium (YPD or YE5S) or minimal supplemented media. G418 was used in S. pombe strain construction at concentration of 0.15 mg/ml. Gene disruptions and tagging in S. pombe were done as described(1). Correct cassette integrations were confirmed by PCR and western blot analysis.

All strains used in this work were listed in the following table:

Strains:

Name	Genotype
P41	h-,ura4-D18, leu1-32
Msc1-TAP	P41:Msc1 C-term TAP KanMX6
Pht1-TAP	P41:Pht1 C-term TAP KanMX6
Epe1-TAP	P41:Epe1 C-term TAP KanMX6
Spac25H1.02-TAP	P41:Spac25H1.02 C-term TAP KanMX6
Spcc622.19-TAP	P41:Spcc622.19 C-term TAP KanMX6
Spac1002.05c-TAP	P41:Spac1002.05c C-term TAP KanMX6
dpht1	P41:pht1::KanMX6
dmsc1	P41:msc1::KanMX6
dswr1	P41:swr1::KanMX6
Pht1-TAP dmsc1	P41:Pht1 C-term TAP KanMX6, msc1::pKS-Ura4
Pht1-TAP dswr1	P41:Pht1 C-term TAP KanMX6, swr1::pKS-Ura4
Msc1-TAP dswr1	P41:Msc1 C-term TAP KanMX6, swr1::pKS-Ura4
Arp6-TAP	P41:Sp_Arp6 C-term TAP KanMX6
Swc2-TAP	P41:Swc2 C-term TAP KanMX6
TetR	P41:pEVP11-TetR
Msc1-TAP cond	P41:Msc1 C-term TAP KanMX6, pEVP11-TetR, Msc1 N-term tet Operator pEVP11 BSD

4.5 TAP purification:

The extraction of yeast cells was performed as described for the yeast SWi/SNF complex (2). The TAP tag consists of a calmodulin binding peptide(CBP), a TEV protease cleavage site, and two IgG binding units of Protein A as described(3). TAP purification was performed according to (3), with following modifications: 40 ml supernatant of the 43,000 x g centrifugation (2) was allowed to bind to 500 ul IgG Sepharose (Pharmacia), equilibrated in buffer E (2) for 2 hr at 4 C using a disposable chromatography column (Biorad). Used extract is equivalent to 6 liter yeast culture at OD600 2-3. The IgG sepharose column was washed with 100 ml buffer E lacking protease inhibitors, followed by 25 ml TEV cleavage buffer (3). TEV cleavage was performed using 10 ul (100 U) rTEV (Gibco) in 2 ml TEV cleavage buffer for 2 hr 16 C. Calmodulin Sepharose (Stratagene) purification was as described (3), only difference was to use two times volume for everything. Purified proteins were concentrated as described in (4). After seperation on 7-25 % SDS-PAGE gradient gels, proteins were stained with Coomasie, in-gel digested with trypsin, and identified by MS.

4.6 Mass-spectrometry analysis:

All the mass-spec analysis was carried out by in-house facility. Protein bands were excised and in-gel digested with trypsin (Roche Diagnostics, Mannheim, Germany) as described in (5). Proteins were identified by a combination of MALDI MS peptide mapping and nanoelectrospray tandem mass spectrometric sequencing as described (6). Briefly, 1 ul aliquots were withdrawn from the in-gel digests and analysed on a REFLEX IV mass spectrometer (Bruker Daltonics, Bremen, Germany) on AnchorChip probes (Bruker Daltonics, Bremen, Germany) as described in (7). If no conclusive identification was achieved, gel pieces were extracted with 5% formic acid and acetonitrile. Unseperated mixtures of recovered tryptic peptides were sequenced by nanoelectrospray tandem mass spectrometry as described on a QSTAR Pulsar i quadruple time-of-flight mass spectrometer (MDS Sciex, Concord, Canada). Database searching with MALDI TOF peptide mass maps and with uninterpreted mass spectra was performed against a comprehensive non-redundant database using MASCOT software (Matrix Science Ltd, London, UK), installed on a local server.

4.7 Sequence analysis:

Database searches were performed with Blastp and psi-Blast (8). Previously characterized protein domains were identified with Pfam(9) and SMART (10). Multiple alignments were generated with clustal W.

4.8 Western blot analysis:

Proteins were seperated by SDS-PAGE and blotted onto nitrocellulose membrane (Protran, Schleicher & Schnell). Antibodies used were: PAP (Sigma), rabbit anti-acetyl K9-H3-ab12178 (Abcam), rabbit polyclonal anti-dimethylated K4-H3-ab7766 (Abcam), rabbit polyclonal anti-trimethylated K20-H4-ab9053(Abcam), rabbit polyclonal anti-monomethylated K20-H4-ab9051(Abcam), anti-trimethylated K36-H3-ab9050(Abcam), anti-acetylated K16-H4-ab1762(Abcam), rabbit polyclonal anti-trimethylated K9-H3-ab8898(Abcam). Secondary antibody used is goat-anti-Rabbit-IgG-HRP conjugate (Amersham). Image analysis was done using the AIDA Image analyzing package (Raytest).

4.9 Microarray preparation:

Deletion strains of h2A.z, msc1 and swr1 were used. Gene expression analysis was performed as in Lyne et al. (2003). BMC Genomics 4:27.

4.10 Sensitivity assays:

Sensitivity assays were performed on YPD or YE5S agar plates. For UV sensitivity the number of colonies were counted after UV treatment with UV stratalinker 2400 Stratagene machine. For TSA, Campthotecin, MMS, Hydroxyurea serial dilutions(10 times dilution, 5 spots) of S. pombe cells were spotted on plates with indicated concentrations.

4.11 Chromatin Immunoprecipation:

H2A.z bound DNA was immunoprecipitated in H2A.z-TAP strain usingTAP-tag Chromatin IP Protocol from Cold Spring Harbour Laboratory Summer Course 2004 by Michael Buck. Global and random amplification of DNA was performed using Round A/B/C DNA amplification protocol from Lieb et al. 2001. Amplification of gene specific regions from ChIP DNA was performed using following primers:

Spac23d3.12 up: CAGTCAATTCATCCATAAAAA

dw: GTTCTCCAACAAAAAAATGTAA

Spac15e1.02c up: AATCTGTACCTAAATCCATAA

dw: GTAAAATAAATGTTTTGTAAT

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I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

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