REGULATION OF CHROMATIN BY FACT AND NUA3

by

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Abstract

The presence of nucleosomes over vast regions of genome negatively influences transcription creating a need for temporal and structural regulation of chromatin. The default transcription-repressive state can be countered by addition of post-translational modifications by chromatin modifiers or chromatin alteration by histone chaperones. Chaperones alter chromatin structure before the RNAPII passage and restore it afterwards. How modifiers and chaperones function within chromatin is an area of intense research. Here we show how two complexes, the yeast FACT and NuA3 contribute to chromatin function.

Yeast Facilitates Chromatin Transactions (yFACT) is a histone chaperone that maintains chromatin structure. The model of yFACT function in vivo is a subject of much debate. We provide evidence that yFACT acts by stably binding and altering nucleosomes. We also present the EM structure of yFACT associated with nucleosomes. We find that yFACT-associated nucleosomes are hyper-acetylated and show evidence for it being an effect of a direct interaction between yFACT and NuA3. At the same time, acetylation of the H3K56 residue by the histone acetyltransferase Rtt109, acts to recruit yFACT to chromatin through a nucleosome-dependent mechanism.

To determine the distribution of yFACT-associated nucleosomes we constructed a map of yFACT-nucleosome localization at single-nucleosome resolution. We show that while yFACT-bound nucleosomes are distributed thought the genome they are also positioned over the canonical Nucleosome Depleted Regions (NDR). The yFACT-bound nucleosomes are positioned around TATA-elements and Nhp6-target sequences genome-wide. Deletion of NHP6A/B leads to loss of chromatin at these loci. Our work suggests the first ever sequence-dependent mechanism of histone chaperone action in Saccharomyces cerevisiae.

We also examined NuA3 recruitment to chromatin and showed that Yng1, a subunit of NuA3 with a known affinity for H3K4me3 is a bivalent protein. While as previously shown, the C-terminal PHD finer of Yng1 binds to H3K4me3, the N-terminus of Yng1 can also bind to unmodified chromatin. Although these motifs can bind independently, together they increase the apparent association of Yng1 with chromatin. Yng1 binding to chromatin is regulated by the HDA1 complex.
Preface

Chapter 1: “yFACT binds to nucleosomes in vivo” is based on a first author manuscript currently in preparation. All experiments were designed by myself and Dr. LeAnn Howe. I conducted the majority of experiments, while the supplementary work was done by Nancy Fang, Dr. Calvin Yip and Nicolas Coutin. While I prepared the samples, Nancy Fang performed the MS. The EM images were taken with Dr. Calvin Yip. Nicolas Coutine performed the bioinformatic analysis of EM images.

Chapter 2: “yFACT-bound nucleosomes are organized by Nhp6 binding sequences” is based on a first author manuscript in preparation. All experiments were designed by myself and Dr. LeAnn Howe. I conducted the majority of experiments, while Benjamin Martin, Nancy Fang, Dr. Julie Brind’Amour and Dr. Mark Hills performed supplementary work and provided help with data analysis. Benjamin Martin helped me to establish the bioinformatics platform for sequence analysis and provided intellectual input to the project. Nancy Fang provided experimental expertise. Dr. Julie Brind’Amour constructed the libraries for sequencing and Dr. Mark Hills helped with the processing of fastq files. The draft of the manuscript was written by myself, and then revised with help of Benjamin Martin and Dr. LeAnn Howe.

A version of Chapter 3:” Critical Determinants for Chromatin Binding by Saccharomyces cerevisiae Yng1 Exist Outside of the Plant Homeodomain Finger” was published. Chruscicki AT, MacDonald VE, Young BP, Loewen CJR, and Howe LJ. (2010). Critical
Determinants for Chromatin Binding by *Saccharomyces cerevisiae* Yng1 Exist Outside of the Plant Homeodomain Finger. Genetics. 185(2):469-77. DOI: 10.1534/genetics.110.116285. I conducted the majority of experiments for the project and wrote the first version of the manuscript that was later revised by Dr. Vicki Maltby (MacDonald) and Dr. LeAnn Howe. My exact contributions to this work were Figures 4.1 b, c; 4.3a, b, c; 4.5a, b; 4.6a, I also performed, analyzed and validated the high-throughput Synthetic Dosage Lethality (SDL) screen. The remainder of the Figures was a shared contribution between Dr. Vicki Maltby (MacDonald) and Dr. LeAnn Howe. In addition, Dr. Vicki Maltby (MacDonald) constructed the plasmids used in this study.
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List of Abbreviations

Δ  Delta- signifies deletion
°C  Degree Celsius
µg  Microgram
µM  Micromolar
Ac  Acetylation
ARS  Autonomously Replicating Sequence
Asf  Anti-Silencing Factor
bp  Base pair
CAF-1  Chromatin Assembly Factor
CC  Coiled-coil domain
CD  Catalytically Dead
CDS  CoDing Sequence
ChIP  Chromatin immunoprecipitation
chip-ChIP  ChIP followed by a microarray
co-IP  Co-immunoprecipitation
COMPASS  COMplex of Proteins ASsociated with Set1
CTD  C-Terminal Domain
Dex  Dextrose
DNA  Deoxyribonucleic acid
DNaseI  Deoxyribonucleic acid nuclease 1
Dot1  Disruptor of Telomeric silencing
DTT  Dithiothreitol
EDTA  Ethylenediamine tetraacetic acid
EM  Electron Microscopy
EtBr  Ethidium Bromide
FACS  Fluorescence activated cell sorting
FACT  FAcilitates Chromatin Transactions
g  Gram
GAL  Galactose
Gcn5  General Control Nonderepressible
GST  Glutathione S-Transferase
h  Hour
H3K14ac  Acetylation of Lysine 14 on Histone H3
H3K23ac  Acetylation of Lysine 23 on Histone H3
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<td>HA</td>
<td>HemAgglutinin</td>
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<td>HDA</td>
<td>Histone DeAcetylase Complex</td>
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<td>KDAC</td>
<td>Histone deacetylase complex</td>
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<td>HMG</td>
<td>High-Mobility Group</td>
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<td>HU</td>
<td>hydroxyurea</td>
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<tr>
<td>ING</td>
<td>Inhibitor of Growth</td>
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<td>IP</td>
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<td>KAT</td>
<td>lysine acetyltransferase complex</td>
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<td>kb</td>
<td>Kilo base pair</td>
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<td>kg</td>
<td>Kilogram</td>
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<td>L</td>
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<td>LTR</td>
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<td>Micrococcal nuclease</td>
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<td>MS</td>
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<tr>
<td>MYST</td>
<td>MOZ, YBF2/SAS3, SAS2, Tip60</td>
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<tr>
<td>NDR</td>
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<tr>
<td>ng</td>
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<td>Nhp</td>
<td>Non Histone Protein</td>
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<td>Nucleoplasmin</td>
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<td>NTD</td>
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<td>PcG</td>
<td>Polycombc</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>Abbreviation</td>
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<tr>
<td>pH</td>
<td>Potential of Hydrogen</td>
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<tr>
<td>PHD</td>
<td>Plant HomeoDomain</td>
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<td>PIC</td>
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<td>Pob3</td>
<td>Polymerase 1 Binding</td>
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<td>rpm</td>
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<td>Ribosomal RNA</td>
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<td>sodium dodecylsulfate</td>
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<td>SuPpressor of Ty</td>
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<td>SSRP</td>
<td>Single Structure Recognition Protein</td>
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<tr>
<td>STDEV</td>
<td>Standard deviation</td>
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<tr>
<td>TA</td>
<td>Transcription Activator</td>
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<tr>
<td>TAP</td>
<td>Tandem Affinity Purification</td>
</tr>
<tr>
<td>TBP</td>
<td>Tata binding protein</td>
</tr>
<tr>
<td>TE</td>
<td>Buffer consisting of Tris base, EDTA, and water</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco Etch Virus Protease</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
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<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
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<tr>
<td>TSS</td>
<td>Transcription Start Site</td>
</tr>
<tr>
<td>Tx</td>
<td>Trithorax</td>
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<tr>
<td>Ub</td>
<td>Ubiquitination</td>
</tr>
<tr>
<td>UTR</td>
<td>UnTranslated Region</td>
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w/o    Without
WT     Wild Type
yFACT  Yeast FACT
Yng1   Yeast homolog of mammalian Ing1
α      Alpha-signifies anti
I would like to thank people listed here who have helped me during my PhD studies. Their support was invaluable to completion of this work.

Most importantly, I would like to thanks Dr. LeAnn Howe, my research supervisor, for her mentorship, guidance and insight. I am especially grateful to Dr. Howe for her persistent support and patience for my research ventures, which admittedly often took me off the beaten path. I am convinced that this experience will be essential to my future career.

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Finally, and most importantly, I want to thank my parents, Barbara and Tadeusz Chruscicki as well as my sister, Ewa for their everlasting support, love and encouragement through all these years of work.
Dedication

I dedicate this thesis to my family.

“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. We should not allow it to be believed that all scientific progress can be reduced to mechanisms, machines, gearings, even though such machinery also has its beauty.

Neither do I believe that the spirit of adventure runs any risk of disappearing in our world. If I see anything vital around me, it is precisely that spirit of adventure, which seems indestructible and is akin to curiosity.”

Marie Sklodowska-Curie
1 Chapter: Introduction

1.1 Chromatin

Eukaryotic DNA is packaged into a structure called chromatin. Chromatin consists of proteins and nucleic acids with the nucleosome as its basic, repeating subunit \[^{[1]}\]. The nucleosome is a tetramer of dimers of histones H2A, H2B, H3 and H4 with 146 bp of DNA wrapped around it. The nucleosomal DNA consists of 1.65 turns of left-handed superhelix (a helix wound up in a shape of a helix) with fourteen contacts points to histone proteins \[^{[1]}\]. Chromatin is a regulatory component of DNA transcription, replication and repair. The repressive nature of the nucleosome is regulated and countered by an array of cellular processes that ensure a balance between chromatin accessibility and stability at all times \[^{[2][3]}\]. Thus, chromatin can be broadly categorized as active and repressive, where the active states promote and repressive ones obstruct gene expression. The active and repressive states of chromatin are regulated by histone Post-Translational Modifications (PTMs), DNA methylation, histone chaperones and ATP-dependent chromatin remodeling complexes \[^{[4][3]}\].

1.2 Histone Post-Translational Modifications

Histone post-translational modifications (PTM) play a crucial role in the regulation of chromatin. Not only do some PTMs alter the affinity of histone-DNA interactions, but they also serve as recognition sites for chromatin complexes. Current estimates hold the number of unique PTM sites at 130 \[^{[5]}\]. The histone PTMs are placed by chromatin
modifiers, which often themselves recognize other PTMs. The number and the diversity of the histone PTMs, as well as their ability to recruit other chromatin modifiers and the downstream effectors create regulatory networks [6]. These networks however are not binary, for certain modifications can lead to often vastly different outcomes depending on the site modified. This only emphasizes that the importance of the PTMs in the chromatin function is yet to be fully understood [3][7].

1.2.1 Histone acetylation

The histone proteins interact with the negatively charged DNA and are themselves positively charged. The basic nature of the histone octamer, can be partially neutralized by ε-lysine acetylation. Early on it was recognized that histone acetylation is placed post-translationally and that histone acetylation can facilitate RNA synthesis in vivo [8]. Histone acetylation was shown to not only affect the nucleosome breathing by loosening the histone-DNA contacts, but also to serve as the recruitment mark for bromodomain-containing complexes [4][9].

Some of the acetylated residues in Saccharomyces cerevisiae are the N-terminal lysines 4, 9, 14,18, 23, 27 and 36 on the histone H3 tail and H3K56 within the histone core domain, as well as lysines 5, 8, 12 and 16 on the histone H4. There are two prevalent models for the function of specific acetyl residues. The first one holds that individual residues do not perform any particular function, but it is rather the sum of acetylations, that have a functional implication. This view is supported by the fact that histone acetylation patterns over genes often co-localize and have overlapping roles in chromatin function. For instance acetylation of lysine residues 5, 8 and 12 on histone
H4 have a non site-specific cumulative effect on transcription \[^{10}\][^{3}]. The alternate view is that the individual acetylation events have specific functions. For example, histone H4K16ac, unlike the other H4 acetylations has been shown to be involved in silencing in yeast \[^{11}\]. Most recently histone H3K14ac in Saccharomyces cerevisiae emerged as a mark that in yeast, inhibits the function of the Jhd2 demethylase, thus acting to preserve the H3K4 methylation on genes \[^{12}\]. Thus it appears that histone acetylation of specific residues has both general and unique functions.

Of particular importance to this work is acetylation of histone H3 at K14, 23 and 56. These residues are acetylated by three different lysine-acetyl-transferases (KATs): Sas3, Gcn5 and Rtt109. H3K14 and 23 are sites of Sas3 and Gcn5 enzymatic activity, while H3K56ac is catalyzed by Rtt109 \[^{13}\][^{14}][^{15}\]. Acetylation of H3K14 and H3K23 has been implicated both as a prerequisite and as a consequence of transcription. Gcn5 is targeted upstream of transcription through recruitment by transcriptional activators \[^{16}\]. Both Gcn5 and Sas3 are targeted as a consequence of transcription by binding to co-transcriptional modifications such as H3K4me3 \[^{17,18}\].

While H3K14 and K23 acetylation are implicated mainly in transcription, H3K56ac functions in multiple processes. In Saccharomyces cerevisiae, H3K56ac is recognized as a mark of histone synthesis and deposition. H3K56ac is important for histone deposition for it doubles the histone affinity of the CAF-1 deposition factor and enhances its ability to assemble nucleosomes \[^{19}\][^{20}\]. H3K56ac fluctuates through the cell cycle, and peaks in S-phase during histone synthesis. Upon the transition to G2, H3K56ac is removed in a wave of deacetylation by Hst3/4 \[^{21}\]. H3K56ac has also been shown to function in transcription, where it regulates the binding of the Rtt106 histone
chaperone to chromatin \[^{[22][23]}\]. H3K56ac is implicated in transcription-mediated histone exchange, and its loss has been shown to suppress the cryptic transcription phenotypes in yeast \[^{[24]}\]. In mammalian cells, H3K56ac is catalyzed by the GCN5/KAT2A acetyltransferase and is important for response to DNA damage \[^{[25]}\]. Thus, H3K56ac performs roles in transcription, replication and DNA repair.

Acetylation of H3K56 has marked effects on nucleosome fluidity. Mutation of H3K56 to glutamine, which is used to mimic acetylation, shows no effect on the structure of the nucleosome, however it does affect trans-nucleosome interactions \[^{[26]}\]. A subsequent higher resolution structural characterization of a human K56Q mutant, found that the glutamine substitution disrupts water-mediated hydrogen bonding between K56 and DNA \[^{[27]}\]. Furthermore the H3K56Q mutation also significantly increases nucleosome breathing \textit{in vitro}, thus aiding in protein invasion of nucleosomal DNA, and in this study the Q mutation was found to quantitatively imitate acetylation of K56 \[^{[28]}\]. In concordance with these findings, H3K56ac was shown to enhance protein binding to nucleosomes, through DNA unwrapping \[^{[28]}\]. Therefore, the function of H3K56ac is likely a combination of these mechanisms.

1.2.2 **Histone methylation**

Histone methylation (me) is another important, multi-functional histone post-translational modification in yeast and mammals. Histones can be modified by the addition of one, two or three methyl groups to \(\varepsilon\)-amino groups on lysine residues. The fact that lysines can be mono-, di- or tri-methylated, and these differential methylation states can perform separate functions, adds another layer of complexity \[^{[29][3]}\]. Unlike
histone acetylation, which is primarily a mark of active chromatin, in higher eukaryotes.

Methylation can be a mark of active or repressive states depending on its position on the histone tail. For example, methylation of H3K4 is a mark of transcription, while H3K9 methylation is a well-characterized repressive mark. Histone methylation recruits both activating and repressive chromatin factors with methyl-binding domains [30].

In contrast to metazoans, the yeast *Saccharomyces cerevisiae* contains only H3K4me, H3K36me and H3K79me. H3K79me is implicated in active transcription, gene silencing and DNA damage response. While all other methylations are catalyzed by a conserved family of SET (Su(var)3-9, Enhancer of Zeste, Trithorax) methyltransferases, H3K79 is facilitated by the Dot1 (Disruptor of Telomeric Silencing) methyltransferase [31][3].

H3K36me3 is a mark of productive transcription elongation and is distributed over the gene body towards the 3' end [32]. In yeast, H3K36me3 is placed by the Set2 methyltransferase, a member of the SET family. Set2 associates with the serine 2 phosphorylated form of the RNAPII C-terminal domain (CTD) repeat [33]. H3K36me2/3 functions to recruit the Isw1b chromatin remodeling complex and modulates the activity of Rpd3S deacetylase [34][35]. This serves to suppress the histone exchange over the coding regions of genes [24]. Thus, H3K36me2/3 functions to maintain chromatin integrity and these activities are necessary to prevent transcription initiation from cryptic promoters within transcribed regions.

Another methylation mark associated with RNAPII transcription is methylation of H3K4. H3K4me3 is placed by the Set1 methyltransferase (a member of the SET family). Set1 associates with the serine 5-phosphorylated CTD of the RNAPII, consequently the
pattern of the H3K4me3 resembles that of the serine 5 phosphorylation and is mainly confined to the 5' ends of genes. Set1 functions as a part of the COMPASS (Complex Proteins Associated with Set1). COMPASS's trimethylation activity is dependent on Rad6/Bre1-mediated H2BK123 ubiquitylation and the PAF1C complex. H3K4me recruits HATs, KDACs and chromatin remodelers and thus can have both activating and repressive roles during transcription. Accordingly, methylation plays many roles in chromatin regulation.

1.3 Chromatin Modifying Complexes

Chromatin modifying complexes have the ability to modify the structure of chromatin. There are two broad categories of chromatin modifiers: those that can add and remove histone PTMs and those that can use ATP to actively remodel chromatin structure. Histone PTMs can affect chromatin modifiers in a number of ways, through recruitment and modulation of activity. Thus, chromatin modifiers contain subunits that recognize specific histone PTMs.

1.3.1 Readers

Chromatin modifiers that recognize PTMs are categorized as readers. The ability to read histone PTMs is conferred by subunits that specifically recognize the modifications, and there are over twenty recognized domains within readers that can perform this function. Specific domains within readers interact with methyllysines, methylarginines, acetyllysines, phosphoserines and phosphothreonines.
The Plant Homo Domain (PHD) zinc finger domain is a methyllysine and unmodified histone-tail binding motif. *Saccharomyces cerevisiae* contains eighteen canonical and non-canonical PHD finger-containing proteins, with specificity for the methylation of H3K4, H3K36 and unmodified histones. For example, PHD fingers with the ability to recognize H3K4me are found in NuA3 (Yng1), NuA4 (Yng2), Rpd3L (Pho23) and COMPASS (Spp1) \(^{[41]}\). The NuA3 acetyltransferase also contains Nto1, with a PHD finger that interacts specifically with H3K36me3 \(^{[42]}\).

The PHD fingers are functionally important; for instance the Yng1 PHD finger of NuA3 promotes its activity by recruiting the complex to H3K4me3 chromatin \(^{[43]}\). In higher eukaryotes, H3K4me3 has been shown to be a prominent target for the INhibitor of Growth (ING) family. The ING4 tumor suppressor contains a PHD finger, which specifically recognizes H3K4me3. ING4 is a subunit of the HBO1 KAT, and the H3K4me3 binding stimulates the acetylation of the ING4 target genes \(^{[44]}\).

Other domains that recognize methyllysines are PWWP domains and chromodomains. For example, Ioc4, a subunit of the Isw1b chromatin remodeling complex in yeast, contains a PWWP domain that recognizes H3K36me \(^{[35]}\). Additionally, chromodomain of Eaf3, a subunit of both the NuA4 KAT and Rpd3S KDAC complexes, interacts with H3K36me as well \(^{[45]}\). These domains function to recruit (Ioc4) and modulate the activity (Eaf3) of their respective complexes.

Another prominent histone PTM that is recognized by readers is histone acetylation. Bromodomains and the Pleckstrin Homology (PH) domains recognize acetylysines \(^{[40]}\). Bromodomains in yeast can be found in HATs and ATP-dependent chromatin remodelers. Bromodomains are found in SAGA, RSC and SWI/SNF \(^{[46]}\). The
SAGA complex catalytic subunit Gcn5 contains a bromodomain that promotes association of SAGA with nucleosomes and facilitates its enzymatic activity [47]. The RSC complex contains eight bromodomains. The recognition of acetylated nucleosomes is important for RSC ATP-dependent remodeling activity in gene activation [48]. The Swi/Snf complex activity is also regulated by its bromodomain containing subunit Swi2/Snf2. Similar to RSC, the loss of Swi2/Snf2 acetyllysine binding ability results in reduced remodeling activity [49]. Acetylated histones are also a target of binding by the PH domain. The Rtt106 histone chaperone in yeast contains a PH domain, which recognizes H3K56ac and facilitates chromatin recruitment [23]. While we only highlighted a handful, there are many more PTM binding domains and with increasing number of PTMs being discovered, the number of PTM-binding domains is only set to grow.

1.3.2 Lysine-acetyltransferases

The yeast Saccharomyces cerevisiae contains a number of KATs, which display both histone and non-histone substrate specificity. Histone H3 acetyltransferases include the Sas3-dependent NuA3, the Gcn5-dependent SAGA, SLIK/SALSA, ADA and HAT-A2 and Rtt109. NuA3 acetylates H3K14 and K23, while the Gcn5-dependent KATs can acetylate H3K9, H3K14, H3K18 and H3K27, Rtt109 acetylates H3K4, H3K9, H3K27, H3K36 and H3K56 [50]. The histone H4 tail is acetylated by the Esa1-dependent NuA4 complex and the Sas2-dependent SAS complex. NuA4 acetylates H4K5, K8 and K12 and the SAS complex acetylates H4K16. [51][52][53][54].
The NuA3 complex consists of five proteins: the Sas3 catalytic subunit and accessory subunits Nto1, Eaf6, Yng1, and Taf14 \[^{[55]}^{[56]}\]. NuA3 is recruited to both unmodified and H3K4me3 chromatin through Yng1 \[^{[57]}^{[43]}^{[58]}^{[17]}\]. The NuA3 complex also interacts with the yFACT histone chaperone through the C-terminal domain of Sas3 \[^{[56]}\]. Additionally, NuA3 can associate with RSC and the Nap1 histone chaperone. The role of these interactions is not fully understood \[^{[59]}\].

The Rtt109 acetyltransferase is a fungal-specific enzyme that acetylates primarily H3K56. Rtt109 associates with the Asf1 and Vps75 histone chaperones. Binding to Asf1 is thought to stimulate the activity of the Rtt109-Asf1 complex towards H3K56ac by presenting the H3/H4 tetramers for acetylation. At the same time the Rtt109-Vp75 association shifts the specificity of Rtt109 to H3K9ac and H3K27ac \[^{[60]}\]. Loss of Rtt109 is associated with DNA replication defects \[^{[61]}^{[15]}\]. Rtt109-mediated H3K56ac is also important for heterochromatic RNAPII elongation \[^{[62]}\]. In mammalian cells the Rtt109 function is performed by the p300 KAT and the resulting H3K56ac is important in the DNA damage response \[^{[63]}\].

### 1.3.3 Lysine-methyltransferases

Set1 and Set2 are a part of the SET-domain protein lysine methyltransferase family. *SET1* is a conserved homolog of human *MML*. Set1 functions as part of the COMPASS (Complex Proteins Associated with Set1) complex, composed of Swd11, Swd2, Swd3, Spp1 and Shg1. Set1 methylation is found primarily at the 5' ends of genes. This pattern of H3K4me3 arises in part due to the Set1 interaction with the Serine 5 (S5) phosphorylated version of the RNAPII C-terminal domain (CTD) \[^{[37]}^{[64]}^{[65]}\].
The Spp1 subunit contains a PHD finger with specificity for di and tri-methyllysines. This potentially could lead to a positive-feedback mechanism, where H3K4me facilitates more H3K4me. Furthermore Spp1 interacts with Set1 and is necessary for trimethylation of H3K4\(^{[42][66][67]}\).

Set2 methylates K36 on the histone H3 tail. Set2 is recruited by the Serine 2 (S2)-phosphorylated RNAPII CTD. Because of this association, H3K36me3 is found in the gene body, towards the 3’ ends of genes \(^{[33]}\). Set2 activity rescues yFACT mutant growth defects and suppresses gene-body histone exchange during transcription \(^{[34][68][24][69]}\).

1.4 The Histone Chaperones

Chromatin exists as a dynamic and fluid structure, where the histone proteins are subject to ongoing mobility, eviction, transfer and deposition. These processes are largely mediated through a class of conserved proteins called histone chaperones. Chaperones facilitate both replication-dependent and independent histone deposition and this is essential for chromatin stability. Additionally, chaperones can increase histone mobility, function in histone storage and prevent potentially detrimental interactions of histones with other factors. Perhaps surprisingly, histone chaperones do not use ATP, but function in nucleosome assembly and disassembly through their histone binding activities.

Nucleoplasmin (NP) was the first protein to be recognized as a histone chaperone through its ability to assemble nucleosomes \textit{in vitro}. This chaperone is the
most abundant protein in the oocytes of *Xenopus laevis* as it makes up to 10% of the total nuclear content \[^{70,71}\]. The histone chaperone activity of NP and its abundance in the nucleus has led to its proposed function as a storage for the oocytes during fertilization and early embryogenesis.

Like many histone chaperones, NP is an acidic protein with a pI of around 5. NP contains two domains, an acidic N-terminal domain responsible for the oligomerization and the C-terminal domain that contains the nuclear localization sequence and two acidic tracts. NP oligomerizes into a homo-pentamer with an ellipsoid-like shape, which is thought to be its active form. NP is regulated by phosphorylation, which stimulates its histone binding activity \[^{72,73,74,75}\].

*Saccharomyces cerevisiae* contains a number of histone chaperones with different binding activities and affinities. The chaperones with H2A/H2B binding preference include Nap1, Chz1 and FACT. Spt6, FACT and Nap1 can also bind to H3/H4. Primary association with H3/H4 is a property of Vps75, CAF1, Asf1, Rtt106 and the Hir/Hpc complex \[^{76,77,3,78,20}\]. Thus histone chaperones are a diverse group of proteins with unique and overlapping roles in chromatin maintenance \[^{79}\].

The Nucleosome Assembly Protein 1 (Nap1) histone chaperone was first recognized as a factor that can assemble nucleosomes onto DNA \[^{80}\]. Nap1 exists as a homodimer, with a highly acidic region on the underside of the protein that binds histones \[^{81}\]. Nap1 has histone H2A/H2B and H3/H4 binding ability *in vitro* and is therefore often used for chromatin assembly, where it readily facilitates nucleosome formation \[^{82,83,84}\]. *In vivo*, Nap1 has the ability to bind all core histones as well \[^{85}\]. The ability of Nap1 to bind to H3/H4 was demonstrated to trap these histones in their
tetrameric conformation and help to shuttle them on and off the DNA [86]. Nap1 also transports the histones H2A/H2B from the cytoplasm to the nucleus [87]. In addition to the histone binding ability, Nap1 has been demonstrated to facilitate nucleosome sliding, independent of ATP [88]. As such, Nap1 might be also a contributor to chromatin fluidity within the cell [89]. Thus, Nap1 functions in many aspects of chromatin integrity.

*Saccharomyces cerevisiae* has at least two Nap proteins, the other one being Vps75. Vps75 displays H3/H4 tetramer binding ability. It associates with RNAPII and is proposed to function in transcription [60]. Additionally Vps75 functions to present the bound H3/H4 tetrAMPers to the Rtt109 HAT to promote the acetylation of H3K9 and H3K27 [86][90]. Hence, Vps75 functions in chromatin biology both through its role as a chaperone and by promoting histone acetylation.

Anti-Silencing Factor 1 (Asf1) is a conserved histone chaperone with a propensity to bind H3/H4 dimers, but not tetramers. Asf1 is involved in DNA transcription, replication and repair [91]. During transcription, Asf1 is important for initiation and elongation. At initiation, Asf1 has been demonstrated to remove histones upstream of the *HO* promoter, which is required for subsequent co-activator recruitment. During transcription elongation, Asf1 is responsible for histone H3 eviction and suppression of transcription initiation from cryptic promoters [92][93]. Through its association with Rtt109, Asf1 facilitates acetylation of H3K56 [15]. This process is important in both DNA repair and replication. Asf1-mediated H3K56 acetylation has been shown to be important for chromatin reassembly and completion of double strand break repair [94]. During DNA replication H3K56ac brought about by the Asf1-Rtt109 interaction increases the affinity of the CAF-1 and Rtt106 chaperones for histones and
facilitates nucleosomes assembly \cite{19}. Therefore, Asf1 functions in transcription, replication and DNA repair through both its H3/H4 chaperone activity and through its facilitation of H3K56 acetylation.

The SuPpressor of Ty 6 (Spt6) complex was initially identified in a screen for mutations that suppress the Ty transposon-mediated loss of expression of the \textit{HIS4} gene in \textit{Saccharomyces cerevisiae} \cite{95}. Spt6 is a histone chaperone that displays a preferential affinity towards histones H3/H4 \cite{96}. Spt6 is believed to function through nucleosome disassembly and consequent reconstitution during transcription. Spt6 is recruited to transcribed chromatin in part through the association of its SH2 domain with the phosphorylated RNAPII CTD \cite{97,98}. During transcription, Spt6 is important for H3K36me3, and through this PTM and its histone chaperone activity Spt6 suppresses transcription from cryptic promoters within genes \cite{99,50}.

1.4.1 FACT

The FAcilitates Chromatin Transactions (FACT) complex is a well-conserved histone chaperone. yFACT (yeast FACT) was initially identified through two separate screens. Initially the \textit{CDC68} gene was recognized by its \textit{cdc68-1} mutant, which displayed cell cycle defects at START \cite{100}. It was subsequently discovered that this phenotype was due to a failure to transcribe the G\textsubscript{1} cyclins \textit{CLN1}, \textit{CLN2}, and that \textit{CDC68} is in fact the same gene as an independently identified \textit{SPT16} \cite{101}. Simultaneously \textit{SPT16} was identified as an essential gene and a suppressor of Ty insertion mutations in the 5’ regions of \textit{HIS4} and \textit{LYS2} \cite{102}. We will refer to \textit{CDC68} as \textit{SPT16} for the remainder of this work. Spt16 was subsequently shown to form a
heterodimer with Pob3. The Pob3 subunit was identified as a homologue of the mammalian Single Structure Recognition Protein 1 (SSRP1). The yeast Spt16-Pob3 complex interacts with DNA Polymerase α and to be localized to chromatin, consistent with its role in replication \[^{103}\[^{104}\[^{105}\].

In mammalian cells, FACT was identified as an elongation factor required for \textit{in vitro} and \textit{in vivo} transcription elongation activity. FACT activity was found to be abolished in the presence of a crosslinking agent, which led to the proposal of its function as a chromatin disassembly factor \[^{106}\]. Mammalian FACT consists of hSpt16, a homologue of \textit{Saccharomyces cerevisiae} Spt16, and a homologue of yPob3, the HMG-1-like protein, SSRP1. In yeast Pob3, the HMG group of SSRP1 is absent, but the analogous function is performed through association of yFACT with the Nhp6a/b HMG group proteins.

Subsequently, yFACT was defined as a functional unit consisting of Spt16, Pob3 and Nhp6. Although, Spt16 and Pob3 show strong genetic interactions with Nhp6a/b, they do not form stable heterotrimers. Nhp6a/b are HMG group proteins that bind to nucleosomes and can subsequently recruit yFACT. The binding of Nhp6a/b and yFACT alters nucleosome structure, however it is the initial alteration by Nhp6a/b that is speculated to recruit yFACT to nucleosomes \[^{107}\]( Formosa et al., 2001).

Unlike some other chaperones, yFACT is asymmetrical and forms a stable heterodimer between Spt16 and Pob3. The Spt16 subunit is comprised of four distinct regions, the N-terminal domain (NTD), the dimerization domain, the mid-domain and the C-terminal region. The N-terminal domain was crystallized and found to contain an inactive “aminopeptidase-like” domain, which is a histone H3/H4 binding module \[^{109}\].
The N-terminal domain of Spt16 has overlapping roles with the M-domain of Pob3, where both serve as docking sites for histone H2A\textsuperscript{[110]}. The CTD of the Spt16 subunit is very acidic and is thought to be essential for the ability of FACT to interact with histones\textsuperscript{[78]}. Pob3 (SSRP1) interacts with Spt16 through the N-terminal domain (NTD) of Pob3, and as described above, the M-domain of Pob3 overlaps in function with the Spt16-NTD. The Pob3-NTD contains a Pleckstrin Homology domain, but it is unknown if this domain contains specific histone-binding activity\textsuperscript{[22][110][78]}. Lastly, the C-terminus of the metazoan Pob3 homologue, SSRP1 contains a DNA binding HMG domain\textsuperscript{[78]}. In \textit{Drosophila melanogaster} phosphorylation in the vicinity of the SSRP1 HMG domain, in an acidic patch, was shown to control the binding of FACT to nucleosomal DNA\textsuperscript{[111]}.

The ability of yFACT to bind various histone substrates through multiple domains lent support to the idea of the complex acting to reorganize nucleosomes. In recent work, FACT was shown to act synergistically by binding nucleosomal DNA and histones \textit{in vitro}\textsuperscript{[112]}. In this study, FACT displayed preferential binding to nucleosomes over histones and this was enhanced by the presence of the highly basic histone tails\textsuperscript{[112]}. These findings support the idea that FACT functions \textit{in vivo} through nucleosome binding.

While FACT is important for transcription and nucleosome alteration, multiple lines of evidence show that it also serves to maintain chromatin stability. Loss of yFACT results in loss of histones from transcribed regions and transcription initiation from cryptic promoters\textsuperscript{[113]}. For example, work on the regulation of the \textit{SER3} promoter in yeast uncovered that transcription from the upstream \textit{SRG1} locus maintains \textit{SER3} in a repressed state\textsuperscript{[114]}. Non-coding transcription from \textit{SRG1} directs nucleosome
occupancy over the SER3 promoter. Similar to cryptic transcription initiation phenotypes, disruption of yFACT function results in loss of histone occupancy and transcription from the SER3 promoter [115][116][117]. In addition to the suppression of cryptic transcription initiation, yFACT represses histone exchange during transcription. The loss of Spt16 function results in increased incorporation of newly synthesized histones as well as the overall loss of nucleosomes from chromatin [118]. FACT therefore performs a dual function where it facilitates transcription but at the same time maintains nucleosome occupancy. This apparent discrepancy will be the subject of the next chapter.

FACT is important in human disease. Studies of FACT distribution in human and mouse cells found that the complex was not detectable using immuno-staining in differentiated adult cells. Instead, the presence of FACT was confined to undifferentiated and tumor cells [119]. A group of small molecule inhibitors, called curaxins, was identified by their ability to inhibit FACT function. The curaxins are intercalating agents, which seem to trap FACT on chromatin. The inhibition of FACT leads to activation of the p53-mediated apoptotic pathway and loss of NF-κB regulated transcription. Curaxins do not cause the toxicity associated with commonly used genotoxic agents because they do not induce DNA damage. As curaxins work through inhibition of FACT, they primarily target tumor cells, which express FACT and thus avoid to a large extent off-target effects of other cancer agents. In animal models and cell culture curaxins have been shown to be potent anti-cancer agents [120]. In the conclusion section, we discuss how our model of FACT function relates to these findings.
1.5 Nucleosome Positioning

Nucleosomes restrict access to DNA. Recent advances in high-throughput sequencing technologies allowed for the mapping of nucleosome positions genome-wide in yeast and other organisms. Nucleosome maps refined our understanding of the precise positioning of nucleosomes in the cell. This positioning is not random but exhibits defined patterns.

The yeast *Saccharomyces cerevisiae* is estimated to have 70000 positioned nucleosomes that cover up to 81% of the genome [121]. The most defined feature of nucleosome positioning over genes in yeast is the consistent presence of a Nucleosome Depleted Regions (NDRs) just upstream of the TSS. A nucleosome, defined as "+1", is positioned downstream of the NDR, partially occluding the TSS, while another nucleosome, approximately 200bp away, and defined as "-1", is positioned upstream of the NDR [121]. The presence of the NDR is partially explained by the DNA sequence since promoters are not very permissive to nucleosome formation because of nucleosome sequence preferences [122][123]. The NDR is a site of Pre-Initiation Complex (PIC) formation and transcriptional activator recruitment and thus has been implicated in regulating factor binding to DNA [124]. The NDR might have yet another role: the "+1" nucleosome was found to partially occlude the TSS on TFIID-dependent genes in yeast. This positioning is proposed to influence RNAPII start site selection [124].

In metazoans, many of the key features of nucleosome organization in yeast are conserved. Like in yeast, *Drosophila melanogaster* and *Mus musculus* both exhibit pronounced NDR regions upstream of the TSS. However there are subtle differences in how these NDRs are defined. While in yeast the "+1" nucleosome often occludes the
TSS, in flies the “+1” nucleosome is positioned further downstream. This difference suggests that in flies the “+1” nucleosome does not play a role in start-site selection. Another marked difference is the absence of the “-1” nucleosome upstream of NDR. The downstream shift of the “+1” nucleosome is proposed to be due to the RNAPII pausing, a major regulatory mechanisms in higher eukaryotes \[125\]. In mice nucleosomes cover only approximately 60% of the genome, but as in yeast and flies the region immediately before the TSS is depleted in nucleosome occupancy \[126\]. Despite small subtleties, ubiquitous mechanisms of nucleosome positioning appear to be conserved across eukaryotes.

While the NDR seem to be ubiquitously found upstream of the TSS, recent work in yeast shows that the NDR might not be depleted of nucleosomes after all. Studies with limited amounts of MNase digestion of chromatin identified a subset of nucleosomes defined as “fragile” based on their increased MNase sensitivity. The positioning of these nucleosomes correlates with the NDRs upstream of the TSS. Consequently these nucleosomes were abbreviated as “0”, for they are placed between the “-1” and the “+1” nucleosomes, and were found enriched on TATA-containing, inducible genes \[127\][128\]. Thus, the pervasiveness of nucleosomes might be higher than previously thought. How the nucleosome “0” affects PIC formation and transcription regulation remains to be shown.
Chapter: yFACT Binds to Nucleosomes *In Vivo*

2.1 Introduction

The FACilitates Chromatin Transactions (yFACT) complex is a histone chaperone \[^{[129]}\]. Yeast FACT (yFACT) contains multiple histone binding domains, as well as a DNA-binding subunit HMG Nhp6a/b \[^{[130]}^{[131]}\]. Loss of yFACT has been associated with SPT phenotypes, transcription initiation and elongation defects and nucleosome loss from DNA \[^{[132]}^{[118]}\]. How the yFACT complex can facilitate transcription while at the same time, maintain chromatin stability remains unclear. The “dimer-eviction” model followed the discovery that FACT binds H2A/H2B dimers and can facilitate hexasome formation. It stipulates that FACT functions through evicting H2A/H2B dimers in the wake of transcription and subsequently re-assembles them after transcription bubble passage \[^{[133]}\]. The accuracy of the “dimer-eviction” model has however been challenged by various lines of evidence. For example, it has been observed that transcription initiation can occur without loss of H2A/H2B or H3/H4 \[^{[134]}\]. The alternate explanation, the “global accessibility/non-eviction model,” proposes that yFACT functions through altering the nucleosome structure. This model proposes that yFACT weakens internal histone-DNA contacts, allowing access to nucleosomal DNA, but prevents histone loss through tethering of the histones through its multiple histone binding domains \[^{[78]}\]. This model is supported by recent *in vitro* studies that show that yFACT preferentially binds nucleosomes over free histones, which is consistent with the global accessibility model \[^{[112]}\]. Additionally the loss of yFACT has been shown to cause not only loss of core
histone occupancy, which is consistent with the complex acting as a histone octamer tether during transcription.\(^{118}\)

While the model of FACT function has been a subject of previous work, less is known about how the histone binding ability of the complex is regulated, particularly by histone modifications. Mammalian FACT has been shown to be regulated by phosphorylation of H2AX, where H2AX phosphorylation by DNA-PK causes nucleosome instability and facilitates the exchange of this variant.\(^{135}\) The same study also found that FACT is subject to regulation by PARP1, where ADP-ribosylation of Spt16 inhibited H2AX exchange.\(^{135}\)

In yeast, H3K56ac has been implicated in yFACT function. The Pob3 subunit of yFACT contains a Pleckstrin Homology (PH) domain, homologous to the one found in another chaperone- Rtt106. While both chaperones bind histones, Rtt106 has been conclusively shown to have increased affinity for H3K56ac histones.\(^{23}\) Whether any other mechanisms of regulation of yFACT exist remains to be shown.

In this chapter, we addressed the mode of yFACT function \textit{in vivo} and provided evidence for yFACT acting through the “non-eviction” model. We determined the chromatin environment associated with yFACT and showed that yFACT preferentially associates with hyper-acetylated nucleosomes. We also showed that recruitment of the complex to chromatin is stimulated by H3K56ac \textit{in vivo}. Lastly, we tested the functional implications of the interaction of yFACT with NuA3 and showed that NuA3 acetylates yFACT-associated nucleosomes.
2.2 Methods

2.2.1 Yeast cell culture

All cultures were grown in Yeast Peptone Dextrose (YPD) unless indicated otherwise at 30°C to an OD$_{600}$ of 0.6-0.8. A detailed list of strains used can be found in Table 1.

Table 1 Yeast strains used in this study

<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Mating Type</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>YLH 101</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63</td>
</tr>
<tr>
<td>YLH 498</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 POB3TAP::URA3</td>
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<tr>
<td>YLH 534</td>
<td>Mat a</td>
<td>leu2-3,112 trp1-1 can1-100 ura3-1 his3-11,15 Pob3TAP::URA3</td>
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<tr>
<td>YLH 544</td>
<td>Mat a</td>
<td>leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 rtt109::KAN Pob3TAP::URA3</td>
</tr>
<tr>
<td>YAC 50</td>
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<td>YAC 51</td>
<td>Mat a</td>
<td>his3D200 leu2D1 lys2-128d ura3-52 trp1D63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912d35::his4 phht2K56Q.HHF2</td>
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<tr>
<td>YAC 52</td>
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<td>his3D200 leu2D1 lys2-128d ura3-52 trp1D63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912d35::his4 phht2K56R.HHF2</td>
</tr>
<tr>
<td>YLH500</td>
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<td>his3D200 leu2D1 lys2-128d ura3-52 trp1D63 sas3::HISMX6 POB3TAP::URA3 pFSM1 (pSas3CD)</td>
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<td>his3D200 leu2D1 lys2-128d ura3-52 trp1D63 sas3::HISMX6 POB3TAP::URA3 pSas3dCFLG(LEU) (pSas3ΔCTD)</td>
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<td>YLH500</td>
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<td>his3D200 leu2D1 lys2-128d ura3-52 trp1D63 sas3::HISMX6 POB3TAP::URA3 pSas3FLG(LEU) (pSas3)</td>
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<td>YLH500</td>
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<td>Yeast Strain</td>
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<td>Genotype</td>
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<td>YLH140</td>
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<td>Mat α</td>
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</tr>
<tr>
<td>YLH140</td>
<td>Mat α</td>
<td>his3D200 leu2D1 lys2-128d ura3-52 trp1D63 NTO1HA::HISMX6 sas3::HISMX6 pSas3dCFLG(LEU) (pSas3ΔCTD)</td>
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<tr>
<td>YLH140</td>
<td>Mat α</td>
<td>his3D200 leu2D1 lys2-128d ura3-52 trp1D63 NTO1HA::HISMX6 sas3::HISMX6 pSas3FLG(LEU) (pSas3)</td>
</tr>
</tbody>
</table>

### 2.2.2 Antibodies

The antibodies used are listed in Table 2. All antibodies were used at 1:500 to 1:4000 dilutions for immunoblots and 2ug per sample for chromatin immunopurifications.

#### Table 2 Antibodies used in this study

<table>
<thead>
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<th>Antibody</th>
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<th>Type</th>
<th>Lot #</th>
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<td>H3K23ac</td>
<td>Active Motif</td>
<td>Rabbit polyclonal: 39131</td>
<td>104</td>
</tr>
<tr>
<td>IgG</td>
<td>Millipore</td>
<td>PP64</td>
<td>LV1602263</td>
</tr>
<tr>
<td>H3K14ac</td>
<td>GeneScript</td>
<td>Affinity-purified polyclonal antibody</td>
<td>Rasied against H3K14ac peptide</td>
</tr>
<tr>
<td>H3</td>
<td>GeneScript</td>
<td>Affinity-purified polyclonal antibody</td>
<td>Raised against scH3 peptide</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>abcam</td>
<td>Mouse monoclonal: ab1012 (isotype IgG2b)</td>
<td>1276040</td>
</tr>
<tr>
<td>H3K79me3</td>
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<td>rabbit polyclonal: ab2621</td>
<td>949429</td>
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<tr>
<td>H2b</td>
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<td>Rabbit polyclonal</td>
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<td>abcam</td>
<td>Rabbit monoclonal: ab76307</td>
<td>EPR996Y</td>
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</tbody>
</table>

### 2.2.3 Chromatin ImmunoPrecipitation (ChIP)

We used Q-PCR to amplify immunopurified DNA with sets of primers listed in Table 3. ChIP experiments were performed as previously described $^{[136]}$. 

---

22
Table 3 Primers used for ChIP-QPCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA1 + 2436s</td>
<td>GCTGGTCCATTCTGGTCTTC</td>
</tr>
<tr>
<td>PMA1 + 2590a</td>
<td>CGATAGACCAGATCCAGACAC</td>
</tr>
</tbody>
</table>

### 2.2.4 Pob3\textsubscript{TAP} based FACT-purification and MNase digestion

Pob3\textsubscript{TAP} and its associated proteins were purified using a one-step TAP-tag based approach based on Lambert et al.\textsuperscript{[137]} Briefly, 1L of each WT and Pob3\textsubscript{TAP} strains were grown to an OD of 0.8 in YPD at 30\textdegree C. Cells were re-suspended in lysis buffer (100mM HEPES pH 8, 20mM Mg acetate, 300mM Na acetate, 10% glycerol, 10mM EGTA, 0.1mM EDTA), lysed using bead beating and gently sonicated (three cycles of 20 sec on, 30 sec off). The proteins were extracted by adding NP40 to 1% and rotating the lysates for 10 minutes at 4\textdegree C, spin at 3000rpm for 5 min, followed by re-extraction of the pellet with lysis buffer with 1% NP-40 and subsequent rotation. The affinity purification was performed using IgG crosslinked to magnetic beads (Invitrogen Dynabeads M-270 Epoxy) and rotation of the beads and lysates for 5 hours at 4\textdegree C. The beads were then washed 3 times in the lysis buffer with 1% NP-40, 3 times with lysis buffer and eluted with 35ul of SDS loading buffer. For the purpose of the MNase digest the purified FACT-nucleosome complexes were washed in MNase digestion buffer (50mM Tris pH8, 4mM MgCl\textsubscript{2}, 2mM CaCl\textsubscript{2}) and digested with limited amounts of MNase. The reactions were stopped by washing the beads with STOP buffer (50mM Tris pH8, 4mM MgCl\textsubscript{2}, 10mM
EDTA). The DNA was purified using phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation.

2.2.5 FACT fractionation

Purified and MNase digested FACT-nucleosomes were washed with TEV buffer (50mM Tris pH 8, 150mM NaCl, 5% glycerol, 0.01% NP-40) and eluted with TEV overnight at 4°C. TEV eluates were then loaded on pre-equilibrated size exclusion chromatography column (GE Superose 6) and run while 1mL fractions were collected.

2.2.6 Electron microscopy

Aliquots (50ul) of fractionated, purified FACT were spotted on carbon grids. Negatively stained specimens were prepared as previously described [138]. In brief, samples were adsorbed to carbon coated copper grids and stained with uranyl formate. Samples were examined using a Tecnai Spirit transmission electron microscope (FEI) operated at an accelerating voltage of 120 kV. Images were recorded at a nominal magnification of 49,000x using a 4K x 4K Eagle charge-coupled device (CCD) camera (FEI). The digitized micrographs were analyzed using the EMAN2 processing suite {Tang:2007ft}. Raw micrographs were imported and their 2D power spectra evaluated based with the following parameters: 2.3 angstroms per pixel, 120 KiloVolts, 2.0 mm spherical aberration, 0.15 amplitude contrast and a box size of 512. Particles were then picked out in semi-automated fashion using a combination of the “swarm” and Gaussian peak finders. Box sizes of 140 pixels with an expected object size of 70 pixels were held constant throughout. Each windowed particle was then windowed out normalized with the “norm.ramp.normvar” setting. The generated particles sets were then grouped into
12 classes using the reference-free “e2refine2d” tool. Here each particle is rotated, translated and flipped and a cross-correlation coefficient (CCC) is calculated relative to all other particles and their transforms over 8 iterations. The average structure for a group is generated from the edge-mean normalized particles within each group.

2.2.7 Mass spectrometry

Purified FACT-nucleosome elutions were run on a 4-20% gradient protein gel (Bio-Rad) for in-gel trypsin digestion prior to mass spectrometry analysis. The protein gel was fixed and stained in fixation buffer containing coomassie blue G (5% acetic acid, 47.5% methanol) for 30 minutes followed by 4-hour wash in nano-pure water. Gel pieces from control sample (WT) were divided into two fractions: 1) non-IgG fraction; and 2) IgG heavy chain and light chain (50kDa and 25kDa bands). Gel pieces from IP sample (Pob3_TAP) were divided into 4 fractions: 1) above 50kDa; 2) between 0kDa to 50 kDa fraction without IgG and histone bands; 3) IgG heavy and light chains; 4) the 4 histone bands. The in-gel trypsin digestion procedure is essentially as described in Shevchenko et al with minor changes: digestion buffer contained 50mM ammonium bicarbonate, and gel pieces were dehydrated using net ethanol instead of acetonitrile \( ^{139} \). The control and IP samples (fractions 1-3) were incubated at 56°C for 45 minutes in 10mM DTT followed by 30-minute incubation in 55mM chloroacetamide at room temperature. The histone fraction was propionylated prior to trypsin digestion using the method described in Ouvry-Patat et al. except, gel pieces were incubated with 20µl 100mM propionic anhydride in acetylation buffer (2M NaCl, 50mM NaHCO3, pH8) for 5 minutes on ice followed by adding an additional 180µl of acetylation buffer for another 25 minutes of
incubation on ice \textsuperscript{140}. The propionylation reaction was quenched with 400 µl of 50mM Tris buffer, pH8. Gel pieces were washed three times with 50mM ammonium bicarbonate before dehydration for trypsin digestion. All samples were incubated with 10 µg/µl trypsin in 50mM ammonium bicarbonate for 90 min on ice for complete absorption of trypsin before a 24 hours incubation at 37\degree C. Peptides were extracted once with extraction buffer A (0.5% acetic acid), twice with extraction buffer B (30% acetonitrile, 0.5% acetic acid) and twice with extraction buffer C (100% acetonitrile). Dry peptide-extraction samples were then resuspended in 0.5% acetic acid solution and acetified to a pH below 2 using extra 100% acetic acid. Peptide samples were then purified by solid phase extraction on C-18 stage tips \textsuperscript{141}\textsuperscript{,142}.

Purified peptides were analysed using a linear-trapping quadrupole - Orbitrap mass spectrometer (LTQ-Orbitrap Velos; ThermoFisher Scientific) on-line coupled to an Agilent 1290 Series HPLC using a nanospray ionization source (ThermoFisher Scientific) including a 2-cm-long, 100-µm-inner diameter fused silica trap column, 50-µm-inner diameter fused silica fritted analytical column and a 20-µm-inner diameter fused silica gold coated spray tip (6-µm-diameter opening, pulled on a P-2000 laser puller from Sutter Instruments, coated on Leica EM SCD005 Super Cool Sputtering Device). The trap column was packed with 5 µm-diameter Aqua C-18 beads (Phenomenex, www.phenomenex.com) while the analytical column was packed with 1.9 µm-diameter Reprosil-Pur C-18-AQ beads (Dr. Maisch, www.Dr-Maisch.com). Buffer A consisted of 0.5% acetic acid, and buffer B consisted of 0.5% acetic acid and 80% acetonitrile. Standard 90 min gradients were run from 0% B to 32% B over 51 min, then from 32% B...
to 40% B in the next 5 min, then increased to 100% B over 2 min period, held at 100% B for 2.5 min, and then dropped to 0% B for another 20 min to recondition the column. The HPLC system included Agilent 1290 series Pump and Autosampler with Thermostat. The thermostat temperature was set at 6°C. The sample was loaded on the trap column at 5 μL/min and the analysis was performed at 0.1 μL/min. The LTQ-Orbitrap was set to acquire a full-range scan at 60,000 resolution from 350 to 1600 Th in the Orbitrap and to simultaneously fragment the top ten peptide ions by CID and top 5 by HCD in each cycle in the LTQ (minimum intensity 1000 counts). Parent ions were then excluded from MS/MS for the next 30 sec. Singly charged ions were excluded since in ESI mode peptides usually carry multiple charges. The Orbitrap was continuously recalibrated using lock-mass function \cite{143}. Mass accuracy: error of mass measurement was typically within 5 ppm and was not allowed to exceed 10 ppm.

For the analysis of mass spectrometry data the centroided fragment peak lists were processed with Proteome Discoverer v. 1.2 (ThermoFisher Scientific) to produce Mascot generic format (mgf) files. The headers of the peak lists in the mgf files were corrected with a custom Pearl script (to be consistent with DTASuper-Charge8 generated mgf files and to allow later processing with MSQuant8, 9 or other software). The Mascot search was performed with Mascot algorithm against a database comprised of the protein sequences from SGD using the following parameters: peptide mass accuracy 10 parts per million; fragment mass accuracy 0.6 Da; trypsin enzyme specificity; ESI-TRAP fragment characteristics; For all samples except histones, Mascot search modifications included fixed modifications – carbamidomethyl; variable
modifications - methionine oxidation, lysine acetylation. For the histone fraction, mascot searches were done with different combination of plausible modifications on histone tails in groups to reduce the FPR introduced by increasing numbers of variable modifications. The modification groups used to generate acetylated histone tail peptide list included: 1) acetyl (K) and propionyl (K); 2) acetyl (K), methyl (K) and propionyl (K). Only those peptides with IonScores exceeding the individually calculated 99% confidence limit for each sample (as opposed to the average limit for the whole experiment) were considered as accurately identified.
2.3 Results

2.3.1 yFACT stably binds nucleosomes in vivo

The *Saccharomyces cerevisiae* FACilitates Chromatin Transactions (yFACT) promotes transcription, while at the same time maintaining chromatin stability \[^{144}/^{134}\]. Thus, yFACT performs two very different functions in the cell: it disrupts chromatin to promote transcription and it also functions to maintain chromatin integrity.

To investigate the role of yFACT in vivo we employed a previously published chromatin purification technique to isolate yFACT and associated proteins \[^{137}\]. We hypothesized that if yFACT acts through the “dimer eviction” model as opposed to the “global accessibility” model, then purified yFACT should primarily bind histones H2A/H2B. If yFACT however, acts by stable association with nucleosomes we should observe stoichiometric amounts of bound histones H2A/H2B and H3/H4. In support of the “global accessibility” model, we found that the two subunits of yFACT- Spt16 and Pob3 co-purify with stoichiometric amounts of the four core histones indicating that yFACT does not show a preferential binding to H2A/H2B in vivo (Figure 2.1a).

The presence of equal amounts of core histones within our co-purifications suggests that yFACT binds nucleosomes in vivo. To explore this possibility we tested for the presence of MNase resistant-DNA in the co-purifications. We found that yFACT-histone complexes protected 100-200 nucleotide fragments of DNA, which was indicative of a nucleosome-like structure (Figure 2.1b).

FACT binding to nucleosomes in vitro is mediated through multiple histone binding domains, but it remains to be shown if this is the case in vivo \[^{112}\]. The “dimer
eviction” hypothesis postulates that yFACT preferentially binds H2A/H2B dimers \cite{ref1, ref2, ref3}. If yFACT however acts by disrupting and tethering nucleosomes, then even in the absence of DNA it would be expected to bind to all core histones through its multiple histone binding subunits. To test the binding preference of *in vivo* purified yFACT-histone complexes we performed the co-purifications in the presence of ethidium bromide (EtBr). EtBr has the ability to intercalate DNA and thus disrupt the DNA-dependent protein interactions allowing for detection of DNA-independent protein-protein contacts \cite{ref4}. In concordance with the latter hypothesis we found that even in the presence of EtBr, and absence of DNA yFACT associated with all core histones (Figure 2.1c). This result was consistent with FACT binding to nucleosomes *in vivo* through multiple histone contacts.
Figure 2.1 yFACT stably binds nucleosomes \textit{in vivo}

a) \textit{In vivo} Pob3\textsubscript{TAP} based purification. Pob3\textsubscript{TAP} co-purifies with Spt16 and core histones H2A, H2B, H3 and H4 in stoichiometric amounts. Samples were imaged using coomassie-stained SDS-PAGE. (−) is WT and (+) Pob3\textsubscript{TAP} strain. b) MNase digestion of Pob3\textsubscript{TAP} purified DNA. Pob3\textsubscript{TAP} associated DNA was purified using phenol:chloroform:isoamyl alcohol extraction and visualized on an agarose gel stained with SYTO60 c) Pob3\textsubscript{TAP} based purification performed with the indicated amounts of ethidium bromide. Samples were imaged using SDS-PAGE followed by coomassie staining.
yFACT facilitates transcription without the need for nucleosome disassembly [134]. Additionally the binding of yFACT \textit{in vitro} alters DNaseI and hydroxyl-radical footprinting of FACT-bound nucleosomes, and these effects were independent of dimer eviction [108]. We therefore set out to test if \textit{in vivo} purified yFACT-nucleosomes have a propensity to lose H2A/H2B dimers. To test whether nucleosomes co-purified with FACT \textit{in vivo} exhibit an increased level of instability we performed a series of NaCl washes of purified FACT-nucleosome complexes. While yFACT-nucleosome complexes dissociated at 600mM NaCl, we did not observe any partial nucleosome disassembly at lower salt concentrations suggesting that yFACT-associated with nucleosomes as a unit (Figure 2.2a).

Nucleosome disassembly progresses through a step-wise process, where H2A/H2B dimers leave first, followed by the H3/H4 tetramer [147]. To test whether yFACT might facilitate such a mode of disassembly, we purified \textit{in vivo} yFACT-nucleosome complexes, performed a complete MNase digest, and cleaved the TAP tagged complex off beads with TEV. We then re-immunoprecipitated the TEV eluates with an anti-H2B antibody and analyzed the purified contents. Our results showed that H2B re-purified complexes contained yFACT as well all core histones (Figure 2.2b). This data thus suggested that yFACT does not facilitate nucleosome disassembly. More importantly, however it showed that there is no population of yFACT \textit{in vivo}, which is exclusively bound to just H2A/H2B dimers as opposed to full nucleosomes. Taken together these results provided support for the “global-accessibility” model of yFACT function, and showed that \textit{in vivo} yFACT is stably associated with nucleosomes.
Figure 2.2 yFACT bound nucleosomes maintain original histone composition

a) Salt wash titration of Pob3\textsubscript{TAP} purified yFACT-nucleosome complexes. Bead-immobilized FACT-associated nucleosomes were washed 3x10min in lysis buffer containing the indicated amounts of NaCl. Samples were eluted off beads with SDS-loading buffer and imaged using coomassie-stained PAGE. b) Re-ChIP of Pob3\textsubscript{TAP} purified yFACT-associated nucleosome complexes. INP: 5% of TEV elution from purified, MNase digested Pob3\textsubscript{TAP} purification. TEV elution due to TAP cleavage resulted in a decrease in MW of Pob3. Lanes 2 and 3: 33% of re-immunoprecipitated INP with αH2B Ab (+) or beads only (-). Samples were imaged using SDS-PAGE stained with coomassie. Top panel: yFACT, bottom panel: yFACT-associated histones.
2.3.2 yFACT-associated nucleosome particles display altered structure

We found that in vivo yFACT binds to nucleosomes. One of the proposed models of FACT function, the “global accessibility” model suggests that yFACT alters the nucleosome structure in the process of binding nucleosomes\(^7\). Consistent with this postulate, the yFACT alters the DNaseI and hydroxyl-radical footprinting of yFACT-bound nucleosomes in vitro, while retaining the nucleosome protein composition\(^1\). We therefore hypothesized that the nucleosomes in our Pob3\(_T\)\(_{\text{AP}}\) purifications might display an altered structure as well. To directly compare the structure of yFACT bound nucleosomes to canonical nucleosomes, we imaged the yFACT-nucleosomes using Electron Microscopy (EM).

To isolate a homogenous population of the yFACT-bound nucleosomes, we first affinity purified yFACT-associated chromatin onto magnetic beads. A limited on-bead MNase digest, optimized to preserve even the fragile nucleosomes, followed the purification. We eluted the yFACT bound nucleosomes off beads using TEV protease, and fractionated the eluate on a GE Superose6 sizing column. We found that yFACT associated with nucleosomal and sub-nucleosomal sized DNA in Fractions (F) 13, 14 and 15 (Figure 2.3c). In the same fractions, yFACT also associated with all core histone proteins (Figure 2.3b). This was consistent with the predicted elution profile for a \(~500\text{kDa}\) complex (Figure 2.3a). The fraction aliquots were then spotted on the grids and treated with a negative stain\(^\text{13}\). The EM images from F13 to 15 as well as control, purified chicken mono-nucleosome sample were analyzed using the EMAN2 processing suite \(^\text{14}\). The particles were generated using semi-automated swarm and Gaussian peak detection. The averaged particles were grouped into eleven classes for both the
control and yFACT-nucleosomes. The average structures were generated using edge-mean normalized entities within each group.

We found that the yFACT-associated nucleosome particles differed in shape as compared to canonical, chicken mono-nucleosomes (Figure 2.4a). Quantitative analysis of each of the eleven classes showed that yFACT-associated nucleosome particles clustered into two groups, and were on average more elongated in shape than the canonical nucleosomes (Figure 2.4b). Measurement of individual yFACT-nucleosome particles revealed that they were also elongated in shape as compared to the control (Figure 2.4c). Consistent with our expectations we found that the yFACT-associated nucleosome particles had a larger area as compared to the control, presumably due to bound yFACT. We also found that ellipticity was not correlated with area, suggesting that the effects we observed are not due to just the increased size of particles (Figure 2.4d). Together this unprecedented look at yFACT suggested that the yFACT-nucleosome particles are elliptical in shape. This data further supported the notion that \textit{in vivo} yFACT binds specifically to nucleosomes.
Figure 2.3 Isolation of yFACT-associated nucleosome population

The in vivo purified Pob3\textsubscript{TAP} samples were subjected to partial MNase digest and TEV elution off beads. The resulting yFACT-nucleosome population was resolved on GE Superose 6 gel filtration column. a) The UV\textsubscript{280} absorbance reading of Superose 6 fractions. b) Silver stain and immunoblot of SDS-PAGE of TCA-precipitated gel filtration fractions. INP: 5% of TEV elution from purified, MNase digested Pob3\textsubscript{TAP} purification, 7-17: collected fractions. c) The DNA content analysis of column fractions. Samples are as described in “B”. Image of an agarose gel stained with Syto60.
Figure 2.4 The EM image of yFACT-associated nucleosome particles

The in vivo purified Pob3\textsubscript{TAP} samples were subjected to partial MNase digest and TEV elution off beads. The resulting yFACT-associated nucleosome population was resolved on GE Superose 6 gel filtration column. The yFACT-nucleosomes (FACT) from fractions 13, 14 and 15 were imaged using Electron Microscope at 49000x magnification. The particles were analyzed using EMAN2 processing suite. Sample of purified chicken mono-nucleosomes was used as control (Nuc). Note: due to its small size and insufficient electron dispersion, we were unable to image the yFACT alone. a) Clustering of yFACT and control particles into the eleven different classes each based on shape and area. b) Bean plots of quantification of the ratio of major to minor axis of the yFACT-nucleosome classes as compared to control, “1” indicates the ratio of one. The edges of the plot represent a Gaussian probability distribution of a given ratio c) Same as “b” for individual particles. d) Smooth scatter plots of the individual particle area correlated with the ellipticity as measured by the ratio of the major to minor axis.
2.3.3 yFACT binds to hyper-acetylated nucleosomes *in vivo*

Chromatin is dynamically regulated through numerous, post-translational modifications such as histone acetylation \[^3\]. These modifications affect both the inherent nucleosome structure and the recruitment of chromatin-modifying enzymes. The yFACT complex interacts with enzymes regulating histone modifications, such as the KAT, NuA3, but the functional implications of these interactions remain elusive \[^{56}\]. Given our findings that yFACT stably binds to nucleosomes *in vivo*, we set out to uncover the modification status of the yFACT bound chromatin.

To determine the yFACT-associated histone modifications, we purified yFACT bound nucleosomes and performed tandem MS/MS on the affinity-purified material. To aid with identification of histone modifications, we propionylated the lysine residues on purified poly-peptides prior to the proteolytic cleavage with trypsin. Propionylation blocks cleavage of lysine residues by trypsin, thus preserving the lysine-rich histone tail. The analysis of the MS/MS data revealed that the yFACT-associated nucleosomes contained a number of mono- and poly-acetylated peptides (Figure 2.5a, Table 4). For example, we found tri- and tetra- acetylated peptides corresponding to the N-terminal tail of histone H4, as well as di- and tri- acetylated peptides corresponding to the histone H3 N-terminal tail. Interestingly, we also found numerous peptides corresponding to H3K56ac. Altogether, the MS/MS analysis showed that yFACT co-purifies with acetylated histones.

The co-purification of acetylated histone peptides with yFACT suggests that the yFACT-associated histones might be hyper-acetylated with respect to bulk chromatin. To test this we compared the histone PTMs of bulk chromatin to yFACT-associated
histones by Western blotting using antibodies that recognize specific histone acetylation marks. Consistent with our prediction, we observed an enrichment of acetylation on the yFACT-associated histones. Specifically H3K14ac, H3K23ac and H3K56ac were enriched on the yFACT-bound histones as compared to bulk chromatin. This effect was specific as two other histone marks, H3K4me3 and H3K79me3 were not enriched (Figure 2.5b). In summary, our results showed that yFACT-bound nucleosomes are hyper-acetylated as compared to bulk chromatin.
Figure 2.5 yFACT-associated nucleosomes are hyper-acetylated

a) Pob3\textsubscript{TAP} purified yFACT-associated nucleosomes were subjected to MS/MS. The propionylated polypeptides were digested with trypsin to preserve the lysine-rich histone tails thus allowed for detection of the histone-tail modifications. The graph represents the total number of acetylated, N-terminal tail peptides as compared to unmodified (propionylated only) peptides.

b) Western blot of the contents of Pob3\textsubscript{TAP} purifications that were eluted off beads using SDS and run on PAGE. Histone levels were normalized between the bulk chromatin (INP) and the yFACT-nucleosomes (IP). The levels of histone modifications were determined using the indicated antibodies.
Table 4 List of acetylated peptides from a Pob3\textsubscript{TAP}-based nucleosome purification identified by MS/MS

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<th>Protein</th>
<th>Peptide Score*</th>
<th>Peptide Sequence</th>
<th>Modification</th>
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* Peptide Score: Measure of Confidence, higher score indicates more confident hit. Peptide Score cutoff is set to maintain False Positive Rate (FDR) below 1%.
2.3.4 yFACT is recruited to chromatin by H3K56ac

We found that H3K56ac is highly enriched on the yFACT-bound nucleosomes as compared to bulk chromatin. H3K56ac is a mark of histone synthesis in S-phase, and has been linked to histone deposition on gene promoter regions outside of DNA replication. H3K56ac has been shown to facilitate transcription factor binding to DNA through partial-unwrapping of nucleosomes, thereby allowing access to the nucleosomal DNA. The yFACT complex contains the HMG-group Nhp6a/b accessory subunits, which bind DNA. Given the high number of H3K56ac peptides identified by MS on the yFACT-bound nucleosomes, we hypothesized that H3K56ac might play a role in recruitment of yFACT to chromatin. To test this hypothesis we purified yFACT-nucleosome complexes from Wild Type (WT) and rtt109 strains. Rtt109 is the major lysine acetyl-transferase that modifies H3K56ac in yeast and thus we reasoned that its disruption should allow us to mimic the loss of H3K56ac. In concordance with our hypothesis, we found that in the absence of Rtt109, yFACT associated with fewer H3/H4 molecules in vivo (Figure 2.6a, Table 5). This suggested that Rtt109, and presumably H3K56 acetylation, enhance yFACT binding to chromatin.

To quantify the contribution of Rtt109 in yFACT recruitment to chromatin, we measured the histone loss of yFACT-associated chromatin with SILAC mass spectrometry. We differentially labeled the WT and rtt109 strains with heavy and light nitrogen (15N and 14N), purified yFACT-nucleosome complexes and subjected the contents to MS/MS. Using histone peptide numbers, normalized to the levels of Pob3 and Spt16, we found that in rtt109 there were approximately 50% fewer histones H2A, H2B, H3 and H4 than in WT (Figure 2.6b). Given the stoichiometric loss of all four
histones in the mutant, these results suggested that Rtt109 positively affects the recruitment of yFACT to nucleosomes as opposed to just H3. Additionally the even loss of all histones as a result of loss of modification on only one of the four histones, served as yet another confirmation of yFACT stably binding to nucleosomes in vivo.
Figure 2.6 H3K56ac promotes yFACT binding to nucleosomes

a) Western blot of the contents of Pob3_TAP purifications that were eluted off beads using SDS and run on PAGE. The samples were normalized for Pob3_TAP and the amount of histones was determined using the indicated antibodies. b) WT and rtt109Δ strains carrying Pob3_TAP were differentially labeled with heavy (N_{15}) or light (N_{14}) nitrogen. Following a Pob3_TAP-based purification, the contents were mixed, in-gel digested with trypsin and subjected to MS/MS. The levels of histone peptides were normalized to the peptides from Pob3 and Spt16, as well as between the samples. The peptide numbers from WT and rtt109Δ were added together and their respective ratios were plotted as a fraction of “1”.

Table 5 Comparison of Histone Levels from Pob3_TAP purification in WT and rtt109Δ by SiLAC MS/MS

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We next analyzed the effect of H3K56ac on the locus-specific recruitment of yFACT to chromatin by ChIP-QPCR. In agreement with our findings regarding the recruitment of yFACT to bulk chromatin, the cross-linking of yFACT to the 3’ region of PMA1 was severely impaired in the rtt109Δ strain as measured by ChIP (Figure 2.7a). Furthermore, mutation of H3K56 to glutamine or arginine also resulted in a decrease in the yFACT cross-linking at PMA1 3’ (Figure 2.7b). These results showed that the histone H3K56ac positively regulates yFACT recruitment to chromatin.

Recently, another histone chaperone – Rtt106 is recruited to chromatin through a Pleckstrin Homology (PH) domain [23]. The Pob3 subunit of yFACT contains a homologous PH domain, but H3K56ac has little effect on Pob3 binding to histone H3 [23]. However, our results clearly argue the case where H3K56ac has an effect on yFACT recruitment to nucleosomes. While the PH domain of Pob3 might not increase the affinity of yFACT to histone H3, the H3K56ac-facilitated unwrapping of nucleosomes could serve as a mechanism for Nhp6a/b (and yFACT) recruitment to DNA [28]. We therefore hypothesized that H3K56ac does not directly increase the affinity of yFACT for binding of H3, but acts through an indirect, DNA-mediated mechanism. To test our hypothesis we purified yFACT in the presence of EtBr to disrupt nucleosomes and isolate yFACT-histone complexes. We then assayed for the enrichment of H3K56ac in our purifications and compared its levels to bulk chromatin in the absence and presence of EtBr using quantitative Western Blotting. We found that the EtBr fraction lost all of the enrichment of H3K56ac on the yFACT-bound histones (Figure 2.7c). This result indicated that yFACT does not have a direct preference for K56ac histone H3, but rather that H3K56ac enhances yFACT binding through a nucleosome-dependent mechanism.
Taken together our results showed that the association of yFACT with nucleosomes was positively influenced by H3K56ac and that this enhancement required the presence of nucleosomes.
Figure 2.7 H3K56ac promotes yFACT recruitment to chromatin.

a) WT and rtt109Δ cells carrying Pob3TAP were cross-linked with formaldehyde and lysed. The resulting lysates were immune-precipitated with IgG rabbit serum coupled to beads. The DNA levels were determined using Q-PCR with primers to 3’ of PMA1. b) Cells expressing a single copy of WT, H3K56Q or H3K56R histone H3 and Pob3TAP were processed as described in “a” c) Pob3TAP purifications were done in triplicate in the presence or absence of 400ug/ml of EtBr. The resulting proteins were resolved with SDS-PAGE. The levels of H3K56ac and H3 were determined using specific antibodies. The densitometry-quantified levels of H3K56 were normalized to their respective inputs. The error bars represent the standard deviation from the mean (n=3).
2.3.5 The effect of the cell cycle on yFACT-associated chromatin

H3K56ac varies with the cell cycle \(^{[21]}\). We found that yFACT-preferentially associated with H3K56ac chromatin. This led us to ask whether the association of yFACT with chromatin varies though the cell cycle with changes in H3K56ac. To test this we arrested the cells in G1, S and G2/M using alpha-factor, hydroxyurea (HU) and nocodazole respectively. We purified the yFACT-nucleosome complexes, normalized the samples for the levels of histone H3 and analyzed the yFACT levels in our purifications. We found that yFACT association with histone H3 through the cell cycle remained constant (Figure 2.8 a). We thus did not observe any cell-cycle dependent changes of yFACT association with chromatin. These results can be explained by the existence of another targeting mechanism, independent of H3K56ac. If there is another targeting mechanism for yFACT other than H3K56ac then we should observe cell-cycle dependent fluctuations of H3K56ac on the yFACT-associated chromatin. To test this we purified the yFACT-associated nucleosomes and compared the H3K56ac levels to bulk chromatin by western blot. We observed an increase in H3K56ac in S-phase, while H3K56ac also persisted in G1 on yFACT-associated histones however to a lesser degree (Figure 2.7b top panel). These results confirmed that there is likely to be another mechanism targeting yFACT to chromatin that functions independently of the H3K56ac.

Histone post-translational modifications (PTM) can be used to probe for nucleosome localization on the ORFs of genes. For example H3K4me3 is primarily found at the 5’ ends of genes, while H3K79me3 occupies the entire gene \(^{[3]}\). To indirectly test for changes in yFACT localization, we asked whether the other yFACT-associated histone modifications change with the cell cycle. To test this we assayed the
levels of histone modifications in the yFACT-nucleosome purifications from cells arrested at different stages of the cell-cycle. In contrast to H3K56ac, we found that the levels of H3K14ac, H3K4me3 and H3K79me3 remained constant throughout the cell-cycle, indicating that yFACT binds to chromatin regions bearing similar PTM signatures throughout the cell cycle (Figure 2.7b, bottom panels). To confirm that our arrests resulted in synchronous cell populations, we performed FACS analysis on the cell inputs for purifications. We found that the assayed populations displayed profiles consistent with the expected cell-cycle stage (Figure 2.7c). Taken together we interpret these results to mean that while H3K56ac increases yFACT recruitment to chromatin, yFACT binding does not alter during the cell-cycle, indicating that other recruitment mechanisms of yFACT maintain its occupancy.
**Figure 2.8 yFACT-nucleosome associated H3K56ac is dependent on the cell cycle**

a) b) Cells were arrested in G1 using alpha-factor, S using hydroxyurea and G2/M with nocodazole. Western Blots of the contents of Pob3\textsubscript{TAP} purifications, eluted off beads with SDS and run on PAGE were probed using indicated antibodies. Samples were normalized for yFACT and histone H3 levels. INP signifies bulk chromatin fraction. IP stands for the Pob3\textsubscript{TAP} based purification c) FACS analysis of cells used in a) and b). The DNA was stained with propidium iodide.
2.3.6 The yFACT-bound nucleosomes are directly acetylated by NuA3

We found that yFACT associated with nucleosomes that were hyper-acetylated at H3K14 and H3K23. H3K14ac and H3K23ac have been shown to be the marks of NuA3 activity in vitro\textsuperscript{[14]}. The Spt16 subunit of yFACT was previously shown to directly interact with NuA3 thorough the Sas3 C-terminal domain\textsuperscript{[56]}. We therefore hypothesized that an explanation for the observed hyper-acetylation of the H3K14ac and H3K23ac could be a direct interaction between yFACT and NuA3. NuA3 is targeted through multiple mechanisms\textsuperscript{[43][17][57][55]}. Therefore in order to directly test for the functional implication of the yFACT-NuA3 interaction we used a truncated version of Sas3 lacking the C-terminal interaction domain (ΔCTD). The deletion of Sas3CTD should only disrupt yFACT-NuA3 interaction thus preserving other NuA3 activities.

We first tested the stability of the NuA3-complex in strains expressing Sas3ΔCTD. Nto1 is a subunit of NuA3 and its association with Sas3 has been shown to be an indicator of complex stability\textsuperscript{[59]}. We performed an anti-FLAG co-IP from sas3ΔNTO1HA cells that carried empty plasmid, or expressed a FLAG-tagged catalytically dead (CD) point mutant of Sas3-CD, FLAG-sas3ΔCTD or FLAG-SAS3. We found that while in the strain lacking Sas3 we did not detect any FLAG or HA signal, Sas3CDFLAG, Sas3ΔCTDFLAG or Sas3FLAG co-immunoprecipitated with equal amounts of Nto1HA (Figure 2.9a). We interpreted this result to mean that deletion of the CTD of Sas3 did not affect NuA3 stability.

To test whether the Spt16-Sas3 interaction has any direct functional implications on either the bulk chromatin or the yFACT-associated nucleosomes we assayed the levels of H3K14 and H3K23 acetylation on the inputs and purified yFACT-nucleosomes,
in sas3Δ strains carrying plasmids expressing wild type Sas3, catalytic dead Sas3M1 or Sas3∆CTD. We found that while on bulk chromatin only, the catalytically dead version of Sas3 showed a significant loss of H3K14ac and H3K23ac, the yFACT-associated nucleosomes showed loss of acetylation in both the Sas3CD and the Sas3∆CTD mutants (Figure 2.9b, c). This result suggested that NuA3 acetylation was more prominent on yFACT-associated nucleosomes as compared to bulk chromatin, and that the interaction between NuA3 and yFACT mediated acetylation of the yFACT-associated nucleosomes.
Figure 2.9 yFACT-associated nucleosomes are directly acetylated by NuA3

a) The co-immunoprecipitation of FLAG tagged Sas3M1, Sas3∆CTD and Sas3 with Nto1HA. Cell lysates from indicated strains (INP) were incubated with the FLAG agarose beads. The resulting immunoprecipitated proteins were eluted off beads with 1% SDS and run on a SDS-PAGE. The blotted membrane was probed using αHA and αFLAG antibodies. b) The sas3Δ Pob3TAP strains carrying plasmids expressing Sas3CD catalytically dead mutant, Sas3∆CTD and WT Sas3 were used for yFACT-nucleosome purifications. The Pob3TAP associated histones as well as bulk chromatin were assayed for levels of the H3K14ac and H3K23ac with Western Blotting using indicated antibodies. c) Preparation as in “b”, Pob3TAP purifications and Western Blots were performed in triplicate (n=3) and quantified using densitometry. The ratio of Sas3∆CTD/WT ac was expressed as fraction of “Normal”, which corresponds to acetylation levels per histone observed in WT strain.
2.4 Discussion

The mode of yFACT function *in vivo* has been a long-standing point of contention. yFACT has been proposed to work either by recycling histones H2A/H2B around the RNAPII bubble or alternatively by binding nucleosomes and altering their structure [78]. Our work showed that yFACT most likely functions through stably binding nucleosomes and altering their structure in accordance with the “global accessibility” model.

The RNAPII-mediated transcription of DNA is generally considered as a process that is disruptive to nucleosomal structure [3]. However, a body of evidence argues that chromatin is surprisingly stable and histones might not dissociate from DNA during the RNAPII passage. It is estimated that 81% of nuclear DNA is covered in nucleosomes, and even more so, recent studies showed that even the canonical NDR are occupied by nucleosomes that display increased sensitivity to MNase [121][128][127]. Additionally, the passage of RNAPII itself has been found to be less disruptive than previously thought. RNAPII progresses through nucleosomes with high survival of nucleosomes and low histone H3/H4 exchange rates [151]. Our data that yFACT acts through stable association with nucleosomes further supports these findings. yFACT is an elongation factor and thus by acting alongside the RNAPII it might further contribute to this observed chromatin stability. Indeed loss of the yFACT complex causes nucleosome depletion and increased histone turnover on both promoters and ORFs [118]. This notion was further supported by our EM analysis. We found that yFACT-nucleosome particles display an altered, elongated structure as compared to the chicken mono-nucleosome control. Despite the alteration of structure, we did not observe a decrease in the area of the yFACT-nucleosome particles indicative of histone loss, but on the contrary see it
increase. If yFACT were to disassemble nucleosomes we would observe particles with a smaller area. We therefore speculate that the observed increase in area is indicative of an intact nucleosome. In summary, this data supports the notion that yFACT stabilizes nucleosome structure on actively transcribed genes.

Transcription-induced histone exchange provides a challenge for histone-mark conservation. If modified histones are lost and not replaced by the same particles, the chromatin landscape will become altered. The action of the histone chaperones, which bind and actively replace lost histones has been proposed to counter this effect \(^3\)[79]. The yFACT-induced nucleosomal stability has likely implications on the histone-mark landscape. If the nucleosomes are not evicted during transcription, it provides a convenient way for histone-mark stability through the process. By preventing histone exchange, yFACT might ensure that modifications, such as acetylation, stay associated with their respective regions. This in turn might be important for subsequent rounds of transcription or transcriptional memory.

The stable binding of yFACT to nucleosomes contrasts the mode of action of other known histone chaperones, such as Spt6 \(^{79}\). Spt6 facilitates histone removal and recycling, particularly at very highly transcribed genes where presumably the stabilizing role of yFACT is not sufficient to withstand multiple RNAPII moving through chromatin \(^{152}\). The Hir/Hpc complex on the other hand, functions by depositing histones \(^{79}\). A possible model, which reconciles these multiple mechanisms, is one where a concerted effort is used to maintain nucleosomal occupancy. For example, during transcription the nucleosomes, which become too unstable to be anchored by yFACT, become recycled by Spt6. If histones fail to be recycled, they are replaced by Hir/Hpc with newly
synthesized H3K56ac histones thus facilitating subsequent yFACT recruitment \[^{21}\]. Such multi-tiered mechanism would ensure that the chromatin is maintained with high fidelity.

Our data showed that H3K56ac acts to recruit yFACT to chromatin. This recruitment was likely not mediated by Pob3PH interaction with H3K56ac \[^{23}\]. We found that yFACT preferentially binds to H3K56ac nucleosomes throughout the cell cycle. H3K56ac is a mark of histone deposition and is responsible for unwrapping of the nucleosomal DNA, while retaining nucleosomal stability \[^{26}\][\(^{21}\)]. The unwrapping of DNA facilitates increased protein binding, due to increased target site availability, as in the case of LexA \[^{28}\]. We speculate that a similar mechanism takes place in yFACT recruitment. The DNA surrounding H3K56ac in the nucleosomes is subject to increased unwrapping. The unwrapping in turn recruits Nhp6a/b to DNA and thus yFACT. Such a mode of recruitment could complement other mechanisms of sequestering yFACT- such as the physical interaction with Paf1, which is transcription-dependent \[^{115}\]. A multivalent mode of recruitment of yFACT might mean that this complex even with a singular activity can perform very diverse functions due to spatial and temporal variation in its placement across the genome.

yFACT interacts with the NuA3 acetyl-transferase \[^{56}\]. The yFACT-NuA3 interaction is dependent on the CTD of Sas3 \[^{153}\]. It however remained unknown whether this interaction had any functional significance. Our data showed that while the changes to bulk chromatin acetylation in the sas3\(\Delta\)CTD mutant are negligible, loss of the CTD conferred a marked loss of acetylation when only the nucleosomes associated with yFACT were taken into consideration. The inability to detect changes in acetylation in bulk chromatin likely arises from the fact that NuA3 contains multiple targeting
mechanisms, which compensate for the loss of the CTD $^{18\[17\]}^{57}$. As for the function of this acetylation, it can only be speculated, but it might play a role in transcription by facilitating RNAPII passage through alteration of nucleosome structure, as we observed by EM. In summary, this work furthers the understanding of yFACT function in vivo as well as provides insight into how chromatin stability can be maintained despite transcription and high rates of acetylation.
3 Chapter: yFACT-Bound Nucleosomes are Positioned Around Nhp6 Binding Sequences

3.1 Introduction

While the mechanism of yFACT function has been partially solved, the mode of its recruitment to chromatin remains elusive \cite{78}. Although yFACT has been proposed to be recruited to chromatin by means such as interaction with the Paf1 components or one of the polymerases, there is no other, defined mechanism of yFACT targeting \textit{in vivo} \cite{115}. Earlier in this work we showed that yFACT is recruited to chromatin with the help of H3K56ac. This mode of recruitment however does not seem to depend directly on yFACT affinity for H3K56ac modified chromatin, but rather on yFACT recognizing a property of a H3K56ac nucleosome, such as an altered structure or increased DNA unwrapping.

yFACT is comprised of Spt16, Pob3 and Nhp6a/b that associates with the two othersubunits in non-stoichiometric amounts. The mammalian counterpart of yFACT is a heterodimer of Spt16 and SSRP1. SSRP1 contains a HMG box, Nhp6-like domain at its C-terminus \cite{104,106}. Nhp6a and Nhp6b are small, abundant High Mobility Group (HMG) proteins. They have demonstrated ability to bind DNA and induce a sharp, 70° bend in the minor groove through their L-shaped HMG box fold. At the same time the N-terminus of the Nhp6a protein can make contacts with the major groove of DNA \cite{155}. Recently the Nhp6a/b proteins have been demonstrated to display sequence preference for TATATAA-containing DNA sequences \cite{156}. As many as ten molecules of Nhp6a can bind to a nucleosome and that this facilitates the recruitment of FACT (Spt16 and Pob3)
*in vitro*[^107]. Nhp6a/b, although not essential, are required for proper induction of RNAPII transcripts. Gel shift studies show that Nhp6a/b facilitate complex formation between TBP and TFIIA at the TATA box[^157]. The action of Nhp6a/b has an effect on RNAPIII mediated transcription, for example, of the *SNR6* gene expression depended on the presence of Nhp6a/b. At the same time, the loss of Nhp6a/b results in a decrease of MNase protection at the *SNR6* TATA suggesting that Nhp6a/b helps to maintain chromatin structure over the promoter[^158].

*Saccharomyces cerevisiae* promoters are often characterized by a Nucleosome Depleted Region (NDR), preceding the “+1” nucleosome. The NDR is thought to be the site of Pre-Initiation Complex (PIC) formation and contains the TATA box sequence[^159]. The TATA box consensus sequence- TATAWAWR, is bound by the TATA Binding Protein (TBP). TBP binding is the first step of an orchestrated PIC recruitment. However only 10% of genes contain a canonical TATA box sequence, while the other 90% do not[^124]. TBP binding to the canonical TATA sequences is regulated by the SAGA complex, while the other 90% of genes see TBP bound as a part of a TFIID (Taf1-containing) complex[^160][^124]. The TBP contained within TFIID has the ability to recognize the TATA sequences that deviate from the canonical TATAWAWR. Bioinformatic analysis shows that 99% of all promoters contain a sequence that is two mismatches or less similar to TATAWAWR[^124]. From here on, we will use the term “TATA element” to refer to both canonical TATA-box consensus and TATA-like elements. The TFIID dependence manifests itself in the architecture of these promoters, where the Taf1-enriched promoters contain a well positioned “+1” nucleosome, and canonical TATA-box containing Taf1-depleted promoters do not. TFIID has been speculated to be
responsible for this positioning\cite{124}. The lack of a well positioned “+1” nucleosome at the Taf1-independent promoters has been attributed to increased gene plasticity, for these promoters are SAGA regulated and more likely to be inducible\cite{124}.

Although gene promoters have been generally thought to contain only the “+1” and “-1” nucleosomes flanking the NDR, more recent studies showed that nucleosome occupancy might be more pervasive than previously thought. The protocols utilized to study the chromatin landscape use MNase conditions optimized to obtain uniform \(\sim 150\text{bp}\) DNA fragments. In this process however, a portion of the DNA becomes lost due to higher MNase susceptibility as an effect of sequence bias or insufficient protection. Work done with MNase conditions optimized to preserve these “fragile nucleosomes” revealed that the canonical NDRs are actually occupied by chromatin, including the promoter regions of genes. The fragile nucleosome at the NDR is termed “0” and its occupancy is correlated with the presence of the TATA box\cite{127}\cite{128}. The presence of nucleosomes over gene promoters raises questions about the co-occupancy of nucleosomes and the PIC, and their spatial arrangement. While the majority of the yeast genome is thought to be transcribed or active, in actuality, only about 5% of genes account for almost 25% of all mRNA in the cell\cite{161}. What this likely means is that the PIC and nucleosome co-occupancy at the NDR might be a population effect and never does the PIC actually physically share the promoter sequence with a nucleosome.

In a previous chapter, we showed that yFACT binds hyper-acetylated nucleosomes. We were wondering if this means that yFACT stabilizes fragile nucleosomes so we mapped the positions of yFACT-associated nucleosomes. Here we
present an *in vivo* map of yFACT-nucleosome interactions at a single nucleosome resolution. We show that yFACT-bound nucleosomes are localized over canonical NDR and NDR promoters. Moreover, the yFACT-nucleosome positioning is defined by the TATA box sequence at promoter regions, while outside of the promoters it is defined by the Nhp6a/b consensus binding site TATATAA. We show for the first time in *Saccharomyces cerevisiae* that yFACT bound nucleosomes occupy the regions of DNA around the Nhp6a/b binding sites. The presence of yFACT-nucleosomes correlates with TBP and TFIIA occupancy but not with other PIC components. Using meta-analysis of data from Celona et al. we show that the loss of Nhp6a/b causes chromatin collapse at loci containing yFACT-nucleosomes [162].
3.2 Methods

3.2.1 Yeast cell culture

All cultures were grown in YPD unless indicated otherwise at 30\(^\circ\)C to an OD\(_{600}\) of 0.6-0.8. A detailed list of strains used can be found in Table 6.

**Table 6 Yeast strains used in this study**

<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Mating Type</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>YLH 101</td>
<td>Mat a</td>
<td>\textit{his3}\Delta 200\textit{leu2}\Delta 1\textit{lys2-128d}\textit{ura3-52}\textit{trp1}\Delta 63</td>
</tr>
<tr>
<td>YLH 498</td>
<td>Mat a</td>
<td>\textit{his3}\Delta 200\textit{leu2}\Delta 1\textit{lys2-128d}\textit{ura3-52}\textit{trp1}\Delta 63 \textit{POB3TAP::URA3}</td>
</tr>
</tbody>
</table>

3.2.2 Antibodies

We obtained IgG from Milipore (Cat # PP64, Lot# LV1602263).

3.2.3 \textit{Pob3}\textit{TAP} based FACT-purification and MNase digestion

\textit{Pob3}\textit{TAP} and its associated proteins were purified using a one-step TAP-tag based approach based on Lambert et al.. Briefly, 1L of each WT and \textit{Pob3}\textit{TAP} strain were grown to an OD of 0.8 in YPD at 30\(^\circ\)C. Cells were re-suspended in lysis buffer (100mM HEPES pH 8, 20mM Mg acetate, 300mM Na acetate, 10% glycerol, 10mM EGTA, 0.1mM EDTA), lysed using bead beating and gently sonicated (three cycles of 20 sec on, 30 sec off). The proteins were extracted by adding NP40 to 1% and rotating the lysates for 10 minutes at 4\(^\circ\)C, centrifuged at 3000rpm for 5 min, followed by re-extraction of the pellet with lysis buffer with 1% NP-40. The affinity purification was performed using rabbit IgG cross-linked to magnetic beads (Invitrogen Dynabeads M-270 Epoxy) and rotation of the beads and lysates for 5 hours at 4\(^\circ\)C. The beads were then washed 3 times in the lysis buffer with 1% NP-40, 3 times with lysis buffer and
eluted with 35ul of SDS loading buffer. For the purpose of the MNase digestion the purified FACT-nucleosome complexes were washed in MNase digestion buffer (50mM Tris pH8, 4mM MgCl₂, 2mM CaCl₂) and digested with limited amounts of MNase (0.05-0.2U/reaction). The reactions were stopped by washing the beads with STOP buffer (50mM Tris pH8, 4mM MgCl₂, 10mM EDTA). The DNA was purified using phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation.

3.2.4 Illumina sequencing and data analysis

The library construction protocol was performed as described in Maltby et al. Library construction for the Illumina platform was performed using a custom procedure for paired-end sequencing. Briefly, 2–10 ng of ChIP material was end-repaired and A-tailed before being ligated to TruSeq PE adaptors. In between each reaction, the material was purified using phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation. The resulting material was then amplified in the Phusion HF master mix (NEB) using TruSeq PE PCR primer 1.0 and custom indexed multiplexing primers [5′ AAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′, where “NNNNNN” corresponds to unique hexamer barcodes]. PCR amplification was performed as follows: denaturation at 98 °C for 60 s; eight cycles of (98 °C, 30 s; 65 °C, 30 s; 72 °C, 30 s), and a final extension at 72 °C for 5 min. Amplified libraries were purified using 0.8 (vol) Agencourt AMPure XP solid phase reversible immobilization paramagnetic beads and eluted in 10 mM Tris-HCl pH 8.5. An aliquot of each library was run on an Agilent High Sensitivity chip to check the size distribution and molarity of the PCR products. Equimolar amounts of indexed, amplified
libraries were pooled, and fragments in the 200–600 bp size range were selected on an 8% (wt/vol) Novex TBE PAGE gel (Invitrogen). An aliquot (1 μL) of the library pool was run on an Agilent High Sensitivity chip to confirm proper size selection and measure DNA concentration. The pooled libraries were diluted to 15 nM and their concentration was confirmed using the Quant-iT dsDNA HS assay kit and Qubit fluorometer (Invitrogen). Libraries were sequenced on the Illumina HiSeq platform at the UBC Biodiversity Research Centre NextGen Sequencing Facility. Clusters were generated on the cBOT (HiSeq2000) and paired-end 100 nucleotide reads generated using v3 sequencing reagents on the HiSeq2000 (SBS) platform. The hexamer barcode was sequenced using the following primer [5’ GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3’]. Image analysis, base-calling, and error calibration were performed using Casava 1.8.2 (Illumina). Reads were aligned to the *S. cerevisiae* genome using BWA [164]. The samples were normalized for the number of reads and the peaks were called using the GeneTrack algorithm on Galaxy (galaxy.psu.edu) service [165]. The peaks were visualized using SeqMonk (v0.21.0: www.bioinformatics.bbsrc.ac.uk/projects/seqmonk). The total peaks were normalized for the number of probes and aligned around various genomic features. The k-means clustering analysis was done using “Seqminer” platform with default parameters, except for no read extension and 600bp window size (http://bips.u-strasbg.fr/seqminer/tiki-index.php).
3.3 Results

3.3.1 The yFACT bound nucleosomes are positioned over nucleosome-depleted regions.

In the previous chapter we showed that yFACT stably binds to nucleosomes in vivo. To test where the yFACT-associated nucleosomes are localized on the genome, we sequenced the nucleosomal DNA associated with yFACT using an Illumina sequencer. To isolate the DNA from yFACT-bound nucleosomes, we first affinity-purified the yFACT-chromatin onto magnetic beads. The purification was followed by an on-bead MNase digest. We then eluted the yFACT-bound nucleosomes off beads with TEV protease, and purified the DNA, which was sequenced on an Illumina Hi-Seq instrument. The reads were aligned to the *Saccharomyces cerevisiae* genome using the Burows-Wheeler Aligner (BWA) mapping, normalized for the total number of reads, and the read-peaks were called with the GeneTrack algorithm. Thus through our approach we were able to map the peaks (regions of enrichment) of the yFACT-bound nucleosomes across the yeast genome.

We aligned the yFACT-bound nucleosome peaks (Figure 3.1a lower panel, black line) over the 4393 annotated Transcription Start Sites (TSS), and compared them to the peak-calls from formaldehyde-crosslinked MNase digestion of bulk chromatin (grey line). We found that the yFACT-associated nucleosomes were positioned over the entire ORF and did not show any depleted regions (lower panel). More so relative to all nucleosomes they were enriched over the canonical Nucleosome Depleted Region (NDR) flanked by the “-1” and “+1” nucleosomes (Figure 3.1a, upper panel).
The peak mapping methods, such as GeneTrack, used in this study rely on the ability to determine the exact dyad of the nucleosome \[^{165}\]. In the previous chapter, we showed that the nucleosomes associated with yFACT are highly acetylated. Histone acetylation has been correlated with increased nucleosomal DNA unwrapping \[^{9}\]. If a nucleosome is more prone to unwrapping, it might also cause it to be more MNase sensitive, thus altering the expected MNase footprint. Additionally the FACT (Spt16, Pob3) complex is an asymmetrical heterodimer and binding of yFACT to nucleosomes produces a footprint, which is uneven with respect to the nucleosome dyad. This suggested that yFACT, when bound to nucleosomes, might differentially affect the directionality of DNA protection from MNase \[^{129}][^{108}\]. Thus, the nucleosome dyad of the species bound by yFACT might vary in its positioning and present a challenge for accurate peak mapping. Therefore, instead of mapping the dyad peaks we called the peaks on the read edges, which define the borders of a nucleosome. Using this method, we can accurately predict the edges of nucleosomes or any other feature and bypass the need for dyad determination. Mapping the edges produces two peaks, corresponding to places of increased MNase sensitivity around the feature, each peak positioned on either side of the nucleosome, with the feature itself spanning the space in-between.

To confirm that the mapping of the read edges is a reliable way of determining nucleosome positioning we tested how well this method can predict the placement of a well positioned “+1” nucleosome. The “+1” nucleosome at the majority of genes has been shown to be positioned downstream of a TATA element in \textit{S. cerevisiae} \[^{124}\]. In agreement with the previously published work we found that we were able to map both
the TATA proximal and distal edges of the “+1” nucleosome, positioned immediately downstream of the TATA element. Upstream of the “+1” nucleosome was the previously characterized NDR (Figure 3.1 b, bottom panel). This data confirmed the validity of our approach.

We hypothesized that the yFACT-associated nucleosomes might be subject to the same positioning as the “+1” nucleosome. However, when aligned around the 5946 TATA sequences we found that the read edges of the NDR-localized yFACT nucleosomes were flanking both sides of the TATA elements (TATAWAWR) in positions “0” and “+1” (Figure 3.1b upper panel). The nucleosome “0” has been identified as the “fragile” nucleosome, and has been found to correspond to the TATA enriched genes and genes displaying transcription plasticity [127].

We found that the nucleosomes positioned around the TATA elements showed read-direction corrected peaks at the TATA-proximal end only, and there were no apparent peaks at the distal end. As mentioned earlier, this might be an effect of the nature of the yFACT binding to nucleosomes. We hypothesized that because the yFACT-bound nucleosomes might be inconsistently susceptible to MNase digestion, the distal ends were absent in the total peak ensemble due to the varying DNA fragment lengths [129] [127][128]. Indeed, during the preparation of the DNA for sequencing we observed that the MNase digestion of the yFACT-associated nucleosomes generated a wide variation of fragment sizes (100 to180bp), as opposed to the ~140 bp seen with the digestion of canonical nucleosomes (data not shown). Similarly, the TATA-proximal end resistance to MNase digestion, on the other hand, could be due to yFACT binding to the nucleosome, the effects of which have been demonstrated to be asymmetrical
with respect to the nucleosome dyad\textsuperscript{129}. Thus, when the read edges were aligned around the TATA elements, the TATA-proximal edges formed a strong peak because the majority of reads end there. The TATA-distal end on the other hand, consisted of a population of read end positions, which due to their wide distribution did not form defined peaks over background. We hypothesized that if we were to select a subset of fragment sizes and position them around the TATA element, we should then be able to detect both edges of the nucleosome. To test our hypothesis we separated each of the fractions into a subset of 10 bp paired-reads increments. We then mapped the 10 bp increment subsets from each fraction around 5946 TATA element sequences\textsuperscript{124}. As predicted upon subset separation, in addition to the TATA proximal, we were able to distinguish a number of defined TATA distal nucleosome-edge peaks (Figure 3.1c).

While our cumulative peak analysis identified yFACT-bound nucleosomes at the “0” and “+1” positions, we sought to understand the origin of this pattern by analyzing the yFACT-nucleosome positioning at individual TATA-elements. To test this we performed a raw k-means clustering analysis on the read-edge peaks of yFACT-bound nucleosomes. In order to visualize the distribution of the clustered loci we constructed a heatmap centered on the 5946 TATA elements. We set the number of clusters to four to account for the possibility that: a) the nucleosome peaks are not positioned, nucleosome is present b) upstream of the TATA element, c) downstream the TATA element, d) nucleosomes are present on both sides of the TATA element. When clustered around TATA elements, we found 808 (13.5%) loci where yFACT nucleosomes were present at both “0” and “+1” positions, (20.5%) loci where yFACT-nucleosomes were present exclusively at the “0” position, 1228 (21%) with only “+1”
position, and 2658 (44.5%) that were not associated with a positioned yFACT-nucleosome. Together this data showed that 56.5% of TATA elements were surrounded by yFACT-associated nucleosomes and that 34.5% of all TATA elements contained an yFACT-associated nucleosome at position “0” (Figure 3.2). We take this result to provide support for the idea that yFACT binds nucleosomes surrounding TATA elements.
Figure 3.1 The yFACT-nucleosomes are positioned over canonical nucleosome depleted regions

Purified Pob3\textsubscript{TAP} samples were subjected to partial MNase digestion and TEV elution off beads. The purified DNA was sequenced using Illumina-based sequencing. The reads were mapped to the \textit{Saccharomyces cerevisiae} genome using BWA and the yFACT-nucleosome peaks were called using GeneTrack. The nucleosome map of cross-linked, bulk chromatin was used as a control (MNase). a) The plot of read-number normalized peak count centered on the 4339 Transcription Start Sites (TSS) in yeast with -500bp upstream and +2000bp downstream. Top: ratio of the yFACT-nucleosome/MNase peaks, bottom: the yFACT-nucleosome peak distribution as compared to canonical MNase peak pattern. The grey bar represents the canonical NDR region. b) The plot of read-number normalized, direction corrected peak counts centered on the 5946
TATAWAWR sequences in yeast (-300bp to +300bp around TATA). Top: the yFACT-nucleosomes, bottom: the MNase control fraction. c) Same as “b”, the paired-reads were parsed based on the pair distance in 10bp increments. The grey bars represent TATA elements.

Figure 3.2 yFACT-nucleosome patterns around TATA elements.

Heatmap of -300bp to +300bp around 5964 TATA elements of k-means clustered nucleosome edge peaks of a) yFACT-associated nucleosomes. The number of clusters was set to four. The gray bar represents the TATA elements. Red indicates presence of peaks, white absence.
3.3.2 yFACT-nucleosomes are positioned around the TATA-box sequence.

The positioning of the “+1” nucleosome downstream of the TATA element is a unique property of “Taf1-enriched” genes. To determine whether the “+1” yFACT-associated nucleosomes are subject to the same dependency, we divided the mapped TATA elements into “Taf1-depleted” and “Taf1-enriched” as described in Rhee et al. [124]. We first confirmed that, the bulk (MNase) “+1” nucleosome positioning around TATA elements was correlated with Taf1 binding (Figure 3.3a bottom panel). This was however, not the case for the yFACT-associated nucleosomes, which were more abundant on the Taf1-depleted genes. (Figure 3.3a top panel).

We observed that the occupancy of the yFACT-nucleosomes around the TATA elements was independent of Taf1. The TFIID independent promoters are more likely to contain canonical TATA sequences. We therefore asked whether it could be the TATA sequence itself that was responsible for the positioning of the yFACT-associated nucleosomes. To test this we determined the read-direction corrected peak distribution of the yFACT-bound nucleosomes around the TATA sequences containing zero, one or two mismatches from TATAWAWR consensus [124]. We found that the positioning of yFACT-nucleosomes (“0” and “+1”) around the TATA elements was inversely correlated with the number of mismatches to TATAWAWR (Figure 3.3b upper panel). The opposite correlation was observed, however to a significantly lesser degree (25% versus 100% drop), for the bulk MNase-chromatin fraction at the “+1” position (Figure 3.3b lower panel). There were no changes at the “+2” position in the control. These results indicated that the yFACT-nucleosome positioning at “0” and “+1” is correlated with the consensus of the TATA box DNA sequence. Taken together, this data showed that the
yFACT-bound nucleosomes were strongly positioned on either side of the TATA consensus sequence and that the yFACT-mediated nucleosome positioning depended on the TATA element consensus \textit{in vivo}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{\textbf{yFACT-nucleosomes are organized by the TATA elements.}}\label{fig:3}
\end{figure}

The plot of direction corrected, read-number normalized peak count centered on the 5946 TATA-element sequences in yeast (-300bp to +300bp around TATA) that a) are or are not enriched for Taf1 binding. Top: the yFACT-nucleosomes, bottom: the MNase control fraction. The grey bars represent the TATA-element. b) contain 0, 1 or 2 mismatches to the consensus sequence at any bp position. The gray bar again represents the TATA element.
3.3.3 yFACT-nucleosomes at the TATA elements are positioned independently of the transcription rate

The yFACT complex is involved in transcription elongation and initiation \cite{115,167,144}. The loss of yFACT causes transcription defects and the complex has been shown to associate at higher levels with the more highly transcribed genes \cite{168,167}. Therefore, while yFACT contributes to transcription, transcription is also correlated with yFACT occupancy. Does then, a high level of transcription always correlate with the high occupancy of yFACT-nucleosomes? To address this question we asked whether the positioning of yFACT-nucleosomes around TATA elements is dependent on the rate of transcription. To test this we parsed our data based on the previously published transcription rate dataset \cite{169}. We grouped the TATA-organized yFACT-nucleosomes and the control MNase nucleosome into four groups of low (0.02-0.049 mRNA/min), medium (0.05-0.099 mRNA/min), high (0.1-0.5 mRNA/min) and very high transcription rates (0.5-0.99mRNA/min). We then determined the probe number normalized peaks for both yFACT-nucleosomes and the MNase control. We found that neither yFACT-associated nucleosomes “0” and “+1”, nor the MNase control “+1” nucleosomes changed with the transcription rate (Figure 3.4). This result showed that the positioning of yFACT-bound nucleosomes on gene promoters is independent of the transcription rate from their respective genes.
Figure 3.4 yFACT-nucleosome positioning is independent of RNAPII

The plot of direction corrected, read-number normalized peak count centered on the 5946 TATA-element sequences in yeast (-300bp to +300bp around the TATA) for the subsets of data, organized by the transcription rate (mRNA/min)\textsuperscript{[169]}.
3.3.4 The positioning of yFACT-bound nucleosomes around the TATA elements correlates with TBP and TFIIA occupancy

We found that the yFACT-nucleosomes, organized around the TATA sequence are positioned around it, but never over it. Disruption of yFACT by mutating the SPT16 gene has been shown to enhance the phenotypes of TBP and TFIIA mutants, as well as reduce their binding to the GAL1 promoter\(^{[68]}\). This is consistent with the published function of yFACT \textit{in vitro}, where yFACT has been shown to enhance TBP access to its nucleosomal TATA binding site\(^{[144]}\).

We hypothesized that by positioning nucleosomes around TATA, yFACT enhances binding of TBP and TFIIA. If our hypothesis is true, then presence yFACT-bound nucleosomes should correlate with TBP and TFIIA binding \textit{in vivo}. We therefore tested whether yFACT-mediated nucleosome occupancy correlated with increased PIC-component occupancy \textit{in vivo}. We compared the localization of the yFACT-nucleosome peaks at individual loci with PIC-component occupancy around the 5946 TATA elements\(^{[124]}\). We found that the yFACT-nucleosome positioning showed a statistically significant correlation (p <0.05) with TFIIA and TBP, the TATA sequence binding factors but not other PIC components (Figure 3.5a). We also found that yFACT-nucleosome binding was not correlated with the distance of TATA to TSS, it was however as we previously showed in this work correlated with the TATA element consensus sequence (Figure 3.5b). This data suggested that yFACT might function at the gene promoters by spacing the “0” and “+1” nucleosomes thus promoting TBP and TFIIA binding.
Figure 3.5 yFACT-nucleosome occupancy correlates with TBP and TFIIA association at promoters

Analysis of the yFACT-nucleosome correlation to a) PIC occupancy b) DNA sequence. yFACT-nucleosome peak occupancy at individual TATA elements was correlated with the previously published factor binding [124]. (*) denotes a statistically significant difference (p<0.05) calculates using Student’s t-test. The correlations were calculated between each subset individually. Total-“factor”: regions that do not contain “factor” binding. FACT+: regions with yFACT-nucleosome peaks, FACT-: regions without the yFACT-nucleosome peaks. The error bars represent the standard deviation from the mean.
3.3.5 yFACT-associated nucleosomes are positioned around Nhp6 binding sites genome-wide

The defined positioning of yFACT-nucleosomes around the TATA elements suggested that yFACT might be recruited to chromatin in a sequence-dependent manner. yFACT contains a DNA binding subunit – the Nhp6a/b proteins [108]. Nhp6a is anywhere from three to ten times more abundant than Nhp6b [170]. Nhp6a/b, like other HMG proteins have a propensity to bind bent DNA, and recent work has demonstrated that Nhp6a/b displays preference for binding of the TATATWA consensus sequence [156]. The striking similarity between the Nhp6a/b binding sequence and the TATAWAWR of the TATA element suggested that it might be the Nhp6 sequence specificity that directs the yFACT-nucleosome positioning around the TATA boxes. To test this, we separated the known 5946 TATAWAWR sequences into two groups, one containing the TATATAA (Nhp6a consensus) sequence and the others that did not. We then mapped the read-direction corrected peak-edges of the yFACT-nucleosomes around these two features. Our results showed that in the absence of the TATATAA sequences the positioning of yFACT-nucleosomes (“0” and “+1”) was lost (Figure 3.6a upper panel). Such loss was not observed in the bulk-MNase control fraction (Figure 3.6a lower panel). This data showed that Nhp6a/b binding sequence is important for yFACT-mediated nucleosome positioning and presumably its recruitment.

We next asked whether the positioning of yFACT-nucleosomes around the Nhp6a/b binding sites is confined to the TATA box NDR sequences only, or whether it is a genome-wide phenomenon. To answer this question we determined the positioning of all the TATATAA sequences in the yeast genome and plotted the peak edges of the
surrounding yFACT-nucleosomes. We found that while the bulk, MNase digested control chromatin did not show a significant positioning around these sites, the yFACT-nucleosomes on the other hand were defined by these Nhp6 binding sequences (Figure 3.6b). The TATATAA sequences match the consensus sequence of TATA elements-TATAWAWR. To eliminate the possibility that this nucleosome positioning was a promoter-specific effect we analyzed the positioning of yFACT-bound and MNase control nucleosomes around 2892 TATATAA sequences that do not overlap with gene promoters. We found that while the bulk, MNase digested chromatin did not show a significant nucleosome positioning around these sites, the yFACT-associated nucleosomes were still defined by these sequences. Thus, the positioning of yFACT-nucleosomes around the Nhp6a binding TATATAA sequences was not a promoter-specific phenomenon (Figure 3.6c). Analysis of individual TATATAA sequences showed that while 37% of these loci do not contain a well-positioned yFACT-nucleosome, the other 63% contain a well-positioned yFACT-associated nucleosome on either or both sides (data not shown). The TATATAA sequences distribution was not uniform through the genome and we found that the TATATAA were preferentially localized to certain loci, such as the 3’ UTR (Table 8).

Similar, to the TATA element analysis (Figure 3.1b), we only observed the Nhp6a binding site proximal end of reads, as the TATATAA distal end was not apparent. We again hypothesized that this is due to the yFACT binding causing differential nucleosome MNase sensitivity [129]. To test this we separated the paired reads into 10bp increments and mapped them around all Nhp6a TATATAA sites genome-wide. As expected, we were able to observe both the TATATAA proximal and distal ends,
indicative of nucleosomes (Figure 3.6d). These results showed that yFACT-nucleosome positioning was organized by TATAAA sequences genome-wide. In summary, we demonstrated for the first time that yFACT and its substrates are distributed on the genome in a sequence-dependent manner and that the yFACT-nucleosomes are positioned specifically around the Nhp6 binding sequences.
Figure 3.6 yFACT-nucleosomes are positioned around Nhp6a/b binding sites genome-wide.

Purified Pob3_{TAP} samples were subjected to partial MNase digestion and TEV elution off beads. The purified DNA was sequenced using Illumina-based sequencing. The reads were mapped to the *Saccharomyces cerevisiae* genome using BWA and the FACT-nucleosome peaks were called using GeneTrack. The nucleosome map of cross-linked,
MNase-digested bulk chromatin was used as a control (MNase). a) The plot of direction corrected, read-number normalized peak count centered on the 5946 TATAAWWR sequences in yeast (-300bp to +300bp around TATA) that do or do not contain the TATATAA Nhp6a/b consensus sequence. Top: the yFACT-nucleosomes, bottom: the MNase control fraction. Grey bar represents the TATA-element. b) Same as in “a” centered on all the Nhp6a/b TATATAA consensus sites through the genome. c) Same as in “a”, centered on the TATATAA sites that do not overlap with gene promoters. d) Same as “b”, the paired-reads were parsed based on the read pair-distance in 10bp increments.

Table 7 Distribution of the Nhp6a/b TATATAA binding sites across various genomic features in Saccharomyces cerevisiae.

<table>
<thead>
<tr>
<th>Genome TATATAA elements: 4996 total</th>
<th>Number</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overlapping CDS</td>
<td>1524</td>
<td>30.5</td>
</tr>
<tr>
<td>Overlapping of long terminal repeat</td>
<td>156</td>
<td>3.1</td>
</tr>
<tr>
<td>Overlapping of LTR retrotransposon</td>
<td>59</td>
<td>1.2</td>
</tr>
<tr>
<td>Overlapping of Pugh histones</td>
<td>2419</td>
<td>48.4</td>
</tr>
<tr>
<td>Overlapping of ARS</td>
<td>137</td>
<td>2.7</td>
</tr>
<tr>
<td>Overlapping of SnoRNA</td>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td>Overlapping of TATA elements</td>
<td>680</td>
<td>13.6</td>
</tr>
<tr>
<td>Overlapping of transposable element gene</td>
<td>34</td>
<td>0.7</td>
</tr>
<tr>
<td>Overlapping of TSS</td>
<td>24</td>
<td>0.5</td>
</tr>
<tr>
<td>Overlapping of X element</td>
<td>20</td>
<td>0.4</td>
</tr>
<tr>
<td>Overlapping of Yassour antisense transcript</td>
<td>379</td>
<td>7.6</td>
</tr>
<tr>
<td>Overlapping of Yassour 5’UTR</td>
<td>768</td>
<td>15.4</td>
</tr>
<tr>
<td>Overlapping of Yassour 3’UTR</td>
<td>1428</td>
<td>28.6</td>
</tr>
<tr>
<td>Within 500bp upstream TSS</td>
<td>953</td>
<td>19.1</td>
</tr>
</tbody>
</table>
3.3.6 The positioning of yFACT-bound nucleosomes around Nhp6a/b binding sites genome-wide is independent of the transcription rate

We found that the yFACT-bound nucleosomes at TATA elements and outside of the promoter regions are positioned around TATATAA Nhp6a/b consensus binding sequence. At the same time, the nucleosomes positioned at TATA boxes show no dependence on the transcription rate. We wanted to test whether the same is true at all Nhp6a/b binding loci. Again, we parsed the peaks mapped around all TATATAA sites by transcription rate and compared the peak intensity. We found that there was no relationship between the transcription rate and the yFACT-nucleosome occupancy at these loci (Figure 3.7).

Nucleosome levels can decrease at very highly transcribed genes [121]. Even though yFACT has been clearly linked to transcription we found that the nucleosomes associated with the complex and positioned around the Nhp6a/b sites did not conform to this correlation. The Nhp6a/b organized nucleosome population might then represent a unique subset of chromatin, which is always stable, regardless of the surrounding transcriptome.
Figure 3.7 Positioning of yFACT-nucleosomes and is independent of transcription rate around the Nhp6a/b binding sites genome-wide

a) The plot of direction corrected, read-number normalized peak count centered on the 5946 TATAAWWR sequences in yeast (-300bp to +300bp around TATA)
3.3.7 The loss of Nhp6a/b causes loss of chromatin at the NDR and Nhp6 TATATAA sites genome-wide

One of the canonical phenotypes of loss of yFACT function is the SuPpressor of Ty phenotype (SPT), which is a result of chromatin instability \[171\]. Given that Nhp6 binding sites organize yFACT-nucleosome localization genome-wide, we hypothesized that the loss of Nhp6a/b and thus yFACT-nucleosome positioning around the TATATAA sequences would result in decrease in nucleosome occupancy at these loci. To test our hypothesis we performed a meta-analysis of a previously published dataset that contained a MNase nucleosome map from WT and \( nhp6a/b\)Δ strains \[162\].

First, we plotted the distribution of the nucleosome peaks in WT and \( nhp6a/b\)Δ strains organized by the 5949 TATA box sequences in the genome \[124\]. We found that there was little difference in the positioning or the amount of nucleosomes over the coding regions between the two strains. However, in concordance with our hypothesis, the TATA element-containing NDR in the \( nhp6a/b\)Δ strain was depleted of chromatin as compared to WT (Figure 3.8a). This suggested that the yFACT localization to TATA sequences is important for the maintenance of chromatin integrity at the promoter NDR.

We found that the positioning of yFACT-nucleosomes around TATA elements was dependent on the TATAWAWR consensus \[124\]. We therefore hypothesized that the magnitude of chromatin loss in \( nhp6a/b\)Δ is inversely correlated to the number of mismatches to TATAWAWR. To test our hypothesis, we determined the levels of chromatin in the WT and \( nhp6a/b\)Δ strains over the TATAWAWR sequences with zero, one or two mismatches. We found that the chromatin loss in \( nhp6a/b\)Δ strain was inversely correlated to mismatch number, where lower number of mismatches resulted
in a greater difference (Figure 3.8b). This suggested that function of Nhp6a/b depends on the TATA box consensus.

Given that we observed the TATAWAWR consensus-dependent chromatin loss in \( nhp6a/b\Delta \) at the TATA elements, we asked if the Nhp6a/b binding sequence also helps to maintain chromatin genome-wide. To address this question we plotted the chromatin distribution in the WT and \( nhp6a/b\Delta \) strains over all genomic TATATAA Nhp6a/b binding sites and compared the levels of chromatin between the two strains. In accordance with our expectations, we observed a marked loss of the nucleosome levels over the Nhp6 binding loci genome-wide (Figure 3.8c). The Nhp6a/b binding sequences then, not only had the yFACT-nucleosomes positioned around them genome-wide, but were also responsible for chromatin stability. In conclusion, our data showed an unprecedented insight into the yFACT function. The yFACT-bound fragile nucleosomes, are positioned around specific DNA sequences, and yFACT maintains chromatin stability at the Nhp6a/b TATATAA binding sequence over the canonical NDR regions.
Figure 3.8 The loss of Nhp6a/b causes chromatin loss around the regions containing yFACT-nucleosomes

The meta-analysis of data from Celona et al (2011). a) A comparison of the normalized nucleosome peak count from WT and nhp6a/bΔ strains organized around 5496 TATA element sequences in the yeast genome. b) The probe-normalized, nucleosome peak count difference between the WT and nhp6a/bΔ strain plotted 150bp around the TATA element sequences that contained 0, 1 or 2 mismatches from the TATAWAWR sequence. c) The normalized nucleosome peak count from WT and nhp6a/bΔ organized by all Nhp6a/b TATATAA containing sequences genome-wide.
3.4 Discussion

The initial MNase-based characterization of the nucleosome occupancy on genes revealed a consistent nucleosome depletion over the promoter regions (NDR) \cite{121}. Recent work though, that used a limited MNase digest, revealed that the nucleosome-depleted regions (NDR) do host chromatin, however since this chromatin is MNase sensitive it had been digested away in the previous studies \cite{127,128}. One of the most striking findings in our work was that yFACT-bound nucleosomes are positioned over the NDRs. These yFACT-nucleosomes are positioned around, but not over, the TATA-box and the Nhp6a binding sequence- TATATAA. An immediate implication of these findings is that nucleosome occupancy is maintained over the majority of the genome, and that the nucleosome-free regions might not actually exist in the cell but rather are fragile, MNase-sensitive sites.

We find that yFACT-bound nucleosomes are positioned across genes and over canonical NDR. Previously, Patrick Cramer's group used chip-ChIP to probe the localization of Spt16 genome-wide. Mayer et al. found that Spt16 was localized over the open reading frames (ORFs) of genes and was depleted over the NDR regions \cite{168}. Thus, our results diverge from their findings. There are several reasons for why that might be. The Cramer group looked at Spt16 directly, whereas we constructed a map of yFACT-associated nucleosomes. Therefore, while Mayer et al. looked at Spt16 localization, we determined the yFACT-substrate association using a tagged version of the Pob3 subunit. We speculate that it is possible that Spt16 and Pob3 are might not always associated together or and thus our results differ as the result of various methodologies.
The exact function of the fragile nucleosomes at the NDR is unknown, but we speculate that they represent a form of chromatin, which is poised for transcription. The acetylation-facilitated DNA unwrapping likely exposes DNA binding sites and allows for access of the transcription machinery. This process is likely further facilitated by the binding of yFACT, which has been shown to increase the DNaseI sensitivity of nucleosomes, our data shows that yFACT-bound nucleosomes are also more MNase sensitive and have an altered structure by EM \(^{129}\). Unlike acetylation, yFACT-induced nucleosome alteration does not depend on the presence of transcription factor-recruited KAT and keeps the promoters accessible independent of activation signal. At the same time the presence of a yFACT-bound nucleosome particle in a NDR region could prevent spurious transcription and off-target binding of the PIC components. Consistent with this hypothesis, recent work has found that in yeast transcription activators tend to have intrinsic sequence specificity that overlaps positioned nucleosomes. Consequently, the binding of transcriptional activators induces more nucleosome disruption than binding of the repressors. This data implies that nucleosomes might be required to prevent spurious transcription or block off-target PIC assembly \(^{172}\).

We found that the occupancy of both TBP and TFIIA was positively correlated with the presence of yFACT-bound nucleosomes. This data was consistent with other studies that showed that yFACT was important for TBP binding to the nucleosomal TATA elements \(^{68}\). yFACT and its subunit Nhp6a/b have been shown to genetically interact with the TBP, and in certain cases suppress TBP phenotypes by helping to maintain PIC formation and transcription fidelity \(^{144}\). We speculate that by positioning the nucleosomes “0” and “+1” around the TATA element, yFACT ensures that the TBP
target binding site remained accessible, as indicated by the position of the read edge peaks in our sequencing data. The lack of MNase protection of DNA at TATA elements by PIC, is consistent with previous work that showed that the transcription machinery is unable to provide MNase protection of DNA \cite{173}.

Another aside to our findings was that yFACT-nucleosomes might promote transcription through topological means. Nucleosomes store negative supercoils. At the same time, promoter melting during initiation is enhanced by negative supercoiling \cite{174,175}. Thus, the yFACT-mediated stabilization of fragile nucleosomes could serve as a "topological sink" that stores the negative supercoils to aid initiation. DNA topology is also regulated by topoisomerases. Topoisomerase 1 and 2 (Top) are involved in regulation of gene expression. The gene targets of Top1/2 share some common properties. Top1/2 regulated genes are inducible, contain a TATA box and show enrichment in nucleosome positioning over their promoters \cite{176}. The properties of Top1/2 regulated genes are shared by loci that house yFACT-nucleosomes. Interestingly, yFACT physically interacts with Top2 suggesting that Top2 and yFACT act on the same gene targets \cite{85}. Thus, we speculate that Nhp6 could not only recruit yFACT but also be responsible for Top recruitment. In this model yFACT and Top2 would mediate the maintenance of inducible gene promoters by positioning nucleosomes and maintaining appropriate topology that is primed for induction.

Our findings agree with studies in other organisms, such as *Drosophila melanogaster*. It has been recently found that the nucleosome depleted regions of chromatin in flies are subject to rapid histone H3.3 deposition (a homologue of yeast H3), through the Hira and Xnp-dependent pathways \cite{177}. Interestingly, another line of
evidence implicated the fly-FACT homologue (dFACT) in the maintenance of gene expression in heterochromatic regions by interaction with Hira and replacement of H3 with the H3.3 on transcribed regions at GAGA elements suggesting that dFACT also might function in a sequence-dependent manner \[^{178}\]. Replacement of H3 by H3.3 in a sequence-specific manner presents an analogous mechanism to the one observed in our work. In summary, our work demonstrated a novel recruitment mechanism for yFACT to chromatin \textit{in vivo}, where the Nhp6a/b consensus sequence serves to position yFACT-bound nucleosomes. This positioning served to maintain nucleosomes over the canonical NDRs at both promoters and genome-wide.
Chapter: Critical Determinants for Chromatin Binding by *Saccharomyces cerevisiae* Yng1 Exist Outside of the Plant Homeodomain Finger

### 4.1 Introduction

Chromatin is a nucleoprotein structure consisting of DNA, histones, and nonhistone proteins. It has been found to regulate numerous processes including transcription, DNA replication, and repair. Histones are subjected to numerous post-translational modifications (PTMs), including acetylation and methylation, that are proposed to act as docking sites for various chromatin-remodeling complexes \(^{179}\). As such, histone post-translational modification is one mechanism for modifying chromatin structure.

One family of proteins that specifically bind histone PTMs is the Inhibitor of Growth (ING) group of proteins. These proteins share highly conserved carboxyl-terminal plant homeodomain (PHD) fingers and less conserved amino-terminal regions \(^{180}\).

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The first ING protein discovered was ING1 (p33), which was later shown to act as a tumor suppressor \[181\]. The ING family is now thought to include five proteins in humans: hING1, hING2, hING3, hING4, and hING5 \[180\]. ING1 and ING2 are found in related histone deacetylase complexes, whereas ING3–5 are associated with MYST family histone acetyltransferases (HATs) \[182\][183][184]. The ING proteins interact with p53, regulate transcription, and promote cell senescence and death when overexpressed \[185][186\].

There are three ING proteins in the yeast \textit{Saccharomyces cerevisiae}, including Yng1, Yng2, and Pho23 \[187\]. Each of these proteins is found associated with a different multiprotein complex that post-translationally modifies histones. Yng1 and Yng2 are associated with the NuA3 and NuA4 HAT complexes, respectively \[187\][188][189][55\]. In contrast, Pho23 is found associated with the Rpd3L KDAC complex \[188\][190\]. Although the ING proteins are not required for the structural integrity of their respective complexes, both Yng1 and Yng2 are required for the ability of their complexes to bind and acetylize nucleosome substrates \[187\][189][55\]. Additionally, loss of \textit{PHO23} causes a reduction in the KDAC activity of the Rpd3L complex \[188\]. These data suggest that members of the ING protein family serve general roles in permitting various enzymatic activities to interact with nucleosomes and modify histone tails.

Similar to their mammalian counterparts, the yeast ING proteins share a conserved PHD finger that preferentially interacts with H3K4me3-modified histones \[18\][191][41][17\]. However, while the ING proteins are important for the function of their cognate complexes, the PHD fingers are surprisingly dispensable \[192][55\]. As a result, whether these highly conserved methyl–histone binding domains actually have a role in...
targeting complexes to specific regions of the genome is in question. In this study we have used both biochemical and genetic approaches to show that Yng1 sequences outside of the PHD finger can directly interact with the histone H3 tail and this interaction is not dependent on the presence of H3K4me3. These results demonstrate that Yng1 possesses bivalent histone-binding motifs that function together to increase the apparent association of Yng1 for the histone H3 tail.
4.2 Methods

4.2.1 Yeast strains, plasmids, and genetic methods

All strains used in this study are isogenic to S288C. Yeast culture and genetic manipulations were carried out using standard protocols \[^{193}\]. Genomic deletions were verified by PCR analysis and whole-cell extracts were generated as previously described \[^{193,194}\]. The plasmids pGALYNG1, pGALYNG1ΔPHD, pGALYNG1W180A, pGALYNG1Δ2-28, pGALYNG2, and pGALPHO23 were generated by cloning sequences encoding YNG1, YNG2, and PHO23 into the BamHI sites of pGAL.415, pGAL.425, or pGAL.416 \[^{195}\]. pGALYNG1HA3, pGALYNG2HA3, and pGALPHO23HA3 were generated by replacing the CYC1 terminator of the above plasmids with an HA3-CYC1 terminator cassette \[^{39}\]. To construct the PHD finger swap plasmids we introduced silent mutations into codons 145 and 146 of YNG1, introducing an AatII restriction site. We then PCR amplified codons 147–219 of Yng1, codons 214–282 of Yng2, and codons 272–330 of Pho23 as AatII/NotI fragments and cloned them into the AatII/NotI-digested plasmid. To construct pHDA1 the HDA1 open reading frame, along with 230 bp of promoter and 651 bp of terminator sequences, was amplified from genomic DNA and cloned into pRS415 using XhoI and SacI restriction sites.

4.2.2 Synthetic dosage resistance genomewide screen

The synthetic genetic array (SGA) starting strain Y7092 (MATα can1Δ::STE2pr-Sp-his5 lypΔ1 his3Δ1 leu2Δ0 met15Δ ura3Δ0) was transformed with pGALYNG1. The resulting query strain was mated to the MATα deletion mutant array. SGA methodology,
previously described for a plasmid-based synthetic dosage resistance screen \cite{18}, was used with the following modifications: (i) medium lacking uracil was used to maintain the plasmid, and (ii) hits were scored against strains containing \textit{pGALYNG1} grown on dextrose. The screen was performed in triplicate and all hits were confirmed using traditional transformation and dilution plating. Confirmed hits were followed up with Western blots with anti-HA antibodies (Santa Cruz, catalog no. sc-57529) to determine the levels of Yng1HA3 expression and anti-H3K4me3 (Active Motif, catalog no. 39159) antibodies to check for changes in H3K4 methylation.

4.2.3 Peptide pull-down assays

Biotinylated histone peptides were synthesized by Anaspec. For pull-down assays, \( \sim 1 \, \mu \text{g} \) of GST-tagged recombinant protein was incubated with \( 1 \, \mu \text{g} \) of biotinylated histone peptides in binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05\% NP-40) overnight at 4\(^\circ\) with rotation. After a 1-hr incubation with streptavidin sepharose beads (Amersham Biosciences) and extensive washing, bound proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting with anti-GST antibodies (Santa Cruz, catalog no. sc-459).
4.3 Results

4.3.1 Sequences outside of the Yng1 PHD finger are required for growth inhibition by YNG1 overexpression.

We have previously reported that full-length YNG1 inhibits yeast growth when overexpressed and this effect requires both the Yng1 PHD finger and histone H3K4 methylation [18]. We hypothesized that growth inhibition is due to excess levels of Yng1 sterically hindering the access of other factors to the H3 tail. If this is true, then other proteins that show similar methyl–histone binding properties should be toxic when expressed at similar levels. The PHD fingers of the three yeast ING proteins, Yng1, Yng2, and Pho23, share significant sequence similarity and H3K4me3 binding properties (Figure 4.1a) [19][41]. It is therefore expected that YNG2 and PHO23 would inhibit growth when overexpressed as well. To address this question we fused the YNG2 and PHO23 open reading frames to the galactose inducible promoter of the GAL1 gene on a URA3-based centromeric plasmid (pGALYNG2, pGALPHO23). The resulting plasmids were transformed into wild-type cells and plated in 10-fold dilution series on media lacking uracil and containing dextrose or galactose as carbon sources (Figure 4.1b). Vectors carrying only the GAL1 promoter alone (pGAL) and the GALYNG1 construct were used as negative and positive controls for growth inhibition, respectively. Consistent with previously published results, cells carrying pGALYNG1 grew on dextrose, but failed to grow on galactose, confirming that overexpression of YNG1 inhibits cell growth. In contrast, yeast carrying empty vector (pGAL), pGALYNG2, or pGALPHO23 grew well on both dextrose and galactose, indicating that neither
overexpression of YNG2 nor that of PHO23 is inhibitory to cell growth (Figure 4.1b). To verify that YNG2 and PHO23 are expressed at similar levels to YNG1, we introduced sequences encoding HA tags into each of our pGAL plasmids such that each ING protein would be expressed with a C-terminal triple-HA tag. Fortuitously, HA-tagged Yng1 is not toxic when overexpressed, allowing us to compare the levels of all of the ING protein in cells grown on galactose. Figure 4.1c demonstrates that Yng1HA3, Yng2HA3, and Pho23HA3 expressed from a GAL1 promoter are present in similar levels.
Figure 4.1 Overexpression of YNG1, but not YNG2 nor PHO23 inhibits yeast cell growth.

a) CLUSTAL 2.0.12 multiple sequence alignment of the ING proteins in S. cerevisiae. "**" indicates that the residues in that column are identical in all sequences in the alignment. "." and ",," means that conserved and semi-conserved substitutions are observed respectively. Sequences corresponding to the PHD finger are indicated by a black bar and amino terminal residues important for unmodified tail binding are shaded gray.

b) Ten-fold serial dilutions of a wild type yeast strain containing the indicated low copy plasmids were plated on synthetic complete medium lacking leucine with either dextrose or galactose as a carbon source and incubated at 30°C for 3 days.

c) Yeast YNG1, YNG2 and PHO23 are equivalently expressed in cells. Shown is an αHA western blot analysis of whole cell extracts from strains grown in galactose and expressing HA tagged versions of YNG1, YNG2 or PHO23 from a GAL1 promoter.
The fact that overexpression of \textit{YNG1} inhibits growth, while \textit{YNG2} and \textit{PHO23} do not, was surprising as we have previously shown that the level of growth inhibition by \textit{YNG1} correlates with the methyl–histone binding ability of the Yng1 PHD finger and that the PHD fingers of all ING proteins share similar methyl–histone binding specificities\cite{18,19,41}. There are two possible explanations as to why this is the case. First, it is feasible that there is something distinct about the Yng1 PHD finger that makes it toxic when overexpressed. Indeed, it has been previously shown that the Yng1 PHD finger binds methylated peptides with a slightly higher affinity than the Yng2 or Pho23 fingers\cite{41}. A second possibility is that while the PHD finger is required for the growth-inhibiting properties of Yng1, additional sequences outside of the PHD finger may also be required. These sequences are significantly divergent from those in Yng2 and Pho23 (Figure 4.1a). To test these possibilities we replaced the PHD finger of Yng1 with the PHD finger of Yng2 or Pho23 and tested whether these chimeric proteins inhibit growth of yeast cells when overexpressed from a GAL1 promoter. While Yng1 lacking the PHD finger does not inhibit growth when overexpressed, addition of either the Yng2 or the Pho23 PHD finger restores the growth-inhibiting properties to Yng1 (Figure 4.2). The fact that the Yng2 and Pho23 PHD fingers do not inhibit growth when fused to their native amino termini, but inhibit growth when fused to Yng1, strongly suggests that the growth inhibition by \textit{YNG1} overexpression is dependent on the amino terminus of Yng1, in addition to the PHD finger.
Figure 4.2 The Yng2 and Pho23 PHD fingers confer inhibition of growth when fused to the amino-terminal domain of Yng1.

Tenfold serial dilutions of a wild type yeast strain transformed with the indicated high copy plasmids were plated on synthetic complete medium lacking uracil containing either dextrose or galactose as a carbon source and incubated at 30°C for 3 days.

4.3.2 Full-length Yng1 binds unmodified chromatin.

The results of this and previously published work indicate that the PHD finger of Yng1 is necessary, but not sufficient to confer growth inhibition when overexpressed. Instead, residues within the amino-terminal region of Yng1 are equally important. This result is reminiscent of other work from our lab demonstrating that while full-length Yng1 is important for NuA3 function in vivo, the PHD finger is dispensable [55]. Additionally, we
have shown that deletion of *YNG1* or the H3 tail disrupts the interaction of NuA3 with chromatin, but we have been unable to show a similar effect due to loss of the Yng1 PHD finger alone \(^{43,18}\). Collectively, these data support the idea that Yng1 may contain bivalent chromatin-binding abilities: a PHD finger that specifically interacts with H3K4me3 and an amino-terminal motif that interacts with some other part of the H3 tail. To test this hypothesis we bacterially expressed and purified full-length Yng1 and the Yng1 PHD finger with amino-terminal GST tags. The purified proteins were incubated with biotinylated peptides corresponding to the H3 tail (residues 1–23) that were either unmodified or trimethylated at K4. The peptides were immobilized on streptavidin sepharose and the bound proteins detected by Western blotting for GST. Figure 4.3a shows that both full-length Yng1 and the Yng1 PHD finger alone are able to interact with trimethylated peptides (lane 4). More importantly, however, full-length Yng1, but not the PHD finger alone, is able to interact with unmodified peptides (lane 3). The fact that full-length Yng1 can bind unmodified tails, while the PHD finger cannot, suggests that residues within the amino terminus of Yng1 are important for recognition of an unmodified H3 tail. These results confirm our hypothesis that Yng1 has two histone-binding modules. The fact that full-length Yng1 binds H3K4me3 tails better than unmodified tails (Figure 4.3a, compare lanes 3 and 4) suggests that these modules work together to increase the strength of interaction of Yng1 for the histone H3 tail.
Full length Yng1 binds unmodified histone H3 tails independently of the PHD finger.

(a and b) Histone tail peptide binding assays were performed with the indicated immobilized peptides and purified GST fusion proteins. Shown are αGST western blots of precipitated material. Input lanes contain 10% of the proteins used for the pulldown.

(c) A gcn5Δ yng1Δ strain expressing GCN5 from a URA3-based plasmid was transformed with the indicated plasmids, plated on synthetic complete medium with and without 5-FOA, and incubated at 30°C for 3 days.

As a final confirmation of the presence of an additional histone-binding activity in Yng1, we mutated a residue within the Yng1 PHD finger that is critical for methyl–histone recognition and tested for binding to both unmodified and methylated H3 tail peptides. Yng1 W180 forms part of an aromatic cage that specifically interacts with H3K4me3 and substitution of this amino acid with an alanine disrupts H3K4me3 binding.
and alleviates Yng1 growth inhibition\textsuperscript{[18]}. We expressed full-length Yng1 with an alanine substitution of W180 and tested the binding of this protein to both unmodified and methylated H3 tail peptides. Figure 4.3b shows that the mutant form of Yng1 can still interact with unmodified H3 tails, but, as expected, lacks the preferential binding to the peptide bearing H3K4me3. Finally, we tested whether this form of Yng1 could rescue a phenotype associated with loss of Yng1. Deletion of \textit{YNG1} results in no significant phenotypes in a wild-type strain, but when deleted in a gcn5Δ strain results in synthetic sickness. This phenotype is due to a redundancy of histone H3 acetylation and acetylation of Rsc4, a subunit of the RSC chromatin remodeling complex, in maintaining cell viability. Introduction of wild-type Yng1 into gcn5Δ yng1Δ restores growth, as does Yng1W180A, reiterating the fact that the PHD finger is not essential for NuA3 function in vivo (Figure 4.3c). These data explain why loss of the PHD finger alone does not disrupt NuA3 function since the interaction of NuA3 with chromatin is presumably still maintained by the interaction of the Yng1 amino terminus with the H3 tail.

We next attempted to map the region of the amino terminus of Yng1 that binds unmodified peptides. To this end we generated deletions of both the amino and the carboxyl regions. Unfortunately, deletion of the PHD finger rendered the protein unstable and we were unable to recover any protein following bacterial expression. In contrast, deletion of residues 2–28 in the amino terminus in Yng1 did not affect protein stability and completely abolished binding to unmodified tails (Figure 4.4a). Concomitant with this, Yng1Δ2–28 is not toxic when overexpressed (Figure 4.4b). These data indicate that the first 28 residues of Yng1 are important for recognition of unmodified histone tails. Moreover, we have been able to separate the unmodified-tail and
methylated-tail binding activities of Yng1 and have shown that each can bind histones independently of the other (Figures 4.3b and 4.4a).

**Figure 4.4 The amino terminus of Yng1 is required for binding of unmodified histone H3 tails.**

a) Histone tail peptide binding assays were performed with the indicated immobilized peptides and purified GST fusion proteins. Shown are αGST western blots of precipitated material. Input lanes contain 10% of the proteins used for the pulldown.

b) Tenfold serial dilutions of a wild type yeast strain containing the indicated low-copy plasmids were plated on synthetic complete medium lacking uracil containing either dextrose or galactose as a carbon source and incubated at 30°C for 3 days.

### 4.3.3 A synthetic dosage resistance screen identifies genes that regulate the binding of Yng1 to chromatin.

Previously we identified the PHD finger of Yng1 as a methyl–histone binding domain, using a synthetic dosage resistance screen \[^{18}\]. In this screen, overexpression of YNG1 in the yeast haploid deletion collection identified a deletion in LGE1 as conferring resistance to YNG1 overexpression. LGE1 is required for methylation of
H3K4 [196]; however, the screen failed to identify numerous other genes known to be required for H3K4me3. Subsequent testing confirmed that all mutants with defects in H3K4me3 are resistant to YNG1 overexpression and thus our initial synthetic dosage resistance screen had a very high false negative rate [18]. With the advent of new instrumentation for SGA analysis, we repeated the screen in an attempt to uncover additional information on the regulation of Yng1 binding to unmodified tails. A pGALYNG1 vector expressing the URA3 marker was introduced to the query strain, which was then crossed with the set of ~5000 nonessential gene deletion mutants. The screen was performed in a 1536-plate format to reduce the plate border effects. The synthetic dosage phenotype was scored by comparing the growth of cells carrying pGALYNG1 on media lacking uracil with either glucose or galactose as the carbon source. Using this approach we identified multiple genes that conferred resistance to YNG1 overexpression (Table 9). Expectedly, we found a large group of genes that encode components of the Set1/COMPASS histone methyltransferase complex, including BRE2, SDC1, SPP1, SWD1, and SWD3 [37][197]. We also identified deletions of BRE1 and RAD6, genes required for ubiquitination of H2B, and RTF1, CDC73, and LEO1, which encode three of the five components of the PAF complex [198][198][197]. Both H2B ubiquitination and the PAF complex are required for H3K4 methylation [199][200][201]. Finally, we identified CTK2 and CTK3, which encode two of the three components of the Ctk1 protein kinase complex that phosphorylates the C-terminal domain (CTD) of RNA Polymerase II [202]. Loss of Ctk1 results in spreading of H3K4 trimethylation across the coding regions of genes, presumably acting as a sink for binding of excess Yng1 [203][204]. Given these results we were confident that this synthetic dosage resistance
screen was useful for identifying genes required for growth inhibition due to \textit{YNG1} overexpression.

### Table 8 Genes required from growth inhibition due to YNG1 over-expression.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Required for normal H3K4 methylation?</th>
<th>Gene identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMPASS complex</td>
<td>yes</td>
<td>\textit{BRE2}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textit{SDC1}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textit{SPP1}</td>
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<td>\textit{SWD1}</td>
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<td>\textit{SWD3}</td>
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<tr>
<td>Rad6-Bre1 complex</td>
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<td></td>
<td>\textit{RAD6}</td>
</tr>
<tr>
<td>PAF complex</td>
<td>yes</td>
<td>\textit{RTF1}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textit{CDC73}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textit{LEO1}</td>
</tr>
<tr>
<td>Ctk1 protein kinase complex</td>
<td>yes*</td>
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<td>\textit{HDA2}</td>
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<td></td>
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<td>\textit{HDA3}</td>
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</tbody>
</table>

*the Ctk1 protein kinase complex restricts the spread of H3K4 methylation into the 3'ends of genes
4.3.4 Deletion of HDA1 confers resistance to YNG1 overexpression.

In addition to genes required for histone H3K4 methylation, we also identified deletions of HDA1, HDA2, and HDA3 as conferring resistance to YNG1 overexpression (Table 9). Hda1, Hda2, and Hda3 compose the tetrameric HDA histone deacetylase complex, which is composed of Hda1 as a catalytic subunit, Hda3, and two molecules of Hda2 [205][206]. The HDA complex has been shown to exhibit activity toward histones H2B, H3, and H4, and deletion of HDA1 results in increased acetylation of these histones. To confirm that deletion of HDA1 confers resistance to YNG1 overexpression, we reintroduced HDA1 with its native promoter on a LEU2-based plasmid (pHDA1) into an hda1Δ mutant. Wild-type (HDA1) and hda1Δ strains transformed with vector alone were used as controls. The resulting strains were tested for resistance to YNG1 overexpression by transforming with either pGAL or pGALYNG1 and spotting in 10-fold dilution series onto plates lacking leucine and uracil and containing dextrose or galactose as the carbon source. Yeast bearing a HDA1 deletion and pGALYNG1 grew on both dextrose and galactose, indicating that deletion of HDA1 confers resistance to YNG1 overexpression. When HDA1 was provided on a plasmid, hda1Δ cells grew in the presence of pGAL on both dextrose and galactose; however, they failed to grow when carrying the pGALYNG1 plasmid, indicating that a functional HDA complex is required for YNG1-mediated inhibition of growth (Figure 4.5a). The two obvious modes of resistance to YNG1 overexpression are a GAL1-transcriptional activation defect, which reduces the levels of Yng1 in the cell, or alternatively disruption of the histone H3K4 methylation pathway. To test whether hda1Δ strains have a GAL1 transcriptional defect, we overexpressed the HA-tagged version of Yng1 in wild-type and hda1Δ cells. The
hda1Δ cells showed no decrease in the levels of Yng1HA, when normalized to levels of H3, indicating that a GAL1 transcription defect is not a likely cause of resistance (data not shown). To test the possibility that deletion of HDA1 causes a decrease in methylation we subjected yeast whole-cell extracts to Western blotting with anti-H3K4me3 antibodies. We found that deletion of HDA1 did not have an effect on histone methylation levels in cells, therefore eliminating it as a mechanism of resistance (Figure 4.5b).

Figure 4.5 Deletion of HDA1 confers resistance to inhibition of growth by YNG1 overexpression.

a) Ten-fold serial dilutions of HDA1 and hda1Δ yeast strains containing the indicated plasmids were plated on synthetic complete medium lacking uracil and leucine containing either dextrose or galactose as a carbon source and incubated at 30°C for 3 days.
b) Western blot of the whole cell extracts from wild type, set1Δ, and hda1Δ strains. The blot was probed with αH3K4me3 and αH3 antibodies.
4.3.5 **Histone H3 acetylation does not affect Yng1 binding to chromatin.**

Deletion of *HDA1* results in increased acetylation of histone H3\(^{112}\). This led us to ask whether Yng1 binding to chromatin might be negatively regulated by acetylation of the histone H3 tail. To test this hypothesis we assayed binding of full-length Yng1 to peptides with and without H3K4me3 and/or acetylation of lysines 9, 14, and 18. In Figure 4.6a we show that full-length Yng1 binds to both unmodified and acetylated peptides (lanes 3 and 5). Although the apparent association of Yng1 increases for methylated peptides as compared to unmodified (compare lanes 3 to 4), we did not observe any change in binding to methylated vs. methylated acetylated peptides (compare lanes 4–6).

To further confirm that the histone acetylation does not block Yng1 binding, we mutated four of the acetylated lysines within the H3 tail to glutamine and tested whether this conferred resistance to Yng1 growth inhibition. Figure 4.6b shows that cells expressing H3 K9, 14, 18, and 23Q showed similar growth to wild type when Yng1 is overexpressed. A strain expressing H3 with an arginine substitution of lysine 4 does rescue growth, consistent with growth inhibition being dependent on methylation of this residue. Collectively these results suggest that the HDA complex does not directly regulate the binding of Yng1 to chromatin.

If histone H3 acetylation does not prevent Yng1 binding, then why does disruption of the HDA complex result in resistance to Yng1 overexpression? The fact that Yng1 contains two independent histone-binding motifs suggests the intriguing possibility that Yng1 could simultaneously bind two H3 tails, resulting in chromatin compaction. If this is the case, then the hyperacetylation associated with loss of the
HDA complex could result in decompaction of chromatin, alleviating the toxic effects of Yng1 overexpression. Consistent with this, mutation of H3 K9, 14, 18, and 23 to arginine, which would be expected to enhance chromatin compaction, renders Yng1 more toxic (Figure 4.6b).

![Figure 4.6](image.png)

**Figure 4.6 Acetylation does not alter the association of Yng1 for the histone H3 tail.**

a) Histone peptide binding assays were performed with the indicated biotinylated peptides and purified GST-Yng1. Shown is an αGST western blot of precipitated material. Input lane contains 10% of the protein amount used for the pulldowns.  

b) Tenfold serial dilutions of a wild type yeast strain containing the indicated plasmids were plated on synthetic complete medium lacking uracil containing either dextrose or galactose as a carbon source and incubated at 30°C for 3 days.
4.4 Discussion

NuA3 is a histone H3-specific HAT complex consisting of Sas3, Nto1, Eaf6, Taf14, and Yng1. Genomewide localization studies have shown that NuA3 is enriched throughout coding sequences, with a modest increase within the 5' half of genes. How this complex is targeted to these regions is unclear. We have previously shown that Yng1 is required for NuA3 association with chromatin in vitro and were interested in determining which part of this protein is required for this function. To this end, we developed two independent assays for Yng1 function in vivo: rescue of an yng1Δ phenotype and inhibition of growth due to overexpression of YNG1 from a GAL1 promoter. Surprisingly, conflicting results were generated when using these two assays. Yng1 lacking the PHD finger rescues a yng1Δ phenotype, suggesting that the PHD finger is dispensable for NuA3 function in vivo. In contrast, the Yng1 PHD finger is essential for the growth-inhibiting properties of this protein when overexpressed. In this study we have been able to reconcile these conflicting observations and generate insight into how this complex is localized to transcribed regions. We show that Yng1 contains two histone-binding motifs: an amino-terminal motif that binds unmodified histone H3 tails and a carboxyl-terminal PHD finger that specifically recognizes H3K4me3. Moreover, our results suggest that the presence of both regions enhances binding of Yng1, and presumably NuA3, to the histone H3 tail.

One phenotype associated with yng1Δ is a synthetic growth defect with loss of GCN5, which encodes an alternate histone H3-specific HAT. We know that this phenotype is due specifically to loss of NuA3 acetyltransferase activity because mutation of the acetyl-CoA binding site of Sas3, the catalytic subunit of NuA3, is lethal in a gcn5Δ
background \cite{14}. Full-length Yng1, Yng1ΔPHD, and Yng1 lacking residues important for methyl–histone recognition fully rescue growth of a \textit{gcn5Δ yng1Δ} mutant, indicating that targeting of NuA3 to H3K4me3 is not essential for NuA3 HAT activity \textit{in vivo} \cite{55}. In this study we demonstrated that amino acids in the amino-terminal region of Yng1 can bind unmodified histone tails and thus Yng1 may be simply required to dock NuA3 on its substrate regardless of the methylation status. We cannot, however, exclude the possibility that the Yng1 PHD finger–H3K4me3 interaction strengthens the binding of the NuA3 complex to chromatin. Indeed the enhanced binding of full-length Yng1 to H3K4me3 vs. unmodified peptide may explain the slight enrichment of NuA3 at the 5′ ends of genes \cite{207}.

Overexpression of \textit{YNG1} inhibits growth of both wild-type and \textit{sas3Δ} strains, indicating that this phenotype is independent of NuA3 HAT activity \cite{18}. This synthetic dosage lethality is dependent on the presence of both the Yng1 amino terminus and the PHD finger. While we do not fully understand why \textit{YNG1} overexpression inhibits growth, we can conclusively state that toxicity is due to the binding of excess Yng1 to the H3 tail. On the basis of the results of this study, we hypothesize that while both the PHD finger and the amino terminus of Yng1 can bind the H3 tail independently, loss of either region weakens the interaction to the extent that the protein is no longer toxic when overexpressed. Whether the enhanced binding activity of the two motifs in combination has a relevant function when expressed at normal levels is unknown, but, as mentioned above, it may explain the modest accumulation of Yng1 observed at the 5′ end of genes \cite{207}.
This study raises a number of interesting questions. First, if Yng1 can bind to unmodified tails, why did genome-localization studies show depletion of Yng1 in intergenic regions \(^{207}\)? We feel that the most likely explanation is that the intergenic regions are depleted for histones and thus have fewer sites for NuA3 to bind \(^{208,209,121}\). Second, why does loss of the HDA complex confer resistance to \textit{YNG1} overexpression? We have eliminated the obvious explanation that acetylation negatively regulates Yng1 binding to chromatin. Interestingly, mutation of the acetylated lysines within the H3 tail to constitutively charged residues enhances growth inhibition by Yng1, which is consistent with the idea that Yng1 may be compacting chromatin. The exact mechanism behind the regulation of Yng1 binding to chromatin, as well as the targeting of NuA3, will be a subject of future studies.
5 Chapter: Conclusions

5.1 Chapter Summary

In Chapter 2, we studied the function of yFACT in vivo. Our data suggested that yFACT stably binds nucleosomes with an altered structure. We also tested the model of yFACT function and provided evidence that in vivo the complex functions through the “global accessibility” model as opposed to the “H2A/H2B dimer eviction” model. Using MS, we determined the PTM status of yFACT-associated nucleosomes. We found that yFACT binds to hyper-acetylated nucleosomes and that H3K56ac promotes the interaction of yFACT with chromatin. Our work revealed that the association of yFACT with hyper-acetylated chromatin was not dependent on cell-cycle fluctuations of H3K56ac suggesting another mechanism exists that regulates the interaction of yFACT with chromatin. Lastly, we showed that a previously published interaction between yFACT and the lysine acetyl-transferase, NuA3, functioned to acetylate the yFACT-associated nucleosomes.

Chapter 3 followed up on the finding that yFACT binds nucleosomes in vivo. We constructed a map of yFACT-nucleosome interactions genome-wide at single-nucleosome resolution and showed that the yFACT-nucleosomes are found across the open reading frames of genes. The distinguishing feature of yFACT-nucleosomes is that, as compared to canonical nucleosomes, they are positioned over the nucleosome depleted regions (NDR) in positions “0” and “+1”. yFACT-associated nucleosomes are enriched on Taf1-depleted genes that contain a strong consensus to the canonical TATAAWWR sequence. The positioning of yFACT-nucleosomes at these loci correlates
with the presence of the Nhp6 binding site -TATATAA. The loss of Nhp6a/b and thus presumably the yFACT recruitment to TATATAA sites causes chromatin loss around the TATATAA sites.

In Chapter 4, we turned our attention to the NuA3 acetyltransferase complex. NuA3 is recruited to chromatin through the Yng1 PHD finger that binds to H3K4me3. However, the deletion of the YNG1_PHD finger sequence does not recapitulate the phenotypes associated with the loss of NuA3 function whereas the full deletion of YNG1 does. Our work showed that Yng1 is a bivalent histone-binding protein. While the C-terminal PHD finger binds H3K4me3, the N-terminus of Yng1 can bind to unmodified chromatin. Together these two domains increase the apparent association of Yng1 with histone H3 tails. We used a genome-wide synthetic dosage lethality screen to uncover novel regulatory mechanisms of Yng1. We showed that the Yng1 binding to chromatin is not only regulated by H3K4me3 but also by the HDA1 KDAC complex, through a yet unknown mechanism.
5.2 Common Discussion

5.2.1 The “dimer eviction” and “global accessibility” models of yFACT function

Ever since its discovery, FACT has been a subject of much speculation. The pleiotropic nature of FACT has sparked an intense debate as to the mechanism of action that would allow it to function in DNA transcription, replication and repair \[101\][102][108][154][130]. Two modes of function were proposed to explain the pleitropic behavior of FACT. The “dimer eviction” model postulates that yFACT removes and recycles H2A/H2B dimers. On the other hand the “global accessibility” model proposes that FACT binds and stabilizes altered, yet intact nucleosomes \[78\]. Recent, careful examination of FACT affinity for its substrates showed that FACT preferentially binds to nucleosomes as opposed to free histones \textit{in vitro}, thus lending support to the “global accessibility” hypothesis \[112\]. The work presented in this thesis addressed what is the preferred substrate of yFACT \textit{in vivo}. We showed that yFACT stably binds to altered nucleosomes inside the cell, consistent with the “global accessibility” model. While the “dimer eviction” hypothesis does not exclude the possibility of yFACT transiently interacting with nucleosomes, we were unable to detect yFACT bound solely to H2A/H2B dimers. Thus, yFACT is unlikely to simply bind and displace H2A/H2B. We conclude that in yeast FACT is most likely to function by binding and altering nucleosome structure, such as to allow the DNA processes to occur. At the same time, through this association yFACT is able to promote nucleosome occupancy and chromatin stability.
5.2.2 The “global accessibility” and yFACT phenotypes

The idea of yFACT functioning by promoting “global accessibility” of chromatin finds support in the published literature. For instance, yFACT was shown to maintain nucleosome levels and suppress incorporation of newly synthesized histones to transcribed chromatin\cite{118}. While both the “global accessibility” and the “dimer eviction” models could explain the maintenance of histone levels during transcription, the “global accessibility” fits better with yFACT acting to suppress histone exchange. If yFACT actively removes and replaces histones from nucleosomes, the exogenous expression of histones ought to result in their incorporation to chromatin. This however, is only observed upon the loss of yFACT function\cite{118}. Hence, yFACT is more likely to stay associated with nucleosomes and stabilize them through transcription, as proposed by the “global accessibility” model.

yFACT localization on genes was proposed to depend on the strength of association between histones H3 and H4. The histone H3L61W mutant was speculated to increase the stability of association between H3 and H4. The H3L61W mutation caused accumulation of yFACT at the 3’ UTRs\cite{211}. Mislocalization of yFACT through such a mechanism requires a stable association between yFACT and nucleosomes. The transient binding required for removal of H2A/H2B, as postulated by the “dimer eviction” model, would be unlikely to result in an interaction stable enough to trap yFACT. Therefore, the “global accessibility” model that explicitly assumes stable association between yFACT and nucleosomes provides a better explanation for these observations.
5.2.3 The recruitment of yFACT to chromatin in yeast and metazoans

yFACT interacts with PAF1C and thus has been proposed to be recruited to chromatin in a transcription-dependent manner \[^{115}\]. Our work proposes two additional mechanisms for yFACT recruitment to chromatin: i) the nucleosome binding affinity of yFACT for H3K56ac and ii) the positioning of yFACT-associated nucleosomes by the Nhp6 consensus binding sequence TATATAA at TATA-elements and genome-wide.

The Pob3 subunit of yFACT and the Rtt106 histone chaperone share a Pleckstrin Homology (PH) domain that was proposed to recognize H3K56ac. Subsequent work showed that while the PH domain of Rtt106 conferred preference for H3K56ac chromatin, the Pob3 PH did not \[^{23}\][\(^{22}\]. Our results however showed that H3K56ac did increase the recruitment of yFACT to chromatin, likely as an indirect consequence of H3K56ac effect on nucleosome structure \[^{28}\]. The H3K56ac-mediated FACT recruitment might not be only a yeast-exclusive mechanism. While metazoans do not have the Rtt109 KAT, H3K56ac is catalyzed by CBP/p300 instead \[^{212}\]. Thus, the H3K56ac-mediated nucleosome alteration might be a universal mode of recruitment of FACT.

The Pleckstrin Homology (PH) domain is widely recognized as a versatile protein-protein interaction domain. Although the Pob3 PH domain did not show a marked affinity for H3K56ac, this does not mean that it does not serve other functions. One of the possible ligands of PH domains are phosphotyrosines \[^{213}\]. Tyrosine 1 of the CTD of RNAPII becomes phosphorylated during transcription and this phosphorylation is observed towards the 3’ ends of genes. Thus, this phosphorylation could act to recruit yFACT to elongating RNAPII \[^{98}\]. Since the Pob3 (SSRP1) PH domain is conserved across different organisms, a PH-phosphotyrosine interaction could be a conserved
mode of FACT recruitment \cite{78}. Since both H3K56ac and Nhp6a/b are absent from some parts of the genome, yet FACT is not, a PH-Tyrosine 1 Phosphorylation interaction could help explain the ubiquitous FACT distribution.

yFACT was shown \textit{in vitro} to bind nucleosomes in a three-step process, first Nhp6 binding results in nucleosomal alteration followed by yFACT recognizing the altered nucleosome and subsequently binding to it \cite{107}\cite{129}. This work provided evidence that yFACT binds intact nucleosomes \textit{in vivo}. Based on our sequencing results we proposed a novel mechanism of yFACT recruitment through the Nhp6-binding sequence, TATATAA. This was the first time a histone chaperone was shown to be arranged on chromatin around specific DNA sequences. While in yeast Nhp6 is present as a separate subunit of yFACT, the metazoan FACT complex contains a Nhp6 homologue as part of its SSRP1 subunit \cite{106}. Consistent with its function as a DNA-binding subunit of FACT, SSRP1 was found to recognize bent DNA structures. Binding of bent DNA is a property shared by many HMG proteins, including Nhp6 \cite{214}\cite{215}. We are not aware whether SSRP1 displays any DNA sequence specificity \cite{156}. Should the SSRP1 HMG domain display sequence-specificity then a recruitment mechanism, analogous to the one observed for yFACT, could occur in metazoans.

\textbf{5.2.4 Recruitment of yFACT to chromatin post-replication}

How chromatin is maintained during DNA replication is a subject of much debate. Because replication doubles the DNA and histone content, it presents a challenge for the conservation of epigenetic marks. Recent work by the Mazo group explored the possibility that epigenetic marks might not be the histone modifications themselves, but
rather their respective chromatin modifiers. Petruk et al. showed that in *Drosophila melanogaster* both the Polycomb (PcG) and Trithorax (TrX) complexes, but not methylated histones, remain associated with their response elements immediately post DNA replication\[^{216}\]. This data suggests that sequence-specificity of chromatin modifiers could be the *de facto* epigenetic mark.

Our work proposed a model for yFACT recruitment to chromatin through Nhp6 binding to specific DNA sequences. It is therefore conceivable that in yeast, in addition to the H3K56ac-mediated binding, post-replication yFACT is recruited to the TATATAA sequences, independently of the presence of transcription. Thus, together with other sequence-dependent factors, yFACT could act to reestablish the chromatin environment following replication. For example, the Spt16-Sas3 interaction could facilitate recruitment of NuA3 to newly-deposited chromatin, and subsequent acetylation of nucleosomes, helping to reestablish transcription\[^{56}\]. Our finding that NuA3 acetylated the yFACT-associated nucleosomes provides support to this notion.

### 5.2.5 FACT in disease

Recently FACT was shown to play a role in cancer progression. The proliferation of tumor cells was demonstrated to depend on FACT overexpression and knockdown of FACT stalled tumor growth. At the same time, immuno-histochemical staining of FACT subunits Spt16 and SSRP1 revealed that FACT is present in undifferentiated cells and tumors in mouse and human tissues, but was undetectable in adult, differentiated cells\[^{217}\][\(^{119}\)]. Thus FACT presents an attractive therapeutic target. The FACT complex was recognized as a target for a novel group of small molecule inhibitors, curaxins. Curaxin-
mediated inhibition of FACT leads to phosphorylation of p53 by FACT-associated CK2 and activation of the apoptotic pathway. At the same time FACT inhibition leads to the loss of NF-κB-mediated transcription, downstream of NF-κB nuclear translocation and DNA binding. Importantly, curaxins, do not damage DNA and thus avoid the toxicity associated with other genotoxic agents. Since FACT is not present in differentiated cells, curaxins avoid the possible side effects of inhibiting FACT in healthy cells \[120\].

Investigation into the mechanism of curaxin action showed that these small molecules deplete the soluble supply of FACT in the cell as measured by the SSRP1 levels, and cause FACT to become completely chromatin-associated. Curaxins are DNA intercalating molecules, and computer modeling showed that they bind to the DNA grooves and alter DNA structure \[120\]. The mechanism of curaxin action is consistent with FACT functioning by the “global accessibility” model. FACT can recognize altered DNA and nucleosome structure \[215][129\], and thus curaxins are likely to mistarget both chromatin-bound and soluble pools of FACT. Since FACT is essential for so many cellular processes, this would cause cell senescence and death. Given the high success rate of curaxins in promoting tumor regression in animal models, curaxins and FACT might just be the so much needed breakthrough in cancer therapy \[120\].

5.2.6 NuA3 targeting to chromatin

NuA3 is a Sas3-dependent lysine acetyl-transferase. Sas3 is a member of the MYST (MOZ, Sas3p/Ybf2p, Sas2p, and Tip60p) family \[218][219\]. NuA3 is recruited to chromatin by the interaction of its subunit, Yng1 with H3K4me3 \[220\]. Our work expands on the understanding of how NuA3 recognizes its substrates. We showed that the N-
terminus of Yng1 could bind to unmodified chromatin. We also showed that the interaction between the catalytic subunit of NuA3, Sas3, and Spt16 of yFACT functions to facilitate acetylation of yFACT nucleosomes. Thus, NuA3 is emerging as a multivalent complex that is targeted to a variety of substrates. What other mechanisms recruit NuA3 remains to be shown.

5.3 Future Directions

The work presented in this thesis raises a number of questions and interesting hypotheses, which should be pursued in the future. We showed that yFACT forms a complex with the nucleosomes in vivo and presented the EM structure of said complex. While the image of yFACT bound to a nucleosome can be informative, a more in-depth understanding would be provided by resolving the exact contact interface between yFACT and the nucleosome. Mapping the contact points between all the proteins involved would allow for a better model of how yFACT alters the nucleosome. A method suitable for such studies would be cross-linked Mass Spectrometry of yFACT-histone contacts followed by Bioinformatic modeling, where protein contact points are cross-linked and analyzed on a high throughput scale [221].

We found that yFACT nucleosomes are positioned genome-wide by the Nhp6a/b targeting sequences. We provided meta-analysis of a previously published dataset that looked at the effects of loss of Nhp6a/b on nucleosome positioning. However, the assay used by the authors was performed using a different protocol [162]. Thus, to better characterize the contribution of Nhp6a/b to yFACT positioning around TATATAA
elements, our sequencing experiment should also be repeated in *nhp6a/bΔ* strain. The mapping of yFACT-nucleosomes should be performed in a number of additional mutants. While disruption of Nhp6a/b would provide information as to whether the positioning around the TATATAA sequences is Nhp6a/b-dependent, the *rtt109Δ* and H3K56A, Q and R mutants would uncover the contribution of H3K56 to targeting of yFACT.

We were unable to uncover the molecular mechanism behind the HDA1-mediated regulation of Yng1 binding to chromatin. Loss of Hda1 did not alter the H3K4me3 levels or *GAL1*-mediated overexpression of Yng1. HDA1 might affect Yng1-chromatin association through a number of mechanisms. For example, because of its bivalent activity Yng1 may bridge inter-nucleosomal contacts. An increase in histone acetylation in the *hda1Δ* strain could disrupt this interaction and thus provide resistance to Yng1 overexpression. *In vitro* studies of Yng1 binding to acetylated and unmodified nucleosomal arrays would be a way of testing this hypothesis.
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