DISSERTATION

SPN1, SPT4, SPT5, AND SPT6 PRESERVE CHROMATIN STRUCTURE OVER PROMOTERS AND OPEN READING FRAMES

Submitted by

Andrew Jordan Tonsager

Department of Biochemistry and Molecular Biology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2024

Doctoral Committee:

Advisor: Laurie A. Stargell

Jeffrey C. Hansen
Thomas Santangelo
Juan Lucas Argueso

ABSTRACT

SPN1 SPT4, SPT5, AND SPT6 PRESERVE CHROMATIN STRUCTURE OVER PROMOTERS AND OPEN READING FRAMES

The eukaryotic chromatin landscape presents formidable nucleosomal barriers for processes that require access to DNA, such as transcription. These barriers are overcome through the action of many factors, including histone chaperones Spn1, Spt5, Spt6, and FACT and transcription elongation factor Spt4. However, it is poorly understood how each contributes to this process. To ascertain the role that these factors play on preserving chromatin structure over the genome, this thesis has utilized micrococcal nuclease digestion followed by sequencing (MNase-seq) to analyze chromatin protections in the yeast genome in cells expressing numerous mutant alleles of these factors. Extensive characterization of MNase-protected fragments in a wide range of sizes established that the essential histone chaperone Spn1 preserves both nucleosomal and subnucleosomal structures over both promoters and open reading frames across the genome. Additional analyses from existing MNase-seq datasets demonstrated the extent to which Spn1 and other RNAPII-associated factors maintain nucleosome features over genes of varied characteristics. The study of factors described in this thesis is performed in living cells, which have been genetically modified to express mutant alleles of chromatin factors. This thesis also describes a course-based undergraduate research experience (CURE) developed to introduce upper-level biochemistry students to techniques in yeast genome engineering in an authentic research setting.

ACKNOWLEDGEMENTS

My time as a graduate student in the Department of Biochemistry and Molecular Biology has been the most challenging, yet most fulfilling chapter of my life, and I wouldn't be at the place I am today without the support of so many amazing individuals.

To my advisor and mentor Laurie Stargell – It's impossible to adequately describe in words just how substantial your impact on my life has been. You took a chance by accepting me into your laboratory at a time when I was more unsure of myself and my capabilities than ever. I can't thank you enough for the countless hours you've spent working with me. Thank you for believing in me during my lowest moments and guiding me through the wonderfully arduous process of earning a Ph.D. I have never had a mentor who engaged with me quite in the way that you have. I'm incredibly grateful to have been your student; I am the scientist, educator, and overall person I am today in such a large part because of you.

To my committee members: Jeff Hansen, Tom Santangelo and Lucas Argueso – You have all been so incredibly instrumental in your own ways during my time here at Colorado State University. Thank you all for modeling the kind of scientist and educator that I strive to be. Jeff – Thank you for believing in me and being such an avid advocate for me; you're the most amazing teaching partner I could ask for. Tom – Thank you for always challenging me in so many ways to be the best scientist I can be and for keeping an open door for me during wonderful and hard times. Lucas – Thank you for always fielding such insightful questions and for being so incredibly pleasant to work with.

To my mentor at the bench, Cathy Radebaugh – I embarked on a research project at the bench feeling apprehensive and unsure of myself. Thank you for your time, dedication, and patience in teaching me how to be a scientist. I've learned so much from you over these wonderful years.

Thanks for sticking this out with me, especially during the many group meetings filled with more bioinformatics than you ever imagined signing up for!

To my other supportive lab mates (past and present): Leah, Bridget, and Jacob – Thank you for enduring my many long presentations and for your feedback.

To my many other mentors in the classroom, namely: Farida Safadi-Chamberlain, Corey Rosenberg, Brian Kalet, Erin Osborne Nishimura and many others – I came to graduate school thinking that I wanted to continue pursuing a career in education. Thank you all in your own ways for the many experiences that validated that the life of an educator is the one for me.

To my family –

Alyssa – Thank you for helping me navigate so many challenging situations in my life and for your consistent encouragement and support of all my endeavors.

Mom – Thank you for your everlasting love and belief in me and for always being only a phone call away for anything that I could possibly need.

Dad – You're why I embarked on my biochemistry journey. Thank you for loving me and sticking with me through this journey.

To all my other family and friends – Thank you all in your own ways for supporting me through this amazing chapter of my life. I couldn't have gotten luckier to be surrounded by such supportive and wonderful people.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Introduction

Eukaryotes package their genomes by wrapping ~147bp of DNA in about 1.7 superhelical turns around an octamer of histone proteins to form nucleosomes[1-3]. This process occurs in a stepwise manner, as DNA initially wraps around a tetramer of histones H3–H4 to form a tetrasome, followed by sequential additions of two H2A-H2B dimers to first form a hexasome, then a nucleosome[4]. Tetrasome and hexasome subnucleosomal structures have been observed both *in vitro*[5] and *in vivo*[6] and represent stable intermediates in nucleosome assembly and disassembly. Nucleosomes regulate DNA access to many proteins that perform essential processes such as DNA replication[7, 8], repair[7, 9], recombination[10], and transcription. Nucleosomes regulate transcription in a variety of ways. Positioning of nucleosomes at promoters can hinder transcription initiation by preventing the binding of transcription factors[11, 12] while positioning of nucleosomes within coding regions can play an important role in preventing intragenic transcription initiation by reducing the accessibility of factors to intragenic initiation sites[13] (Figure 1.1A). Nucleosomes are inhibitory to transcription elongation of protein coding genes, as they pose a significant barrier to RNA polymerase II (RNAPII) and reduce its transcription rate[14, 15] (Figure 1.1B). Transcription through chromatin requires at least transient disassembly of downstream nucleosomes which must be reassembled in the wake of RNAPII to maintain proper chromatin structure[6].

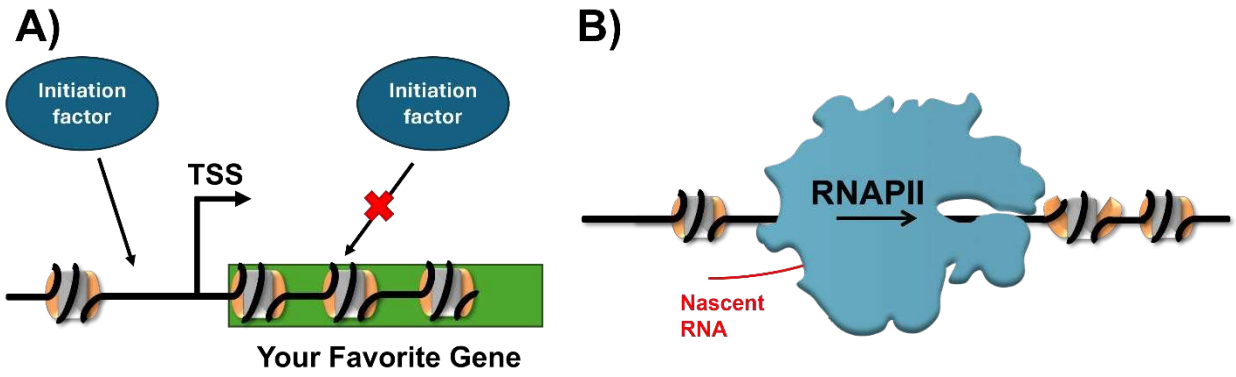
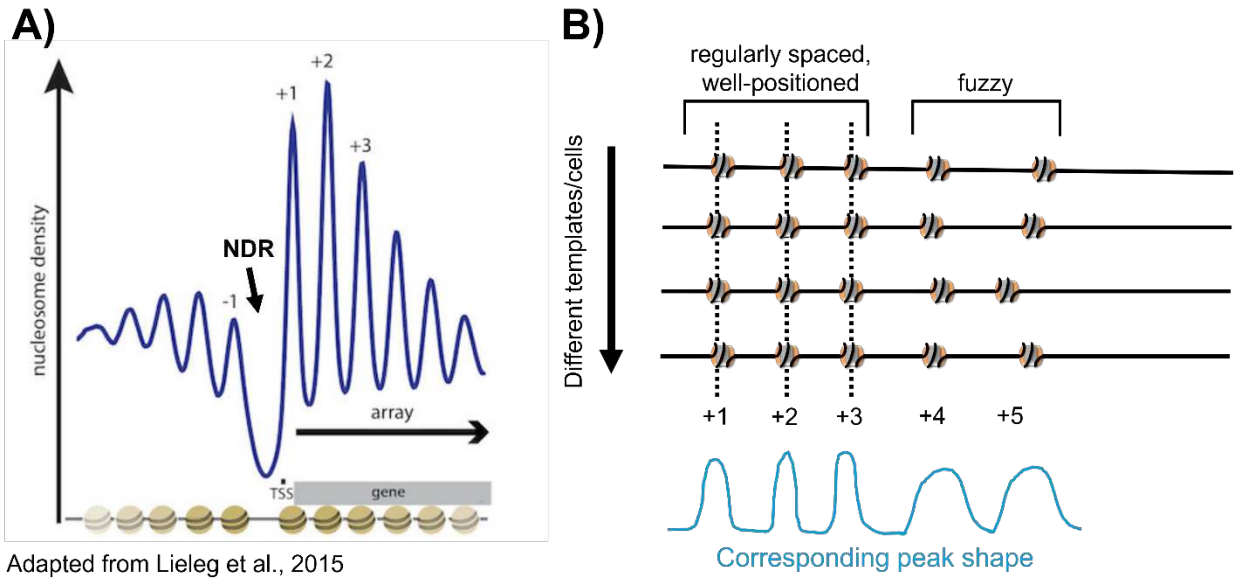


Figure 1.1. Nucleosomes are a major regulator of processes that require access to DNA, like transcription. (A) Nucleosomes occlude transcription initiation factor recognition sequences and regulate binding. Sequences upstream of transcription start sites need to be accessible for transcription factors to bind and initiate transcription. Nucleosomes also prevent intragenic transcription initiation by inhibiting transcription initiation factor binding at sequences within gene bodies. (B) Transcription by the RNAPII elongation complex through chromatin requires at least transient disassembly of downstream nucleosomes followed by reassembly of nucleosomes in the wake of RNAPII.

In wild-type cells, metagene analyses have demonstrated that well-positioned nucleosomes flank transcription start sites (TSSs) and are followed by a regularly spaced array of nucleosomes downstream (reviewed in[16]) (Figure 1.2A). The first nucleosome downstream of the TSS is referred to as the +1 nucleosome with subsequent positions named in ascending numerical order. The first nucleosome encountered upstream of the TSS is referred to as the -1 nucleosome, and the trough in between the +1 and -1 nucleosome positions is known as the nucleosome-depleted region (NDR). This region hosts promoter sequences and its nucleosome depletion is maintained by chromatin remodelers like the RSC complex[17]. This region is bound by many other factors, such as activators and enhancers that bind and compete with nucleosome formation within NDRs, contributing to the low nucleosome density in these regions[18]. Nucleosomes downstream of TSSs are increasingly less well-positioned with distance from the TSS and are referred to as “fuzzier” (Figure 1.2B). Nucleosomes that are “fuzzier” exhibit a wider range of DNA positions when sampled over many different genes.



Adapted from Lieleg et al., 2015

Figure 1.2. Nucleosomes form regularly spaced arrays over genes. (A) Diagram of the average nucleosome density profile over eukaryotic genes adapted from [16]. Nucleosomes are numbered based on their proximity to TSSs and become increasingly less well-positioned with increased distance from the TSS. The +1 and -1 nucleosomes flank nucleosome-depleted regions (NDRs) which allow for transcription initiation factors to access recognition sequences within this region. (B) Diagram of how differences in nucleosome positioning at different positions produce distinct peak shapes. Nucleosomes proximal to the TSS (+1, +2, +3) tend to exhibit little variability in their occupied DNA positions across different templates, resulting in regularly spaced and well-positioned nucleosomes. In metagene analyses, this produces a sharp peak shape. Nucleosomes occupy a wider range in positions over different templates and become "fuzzier" with increased distance from the TSS. Metagene analyses produce shorter and wider peaks over these positions.

Metagene analyses with sufficiently high sequencing depth have revealed that nucleosome peaks in density arise from alternative rotational positions, separated by a 10bp periodicity on either side of the dyad[19]. These peaks in occupancy correlate strongly with an oscillatory pattern of AT-containing dinucleotides spaced ~10bp apart and in counter-phase with GC-containing dinucleotides over nucleosome positions in the yeast genome[20, 21]. Peaks corresponding to favorable nucleosome positioning occur when AT-containing dinucleotides face toward histones and GC-containing dinucleotides face away from them[21, 22].

Histone chaperones are a class of proteins that contribute to chromatin structure, by maintaining nucleosome positioning and occupancy through deposition, removal, and exchange of histones

from DNA in an ATP-independent manner[23]. A multitude of histone chaperones have been identified that assist RNAPII during transcription by assisting in the transient disassembly and subsequent reassembly of nucleosomes during RNAPII passage[24]. Many of these histone chaperones are essential for cell viability across eukaryotes, including Spn1, Spt5, Spt6, and FACT[25-28]. These specific factors physically engage with RNAPII as recently revealed through cryo-electron microscopy (cryo-EM) [29] (Figure 1.3). However, how these proteins compare in their functional maintenance of proper chromatin structure has not yet been undertaken.

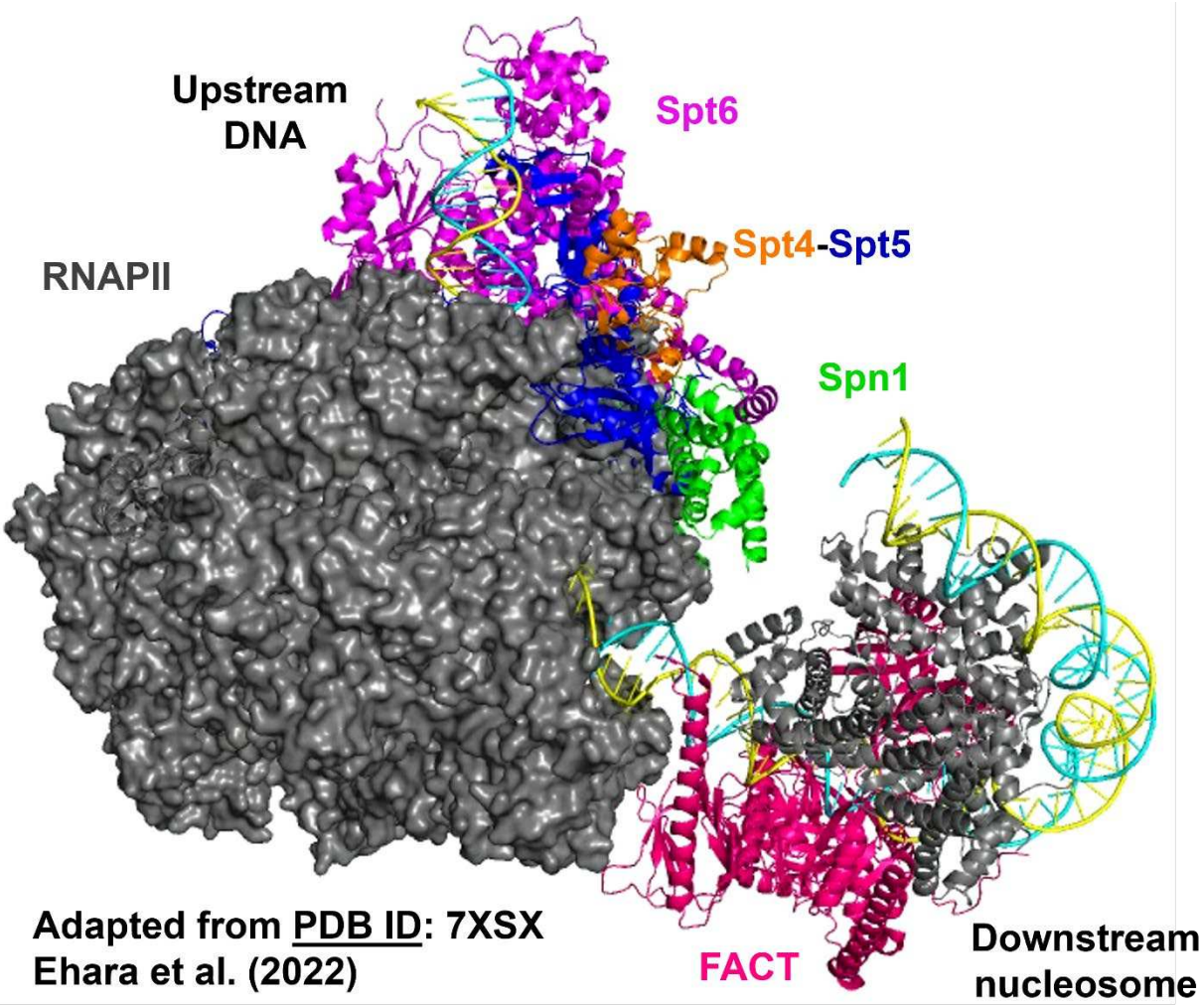


Figure 1.3. Several essential histone chaperones physically associate with the RNAPII elongation complex. Cryo-electron microscopy (cryo-EM) data adapted from the published EC49 structure published in [29] depicts Spn1 (green), Spt4-Spt5 (orange, blue), and Spt6 (purple) physically engaged with RNAPII (grey) with distinct interacting surfaces. FACT (pink) is engaged with the downstream nucleosome in this structure.

Spn1 is a multifunctional and essential histone chaperone that is highly conserved across eukaryotes. *S. cerevisiae* Spn1 preferentially binds H3-H4 histones[30] via its N-terminal region, with high binding affinity (K_D ~ 10nM). Spn1 affinity to H3-H4 is at least 50-fold higher than that to H2A-H2B dimers[31]. Spn1 binds nucleosomes with its C-terminal region[30, 31]. Spn1 can deposit H3-H4 onto DNA and facilitate the assembly of H3-H4 tetrasomes *in vitro*[30]. Spn1 also has a well-characterized interaction with another essential histone chaperone Spt6; they bind each other with high affinity (K_D = 10-100nM) to form a heterodimer[32-35]. Depletion of either protein through the auxin-inducible degron system results in a reduction in recruitment of the other to chromatin[36]. The central domain of Spn1 is a highly conserved eight helix bundle and is primarily responsible for binding Spt6[31].

Cells with endogenous Spn1 replaced with the truncated *spn1*$^{141-305}$ mutant are viable and grow comparably to cells with wild-type Spn1 when in rich media[27, 30]. However, *spn1*$^{141-305}$ is unable to directly bind DNA, nucleosomes, and histones, though it retains the ability to bind Spt6 and RNAPII[30, 31]. In contrast, yeast expressing *spn1*^{K192N} are temperature sensitive[27], and have reduced Spn1 recruitment to the *CYC1* locus and fail to recruit Spt6 to *CYC1*[37]. The *spn1*^{K192N} mutant also has reduced Spn1 binding to RNA Polymerase II (RNAPII) by co-immunoprecipitation[37]. As recent work demonstrates that Iws1, the human ortholog of Spn1, serves as a hub that maintains association of numerous transcription elongation factors[38], it remains unclear which interactions are impaired that lead to loss of RNAPII recruitment.

Spt6 is similarly highly conserved across eukaryotes but binds H3-H4 and H2A-H2B with comparable affinity[39] and similarly can bind nucleosomes, though only in the presence of Nhp6, a small high-mobility group (HMG) protein[32]. Spn1 and Spt6 binding to nucleosomes are mutually exclusive[31, 32]. Loss of Spt6 function has been widely shown to produce defects in chromatin features, such as loss of nucleosomes over transcribed regions, which can drive cryptic transcription initiation or improper initiation over certain gene promoters[13, 40-43].

FACT (FAcilitates Chromatin Transcription/Transactions), comprised of subunits Spt16 and Pob3, is functionally like Spt6 in many ways. FACT is both an H2A-H2B histone chaperone[44] and H3-H4 chaperone[45] and similarly requires Nhp6 for binding nucleosomes[25]. Both factors play a role in regulating core histone levels[46, 47] as well as proper maintenance of non-canonical histone H2A.Z localization[48]. Mutations in FACT also produce striking defects in chromatin structure, including loss of nucleosome occupancy over genes[41, 49-51].

Histone chaperone Spt5 also binds H3-H4 histones, and this function is essential for cell viability in yeast[52]. It is the only factor that is physically associated with RNA polymerase that is universally conserved across life, including both bacteria and archaea[53]. Spt5 forms a well-characterized complex with non-essential transcription elongation factor Spt4 (DSIF in humans), controlling RNAPII processivity and chromatin structure[35, 54-57]. Intriguingly, mutation of Spt4 knockout combined with mutation of Spn1 leads to synthetic lethality[37]. Mutations in all the aforementioned factors have been informative in understanding their wild-type functions through characterization of their defects (Table 1.1).

Table 1.1. Mutant alleles of chromatin factors produce distinct defects in their functions. Mutations in factors associated with RNAPII and mutations in RNAPII itself produce varied defects in their functions, including disrupting characterized physical interactions.

Chromatin Factor	Mutant Allele	Description
Spn1	*spn1-141-305*	Truncated mutant lacking N-terminal and C-terminal regions and cannot bind histones, nucleosomes, or DNA[30, 31]
	spn1-K192N	Temperature-sensitive allele with defective recruitment to RNAPII complex[37], potentially through lost Spt6 and/or Spt5 interactions[29]
Spt6	*spt6-YW*	Temperature-sensitive allele with two point mutations (Y255A, W257A) which impairs the Spn1-Spt6 interaction[58]
	spt6-1004	Spt6 allele with deletion of helix-hairpin-helix motif (931-994); temperature-sensitive allele whose Spt6 levels reduce dramatically upon shift to a non-permissive temperature of 37°C[43]
	spt6-R,KK	Contains point mutations (R1282H,K1355A,K1435A) which disrupt Spt6-RNAPII binding *in vitro*[59]
Spt4	*spt4Δ*	Cells with *SPT4* deleted are viable[60]
Spt5	*spt5-QS*	Mutation obtained in a screen that suppresses spt6-YW temperature-sensitivity[58]. Mutations are located distally from Spt4 and putative Spn1 interaction surfaces (Q342H,S343del [3 AA deleted])
	spt5-AID	Auxin-inducible degron of Spt5
	spt5-3A	Point mutations in the unstructured N-terminal region which binds histones (F180A,E184A,V187A); mutations produce lethal phenotype[52]
FACT	*spt16-G132D*	Temperature-sensitive allele of Spt16 depleted by raising to non-permissive temperature of 37°C[61]
	pob3-Q308K	FACT mutant defective for resolving nucleosome organization; displays inefficient dissociation of FACT-nucleosome complexes[62]
RNAPII (Rpb1)	*rpb1-1*	Temperature-sensitive allele of Rpb1, the catalytic subunit of RNAPII[63]
	rpb1-TPY,FSP	Substitutions from 1490-1496 (LMFSPLV to KMKRRK) which disrupt Spt6 interaction *in vitro*[59]

Study of the role of factors on their contributions to chromatin structure maintenance often employ digestion with micrococcal nuclease followed by sequencing (MNase-seq) to quantify nucleosomal protections across the genome (reviewed in [64]) (Figure 1.4). The exo-endonuclease activity of MNase digests linker DNA at 25 times the rate of nucleosomal DNA[65] which generates a library of DNA fragments protected by proteins. This DNA can be sequenced and mapped to genomes leading to interpretations where the length and abundance of these fragments can be interpreted to examine changes in chromatin protections across the genome.

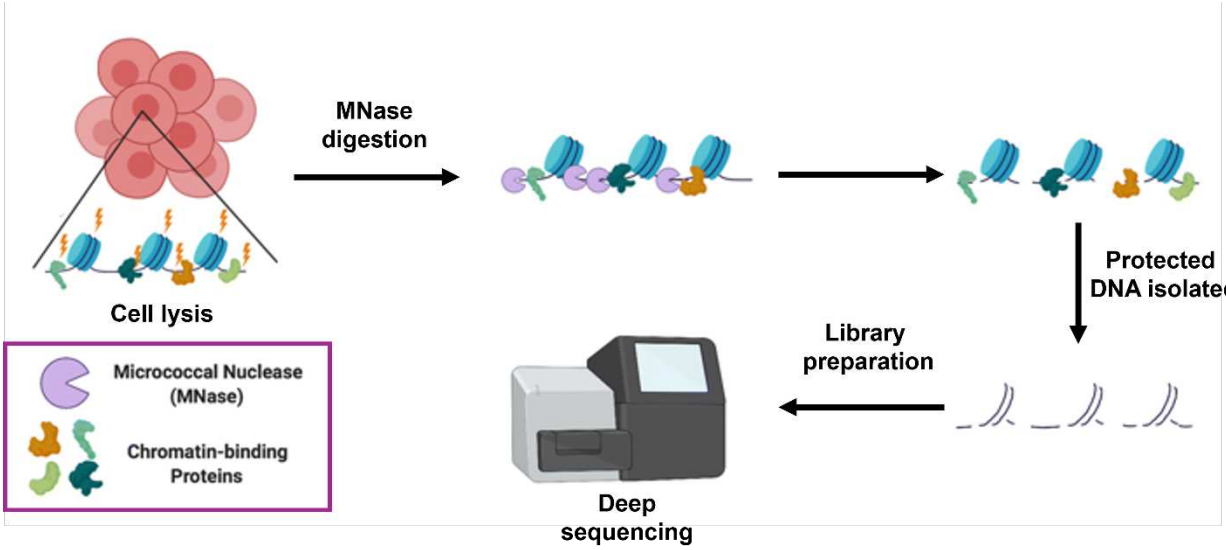


Figure 1.4. MNase-seq overview. Diagram of the major steps performed during MNase-seq adapted from [64]. Cells are lysed and can be subjected to formaldehyde crosslinking. MNase digests DNA not protected by proteins and the protected DNA can be isolated. These DNA fragments range in size based on the footprint of the protein protection. Libraries are prepared from the isolated DNA fragments and subjected to sequencing.

Thesis rationale

While we and others have previously demonstrated that Spn1 possesses multiple interactions regarding chromatin functions, the role of Spn1 *in vivo* is not well-understood. Additionally, while individual studies of other RNAPII-interacting factors like Spt4, Spt5, Spt6, and FACT have elucidated much about their roles in maintaining chromatin structure, how these factors and

Spn1 functionally compare to each other has not been determined. The work performed in this thesis revealed that Spn1 preserves both nucleosomal and subnucleosomal structures across the yeast genome. This was accomplished through a series of analyses of MNase-protected fragments from cells expressing the functionally different mutant alleles of Spn1, *spn1*^{K192N} and *spn1*$^{141\text{-}305}$. These findings are distinct from prior work by quantifying chromatin protections of a wide range of sizes, which led to the discovery that Spn1 maintains subnucleosomal protections in nucleosome-depleted regions (NDRs). Additionally, this work employs novel techniques in quantifying shifts in nucleosomes and subnucleosomal intermediates to reveal that Spn1 maintains the positioning of nucleosomes, hexasomes, and tetrasomes over open reading frames genome wide. The discoveries described in this thesis provide new tools for quantification of changes in chromatin and reveal the importance of future studies on how histone chaperones contribute to maintenance of NDRs and subnucleosomal protections across the genome.

The thesis also seeks to further compare the functions of RNAPII-interacting factors Spn1, Spt4, Spt5, Spt6, and FACT with each other. Application of a custom MNase-seq pipeline with existing MNase-seq datasets was performed to examine the role of each factor on maintenance of key features of chromatin including nucleosome occupancy, positioning, and NDR width, and the extent in which these factors function differentially at genes of varied characteristics. Notably, this analysis revealed that each factor preserves chromatin features over genes in a manner that generally positively correlates with both RNAPII occupancy over genes and gene length. Additionally, this work also provides more evidence that the role of RNAPII-associated factors extends beyond transcribed regions.

The study of factors described in this thesis is performed in living cells, which have been genetically modified to express mutant alleles of chromatin factors. The ability to generate targeted and specific genetic alterations into an organism’s genome is a powerful tool for life

scientists. As such, it is critical that emerging scientists become trained in working with this technology for future studies in living systems. This thesis also describes a novel course-based undergraduate research experience (CURE) that has been designed to teach upper division biochemistry students how to perform genome engineering, specifically with the CRISPR-Cas9 approach. This CURE engages students in the entire process of CRISPR-Cas9 genome in an authentic research setting through a project involving the engineering of yeast with a mutation in an essential gene. This course advances the field of biochemistry and molecular biology education by providing rigorous training in mastering fundamental and transferable skills for success in life sciences laboratories.

REFERENCES

1. Davey CA, Sargent DF, Luger K, Maeder AW, Richmond TJ. Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. J Mol Biol. 2002;319(5):1097-113.
2. Flaus A, Luger K, Tan S, Richmond TJ. Mapping nucleosome position at single base-pair resolution by using site-directed hydroxyl radicals. Proc Natl Acad Sci U S A. 1996;93(4):1370-5.
3. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature. 1997;389(6648):251-60.
4. Polo SE, Almouzni G. Chromatin assembly: a basic recipe with various flavours. Curr Opin Genet Dev. 2006;16(2):104-11.
5. Brower-Toland BD, Smith CL, Yeh RC, Lis JT, Peterson CL, Wang MD. Mechanical disruption of individual nucleosomes reveals a reversible multistage release of DNA. Proc Natl Acad Sci U S A. 2002;99(4):1960-5.
6. Ramachandran S, Ahmad K, Henikoff S. Transcription and Remodeling Produce Asymmetrically Unwrapped Nucleosomal Intermediates. Mol Cell. 2017;68(6):1038-53 e4.
7. Groth A, Rocha W, Verreault A, Almouzni G. Chromatin challenges during DNA replication and repair. Cell. 2007;128(4):721-33.
8. Eaton ML, Galani K, Kang S, Bell SP, MacAlpine DM. Conserved nucleosome positioning defines replication origins. Genes Dev. 2010;24(8):748-53.
9. Adkins NL, Niu H, Sung P, Peterson CL. Nucleosome dynamics regulates DNA processing. Nat Struct Mol Biol. 2013;20(7):836-42.
10. Bevington S, Boyes J. Transcription-coupled eviction of histones H2A/H2B governs V(D)J recombination. EMBO J. 2013;32(10):1381-92.
11. Henikoff JG, Belsky JA, Krassovsky K, MacAlpine DM, Henikoff S. Epigenome characterization at single base-pair resolution. Proc Natl Acad Sci U S A. 2011;108(45):18318-23.
12. Kubik S, Bruzzone MJ, Jacquet P, Falcone JL, Rougemont J, Shore D. Nucleosome Stability Distinguishes Two Different Promoter Types at All Protein-Coding Genes in Yeast. Mol Cell. 2015;60(3):422-34.
13. Kaplan CD, Laprade L, Winston F. Transcription elongation factors repress transcription initiation from cryptic sites. Science. 2003;301(5636):1096-9.
14. Selth LA, Sigurdsson S, Svejstrup JQ. Transcript Elongation by RNA Polymerase II. Annu Rev Biochem. 2010;79:271-93.
15. Brahma S, Henikoff S. Epigenome Regulation by Dynamic Nucleosome Unwrapping. Trends Biochem Sci. 2020;45(1):13-26.
16. Lieleg C, Krietenstein N, Walker M, Korber P. Nucleosome positioning in yeasts: methods, maps, and mechanisms. Chromosoma. 2015;124(2):131-51.
17. Lorch Y, Maier-Davis B, Kornberg RD. Role of DNA sequence in chromatin remodeling and the formation of nucleosome-free regions. Genes Dev. 2014;28(22):2492-7.
18. Rando OJ, Winston F. Chromatin and transcription in yeast. Genetics. 2012;190(2):351-87.
19. Brogaard K, Xi L, Wang JP, Widom J. A map of nucleosome positions in yeast at base-pair resolution. Nature. 2012;486(7404):496-501.
20. Albert I, Mavrich TN, Tomsho LP, Qi J, Zanton SJ, Schuster SC, Pugh BF. Translational and rotational settings of H2A.Z nucleosomes across the Saccharomyces cerevisiae genome. Nature. 2007;446(7135):572-6.
21. Segal E, Fondufe-Mittendorf Y, Chen L, Thastrom A, Field Y, Moore IK, et al. A genomic code for nucleosome positioning. Nature. 2006;442(7104):772-8.

22. Satchwell SC, Drew HR, Travers AA. Sequence periodicities in chicken nucleosome core DNA. J Mol Biol. 1986;191(4):659-75.
23. Bai L, Morozov AV. Gene regulation by nucleosome positioning. Trends Genet. 2010;26(11):476-83.
24. Kujirai T, Kurumizaka H. Transcription through the nucleosome. Curr Opin Struct Biol. 2020;61:42-9.
25. Wittmeyer J, Formosa T. The Saccharomyces cerevisiae DNA polymerase alpha catalytic subunit interacts with Cdc68/Spt16 and with Pob3, a protein similar to an HMG1-like protein. Mol Cell Biol. 1997;17(7):4178-90.
26. Swanson MS, Malone EA, Winston F. SPT5, an essential gene important for normal transcription in Saccharomyces cerevisiae, encodes an acidic nuclear protein with a carboxy-terminal repeat. Mol Cell Biol. 1991;11(6):3009-19.
27. Fischbeck JA, Kraemer SM, Stargell LA. SPN1, a conserved gene identified by suppression of a postrecruitment-defective yeast TATA-binding protein mutant. Genetics. 2002;162(4):1605-16.
28. Clark-Adams CD, Winston F. The SPT6 gene is essential for growth and is required for delta-mediated transcription in Saccharomyces cerevisiae. Mol Cell Biol. 1987;7(2):679-86.
29. Ehara H, Kujirai T, Shirouzu M, Kurumizaka H, Sekine SI. Structural basis of nucleosome disassembly and reassembly by RNAPII elongation complex with FACT. Science. 2022;377(6611):eabp9466.
30. Li S, Almeida AR, Radebaugh CA, Zhang L, Chen X, Huang L, et al. The elongation factor Spn1 is a multi-functional chromatin binding protein. Nucleic Acids Res. 2018;46(5):2321-34.
31. Li S, Edwards G, Radebaugh CA, Luger K, Stargell LA. Spn1 and Its Dynamic Interactions with Spt6, Histones and Nucleosomes. J Mol Biol. 2022;434(13):167630.
32. McDonald SM, Close D, Xin H, Formosa T, Hill CP. Structure and biological importance of the Spn1-Spt6 interaction, and its regulatory role in nucleosome binding. Mol Cell. 2010;40(5):725-35.
33. Krogan NJ, Kim M, Ahn SH, Zhong G, Kobor MS, Cagney G, et al. RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach. Mol Cell Biol. 2002;22(20):6979-92.
34. Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature. 2002;415(6868):141-7.
35. Lindstrom DL, Squazzo SL, Muster N, Burckin TA, Wachter KC, Emigh CA, et al. Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. Mol Cell Biol. 2003;23(4):1368-78.
36. Reim NI, Chuang J, Jain D, Alver BH, Park PJ, Winston F. The conserved elongation factor Spn1 is required for normal transcription, histone modifications, and splicing in Saccharomyces cerevisiae. Nucleic Acids Res. 2020;48(18):10241-58.
37. Zhang L, Fletcher AG, Cheung V, Winston F, Stargell LA. Spn1 regulates the recruitment of Spt6 and the Swi/Snf complex during transcriptional activation by RNA polymerase II. Mol Cell Biol. 2008;28(4):1393-403.
38. Cermakova K, Demeulemeester J, Lux V, Nedomova M, Goldman SR, Smith EA, et al. A ubiquitous disordered protein interaction module orchestrates transcription elongation. Science. 2021;374(6571):1113-21.
39. Bortvin A, Winston F. Evidence that Spt6p controls chromatin structure by a direct interaction with histones. Science. 1996;272(5267):1473-6.

40. Adkins MW, Tyler JK. Transcriptional activators are dispensable for transcription in the absence of Spt6-mediated chromatin reassembly of promoter regions. Mol Cell. 2006;21(3):405-16.
41. Hainer SJ, Pruneski JA, Mitchell RD, Monteverde RM, Martens JA. Intergenic transcription causes repression by directing nucleosome assembly. Genes Dev. 2011;25(1):29-40.
42. Thebault P, Boutin G, Bhat W, Rufiange A, Martens J, Nourani A. Transcription regulation by the noncoding RNA SRG1 requires Spt2-dependent chromatin deposition in the wake of RNA polymerase II. Mol Cell Biol. 2011;31(6):1288-300.
43. Doris SM, Chuang J, Viktorovskaya O, Murawska M, Spatt D, Churchman LS, Winston F. Spt6 Is Required for the Fidelity of Promoter Selection. Mol Cell. 2018;72(4):687-99 e6.
44. Hondele M, Stuwe T, Hassler M, Halbach F, Bowman A, Zhang ET, et al. Structural basis of histone H2A-H2B recognition by the essential chaperone FACT. Nature. 2013;499(7456):111-4.
45. Kemble DJ, Whitby FG, Robinson H, McCullough LL, Formosa T, Hill CP. Structure of the Spt16 middle domain reveals functional features of the histone chaperone FACT. J Biol Chem. 2013;288(15):10188-94.
46. Jeronimo C, Poitras C, Robert F. Histone Recycling by FACT and Spt6 during Transcription Prevents the Scrambling of Histone Modifications. Cell Rep. 2019;28(5):1206-18 e8.
47. Jamai A, Puglisi A, Strubin M. Histone chaperone spt16 promotes redeposition of the original h3-h4 histones evicted by elongating RNA polymerase. Mol Cell. 2009;35(3):377-83.
48. Jeronimo C, Watanabe S, Kaplan CD, Peterson CL, Robert F. The Histone Chaperones FACT and Spt6 Restrict H2A.Z from Intragenic Locations. Mol Cell. 2015;58(6):1113-23.
49. Feng J, Gan H, Eaton ML, Zhou H, Li S, Belsky JA, et al. Noncoding Transcription Is a Driving Force for Nucleosome Instability in spt16 Mutant Cells. Mol Cell Biol. 2016;36(13):1856-67.
50. Biswas D, Dutta-Biswas R, Stillman DJ. Chd1 and yFACT act in opposition in regulating transcription. Mol Cell Biol. 2007;27(18):6279-87.
51. Ransom M, Williams SK, Dechassa ML, Das C, Linger J, Adkins M, et al. FACT and the proteasome promote promoter chromatin disassembly and transcriptional initiation. J Biol Chem. 2009;284(35):23461-71.
52. Evrin C, Serra-Cardona A, Duan S, Mukherjee PP, Zhang Z, Labib KPM. Spt5 histone binding activity preserves chromatin during transcription by RNA polymerase II. EMBO J. 2022;41(5):e109783.
53. Werner F, Grohmann D. Evolution of multisubunit RNA polymerases in the three domains of life. Nat Rev Microbiol. 2011;9(2):85-98.
54. Hartzog GA, Fu J. The Spt4-Spt5 complex: a multi-faceted regulator of transcription elongation. Biochim Biophys Acta. 2013;1829(1):105-15.
55. Crickard JB, Lee J, Lee TH, Reese JC. The elongation factor Spt4/5 regulates RNA polymerase II transcription through the nucleosome. Nucleic Acids Res. 2017;45(11):6362-74.
56. Uzun U, Brown T, Fischl H, Angel A, Mellor J. Spt4 facilitates the movement of RNA polymerase II through the +2 nucleosomal barrier. Cell Rep. 2021;36(13):109755.
57. Ehara H, Kujirai T, Fujino Y, Shirouzu M, Kurumizaka H, Sekine SI. Structural insight into nucleosome transcription by RNA polymerase II with elongation factors. Science. 2019;363(6428):744-7.

58. Viktorovskaya O, Chuang J, Jain D, Reim NI, Lopez-Rivera F, Murawska M, et al. Essential histone chaperones collaborate to regulate transcription and chromatin integrity. Genes Dev. 2021;35(9-10):698-712.
59. Sdano MA, Fulcher JM, Palani S, Chandrasekharan MB, Parnell TJ, Whitby FG, et al. A novel SH2 recognition mechanism recruits Spt6 to the doubly phosphorylated RNA polymerase II linker at sites of transcription. Elife. 2017;6.
60. Malone EA, Fassler JS, Winston F. Molecular and genetic characterization of SPT4, a gene important for transcription initiation in Saccharomyces cerevisiae. Mol Gen Genet. 1993;237(3):449-59.
61. Malone EA, Clark CD, Chiang A, Winston F. Mutations in SPT16/CDC68 suppress cis- and trans-acting mutations that affect promoter function in Saccharomyces cerevisiae. Mol Cell Biol. 1991;11(11):5710-7.
62. McCullough L, Poe B, Connell Z, Xin H, Formosa T. The FACT histone chaperone guides histone H4 into its nucleosomal conformation in Saccharomyces cerevisiae. Genetics. 2013;195(1):101-13.
63. Nonet M, Scafe C, Sexton J, Young R. Eucaryotic RNA polymerase conditional mutant that rapidly ceases mRNA synthesis. Mol Cell Biol. 1987;7(5):1602-11.
64. Klein DC, Hainer SJ. Genomic methods in profiling DNA accessibility and factor localization. Chromosome Res. 2020;28(1):69-85.
65. Chereji RV, Ocampo J, Clark DJ. MNase-Sensitive Complexes in Yeast: Nucleosomes and Non-histone Barriers. Mol Cell. 2017;65(3):565-77 e3.

CHAPTER 2: THE HISTONE CHAPERONE SPN1 PRESERVES SUBNUCLEOSOMAL STRUCTURES AT PROMOTERS AND NUCLEOSOME POSITIONING IN OPEN READING FRAMES

Summary

Spn1 is a multifunctional histone chaperone essential for life in eukaryotes. While previous work has elucidated regions of the protein important for its many interactions, it is unknown how these domains contribute to the maintenance of chromatin structure. Here, we employ digestion by micrococcal nuclease followed by single-stranded deep sequencing (MNase-SSP) to characterize chromatin structure in yeast expressing wild-type or mutants of Spn1 by mapping nucleosome and subnucleosomal protections genome-wide. Surprisingly, we observed a genome-wide loss of subnucleosomal protection over nucleosome-depleted regions (NDRs) in the Spn1-K192N-containing strain, indicating critical functions of Spn1 in maintaining normal chromatin architecture in promoter regions. Additionally, alterations in nucleosome and hexasome positioning were observed in markedly different mutant Spn1 strains, demonstrating that multiple functions of Spn1 are required to maintain proper chromatin in open reading frames. Moreover, these functions become increasingly important with greater gene expression and greater gene length. Taken together, our results reveal a previously unknown role of Spn1 in the maintenance of NDR architecture and deepen our understanding of Spn1-dependent chromatin maintenance over transcribed regions.

---

[1] Most of this chapter was previously submitted for publication and pre-printed under the following title with a few updates: Tonsager AJ, Zukowski A, Radebaugh CA, Weirich A, Stargell LA, Ramachandran S. The Histone Chaperone Spn1 Preserves Subnucleosomal Structures at Promoters and Nucleosome Positioning in Open Reading Frames. *bioRxiv*. 2024.

Introduction

Eukaryotes package their genomes by wrapping ~147bp of DNA in about 1.7 superhelical turns around an octamer of histone proteins to form nucleosomes[1-3]. This process occurs in a stepwise manner, as DNA initially wraps around a tetramer of histones H3–H4 to form a tetrasome, followed by sequential additions of two H2A-H2B dimers to first form a hexasome, then a nucleosome[4]. Tetrasome and hexasome subnucleosomal structures have been observed both *in vitro*[5] and *in vivo*[6] and represent stable intermediates in nucleosome assembly and disassembly. Nucleosomes regulate DNA access to many proteins that perform essential processes such as DNA replication[7, 8], repair[7, 9], recombination[10], and transcription. Nucleosomes regulate transcription in a variety of ways. Positioning of nucleosomes at promoters can hinder transcription initiation by preventing the binding of transcription factors[11, 12] while positioning of nucleosomes within coding regions can play an important role in preventing intragenic transcription initiation by reducing the accessibility of factors to intragenic initiation sites[13]. Nucleosomes are inhibitory to transcription elongation of protein coding genes, as they pose a significant barrier to RNA polymerase II (RNAPII) and reduce its transcription rate[14, 15]. As a result, chromatin structure must be dynamic to allow for selective gene expression. An important class of proteins responsible for modifying chromatin structure is histone chaperones, which bind histones and facilitate histone deposition, exchange, or removal from chromatin, independent of ATP[16].

Spn1 is a multifunctional and essential histone chaperone that is highly conserved across eukaryotes. *S. cerevisiae* Spn1 preferentially binds H3-H4 histones[17] via its N-terminal region, with high binding affinity (K_D ~ 10nM). Spn1 affinity to H3-H4 is at least 50-fold higher than that to H2A-H2B dimers[18]. Spn1 binds nucleosomes with its C-terminal region[17, 18]. Spn1 can deposit H3-H4 onto DNA and facilitate the assembly of H3-H4 tetrasomes *in vitro*[17]. Spn1 also has a well-characterized interaction with another essential histone chaperone Spt6; they bind each

other with high affinity (K_D = 10-100nM) to form a heterodimer[19-22]. Depletion of either protein through the auxin-inducible degron system results in a reduction in recruitment of the other to chromatin[23]. The central domain of Spn1 is a highly conserved eight helix bundle and is primarily responsible for binding Spt6[18].

Cells with endogenous Spn1 replaced with the truncated *spn1*$^{141-305}$ mutant are viable and grow comparably to cells with wild-type Spn1 when in rich media[17, 24]. However, *spn1*$^{141-305}$ is unable to directly bind DNA, nucleosomes, and histones, though it retains the ability to bind Spt6 and RNAPII[17, 18]. In contrast, yeast expressing *spn1*^{K192N} are temperature sensitive[24], and we have previously demonstrated that these cells have reduced Spn1 recruitment to the *CYC1* locus and fail to recruit Spt6 to *CYC1*[25]. The *spn1*^{K192N} mutant also has reduced Spn1 binding to RNA Polymerase II (RNAPII) by co-immunoprecipitation[25]. As recent work demonstrates that Iws1, the human ortholog of Spn1, serves as a hub that maintains association of numerous transcription elongation factors[26], it remains unclear which interactions are impaired that lead to loss of RNAPII recruitment.

While we and others have previously demonstrated that Spn1 possesses multiple interactions regarding chromatin functions, the role of Spn1 *in vivo* is not well-understood. Here, micrococcal nuclease digestion followed by single-stranded deep sequencing (MNase-SSP) is used to characterize chromatin structure in yeast expressing wild-type or mutant Spn1 alleles. MNase-SSP allows for analysis of nucleosome, subnucleosomal and transcription factor protections genome-wide. The results indicate that despite seemingly distinct functional defects, both *spn1*^{K192N} and *spn1*$^{141-305}$ expression manifest similar chromatin defects across genes; namely a progressive 3' downstream shift in nucleosomes and intermediates over gene bodies. However, expression of *spn1*$^{141-305}$, but not *spn1*^{K192N}, produces a unique molecular mutant phenotype of a shift in the +1 and +2 nucleosome positions. Mapping the midpoints of the sequenced fragments reveals that shifts in nucleosomes and subnucleosomes manifest as a downstream

redistribution of occupancy at alternative rotational positions previously observed in the yeast genome[27]. This molecular mutant phenotype of the downstream shift increases with gene expression and gene length. Intriguingly, MNase-SSP also revealed a molecular mutant phenotype specific to *spn1*^{K192N}: the loss of subnucleosomal protections over nucleosome-depleted regions (NDRs) in promoters. Taken together, these studies reveal the importance of both the central domain of Spn1 and chromatin-binding disordered regions of Spn1 in preserving chromatin structure across the yeast genome.

Materials and Methods

Yeast Culturing and Strains

All *Saccharomyces cerevisiae* strains were grown in yeast extract, peptone, dextrose (YPD; 2% dextrose) media at 30°C. A description of yeast strains and plasmids is provided in Table A.1. Strains used in this study were previously created as described[17, 25, 28]. In summary, strains were transformed with a covering plasmid expressing wild-type *SPN1* flanked by the *TOA1* promoter and terminator. Endogenous *SPN1* was replaced by a *LEU2* fragment flanked by the *SPN1* promoter and terminator through homologous recombination. Plasmids containing wild-type *SPN1*, *spn1*^{K192N}, or *spn1*$^{141\text{-}305}$ under the control of their native promoter and terminator were introduced into strains by plasmid shuffling.

Micrococcal nuclease digestion followed by deep single-strand sequencing (MNase-SSP)

Yeast cultures (3 ml) were grown to OD_{600} = 0.7-1.0 at 30°C, then used to inoculate yeast cultures (400 ml) grown to OD_{600} = 1.0 at 28°C. Cells are centrifuged at 5000 x rpm for 10 minutes at 4°C and the supernatant is decanted. The cell pellets are resuspended in 30 ml of sorbitol buffer (50mM Tris-Cl pH 7.5, 1M sorbitol, 10mM $MgCl_2$) containing 10mM β-

mercaptoethanol (βME) and 1mM phenylmethylsulfonyl fluoride (PMSF). The cell pellets are resuspended in sorbitol buffer with βME and PMSF and centrifuged at max rpm for 5 minutes at 4°C twice, and the cell pellets are weighed afterwards. Pellets are then suspended in 0.66ml sorbitol buffer with βME and PMSF per gram of cells. 20 µl of the cell suspension is set aside for monitoring cell wall digestion, and the remainder is incubated at 30°C with gentle agitation for 10 minutes.

Spheroplasting is performed by adding 0.34 ml of Zymolase (10mg/ml) per gram of cells to the sorbitol-cell suspension, followed by incubation at 30°C. Spheroplasting is monitored by determining the OD_{600} of 4 µl of cell suspension with or without Zymolase combined with 4 µl of 1% SDS and 992 µl of ddH_2O. When the OD_{600} of the cells with Zymolase is ~10% of the cells without Zymolase, sorbitol buffer with PMSF is slowly added until the final volume is 30 ml. Spheroplasts are collected by centrifugation at max rpm for 5 minutes, followed by decanting of the supernatant. The spheroplasts are washed in 5 ml of sorbitol buffer two additional times, then suspended in Ficoll buffer (18% Ficoll 400, 20mM Tris-Cl pH 7.5, 0.5mM $MgCl_2$). Each spheroplast suspension is transferred to a 50 ml Potter-Elvehjem homogenizer and homogenized on ice with six up-down even strokes with a handheld drill at max rpm. The suspensions are transferred to chilled 25 ml centrifuge tubes, and the remaining cells are pelleted at 6400 x rpm for 5 minutes at 4°C. The supernatant containing yeast nuclei is transferred to new centrifuge tubes and pelleted at 15,000 x rpm at 4°C. Nuclei are suspended in 10 ml of MNase digestion buffer (20mM Tris-Cl pH 8.0, 1.5mM $CaCl_2$, 150mM NaCl, and 1mM PMSF). The OD_{600} of the nuclei suspension is determined by a 1:10 dilution in digestion buffer, which should be ~0.2 for nuclei from 1 gram of cells. Nuclei are pelleted at 15,000 x rpm for 30 minutes at 4°C and suspended in digestion buffer at a ratio of 2.4 ml for OD_{600} = 0.2. 400 µl aliquots of the nuclei suspension are prepared for MNase digestion and incubated at 37°C for 10 minutes.

Nuclei are digested with a range of MNase concentrations (0U, 32U, 64U, 128U, and 256U) at 37°C for 10 minutes. 100 µl of stop solution (50mM EDTA, 2.5% SDS, 0.25mg/ml Proteinase K) is added to each reaction, then samples are incubated at 37°C overnight. Samples are extracted twice with phenol:chloroform, once with chloroform, and precipitated with ethanol. The DNA was washed with 70% ethanol, dried at room temperature, then suspended in 100 µL of TE buffer (pH 7.5) and 3 µL of RNaseA (10 mg/mL). Reactions were incubated overnight at 37°C, followed by ethanol precipitation, wash with 70% ethanol and drying at room temperature. The precipitated DNA is suspended in 100µl of TE buffer (10mM Tris-Cl, pH 8.0 and 1mM EDTA, pH 8.0) and 10µl is subjected to agarose gel electrophoresis.

Sequencing

The capture of MNase-protected DNA fragments prior to sequencing was performed as previously described[29, 30]. In brief, 10ng DNA was dephosphorylated using FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific cat. EF0651), denatured, and incubated overnight with CircLigaseII (Lucigen cat. CL9025K) and 0.093-0.125 µM biotinylated CL78 primer[29] at 60ºC with shaking every 5 minutes. Captured DNA fragments were denatured and then bound to magnetic streptavidin M-280 beads (Invitrogen cat. 11205D) for 30 minutes at room temperature with nutation. Beads were washed and second-strand synthesis was performed using Bst 2.0 DNA polymerase (NEB cat. M0537) with an increasing temperature gradient 15-31ºC with shaking at 1750 rpm. Beads were washed and a 3’ gap fill was performed using T4 DNA polymerase (Thermo Scientific cat. EL0011) for 30 minutes at room temperature. Beads were washed and a double-stranded adapter was ligated using T4 DNA ligase (Thermo Scientific cat. EP0062) for 2 hours at room temperature with shaking at 1750 rpm. Beads were washed and resuspended in 30 µL 10 mM TET buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.05% Tween-20). DNA was denatured at 95ºC for 3 min and DNA libraries were collected after immediate magnetic separation.

Quantitative real-time PCR was performed on DNA libraries using iTAQ Supermix (Bio-Rad cat. 1725124) and Ct values were used to determine the number of PCR cycles needed to amplify each library. PCR was performed with KAPA HiFi DNA polymerase (Kapa Biosystems cat. KK2502) using barcoded indexing primers for Illumina. Primer dimers were removed from the libraries using AMPure beads (Beckman Coulter cat. A63881). Libraries were eluted in 0.1X TE and concentrations were determined using Qubit. The length distribution of each library was assessed by the Agilent Bioanalyzer using the D1000 or HSD1000 cassette. Libraries were sequenced for 150 cycles in paired-end mode on NovaSeq 6000 system at University of Colorado Cancer Center Genomics Shared Resource.

MNase-SSP analysis

Sequencing reads were trimmed using Cutadapt[31]: Illumina adapter sequences were removed, and reads were trimmed to 140 bp. Reads less than 35 bp were discarded. Trimmed reads were aligned to the sacCer3 version of *Saccharomyces cerevisiae* genome using bowtie2[32]. Samtools[33] and bedtools[34] were used for processing aligned reads from sam to bed files. Coverage at 1 bp windows genome-wide for specific fragment lengths was calculated as the number of reads that mapped at that window, normalized by the factor N: N = 139712364/(Total number of mapped reads). 139712364 was a number chosen arbitrarily. Coverage of fragment centers for specific fragment lengths were also calculated similarly at 1 bp windows. V-plots were generated using custom scripts publicly available (https://github.com/srinivasramachandran/VPLOTS/). Nucleosome positions were obtained from nucleosome dyad mapping data[27]. Transcription start site positions were obtained from published TSS mapping data[35]. Genes were stratified by expression using published NET-seq data[36]. NET-seq normalized read counts were summed between TSS+100 and TSS+200. Reb1, Abf1, and Rap1 binding sites were obtained from published ChEC-seq data[37].

Results

MNase-SSP probes nucleosomal and subnucleosomal structures across the yeast genome

To study the role of both the central and disordered chromatin-binding domains of Spn1 on maintaining chromatin structure, wild-type Spn1, *spn1*^{K192N}, or *spn1*$^{141-305}$ was expressed in yeast with endogenous *SPN1* deleted. Micrococcal nuclease digestion followed by single-stranded deep sequencing (MNase-SSP) was performed on nuclei with increasing levels of MNase, which digests linker DNA at 25 times the rate of nucleosomal DNA and produces protected genomic fragments of varied sizes[38]. Samples were subjected to agarose gel electrophoresis to qualitatively assess the population of polynucleosomal, nucleosomal, and smaller fragment sizes after MNase digestion (Figure A.1A). Similar distributions of fragment lengths were observed for each sample (Figure A.1B), and libraries were constructed with fragments as small as 35 bp. Fragments of all lengths were sequenced and mapped to the *S. cerevisiae* genome. To map nucleosome and subnucleosome positioning in WT and Spn1 mutants, a metagene analysis was performed to generate plots of fragment midpoint vs. length ("V-plots")[11] centered at the dyad of each nucleosome downstream of the transcription start site from the +1 to +10 positions. Dyad definitions from chemical cleavage mapping were used to define nucleosome dyad positions spanning 4688 yeast genes[27] (Figure A.2). The density at position 0 and fragment length 147bp corresponds to the strongest dyad signal in chemical cleavage mapping. V-plots centered at +1, +2, and +5 exemplify that plots at each nucleosome position show a checkered pattern which appears similarly across both wild-type and mutant samples (Figure 2.1A). At nucleosomal size fragments (~150bp), this arises from peaks in density separated by a 10bp periodicity on either side of the dyad, which corresponds to nucleosome occupancy at previously mapped rotational positions surrounding yeast nucleosomes[27, 39]. Strikingly, the V-plots also reveal the existence of rotational positioning of

subnucleosomal structures, which is the first time this phenomenon has been observed *in vivo.* As expected, the V-plot analysis also demonstrates that nucleosome positions become fuzzier with each downstream nucleosome from the TSS.

While the V-plots provide an extensive picture of chromatin protections of varying sizes across the yeast genome, it is difficult to make comparisons between datasets from wild-type and mutant Spn1 expressing yeast. To analyze the change in the chromatin landscape in mutant Spn1 expressing yeast compared to wild-type yeast, we generated difference V-plots by subtracting mutant V-plots from wild-type (Figure A.3). The *spn1*^{K192N} mutation, but not *spn1*$^{141-305}$, produces a loss in short fragments 50-150 bp upstream of the +1 dyad, corresponding to a decrease in protection over nucleosome-depleted regions (NDR) (Figure 2.1B). In contrast, the expression of *spn1*$^{141-305}$ produces a pattern of fragment loss directly over the dyad with a corresponding increase in fragments immediately downstream. This shift in fragment distribution is consistent across fragment lengths 50 – 150bp, corresponding to shifts in both nucleosomal and subnucleosomal protections. The pattern of a decrease in fragment density over the dyad and a corresponding increase downstream is observed at subsequent downstream dyad positions in both *spn1*$^{141-305}$ and *spn1*^{K192N}. These results reveal that both the central and unstructured N- and C-terminal regions of Spn1 have overlapping roles in maintaining chromatin structure over genes, but with functional differences proximal to transcription start sites.

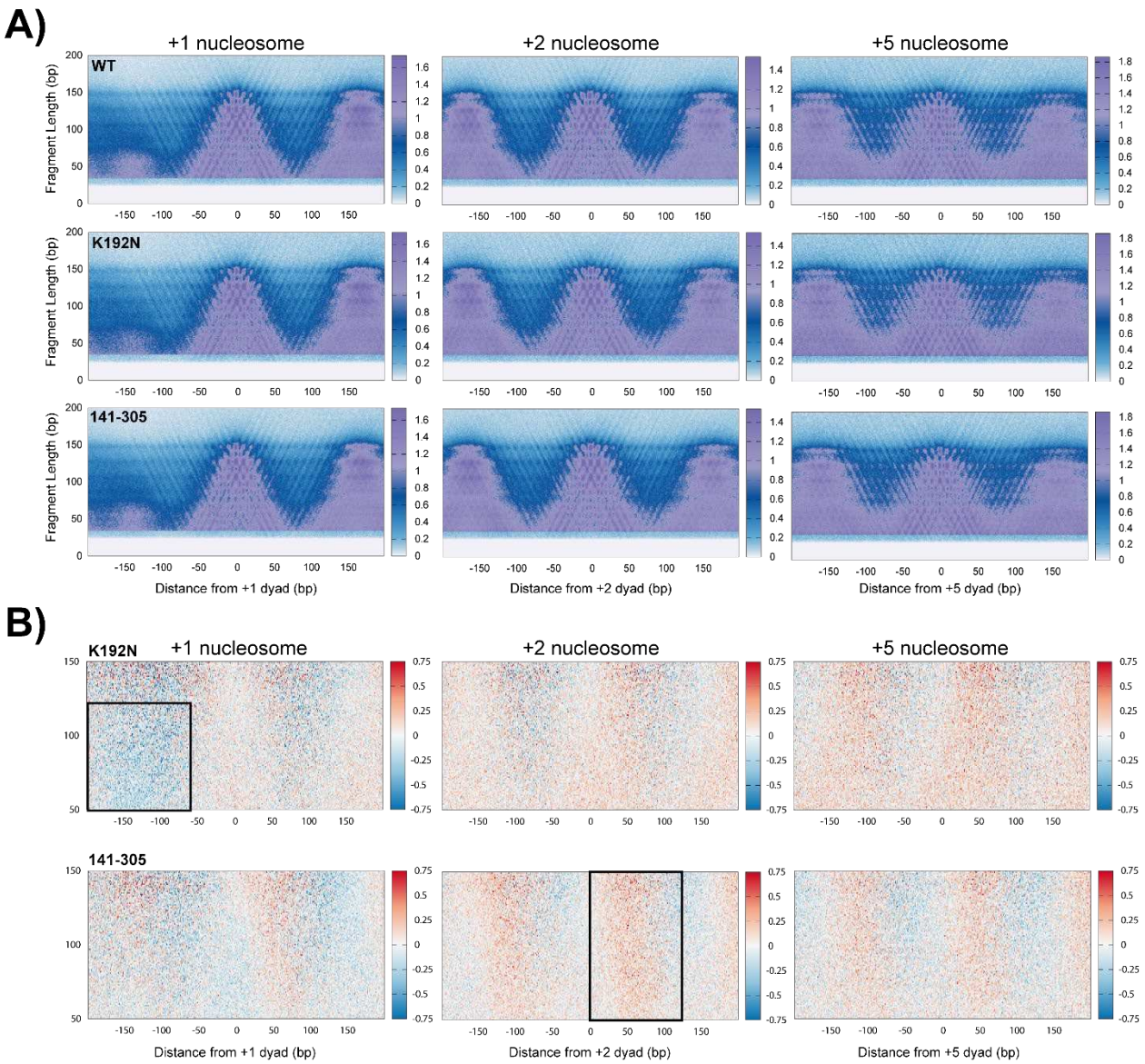


Figure 2.1. An extensive characterization of MNase produced fragments suggests shifts in chromatin protections in Spn1 mutant yeast across fragment lengths. (A) Nuclei harvested from two biological replicates of wild-type and mutant Spn1 expressing yeast grown to log phase were digested with 128U MNase and protected fragments were isolated and sequenced. V-plots were produced by mapping fragment midpoints ~30 - 180bp in length to the yeast genome anchored at +1, +2, and +5 nucleosome dyad positions from 4688 genes. (B) Difference V-plots produced by quantifying differences between wild-type and *spn1*^{K192N} (left hand panels) and *spn1*$^{141-305}$ (right hand panels) fragment midpoint occupancies in (A). Regions in red come from an increase in fragment density in the mutant Spn1 expressing yeast while regions in blue come from a decrease in fragment density in the mutant Spn1 expressing yeast. The box over the *spn1*^{K192N} difference V-plot highlights a reduction in protection over NDRs while the box over the *spn1*$^{141-305}$ difference plot highlights the shift in chromatin protections.

Spn1 is critical for accurate nucleosome positioning over genes, correlated positively with gene length and expression

The previous figure showed V-plots that were generated by averaging the change in mapped fragment occupancy over all genes. To further the analysis from average changes to those of individual genes, fragments were mapped and plotted on a heatmap (Figure A.4). Genes are aligned by their +1-nucleosome dyad position and ranked by length. To determine if wild-type and mutant strains possess different patterns, the wild-type occupancy scores were subtracted from the *spn1*^{K192N} and *spn1*$^{141-305}$ samples to generate a difference heatmap. Strikingly, this analysis reveals that loss of protection across NDRs in the *spn1*^{K192N} mutant is pervasive across the yeast genome (Figure 2.2A). Additionally, the difference heatmap reveals a striated pattern across the open reading frames of genes in both mutants, consistent with a downstream shift in nucleosome positioning. This result is consistent with the metagene analysis performed in the V-plots and additionally demonstrates that the defects observed in the V-plots occur consistently across genes. The fragment size bin used for the difference heatmap is sufficiently wide that it includes fragments protected by subnucleosomes[6].

To further investigate nucleosome positioning, an additional metagene analysis was performed by averaging the normalized read count over genes and plotting the result as a profile. The wild-type strain produces the expected profile of regularly phased and well-positioned nucleosomes (Figure 2.2B). This analysis reveals that expression of both *spn1*^{K192N} and *spn1*$^{141-305}$ mutant alleles produced a progressive 3’ downstream shift in nucleosome positioning, despite their extensive functional differences. To further examine differences between the mutant and wild-type nucleosome profiles, the difference in reads was quantified between wild-type and mutant samples and plotted alongside the wild-type nucleosome density (Figure 2.2C). This difference plot demonstrates that while nucleosome protections shift in both mutant strains, nucleosome positions +1 and +2 shift specifically in cells expressing the *spn1*$^{141-305}$ allele. This result reveals

that Spn1 maintains nucleosomes across genes, but the contributions of its functional domains differ in the maintenance of nucleosomes at the 5' ends of genes versus over the rest of gene bodies.

As Spn1 is part of the RNAPII elongation complex, defects in chromatin structure observed in Spn1 mutant expressing strains may be largest at the most highly transcribed genes, especially since Spn1 occupancy over coding genes is highly correlated to RNAPII occupancy (Rpb1) over those genes[23, 40, 41]. To test this, genes were first ranked by Rpb3 occupancy (a subunit of RNAPII) using published NET-seq data[36]. Across all quartiles, the number of genes with nucleosomes at each position was relatively consistent (Figure A.5A). Mapping the sequenced protected fragments 80-170bp in length over each set of genes demonstrated that the highest quartile of genes ranked by expression experience larger shifts compared to the average gene in both *spn1*^{K192N} and *spn1*$^{141-305}$ expressing yeast (Figure 2.2D, Figure A.5B). In contrast, genes in the lowest quartile of expression experienced little shift in nucleosome positioning on average.

The prior analyses revealed that expression of *spn1*$^{141-305}$, but not *spn1*^{K192N}, produces a shift in the +1 and +2 nucleosome positions. This reveals that maintenance of promoter proximal nucleosomes (+1 and +2), and promoter distal nucleosomes (+3 to +10), may be functionally distinct. Genes across the yeast genome vary in length, and this phenomenon could change depending on length. This prompted an investigation of gene length as a parameter for nucleosome shift. To begin examining this relationship, the gene list was ranked by length into quartiles of equal gene numbers (Figure A.6A). Mapping the sequenced protected fragments 80-170bp in length over each set of genes demonstrated that the furthest downstream nucleosomes present in the longest quartile of genes exhibit the largest shift in nucleosome position (Figure 2.2E, Figure A.6B). Strikingly, the set of shortest genes experienced, on average, no defects in nucleosome positioning, even at nucleosome positions that were shifted

in the longer genes. To verify these changes in chromatin protections are present specifically in nucleosomes and that the results are not driven exclusively by smaller subnucleosomal protections, the analysis was repeated with the 142-152bp fragments. We observed a similar positive correlation between the extent of nucleosome positioning shift and both gene expression and gene length (Figure A.7, A.8). Taken together, these results suggest that Spn1 maintenance of chromatin structure becomes increasingly critical as gene expression and gene length increases.

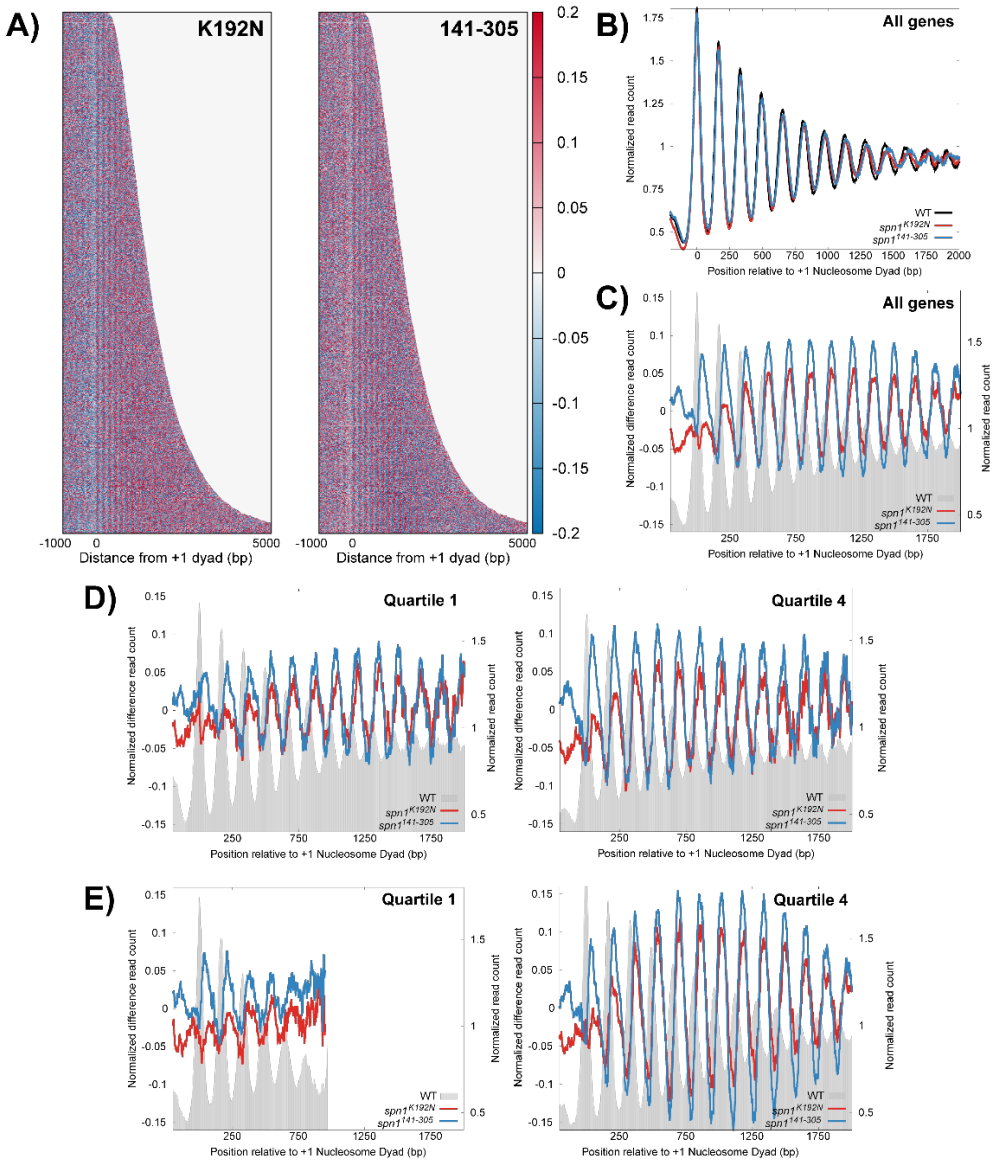


Figure 2.2. Mutations in Spn1 produce a downstream shift in nucleosome positioning over open reading frames genome wide. (A) Difference heatmap between wild-type and mutant Spn1 expressing yeast strains scored by changes in nucleosome occupancy. Genes are aligned by their +1 nucleosome dyad position and ranked by length. Scores are calculated by subtracting wild-type nucleosome occupancy data from mutant nucleosome occupancy. Regions in red are enriched for nucleosomes at that position while blue regions are depleted for nucleosomes. (B) Profiles of MNase protected fragments (80-170bp) mapped over 4688 genes aligned to the +1 nucleosome dyad in wild-type, *spn1*^{K192N}, and *spn1*$^{141-305}$ expressing yeast. (C) Difference profile plot between wild-type and mutant samples. Average nucleosome density from wild-type expressing yeast is plotted in grey. Differences between wild-type and mutants (*spn1*^{K192N}, red and *spn1*$^{141-305}$, blue) against wild-type replicates are plotted. (D) Difference profile plots between wild-type and mutant samples over 4688 genes binned into quartiles by published NET-seq data[36], with Q1 representing the lowest levels of Rpb3 (left panel) and Q4 representing the highest (right panel). (E) Difference profile plots between wild-type and mutant samples over 4688 genes binned into quartiles by length, with Q1 representing the shortest genes (left panel), and Q4 representing the longest genes (right panel).

The *spn1*^{K192N} mutation reduces stability over nucleosome-depleted regions

The MNase-SSP results demonstrate that expression of either *spn1*^{K192N} or *spn1*$^{141-305}$ generates a downstream shift in chromatin protections over yeast open reading frames. In contrast, a loss in MNase-protected fragments in the nucleosome-depleted regions (NDRs) was observed specifically in the *spn1*^{K192N} mutant yeast strain (Figure 2.1A, 2.1B). NDRs are flanked by well-positioned -1 and +1 nucleosomes, which reside directly upstream and downstream of this region. Chromatin remodelers, such as the RSC complex, actively maintain NDRs after nucleosome deposition[42]. This region is bound by many other factors, such as activators and enhancers that bind and compete with nucleosome formation within NDRs, contributing to the low nucleosome density in these regions[43]. Nucleosome depleted regions upstream of transcription start sites contain promoter sequences bound by transcription initiation factors. Overall, NDRs are protected by a variety of factors, many of which have smaller footprints (<60bp). To explore the finding that *spn1*^{K192N} cells have reduced protection over NDRs, the bin containing short (<60bp) fragments was mapped to the yeast genome, revealing a dramatic loss in fragments in *spn1*^{K192N} over NDRs (Figure 2.3A). There are a variety of protein-DNA interactions at NDRs that protect small segments of DNA from MNase. These include TATA-binding protein (TBP), pre-initiation complex (PIC) members, remodeling complexes, and general regulatory factors (GRFs) like Reb1, Rap1, and Abf1. Loss of any of these interactions with chromatin could explain the decrease in MNase-protected fragments at NDRs. One possibility explored was if the loss of protection over NDRs is due to a reduction in GRF binding. To begin investigating this possibility, short, protected fragments were centered over Reb1 binding sites from published ChEC-seq data[37]. The level of MNase-protected fragments did decrease over the Reb1 binding sites in *spn1*^{K192N} (Figure 2.3B), and similar results were observed for Rap1 and Abf1 (Figure A.9A). As these binding sites highly correlate with NDRs[39],

these results support that *spn1*^{K192N} reduces very short protections in the NDR, which may include GRFs or other factors.

Nucleosome-depleted regions are maintained and protected by a wide range of proteins, including those sensitive to MNase, such as fragile nucleosomes[11, 12, 38]. One possibility for the change in NDR protection in *spn1*^{K192N} cells is an increased sensitivity to MNase, which would produce a decrease in protection. To investigate this possibility, short fragments from samples digested with a low amount of MNase were mapped to the yeast genome, revealing that the NDR is largely unaffected in these samples (Figure 2.3C). This demonstrates that *spn1*^{K192N} cells exhibit a defect in nucleosome-depleted regions which render them significantly less stable than in wild-type cells. To further investigate this phenomenon, the analysis over GRF binding sites was repeated, revealing the footprint for Reb1, Rap1, and Abf1 binding was unchanged in *spn1*^{K192N} cells (Figure 2.3D, Figure A.9B). This indicates that while GRF binding may not be changing in the NDR, the stability of this binding, or more generally the overall stability of the NDR, is compromised in *spn1*^{K192N} cells.

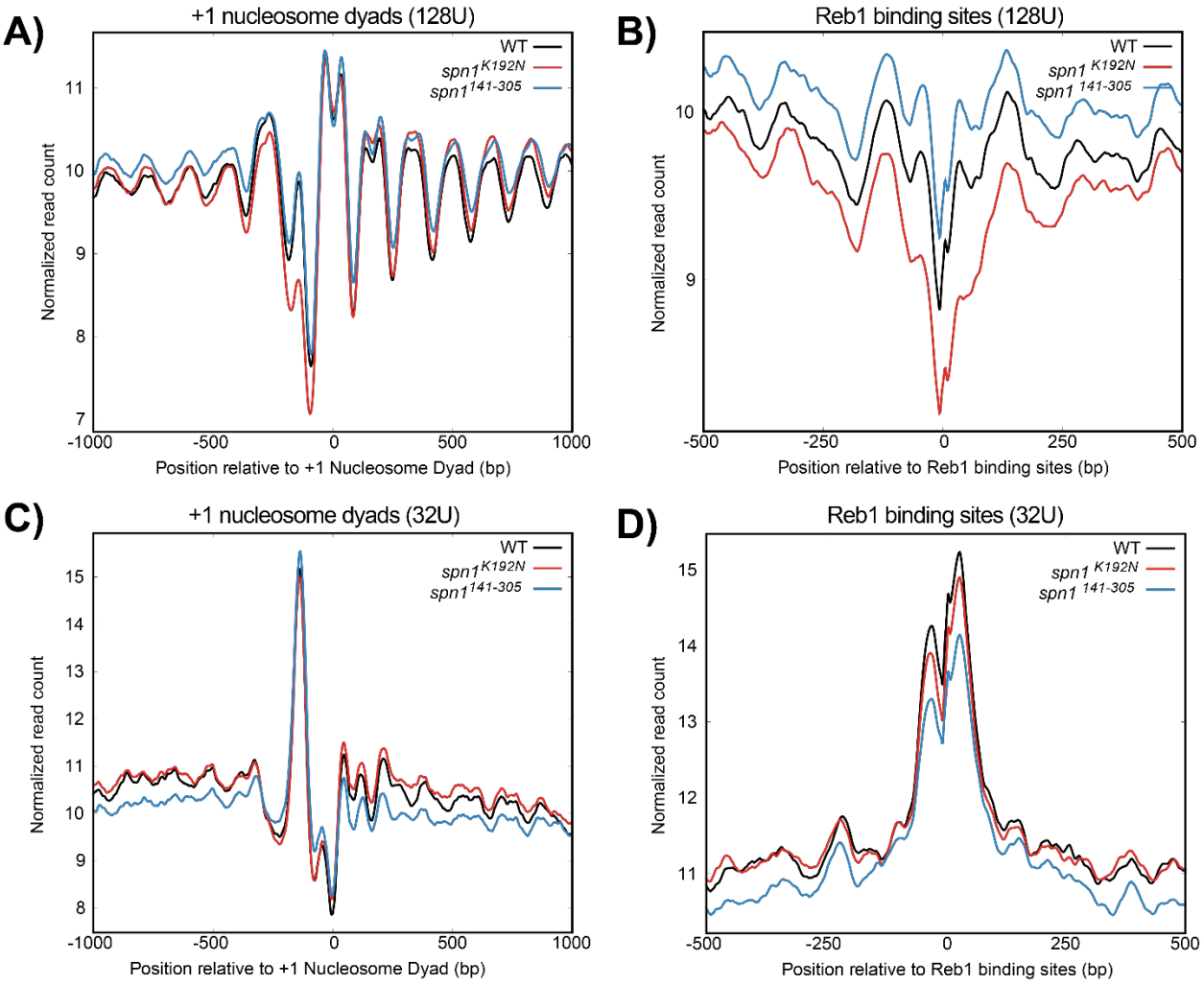


Figure 2.3. The *spn1*^{K192N} mutation reduces the stability of nucleosome-depleted regions. (A) Profiles of short MNase protected fragments (0-60bp) mapped over 4688 genes aligned to the +1 nucleosome dyad in wild-type, *spn1*^{K192N}, and *spn1*$^{141-305}$ expressing yeast. (B) Profiles of short MNase protected fragments (0-60bp) mapped over Reb1 binding sites from existing ChEC-seq data[37]. (C) Profiles of short MNase protected fragments (0-60bp) from low MNase-digested samples (32U) mapped over 4688 genes aligned to the +1 nucleosome dyad in wild-type, *spn1*^{K192N}, and *spn1*$^{141-305}$ expressing yeast. (D) Profiles of short MNase protected fragments (0-60bp) from low MNase-digested samples (32U) mapped over Reb1 binding sites from existing ChEC-seq data[37].

Quantification of nucleosome positioning shifts reveals that nucleosomal rotational position preference is redistributed in Spn1-mutant cells

This analysis of MNase-SSP data from wild-type and mutant Spn1 expressing yeast thus far has revealed global changes in nucleosome positioning across open reading frames genome-wide. An important characteristic of the data featured previously in the V-plots is the ability to capture alternate rotational positions occupied by nucleosomes around their mapped dyad locations (Figure 2.1). As these positions correspond to DNA sequences with favorable histone-DNA interactions, it seemed likely that nucleosome positioning shifts would occur in discrete steps at alternative, favorable DNA sequences in the mutants. To test this, the changes in density of 142 – 152 bp fragment midpoints were mapped relative to each nucleosome position downstream of the TSS individually. The results of this analysis are consistent with the prior results that shift in nucleosome positioning occur most dramatically at downstream nucleosome positions as a function of gene expression and gene length (Figure 2.4A, 2.4B, Figure A.10, Figure A.11). Notably, the analysis also reveals that shifted nucleosomes occupy the same rotational positions as wild-type cells, but with higher occupancy at rotational positions downstream of the dyad with a loss of occupancy at positions upstream of the dyad location.

As the shift in nucleosomes manifests as a change in nucleosome occupancy at discrete nucleosome positions, nucleosome positioning shift can be quantified by calculating the change in occupancy at each rotational position at each nucleosome. To accomplish this, the Z-scores across -5 to +5 rotational positions were calculated for each mapped nucleosome dyad, for each gene. A positive Z-score denotes a preferred rotational position at a nucleosome position. The Z-scores of wild-type Spn1 were then subtracted from Spn1 mutants to obtain changes in rotation position-preference due to Spn1 mutations. The median difference was plotted across all genes at each rotational position of each nucleosome position as a dyad. The previous analysis indicates that the shifts in nucleosome positioning were correlated with gene

expression and gene length. To evaluate the extent to which these correlations are significant, Z-scores were calculated and p-values (using the Wilcoxon Rank Sum test) for the changes in fragment midpoint occupancy at each rotational position around each nucleosome dyad for the genes in our previously determined quartiles of expression and length. This revealed that the number of rotational positions with significant changes in nucleosome occupancy increases with gene expression and length for both Spn1 mutant-expressing cells (Figure 2.4C, Figure 2.4D). Additionally, while both *spn1*^{K192N} and *spn1*$^{141\text{-}305}$ mutations shift nucleosome positions, *spn1*$^{141\text{-}305}$ mutant cells have more rotational positions with significant changes in nucleosome occupancy, including in the +1 and +2 nucleosome positions. Taken together, these results support the prior finding that both the central and disordered regions of Spn1 preserve nucleosome positioning over genes, and this function becomes increasingly critical with gene expression and gene length. Additionally, the disordered regions of Spn1 specifically play a role in the proper maintenance of nucleosomes at the 5' end of open reading frames.

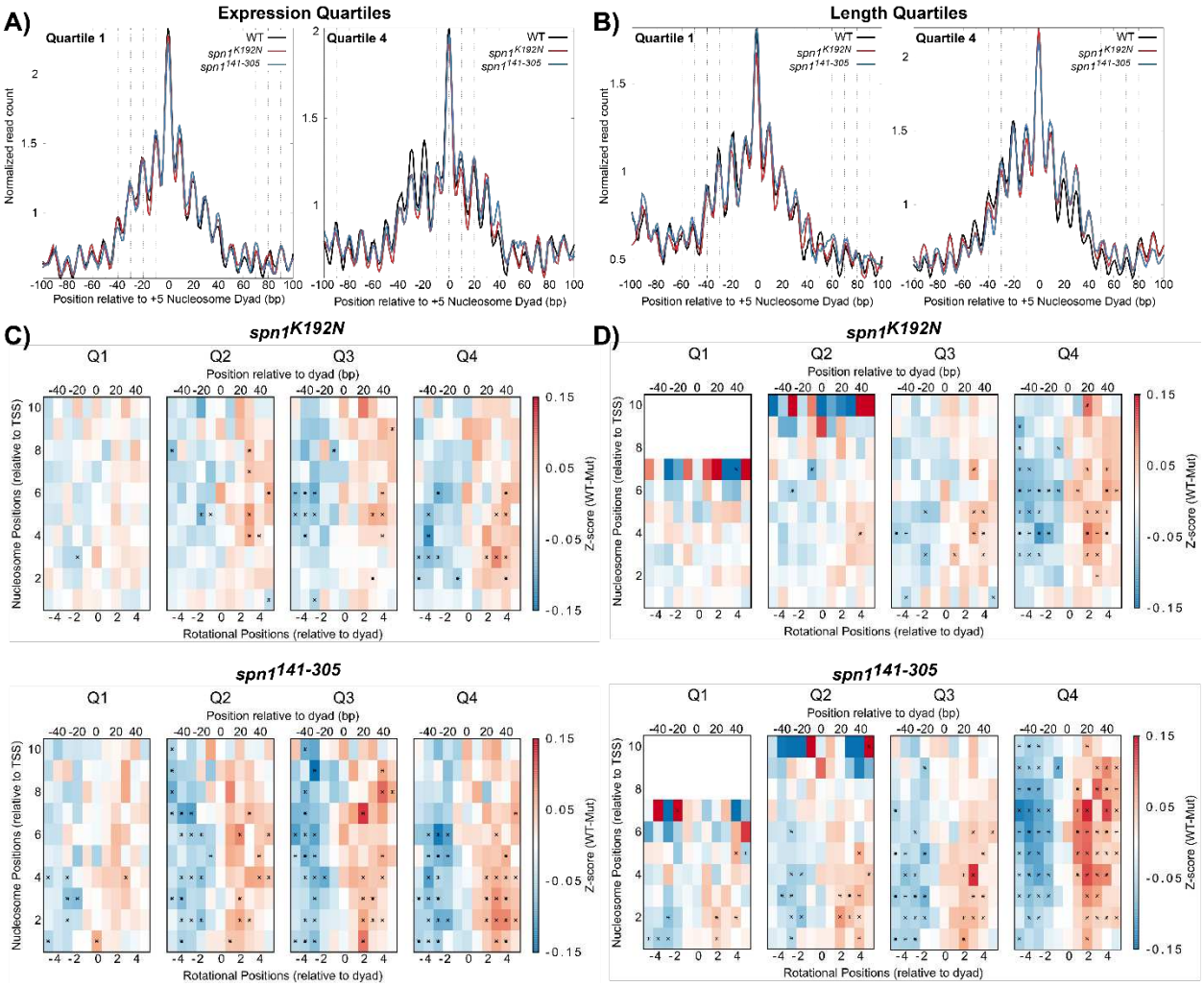


Figure 2.4. Quantification of nucleosome positioning shifts in Spn1-mutant cells reveals that nucleosomes are redistributed at rotational positions. (A) Metagene plots of fragment midpoints 142 - 152bp in length mapped to the +5 nucleosome dyad at gene quartiles ranked by expression. (B) Metagene plots of fragment midpoints 142 - 152bp in length mapped to the +5 nucleosome dyad at gene quartiles ranked by length. (C) Difference heatmaps between wild-type and mutant Spn1 expressing yeast strains scored by changes in occupancy at rotational positions surrounding each nucleosome dyad position at genes ranked by published NET-seq data[36]. (D) Difference heatmaps between wild-type and mutant Spn1 expressing yeast strains scored by changes in occupancy at rotational positions surrounding each nucleosome dyad position at genes ranked by length.

Subnucleosomal intermediates are mispositioned in Spn1 mutants

An advantage to the MNase-SSP approach is the retention of small fragments in MNase-digested samples (Figure A.1B), which can be mapped to the genome to determine the occupancy and positioning of other proteins[6, 11]. For instance, the abundance of protected fragments 102 – 112 bp in size can be interpreted as DNA bound by hexasomes[6]. As hexasomes are a subnucleosomal intermediate produced during transcription, Spn1 mutation may also generate mispositioned hexasomes. To test this hypothesis, the midpoints of protected fragments 102 – 112 bp in size were mapped over the top and bottom quartiles of genes ranked by expression or length. As was observed in the V-plots, plotting the midpoints of these fragments demonstrates the existence of alternative hexasome rotational positions around mapped nucleosome dyad positions with 10bp periodicity on either side of the dyad (Figure 2.5A, Figure 2.5B, Figure A.12, Figure A.13). In contrast to the analysis with nucleosome-protected fragments, the relative occupancy of hexasome protection is distributed more equally across the rotational positions upstream and downstream of the dyad position, most probably because loss of contacts to an H2A-H2B dimer shifts the location of the midpoint on the DNA protected by a hexasome[6]. As transcription through nucleosomes generates hexasomes with transient loss of contacts to both proximal and distal H2A-H2B dimers, the protected fragments have midpoints shifted on either side of the dyad that result in a wider distribution of hexasome-occupied rotational positions. Length- and expression-dependent downstream shift in rotational positioning preference of hexasomal protections was observed in Spn1 mutants compared to wild-type (Figure 2.5A, Figure 2.5B, Figure A.12, Figure A.13).

To quantify and statistically validate the shifts observed in *spn1*^{K192N} and *spn1*$^{141\text{-}305}$ cells, Z-scores and p-values were calculated at rotational positions around each mapped dyad location as described before with gene lists ranked by expression and length (Figure 2.5C, Figure 2.5D). This demonstrated that mutation of Spn1 produces a similar trend to the one found with

nucleosome positioning, where hexasomes are redistributed from upstream rotational positions to downstream positions in both *spn1*^{K192N} and *spn1*$^{141\text{-}305}$ cells. As seen before, more positions had significant changes in occupancy in *spn1*$^{141\text{-}305}$ cells than *spn1*^{K192N}, including shifts at the +1 and +2 dyad locations in exclusively *spn1*$^{141\text{-}305}$ cells, with more positions experiencing significant changes in hexasome occupancy at higher expressed and longer genes (Figure 2.5C, Figure 2.5D).

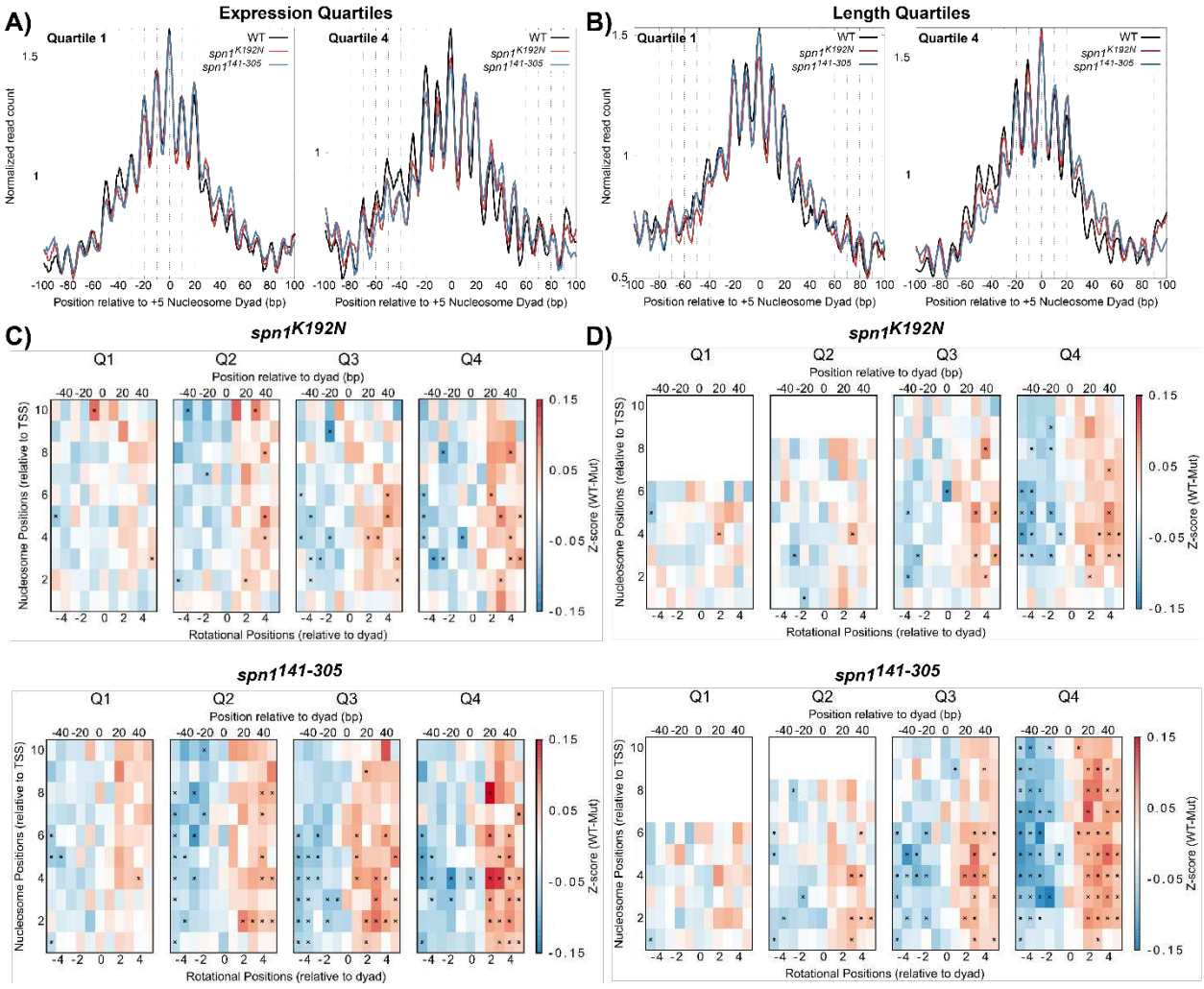


Figure 2.5. Mutations in Spn1 produce a downstream shift in hexasome positioning over open reading frames genome wide. (A) Metagene plots of fragment midpoints 102 - 112bp in length mapped to the +5 nucleosome dyad at gene quartiles ranked by expression. (B) Metagene plots of fragment midpoints 102 - 112bp in length mapped to the +5 nucleosome dyad at gene quartiles ranked by length. (C) Difference heatmaps between wild-type and mutant Spn1 expressing yeast strains scored by changes in occupancy at rotational positions surrounding each nucleosome dyad position at genes ranked by published NET-seq data[36]. (D) Difference heatmaps between wild-type and mutant Spn1 expressing yeast strains scored by changes in occupancy at rotational positions surrounding each nucleosome dyad position at genes ranked by length.

Loss of both H2A-H2B dimers produces tetrasomes, and their genome-wide occupancy and positioning can be interpreted by mapping protected fragments 61 – 71bp in length over the yeast genome[6]. For 61-71 bp fragments, rotational positions were even more equally distributed compared to hexasomes due to the larger shifts in their midpoints compared to nucleosomal fragments (Figure 2.6A, Figure 2.6B, Figure A.14, Figure A.15). Tetrasomal protections in *spn1*^{K192N} have minimal changes compared to wild-type, even at highly expressed genes or longer genes. However, tetrasomal protections in *spn1*$^{141\text{-}305}$ show significant shifts to downstream rotational positions like hexasomes as a function of length and expression (Figure 2.6C, Figure 2.6D). In summary, *spn1*$^{141\text{-}305}$ but not *spn1*^{K192N} results in significant shifts in tetrasomal rotational position preferences as a function of length and expression.

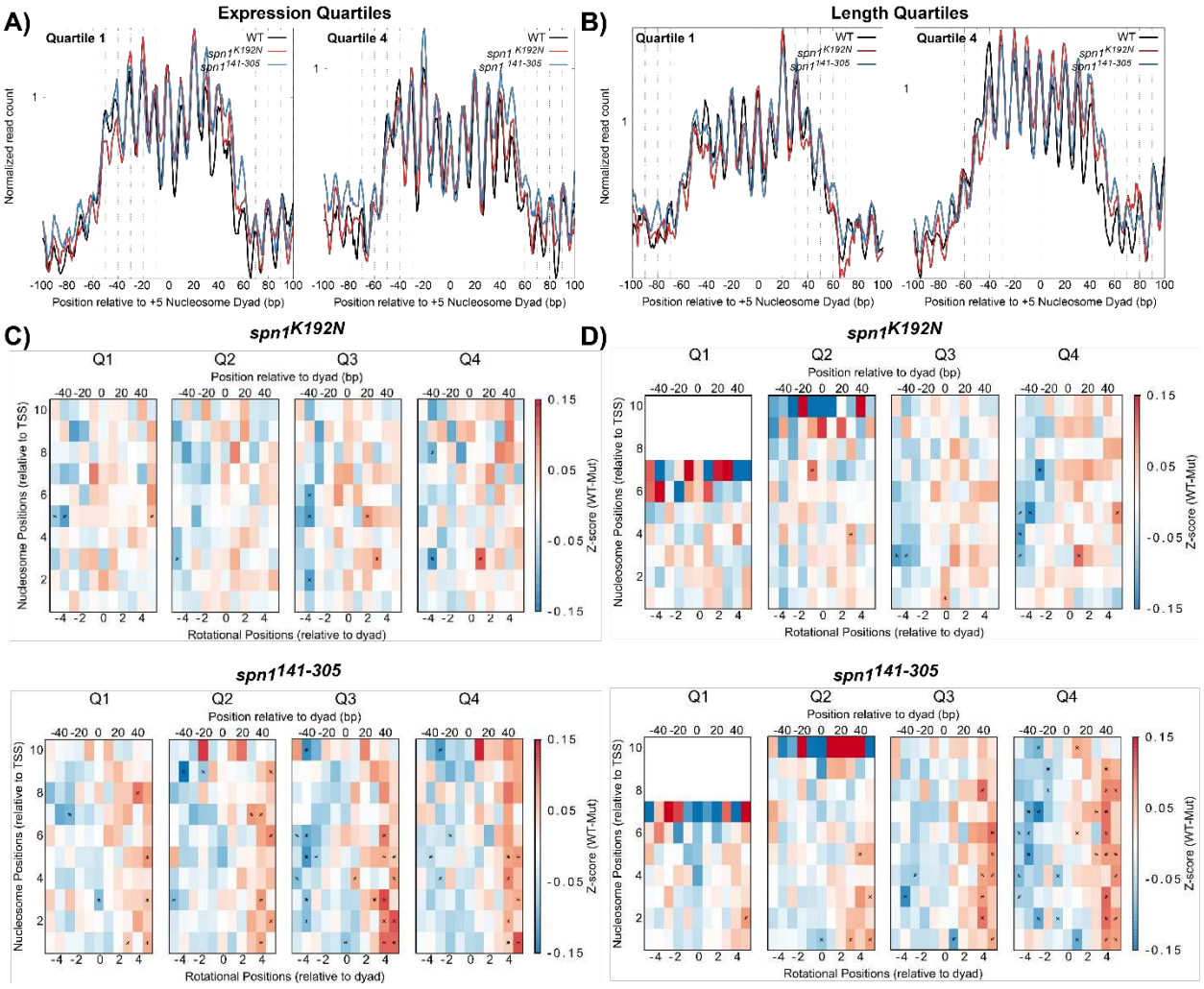


Figure 2.6. Mutations in Spn1 produce slight perturbations in tetrasome positioning over open reading frames genome wide. (A) Metagene plots of fragment midpoints 61 - 71bp in length mapped to the +5 nucleosome dyad at gene quartiles ranked by expression. (B) Metagene plots of fragment midpoints 61 - 71bp in length mapped to the +5 nucleosome dyad at gene quartiles ranked by length. (C) Difference heatmaps between wild-type and mutant Spn1 expressing yeast strains scored by changes in occupancy at rotational positions surrounding each nucleosome dyad position at genes ranked by published NET-seq data[36]. (D) Difference heatmaps between wild-type and mutant Spn1 expressing yeast strains scored by changes in occupancy at rotational positions surrounding each nucleosome dyad position at genes ranked by length.

Discussion

In this study, an extensive mapping of chromatin protections in yeast expressing functionally distinct mutant alleles of Spn1 was performed to assess the role of this essential histone chaperone in maintaining chromatin structure. By retaining fragments of a wide range of sizes, the MNase-SSP approach demonstrates that Spn1 preserves both nucleosomal and subnucleosomal protections across the yeast genome. The analysis revealed that this function of Spn1 is critical for maintaining chromatin over NDRs, the 5' ends of genes, and the rest of the gene bodies, through distinct mechanisms.

The findings from this work revealed a previously unknown role of Spn1 in the maintenance of NDR architecture. While defects in chromatin in Spn1 mutants have been primarily characterized over gene bodies during transcription elongation, the *spn1*^{K192N} mutation clearly reduces subnucleosomal structures in the NDR. The mechanism by which this mutation leads to this defect remains unclear. The *spn1*^{K192N} mutant is defective for recruitment to the RNAPII elongation complex, likely in part through a disruption of interaction with Spt5 and Spt6[25, 44]. Much of the study of Spn1 and its interactions is primarily focused on its role as an elongation factor over gene bodies. For example, previous work with other mutants that disrupt specifically the Spn1-Spt6 complex have implicated the complex in surveilling nucleosome assembly over genes[40, 45] and weakening this interaction decreases the quality of chromatin. Additionally, compromise of Spt6 function has been shown to produce aberrations in chromatin over gene bodies, namely the misincorporation of histone variant H2A.Z[46]. Taken together, these findings all support the notion that surveillance of proper chromatin structure by the Spn1-Spt6 complex extends to the NDRs.

While both *spn1*^{K192N} and *spn1*$^{141\text{-}305}$ produced a downstream shift in nucleosome positioning over gene bodies, *spn1*$^{141\text{-}305}$ exclusively exhibited defects in the positioning of the +1 and +2

nucleosomes. This finding reveals that the mechanism of chromatin maintenance at the 5' ends of genes is functionally distinct from that over the rest of the gene and that the histone- and nucleosome-binding functions of Spn1 are part of this mechanism. As *spn1*^{K192N} did not produce significant shifts in nucleosome or subnucleosome positioning at the 5' ends of genes, one possible interpretation is that the mechanism of maintenance of this portion of genes requires Spn1 to directly bind chromatin, whose binding regions are unaffected in *spn1*^{K192N}. In contrast, maintenance of chromatin over the rest of the body of the gene requires both Spn1 recruitment to the RNAPII complex and its histone- and nucleosome-binding capabilities, as evidenced by defects in nucleosome and subnucleosome positioning at nucleosomes distal from the promoter in both mutants.

The defects in nucleosome positioning observed in Spn1 mutants is reminiscent of work performed by others demonstrating that proper function of Spn1, Spt6, and Spt4 are required for maintenance of chromatin structure[40, 47]. Each of these factors are physically associated with RNAPII during transcription elongation, with Spn1 and Spt4 positioned at the tip of the RNAPII clamp facing the downstream DNA[44]. In contrast, while the Spt6 N-terminal helices that bind Spn1 are also proximal to the downstream DNA, the main body of Spt6 is docked near the upstream DNA exit channel[44]. Spt6 binds histones and nucleosomes if the HMG-box like protein Nhp6 is present[45]. Spt6 is commonly attributed as a histone chaperone necessary for nucleosome reassembly in the wake of RNAPII, which is supported by its proximity to upstream DNA. While Spt4 has not been shown to bind chromatin, it oscillates on and off RNAPII along with its binding partner Spt5 based on nucleosome positions and its deletion produces increased stalling and backtracking during early transcription elongation[47]. Despite the differences in their transcription-associated functions, each of these factors are critical for maintenance of proper chromatin structure and strikingly, defects in each produced a similar pattern of aberrant nucleosome positioning. One explanation for this commonality is that all

participate in this process of co-transcriptional nucleosome disassembly and reassembly, and a defect in any step of this process might produce a similar defect in chromatin. Others have posed that this defect in passage through nucleosomes may produce an elongation complex that transcribes through chromatin more slowly than in wild-type cells, which is what produces mispositioned nucleosomes[47]. Indeed, there is evidence that loss of RNAPII transcription shifts nucleosome positioning, as both anchoring away of Rpb1[48] and growth of the temperature sensitive *rpb1-1* allele at a non-permissive temperature[49] produces a downstream nucleosome shift.

An important feature of this work came from mapping the midpoints of MNase-protected fragments to the yeast genome, where alternative rotational positions were observed around nucleosome dyad positions. This property of our dataset was utilized to demonstrate that shifts in nucleosome positioning in Spn1 mutants occur in discrete steps at these alternate favored rotational positions. By quantifying the occupancy of nucleosomes at each rotational position, this work demonstrated an alternative method for quantifying and statistically validating nucleosome positioning shift. This approach in quantifying shift in nucleosome positions may prove to be useful for other datasets of sufficiently high sequencing depth in organisms with small genomes like *S. cerevisiae*. The mapping of smaller bins of fragments protected by subnucleosomal intermediates also demonstrated the first *in vivo* evidence of alternate rotational positions occupied by hexasomes and subnucleosomes. These positions also occurred in discrete steps of 10bp on either side of the dyad and hexasome and tetrasome occupancy were distributed more evenly across the positions surrounding the mapped nucleosome dyad position.

A puzzling question that remains is how $spn1^{K192N}$ and $spn1^{141\text{-}305}$ produce such similar changes in nucleosome and hexasome positioning over nucleosomes distal to promoters despite their vast functional differences. Both Spn1 recruitment to the elongating RNAPII complex as well as

its ability to bind histones and nucleosomes is critical for preserving chromatin structure, likely co-transcriptionally. Wild-type Spn1 is poised near the downstream nucleosome in the RNAPII elongation complex[44]. Spn1 can directly bind histones and nucleosomes, a function that may be important for disassembly of downstream nucleosomes during transcription. If recruitment of Spn1 to sites of transcription is diminished, the process of disassembling the downstream nucleosome may be impacted. Conversely, the *spn1*$^{141\text{-}305}$ mutant is likely still recruited to sites of transcription. However, its loss of histone- and nucleosome-binding regions renders it unable to participate in the process of downstream nucleosome disassembly. In either mutant, the loss of Spn1 function results in defects in chromatin preservation during transcription elongation, resulting in a shift in nucleosome and hexasome positioning. Taken together, these findings deepen our understanding of Spn1-dependent chromatin maintenance over the yeast genome and provide insight into mechanisms underlying this process.

REFERENCES

1. Davey CA, Sargent DF, Luger K, Maeder AW, Richmond TJ. Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. J Mol Biol. 2002;319(5):1097-113.
2. Flaus A, Luger K, Tan S, Richmond TJ. Mapping nucleosome position at single base-pair resolution by using site-directed hydroxyl radicals. Proc Natl Acad Sci U S A. 1996;93(4):1370-5.
3. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature. 1997;389(6648):251-60.
4. Polo SE, Almouzni G. Chromatin assembly: a basic recipe with various flavours. Curr Opin Genet Dev. 2006;16(2):104-11.
5. Brower-Toland BD, Smith CL, Yeh RC, Lis JT, Peterson CL, Wang MD. Mechanical disruption of individual nucleosomes reveals a reversible multistage release of DNA. Proc Natl Acad Sci U S A. 2002;99(4):1960-5.
6. Ramachandran S, Ahmad K, Henikoff S. Transcription and Remodeling Produce Asymmetrically Unwrapped Nucleosomal Intermediates. Mol Cell. 2017;68(6):1038-53 e4.
7. Groth A, Rocha W, Verreault A, Almouzni G. Chromatin challenges during DNA replication and repair. Cell. 2007;128(4):721-33.
8. Eaton ML, Galani K, Kang S, Bell SP, MacAlpine DM. Conserved nucleosome positioning defines replication origins. Genes Dev. 2010;24(8):748-53.
9. Adkins NL, Niu H, Sung P, Peterson CL. Nucleosome dynamics regulates DNA processing. Nat Struct Mol Biol. 2013;20(7):836-42.
10. Bevington S, Boyes J. Transcription-coupled eviction of histones H2A/H2B governs V(D)J recombination. EMBO J. 2013;32(10):1381-92.
11. Henikoff JG, Belsky JA, Krassovsky K, MacAlpine DM, Henikoff S. Epigenome characterization at single base-pair resolution. Proc Natl Acad Sci U S A. 2011;108(45):18318-23.
12. Kubik S, Bruzzone MJ, Jacquet P, Falcone JL, Rougemont J, Shore D. Nucleosome Stability Distinguishes Two Different Promoter Types at All Protein-Coding Genes in Yeast. Mol Cell. 2015;60(3):422-34.
13. Kaplan CD, Laprade L, Winston F. Transcription elongation factors repress transcription initiation from cryptic sites. Science. 2003;301(5636):1096-9.
14. Selth LA, Sigurdsson S, Svejstrup JQ. Transcript Elongation by RNA Polymerase II. Annu Rev Biochem. 2010;79:271-93.
15. Brahma S, Henikoff S. Epigenome Regulation by Dynamic Nucleosome Unwrapping. Trends Biochem Sci. 2020;45(1):13-26.
16. Bai L, Morozov AV. Gene regulation by nucleosome positioning. Trends Genet. 2010;26(11):476-83.
17. Li S, Almeida AR, Radebaugh CA, Zhang L, Chen X, Huang L, et al. The elongation factor Spn1 is a multi-functional chromatin binding protein. Nucleic Acids Res. 2018;46(5):2321-34.
18. Li S, Edwards G, Radebaugh CA, Luger K, Stargell LA. Spn1 and Its Dynamic Interactions with Spt6, Histones and Nucleosomes. J Mol Biol. 2022;434(13):167630.
19. McDonald SM, Close D, Xin H, Formosa T, Hill CP. Structure and biological importance of the Spn1-Spt6 interaction, and its regulatory role in nucleosome binding. Mol Cell. 2010;40(5):725-35.
20. Krogan NJ, Kim M, Ahn SH, Zhong G, Kobor MS, Cagney G, et al. RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach. Mol Cell Biol. 2002;22(20):6979-92.

21. Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature. 2002;415(6868):141-7.
22. Lindstrom DL, Squazzo SL, Muster N, Burckin TA, Wachter KC, Emigh CA, et al. Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. Mol Cell Biol. 2003;23(4):1368-78.
23. Reim NI, Chuang J, Jain D, Alver BH, Park PJ, Winston F. The conserved elongation factor Spn1 is required for normal transcription, histone modifications, and splicing in Saccharomyces cerevisiae. Nucleic Acids Res. 2020;48(18):10241-58.
24. Fischbeck JA, Kraemer SM, Stargell LA. SPN1, a conserved gene identified by suppression of a postrecruitment-defective yeast TATA-binding protein mutant. Genetics. 2002;162(4):1605-16.
25. Zhang L, Fletcher AG, Cheung V, Winston F, Stargell LA. Spn1 regulates the recruitment of Spt6 and the Swi/Snf complex during transcriptional activation by RNA polymerase II. Mol Cell Biol. 2008;28(4):1393-403.
26. Cermakova K, Demeulemeester J, Lux V, Nedomova M, Goldman SR, Smith EA, et al. A ubiquitous disordered protein interaction module orchestrates transcription elongation. Science. 2021;374(6571):1113-21.
27. Brogaard K, Xi L, Wang JP, Widom J. A map of nucleosome positions in yeast at base-pair resolution. Nature. 2012;486(7404):496-501.
28. Thurston AK, Radebaugh CA, Almeida AR, Argueso JL, Stargell LA. Genome Instability Is Promoted by the Chromatin-Binding Protein Spn1 in Saccharomyces cerevisiae. Genetics. 2018;210(4):1227-37.
29. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. Cell. 2016;164(1-2):57-68.
30. Ramani V, Qiu R, Shendure J. High Sensitivity Profiling of Chromatin Structure by MNase-SSP. Cell Rep. 2019;26(9):2465-76 e4.
31. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. The EMBnet J. 2011;17(3).
32. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9(4):357-9.
33. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078-9.
34. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics. 2010;26(6):841-2.
35. Park D, Morris AR, Battenhouse A, Iyer VR. Simultaneous mapping of transcript ends at single-nucleotide resolution and identification of widespread promoter-associated non-coding RNA governed by TATA elements. Nucleic Acids Res. 2014;42(6):3736-49.
36. Churchman LS, Weissman JS. Nascent transcript sequencing visualizes transcription at nucleotide resolution. Nature. 2011;469(7330):368-73.
37. Zentner GE, Kasinathan S, Xin B, Rohs R, Henikoff S. ChEC-seq kinetics discriminates transcription factor binding sites by DNA sequence and shape in vivo. Nat Commun. 2015;6:8733.
38. Chereji RV, Ocampo J, Clark DJ. MNase-Sensitive Complexes in Yeast: Nucleosomes and Non-histone Barriers. Mol Cell. 2017;65(3):565-77 e3.
39. Chereji RV, Ramachandran S, Bryson TD, Henikoff S. Precise genome-wide mapping of single nucleosomes and linkers in vivo. Genome Biol. 2018;19(1):19.
40. Viktorovskaya O, Chuang J, Jain D, Reim NI, Lopez-Rivera F, Murawska M, et al. Essential histone chaperones collaborate to regulate transcription and chromatin integrity. Genes Dev. 2021;35(9-10):698-712.

41. Mayer A, Lidschreiber M, Siebert M, Leike K, Soding J, Cramer P. Uniform transitions of the general RNA polymerase II transcription complex. Nat Struct Mol Biol. 2010;17(10):1272-8.
42. Lorch Y, Maier-Davis B, Kornberg RD. Role of DNA sequence in chromatin remodeling and the formation of nucleosome-free regions. Genes Dev. 2014;28(22):2492-7.
43. Rando OJ, Winston F. Chromatin and transcription in yeast. Genetics. 2012;190(2):351-87.
44. Ehara H, Kujirai T, Shirouzu M, Kurumizaka H, Sekine SI. Structural basis of nucleosome disassembly and reassembly by RNAPII elongation complex with FACT. Science. 2022;377(6611):eabp9466.
45. McCullough L, Connell Z, Petersen C, Formosa T. The Abundant Histone Chaperones Spt6 and FACT Collaborate to Assemble, Inspect, and Maintain Chromatin Structure in Saccharomyces cerevisiae. Genetics. 2015;201(3):1031-45.
46. Jeronimo C, Poitras C, Robert F. Histone Recycling by FACT and Spt6 during Transcription Prevents the Scrambling of Histone Modifications. Cell Rep. 2019;28(5):1206-18 e8.
47. Uzun U, Brown T, Fischl H, Angel A, Mellor J. Spt4 facilitates the movement of RNA polymerase II through the +2 nucleosomal barrier. Cell Rep. 2021;36(13):109755.
48. Tramantano M, Sun L, Au C, Labuz D, Liu Z, Chou M, et al. Constitutive turnover of histone H2A.Z at yeast promoters requires the preinitiation complex. Elife. 2016;5.
49. Feng J, Gan H, Eaton ML, Zhou H, Li S, Belsky JA, et al. Noncoding Transcription Is a Driving Force for Nucleosome Instability in spt16 Mutant Cells. Mol Cell Biol. 2016;36(13):1856-67.

CHAPTER 3: RNAPII-ASSOCIATED FACTORS MAINTAIN NUCLEOSOME FEATURES OVER THE YEAST GENOME

Summary

The eukaryotic chromatin landscape presents a formidable barrier for processes that require access to DNA. One such process is transcription, where open reading frames contain nucleosomal barriers that must be transiently disassembled and reassembled to allow for gene expression to occur. These barriers are overcome through the action of many factors, including histone chaperones Spn1, Spt5, Spt6, and FACT and transcription elongation factor Spt4. However, it is poorly understood how each contributes to this process. To further explore the relationship between these factors, existing MNase-seq datasets were analyzed from cells expressing mutant alleles of Spn1, Spt4, Spt5, Spt6, FACT, and RNA Polymerase II itself. This study differs from previous work by using the same pipeline with the same master gene list for all samples, querying for changes in chromatin features of nucleosome positioning and occupancy over gene bodies, as well as width of nucleosome-depleted regions (NDRs) in promoters. This analysis was further refined to determine whether these features change depending on the expression or length of genes in the master gene list. The results reveal that most factors in the study maintain nucleosome positioning with a greater role at longer genes and genes with higher RNAPII occupancy. Conversely, the factors that maintain nucleosome occupancy and NDR width do so in a manner largely independent of gene length or RNAPII occupancy. Taken together, this work furthers the understanding of the relationship between these factors in maintaining proper chromatin structure.

Introduction

Eukaryotes package their DNA with histone proteins to form nucleosomes, whereby ~147bp of DNA is wrapped in about 1.7 superhelical turns around an octamer of histone proteins[1-3]. An important functional role of nucleosomes *in vivo* is that they regulate DNA access to many proteins that perform essential processes such as DNA replication[4, 5], repair[4, 6], recombination[7], and transcription. Nucleosomes regulate transcription in a variety of ways. Nucleosomes are inhibitory to transcription elongation of protein coding genes, as they pose a significant barrier to RNA polymerase II (RNAPII) and reduce its transcription rate[8, 9] Additionally, nucleosomes present at promoter sequences hinder transcription initiation by preventing the binding of transcription factors[10, 11]. In wild-type cells, metagene analyses have demonstrated that well-positioned nucleosomes flank transcription start sites (TSSs) and are followed by a regularly spaced array of nucleosomes downstream (reviewed in [12]). These nucleosomes downstream of TSSs are less well-positioned and are referred to as “fuzzier”.

Histone chaperones are a class of proteins that contribute to chromatin structure, by maintaining nucleosome positioning and occupancy through deposition, removal, and exchange of histones from DNA in an ATP-independent manner[13]. A multitude of histone chaperones have been identified that assist RNAPII during transcription by assisting in the transient disassembly and subsequent reassembly of nucleosomes during RNAPII passage[14]. Many of these histone chaperones are essential for cell viability across eukaryotes, including Spn1, Spt5, Spt6, and FACT[15-18]. These specific factors physically engage with RNAPII as recently revealed through cryo-electron microscopy (cryo-EM)[19]. However, how these proteins compare in their functional maintenance of proper chromatin structure has not yet been undertaken.

Spn1 is a multifunctional and essential histone chaperone that is highly conserved across eukaryotes. It preferentially binds H3-H4 histones over H2A-H2B[20] via its N-terminal region, with

high binding affinity (K_D ~ 10nM). Spn1 binds nucleosomes with its C-terminal region[20, 21] a function which is not present among all histone chaperones. Spn1 also has a high affinity interaction (K_D = 10-100nM) with fellow essential histone chaperone Spt6[22-25]. Spt6 is similarly highly conserved across eukaryotes but binds H3-H4 and H2A-H2B with comparable affinity[26] and similarly can bind nucleosomes, though only in the presence of Nhp6, a small high-mobility group (HMG) protein[22]. Spn1 and Spt6 binding to nucleosomes are mutually exclusive[21, 22].

FACT (FAcilitates Chromatin Transcription/Transactions), comprised of subunits Spt16 and Pob3, is functionally like Spt6 in many ways. FACT is both an H2A-H2B histone chaperone[27] and H3-H4 chaperone[28] and similarly requires Nhp6 for binding nucleosomes[15]. Both factors play a role in regulating core histone levels[29, 30] as well as proper maintenance of non-canonical histone H2A.Z localization[31]. Histone chaperone Spt5 also binds H3-H4 histones, and this function is essential for cell viability in yeast[32]. It is the only factor that is physically associated with RNA polymerase that is universally conserved across life, including both bacteria and archaea[33]. Spt5 forms a well-characterized complex with non-essential transcription elongation factor Spt4 (DSIF in humans), controlling RNAPII processivity and chromatin structure[25, 34-37]. Intriguingly, mutation of Spt4 knockout combined with mutation of Spn1 leads to synthetic lethality[38].

While individual studies of Spn1, Spt4, Spt5, Spt6, and FACT have elucidated much about their roles in maintaining chromatin structure, how these factors functionally compare to each other has not been determined. Additionally, gaps remain in the individual studies of how these RNAPII-interacting factors maintain specific features of chromatin structure. Study of the role of factors like these often employ digestion with micrococcal nuclease followed by sequencing (MNase-seq) to quantify nucleosomal protections across the genome. The exo-endonuclease activity of MNase digests linker DNA at 25 times the rate of nucleosomal DNA[39] which generates a library of DNA fragments protected by proteins. This DNA can be sequenced and

mapped to genomes leading to interpretations where the length and abundance of these fragments can be interpreted to examine changes in chromatin protections across the genome. In this study, existing MNase-seq datasets were analyzed using a custom pipeline to compare the functions of RNAPII-interacting factors Spn1, Spt4, Spt5, Spt6, and FACT. This analysis consisted of examination of factor maintenance of key features of chromatin including nucleosome occupancy, positioning, and NDR width, and the extent in which these factors function differentially at genes of varied characteristics. Multiple mutant alleles were analyzed for each factor when available, allowing for the detection of additional functions. Our results demonstrate that each factor preserves chromatin features over genes in a manner that generally positively correlates with both RNAPII occupancy over genes and gene length.

Materials and Methods

Acquisition of published MNase-seq datasets

MNase-seq datasets were obtained from the Gene Expression Omnibus (GEO) repository as outlined in Table B.1. This study focused on factors involved in the maintenance of chromatin during transcription, specifically Spn1, Spt4, Spt5, Spt6, FACT, and RNAPII (Rpb1) itself. In all but the MNase-seq experiment with *spn1-K192N* and *spn1-141-305*, sequencing libraries were prepared with gel-excised, primary mono-nucleosomal DNA. As a result, the MNase-protected fragments contained within each of these samples are interpreted as protected specifically by nucleosomes. MNase-seq data from *spn1-K192N* and *spn1-141-305* expressing yeast contained fragment lengths in a wide range of sizes (35 – 180bp) and a bin of 80 – 170bp was utilized for this analysis[40]. All biological replicates were obtained for both samples from mutant-expressing yeast and the samples from control strains.

MNase-seq analysis

MNase-seq analysis was performed as outlined in a custom script (https://github.com/ajtonsager/2024_MNase-seq). First, replicate bigWig files from mutant-expressing yeast and their controls were obtained and analyzed separately or averaged using WiggleTools[41], storing the median scores into a new bigWig prior to analysis. Scores from the bigWigs were calculated over genes anchored by their transcription start site (TSS) to count their values over each genome region using deepTools2[42]. The resulting scores are stored in a matrix and are used to plot profiles. These profiles are then interpreted to qualitatively score changes in chromatin features: namely, nucleosome occupancy, positioning, and nucleosome-depleted region (NDR) width in mutant samples compared to the control samples. When assessing the relationship between RNAPII occupancy or gene length on changes in these features, this process is repeated with the corresponding gene list divided into quartiles.

Gene list ranking by RNAPII density

A comprehensive list of gene transcription start and end sites in yeast were obtained from a published annotation[43]. Only the transcript annotations for protein coding genes were included in the analysis (n = 4928 genes). To rank these genes by RNAPII density, published Rpb3 ChIP-seq data was downloaded[44] and the scores were mapped over regions spanning 500bp upstream of the mapped TSS locations as depicted in Figure B.1. These regions and their corresponding downstream genes were ranked by occupancy of Rpb3 and divided into quartiles. Quartile Q1 represented the lowest Rpb3 and Q4 represented the highest Rpb3 occupancy.

Analysis of factor occupancy over genomic locations

Analysis of factor occupancy over genomic locations was performed as outlined in a custom script (https://github.com/ajtonsager/2024_ChIP_exo). In summary, BAM files containing mapped sequencing reads from ChIP-exo was acquired for cells expressing TAP-tagged Spn1,

Spt4, Spt5, Spt6, Spt16, Pob3, and Rpb3[45]. The number of reads were scored over the genome and a coverage track (bedGraph) was generated. Scores were computed over mapped TSS locations[43] from quartiles ranked by Rpb3 occupancy and gene length and the results were plotted in a profile.

Results

MNase-seq datasets were analyzed in parallel to probe the role of chromatin factors in maintaining nucleosome features over different classes of genes

The workflow began with published MNase-seq datasets bigWig files that contain scores of sequenced reads mapped to the yeast genome. The bigWig files were subjected to a custom analysis pipeline comprised of calculating average scores over genomic regions anchored at 4928 yeast transcription start sites residing upstream of protein-coding genes (Figure 3.1A). After the scores are averaged, they are visualized by plotting a profile of occupancy score versus region of the genome relative to transcription start sites for the wild-type strain control and the mutant strain dataset. These included shifts in nucleosome positioning, changes in nucleosome occupancy, and changes in NDR width (Figure 3.1B). Nucleosome positioning was defined as the average genomic location of the maxima of nucleosome peaks. In cases where nucleosome positioning was shifted, the maxima of nucleosome peaks and the distribution of the peak was shifted on average to a different position. For this analysis, nucleosome occupancy is defined as the distance from the maxima of nucleosome peaks to the minima in the regions between nucleosome peaks, or the “height” of the peaks. Instances where this distance increased or decreased were interpreted as a corresponding increase or decrease in nucleosome occupancy. Finally, the feature of NDR width was defined as the distance and depth of the region between the +1 and -1 nucleosome peaks. In this analysis, any change in

the distance between -1 and +1 nucleosome peaks and/or the depth of the NDR was interpreted as a change in NDR width.

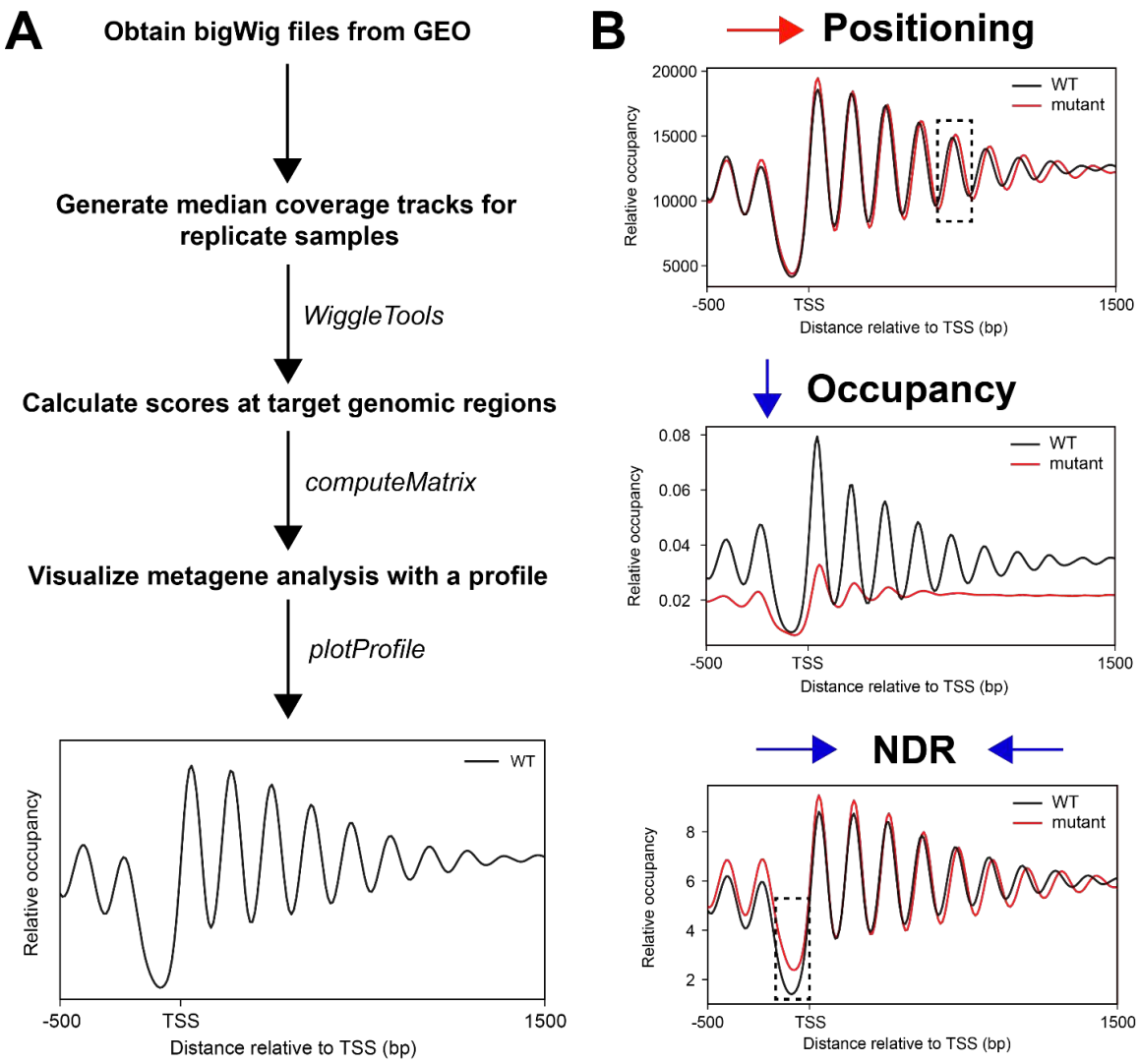


Figure 3.1. Overview of MNase-seq analysis to probe for changes in chromatin features in mutant yeast. (A) Diagram of analysis pipeline. Processed bigWigs were obtained from the Gene Expression Omnibus (GEO) repository, including all control samples, and median coverage tracks were generated for replicates. Scores were calculated at target genomic regions anchored by mapped transcription start sites (TSS) and visualized with a profile. (B) Profiles showcasing instances of deviations in chromatin features relative to wild-type. Mutants with shifts in nucleosome positioning are identified by a shift in the position of nucleosome peaks as shown in the dotted box. Mutants with changes in nucleosome occupancy are identified by an increase or decrease in the height of nucleosome peaks. Mutants with changes in NDR width are identified by a change in either the distance between -1 and +1 nucleosome peaks or the depth of the NDR as shown in the dotted box.

This custom pipeline also was used to compare the role of RNAPII-interacting factors in maintaining chromatin over 4928 genes of varying expression and length. To do so, genes were first ranked separately into quartiles by RNAPII occupancy and gene length (Figure 3.2). In yeast, RNAPII does not experience promoter-proximal pausing[46] and its occupancy is relatively uniform across promoters and their downstream genes[47]. This occupancy is often measured by ChIP-seq analysis of the RNAPII subunit Rpb3. This can be used as a proxy for expression, as Rpb3 occupancy over genes correlates relatively well with active transcription of those genes[48]. Genes within quartile 1 ranked by RNAPII occupancy have the lowest levels of Rpb3 over their 5' ends while genes in quartile 4 have the highest levels of Rpb3 (Figure 3.2A, Figure 3.2B). Genome-wide mapping of the factors over the master TSS list incorporated in this analysis demonstrates that Spn1, Spt4, Spt5, Spt6, and FACT levels correlate with RNAPII (Figure B.2A), as previously observed[45, 49]. Additionally, the occupancy of each factor over genes ranked by RNAPII occupancy all have similar patterns, with the lowest levels over quartile 1 genes and the highest levels over quartile 4 genes (Figure B.2B).

A separate ranking of genes into quartiles by length was performed by measuring the distance between TSS and TES. Genes within quartile 1 ranked by length are the shortest while genes in quartile 4 are the longest. Mapping of wild-type nucleosome-protected fragments from MNase-seq of wild-type samples demonstrates that nucleosome occupancy persists over the entirety of the gene body, with depletion proximal to the TSS and TES (Figure 3.2C, Figure 3.2D). Intriguingly, the occupancy of Rpb3 and the RNAPII-associated factors is negatively correlated with gene length (Figure B.3). Comparison of the gene lists ranked by RNAPII occupancy and gene length reveals that while the lists are distinct, there is a slight overrepresentation of long genes in the lowest Rpb3 quartile of genes and conversely, a slight overrepresentation of short genes in the highest Rpb3 quartile of genes (Figure B.4).

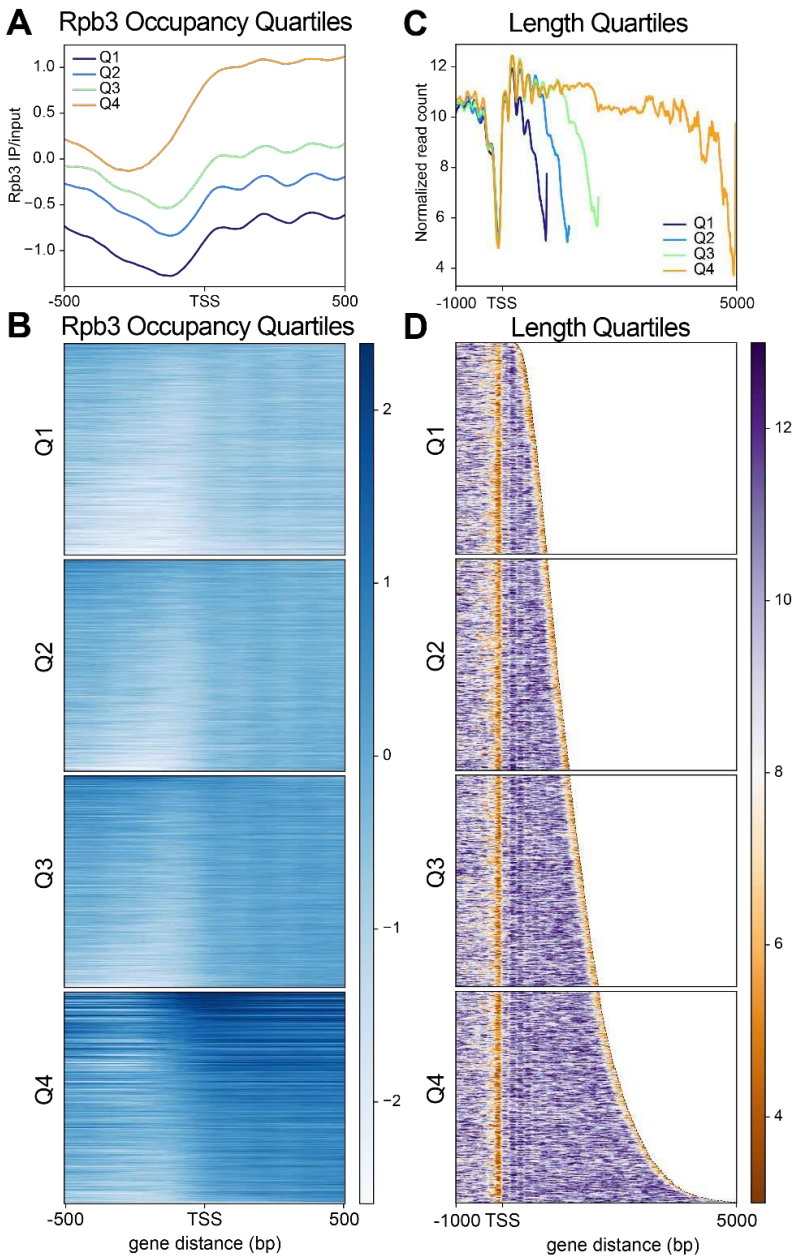


Figure 3.2. Genes ranked into bins by expression and length using existing datasets. (A) Metagene analysis of Rpb3 ChIP-seq (IP/input) data[44] mapped over yeast transcription start sites[43] ranked by Rpb3 occupancy. (B) Rpb3 ChIP-seq data from (A) visualized as a heatmap over genes within each quartile ranked by Rpb3 occupancy. (C) Metagene analysis of MNase-seq protected fragments (142 – 152bp) from wild-type yeast[40] mapped over yeast transcription start sites ranked by gene length. (D) MNase-seq protected fragments from (C) visualized as a heatmap over genes within each quartile ranked by gene length.

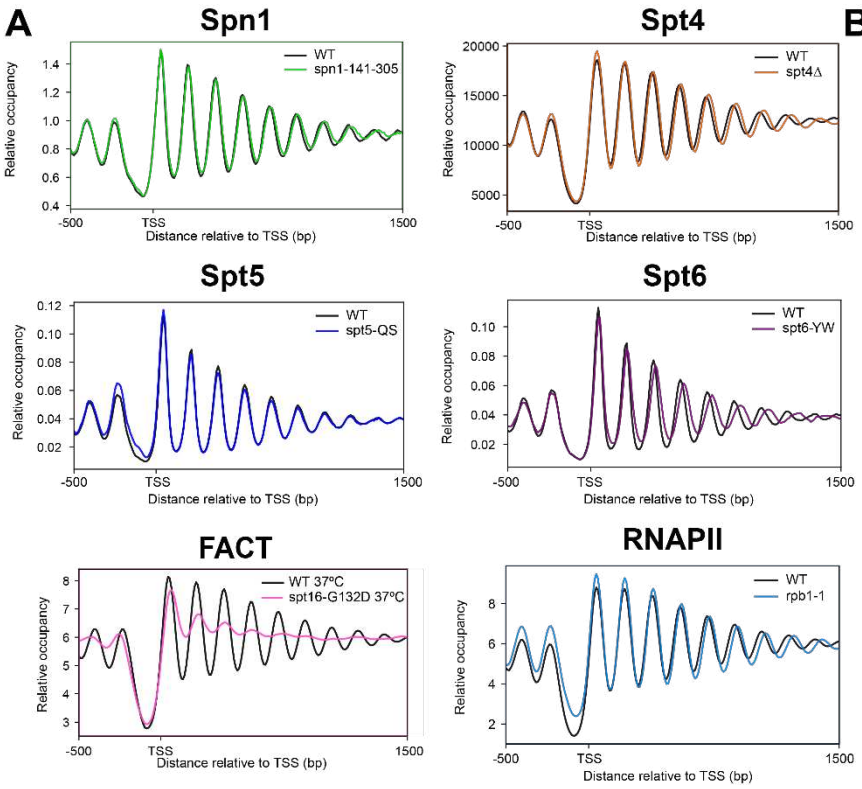
Spn1, Spt4, Spt6, FACT, and RNAPII maintain nucleosome positioning over open reading frames with a greater role at longer genes and genes with higher RNAPII occupancy

One goal of this study was to utilize a uniform pipeline to analyze available datasets for analyses of Spn1, Spt4, Spt5, Spt6, FACT and RNAPII mutants. To first examine the role in these factors in maintaining nucleosome positioning using this custom MNase-seq analysis pipeline, average bigWig files from mutant and control samples were mapped over the entire master TSS list (n = 4928 genes). Changes in nucleosome positioning were identified by determining if the genomic location of nucleosome peaks shifted to a different position as described previously. Plotting of the average scores demonstrates that mutations in Spn1, Spt4, Spt6, FACT, and RNAPII, but not Spt5, produce genome-wide shifts in nucleosome positioning (Figure 3.3A), as previously observed[36, 40, 50-54]. The nucleosome positioning shift observed in the mutants was observed as a progressive downstream shift, with the most shifted nucleosomes distal to the TSS. In some cases, such as with depletion of FACT through the temperature-sensitive *spt16-G132D* allele, this loss of positioning manifests primarily as an increase in fuzziness, when nucleosome positionings within a peak have an increase in variability[51]. Others have previously attributed the Spn1-Spt6 interaction in also preventing fuzziness, as both *spn1-K192N* and *spt6-YW* were shown to produce more variable nucleosome positions over genes, especially when assayed after growth in a non-permissive temperature[50].

To determine whether the identified shifts in nucleosome positioning in the mutant samples are more dramatic on a class of genes with specific RNAPII occupancy levels, the analysis was repeated and mapped separately for mutants of Spn1 (Figure B.5), Spt4 (Figure B.6), Spt5 (Figure B.7), Spt6 (Figure B.8), FACT subunits (Figure B.9) and RNAPII (Figure B.10). In most instances where a downstream shift in nucleosome positioning was observed, these changes were highly positively correlated with RNAPII occupancy (Figure 3.3B). While not every allele represented in the analysis produced nucleosome positioning shifts correlated with RNAPII

occupancy, at least one allele from each RNAPII-associated factor did. While the findings from Chapter 2 demonstrated that *spn1*^{K192N} and *spn1*$^{141\text{-}305}$ produce nucleosome shifts correlating with gene expression, the analysis in this Chapter did not reveal a correlation using genes ranked by Rpb3 occupancy. Ranking genes by Rpb3 and by NET-seq may produce slightly different gene lists. These findings support a model in which proper nucleosome positioning is maintained by the combined efforts of Spn1, Spt4, Spt6, FACT, and RNAPII, which becomes increasingly critical with gene expression.

To determine whether the identified shifts in nucleosome positioning in the mutant samples correlate with genes of varied length, the analysis was repeated and mapped separately for mutants of Spn1 (Figure B.5), Spt4 (Figure B.6), Spt5 (Figure B.7), Spt6 (Figure B.8), FACT subunit mutants (Figure B.9) and RNAPII (Figure B.10). In every instance where a downstream shift in nucleosome positioning was observed except for cells expressing *spt6-1004*, these changes were highly positively correlated with gene length (Figure 3.3B). These findings support a model in which proper nucleosome positioning is maintained by the combined efforts of Spn1, Spt4, Spt6, FACT, and RNAPII, which becomes increasingly critical over longer genes.



B

	Strain	Positioning	RNAPII occupancy trends	Length trends
Spn1	*spn1-141-305*	→	o	+
	spn1-K192N	→	o	+
	spn1-K192N (37°C)	→	+	+
Spt4	*spt4Δ*	→	+	+
Spt5	*spt5-QS*	N.C.	o	o
	spt5-QS (37°C)	N.C.	o	o
	spt5-AID	N/A	o	o
	spt5-3N	N/A	o	o
Spt6	*spt6-YW*	→	+	+
	spt6-YW (37°C)	→	+	+
	spt6-1004 (37°C)	→	+	o
	spt6-R,KK	→	o	-
FACT	*spt16-G132D*	N.C.	o	o
	spt16-G132D	→	o	o
	pob3-Q308K	→	o	+
RNAPII	*rpb1-1*	→	+	+
	rpb1-TPY,FSP	→	-	+

Figure 3.3. Spn1, Spt4, Spt6, FACT, and RNAPII maintenance of nucleosome positioning over open reading frames highly correlates with RNAPII occupancy and gene length. (A) Representative plots from MNase-seq analyzed samples (*spn1-141-305, spt4Δ, spt5-QS, spt6-YW, spt16-G132D* shifted to a non-permissive temperature, and *rpb1-1*) featuring factors whose mutation produces a shift in nucleosome positioning. Replicate samples from wild-type control samples and mutant samples were averaged prior to scoring over the entire list of transcription start sites utilized in the analysis. Only fragments 80-170bp in length are plotted for the *spn1-141-305* mutant and its corresponding control while the entirety of the mononucleosomal fraction was included for the remaining datasets. (B) Summary of effects on nucleosome positioning caused by expression of factor mutants and their correlation with genes ranked by RNAPII occupancy and gene length. **The dataset containing *spt5-AID* and *spt5-3A* samples produced profiles with poor nucleosome positioning and were not scored for nucleosome positioning changes.

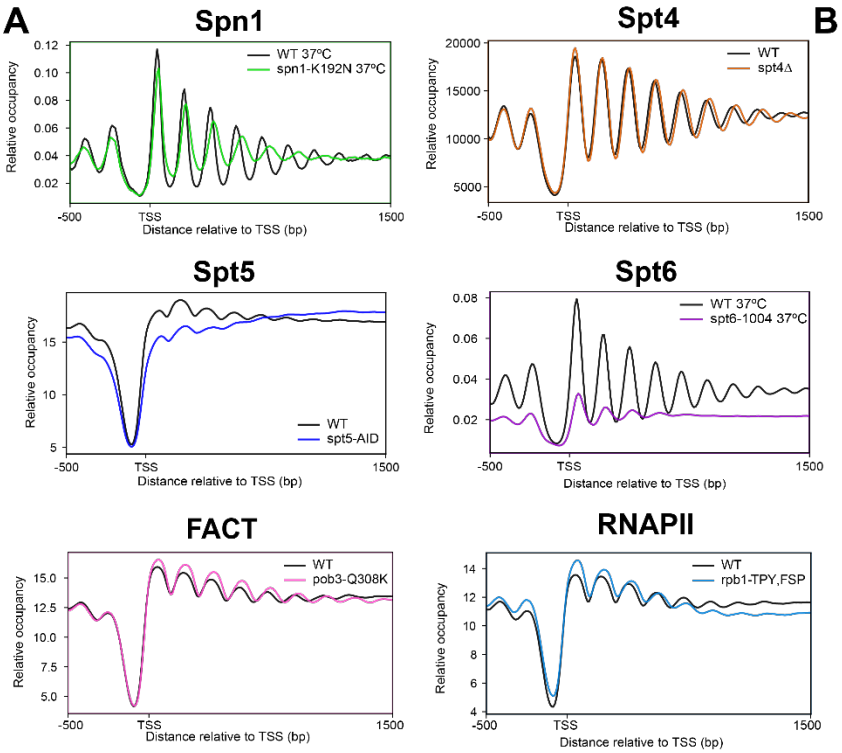
Histone chaperones Spn1, Spt5, Spt6, and FACT generally promote nucleosome occupancy while RNAPII activity inhibits occupancy over genes largely independent of gene expression or length.

The feature of nucleosome occupancy is defined in this study as the distance from the maxima of nucleosome peaks to the minima in the regions between nucleosome peaks, or the “height”

of the peaks as previously defined. To identify instances in which nucleosome occupancy is changed in the factors included in this study, average bigWig files from mutant and control samples were first mapped over the master TSS list used in this study recapitulated results in which mutations of histone chaperones Spn1, Spt5, Spt6, and FACT generally promote nucleosome occupancy over genes (Figure 3.4A), as previously observed[32, 50-53, 55]. In contrast, loss of Spt4 does not produce a change in occupancy as previously observed[36]. In most cases, mutations in histone chaperones led to a decrease in occupancy. An exception is in the *pob3-Q308K* mutant strain, which shows an increase in nucleosomes over genes as previously observed[55]. The role of RNAPII itself also is antagonistic to that of histone chaperones, as expression of the *rpb1-TPY,FSP* allele also increases nucleosome occupancy over genes. Intriguingly, the loss of nucleosome occupancy in histone chaperone mutants was typically observed in the mutants whose expression results in depletion of the factor, such as with temperature sensitive alleles of *spn1-K192N*, *spt6-YW*, and *spt6-1004*. As these histone chaperones are all essential for cell viability, this suggests that maintaining nucleosome occupancy is part of their essential function. While the histone chaperones had commonalities in the loss of nucleosome occupancy upon their mutation, the profiles also demonstrate key differences in how the change in nucleosome occupancy manifests. In the case of *spn1-K192N* shifted to a non-permissive temperature, this decrease was consistent across the entire gene body and the upstream -1 nucleosome. In contrast, loss of Spt5 through auxin-induced degradation (*spt5-AID*) produced a loss in nucleosome occupancy primarily over the 5’ ends of genes.

To determine if these changes are more dramatic with genes ranked by RNAPII occupancy or gene length, the analysis was extended to the ranked TSS quartiles. This analysis revealed that most occupancy changes occur globally, without significant correlation with RNAPII occupancy or gene length (Figure 3.4B, Figures B.5 – B.10). This demonstrates that unlike maintenance of

nucleosome positioning, histone chaperones are equally critical for maintaining nucleosome occupancy over all genes, regardless of gene length, including those experiencing varied levels of ongoing transcription.



B

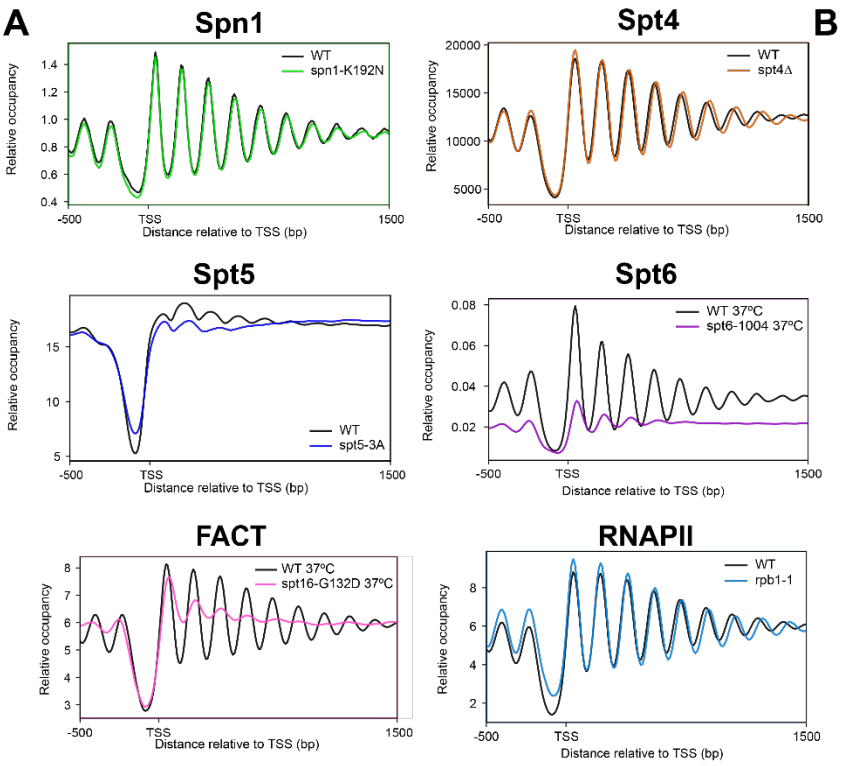
	Strain	Occupancy	RNAPII occupancy trends	Length trends
Spn1	*spn1-141-305*	N.C.	o	o
	spn1-K192N	N.C.	o	o
	spn1-K192N (37°C)	↓	o	o
Spt4	*spt4Δ*	N.C.	o	o
Spt5	*spt5-QS*	N.C.	o	o
	spt5-QS (37°C)	N.C.	o	o
	spt5-AID	↓	o	o
	spt5-3N	↓	o	o
Spt6	*spt6-YW*	↓	o	o
	spt6-YW (37°C)	↓	o	o
	spt6-1004 (37°C)	↓	o	o
	spt6-R,KK	↓	o	-
FACT	*spt16-G132D*	↓	o	o
	spt16-G132D	↓	o	+
	pob3-Q308K	↑	o	+
RNAPII	*rpb1-1*	N.C.	o	o
	rpb1-TPY,FSP	↑	-	+

Figure 3.4. Histone chaperones Spn1, Spt5, Spt6, and FACT generally promote nucleosome occupancy while RNAPII activity inhibits occupancy over genes largely independent of gene expression or length. (A) Representative plots from MNase-seq analyzed samples (*spn1-K192N* shifted to a non-permissive temperature*, spt4Δ, spt5-AID, spt6-1004* shifted to a non-permissive temperature*, pob3-Q308K*, and *rpb1-TPY,FSP*) featuring factors whose mutation produces a change in nucleosome occupancy. Replicate samples from wild-type control samples and mutant samples were averaged prior to scoring over the entire list of transcription start sites utilized in the analysis. The entirety of the mononucleosomal fraction was included for each dataset. (B) Summary of effects on nucleosome occupancy caused by expression of factor mutants and their correlation with genes ranked by RNAPII occupancy and gene length.

NDR width is maintained in part by components of the RNAPII elongation complex

While study of chromatin structure maintenance by the factors in this study has mostly focused over gene bodies in previous studies, metagenome analyses demonstrate that each factor does

occupy regions upstream of transcription start sites, albeit at lower levels (Figure B.2, Figure B.3). Additionally, some early evidence demonstrated that RNAPII itself plays a role in maintaining NDR width, potentially through eviction of the -1 nucleosome[56]. NDR width in this study is defined as both the length and depth of the region between the +1 and -1 nucleosome peaks. To first elucidate the role of each factor in maintaining NDR width, average bigWig files from mutant and control samples were mapped over the master TSS list as performed previously. This analysis demonstrated that while less common, some mutations in histone chaperones alter the width of the NDR (Figure 3.5). This finding includes Spt5, where expression of either the *spt5-3A* or *spt5-QS* mutant alleles decrease the width of NDRs across the genome. In general, changes in NDR width were more subtle among histone chaperone mutants, including Spt6, where expression of the temperature-sensitive allele *spt6-1004* produces a slightly wider NDR with subtle shifts in -1 and +1 nucleosome positions. To determine if changes in NDR width in the mutants correlate with RNAPII occupancy or gene length, the analysis was repeated with the ranked TSS quartiles. The results show that the NDR width changes observed do not correlate with these gene characteristics (Figure 3.5B, Figures B.5 – B.10). This provides evidence that these factors, including RNAPII itself, preserve NDRs globally, even at genes with the lowest amount of Rpb3.



B

	Strain	NDR width	RNAPII occupancy trends	Length trends
Spn1	*spn1-141-305*	N.C.	O	O
	spn1-K192N	N.C.	O	O
	spn1 K192N (37°C)	N.C.	O	O
Spt4	*spt4Δ*	N.C.	O	O
Spt5	*spt5-QS*	N.C.	O	O
	spt5-QS (37°C)	→ ←	O	O
	spt5-AID	N.C.	O	O
	spt5-3N	→ ←	O	O
Spt6	*spt6-YW*	N.C.	O	O
	spt6-YW (37°C)	N.C.	O	O
	spt6-1004 (37°C)	← →	O	O
	spt6-R,KK	N.C.	O	O
FACT	*spt16-G132D*	N.C.	O	O
	spt16-G132D (37°C)	N.C.	O	O
	pob3-Q308K	N.C.	O	O
RNAPII	*rpb1-1*	→ ←	O	O
	rpb1-TPY,FSP	→ ←	O	O

Figure 3.5. NDR width is maintained in part by components of the RNAPII elongation complex. (A) Representative plots from MNase-seq analyzed samples (*spn1-K192N*, *spt4Δ, spt5-3A, spt6-1004* shifted to a non-permissive temperature, *spt16-G132D* shifted to a non-permissive temperature, and *rpb1-1*) featuring factors whose mutation produces a change in NDR width. Replicate samples from wild-type control samples and mutant samples were averaged prior to scoring over the entire list of transcription start sites utilized in the analysis. Only fragments 80-170bp in length are plotted for the *spn1-K192N* mutant and its corresponding control while the entirety of the mononucleosomal fraction was included for the remaining datasets. (B) Summary of effects on NDR width caused by expression of factor mutants and their correlation with genes ranked by RNAPII occupancy and gene length.

Discussion

In this study, extensive analysis of existing MNase-seq datasets was performed to provide a more comprehensive view of the contributions of Spn1, Spt4, Spt5, Spt6, FACT, and RNAPII on preservation of chromatin structure. By re-examining the role of each using the identical pipeline with emphasis on their maintenance of key features of nucleosome occupancy, positioning, and NDR width, this analysis reveals insights into the similarities and differences in their function. Additionally, this study sought to elucidate previously unknown correlations between these

factors and the extent to which their function is important at genes ranked by different characteristics. The analysis conducted in this study demonstrated that many chromatin changes in cells expressing mutant alleles of Spn1, Spt4, Spt5, Spt6, FACT, and RNAPII correlate strongly with both RNAPII occupancy and gene length (Figure B.11).

Our findings further the relationships between the factors incorporated in this analysis and how they function in maintaining normal chromatin features (Figure 3.6). Nucleosome positioning specifically is maintained through the combined efforts of all factors in the analysis excluding Spt5. This comes from the observation that numerous mutant alleles in these factors produce a downstream shift in nucleosome positioning, with some instances in which positioning becomes “fuzzier”. While Spt5 was not directly interpreted to maintain nucleosome positioning through this analysis, the *spt5-AID, spt5-3A*, and corresponding wild-type control datasets exhibited poor resolution of nucleosome positioning. Spt5 may contribute to this process, though future work is required to determine this possibility. Control of this chromatin feature was highly positively correlated with both RNAPII occupancy and gene length, as the largest shifts in positioning were observed at the longest genes and genes with the highest Rpb3 occupancy.

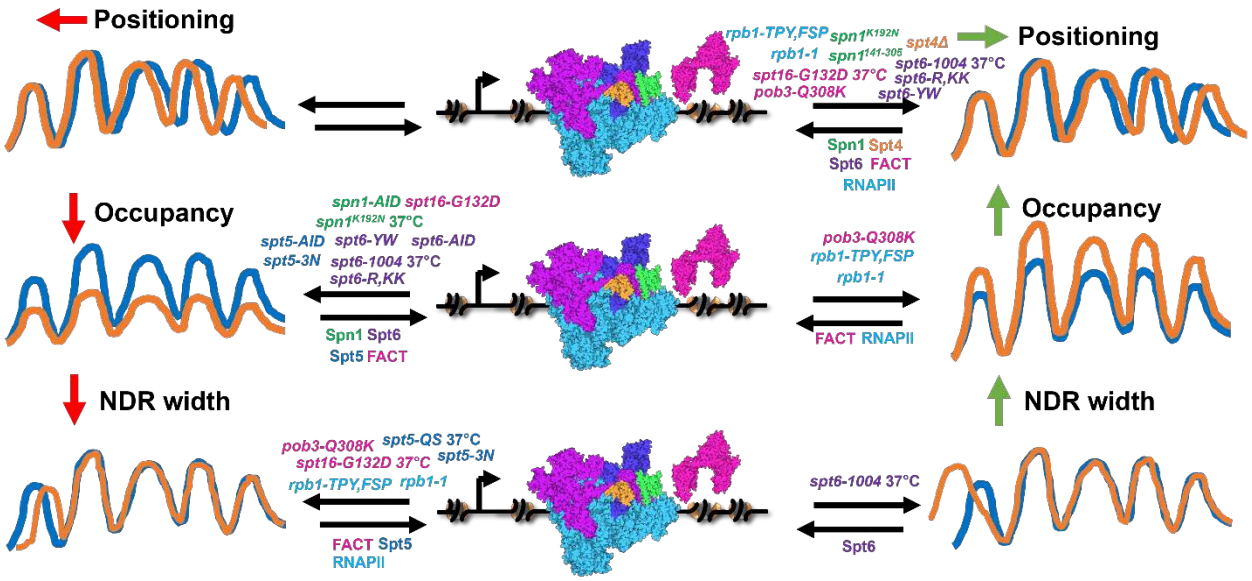


Figure 3.6. Clustering of factors by chromatin changes upon mutation reveal classes of factors responsible for maintaining chromatin features. Schematic depicting the relationship between chromatin factors incorporated in the study and their role in maintaining chromatin structure. Mutants in factors produce changes in nucleosome positioning, nucleosome occupancy, and NDR width indicated by arrows pointing toward aberrant chromatin states. The wild-type proteins function by maintaining these chromatin features, indicated by arrows pointed towards a wild-type chromatin state.

Similarly with nucleosome positioning, nucleosome occupancy is also maintained by most of the factors in this analysis, namely each histone chaperone and RNAPII itself. Most of the histone chaperone mutants exhibited a loss in nucleosome occupancy while mutations in Rpb1 resulted in a nucleosome occupancy increase. Intriguingly, expression of the *pob3-Q308K* mutant allele similarly produced an increase in nucleosome occupancy, implicating FACT has both positive and negative regulatory roles in maintaining this chromatin feature. Our analysis also revealed that most occupancy changes did not correlate with genes ranked by Rpb3 occupancy or gene length, in contrast to what was observed with nucleosome positioning. This demonstrates that these RNAPII-associated histone chaperones and RNAPII itself maintain nucleosome occupancy over all genes, regardless of length, including those not undergoing active transcription.

This study also examined the role that these RNAPII-associated factors play in maintenance of NDR width, which is largely thought to be controlled by other factors, such as chromatin remodelers. Some mutations in histone chaperones exhibited changes in NDR width, with opposing roles revealed between Spt6 and both Spt5 and RNAPII. Mutation in Spt6 produced wider NDRs while mutations in Spt5 and RNAPII produce narrower NDRs. This provides more evidence that the role of RNAPII-associated factors extends beyond transcribed regions and requires further work to determine the role of these factors in maintaining normal NDR architecture.

REFERENCES

1. Davey CA, Sargent DF, Luger K, Maeder AW, Richmond TJ. Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. J Mol Biol. 2002;319(5):1097-113.
2. Flaus A, Luger K, Tan S, Richmond TJ. Mapping nucleosome position at single base-pair resolution by using site-directed hydroxyl radicals. Proc Natl Acad Sci U S A. 1996;93(4):1370-5.
3. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature. 1997;389(6648):251-60.
4. Groth A, Rocha W, Verreault A, Almouzni G. Chromatin challenges during DNA replication and repair. Cell. 2007;128(4):721-33.
5. Eaton ML, Galani K, Kang S, Bell SP, MacAlpine DM. Conserved nucleosome positioning defines replication origins. Genes Dev. 2010;24(8):748-53.
6. Adkins NL, Niu H, Sung P, Peterson CL. Nucleosome dynamics regulates DNA processing. Nat Struct Mol Biol. 2013;20(7):836-42.
7. Bevington S, Boyes J. Transcription-coupled eviction of histones H2A/H2B governs V(D)J recombination. EMBO J. 2013;32(10):1381-92.
8. Selth LA, Sigurdsson S, Svejstrup JQ. Transcript Elongation by RNA Polymerase II. Annu Rev Biochem. 2010;79:271-93.
9. Brahma S, Henikoff S. Epigenome Regulation by Dynamic Nucleosome Unwrapping. Trends Biochem Sci. 2020;45(1):13-26.
10. Henikoff JG, Belsky JA, Krassovsky K, MacAlpine DM, Henikoff S. Epigenome characterization at single base-pair resolution. Proc Natl Acad Sci U S A. 2011;108(45):18318-23.
11. Kubik S, Bruzzone MJ, Jacquet P, Falcone JL, Rougemont J, Shore D. Nucleosome Stability Distinguishes Two Different Promoter Types at All Protein-Coding Genes in Yeast. Mol Cell. 2015;60(3):422-34.
12. Lieleg C, Krietenstein N, Walker M, Korber P. Nucleosome positioning in yeasts: methods, maps, and mechanisms. Chromosoma. 2015;124(2):131-51.
13. Bai L, Morozov AV. Gene regulation by nucleosome positioning. Trends Genet. 2010;26(11):476-83.
14. Kujirai T, Kurumizaka H. Transcription through the nucleosome. Curr Opin Struct Biol. 2020;61:42-9.
15. Wittmeyer J, Formosa T. The Saccharomyces cerevisiae DNA polymerase alpha catalytic subunit interacts with Cdc68/Spt16 and with Pob3, a protein similar to an HMG1-like protein. Mol Cell Biol. 1997;17(7):4178-90.
16. Swanson MS, Malone EA, Winston F. SPT5, an essential gene important for normal transcription in Saccharomyces cerevisiae, encodes an acidic nuclear protein with a carboxy-terminal repeat. Mol Cell Biol. 1991;11(6):3009-19.
17. Fischbeck JA, Kraemer SM, Stargell LA. SPN1, a conserved gene identified by suppression of a postrecruitment-defective yeast TATA-binding protein mutant. Genetics. 2002;162(4):1605-16.
18. Clark-Adams CD, Winston F. The SPT6 gene is essential for growth and is required for delta-mediated transcription in Saccharomyces cerevisiae. Mol Cell Biol. 1987;7(2):679-86.
19. Ehara H, Kujirai T, Shirouzu M, Kurumizaka H, Sekine SI. Structural basis of nucleosome disassembly and reassembly by RNAPII elongation complex with FACT. Science. 2022;377(6611):eabp9466.

20. Li S, Almeida AR, Radebaugh CA, Zhang L, Chen X, Huang L, et al. The elongation factor Spn1 is a multi-functional chromatin binding protein. Nucleic Acids Res. 2018;46(5):2321-34.
21. Li S, Edwards G, Radebaugh CA, Luger K, Stargell LA. Spn1 and Its Dynamic Interactions with Spt6, Histones and Nucleosomes. J Mol Biol. 2022;434(13):167630.
22. McDonald SM, Close D, Xin H, Formosa T, Hill CP. Structure and biological importance of the Spn1-Spt6 interaction, and its regulatory role in nucleosome binding. Mol Cell. 2010;40(5):725-35.
23. Krogan NJ, Kim M, Ahn SH, Zhong G, Kobor MS, Cagney G, et al. RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach. Mol Cell Biol. 2002;22(20):6979-92.
24. Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature. 2002;415(6868):141-7.
25. Lindstrom DL, Squazzo SL, Muster N, Burckin TA, Wachter KC, Emigh CA, et al. Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins. Mol Cell Biol. 2003;23(4):1368-78.
26. Bortvin A, Winston F. Evidence that Spt6p controls chromatin structure by a direct interaction with histones. Science. 1996;272(5267):1473-6.
27. Hondele M, Stuwe T, Hassler M, Halbach F, Bowman A, Zhang ET, et al. Structural basis of histone H2A-H2B recognition by the essential chaperone FACT. Nature. 2013;499(7456):111-4.
28. Kemble DJ, Whitby FG, Robinson H, McCullough LL, Formosa T, Hill CP. Structure of the Spt16 middle domain reveals functional features of the histone chaperone FACT. J Biol Chem. 2013;288(15):10188-94.
29. Jeronimo C, Poitras C, Robert F. Histone Recycling by FACT and Spt6 during Transcription Prevents the Scrambling of Histone Modifications. Cell Rep. 2019;28(5):1206-18 e8.
30. Jamai A, Puglisi A, Strubin M. Histone chaperone spt16 promotes redeposition of the original h3-h4 histones evicted by elongating RNA polymerase. Mol Cell. 2009;35(3):377-83.
31. Jeronimo C, Watanabe S, Kaplan CD, Peterson CL, Robert F. The Histone Chaperones FACT and Spt6 Restrict H2A.Z from Intragenic Locations. Mol Cell. 2015;58(6):1113-23.
32. Evrin C, Serra-Cardona A, Duan S, Mukherjee PP, Zhang Z, Labib KPM. Spt5 histone binding activity preserves chromatin during transcription by RNA polymerase II. EMBO J. 2022;41(5):e109783.
33. Werner F, Grohmann D. Evolution of multisubunit RNA polymerases in the three domains of life. Nat Rev Microbiol. 2011;9(2):85-98.
34. Hartzog GA, Fu J. The Spt4-Spt5 complex: a multi-faceted regulator of transcription elongation. Biochim Biophys Acta. 2013;1829(1):105-15.
35. Crickard JB, Lee J, Lee TH, Reese JC. The elongation factor Spt4/5 regulates RNA polymerase II transcription through the nucleosome. Nucleic Acids Res. 2017;45(11):6362-74.
36. Uzun U, Brown T, Fischl H, Angel A, Mellor J. Spt4 facilitates the movement of RNA polymerase II through the +2 nucleosomal barrier. Cell Rep. 2021;36(13):109755.
37. Ehara H, Kujirai T, Fujino Y, Shirouzu M, Kurumizaka H, Sekine SI. Structural insight into nucleosome transcription by RNA polymerase II with elongation factors. Science. 2019;363(6428):744-7.
38. Zhang L, Fletcher AG, Cheung V, Winston F, Stargell LA. Spn1 regulates the recruitment of Spt6 and the Swi/Snf complex during transcriptional activation by RNA polymerase II. Mol Cell Biol. 2008;28(4):1393-403.

39. Chereji RV, Ocampo J, Clark DJ. MNase-Sensitive Complexes in Yeast: Nucleosomes and Non-histone Barriers. Mol Cell. 2017;65(3):565-77 e3.
40. Tonsager AJ, Zukowski A, Radebaugh CA, Weirich A, Stargell LA, Ramachandran S. The Histone Chaperone Spn1 Preserves Subnucleosomal Structures at Promoters and Nucleosome Positioning in Open Reading Frames. bioRxiv. 2024.
41. Zerbino DR, Johnson N, Juettemann T, Wilder SP, Flicek P. WiggleTools: parallel processing of large collections of genome-wide datasets for visualization and statistical analysis. Bioinformatics. 2014;30(7):1008-9.
42. Ramirez F, Ryan DP, Gruning B, Bhardwaj V, Kilpert F, Richter AS, et al. deepTools2: a next generation web server for deep-sequencing data analysis. Nucleic Acids Res. 2016;44(W1):W160-5.
43. Pelechano V, Wei W, Steinmetz LM. Extensive transcriptional heterogeneity revealed by isoform profiling. Nature. 2013;497(7447):127-31.
44. Swygert SG, Kim S, Wu X, Fu T, Hsieh TH, Rando OJ, et al. Condensin-Dependent Chromatin Compaction Represses Transcription Globally during Quiescence. Mol Cell. 2019;73(3):533-46 e4.
45. Rossi MJ, Kuntala PK, Lai WKM, Yamada N, Badjatia N, Mittal C, et al. A high-resolution protein architecture of the budding yeast genome. Nature. 2021;592(7853):309-14.
46. Stargell LA, Struhl K. Mechanisms of transcriptional activation in vivo: two steps forward. Trends Genet. 1996;12(8):311-5.
47. Steinmetz EJ, Warren CL, Kuehner JN, Panbehi B, Ansari AZ, Brow DA. Genome-wide distribution of yeast RNA polymerase II and its control by Sen1 helicase. Mol Cell. 2006;24(5):735-46.
48. Pelechano V, Chavez S, Perez-Ortin JE. A complete set of nascent transcription rates for yeast genes. PLoS One. 2010;5(11):e15442.
49. Mayer A, Lidschreiber M, Siebert M, Leike K, Soding J, Cramer P. Uniform transitions of the general RNA polymerase II transcription complex. Nat Struct Mol Biol. 2010;17(10):1272-8.
50. Viktorovskaya O, Chuang J, Jain D, Reim NI, Lopez-Rivera F, Murawska M, et al. Essential histone chaperones collaborate to regulate transcription and chromatin integrity. Genes Dev. 2021;35(9-10):698-712.
51. Feng J, Gan H, Eaton ML, Zhou H, Li S, Belsky JA, et al. Noncoding Transcription Is a Driving Force for Nucleosome Instability in spt16 Mutant Cells. Mol Cell Biol. 2016;36(13):1856-67.
52. Connell Z, Parnell TJ, McCullough LL, Hill CP, Formosa T. The interaction between the Spt6-tSH2 domain and Rpb1 affects multiple functions of RNA Polymerase II. Nucleic Acids Res. 2022;50(2):784-802.
53. Doris SM, Chuang J, Viktorovskaya O, Murawska M, Spatt D, Churchman LS, Winston F. Spt6 Is Required for the Fidelity of Promoter Selection. Mol Cell. 2018;72(4):687-99 e6.
54. Weiner A, Hughes A, Yassour M, Rando OJ, Friedman N. High-resolution nucleosome mapping reveals transcription-dependent promoter packaging. Genome Res. 2010;20(1):90-100.
55. McCullough LL, Pham TH, Parnell TJ, Connell Z, Chandrasekharan MB, Stillman DJ, Formosa T. Establishment and Maintenance of Chromatin Architecture Are Promoted Independently of Transcription by the Histone Chaperone FACT and H3-K56 Acetylation in Saccharomyces cerevisiae. Genetics. 2019;211(3):877-92.
56. Venters BJ, Pugh BF. A canonical promoter organization of the transcription machinery and its regulators in the Saccharomyces genome. Genome Res. 2009;19(3):360-71.

CHAPTER 4: AN UNDERGRADUATE RESEARCH EXPERIENCES IN CRISPR-CAS9 MEDIATED EUKARYOTIC GENOME EDITING TO TEACH FUNDAMENTAL BIOCHEMISTRY TECHNIQUES

Summary

CRISPR-Cas9 technology is an established, powerful tool for genome editing through the ability to target specific DNA sequences of interest for introduction of desired genetic modifications. CRISPR-Cas9 is utilized for a variety of purposes, ranging from a research molecular biology tool to treatment for human diseases. Due to its prominence across a variety of applications, it is critical that undergraduates in the life sciences are educated on CRISPR-Cas9 technology. To this end, we created an intensive eight-week long course-based undergraduate research experience (CURE) designed for students to understand CRISPR-Cas9 genome editing and perform it in *Saccharomyces cerevisiae*. Students enrolled in the CURE participate in two three-hour sessions a week and are engaged in the entire process of CRISPR-Cas9 genome editing, from preparation of genome editing reagents to characterization of mutant yeast strains. During the process, students master fundamental techniques in the life sciences, including sterile technique, Polymerase Chain Reaction (PCR), primer design, sequencing requirements, and data analysis. The course is developed with flexibility in the schedule for repetition of techniques in the event of a failed experiment, providing an authentic research experience for the students. Additionally, we have developed the course to be easily modified for the editing of any yeast gene, offering the potential to expand the course in research-driven classroom or laboratory settings.

---

[2] Most of this chapter was previously submitted for publication under the following title with a few updates: Tonsager AJ, Stargell LA. An undergraduate research experience in CRISPR-Cas9 mediated eukaryotic genome editing to teach fundamental biochemistry techniques. *Submitted to Biochemistry and Molecular Biology Education* (2024)

Introduction

The ability to introduce targeted and specific genetic alterations into an organism's genome is a powerful tool for life scientists from molecular biologists to physicians. Clustered regularly interspaced short palindromic repeats with CRISPR-associated protein 9 (CRISPR-Cas9) gene-editing technology has emerged as a powerful strategy for genomic DNA editing across domains of life due to its low cost and widespread applications. This strategy utilizes the Cas9 endonuclease, which generates a double-stranded break in a genome targeted by a short guide RNA (gRNA) sequence. In the wild, CRISPR-Cas9 is employed by prokaryotes as a defense mechanism against viral infections[1]. In the laboratory or clinic this system has been adapted to genome editing through programming the gRNA target to a genomic location of interest[2]. This double-stranded break can then be repaired using the endogenous Non-Homologous End Joining (NHEJ) pathway or Homology-Directed Repair (HDR) by providing a donor template. This technology has been employed in the laboratory to successfully edit genomes in a wide range of eukaryotes, from yeast[3] to plants[4] to mice[5] and many other organisms. In the clinic this technology has numerous promising applications in the treatment of human diseases, such as cardiovascular diseases[6], sick cell anemia[7], and cancers (reviewed in [8]).

With its widespread applications, it is essential for undergraduates in the life sciences to be familiar with CRISPR-Cas9 technology. Others have designed Course-based Undergraduate Research Experiences (CUREs) designed around teaching students how to perform CRISPR-Cas9 genome editing in several model systems, including bacteria[9, 10], yeast[11, 12], plants[13], flies[14], zebrafish[15], and butterflies[16, 17]. Yeast is an attractive eukaryotic organism for teaching CRISPR-Cas9 genome editing due to the low cost of its culturing, rapid growth rate and safety of use. Previous CUREs performing CRISPR-Cas9 in yeast have been used to introduce stop codons in the yeast *ADE2* and *STE12* genes, creating nonfunctional products conferring visual changes in phenotype[12]. Another laboratory module was developed to inactivate the yeast

CAN1 gene, whose mutant phenotype can be selected for on media containing canavanine[11]. Here we advance the field by designing a new CURE for upper division biochemistry students designed to generate CRISPR-Cas9 mediated mutation in the ATPase active site in the yeast gene encoding *SNF2*. Students participating in the CURE are engaged in the entire process of CRISPR-Cas9 genome editing, starting from preparation of genome editing reagents, transformation into bacteria to create reagents and then into yeast for genome editing, to characterization of mutant yeast strains. During this process, students master fundamental techniques like Polymerase Chain Reaction (PCR), cell transformations, and sterile technique to perform CRISPR-Cas9 genome editing successfully. To this end, a major focus of the CURE was to train undergraduates rigorously in mastering techniques by providing them with the opportunity to repeat procedures during the course. Students also prepare many of their own reagents to better simulate their future experiences working in laboratory settings. By the end of the course, students performed CRISPR-Cas9 genome editing in an authentic setting and are equipped with fundamental and transferable skills for success in life sciences laboratories. Moreover, the methods described here are easily modified to any other gene of interest in the yeast genome.

Materials and Methods

Detailed protocols and preparation notes for all labs and experiments can be found in the Supplementary Material located in Appendix C. An instructor manual with instructions on designing each reagent for the course, protocols with notes for instructors, daily check-ins, and student assignments are in Appendix D.

Yeast Strain and Media

The starting *S. cerevisiae* strain (LZ0-1) used is derived from BY4741 and does not require selection[18]. Yeast were grown in yeast extract, peptone, and dextrose (YPD; 2% dextrose) media at 30°C prior to transformations. Cells transformed with the Cas9 plasmid were grown on YPD agar plates containing 100µg/ml nourseothricin. Cas9 expressing yeast transformed with the guide RNA plasmid and donor DNA were grown on YPD agar plates containing 500µg/ml hygromycin and 100µg/ml nourseothricin. After isolating colonies from this transformation, cells were grown in YPD media for all subsequent experiments.

PCR Amplification of donor DNA

PCR was prepared as described in the Supplementary Material. Donor DNA was amplified with forward (snf2-K798A-F) and reverse (snf2-K798A-R) primers. After confirmation of successful amplification by gel electrophoresis, the PCR product was precipitated with ammonium acetate and ethanol.

Engineering of the guide RNA plasmid

The starting guide RNA plasmid (gRNA-ura-HYB) was used as a template for PCR as described in the Supplementary Material using primers that amplify the entire plasmid into a linear product but replace the 20bp guide RNA recognition sequence with a sequence targeting the *SNF2* locus. After PCR, the amplicon is treated with a Kinase-Ligase-DpnI (KLD) enzyme mix for circularization and starting plasmid template removal as described in the Q5® Site-Directed Mutagenesis Kit Manual (New England Biolabs: #E0554S). The resulting plasmid is transformed into chemically competent *E. coli*, isolated from cells, and submitted to Azenta/GENEWIZ (Azenta US, Inc., South Plainfield, NJ, USA) for Sanger sequencing.

Lithium acetate transformation of DNA into yeast

The Cas9 plasmid was transformed into the starting yeast strain using the lithium acetate transformation method[19] as described in Supplementary Material. Cas9 plasmid DNA (1µg) was

transformed into log phase cells (OD_{600} = 0.7 – 1.0) and transformants were plated on a YPD plate containing 100µg/ml nourseothricin to select for cells containing the plasmid. The guide RNA plasmid and donor DNA were transformed into yeast expressing the Cas9 plasmid using a high-efficiency lithium acetate transformation method[20] as described in the Supplementary Material. Log phase cells successfully transformed with the Cas9 plasmid were transformed with 1µg of guide RNA plasmid and 5µg of donor DNA. The transformants were plated on a YPD plate containing 100µg/ml nourseothricin and 500µg/ml hygromycin to select for cells containing both the Cas9 plasmid and the guide RNA plasmid.

Diagnostic test for successful genome editing

Genomic DNA was isolated from *snf2-K798A* candidates with glass beads and phenol:chloroform:isoamyl alcohol extraction as described in the Supplementary Material. To distinguish *SNF2* from the *snf2-K798A* genotypes, PCR products were digested separately with KpnI and BamHI as described in the Supplementary Material. Samples not digested with KpnI but digested with BamHI were submitted to Azenta/GENEWIZ (Azenta US, Inc., South Plainfield, NJ, USA) for Sanger sequencing to confirm the presence of the desired mutations.

Spot assay to assess growth phenotypes

Log phase cultures from wild-type or *SNF2* mutant yeast strains were diluted four times with ten-fold serial dilutions and spotted on YPD + agar media to assay for growth phenotypes as described in the Supplementary Material. Plates were incubated at either room temperature, 30°C, 37°C, or 39°C overnight and imaged followed by scoring of the mutant strains for growth in comparison to the wild-type strain.

Background preparation by instructors to start the course

The pHPH-13myc, Cas9-NAT, and gRNA-ura-HYB plasmids were transformed into DH5α cells (New England Biolabs: C2987H) and plated on LB media containing 100µg/ml ampicillin (Amp).

An aliquot of the overnight cultures from single colonies were mixed with 50% glycerol (v/v) for long-term storage of the bacterial strain at -80°C. The remainder of the culture was used for isolation of large quantities of plasmid using a QIAprep Spin Miniprep Kit (QIAGEN Cat. No. #27104). All other reagents were designed and prepared as described in the Supplementary Material.

Results

Course-based Research Experience (CURE) Organization and Objectives

This CURE has been taught in an eight-week long biochemistry course (BC406B) at Colorado State University. Students attended twice a week for three-hour laboratory periods and are comprised of junior and senior undergraduate students who have taken pre-requisite courses in cell biology, genetics, and biochemistry lecture and laboratory courses. On days when students performed an experimental procedure, class began with a brief introduction to the protocol lasting 20 minutes or less, followed by a short daily check-in to assess mastery of concepts fundamental to the previous class period as well as the upcoming procedure. Some class periods did not involve bench work and instead focused on background material on CRISPR-Cas9, or the yeast genes involved in the course. As many of the experiments required an overnight yeast culture, we opted to perform those steps the afternoon before class periods and offered students the opportunity to attend each of these sessions.

This course has four primary learning objectives as outlined below.

> Learning Objective #1: Students will be able to describe the fundamental aspects of CRISPR-Cas9 genome editing and the steps required in implementation.

Learning Objective #2: Students will be able to maintain a research notebook that can be used by the research lab from which their project originated.

Learning Objective #3: Students will be able to accurately execute several biochemical, genetic, molecular and cell biology experimental techniques.

Learning Objective #4: Students will be able to interpret data from experimental techniques and articulate the relevance of these data to their peers.

To accomplish these learning objectives, students performed a project comprised of four experimental modules (Table 4.1). The overall goal of the course is to introduce a K798A point mutation in the endogenous *SNF2* gene using CRISPR-Cas9 technology. *SNF2* encodes the catalytic subunit of the SWI/SNF chromatin remodeling complex, and this mutation eliminates the ATPase activity of the Snf2 protein[21]. Module I begins with the preparation of the reagents required for transformation of wild-type yeast with the genome editing machinery for both CRISPR-Cas9 based and one-step homologous recombination approaches. In Module II, students transform a starting yeast strain sequentially with the plasmids expressing Cas9, and then the guide RNA plasmid along with donor DNA template, and transformants are selected for antibiotic resistance. In Module III, yeast genomic DNA is isolated and diagnostic tests involving PCR and restriction digestion reactions are performed to identify candidate yeast strains with the desired mutations, which are confirmed by Sanger sequencing. In Module IV, students perform assays to assess any changes in the growth phenotype of their engineered strains.

Table 4.1. Experimental Modules overview. Students perform a project that follows four experimental modules. The days associated with each experimental module are outlined in the table.

Experimental Module	Days
(I) Preparation of CRISPR-Cas9 reagents	• Day 2: Guide RNA plasmid engineering • Day 3: Gel electrophoresis, KLD treatment and transformation in competent *E. coli* cells • Day 4: Plasmid isolation for sequencing • Day 5: Sequencing analysis • Day 6: Yeast media preparation • Day 8: Donor DNA amplification
(II) CRISPR-Cas9 genome editing	• Day 7: Yeast transformation with Cas9 plasmid • Day 9: Yeast transformation with guide RNA plasmid and donor DNA • Day 10: Colony purification, analyze transformation efficiency
(III) Test for successful mutation	• Day 11: Genomic DNA isolation and diagnostic PCR • Day 12: Restriction enzyme digestion of PCR product and DNA sequencing
(IV) Assess growth phenotypes	• Day 13: Assay yeast strains for growth phenotypes • Day 14: Data analysis and preparation for presentations

In addition to these experimental modules, students demonstrate their ability to interpret data from experimental techniques by giving two presentations. Students are tasked with sharing primary literature describing a clinical trial using CRISPR-Cas9. Students describe how the genome editing was used to treat the disease and what experiments were performed to test if the editing was successful. At the end of the course, students give a final presentation where they share their results and interpretations from their project. These presentations provide students with opportunities for peer-to-peer learning by facilitating discussions with each other, as well as developing transferable skills in communications.

Preparation of reagents required for genome editing

Students began the course by performing fundamental techniques like PCR, bacterial transformation, and plasmid isolation to prepare the reagents required for CRISPR-Cas9

mediated genome editing. The primary objective was to engineer both the guide RNA (gRNA)-expressing plasmid that targets Cas9 to the yeast *SNF2* gene as well as produce large amounts of donor DNA encoding the *snf2-K798A* mutation (Figure 4.1A). To accomplish this, students performed PCR to amplify the entire starting guide RNA plasmid (gRNA-ura-HYB) with primers that replace the 20bp gRNA recognition sequence with a sequence targeting the *SNF2* locus (Figure 4.1B). To prepare the donor DNA for CRISPR-Cas9 genome editing, students perform PCR to amplify the ~1kb linear DNA fragment encoding the *snf2-K798A* mutation. This fragment also contains silent mutations that remove the guide RNA target sequence, eliminate a KpnI recognition sequence, and introduce a BamHI recognition sequence (Figure 4.1C).

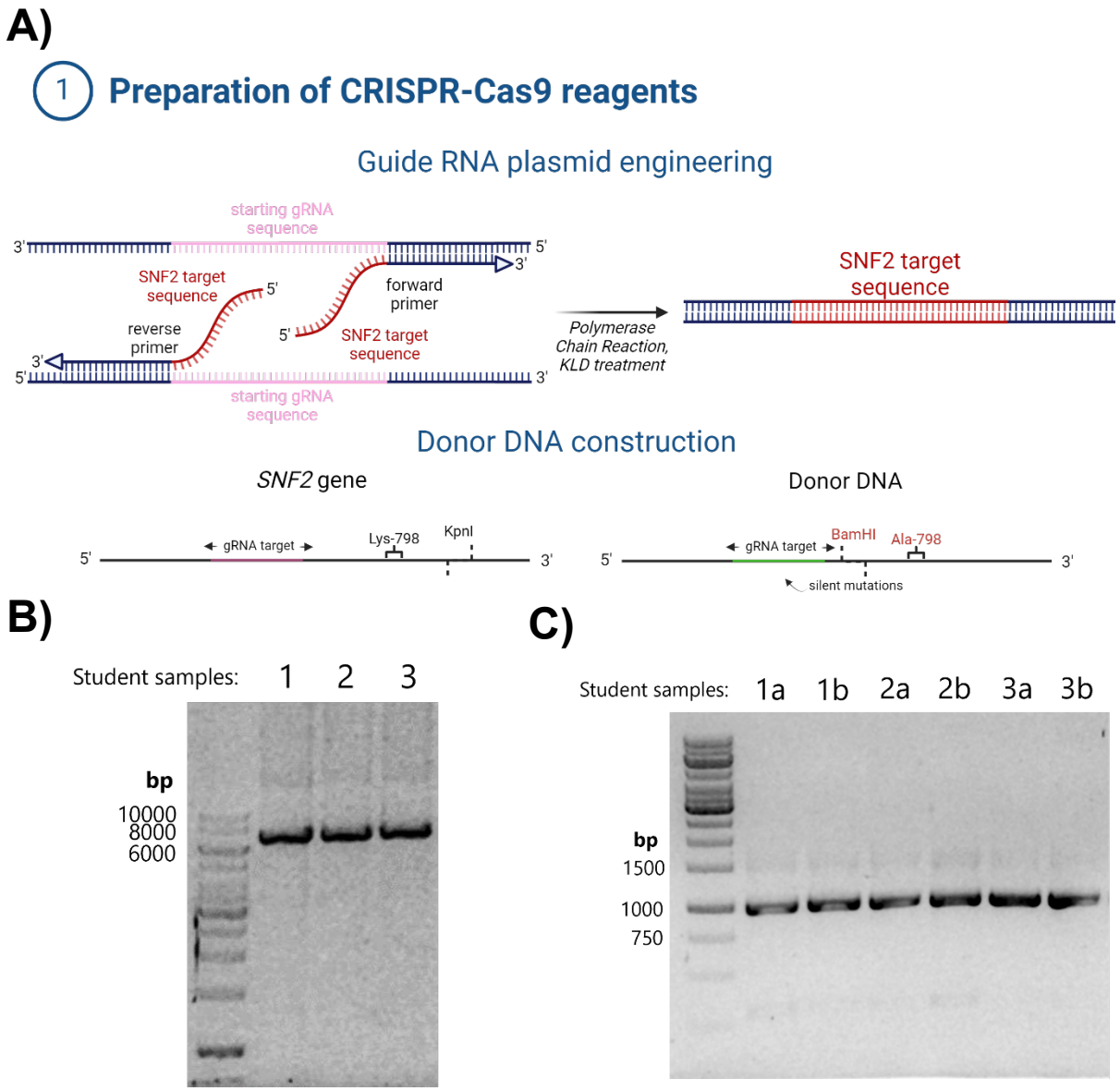


Figure 4.1. Preparation of CRISPR/Cas9 reagents. (A) Schematic overview of guide RNA plasmid and donor DNA design. 20bp of the gRNA-ura-Hyg plasmid containing the gRNA target sequence is replaced with a 20bp target within the *SNF2* gene by PCR. Donor DNA was constructed by modifying a ~1kb region of *SNF2* genomic sequence to encode the K798A mutation, remove the endogenous KpnI site, create a unique BamHI site, and make silent mutations in the gRNA target. Image created with BioRender.com. (B) Representative agarose gel electrophoresis of 3 students' guide RNA plasmid engineering PCR reaction. PCR using forward and reverse primers diagrammed in (A) amplify the entire gRNA-ura-Hyg plasmid and replace the 20bp gRNA target sequence. (C) Representative agarose gel electrophoresis of students' donor DNA PCR amplification. Donor DNA is amplified by PCR to generate sufficiently high quantities of DNA (>5µg) for genome engineering (2 reactions per each of 3 students).

After performing PCR to replace the guide RNA target sequence in the starting gRNA plasmid, students use a Kinase-Ligase-DpnI (KLD) enzyme mix to circularize their PCR products and remove the methylated plasmid template isolated from bacteria. The resulting circular plasmids are transformed into chemically-competent *E. coli* cells, diluted, and plated with dilutions on Luria Broth (LB) media containing ampicillin to select for successful transformation (Figure 4.2A). To confirm the presence of the sequence targeting *SNF2* in the gRNA plasmid, students isolate plasmid DNA from bacterial cultures and submit an aliquot for Sanger sequencing. Afterwards, students analyze their sequencing results by performing a sequence alignment using BLAST® comparing their starting plasmid sequence to the sequence of their newly isolated engineered plasmid. The students who successfully isolated plasmids from ampicillin-resistant transformants were all able to successfully detect the *SNF2* target sequence in their plasmids (Figure 4.2B).

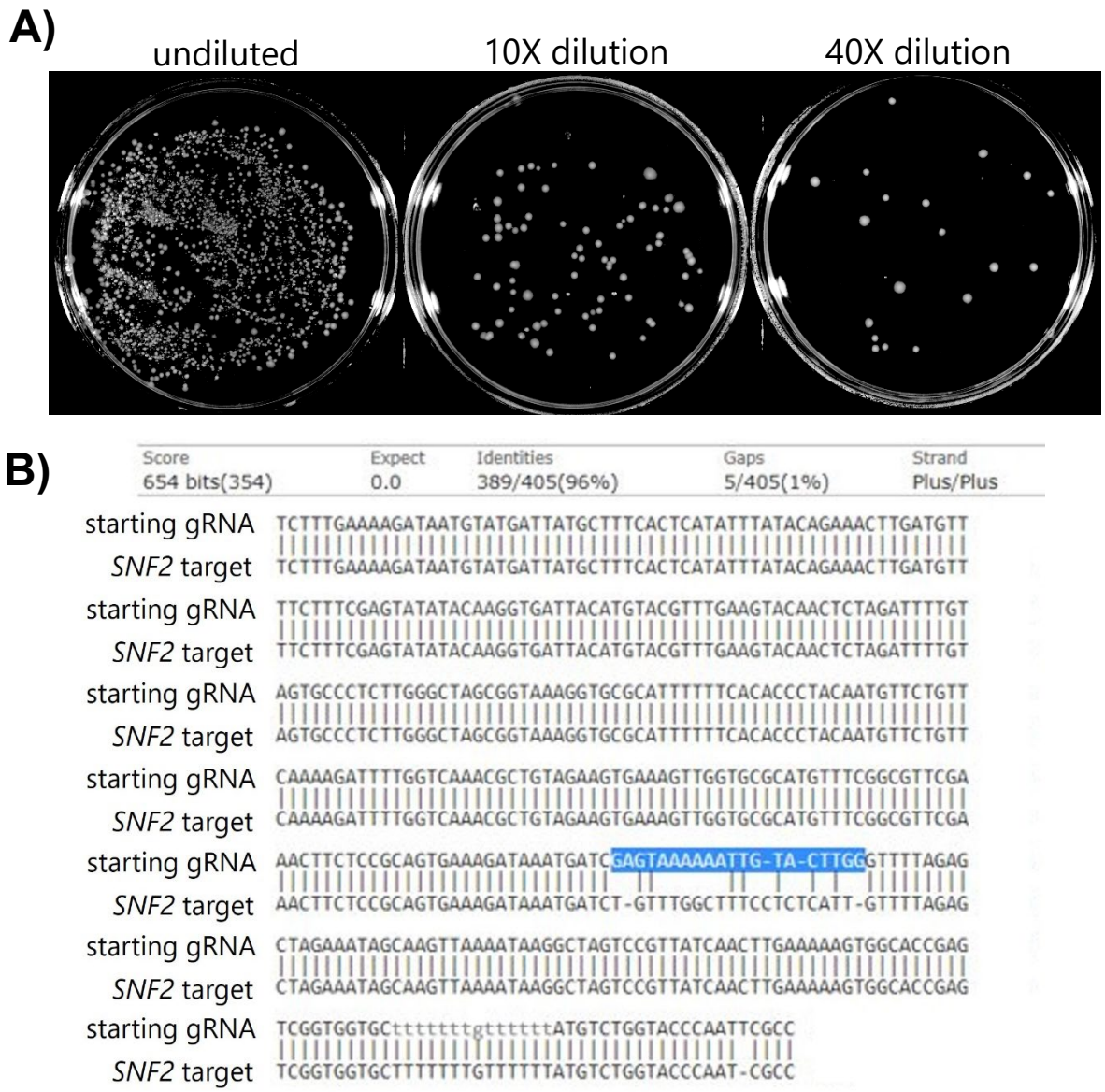


Figure 4.2. Guide RNA plasmid bacterial transformation and sequence confirmation. (A) Images of LB + ampicillin plates plated with dilutions of *E. coli* transformed with gRNA-snf2-HPH. NEB 5-alpha chemically competent cells (New England Biolabs) were transformed with engineered gRNA-snf2-HPH plasmid containing an ampicillin resistance cassette. (B) BLAST alignment between gRNA-ura-Hyg starting plasmid and successfully generated gRNA-snf2-HPH plasmid. Single colonies were isolated and grown overnight in LB + ampicillin prior to plasmid DNA isolation. Plasmids were sequenced by Sanger sequencing and compared to the starting plasmid sequence. The region in blue containing a different sequence corresponds to the region modified through the PCR reaction.

Transformation of yeast with genome editing machinery

Students performed two sequential lithium acetate transformations in yeast to first introduce the Cas9 plasmid, followed by the engineered gRNA plasmid with the donor DNA (Figure 4.3A). Students transformed the starting yeast strain (LZ0-1) with a Cas9-expressing plasmid containing a nourseothricin-resistant cassette. As a control, students prepared reactions without plasmid DNA. Reactions were plated on YPD media containing nourseothricin to positively select for the successful transformation of the Cas9 plasmid (Figure 4.3B). The lithium acetate protocol is straightforward, and all students observed colonies on the nourseothricin-containing media when the plasmid was present in the transformation mix, and no colonies on plates with the negative control. After successfully obtaining nourseothricin-resistant cells, students performed a more arduous, but higher efficiency lithium acetate transformation procedure to co-transform their gRNA plasmid with a hygromycin-resistant cassette and donor DNA into cells expressing the Cas9 plasmid. Students also prepared transformation mixes without the guide RNA plasmid as a control. Reactions were plated on YPD media containing both nourseothricin and hygromycin to select for both plasmids (Figure 4.3C).

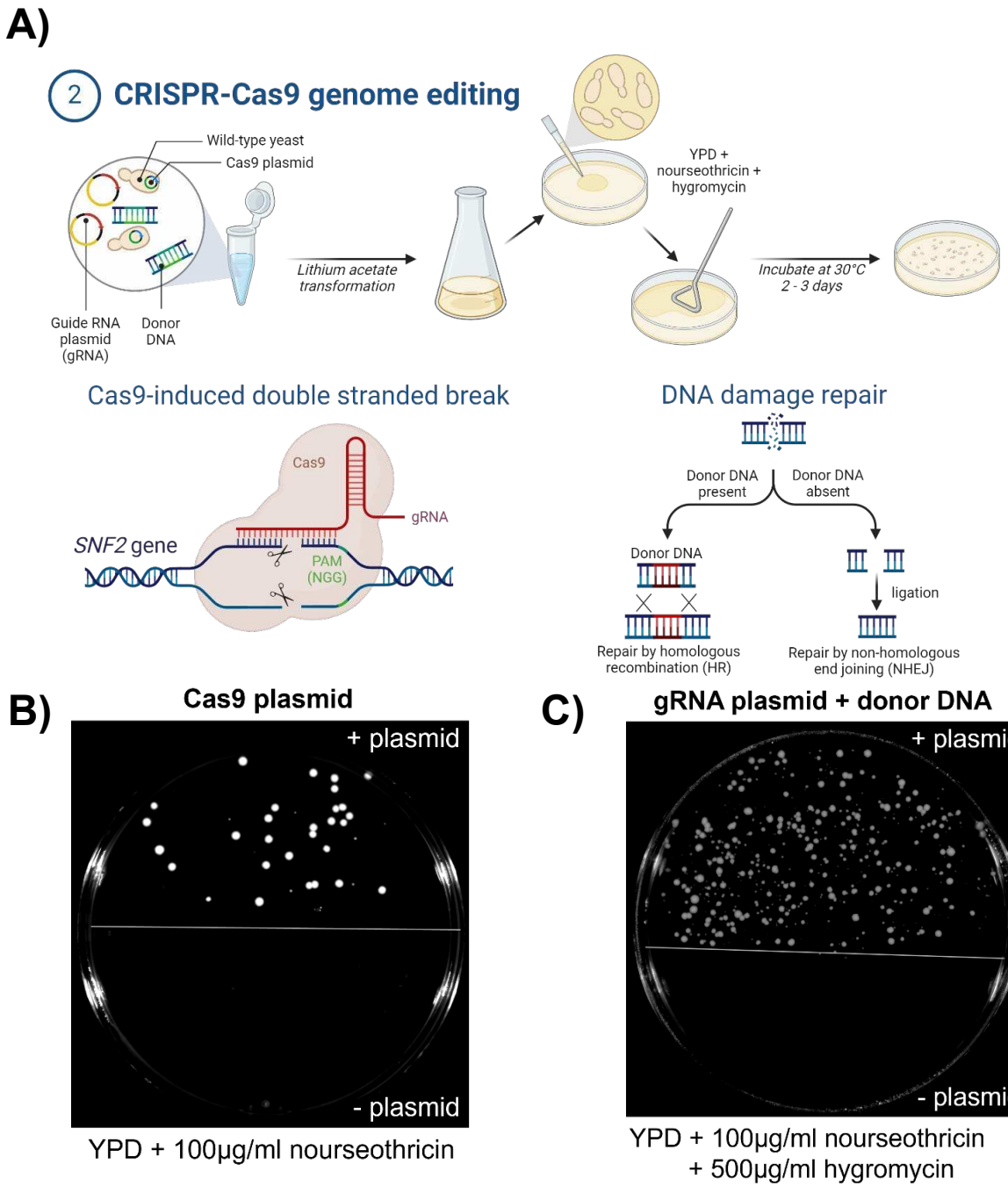


Figure 4.3. CRISPR-Cas9 genome editing of yeast *SNF2* gene. (A) Schematic overview of introducing CRISPR-Cas9 reagents into yeast for genome editing. Yeast with wild-type *SNF2* are first transformed with the Cas9 plasmid and cells resistant to nourseothricin are then transformed with the engineered guide RNA plasmid and donor DNA. Cas9 induces a double stranded break in the *SNF2* gene as targeted by the gRNA. If donor DNA is present, the break is repaired through homologous recombination of the donor. Image created with BioRender.com. (B) Colony formation after plating transformants with the Cas9 plasmid onto YPD with nourseothricin. (C) Colony formation after plating transformants with the guide RNA plasmid onto YPD with hygromycin and nourseothricin.

Test for successful mutation of the *SNF2* gene

We and others have previously observed that deletion of *SNF2* significantly reduces the growth rate of yeast cells[22, 23]. After yeast expressing Cas9 were transformed with the engineered gRNA plasmid and donor DNA, our students observed colonies with varied sizes on their plates with the *snf2-K798A* mutant candidates (Figure 4.3C). We hypothesized that the smaller colonies could be cells that successfully introduced the DNA containing the mutation by homologous recombination into their genome. To test for *SNF2* mutation in the *snf2-K798A* candidate cells, students performed a series of experiments to isolate genomic DNA from candidate cells, perform PCR to amplify their region of interest, and use a restriction enzyme digest diagnostic test to identify *snf2-K798A* candidates (Figure 4.4A). Since we were re-using primers that were used to amplify our donor DNA (which is “pure” since it was synthesized), it was important to determine how well they would work on genomic yeast DNA. We first tested a range of annealing temperatures and observed persistent amplification of non-specific products at 52°C and 55°C with total loss of amplification at 58°C (Figure C.1A). Occasionally, non-specific amplification can be caused by a single primer. To test this possibility, we repeated PCR using a 52°C annealing temperature with each primer individually. Omitting either the forward or reverse primer in the PCR revealed that neither oligonucleotide was responsible for driving the formation of the non-specific products observed in our PCR (Figure C.1B).

Students ultimately performed PCR using the existing primers to amplify a ~1kb fragment of the *SNF2* gene. As the mutations introduced in the donor DNA do not confer a change in size of this region, further testing was required to determine whether a *snf2-K798A* candidate yeast strain was created. To distinguish wild-type *SNF2* from *snf2-K798A*, students digested the PCR separately with KpnI and BamHI restriction enzymes. The donor DNA containing the K798A mutation was also designed to contain silent mutations to both eliminate an endogenous KpnI restriction enzyme cut site and introduce a BamHI site into the *SNF2* gene (Figure 4.1A).

Separate digests of the PCR product with KpnI and BamHI were successful in distinguishing DNA repaired with the *snf2-K798A* donor DNA from wild-type (Figure 4.4B). To confirm the presence of the *snf2-K798A* mutation and the lack of other accumulated mutations in *SNF2*, students submitted the PCR amplified DNA from the putative *snf2-K798A* strain for Sanger sequencing. Alignment to the wild-type *SNF2* sequence using BLAST® confirmed the presence of the donor DNA in the *SNF2* gene (Figure 4.4C). Intriguingly, we observed no correlation between colony size after gRNA plasmid and donor DNA transformation and success rate of obtaining the *snf2-K798A* mutant. An unexpected finding from this process was the presence of yeast cut with both BamHI and KpnI (Figure C.2). Sanger sequencing confirmed these fragments contain the engineered BamHI cut site, but also have the wild-type DNA encoding lysine-798 and the endogenous KpnI cut site (Figure C.3). This is indicative that the crossover event that occurred during homology-directed repair was in the region between the BamHI cut site and the codon encoding lysine-798. While the diagnostic tests with KpnI and BamHI do not provide definitive proof of successful engineering of the *snf2-K798A* yeast, it greatly reduces the number of samples sent for Sanger sequencing.

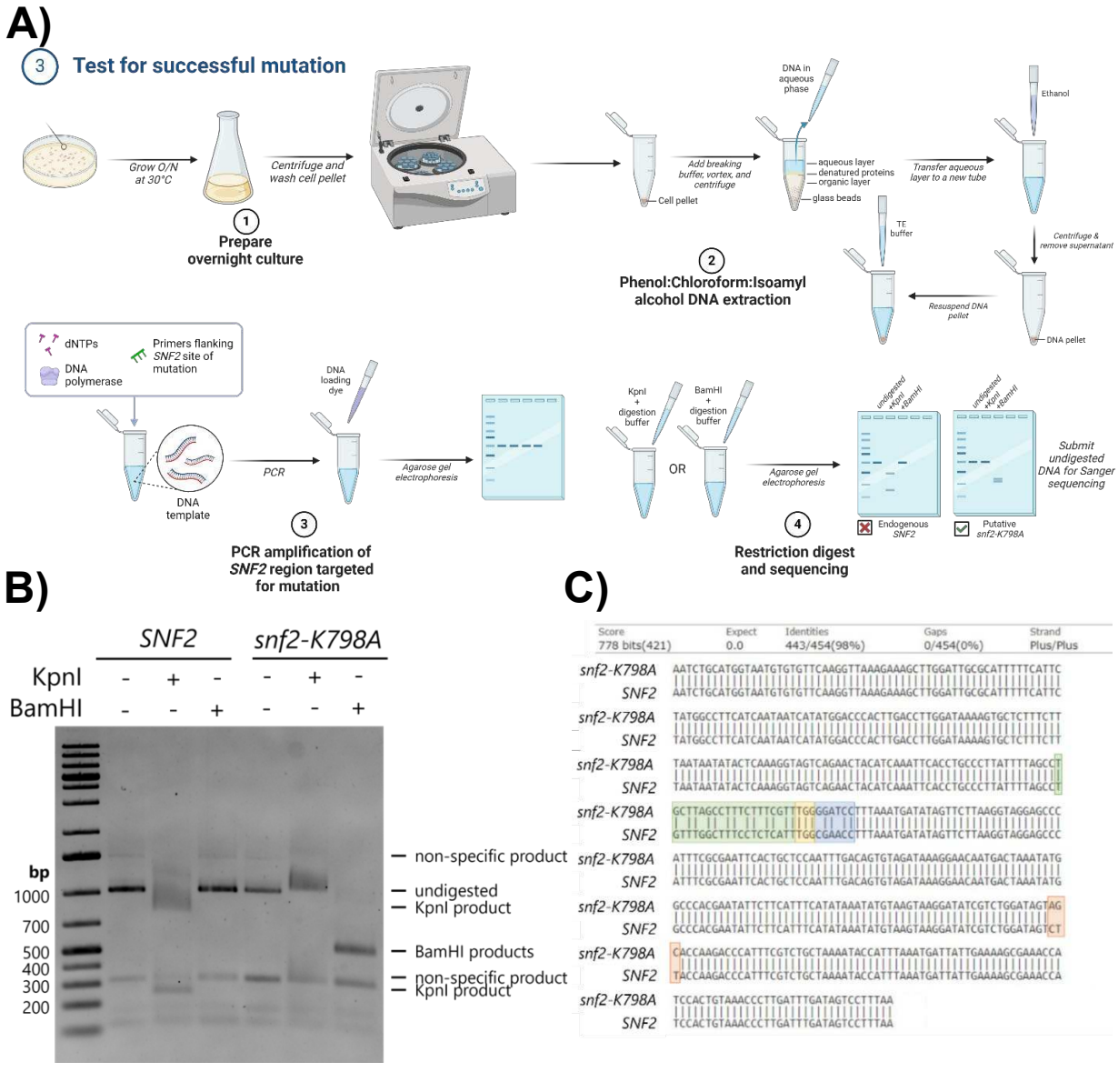


Figure 4.4. Tests for successful mutation of the *SNF2* gene. (A) Schematic overview of diagnostics to test for successful mutation of *SNF2*. Single yeast colonies are grown to stationary phase and extracted for genomic DNA. Genomic DNA is amplified by PCR using primers flanking the site of mutations in *SNF2* and the resulting product is run on an agarose gel. The product is digested with KpnI or BamHI to test if the donor DNA was recombined at *SNF2*. Samples digested with BamHI but not KpnI are submitted for Sanger sequencing. Image created with BioRender.com. (B) Representative restriction enzyme digest of PCR product of genomic DNA from *SNF2* and *snf2-K798A* cells. DNA with the KpnI cut site digested with KpnI yields 243bp and 775bp products, while DNA with the BamHI cut site digested with BamHI yields 523bp and 493bp products. (C) BLAST alignment between sequenced PCR product from a putative *snf2-K798A* yeast and the wild-type *SNF2* gene. Differences in sequence correspond to silent mutations in gRNA recognition sequence (green), introduction of the K798A mutation (orange), and addition of BamHI cut site (blue). The PAM sequence is unchanged (yellow).

Assessment of growth phenotypes in *snf2-K798A* yeast

In the final experimental procedure of the course, students spotted serial dilutions of their mutant yeast (*snf2-K798A*) and a control strain (*SNF2* + Cas9) onto YPD media to test for any growth defects in the genome-edited strain. Students spotted onto separate YPD plates and incubated them at different temperatures and the plates were imaged after 1 – 1.5 days (Figure 4.5A). The yeast spot assay demonstrated that the *snf2-K798A* containing yeast strain grew significantly slower than the control strain across the temperatures tested, including the ideal growth temperature of 30°C (Figure 4.5B). Additionally, the *snf2-K798A* strain exhibited temperature sensitivity, both at cold (22°C) and hot (39°C) temperatures.

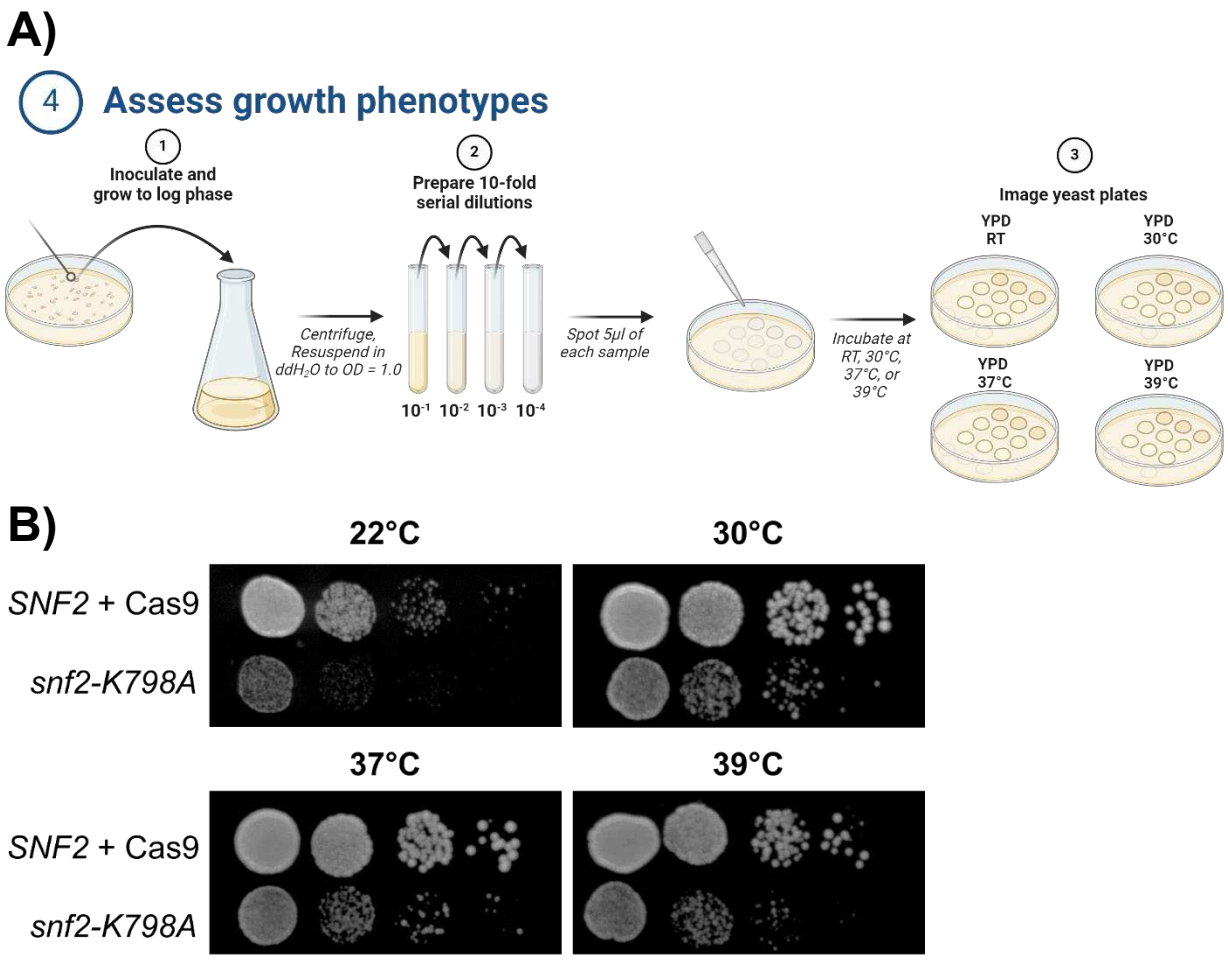


Figure 4.5. Assessment of growth phenotypes. (A) Schematic overview of the spot assay used to compare growth rates of yeast strains at different temperatures. Single yeast colonies are inoculated in a YPD culture and grown to log phase (OD600 = 0.6 - 1.0), pelleted, then resuspended in ddH2O. 10-fold serial dilutions are prepared and an aliquot of each is plated onto YPD media followed by incubation at a range of temperatures for 1-2 days. Image created with BioRender.com. (B) Spot assay from cells expressing either wild-type *SNF2* or snf2-K798A. Serial dilutions of *SNF2* + Cas9 and *snf2-K798A* cells were spotted onto YPD plates and incubated at room temperature (22°C), 30°C, 37°C, and 39°C for ~1.5 days.

Assessment of learning objectives

Over the course of the eight-week period, students are formally and informally assessed in a variety of ways on the daily learning objectives (Table 4.2). These daily learning objectives are associated with our course learning objectives. Prior to each class, students are instructed to

write a purpose and protocol for the experimental procedure to be performed during the class period. Each class period begins with a brief daily check-in to assess their knowledge on both the experimental technique and foundational concepts relevant to both the upcoming and previous experiments. During periods of down time during experiments, students are informally assessed on essential laboratory skills, such as how to pipette properly, perform sterile technique, and measure chemicals. Students are also tasked with keeping a notebook containing the methods, results, and conclusions from each class period. At the end of each lab period, students are instructed to record any observations and results from the class period in their notebook as well as write a conclusion to interpret their data. These notebooks are informally assessed throughout the semester, concluding with them being graded at the end of the course.

Table 4.2. Daily Experiments and Student Learning Objectives for each class period. The experiment and associated student learning objectives for each day of the CURE were communicated to the students in the syllabus presented at the start of class. The daily learning objectives are associated with the overall course learning objectives as outlined in the table.

Day	Experiment	Daily Student Learning Objectives	Associated Course Learning Objectives
Day 1	Introduction to the Course, Student Check-In, Polymerase Chain Reaction	• Be aware of laboratory safety and code of conduct. • Maintain a proper laboratory notebook. • Perform Polymerase Chain Reactions (PCR) and describe how it works. • Explain CRISPR-Cas9 viral defense system, how it is used in bacteria, and its discovery. • Compare NHEJ to HR repair as used in genome editing. • Access peer-reviewed web-based material on current human trials using CRISPR-Cas9 for human genome editing	**1, 2, 3**
Day 2	Agarose gel electrophoresis and guide RNA plasmid engineering	• Analyze PCR products on an agarose gel. • Describe the design and construct of guide RNA plasmids. • Define the function of the SWI/SNF complex.	**1, 2, 3, 4**
Day 3	Gel electrophoresis, KLD treatment and transformation in competent *E. coli* cells	• Locate information about yeast genes using the *Saccharomyces cerevisiae* genome database (SGD). • Define the function of the SWI/SNF complex. • Hypothesize how genome editing may impact the SWI/SNF complex. • Describe the function of each enzyme present in the KLD mix. • Transform plasmids into competent *E. coli* safely with sterile technique.	**1, 2, 3**

Day 4	Plasmid isolation for sequencing, donor DNA PCR amplification	• Describe the purpose of each buffer used in the Qiagen DNA extraction kit. • Extract DNA from bacterial cultures with sufficient yield and purity. • Explain how Sanger sequencing is used to assess the success of plasmid engineering.	**1, 2, 3**
Day 5	Sequencing analysis	• Analyze DNA sequencing results to assess the success of our plasmid engineering.	**2, 4**
Day 6	Yeast media preparation	• Describe the composition of yeast rich media and how antibiotics can be used for selection. • Use sterile technique to prepare yeast media and revive yeast from glycerol frozen stocks.	**1, 2, 3**
Day 7	Yeast transformation with Cas9 plasmids	• Use sterile technique to measure growth of yeast cultures and transform yeast cells with plasmids. • Describe how CRISPR-Cas9 can be employed to edit genomic information. • Explain the underlying principles behind lithium acetate yeast plasmid transformation, including the role of each reagent used in the procedure.	**1, 2, 3**
Day 8	Donor DNA PCR amplification	• Scale up PCR and demonstrate mastery of this technique. • Design a donor DNA sequence for introducing a desired mutation after repairing Cas9-mediated cleavage.	**1, 2, 3**
Day 9	Yeast transformation with guide RNA plasmid and donor DNA	• Perform high efficiency yeast transformations consistently across numerous samples. • Give a concise description on how CRISPR-Cas9 mediated genome editing is accomplished.	**1, 2, 3**
Day 10	Colony purification, analyze transformation efficiency. Clinical trial presentations	• Calculate transformation efficiency from our experiments with the Cas9 plasmid. • Share experimental procedures and interpretations of data from primary literature in an interpretable manner.	**2, 4**
Day 11	Genomic DNA isolation and diagnostic PCR	• Harvest genomic DNA from yeast cell cultures using the phenol:chloroform:isopropyl alcohol (PCI) method. • Describe the purpose of each reagent used during the DNA extraction protocol.	**1, 2, 3**
Day 12	Restriction enzyme digestion of PCR product and DNA sequencing	• Perform a restriction enzyme digestion reaction. • Interpret PCR and restriction enzyme results to make conclusions about the effectiveness of previous transformations.	**1, 2, 3, 4**
Day 13	Assay yeast strains for growth phenotypes	• Use sterile technique to generate an array of yeast spots plated in different growth conditions.	**1, 2, 3**
Day 14	Data analysis and preparation for presentations	• Interpret PCR and restriction enzyme results to make conclusions about the effectiveness of previous CRISPR transformations. • Calculate transformation efficiency from experiments with the Cas9 and gRNA plasmids. • Assess the effect of mutation of the SWI/SNF complex on growth of yeast strains.	**2, 4**
Day 15	Final presentations	• Share student results and interpretations from the experiments through a presentation. • Answer questions regarding your project, including what you would have done differently and what you would do next if we had more time together.	**4**
Day 16	Student Check-Out, Lab Clean-up	• Ensure all unwanted samples are properly disposed of and samples to be saved from the course are properly labeled.	**2, 4**

Assignments were given to the students with the purpose of enhancing student understanding of CRISPR-Cas9 technology. One assignment was for students to demonstrate their understanding of how guide RNA is used to target Cas9 for genome editing by having them design their own reagents to target Cas9 to a different gene from those used in the class. Another required the students to count transformants from their CRISPR-Cas9 transformation results, a practice commonly performed in quantifying transformation efficiency. To expose students to the range of applications where CRISPR-Cas9 technology is being utilized, students were tasked with selecting a peer-reviewed, published paper describing a CRISPR clinical trial and share it with the rest of class; describing the disease treated, how CRISPR is employed in the clinical trial, the method for determining the success of the treatment, and conclusions/future directions. At the end of the course, students prepared final presentations to share their experiments, results, and conclusions from their project. This was meant to further simulate an authentic research experience, as this practice is common during meetings among research laboratory members. All daily check-ins and student assignments are provided in the instructor manual (Appendix D).

Early in the CURE (Daily Check-In 3), students provided feedback on which concepts they felt they understood well at this point as well as concepts that are still unclear to them. This was beneficial to take both their performance and perception of their performance into consideration to best tailor the learning experience for them during the rest of the semester. For example, when asked about a concept from our course that was still unclear to them, one student wrote “how to successfully run a PCR”. Another wrote, “following a detailed protocol where I had to fill in the numbers myself makes this harder”. While our students were juniors and seniors that had taken laboratory courses previously, most had never had an authentic laboratory experience before. As a result, it was understandable that the transition from traditional laboratory classes to a more independent course-based research experience would be challenging. This

experience was comprised of a series of complex experimental procedures and does not follow a conventional “easy bake oven” structure. As a result, it was critical for the students to have the opportunity to perform experiments, make mistakes, receive personalized feedback, and correct them with repeated experiments.

By the end of the eight-week period, all students were able to demonstrate mastery by performing fundamental experiments like PCR independently. The final daily check-in of the semester included a question asking students to identify the class period/experiment that they found to be the most helpful. One student wrote, “from working with *E. coli* and yeast to PCR, I thought [this CURE] was enjoyable and provided skills beyond this class that we can use”. Students also informally commented on their enjoyment of giving the two assigned presentations to the others. Students liked sharing the details of their selected clinical trial utilizing CRISPR-Cas9 in class.

Discussion

With its increased widespread applications and increased usage as an essential tool for molecular biologists, it is critical to provide undergraduate students with thorough instruction on CRISPR-Cas9 technology. To this end, we developed an eight-week long laboratory course targeted towards upper division undergraduates designed to introduce CRISPR-Cas9 genome editing technology and give them an opportunity to perform the process from start to finish. The primary focus was on providing an experience as close to an authentic research experience as possible. To this end, the CURE was designed to consist of multiple instances of the same experimental technique and incorporated sufficient time in the schedule for students to repeat failed experiments. As most students had no prior experience in a research lab, we wanted to

best prepare these students for success in the life sciences by providing ample opportunity for mastery of essential biochemistry techniques.

Course-based undergraduate experiences (CURE) like this are beneficial for student learning in a variety of ways. Student engagement in research experiences positively influences their persistence in science and have positive outcomes in conceptual understanding and skills[24]. Implementing CUREs can also help to make science more inclusive. For example, enrolling in a course can make interactions with research faculty much more approachable, especially for historically underrepresented students[25]. Many students, particularly first-generation students, may also not be as aware of the potential benefits of engaging with research experiences during their time in college[26].

This course was both a challenging but rewarding experience for our students. Students initially found working on an authentic research project to be intimidating, and we observed more instances of experimental mistakes. For example, we observed a situation where a student added the forward primer to their PCR tube twice, rather than adding the forward and reverse primer. We also noted instances in which steps were skipped during long protocols like the bacterial transformation, where students came to realize the importance of following directions and maintaining focus during experiments. As the course went on, we observed much fewer instances of mistakes like this, which we attribute to our personalized feedback and inclusion of repeated experiments. The most challenging experiments for the students were the guide RNA and donor DNA transformation and the isolation of yeast genomic DNA. Both procedures have time-consuming, multi-step procedures and require students to maintain focus. Fortunately, both cohorts of students were able to successfully accomplish the experimental objective of creating the *snf2-K798A* strain, and we believe the process of making mistakes and learning earlier in the semester played a large role in this success.

This course could be modified in several ways to be suitable for other instructors. The design of the CURE is generalized for the modification of any yeast gene. An instructor looking to implement a CURE on CRISPR-Cas9 can use our detailed instructions and course materials in the Supplementary Material to mutate a gene of interest and train students to do so. This CURE can also be shortened substantially by providing students with the CRISPR-Cas9 reagents up front, saving time on earlier experiments. Conversely, if given an entire sixteen-week semester, students could begin the course by designing the reagents used in the laboratory. While we provided an assignment where students designed the guide RNA required for targeting Cas9 to a different yeast gene, we did not have students design the donor DNA due to our eight-week time constraints. The design of CRISPR-Cas9 reagents and interpretation of results could also be the focus of a “dry lab” course. This could allow for students to have an accessible experience with CRISPR-Cas9 genome editing by not requiring a laboratory space, reagents and instrumentation.

REFERENCES

1. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science. 2007;315(5819):1709-12.
2. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337(6096):816-21.
3. DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM. Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. Nucleic Acids Res. 2013;41(7):4336-43.
4. Li J, Xu R, Qin R, Liu X, Kong F, Wei P. Genome editing mediated by SpCas9 variants with broad non-canonical PAM compatibility in plants. Mol Plant. 2021;14(2):352-60.
5. Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, et al. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell. 2014;159(2):440-55.
6. Vermersch E, Jouve C, Hulot JS. CRISPR/Cas9 gene-editing strategies in cardiovascular cells. Cardiovasc Res. 2020;116(5):894-907.
7. Newby GA, Yen JS, Woodard KJ, Mayuranathan T, Lazzarotto CR, Li Y, et al. Base editing of haematopoietic stem cells rescues sickle cell disease in mice. Nature. 2021;595(7866):295-302.
8. Zhan T, Rindtorff N, Betge J, Ebert MP, Boutros M. CRISPR/Cas9 for cancer research and therapy. Semin Cancer Biol. 2019;55:106-19.
9. Trudel L, Frenette M, Moineau S. CRISPR-Cas in the laboratory classroom. Nat Microbiol. 2017;2:17018.
10. Pieczynski JN, Deets A, McDuffee A, Lynn Kee H. An undergraduate laboratory experience using CRISPR-cas9 technology to deactivate green fluorescent protein expression in Escherichia coli. Biochem Mol Biol Educ. 2019;47(2):145-55.
11. de Waal E, Tran T, Abbondanza D, Dey A, Peterson C. An undergraduate laboratory module that uses the CRISPR/Cas9 system to generate frameshift mutations in yeast. Biochem Mol Biol Educ. 2019;47(5):573-80.
12. Sehgal N, Sylves ME, Sahoo A, Chow J, Walker SE, Cullen PJ, Berry JO. CRISPR Gene Editing in Yeast: An Experimental Protocol for an Upper-Division Undergraduate Laboratory Course. Biochem Mol Biol Educ. 2018;46(6):592-601.
13. Ruppel NJ, Estell LE, Jackson RI, Wolyniak MJ. An Undergraduate Research Project Utilizing CRISPR-Cas9 Gene Editing Technology to Study Gene Function in Arabidopsis thaliana. J Microbiol Biol Educ. 2019;20(2).
14. Adame V, Chapapas H, Cisneros M, Deaton C, Deichmann S, Gadek C, et al. An undergraduate laboratory class using CRISPR/Cas9 technology to mutate drosophila genes. Biochem Mol Biol Educ. 2016;44(3):263-75.
15. Jay M. Bhatt AKC. First Year Course-Based Undergraduate Research Experience (CURE) Using the CRISPR/Cas9 Genome Engineering Technology in Zebrafish. Journal of Microbiology & Biology Education. 2018;19:10-1128.
16. Martin A, Wolcott NS, O'Connell LA. Bringing immersive science to undergraduate laboratory courses using CRISPR gene knockouts in frogs and butterflies. J Exp Biol. 2020;223(Pt Suppl 1).
17. Thulluru A, Saad L, Nagah Abdou Y, Martin A, Kee HL. CRISPR in butterflies: An undergraduate lab experience to inactivate wing patterning genes during development. Biochem Mol Biol Educ. 2022;50(6):605-19.
18. Li S, Almeida AR, Radebaugh CA, Zhang L, Chen X, Huang L, et al. The elongation factor Spn1 is a multi-functional chromatin binding protein. Nucleic Acids Res. 2018;46(5):2321-34.

19. Schiestl RH, Gietz RD. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr Genet. 1989;16(5-6):339-46.
20. Gietz RD, Schiestl RH. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc. 2007;2(1):31-4.
21. Richmond E, Peterson CL. Functional analysis of the DNA-stimulated ATPase domain of yeast SWI2/SNF2. Nucleic Acids Res. 1996;24(19):3685-92.
22. Zhang L, Fletcher AG, Cheung V, Winston F, Stargell LA. Spn1 regulates the recruitment of Spt6 and the Swi/Snf complex during transcriptional activation by RNA polymerase II. Mol Cell Biol. 2008;28(4):1393-403.
23. Davie JK, Kane CM. Genetic interactions between TFIIS and the Swi-Snf chromatin-remodeling complex. Mol Cell Biol. 2000;20(16):5960-73.
24. Bell JK, Eckdahl TT, Hecht DA, Killion PJ, Latzer J, Mans TL, et al. CUREs in biochemistry-where we are and where we should go. Biochem Mol Biol Educ. 2017;45(1):7-12.
25. Hurtado S, Eagan MK, Tran MC, Newman CB, Chang MJ, Velasco P. "We Do Science Here": Underrepresented Students' Interactions with Faculty in Different College Contexts. J Soc Issues. 2011;67(3):553-79.
26. Bangera G, Brownell SE. Course-based undergraduate research experiences can make scientific research more inclusive. CBE Life Sci Educ. 2014;13(4):602-6.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The eukaryotic chromatin landscape is critical for proper gene expression yet presents a barrier for processes that require access to DNA, such as transcription. These barriers are overcome through the action of many factors, including histone chaperones Spn1, Spt5, Spt6, and FACT and transcription elongation factor Spt4. However, it is poorly understood how each contributes to this process. The extensive analysis of MNase-protected fragments reported in Chapter 2 established that the essential histone chaperone Spn1 preserves both nucleosomal and subnucleosomal structures over both promoters and open reading frames across the yeast genome. The findings presented in Chapter 3 supplement this work by demonstrating the extent to which Spn1 and other RNAPII-associated factors maintain nucleosome features over genes of varied characteristics. Chapter 4 describes a course-based undergraduate research experience (CURE) developed to introduce upper-level biochemistry students to techniques in yeast genome engineering in an authentic research setting. This chapter will highlight major conclusions and describe future directions for the work presented in this thesis.

Conclusions and Future Directions

The histone chaperone Spn1 preserves subnucleosomal structures at promoters and nucleosome positioning in open reading frames

The findings from Chapter 2 demonstrated that the essential histone chaperone Spn1 is critical for preserving both nucleosomal and subnucleosomal protections across the yeast genome. This was accomplished through extensive mapping of chromatin protections in yeast expressing *spn1*^{K192N} and *spn1*$^{141\text{-}305}$, two functionally distinct mutant alleles of Spn1. The MNase-seq approach utilized in this study facilitated this process by retaining fragments of a wide range of sizes, including very short (<60bp) protections. As a result, this analysis revealed that this function of Spn1 is critical for maintaining chromatin over nucleosome-depleted regions (NDRs), the 5' ends of genes, and the rest of the gene bodies, through distinct mechanisms.

The findings from this work revealed a previously unknown role of Spn1 in the maintenance of NDRs. While defects in chromatin in Spn1 mutants have been primarily characterized over gene bodies during transcription elongation, the *spn1*^{K192N} mutation clearly reduces subnucleosomal structures in the NDR. As this defect was present only in samples digested with high a MNase concentration, and not a low MNase concentration, *spn1*^{K192N} reduces the stability of protections within the NDR (Figure 2.3). While striking, this analysis alone does not reveal the identity of protections lost in cells expressing *spn1*^{K192N}.

A potential future direction of this work is to determine specifically which protections are lost in the NDRs in *spn1*^{K192N} cells. One avenue to follow up this finding comes from the observation that the loss of protection was visible even in the 80-170bp bin of fragments (Figure 2.2). As this window of fragments encompasses both hexasomes and nucleosomes, it remains unknown if an increased fragility of nucleosomes in the NDR is contributing to this process. One follow-up analysis that was done was to specifically assess changes in nucleosome protections in the

Spn1 mutant expressing strains by generating new heatmaps exclusively mapping reads 142-152bp in length from samples digested with high MNase (128U) over the gene list ranked by length utilized in Chapter 2 (Figure 5.1A). To determine if there is a difference in NDR protection in *spn1*^{K192N} in this bin of fragments, a difference heatmap was generated by subtracting the wild-type occupancy scores from the *spn1*^{K192N} and *spn1*$^{141-305}$ samples followed by mapping the difference scores over the gene list (Figure 5.1B). A similar striated pattern was observed in the difference heatmap over open reading frames, which showcases the shift in nucleosome positions described in Chapter 2. However, the loss of protection in the NDR previously seen in *spn1*^{K192N} cells is not present in this fragment size bin, indicating nucleosomes are unaffected in promoter regions of *spn1*^{K192N} cells.

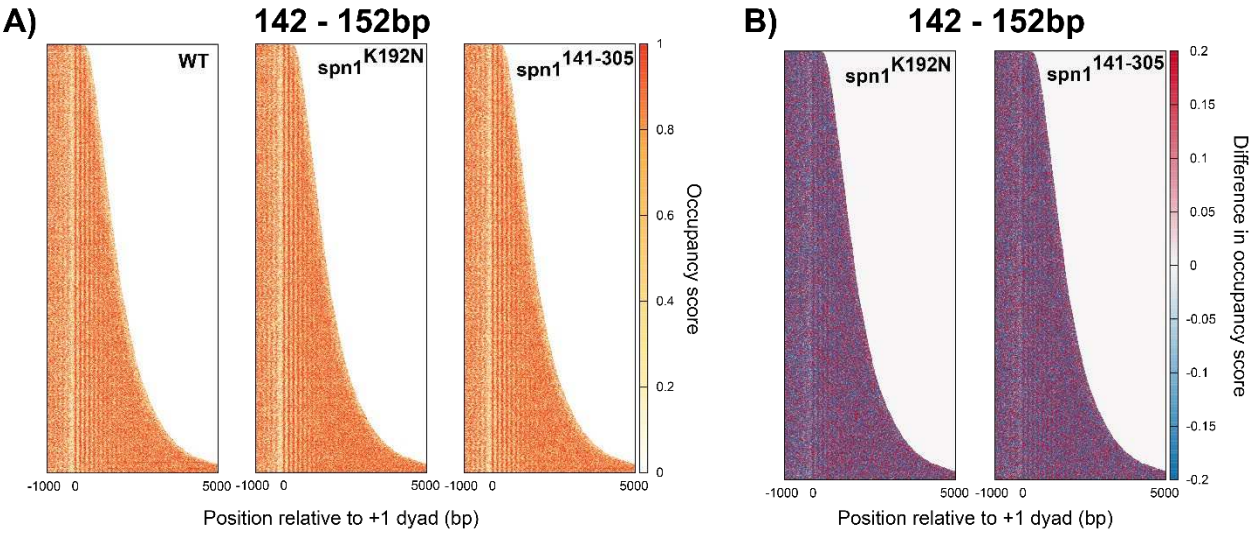


Figure 5.1. Nucleosome protections are largely unaffected in promoter regions in *spn1*^{K192N} and *spn1*$^{141-305}$ cells. (A) Heatmaps displaying MNase protected fragments (142-152bp) quantified by occupancy across 4688 genes ranked by length in wild-type (left), *spn1*^{K192N} (middle), and *spn1*$^{141-305}$ (right) expressing yeast.(B) Difference heatmap between wild-type and mutant Spn1 expressing yeast strains scored by changes in nucleosome occupancy. Genes are aligned by their +1 nucleosome dyad position and ranked by length. Scores are calculated by subtracting wild-type nucleosome occupancy data from mutant nucleosome occupancy. Regions in red are enriched for nucleosomes at that position while blue regions are depleted for nucleosomes.

While nucleosomes are generally depleted in NDRs, recent work has elucidated that some do contain fragile nucleosomes (FNs) which are highly sensitive to MNase[1-3]. While the prior analysis provided evidence that the loss in NDR stability does not arise from nucleosome

protections, it is possible that specifically NDRs containing FNs may be affected in *spn1*^{K192N} cells. To follow-up on this possibility, yeast genomic positions for FNs were obtained from a published dataset and the 142-152bp fragments from each sample digested with either high or low MNase concentrations were mapped to these sites (Figure 5.2). Both the metagene analysis and heatmaps demonstrate that at high concentrations of MNase, the level of 142-152bp MNase-protected fragments is unchanged over fragile nucleosome dyad locations. This provides evidence that fragile nucleosomes are not made even less stable in *spn1*^{K192N} cells, and the loss of protection in the 80-170bp fragment bin most likely arises from the loss of hexasomes, which are included in this bin.

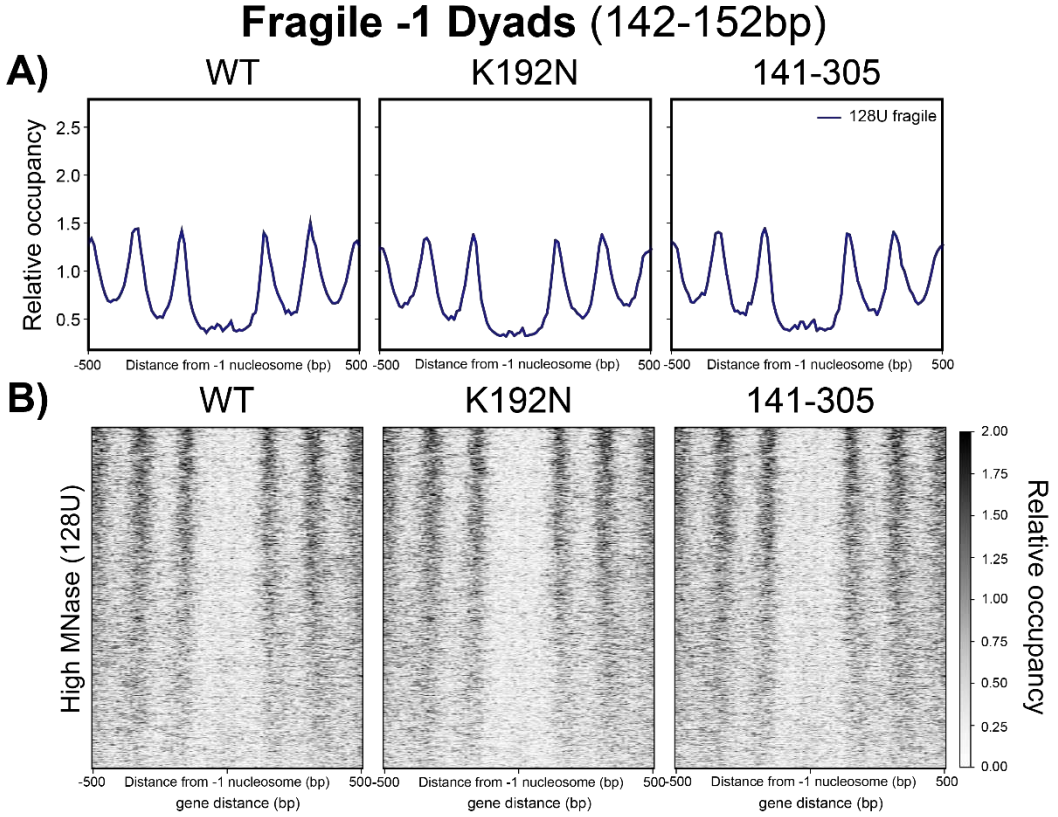


Figure 5.2. Fragile nucleosome protections remain largely unchanged in *spn1*^{K192N} and *spn1*$^{141-305}$ after digestion with both high and low MNase concentrations. Fragments protected from MNase by nucleosomes (142-152bp) were digested with either a high or a low MNase concentration. Data was plotted either as a (A) metagene analysis and as a (B) heatmap over 1953 mapped fragile nucleosome dyads.

A possible future direction of this work is to further explore the identity of very short protections (0-60bp) lost in the high MNase digested *spn1*^{K192N} samples. Analysis from Chapter 2 demonstrated that this loss occurs over general regulatory factor (GRF) Reb1, Rap1, and Abf1 binding sites, but this analysis alone isn't conclusive as to the identity of these lost fragments. As MNase-seq simply digests DNA not protected by proteins, regardless of their identity, the approach has limitations in the extent in which the protected fragments can be identified. These fragments can arise from a wide range of protein protections, such as by components of the pre-initiation complex (PIC), general transcription factors (GTFs) and GRFs as previously explored. Whole genome protein mapping approaches like chromatin immunoprecipitation followed by sequencing (ChIP-seq) or Cleavage Under Targets and Release Using Nuclease (CUT&RUN) could be performed to identify changes in the binding of these different candidates in wild-type Spn1 or *spn1*^{K192N} expressing cells. These results would supplement the MNase-seq work described in this thesis by identifying which factor(s) binding becomes more susceptible to MNase in *spn1*^{K192N}, underlying the loss of stability of the NDR in these cells.

The mechanism by which the *spn1*^{K192N} mutation leads to this defect remains unclear. As *spn1*^{K192N} mutant is defective for recruitment to the RNAPII elongation complex, likely in part through a disruption of interaction with Spt5 and Spt6[4, 5]. This interpretation has evolved over time, as the *spn1*^{K192N} mutation has long thought to reduce recruitment primarily through a loss in interaction with Spt6. The K192 residue resides within the central domain of Spn1, which contains the binding surface for Spt6[6]. However, its location is buried within the core of the Spn1 helical bundle, distal from the Spt6 interacting region[6, 7]. Additionally, recent cryo-EM studies with the elongating RNAPII complex in the *K. pastoris* species of yeast reveals a loop of Spt5 interacting with the core of Spn1, potentially forming a salt bridge with the homologous Spn1 K192 residue[4]. This supports a model that *spn1*^{K192N} cells experience reduced Spn1 recruitment to sites of transcription due in part to impaired Spn1-Spt5 interaction.

Regardless, defective Spn1 recruitment to RNAPII clearly produces widespread defects in chromatin. Previous work with other mutants that disrupt specifically the Spn1-Spt6 complex have implicated the complex in surveilling nucleosome assembly over genes[8, 9] and weakening this interaction decreases the quality of chromatin. While focused more on Spt6, another studied demonstrated that compromise of Spt6 function has been shown to produce aberrations in chromatin over gene bodies, namely the misincorporation of histone variant H2A.Z[10]. A simple interpretation from each of these data is that the Spn1-Spt6 complex surveils chromatin structure and the results from Chapter 2 support the notion that this function extends to the NDRs.

A major conclusion from the work in this chapter is that both *spn1*^{K192N} and *spn1*$^{141\text{-}305}$ produce downstream shifts in nucleosome and subnucleosome positioning over gene bodies. Intriguingly, only *spn1*$^{141\text{-}305}$ expression produced shifts in the +1 and +2 nucleosomes, revealing that the mechanism of chromatin maintenance at the 5' ends of genes is functionally distinct from that over the rest of the gene. These mutants are functionally distinct, as the truncated *spn1*$^{141\text{-}305}$ loses its histone- and nucleosome-binding capabilities while *spn1*^{K192N} has these regions intact. While it is currently unclear why these loss of function Spn1 mutants produce the shifts in chromatin reported in Chapter 2, an important future experiment is to quantify Spn1 occupancy over the yeast genome using ChIP-seq or CUT&RUN in wild-type Spn1, *spn1*^{K192N}, and *spn1*$^{141\text{-}305}$ expressing yeast. As the mechanism proposed in the discussion of this chapter hinges on potential changes in Spn1 recruitment to chromatin in yeast expressing the mutants, this follow-up experiment is a critical one to perform.

RNAPII-associated factors maintain nucleosome features over the yeast genome

The work presented in Chapter 3 provides a more comprehensive view of the contributions of Spn1 as well as other RNAPII-associated factors Spt4, Spt5, Spt6, FACT on preserving

chromatin structure across the yeast genome. This was accomplished by incorporating datasets from yeast expressing different mutant alleles of each factor into a uniform MNase-seq analysis pipeline with emphasis on their maintenance of key features of nucleosome occupancy, positioning, and NDR width. Additionally, this study identified correlations between these factors and the extent to which their function is important at genes ranked by both RNAPII occupancy as a proxy for expression, and gene length. As a result, the analysis implicated all these factors except Spt5 and RNAPII itself in the maintenance of nucleosome positioning, as previously observed[9, 11-16]. This work also demonstrated for the first time that maintenance of nucleosome positioning in each of these factors becomes increasingly critical with both increases in RNAPII occupancy and increase in gene length (Figure 3.3).

The reason why mutation in Spn1, Spt4, Spt6, FACT, and RNAPII produces defects in nucleosome positioning remains unknown. Each of these factors physically engage with RNAPII during transcription elongation, with Spn1 and Spt4 positioned at the tip of the RNAPII clamp facing the downstream DNA[4]. In contrast, while the Spt6 N-terminal helices that bind Spn1 are also proximal to the downstream DNA, the main body of Spt6 is docked near the upstream DNA exit channel[4]. FACT subunits Spt16 and Pob3 are shown to directly bind the downstream nucleosome in the recently cryo-EM structures[4]. As each of these factors all are engage with RNAPII and defects in each can lead to similar downstream shifts in nucleosome position, it is likely that they each participate in the same process of transient nucleosome disassembly prior to RNAPII passage and the subsequent nucleosome reassembly in the wake of RNAPII. Others have posed that this defect in passage through nucleosomes may produce an elongation complex that transcribes through chromatin more slowly than in wild-type cells, which is what produces mispositioned nucleosomes[12]. The analysis from Chapter 3 demonstrates that expression of the *rpb1-1* mutant allele of Rpb1 produces a comparable shift in nucleosomes.

Additionally, anchoring away of Rpb1 produces a similar shift in nucleosome positioning, excacerbated at highly expressed genes[17].

One future direction to test the model that the reason why mutation in these factors produces a change in nucleosome position is to perform new MNase-seq analysis using yeast expressing mutations in RNAPII known to reduce its rate of transcription. If this mutant produces a comparable shift in nucleosome positioning, it supports the notion that these mutations may be slowing the rate of transcription elongation through nucleosomes. To begin assessing this possibility, MNase-seq experiments can be performed from cells expressing either a fast or slow mutant of Rpb1. The E1103G mutation produces a faster, lower fidelity enzyme by stabilizing a triple α-helix of the polymerase trigger loop and bridge helix[18]. The H1085Y mutation disrupts binding with the β-phosphate of nucleotide triphosphate, slowing the rate of elongation[19]. A preliminary experiment has been performed with these mutants by a collaborator, which indicated that expression of the slow mutant produces a downstream nucleosome positioning shift (data not shown). However, this experiment lacked sufficient controls and needs to be repeated with more rigor to provide more conclusive results.

In contrast to changes in nucleosome positioning, changes in nucleosome occupancy were observed in cells expressing mutant alleles from each histone chaperone and RNAPII itself. Most of the histone chaperone mutants exhibited a loss in nucleosome occupancy while mutations in Rpb1 resulted in a nucleosome occupancy increase. Intriguingly, expression of the *pob3-Q308K* mutant allele similarly produced an increase in nucleosome occupancy, implicating FACT has both positive and negative regulatory roles in maintaining this chromatin feature. An additional finding reported in Chapter 3 is that most occupancy changes did not correlate with genes ranked by Rpb3 occupancy or gene length, indicating that these factors maintain nucleosome occupancy over all genes, regardless of length or expression.

The MNase-seq analysis also examined the role that these RNAPII-associated factors play in maintenance of NDR width, which is largely thought to be controlled by other factors, such as chromatin remodelers. Some mutations in histone chaperones exhibited changes in NDR width, with opposing roles revealed between Spt6 and both Spt5 and RNAPII. Mutation in Spt6 produced wider NDRs while mutations in Spt5 and RNAPII produce narrower NDRs. This provides more evidence that the role of RNAPII-associated factors extends beyond transcribed regions and requires further work to determine the role of these factors in maintaining normal NDR architecture. An important caveat is that investigation of the NDR in these datasets is limited to primarily mononucleosomal protections, as fragments of other lengths were discarded during sequencing library preparation in all studies except the MNase-seq experiment described in Chapter 2. Spn1 maintains primarily subnucleosomal structures in the NDR, whose protected fragments are largely absent from the other samples analyzed in this study. Thus, future MNase-seq experiments could be performed in yeast expressing these other factor mutant alleles to assess if subnucleosomal structures are also maintained in the NDR by these other RNAPII-associated factors.

While this study provides new insights into the relationships between these factors and their respective roles on maintaining specific features over the yeast genome, further work is required to examine these roles quantitatively. With respect to nucleosome positioning, the work described in Chapter 2 utilized a custom pipeline consisting of mapping of MNase-protected fragment midpoints to the genome which enabled quantification of nucleosome positioning shift by measuring occupancy at alternative rotational positions. Mapping of the fragment midpoints from these other datasets may similarly allow for quantitative measurements utilizing this approach, but such analysis requires starting with the raw sequencing reads rather than the bigWig files. Additionally, other algorithms exist that enable quantification of changes in nucleosome features, such as DANPOS2, which counts the number of nucleosomes with

changes in positioning, occupancy, and fuzziness[20, 21]. However, application of this algorithm only works if bigWigs were generated directly from the wiggle file type, which was not the case for many of the samples utilized in this analysis. Thus, a future direction is to perform the entire MNase-seq analysis starting with the raw sequencing reads to enable both downstream strategies of quantifying changes in chromatin features. While each of the samples incorporated into this study contained their own internal controls, repeating the analysis from the raw sequencing data ensures that the steps prior to bigWig generation, namely fastq reads processing and mapping to the genome, is performed identically. As inclusion of each of these steps drastically increases the time required to analyze each dataset, this study was conducted first as a survey.

An undergraduate research experience in CRISPR-Cas9 mediated eukaryotic genome editing to teach fundamental biochemistry techniques

With its increased widespread applications and increased usage as an essential tool for molecular biologists, it is critical to provide undergraduate students with thorough instruction on CRISPR-Cas9 technology. As described in Chapter 4, we developed an eight-week long course-based undergraduate research experience (CURE) for upper division undergraduates designed to introduce CRISPR-Cas9 genome editing technology and give them an opportunity to perform the process from start to finish. This course was designed to provide an experience as close to an authentic research experience as possible.

Implementing this course to two cohorts of students was incredibly insightful. Students initially found working on an authentic research project to be intimidating, and many instances of experimental mistakes were observed in the early stages of the course. As the course progressed, students made far fewer mistakes, which we attribute to our personalized feedback and inclusion of repeated experiments.

While the course can be implemented as is into other classrooms using the tools described in the instructor manual (Appendix D), this course could be modified in several ways to be suitable for other instructors. An important feature included in the design of this CURE is that it is generalizable for the modification of any yeast gene. This CURE can also be shortened substantially by providing students with the CRISPR-Cas9 reagents up front, saving time on earlier experiments. Conversely, if given an entire sixteen-week semester, students could begin the course by designing the reagents used in the laboratory. This could include the involved procedure of designing donor DNA, which we were unable to do in our CURE based on time constraints. The design of CRISPR-Cas9 reagents and interpretation of results could also be the focus of a “dry lab” course. This could allow for students to have an accessible experience with CRISPR-Cas9 genome editing by not requiring a laboratory space, reagents and instrumentation.

REFERENCES

1. Henikoff JG, Belsky JA, Krassovsky K, MacAlpine DM, Henikoff S. Epigenome characterization at single base-pair resolution. Proc Natl Acad Sci U S A. 2011;108(45):18318-23.
2. Chereji RV, Ocampo J, Clark DJ. MNase-Sensitive Complexes in Yeast: Nucleosomes and Non-histone Barriers. Mol Cell. 2017;65(3):565-77 e3.
3. Kubik S, Bruzzone MJ, Jacquet P, Falcone JL, Rougemont J, Shore D. Nucleosome Stability Distinguishes Two Different Promoter Types at All Protein-Coding Genes in Yeast. Mol Cell. 2015;60(3):422-34.
4. Ehara H, Kujirai T, Shirouzu M, Kurumizaka H, Sekine SI. Structural basis of nucleosome disassembly and reassembly by RNAPII elongation complex with FACT. Science. 2022;377(6611):eabp9466.
5. Zhang L, Fletcher AG, Cheung V, Winston F, Stargell LA. Spn1 regulates the recruitment of Spt6 and the Swi/Snf complex during transcriptional activation by RNA polymerase II. Mol Cell Biol. 2008;28(4):1393-403.
6. McDonald SM, Close D, Xin H, Formosa T, Hill CP. Structure and biological importance of the Spn1-Spt6 interaction, and its regulatory role in nucleosome binding. Mol Cell. 2010;40(5):725-35.
7. Pujari V, Radebaugh CA, Chodaparambil JV, Muthurajan UM, Almeida AR, Fischbeck JA, et al. The transcription factor Spn1 regulates gene expression via a highly conserved novel structural motif. J Mol Biol. 2010;404(1):1-15.
8. McCullough L, Connell Z, Petersen C, Formosa T. The Abundant Histone Chaperones Spt6 and FACT Collaborate to Assemble, Inspect, and Maintain Chromatin Structure in Saccharomyces cerevisiae. Genetics. 2015;201(3):1031-45.
9. Viktorovskaya O, Chuang J, Jain D, Reim NI, Lopez-Rivera F, Murawska M, et al. Essential histone chaperones collaborate to regulate transcription and chromatin integrity. Genes Dev. 2021;35(9-10):698-712.
10. Jeronimo C, Poitras C, Robert F. Histone Recycling by FACT and Spt6 during Transcription Prevents the Scrambling of Histone Modifications. Cell Rep. 2019;28(5):1206-18 e8.
11. Tonsager AJ, Zukowksi A, Radebaugh CA, Weirich A, Stargell LA, Ramachandran S. The Histone Chaperone Spn1 Preserves Subnucleosomal Structures at Promoters and Nucleosome Positioning in Open Reading Frames. bioRxiv. 2024.
12. Uzun U, Brown T, Fischl H, Angel A, Mellor J. Spt4 facilitates the movement of RNA polymerase II through the +2 nucleosomal barrier. Cell Rep. 2021;36(13):109755.
13. Feng J, Gan H, Eaton ML, Zhou H, Li S, Belsky JA, et al. Noncoding Transcription Is a Driving Force for Nucleosome Instability in spt16 Mutant Cells. Mol Cell Biol. 2016;36(13):1856-67.
14. Connell Z, Parnell TJ, McCullough LL, Hill CP, Formosa T. The interaction between the Spt6-tSH2 domain and Rpb1 affects multiple functions of RNA Polymerase II. Nucleic Acids Res. 2022;50(2):784-802.
15. Doris SM, Chuang J, Viktorovskaya O, Murawska M, Spatt D, Churchman LS, Winston F. Spt6 Is Required for the Fidelity of Promoter Selection. Mol Cell. 2018;72(4):687-99 e6.
16. Weiner A, Hughes A, Yassour M, Rando OJ, Friedman N. High-resolution nucleosome mapping reveals transcription-dependent promoter packaging. Genome Res. 2010;20(1):90-100.
17. Tramantano M, Sun L, Au C, Labuz D, Liu Z, Chou M, et al. Constitutive turnover of histone H2A.Z at yeast promoters requires the preinitiation complex. Elife. 2016;5.

18. Malagon F, Kireeva ML, Shafer BK, Lubkowska L, Kashlev M, Strathern JN. Mutations in the Saccharomyces cerevisiae RPB1 gene conferring hypersensitivity to 6-azauracil. Genetics. 2006;172(4):2201-9.
19. Kaplan CD, Larsson KM, Kornberg RD. The RNA polymerase II trigger loop functions in substrate selection and is directly targeted by alpha-amanitin. Mol Cell. 2008;30(5):547-56.
20. Chen K, Chen Z, Wu D, Zhang L, Lin X, Su J, et al. Broad H3K4me3 is associated with increased transcription elongation and enhancer activity at tumor-suppressor genes. Nat Genet. 2015;47(10):1149-57.
21. Chen K, Xi Y, Pan X, Li Z, Kaestner K, Tyler J, et al. DANPOS: dynamic analysis of nucleosome position and occupancy by sequencing. Genome Res. 2013;23(2):341-51.

APPENDIX A – CHAPTER 2 SUPPLEMENTARIES

Supplemental Data

Table A.1. Plasmids and yeast strains used in the Chapter 2 MNase-seq study.

Plasmid	**Description**	
pCR311	Full length wild type *SPN1* with 403 bp of upstream sequence and 116bp of downstream sequence, myc2 tagged at the amino terminus, pRS313 (CEN, *HIS3*)	
pCR312	*spn1*^{K192N} with 403 bp of upstream sequence and 116bp of downstream sequence, myc2 tagged at the amino terminus, pRS313 (CEN, *HIS3*)	
pAA344	*spn1*$^{141-305}$ with 403 bp of upstream sequence and 116bp of downstream sequence, myc2 tagged at the amino terminus, pRS313 (CEN, *HIS3*)	
Strain	**Genotype**	**Source**
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
LZ0-1	BY4741 *spn1Δ::LEU2* + pCR311	Li et al. (2018), Zhang et al. (2008)
LZ0-2	BY4741 *spn1Δ::LEU2* + pCR312	Li et al. (2018), Zhang et al. (2008)
LZ0-3	BY4741 *spn1Δ::LEU2* + pAA344	Li et al. (2018), Zhang et al. (2008)

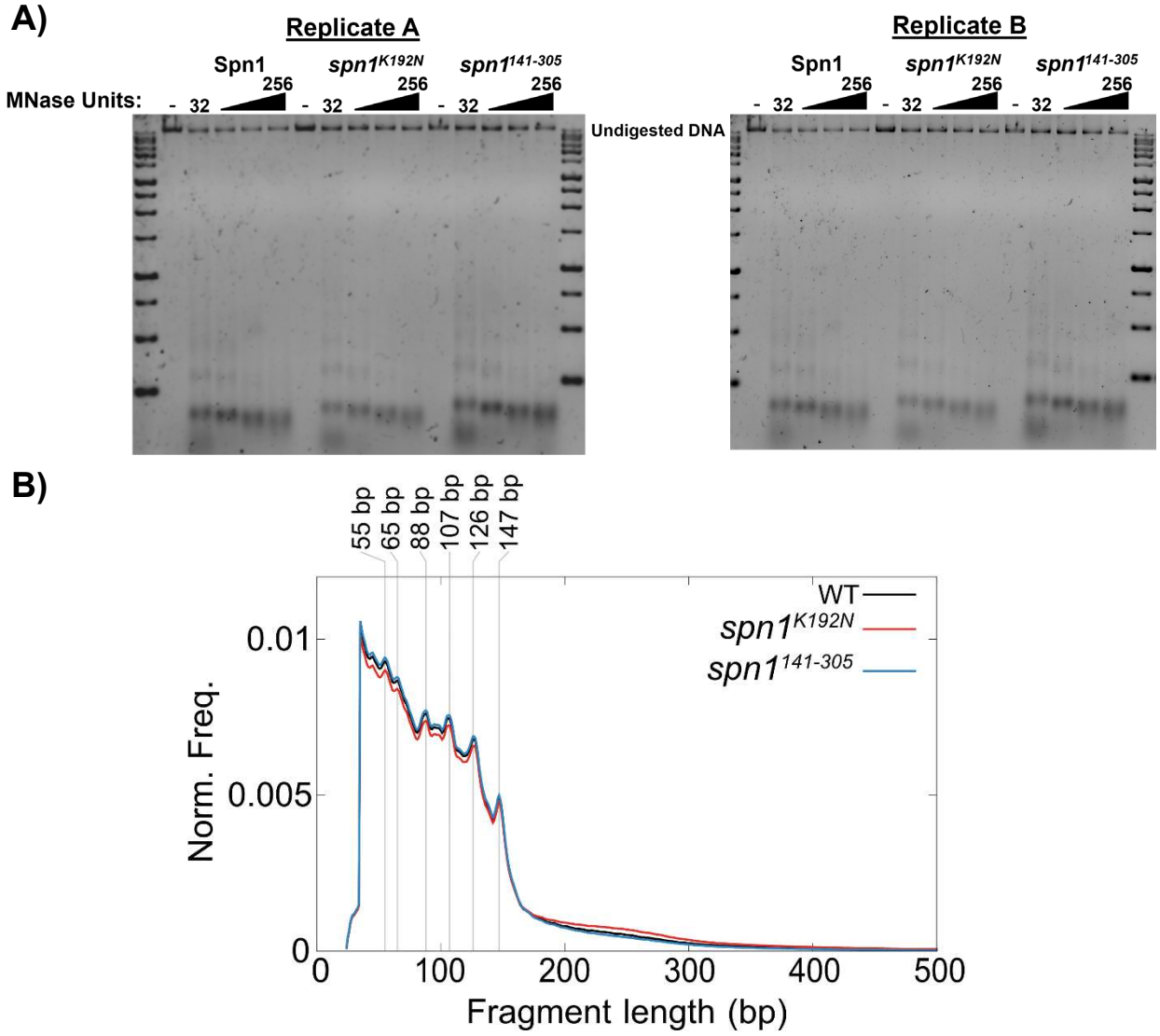


Figure A.1. Characterization of MNase digested samples demonstrates uniform distribution of protected fragments. (A) Yeast nuclei isolated from yeast expressing wild-type Spn1, *spn1*^{K192N} and *spn1*$^{141-305}$ were digested with increasing amounts of MNase then subjected to agarose gel electrophoresis. (B) The normalized fragment length distribution present in each library prepared from MNase digested samples (wild-type: black, *spn1*^{K192N}: red, *spn1*$^{141-305}$: blue). Fragment length distributions were normalized by averaging distributions from each sample followed by resampling.

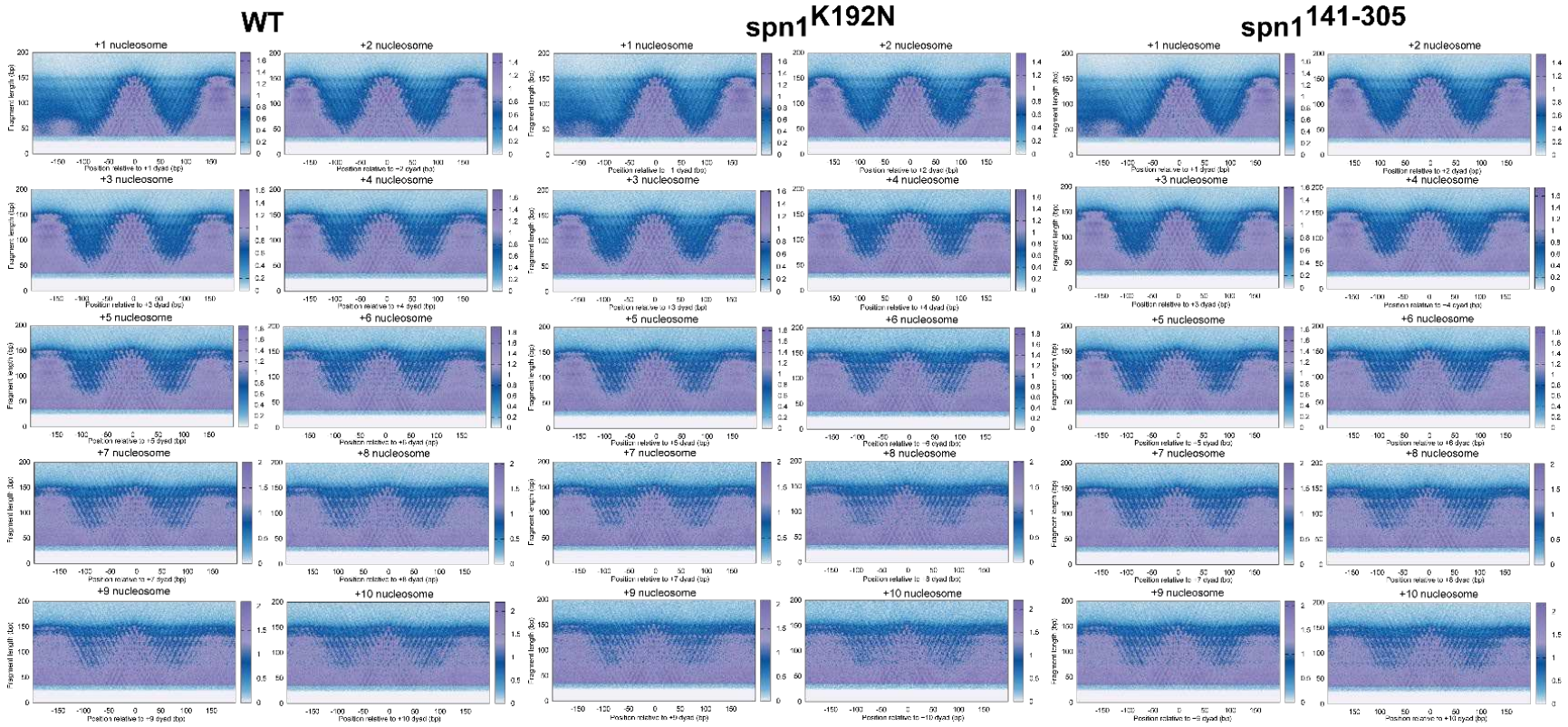


Figure A.2. V-plots over nucleosome dyad positions reveal rotational positioning of chromatin protections across transcribed regions. Nuclei from wild-type and mutant Spn1 expressing yeast were digested with 128U MNase and protected fragments were isolated and sequenced. V-plots were produced by mapping fragment midpoints ~30 - 180bp in length to the yeast genome anchored at each nucleosome dyad position from positions +1 to +10 over 4688 genes

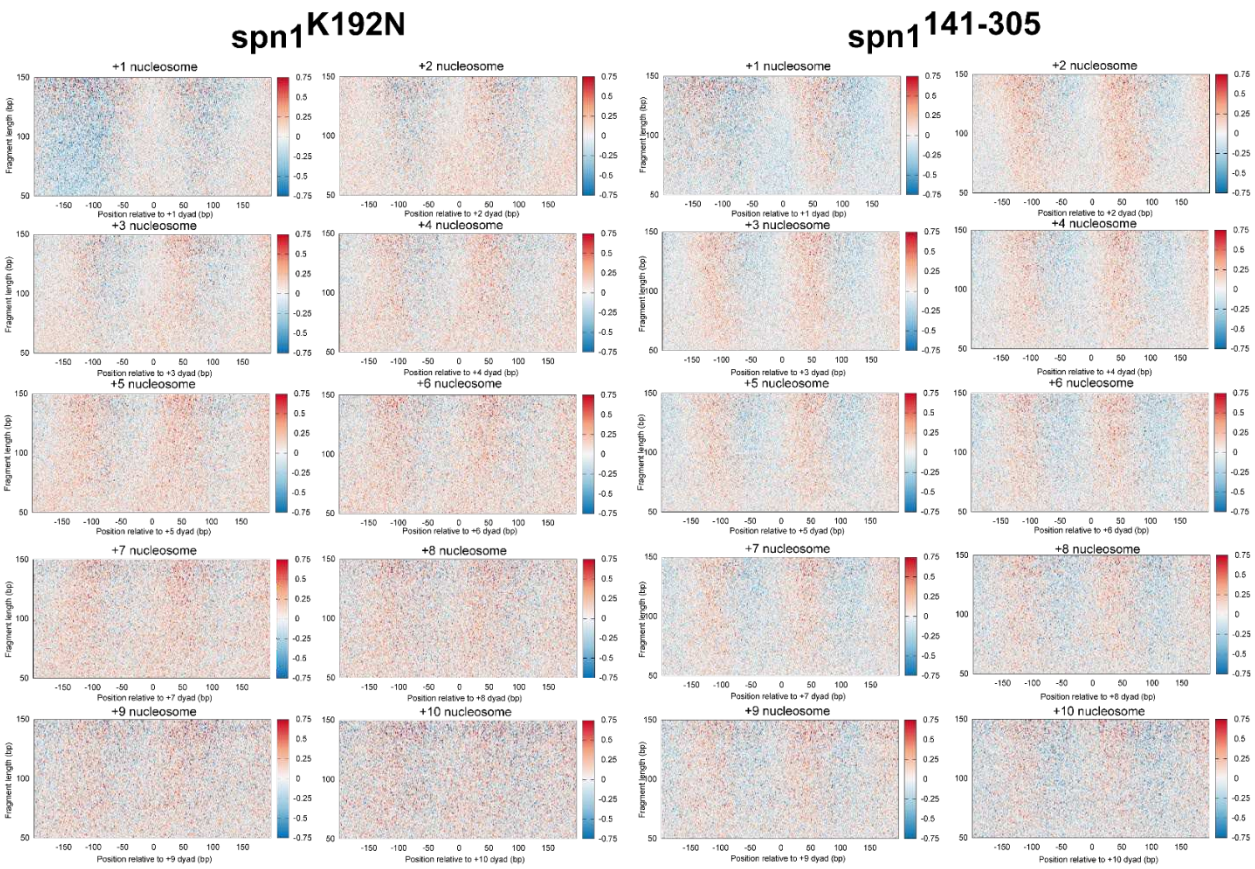


Figure A.3. Mutations in Spn1 produce shifts in chromatin protections across transcribed regions. Difference V-plots produced by quantifying the Z-score differences between wild-type and *spn1*^{K192N} (left hand panels) and *spn1*$^{141-305}$ (right hand panels) fragment midpoint occupancies in Supplemental Figure S2. Regions in red come from an increase in fragment density in the mutant Spn1 expressing yeast while regions in blue come from a decrease in fragment density in the mutant Spn1 expressing yeast.

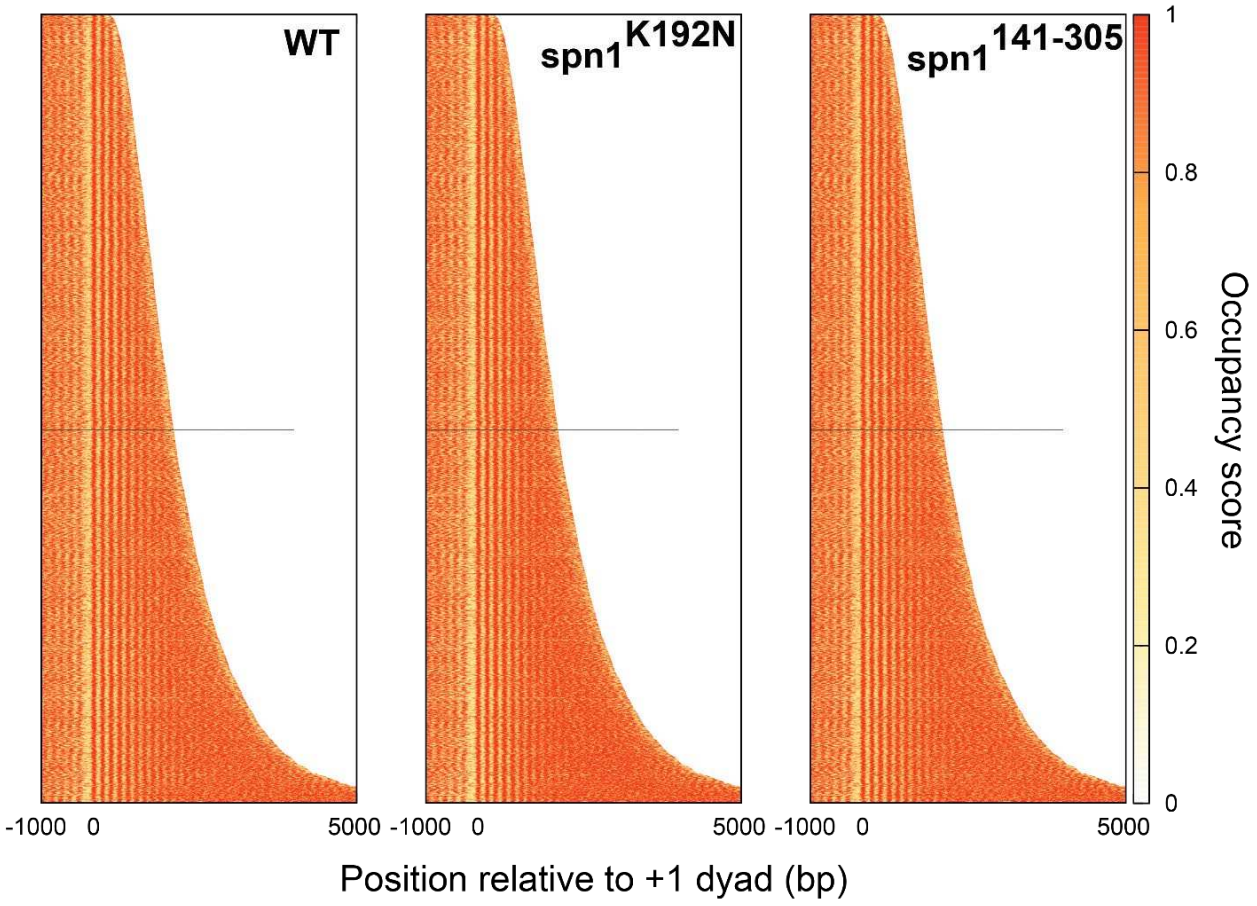


Figure A.4. Mutations in Spn1 propagate a downstream shift in nucleosome positioning over open reading frames genome wide. Heatmaps displaying MNase protected fragments (80-170bp) quantified by occupancy across 4688 genes ranked by length in wild-type (left), *spn1*^{K192N} (middle), and *spn1*$^{141-305}$ (right) expressing yeast.

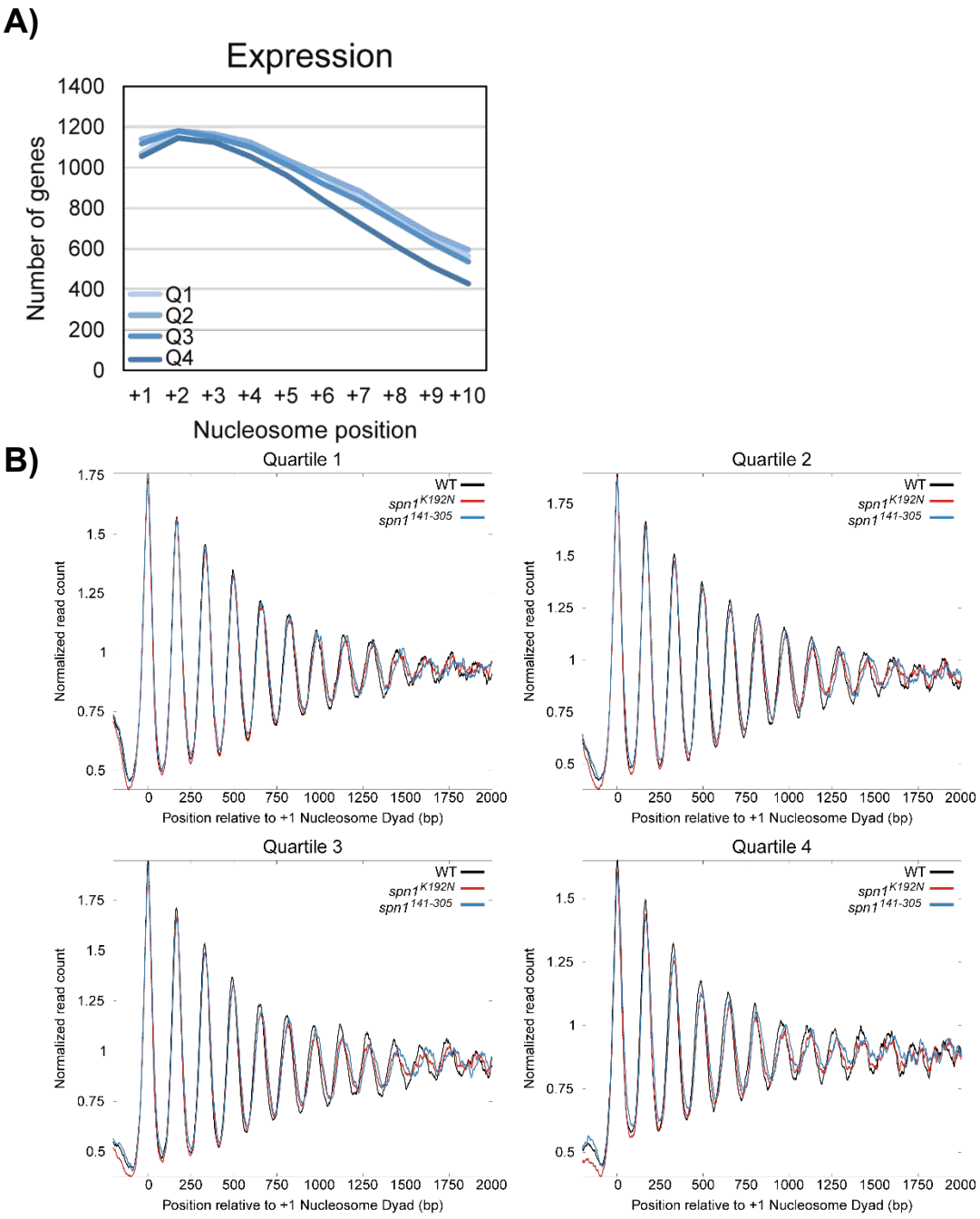


Figure A.5. The highest quartile of genes ranked by expression experience larger shifts compared to the average gene in both *spn1*^{K192N} and *spn1*$^{141\text{-}305}$ expressing yeast. (A) Count of genes containing mapped nucleosomes at each position across quartiles ranked by expression shows a uniform nucleosome count across quartiles at each nucleosome position. (B) Profiles of MNase protected fragments (80-170bp) mapped over 4688 genes binned into quartiles by Rpb3 ChIP occupancy[1], with Q1 containing the lowest levels of Rpb3 and Q4 containing the highest.

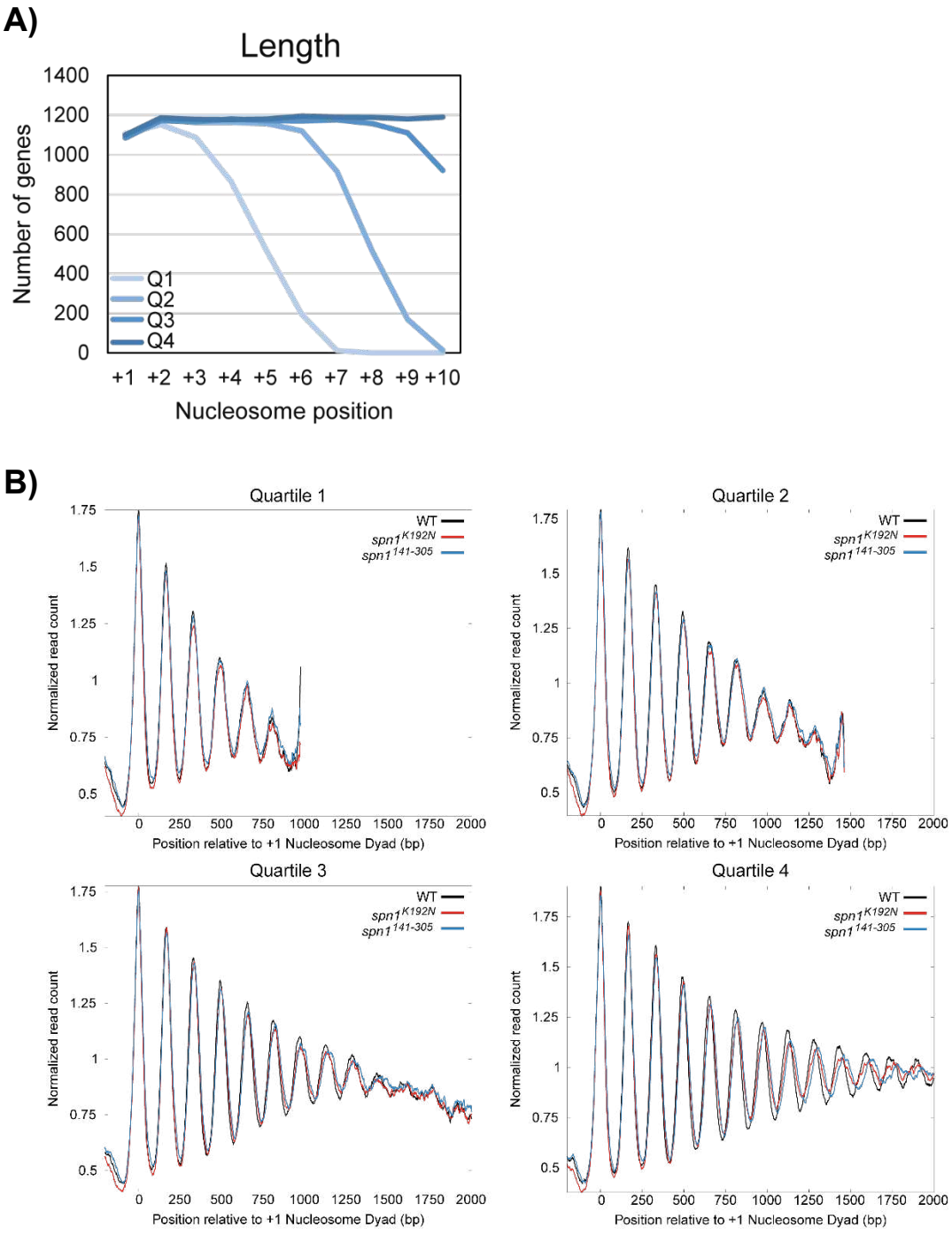


Figure A.6. The longest genes experience larger shifts compared to the average gene in both *spn1*^{K192N} and *spn1*$^{141\text{-}305}$ expressing yeast. (A) Count of genes containing mapped nucleosomes at each position across quartiles ranked by length shows the loss of downstream nucleosome positions in quartiles. (B) Profiles of MNase protected fragments (80-170bp) mapped over 4688 genes binned into quartiles by length, with Q1 containing the shortest genes and Q4 containing the longest genes.

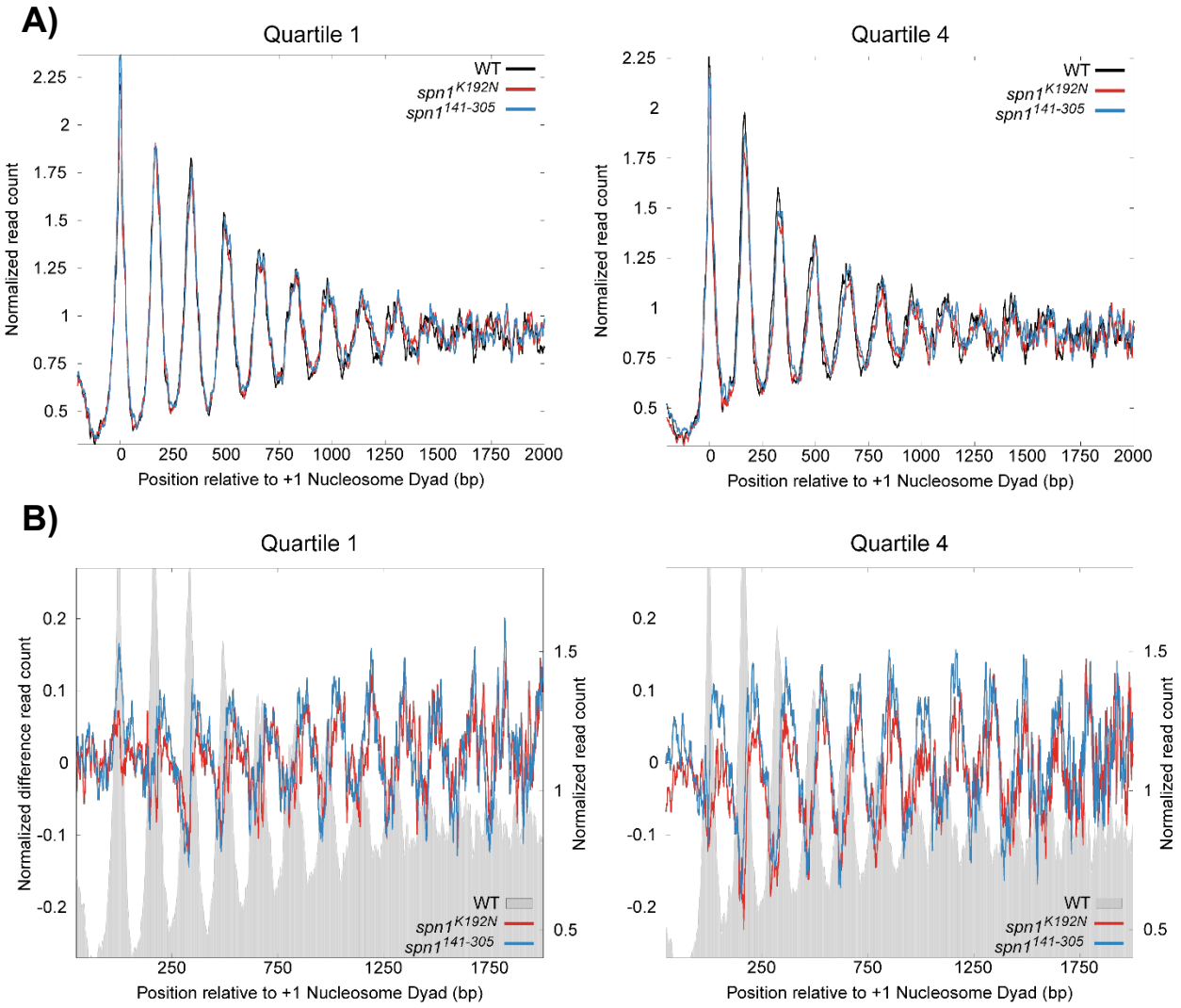


Figure A.7. The highest quartile of genes ranked by expression experience larger shifts in nucleosome positioning compared to the average gene in both *spn1*^{K192N} and *spn1*$^{141\text{-}305}$ expressing yeast. (A) Profiles of MNase protected fragments (142-152bp) mapped over 4688 genes binned into quartiles by Rpb3 ChIP occupancy (reference), with Q1 representing the lowest levels of Rpb3 and Q4 representing the highest. (B) Difference profile plots between wild-type and mutant samples. Average nucleosome density from wild type expressing yeast is plotted in grey. Differences between wild-type and mutants (K192N, red and 141-305, blue) against wild-type replicates are plotted.

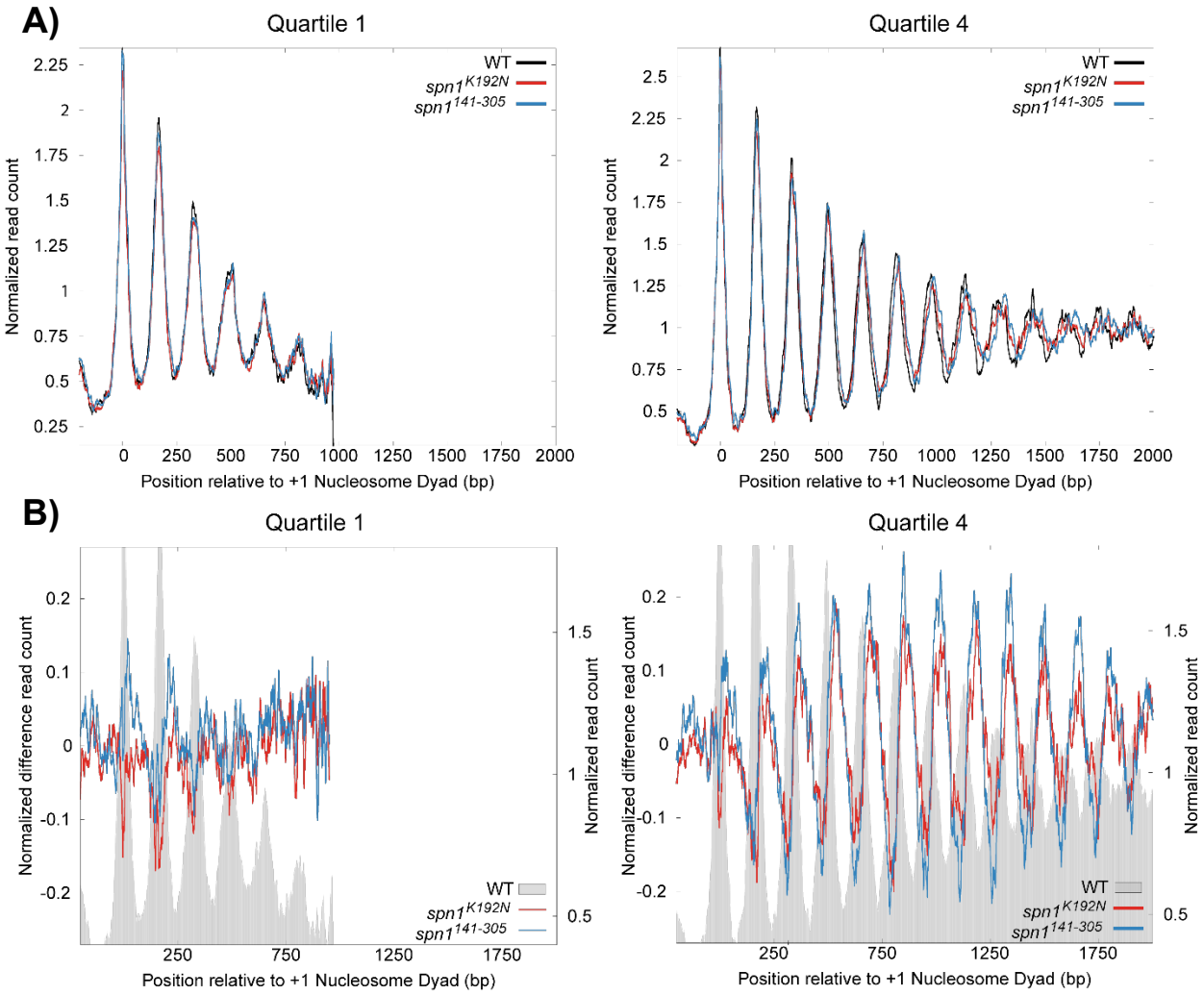


Figure A.8. The longest genes experience larger shifts in nucleosome positioning compared to the average gene in both *spn1*^{K192N} and *spn1*$^{141\text{-}305}$ expressing yeast. (A) Profiles of MNase protected fragments (142-152bp) mapped over 4688 genes binned into quartiles by length, with Q1 representing the shortest genes and Q4 representing the longest genes. (B) Difference profile plots between wild-type and mutant samples. Average nucleosome density from wild type expressing yeast is plotted in grey. Differences between wild-type and mutants (K192N, red and 141-305, blue) against wild-type replicates are plotted.

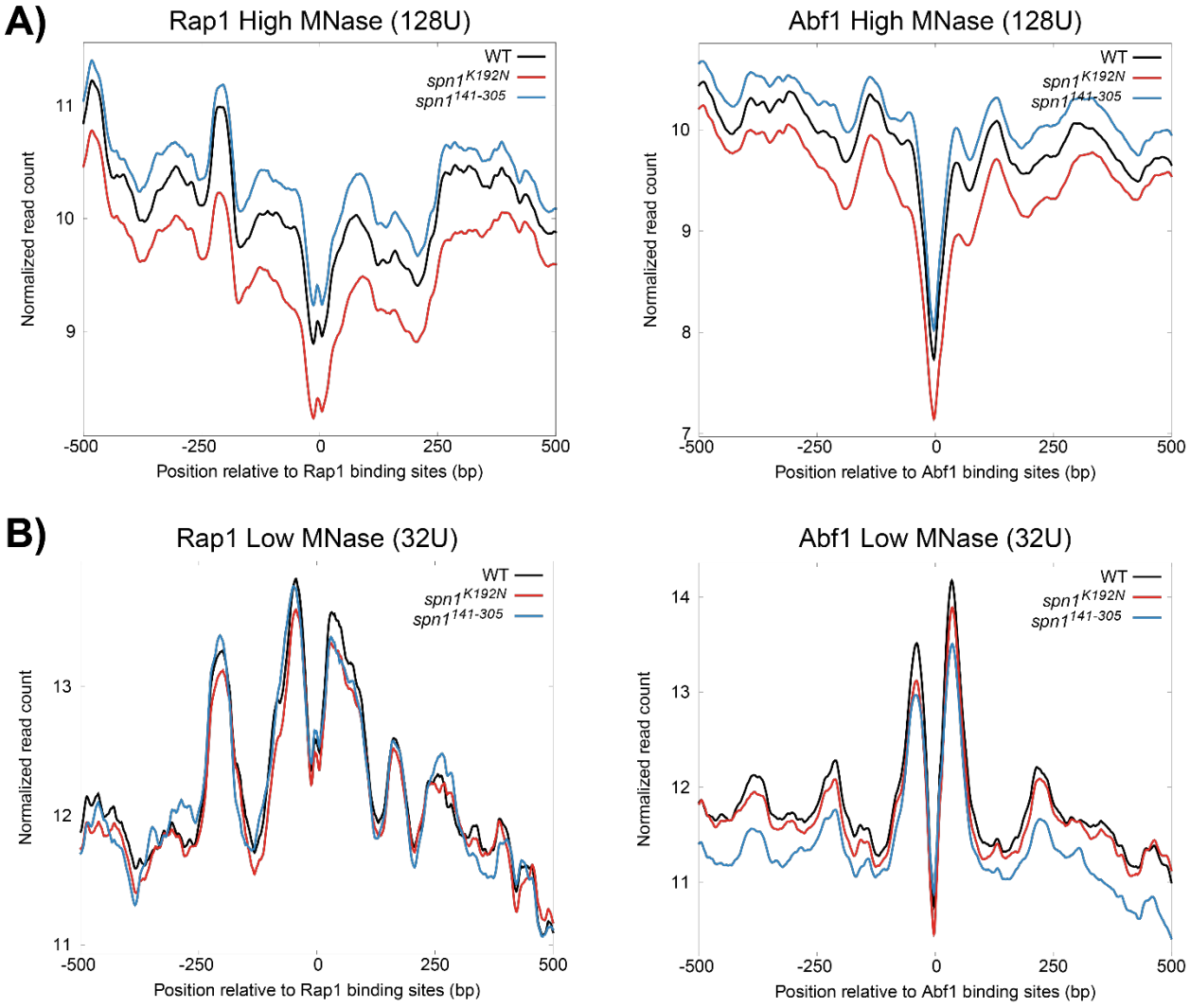


Figure A.9. Mutant *spn1*^{K192N} cells exhibit a loss of protection at Abf1 and Rap1 binding when subjected to a high MNase concentration. Short fragments (0-60bp) from samples digested with (A) high (128U) and (B) low (32U) MNase are mapped over Rap1 and Abf1 binding sites obtained from existing ChEC-seq data[2]

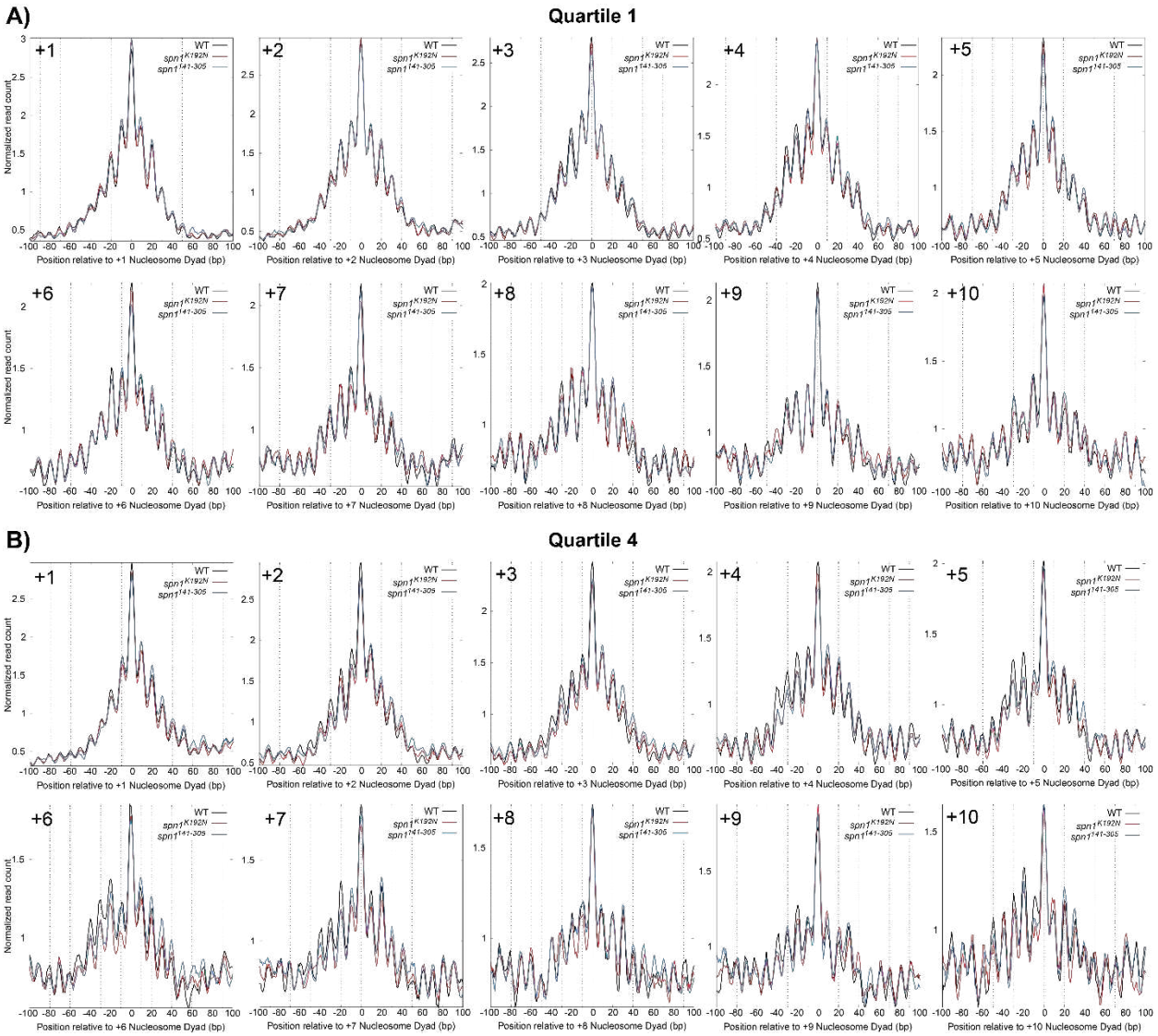


Figure A.10. Shifted nucleosomes in Spn1 mutants have higher occupancy at rotational positions downstream of the dyad with a loss of occupancy at positions upstream of the dyad location positively correlated with gene expression. Metagene plots of fragment midpoints 142 - 152bp in length mapped to each nucleosome dyad at the (A) lowest and (B) highest gene quartiles ranked by expression.

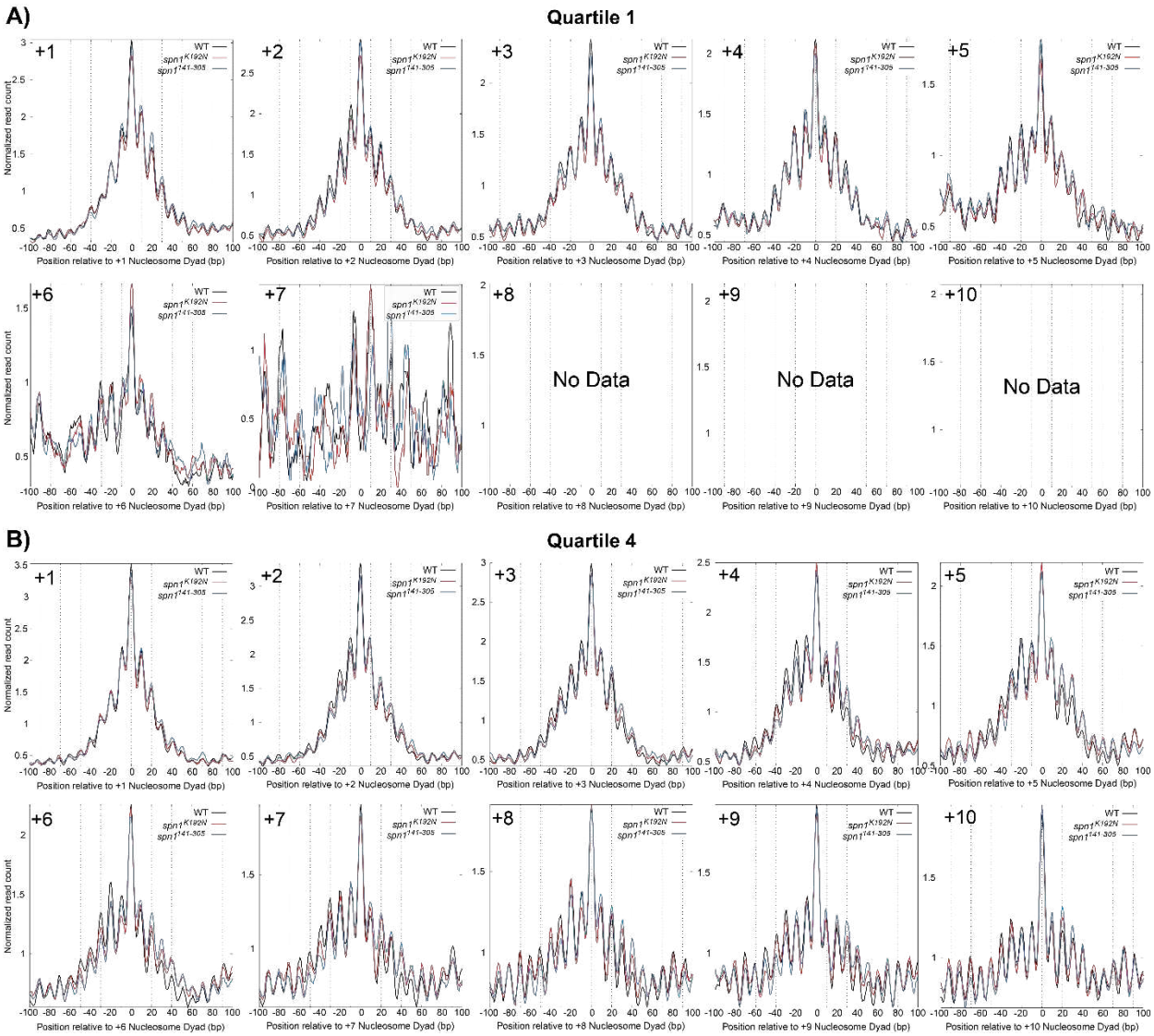


Figure A.11. Shifted nucleosomes in Spn1 mutants have higher occupancy at rotational positions downstream of the dyad with a loss of occupancy at positions upstream of the dyad location positively correlated with gene length. Metagene plots of fragment midpoints 142 - 152bp in length mapped to each nucleosome dyad at the (A) shortest and (B) longest gene quartiles ranked by length.

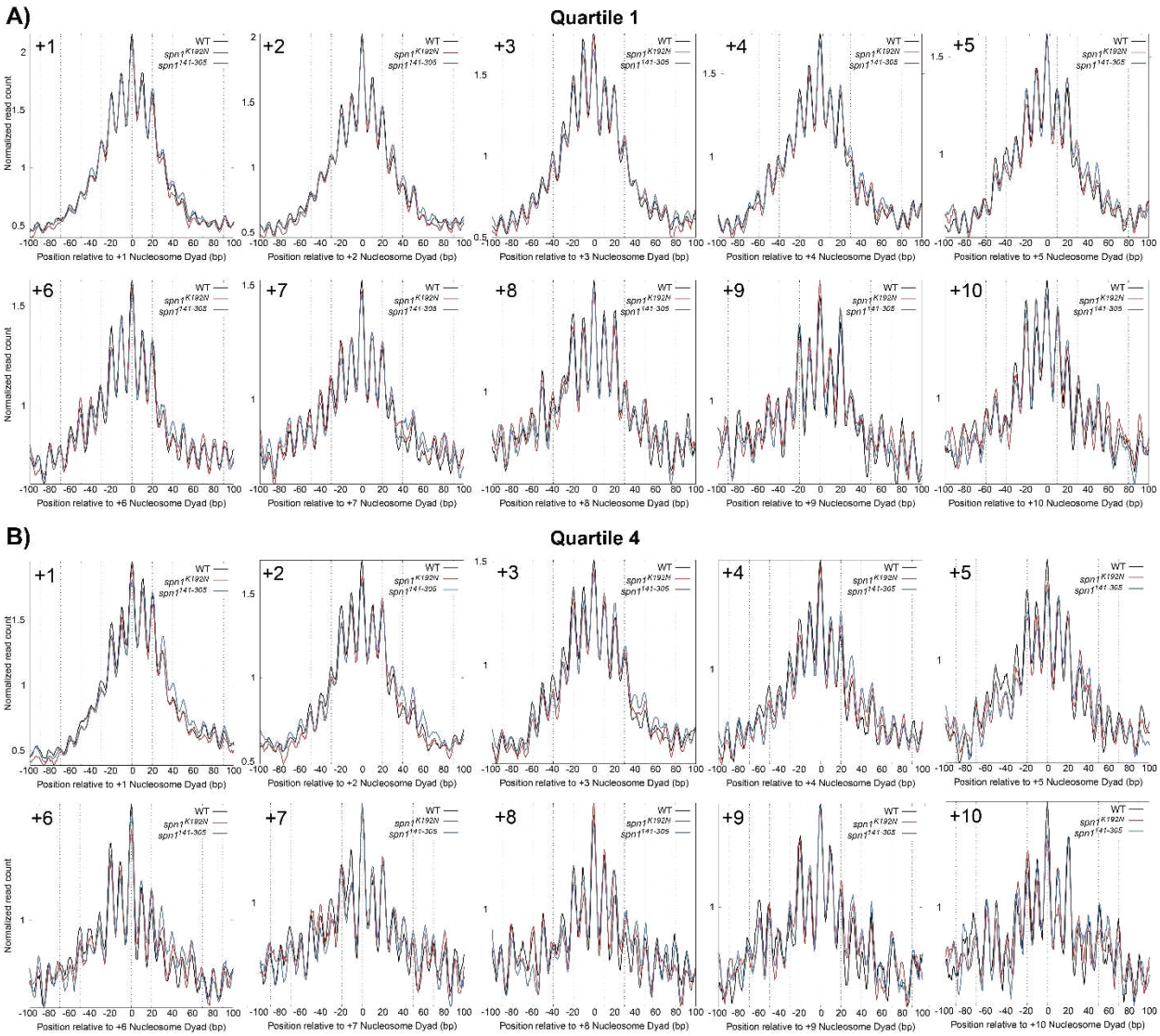


Figure A.12. Shifted hexasomes in Spn1 mutants have higher occupancy at rotational positions downstream of the dyad with a loss of occupancy at positions upstream of the dyad location positively correlated with gene expression. Metagene plots of fragment midpoints 102 - 112bp in length mapped to each nucleosome dyad at the (A) lowest and (B) highest gene quartiles ranked by expression.

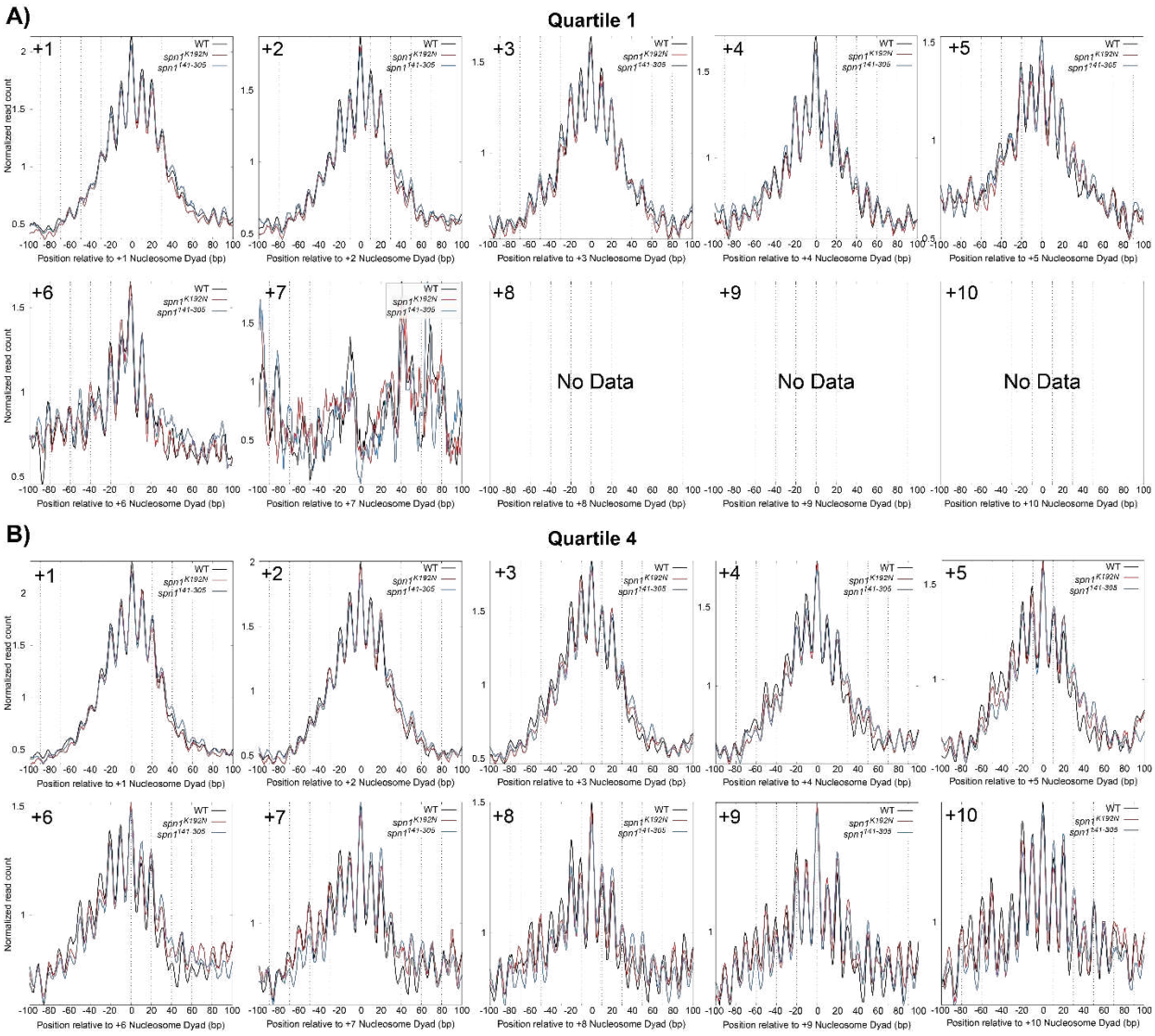


Figure A.13. Shifted hexasomes in Spn1 mutants have higher occupancy at rotational positions downstream of the dyad with a loss of occupancy at positions upstream of the dyad location positively correlated with gene length. Metagene plots of fragment midpoints 102 - 112bp in length mapped to each nucleosome dyad at the (A) shortest and (B) longest gene quartiles ranked by length.

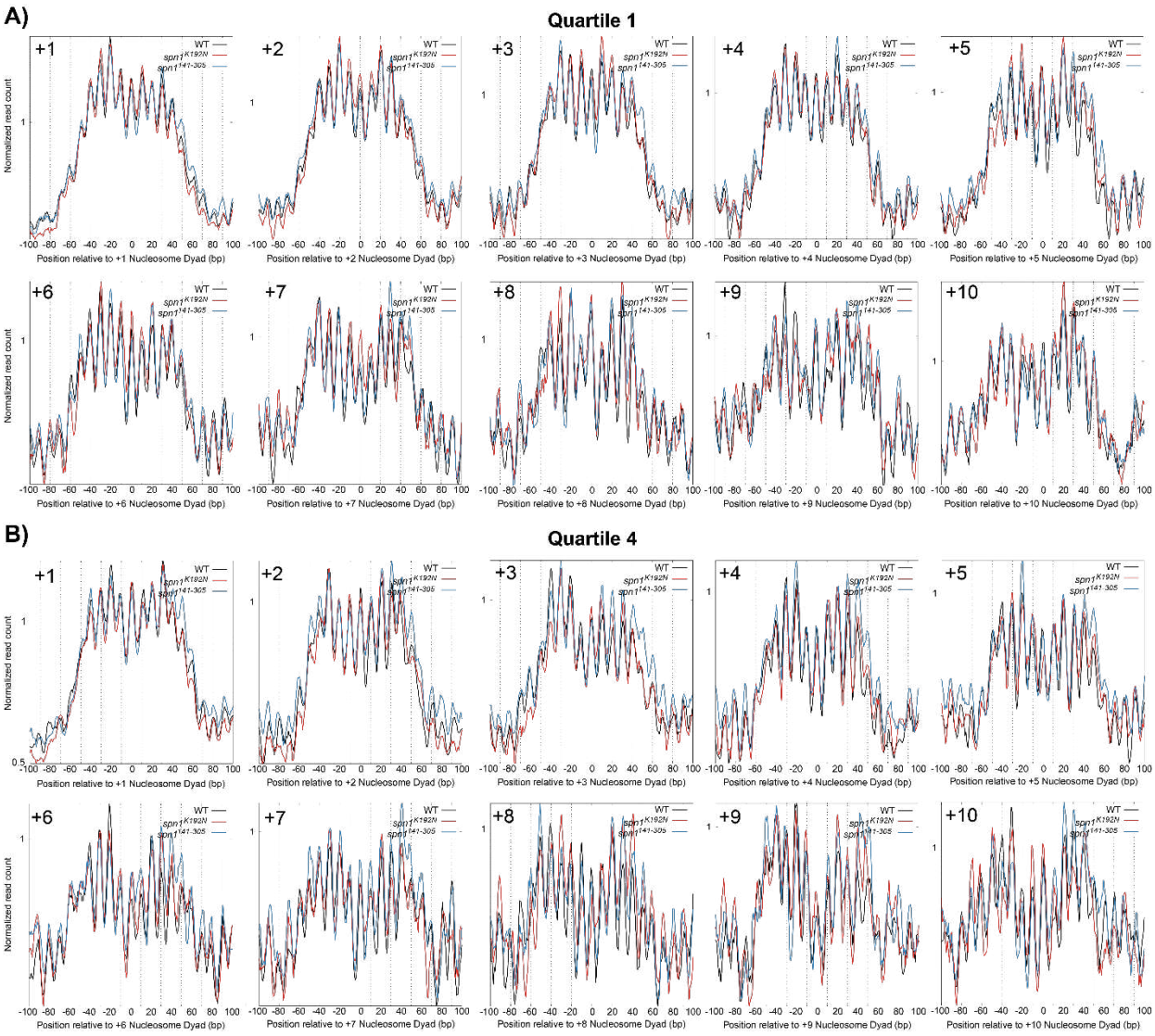


Figure A.14. Tetrasomes in *spn1*$^{141-305}$ have higher occupancy at rotational positions downstream of the dyad with a loss of occupancy at positions upstream of the dyad location positively correlated with gene expression. Metagene plots of fragment midpoints 61 - 71bp in length mapped to each nucleosome dyad at the (A) lowest and (B) highest gene quartiles ranked by expression.

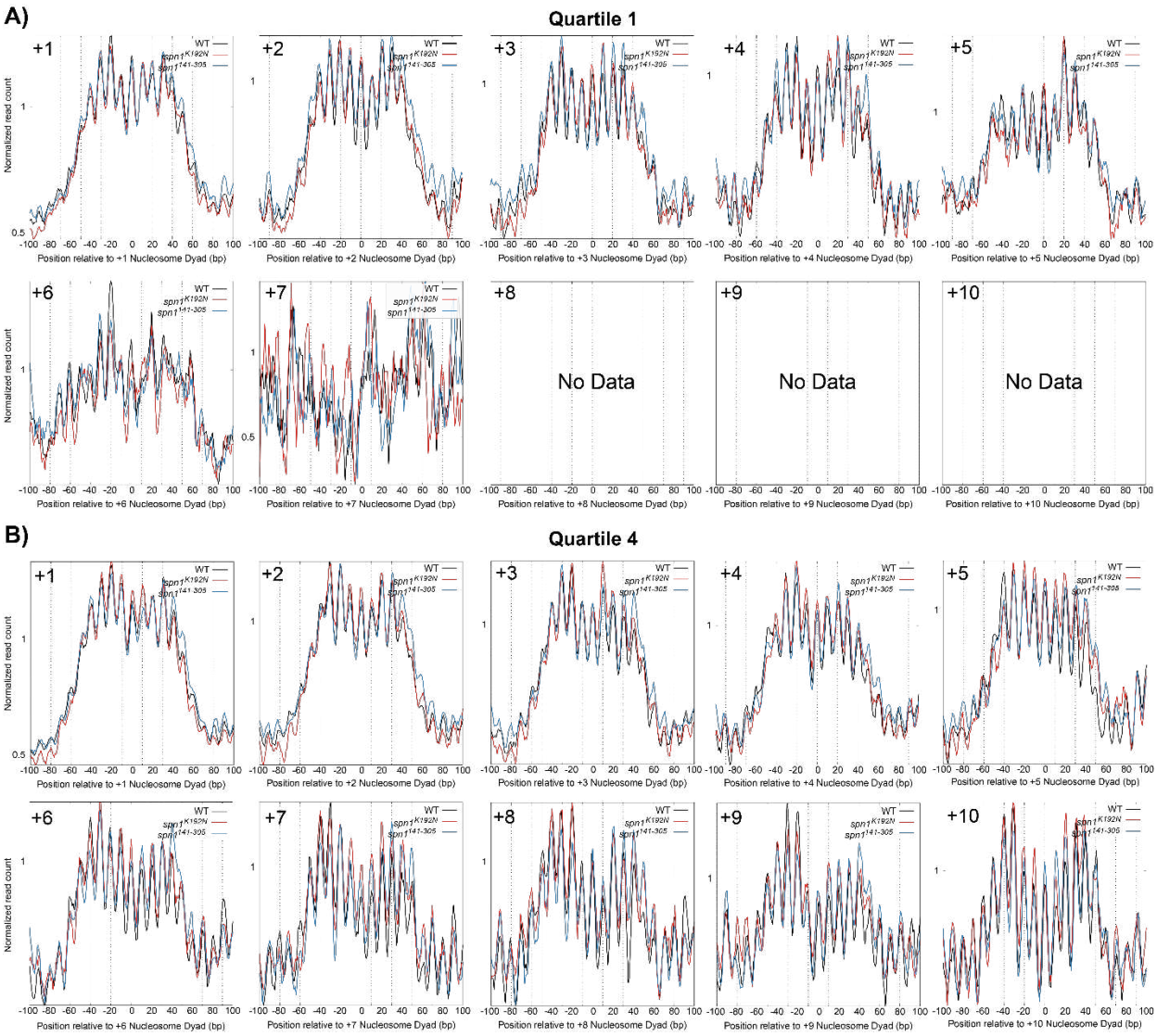


Figure A.15. Tetrasomes in *spn1*$^{141-305}$ have higher occupancy at rotational positions downstream of the dyad with a loss of occupancy at positions upstream of the dyad location positively correlated with gene length. Metagene plots of fragment midpoints 61 - 71bp in length mapped to each nucleosome dyad at the (A) shortest and (B) longest gene quartiles ranked by length.

REFERENCES

1. Churchman LS, Weissman JS. Nascent transcript sequencing visualizes transcription at nucleotide resolution. Nature. 2011;469(7330):368-73.
2. Zentner GE, Kasinathan S, Xin B, Rohs R, Henikoff S. ChEC-seq kinetics discriminates transcription factor binding sites by DNA sequence and shape in vivo. Nat Commun. 2015;6:8733.

Supplemental Data

Table B.1. MNase-seq datasets incorporated into the Chapter 3 study. Data were acquired from the Gene Expression Omnibus (GEO) repository as processed bigWig files. All replicate samples from both mutant and control strains were downloaded and incorporated into the MNase-seq analysis pipeline.

Chromatin Factor	Sample	Reference	GEO Accession Number
Spn1	*spn1-141-305*	(Tonsager et al., 2024)[1]	TBA
	spn1-K192N	(Tonsager et al., 2024)[1]	TBA
	spn1-K192N (37°C)	(Viktorovskaya et al., 2021)[2]	GSE160821
Spt6	*spt6-YW*	(Viktorovskaya et al., 2021)[2]	GSE160821
	spt6-YW (37°C)	(Viktorovskaya et al., 2021)[2]	GSE160821
	spt6-1004 (37°C)	(Doris et al., 2018)[3]	GSE115775
	spt6-R,KK	(Connell et al., 2022)[4]	GSE184955
Spt4	*spt4Δ*	(Uzun et al., 2021)[5]	GSE159291
Spt5	*spt5-QS*	(Viktorovskaya et al., 2021)[2]	GSE160821
	spt5-QS (37°C)	(Viktorovskaya et al., 2021)[2]	GSE160821
	spt5-AID	(Evrin et al., 2022)[6]	GSE181901
	spt5-3A	(Evrin et al., 2022)[6]	GSE181901
FACT	*spt16-G132D*	(Feng et al., 2016)[7]	GSE66215
	spt16-G132D (37°C)	(Feng et al., 2016)[7]	GSE66215
	pob3-Q308K	(McCullough et al., 2019)[8]	GSE118332
RNAPII (Rpb1)	*rpb1-1*	(Feng et al., 2016)[7]	GSE66215
	rpb1-TPY,FSP	(Connell et al., 2022)[4]	GSE184955

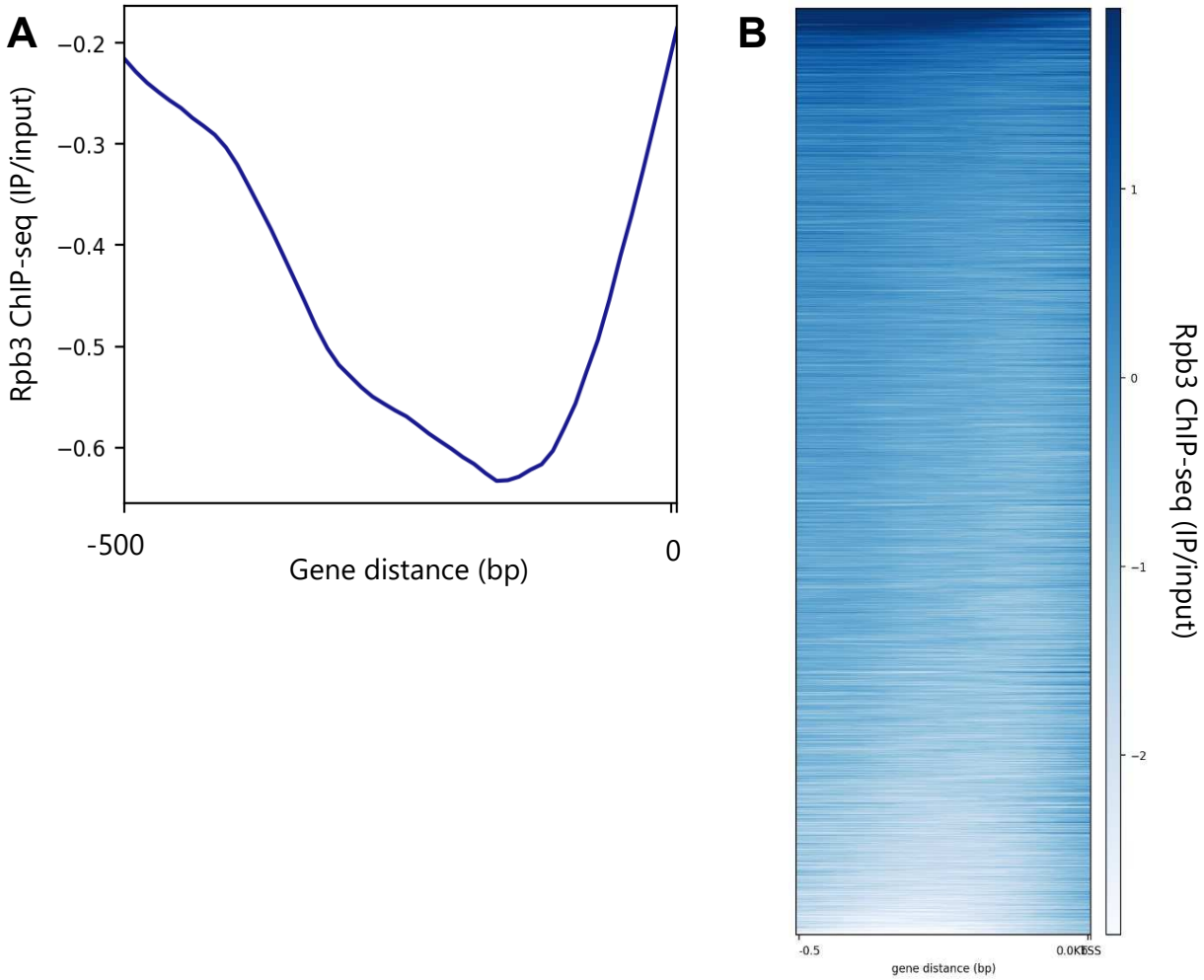


Figure B.1. Gene list ranking by Rpb3 occupancy upstream of transcription start sites. (A) Metagene analysis of Rpb3 ChIP-seq (IP/input) data[9] mapped directly upstream of yeast transcription start sites[10]. (B) Rpb3 ChIP-seq data from (A) visualized as a heatmap over genes ranked by Rpb3 occupancy. The order of genes displayed in the heatmap was retained and divided into four quartiles and saved as a new gene list ranked by Rpb3 occupancy.

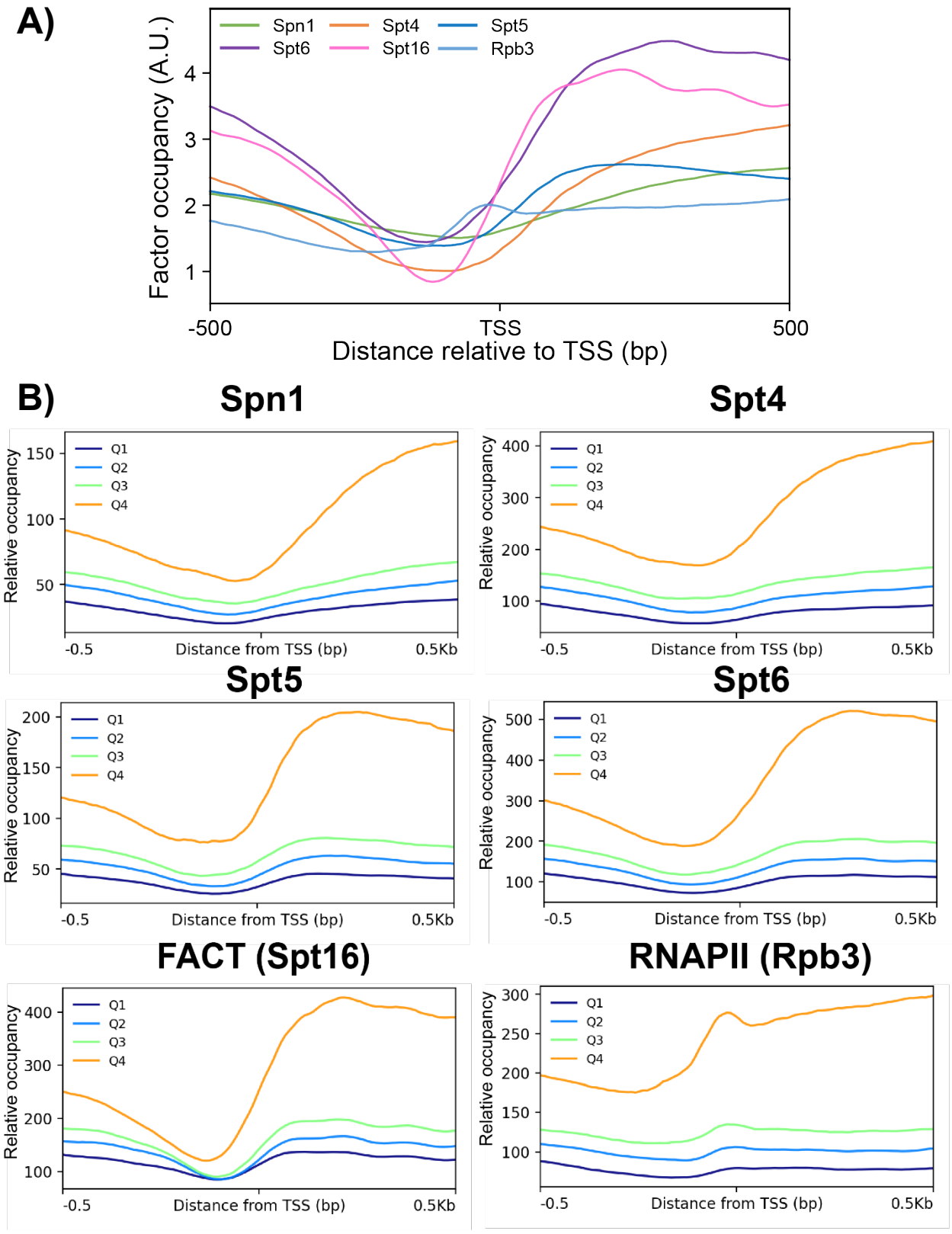

A)
Spn1
Spt4
Spt5
Spt6
Spt16
Rpb3
Factor occupancy (A.U.)
1
2
3
4
-500
TSS
500
Distance relative to TSS (bp)
B)
Spn1
Spt4
Spt5
Spt6
FACT (Spt16)
RNAPII (Rpb3)
Relative occupancy
Q1
Q2
Q3
Q4
-0.5
Distance from TSS (bp)
0.5Kb

Figure B.2. RNAPII-associated factors occupy genes at levels highly correlated with Rpb3. Sequencing data aligned to the yeast genome (BAM files) were obtained from a recently published ChIP-exo data[11] from TAP-tagged wild-type Spn1, Spt4, Spt5, Spt6, Spt16, and Rpb3. BAM files were scored over the genome to create coverage track files (bigWigs). The coverages were scored over transcription start sites and the data was averaged over (A) the master TSS list and (B) the quartile list from genes ranked by RNAPII occupancy.

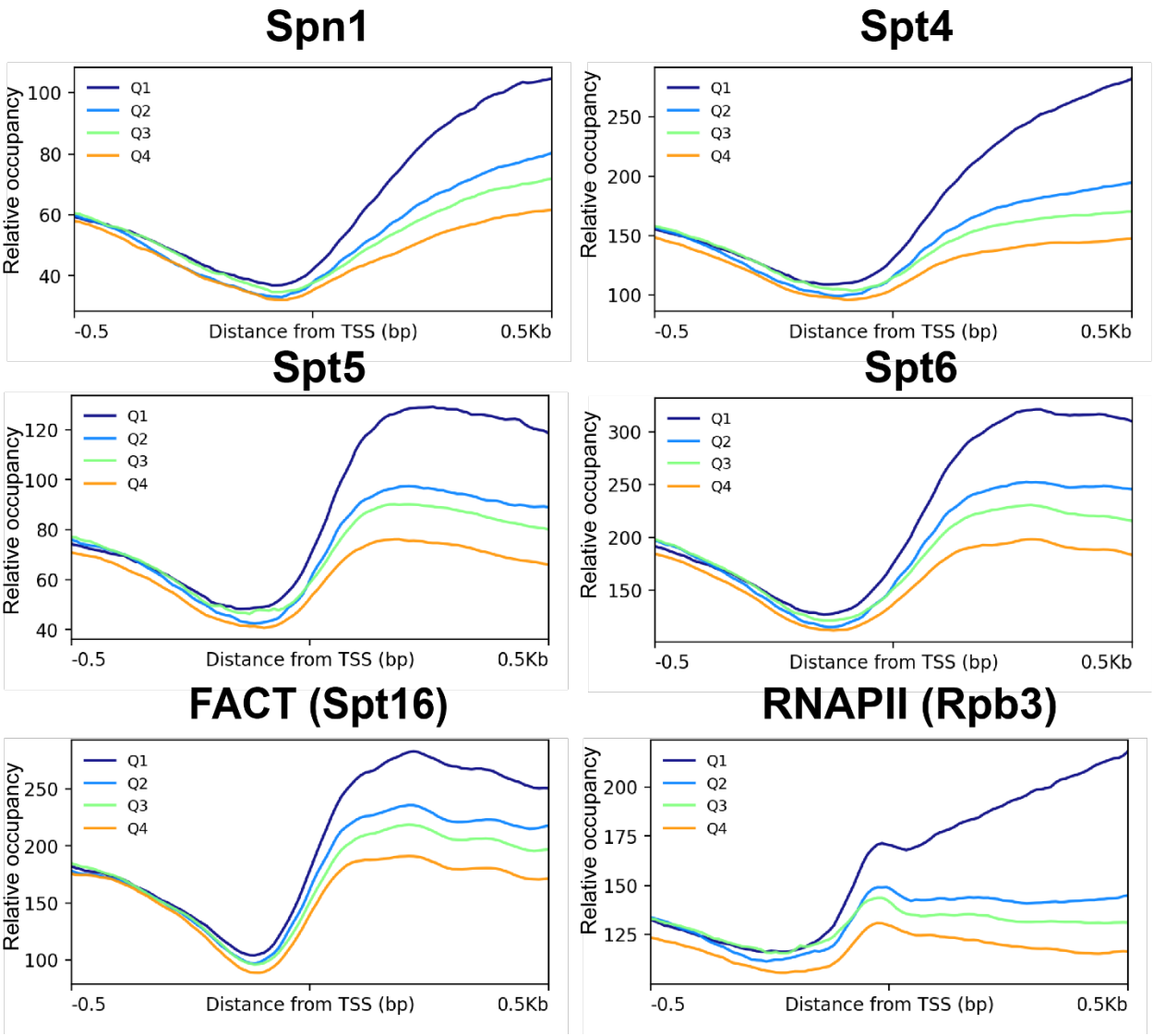


Figure B.3. Genes ranked by length are negatively correlated with occupancy of RNAPII and RNAPII-associated factors. Sequencing data aligned to the yeast genome (BAM files) were obtained from a recently published ChIP-exo data[11] from TAP-tagged wild-type Spn1, Spt4, Spt5, Spt6, Spt16, and Rpb3. BAM files were scored over the genome to create coverage track files (bigWigs). The coverages were scored over transcription start sites and the data was averaged for each quartile from genes ranked by gene length.

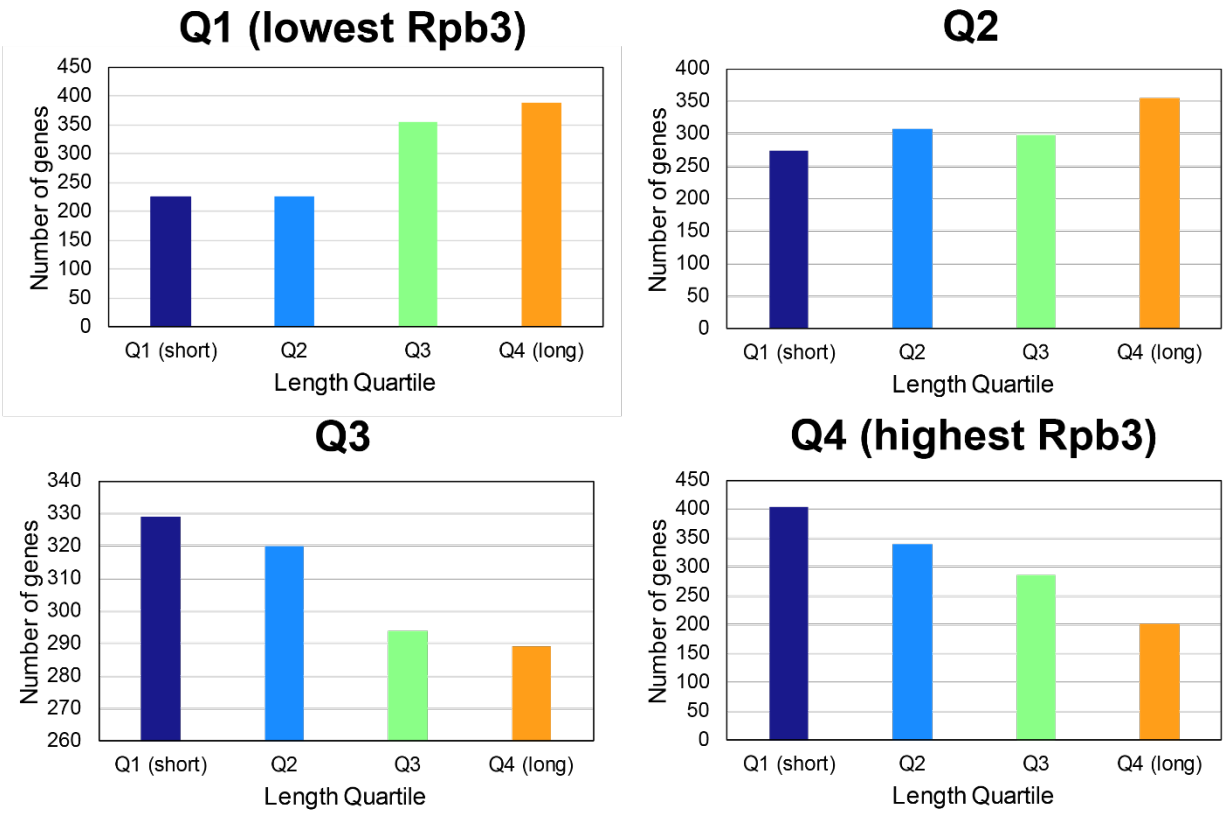


Figure B.4. Comparison of gene quartiles ranked by RNAPII occupancy and length. The number of genes present within each length quartile was quantified in each of the Rpb3 occupancy quartiles. Genes with low RNAPII occupancy are slightly overrepresented with longer genes, while genes with high RNAPII occupancy are slightly overrepresented with shorter genes.

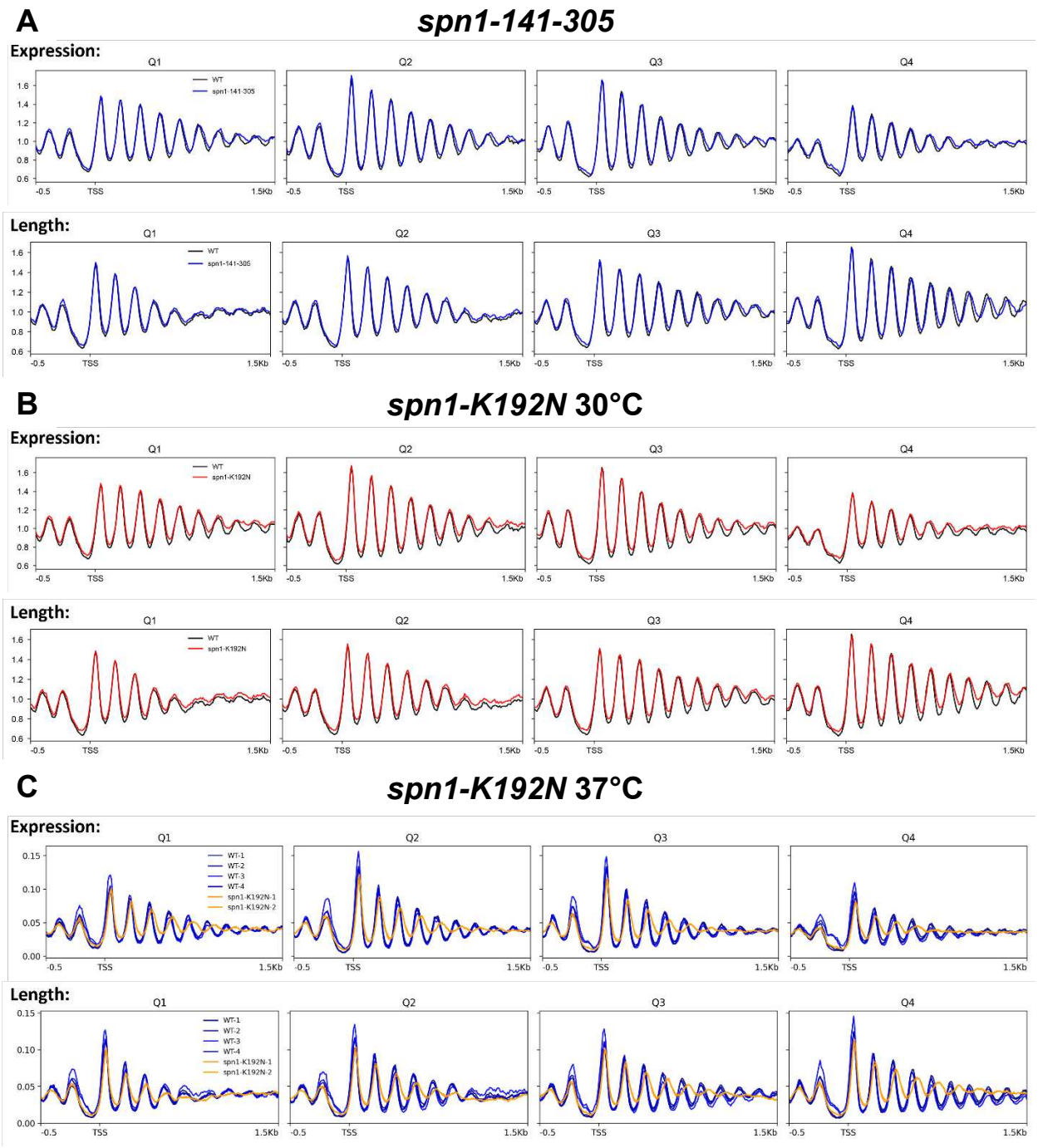


Figure B.5. Spn1 mutants produce changes in nucleosome features that correlate with RNAPII occupancy and gene length. (A) MNase-protected fragments from yeast expressing *spn1-141-305* (Tonsager et al., 2024), (B) *spn1-K192N* (Tonsager et al., 2024), and (C) *spn1-K192N* shifted to a non-permissive temperature of 37°C [2] mapped over transcription start sites in quartiles ranked by Rpb3 occupancy (expression) and gene length.

spt4Δ

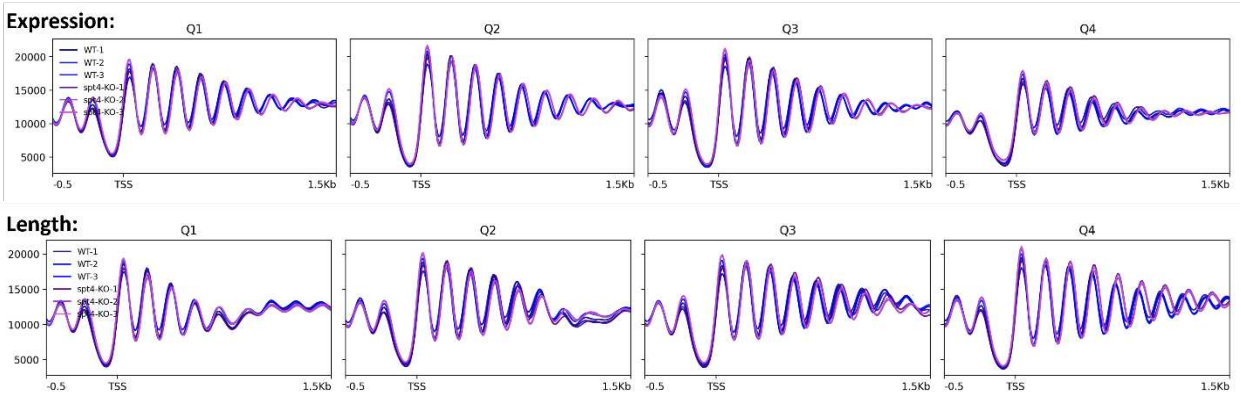


Figure B.6. Deletion of Spt4 produces a downstream nucleosome positioning shift that correlates with RNAPII occupancy and gene length. MNase-protected fragments from yeast with deleted *SPT4* [5] mapped over transcription start sites in quartiles ranked by Rpb3 occupancy (expression) and gene length.

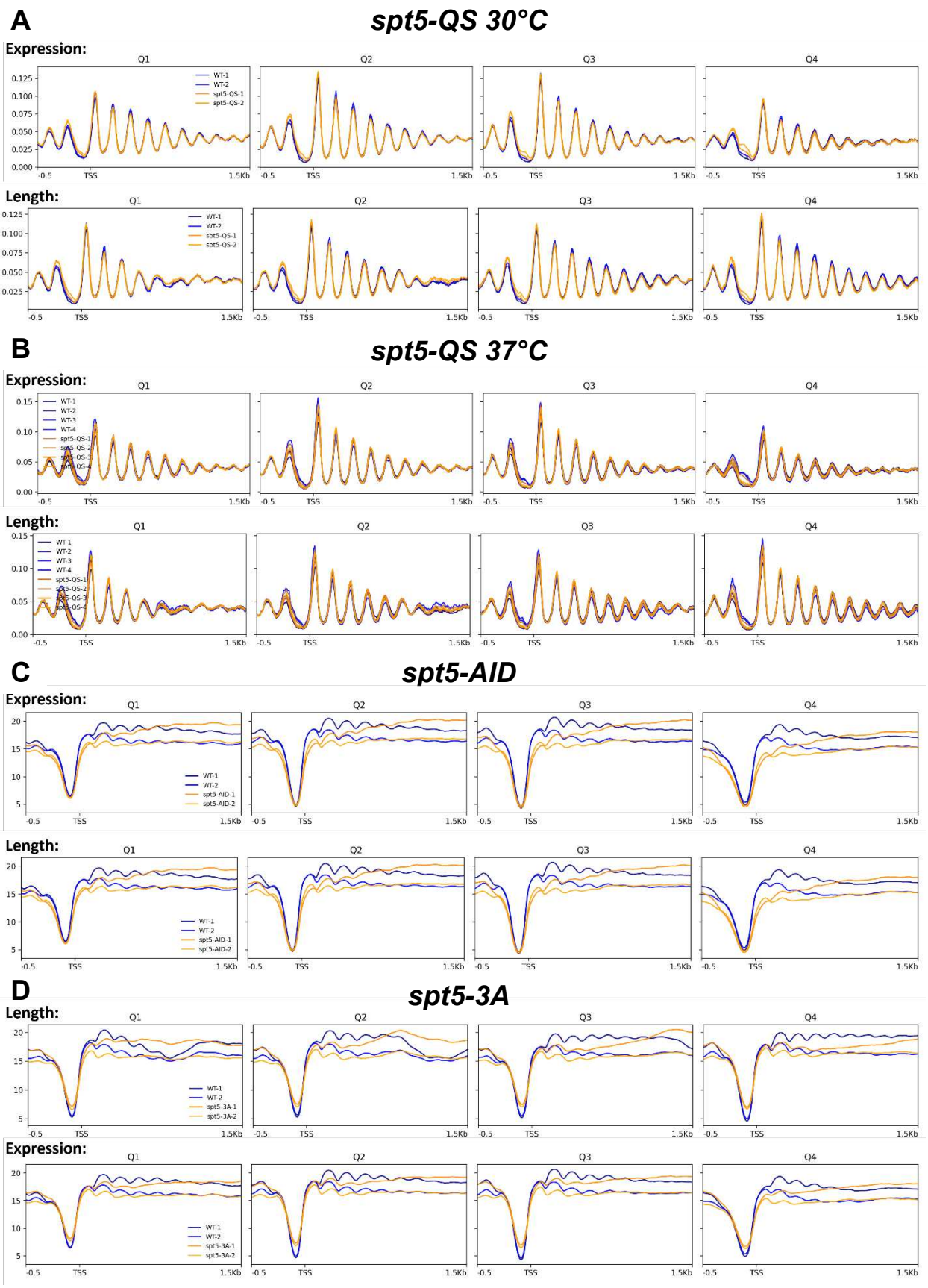

A
spt5-QS 30°C
Expression:
Length:
Q1
Q2
Q3
Q4
WT-1
WT-2
spt5-QS-1
spt5-QS-2
-0.5
TSS
1.5Kb
B
spt5-QS 37°C
WT-3
WT-4
spt5-QS-3
spt5-QS-4
C
spt5-AID
spt5-AID-1
spt5-AID-2
D
spt5-3A
spt5-3A-1
spt5-3A-2

Figure B.7. Mutations in Spt5 reduce nucleosome occupancy and produce a narrower NDR that generally correlates with RNAPII occupancy and gene length. (A) MNase-protected fragments from yeast expressing *spt5-QS* [2], (B) *spt5-QS* shifted to 37°C [2], (C) *spt5-AID* [6], and (D) *spt5-3A* [6] mapped over transcription start sites in quartiles ranked by Rpb3 occupancy (expression) and gene length.

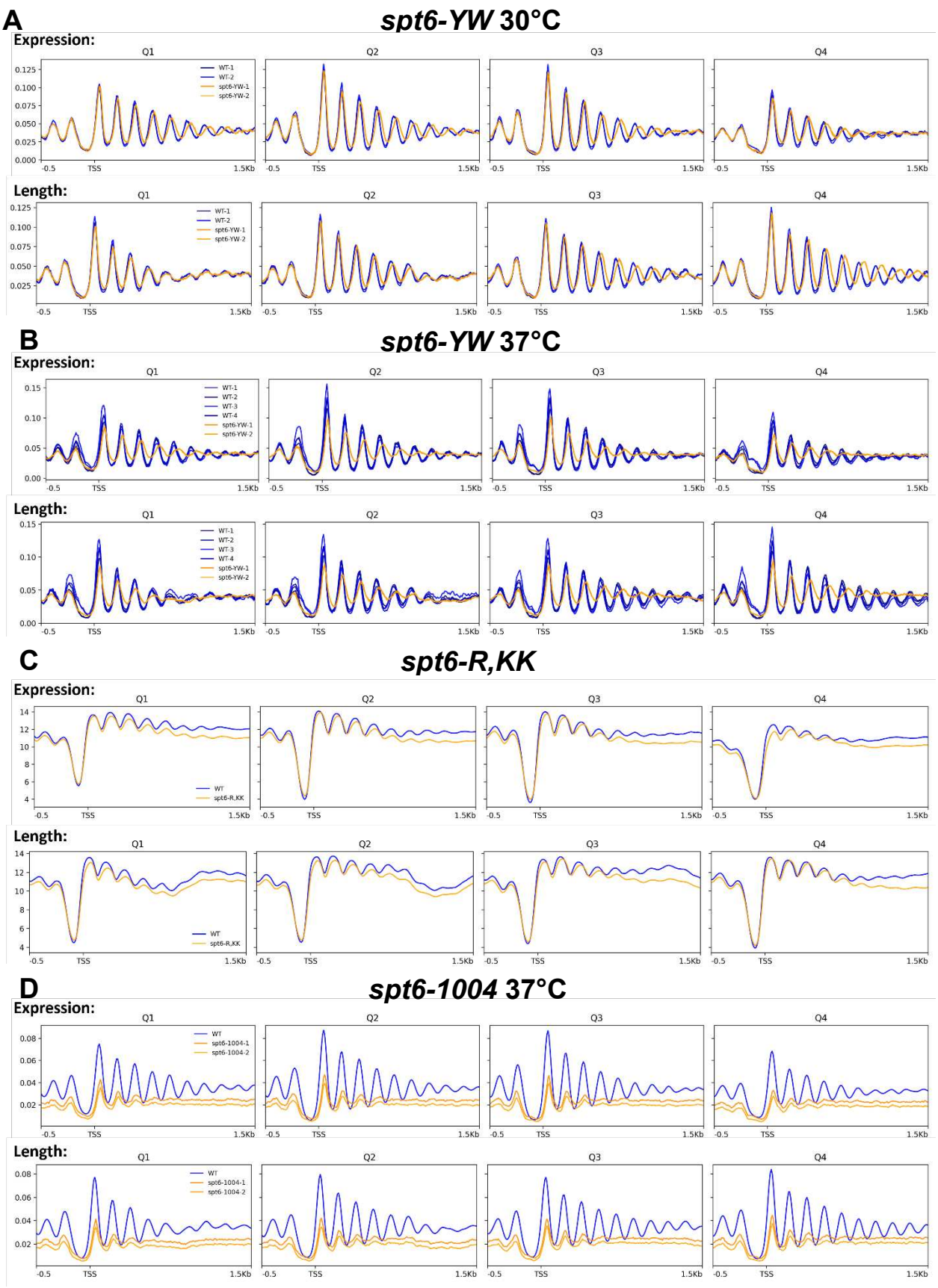

A
spt6-YW 30°C
Expression:
Length:
Q1
Q2
Q3
Q4
WT-1
WT-2
spt6-YW-1
spt6-YW-2
-0.5
TSS
1.5Kb
B
spt6-YW 37°C
WT-3
WT-4
C
spt6-R,KK
WT
spt6-R,KK
D
spt6-1004 37°C
spt6-1004-1
spt6-1004-2

Figure B.8. Mutations in Spt6 shift nucleosome positioning and reduce nucleosome occupancy that correlates with RNAPII occupancy and gene length. (A) MNase-protected fragments from yeast expressing *spt6-YW* [2], (B) *spt5-YW* shifted to a non-permissive temperature of 37°C [2], (C) *spt6-R,KK* [4] and (D) *spt6-1004* shifted to a non-permissive temperature of 37°C [3] mapped over transcription start sites in quartiles ranked by Rpb3 occupancy (expression) and gene length.

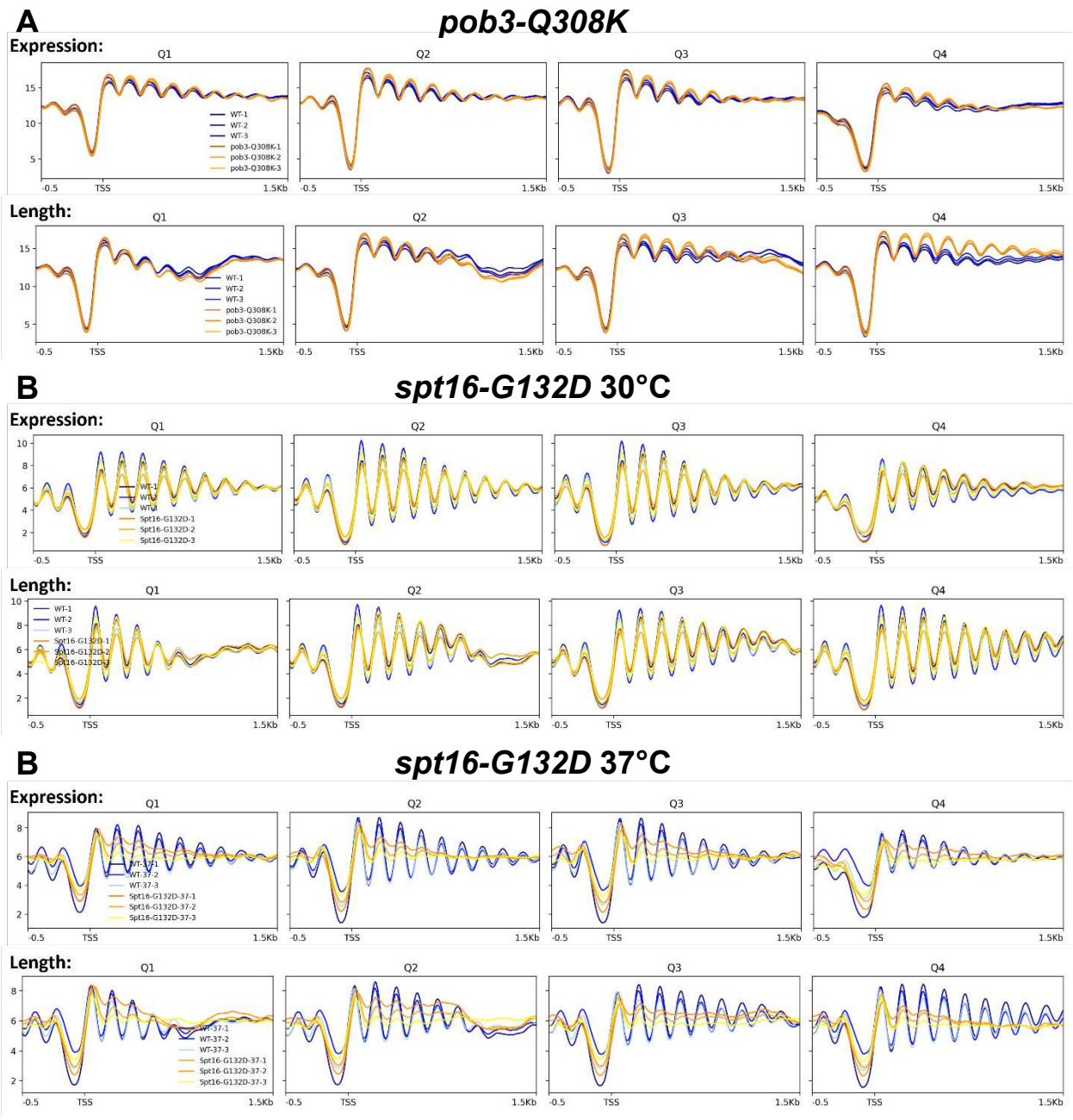


Figure B.9. Mutations in FACT produce changes in nucleosome features that generally correlate with RNAPII occupancy and gene length. (A) MNase-protected fragments from yeast expressing *pob3-Q308K* [8], (B) *spt16-G132D* [7], and (C) *spt16-G132D* shifted to a non-permissive temperature of 37°C [7] mapped over transcription start sites in quartiles ranked by Rpb3 occupancy (expression) and gene length.

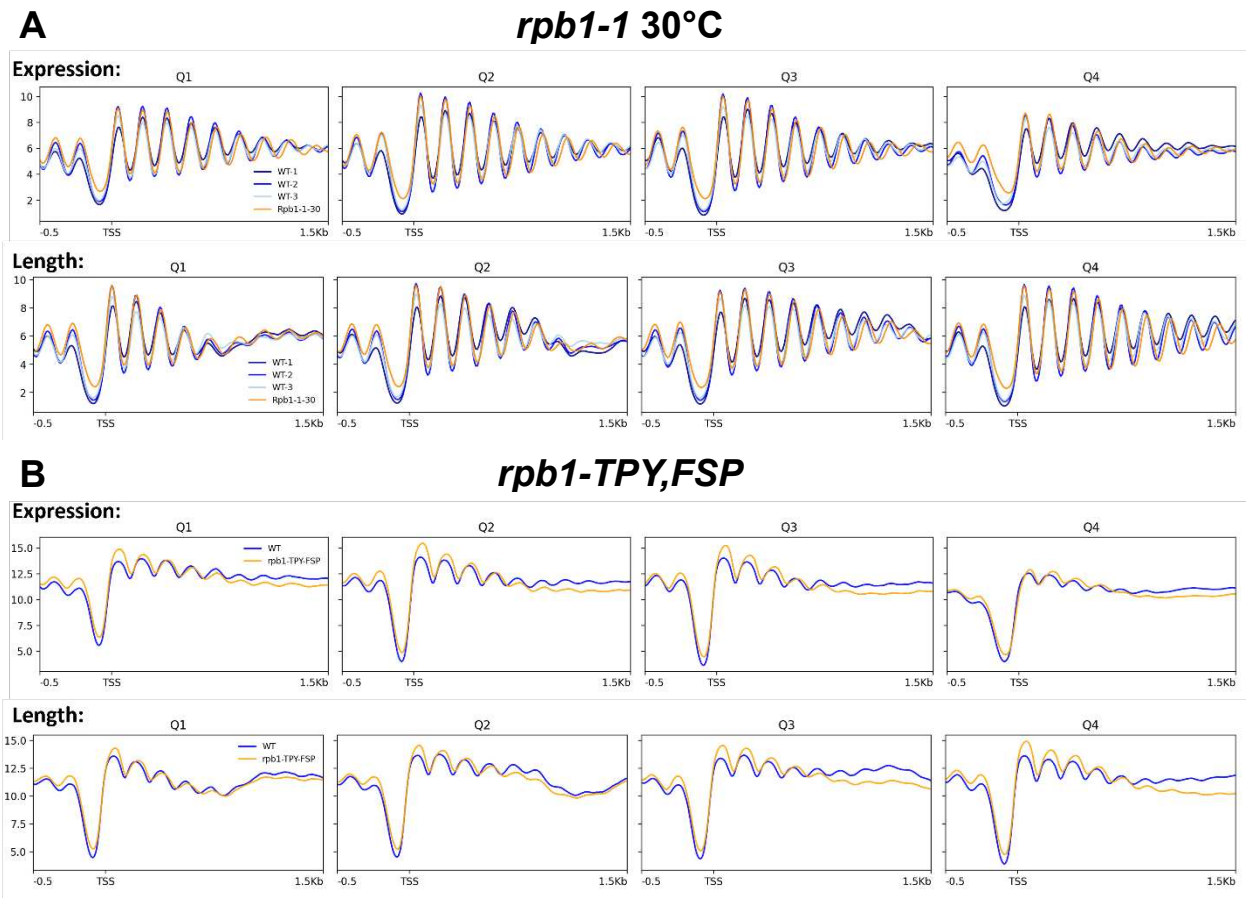


Figure B.10. Mutations in RNAPII (Rpb1) produce changes in nucleosome features that correlate with RNAPII occupancy and gene length. (A) MNase-protected fragments from yeast expressing *rpb1-1* [7] and (B) *rpb-TPY,FSP* [4] mapped over transcription start sites in quartiles ranked by Rpb3 occupancy (expression) and gene length.

	Strain	Positioning	RNAPII occupancy trends	Length trends	Occupancy	RNAPII occupancy trends	Length trends	NDR width	RNAPII occupancy trends	Length trends
Spn1	*spn1-141-305*	→	o	+	N.C.	o	o	N.C.	o	o
	spn1-K192N	→	o	+	N.C.	o	o	N.C.	o	o
	spn1-K192N (37°C)	→	+	+	↓	o	o	N.C.	o	o
Spt4	*spt4Δ*	→	+	+	N.C.	o	o	N.C.	o	o
Spt5	*spt5-QS*	N.C.	o	o	N.C.	o	o	N.C.	o	o
	spt5-QS (37°C)	N.C.	o	o	N.C.	o	o	→ ←	o	o
	spt5-AID	N/A	o	o	↓	o	o	N.C.	o	o
	spt5-3N	N/A	o	o	↓	o	o	→ ←	o	o
Spt6	*spt6-YW*	→	+	+	↓	o	o	N.C.	o	o
	spt6-YW (37°C)	→	+	+	↓	o	o	N.C.	o	o
	spt6-1004 (37°C)	→	+	o	↓	o	o	← →	o	o
	spt6-R,KK	→	o	-	↓	o	-	N.C.	o	o
FACT	*spt16-G132D*	N.C.	o	o	↓	o	o	N.C.	o	o
	spt16-G132D	→	o	o	↓	o	+	N.C.	o	o
	pob3-Q308K	→	o	+	↑	o	+	N.C.	o	o
RNAPII	*rpb1-1*	→	+	+	N.C.	o	o	→ ←	o	o
	rpb1-TPY,FSP	→	-	+	↑	-	+	→ ←	o	o

Figure B.11. Summary of chromatin feature aberrations in factor mutants and their correlations with RNAPII occupancy and gene length. Table summarizing defects in chromatin structure in factor mutants and their correlations with RNAPII occupancy and gene length. Mutations of chromatin factors produce various defects in nucleosome occupancy, positioning, and NDR width. N.C. indicates no change in feature. N/A** indicates that nucleosome positioning change could not be confidently interpreted as the sample contained too low of resolution in nucleosome peaks.

REFERENCES

1. Tonsager AJ, Zukowski A, Radebaugh CA, Weirich A, Stargell LA, Ramachandran S. The Histone Chaperone Spn1 Preserves Subnucleosomal Structures at Promoters and Nucleosome Positioning in Open Reading Frames. bioRxiv. 2024.
2. Viktorovskaya O, Chuang J, Jain D, Reim NI, Lopez-Rivera F, Murawska M, et al. Essential histone chaperones collaborate to regulate transcription and chromatin integrity. Genes Dev. 2021;35(9-10):698-712.
3. Doris SM, Chuang J, Viktorovskaya O, Murawska M, Spatt D, Churchman LS, Winston F. Spt6 Is Required for the Fidelity of Promoter Selection. Mol Cell. 2018;72(4):687-99 e6.
4. Connell Z, Parnell TJ, McCullough LL, Hill CP, Formosa T. The interaction between the Spt6-tSH2 domain and Rpb1 affects multiple functions of RNA Polymerase II. Nucleic Acids Res. 2022;50(2):784-802.
5. Uzun U, Brown T, Fischl H, Angel A, Mellor J. Spt4 facilitates the movement of RNA polymerase II through the +2 nucleosomal barrier. Cell Rep. 2021;36(13):109755.
6. Evrin C, Serra-Cardona A, Duan S, Mukherjee PP, Zhang Z, Labib KPM. Spt5 histone binding activity preserves chromatin during transcription by RNA polymerase II. EMBO J. 2022;41(5):e109783.
7. Feng J, Gan H, Eaton ML, Zhou H, Li S, Belsky JA, et al. Noncoding Transcription Is a Driving Force for Nucleosome Instability in spt16 Mutant Cells. Mol Cell Biol. 2016;36(13):1856-67.
8. McCullough LL, Pham TH, Parnell TJ, Connell Z, Chandrasekharan MB, Stillman DJ, Formosa T. Establishment and Maintenance of Chromatin Architecture Are Promoted Independently of Transcription by the Histone Chaperone FACT and H3-K56 Acetylation in Saccharomyces cerevisiae. Genetics. 2019;211(3):877-92.
9. Swygert SG, Kim S, Wu X, Fu T, Hsieh TH, Rando OJ, et al. Condensin-Dependent Chromatin Compaction Represses Transcription Globally during Quiescence. Mol Cell. 2019;73(3):533-46 e4.
10. Pelechano V, Wei W, Steinmetz LM. Extensive transcriptional heterogeneity revealed by isoform profiling. Nature. 2013;497(7447):127-31.
11. Rossi MJ, Kuntala PK, Lai WKM, Yamada N, Badjatia N, Mittal C, et al. A high-resolution protein architecture of the budding yeast genome. Nature. 2021;592(7853):309-14.

APPENDIX C: CHAPTER 4 SUPPLEMENTARIES

Supplemental Data

Table C.1. Primers, plasmids, and yeast strains used in the Chapter 4 study.

Primer	**Sequence**	
F-snf2-gRNA	5'-tcctctcattGTTTTAGAGCTAGAAATAGC-3'	
R-snf2-gRNA	5'-aagccaaacaGATCATTTATCTTTCACTGC-3'	
snf2-K798A-F	5'-GGTACATAATTTGTTGTAAAGCG-3'	
snf2-K798A-R	5'-CAAACAAACGCATTTTTAGATTC-3'	
snf5-HPH-F	5'AGGGAACATATAGTAAAGAACTACACAAAAGCAA CACAGGGTAATATAGATCTGTTTAGC-3'	
snf5-HPH-R	5'TTTACATCTCCGGTATATTTTATATATGTGTATATAT TTTGAATTCGAGCTCGTTTAAAC-3'	
snf5-up-F	5'-CATCTATTTTGGTTGGGTTG-3'	
snf5-down-R	5'-GCCCTATAAGTAGTTCTTGC-3'	
Plasmid	**Description**	**Source**
Cas9-NAT	*CAS9* gene under control of the *TEF1* promoter with the *CYC1* terminator sequence containing a nourseothricin resistance cassette (natMX6) and ampicillin resistance cassette (AmpR)	(Zhang et al., 2014) [1] Addgene #64329
gRNA-ura-HYB	Starting guide RNA plasmid with gRNA sequence (5'-GAGTAAAAAATTGTACTTGG-3') with scaffold under control of *SNR52* promoter and *SUP4* terminator containing a hygromycin resistance cassette (hphMX6) and ampicillin resistance cassette (AmpR)	(Zhang et al., 2014) [1] Addgene #64330
gRNA-snf2-HPH	gRNA-ura-HYB with the starting guide RNA sequence replaced by the *SNF2* targeting sequence (5'-TGTTTGGCTTTCCTCTCATT-3')	This study
pHPH-13myc	Plasmid containing a hygromycin resistance cassette (hphMX6) and ampicillin resistance cassette (AmpR)	This study
Strain	**Genotype**	**Source**
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Research Genetics
LZ0-1	BY4741 *spn1Δ::LEU2* + pCR311	(Li et al., 2018) [2]
SNF2 + Cas9	LZ0-1 + Cas9-NAT	This study
snf2-K798A	*snf2-K798A* + Cas9-NAT + gRNA-snf2-HPH	This study

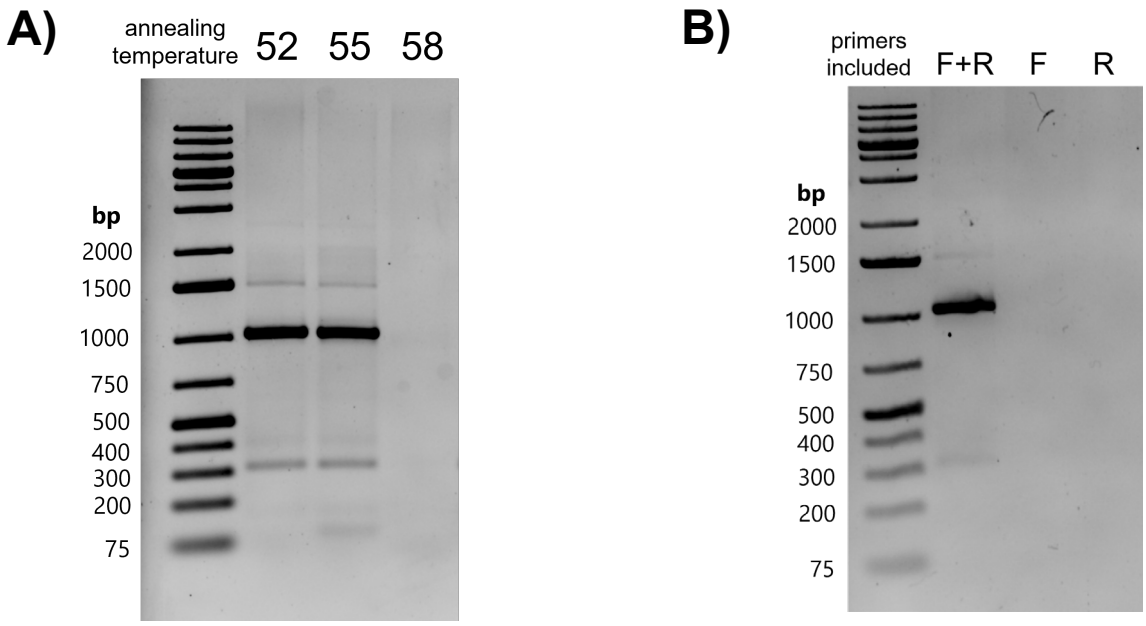


Figure C.1. Testing of PCR cycling conditions and oligonucleotide primers in genomic DNA amplification. (A) Agarose gel electrophoresis of PCR products amplified with a range of annealing temperatures. Genomic DNA from a hygromycin and nourseothricin resistant yeast amplified with the snf2-K798A-F and snf2-K798A-R primers produce the expected 1018bp fragment as well as other non-specific products. (B) Agarose gel electrophoresis from PCR reactions lacking either forward or reverse primers. Genomic DNA were used in PCR reactions lacking forward and reverse primers to assess whether non-specific product formation was driven by either the forward or reverse primer.

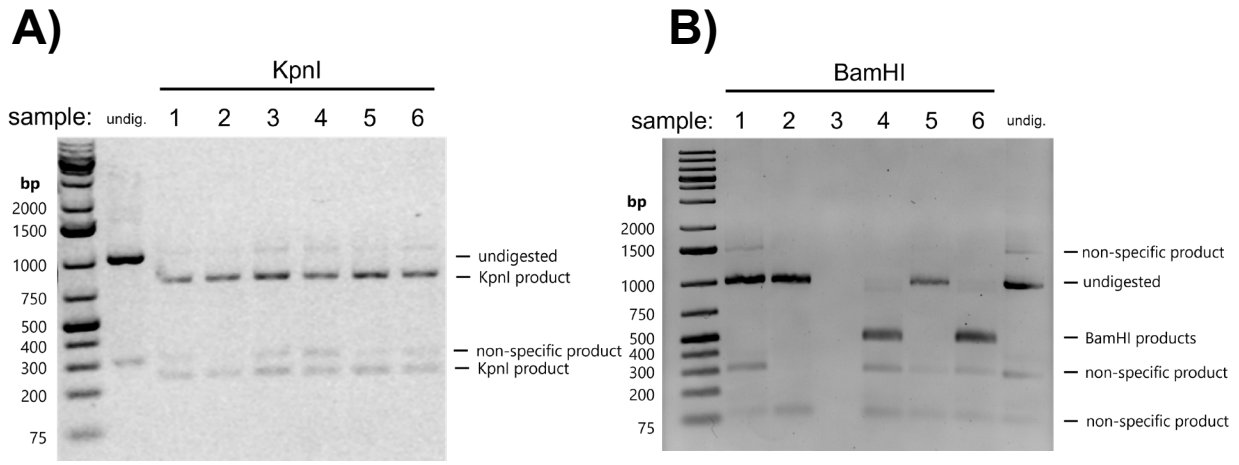


Figure C.2. Student restriction enzyme digests demonstrates cases where yeast genomic DNA samples are digested with both KpnI and BamHI. (A) Student samples (labeled 1-6) are digested with KpnI and loaded into an agarose gel alongside an undigested control. All six samples in this set are digested with KpnI, whose recognition sequence is present in the endogenous *SNF2*. (B) The same student samples in (A) are digested with BamHI and loaded into an agarose gel alongside an undigested control. In this set, samples 4 and 6 are also digested with BamHI, whose recognition sequence is introduced in the *snf2-K798A* donor DNA.

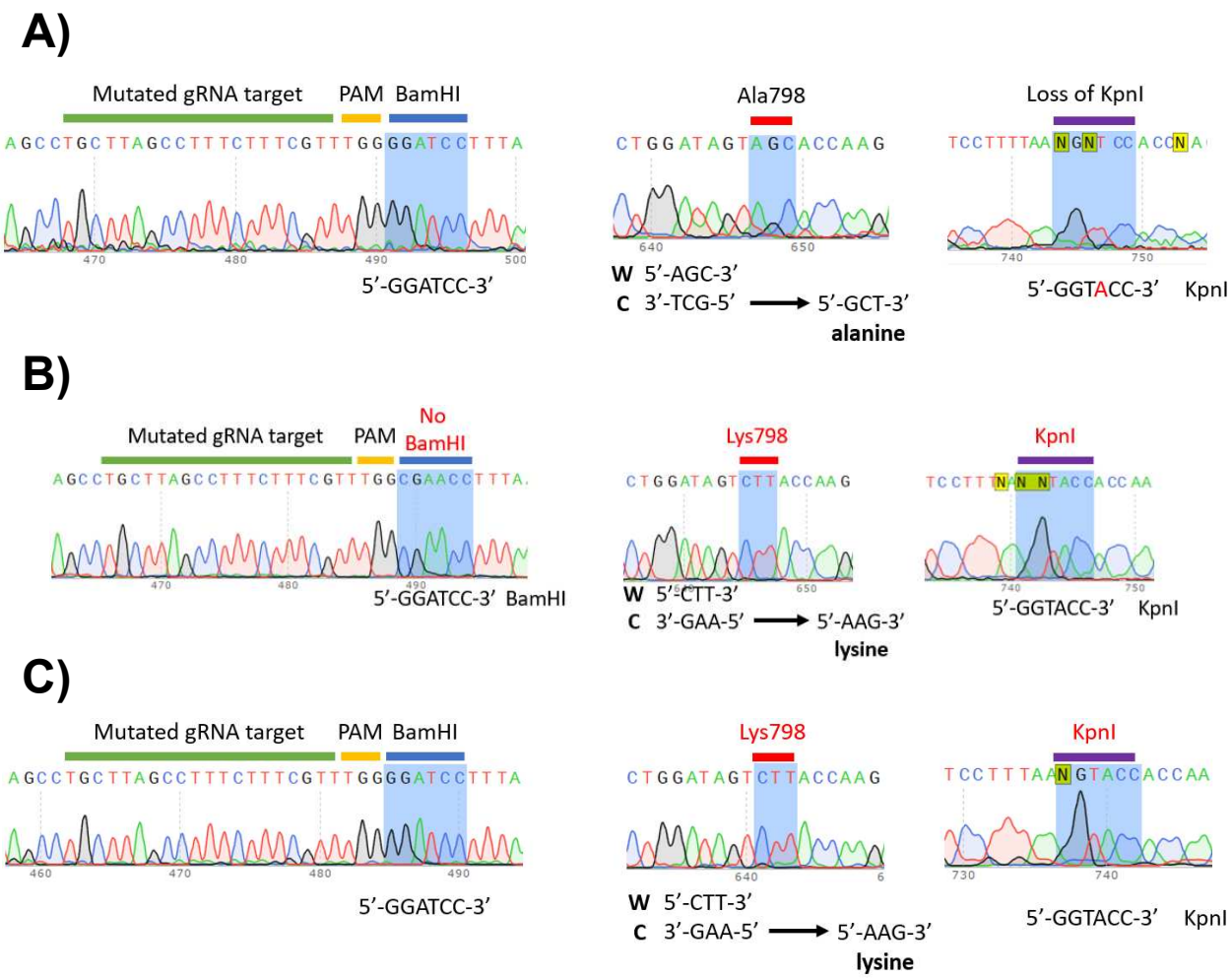


Figure C.3. Chromatogram data received from Sanger sequencing of *snf2-K798A* candidate cells. (A) Portions of the chromatogram obtained from the sequencing of DNA from a *snf2-K798A* cell demonstrates the mutations present in the donor DNA have been incorporated into the yeast *SNF2* gene, including mutations of the gRNA target, introduction of the BamHI cut site, substitution of the codon encoding Lys798 to Ala798, and removal of the KpnI cut site. (B) Portions of the chromatogram obtained from the sequencing of DNA from a false positive (*SNF2*) cell resistant to nourseothricin and hygromycin after transformation. This DNA was successfully cut with KpnI but not with BamHI, and Sanger sequencing confirms the presence of the wild-type *SNF2* sequences for the codon encoding Lys798 and KpnI restriction enzyme. However, a crossover event occurred, as this DNA did contain the mutated gRNA target sequence. (C) Portions of the chromatogram obtained from the sequencing of DNA cut by both KpnI and BamHI. Sanger sequencing confirmed the presence of both BamHI and KpnI cut sites in the genomic DNA from this cell. As this DNA contains the wild-type sequence encoding Lys-798, the crossover event occurring during homology-directed repair must have occurred in the region between the Lys-798 sequence and the newly engineered BamHI sequence.

Extended Materials and Methods

Background Preparation by Instructors

The pHPH-13myc, Cas9-NAT, and gRNA-ura-HYB plasmids were transformed into DH5α cells (New England Biolabs: C2987H) and plated on LB media containing 100µg/ml ampicillin (Amp). Single colonies were inoculated in 3-5ml of LB + Amp and grown at 37°C overnight. 700µl of the overnight culture was mixed with 300µl of 50% glycerol (v/v) for long-term storage of the bacterial strain at -80°C. The remainder of the culture was used for isolation of large quantities of plasmid using a QIAprep Spin Miniprep Kit (QIAGEN Cat. No. #27104). Primers for each PCR reaction and donor DNA were designed as described in the “How to design each reagent required for this course” section of the Supplementary Material.

Yeast Strain and Media

The starting *S. cerevisiae* strain used for the laboratory is derived from BY4741 and has the endogenous *SPN1* gene deleted but contains a plasmid expressing *SPN1* under the control of their native promoter and terminator and was created as previously described[2-4]. Yeast were grown in yeast extract, peptone, dextrose (YPD; 2% dextrose) media at 30°C prior to transformations. After transformation with the HPH DNA, cells were grown on YPD agar plates containing 250µg/ml hygromycin (Gold Biotechnology Cat. No. H-270-1). Cells transformed with the Cas9 plasmid were grown on YPD agar plates containing 100µg/ml nourseothricin (Gold Biotechnology Cat. No. N-500-500). Cas9 expressing yeast transformed with the guide RNA plasmid and donor DNA were grown on YPD agar plates containing 500µg/ml hygromycin and 100µg/ml nourseothricin. After isolating colonies from this transformation, cells were grown in YPD media for all subsequent experiments.

PCR Amplification of donor DNA

PCR reactions were prepared with Phusion® High-Fidelity DNA Polymerase (New England Biolabs: M0530S) in 0.2ml thin-walled PCR tubes. Reactions are comprised of the following: 16.25µl double-distilled Milli-Q water (ddH_2O), 2.5µl 5X Phusion GC buffer, 0.5µl 10mM dNTPs, 1µl 10µM forward primer (snf2-K798A-F), 1µl 10µM reverse primer (snf2-K798A-R), 1µl 5ng/µl donor DNA and 0.25µl Phusion High-Fidelity DNA Polymerase. Reactions were placed in a thermocycler and subjected to the following PCR program unless noted otherwise: Step 1: 98°C for 50 sec. Step 2: 98°C for 10 sec, 52°C for 20 sec, 72°C for 30 sec. Repeat Step 2 for 35 cycles. Step 3: 72°C for 3 min. Step 4: 4°C indefinite hold. PCR products were loaded into wells of a 1% agarose gel containing SYBR® Safe DNA Gel Stain (Thermo Fischer Scientific: S33102) and GeneRuler 1kb DNA Ladder (Thermo Fischer Scientific: SM0312) or GeneRuler 1kb+ DNA Ladder (Thermo Fischer Scientific: SM1331) was used to check for the presence of a 1018bp amplicon (donor DNA).

The remaining PCR product is precipitated with 0.4 volumes of 7.5M ammonium acetate and 2 volumes of 95% ethanol and left at -20°C for minimally 2 hours. Samples are centrifuged for 10 min at 13K RPM and the supernatant is removed by decanting. Pellets are washed with 200µl of 70% ethanol and centrifuged again for 10 min at 13K RPM. The supernatant is removed, and the tubes are left with their lids open until the ethanol fully evaporated. Samples are suspended in 50µl TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0) and DNA concentration is quantified using a ThermoScientific NanoDrop™ One^C.

Engineering of the guide RNA plasmid:

The starting guide RNA plasmid (gRNA-ura-HYB) was used as a template for PCR reactions using primers that amplify the entire plasmid into a linear product but replace the 20bp guide RNA recognition sequence. PCR reactions were prepared as outlined in the Q5® Site-Directed Mutagenesis Kit Manual (New England Biolabs: E0554S). Reactions were comprised of the following: 12.5µL of Q5 Hot Start High-Fidelity 2X Master Mix, 1µL of 5ng/µl plasmid, 9µL of

Milli-Q water, 1.25µL 10µM forward primer (F-gRNA or F-gRNA control), and 1.25µL 10µM reverse primer (R-gRNA or R-gRNA control). Reactions were placed in a thermocycler and subjected to the following PCR program: Step 1: 98°C for 30 sec. Step 2: 98°C for 10 sec, 52°C for 20 sec, 72°C for 30 sec. Repeat Step 2 for 25 cycles. Step 3: 72°C for 2 min. Step 4: 4°C indefinite hold. PCR products were loaded into wells of a 1% agarose gel containing SYBR® Safe DNA Gel Stain (Thermo Fischer Scientific: S33102) and GeneRuler 1kb DNA Ladder (Thermo Fischer Scientific: SM0312) was used to check for the presence of a 6509bp amplicon.

After PCR, the amplificon is added directly to a unique Kinase-Ligase-DpnI (KLD) enzyme mix for circularization and template removal as described in the Q5® Site-Directed Mutagenesis Kit Manual. The resulting plasmid is transformed into chemically competent *E. coli* and plated on LB + 100 µg/ml ampicillin plates. Single colonies were inoculated in 3-5ml of LB + Amp and grown at 37°C overnight. 700µl of the overnight culture was mixed with 300µl of 50% glycerol (v/v) for long-term storage of the bacterial strain at -80°C. The remainder of the culture was used for isolation of large quantities of plasmid using a QIAprep Spin Miniprep Kit (QIAGEN Cat. No. #27104). A 10µl aliquot of 50ng/µl plasmid was sent in to Azenta/GENEWIZ (Azenta US, Inc., South Plainfield, NJ, USA) for Sanger sequencing using the M13 fwd sequencing primer provided by Genewiz. The chromatogram file was viewed using Sequence Scanner Software v2.0, and regions with high quality data were aligned to the starting gRNA-phMX6 sequence using BLAST® to assess the success of the guide RNA plasmid engineering.

Rapid transformation of Cas9 plasmid into yeast

The Cas9 plasmid was transformed into the starting yeast strain using the lithium acetate transformation method[5]. A single colony was inoculated in ~5ml YPD media and diluted 1:2, 1:5, and 1:10 in glass culture tubes then grown overnight at 30°C in a rotator. The following morning the OD_{600} of each culture was measured with a spectrophotometer, and the culture with OD_{600} = 0.7 – 1.0 was used for transformation. 1.2ml of cells were transferred to an Eppendorf tube and

centrifuged at 5,000 x g for 3 min. The supernatant was decanted, then the pellet was resuspended in 1ml of sterile ddH_2O and centrifuged at maximum speed for 1 min. The supernatant was removed by pipetting and the cells were resuspended in a LiAc/TE solution (0.1M lithium acetate, 10mM Tris-HCl, pH 8.0, 1mM EDTA). Cells were centrifuged at maximum speed for 1 minute and the supernatant was removed by pipetting. Cells were resuspended in 100µl of LiAc/TE solution and 10µl of 10mg/ml salmon sperm DNA. Cas9 plasmid DNA (1µg) was added to a 50µl aliquot of the cell suspension followed by addition of 300µl of LiAc/PEG/TE solution (0.1M lithium acetate, 10mM Tris-HCl, pH 8.0, 1mM EDTA, 400g/L PEG 3350). Cells were incubated at 30°C for 30 min, followed by heat shock at 42°C for 20 min. 750µl of YPD was added to the transformation mix and then transferred to sterile glass culture tubes, followed by incubation at 30°C for 1.5 hours on a rotator. Cells were transferred to an Eppendorf tube, centrifuged at maximum speed for 1 min, then resuspended in 250µl of YPD. Half of the transformants were plated on a YPD plate containing 100µg/ml nourseothricin and grown at 30°C for 2 – 4 days.

High efficiency transformation of yeast

The guide RNA plasmid and donor DNA were transformed into yeast expressing the Cas9 plasmid using a high efficiency lithium acetate transformation method[6]. A single colony was inoculated in 3ml YPD media + 100µg/ml nourseothricin then grown overnight at 30°C in a rotator. The following day, the OD_{600} of each culture was measured with a spectrophotometer, and the culture was diluted into fresh media in a volume sufficiently large for >10ml per student and grown overnight at 30°C in a shaker. The starting OD_{600} of the culture was calculated to ensure the culture is at OD_{600} = 0.7 – 1.0 by the next morning. A 10ml aliquot of cells were transferred to sterile 15ml conical tubes then centrifuged at 3000 RPM for 5 min. The supernatant was decanted, and the cells were suspended in 5ml of sterile water and centrifuged again at 3000 RPM for 5 min. The supernatant was decanted, and the cells were resuspended

in 1ml of 100mM LiAc, then transferred to a 1.7ml Eppendorf tube. Cells were pelleted by centrifugation at maximum speed for 1 min, then the supernatant was pulled off by pipetting. Cells were suspended in 100µl of 100mM LiAc by vortexing. Transformation mixes were prepared with the following: 240µl of 50% w/v PEG, 36µl of 1M LiAc, 10µl of 10mg/ml salmon sperm DNA, 1µg of guide RNA plasmid, 5µg of donor DNA, and ddH_2O to a final volume of 360µl. The transformation mix was added to a 50µl aliquot of the cell suspension and suspended by vortexing vigorously. Cells were incubated at 42°C for 40 min, then transferred to glass culture tubes. 1ml of YPD was added to the transformants and cells were then incubated at 30°C for approximately 3 hours on a rotator. Cells were transferred to an Eppendorf tube, centrifuged at maximum speed for 1 min, then resuspended in 250µl of YPD. Half of the transformants were plated on a YPD plate containing 100µg/ml nourseothricin and 500µg/ml hygromycin and grown at 30°C for 2 – 4 days. The starting yeast strain was transformed with HPH DNA using the high efficiency transformation protocol and plated on YPD media containing 250µg/ml hygromycin.

Rapid isolation of yeast genomic DNA

Genomic DNA was isolated from yeast resistant to hygromycin and nourseothricin (*snf2-K798A* candidates) and from yeast resistant to hygromycin (*snf5::hphMX6* candidates). Briefly, single colonies were inoculated in 10ml of YPD and grown overnight at 30°C. 700µl of the overnight cultures were mixed with 300µl of 50% glycerol (v/v) for long-term storage of the yeast candidates at -80°C. The remainder of the cultures were centrifuged at 3000 RPM for 5 min and the supernatant was removed by decanting. The pellet was suspended in 1ml of ddH_2O then transferred to an Eppendorf tube and centrifuged at maximum speed for 30 sec. The supernatant was removed by pipetting and cells were suspended in 200µl of breaking buffer (100mM NaCl, 10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0, 2% Triton X-100, 1% SDS), 0.3g of 0.5mm glass beads (~200µl volume), and 200µl of phenol:chloroform:isoamyl alcohol solution

(25:24:1, v/v). Samples were vortexed for 3 minutes then 200µl of TE buffer was added followed by brief vortexing. Samples were centrifuged at maximum speed for 5 min. The upper aqueous layer (~400µl) was transferred to new Eppendorf tubes, then 1ml of 95% ethanol was added followed by mixing by inversion. Samples were centrifuged at maximum speed for 3 min, then the supernatant was removed by pipetting. Pellets were resuspended in 400µl TE buffer followed by addition of 3µl of 10mg/ml DNase-free RNase A then incubated at 37°C for 5 min. 5.3µl of 7.5M ammonium acetate and 1ml of 95% ethanol were added to the tubes followed with mixing by inversion. Samples were centrifuged at maximum speed for 3 min, and the supernatant was removed by pipetting. Pellets were washed with 1ml of 75% ethanol and centrifuged at maximum speed for 3 min. The supernatant was discarded, and pellets were dried by inversion on a paper towel with the lids open. The DNA pellets were resuspended in 100µl of TE buffer.

Diagnostic test for successful genome engineering

PCR reactions to test for the presence of the *snf2-K798A* point mutation were prepared with identical conditions to the donor DNA PCR amplification, but with 1µl of genomic DNA used as the template. Genomic DNA from *snf5::hphMX6* candidates were amplified with 1µl of genomic DNA, 1µl forward primer (snf5-up-F) and 1µl reverse primer (snf5-down-R), with an extension time of 2 min 30 sec. PCR products were loaded into wells of a 1% agarose gel as described previously to check for the presence of amplification. For the *snf5::hphMX6* candidates, the expected PCR product sizes are 3158 for the *SNF5* genotype, and 2122 for the *snf5::hphMX6* genotype.

To distinguish *SNF2* from the *snf2-K798A* genotypes, PCR products were digested separately with KpnI and BamHI as follows: 8µl of PCR product, 2.5µl of 10X CutSmart buffer, 14µl of ddH_2O, and 0.5µl of either 20,000U/ml KpnI-HF® (New England Biolabs: #R3142) or 20,000U/ml BamHI-HF® (New England Biolabs: #R3136). Samples were incubated at 37°C for

1 hour, then loaded in a 1.5% agarose gel as described previously. Only *SNF2* cells digested with KpnI will yield 243bp and 775bp products, while only *snf2-K798A* cells digested with BamHI will yield 523bp and 493bp products.

Spot assay to assess growth phenotypes

Single colonies from wild-type or mutant yeast strains were inoculated in ~5ml YPD media and diluted 1:2, 1:5, and 1:10 in glass culture tubes then grown overnight at 30°C in a rotator. The following morning the OD_{600} of each culture was measured with a spectrophotometer, and the culture with OD_{600} = 0.7 – 1.0 was used for the spot assay. Cells were collected by centrifugation at maximum speed for 2 minutes. The supernatant was removed by pipetting, then washed with 1ml ddH_2O. Cells were suspended after centrifugation in a volume of ddH_2O to bring its OD_{600} = 1. Four Ten-fold serial dilutions were prepared in glass culture tubes and 5µl of each was spotted onto YPD media, then incubated at either 30°C, 37°C, or 39°C. Plates were incubated for 2 days, imaged, and mutant strains were scored for growth qualitatively in comparison to the wild-type strain.

REFERENCES

1. Zhang GC, Kong, II, Kim H, Liu JJ, Cate JH, Jin YS. Construction of a quadruple auxotrophic mutant of an industrial polyploid saccharomyces cerevisiae strain by using RNA-guided Cas9 nuclease. Appl Environ Microbiol. 2014;80(24):7694-701.
2. Li S, Almeida AR, Radebaugh CA, Zhang L, Chen X, Huang L, et al. The elongation factor Spn1 is a multi-functional chromatin binding protein. Nucleic Acids Res. 2018;46(5):2321-34.
3. Zhang L, Fletcher AG, Cheung V, Winston F, Stargell LA. Spn1 regulates the recruitment of Spt6 and the Swi/Snf complex during transcriptional activation by RNA polymerase II. Mol Cell Biol. 2008;28(4):1393-403.
4. Thurston AK, Radebaugh CA, Almeida AR, Argueso JL, Stargell LA. Genome Instability Is Promoted by the Chromatin-Binding Protein Spn1 in Saccharomyces cerevisiae. Genetics. 2018;210(4):1227-37.
5. Schiestl RH, Gietz RD. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr Genet. 1989;16(5-6):339-46.
6. Gietz RD, Schiestl RH. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat Protoc. 2007;2(1):31-4.

APPENDIX D: INSTRUCTOR MANUAL

Introduction

This manual is written with instructions on how to use CRISPR-Cas9 genome engineering to introduce a mutation into any yeast gene. This manual has been implemented during two semesters of BC406B, an eight-week long research experience for upperclassmen Biochemistry majors at Colorado State University. It contains detailed instructions on how to design each reagent required for this process including PCR primers, guide RNA target sequences, and donor DNA. The manual contains experimental procedures with detailed lists of reagents/supplies required, tips for success, clean-up procedures, as well as additional comments written for instructors. Finally, the manual contains a list of 10 daily check-ins with answer keys and two student assignments that can be implemented into the classroom.

Additionally, this manual includes instructions on how to delete any yeast gene using homologous recombination of the *hphMX6* hygromycin antibiotic resistance cassette. These instructions are contained within the following experimental procedures: Days 1-2.5, 9-12. This procedure was included in the manual to give students an opportunity to learn about other approaches of genome engineering.

How to design each reagent required for this course:

This course has been previously implemented to produce the *snf2-K798A* point mutation in the yeast genome by CRISPR-Cas9 mediated genome editing and delete the *SNF5* gene by a one-step homologous recombination method. The following is a set of instructions for producing the necessary DNA sequences required for implementation of this project for mutation of other yeast genes.

Introduction:

A widely used approach for deletion of yeast genes is through replacement of the endogenous sequence with that of an auxotrophic marker or antibiotic resistance cassette. This method is advantageous as it selects for successful homologous recombination into the yeast genome and is highly effective at deleting the gene of interest. As this approach incorporates a selectable marker directly into the yeast genome, it is limited in its applications.

CRISPR-Cas9 genome editing is versatile by comparison and can be used to produce deletions, point mutations, and other types of edits without introduction of a marker into the genome. However, it requires more reagents to perform which renders it more costly. Additionally, unless CRISPR-Cas9 is used to edit a gene which has a visible change in phenotype upon mutation, selection is only effective at isolating cells successfully transformed with the Cas9 and guide RNA plasmids. Thus, its efficiency is lower and requires screening of more colonies for the desired mutations.

Design of DNA for one-step homologous recombination-based deletion of a yeast gene

This sequence of DNA is engineered with homology to a gene of interest and transformed into yeast. If an auxotrophic marker is used, such as *URA3* or *HIS3*, transformants are plated on synthetic media lacking uracil or histidine respectively to select for the successful incorporation of the marker into the yeast genome. If an antibiotic resistance cassette is preferred, cells transformed with this marker can be grown on media containing the antibiotic for selection.

1) Obtain the sequence of your desired marker as well as a source to amplify it by PCR. For demonstration purposes, the design is shown for amplification of the *hphMX6* cassette from the pHPH-13myc plasmid targeted to delete the yeast *SNF2* gene.

2) Locate the 5’ and 3’ ends of your marker to be included in primers to amplify the marker. Obtain a suitable primer sequence just upstream of the first ~20nt of the 5’ end of the cassette. (HPH forward is the sequence used here.)

3) Obtain a suitable primer sequence just downstream of the last ~20nt of the 3' end of the cassette (HPH reverse is the sequence used here.)
    a. Ensure you obtain the **reverse compliment** for the 3' portion of the marker.

4) Obtain the sequence of your gene of interest and obtain ~40nt of sequence immediately upstream and downstream of the open reading frame.
    a. Ensure you obtain the **reverse compliment** for your downstream sequence.

Forward:

Reverse:

5) Design your forward and reverse primers by combining your marker and gene sequence as follows:

Forward primer: SNF5-HPH-F

5'-AGGGAACATATAGTAAAGAACTACACAAAAGCAACACAGGGTAATATAGATCTGTTTAGC-3'

Reverse primer: SNF5-HPH-R

5'-TTTACATCTCCGGTATATTTTATATATGTGTATATATTTTGAATTCGAGCTCGTTTAAAC-3'

Blue: indicates regions homologous to the *hphMX6* cassette

Green: indicates 40bp region homologous to region immediately outside of the genomic sequence of *SNF5*.

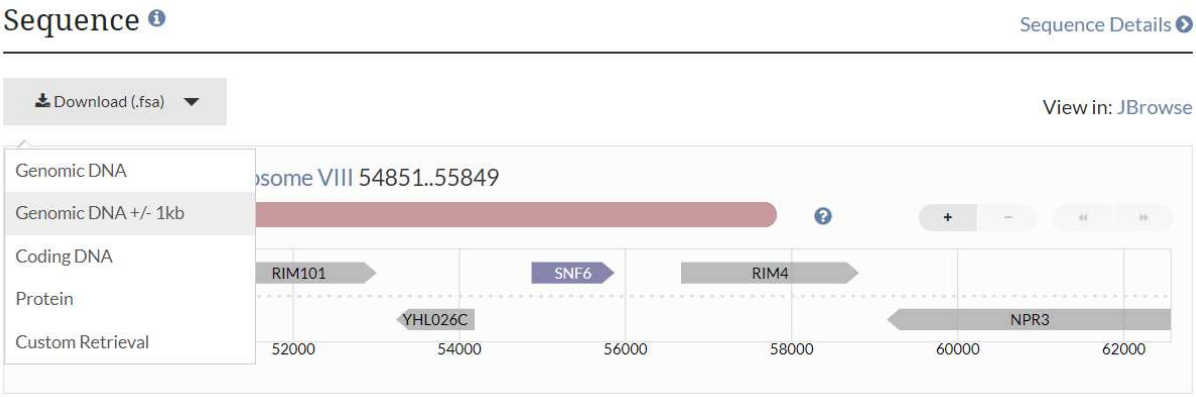
6) Use these primers to amplify the marker from your template by PCR.

Design of guide RNA plasmid

The design of the guide RNA plasmid and the reagents used to engineer it are part of the included student assignment “DNA Design Assignment”, which includes student questions during the procedure. For demonstration purposes, the following instructions will be for designing a guide RNA sequence that targets Cas9 to the *SNF6* yeast gene.

Step 1: Download Snapgene Viewer and obtain the DNA sequence files for *SNF6* and the gRNA plasmid (Addgene #64330).

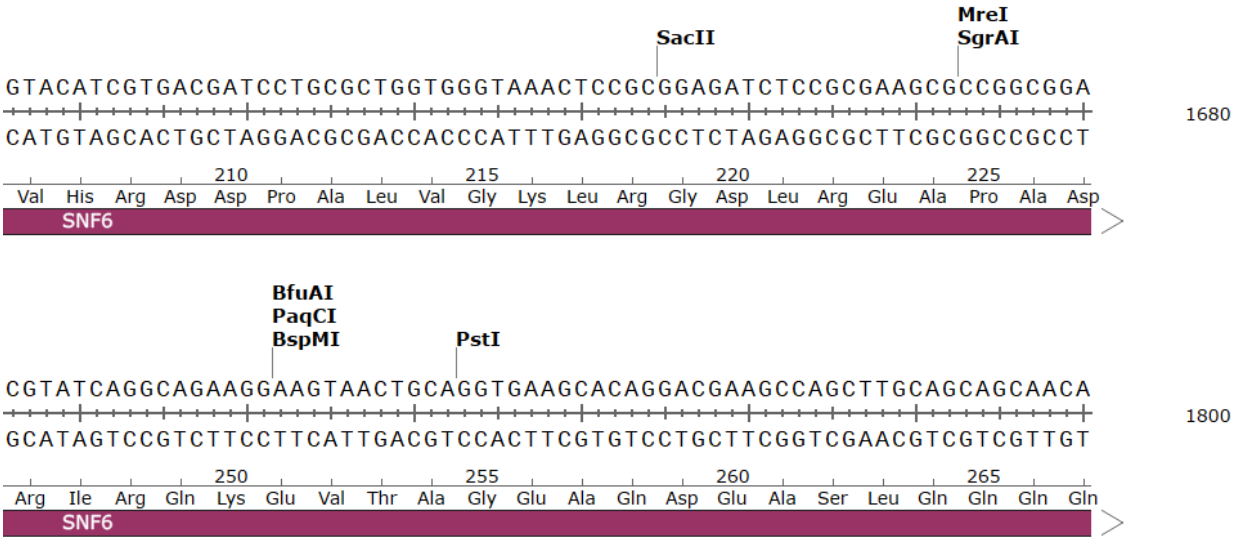
1) Navigate to https://www.snapgene.com/snapgene-viewer to download Snapgene Viewer (if you don’t already have this software on your computer)
    a. This program will allow you to work with the DNA sequences required for engineering a guide RNA for targeting Cas9 to generate a double-stranded break at the *SNF6* gene.
2) Obtain the gRNA-ura-HYB sequence (https://www.addgene.org/64330/sequences/) and SNF6 genomic +/- 1000bp sequence from the Saccharomyces Genome Database. (https://www.yeastgenome.org/locus/S000001017)



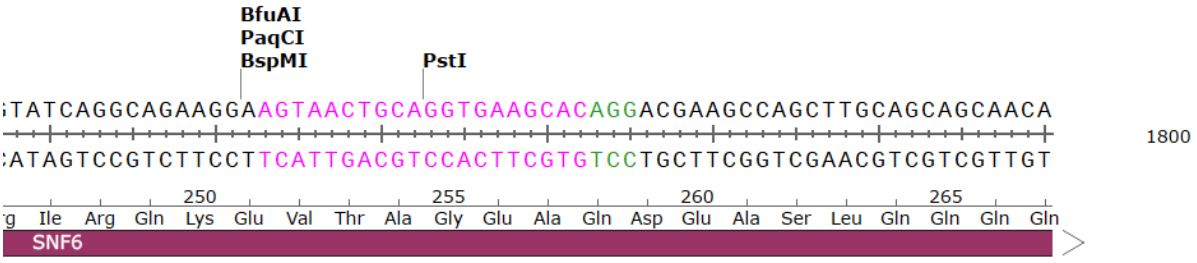
Step 2: Locate a sequence of *SNF6* adjacent to a PAM sequence.

1) Your goal is to find a PAM sequence within the *SNF6* gene near the middle of the gene. (The sequence for Cas9 is **NGG**.)

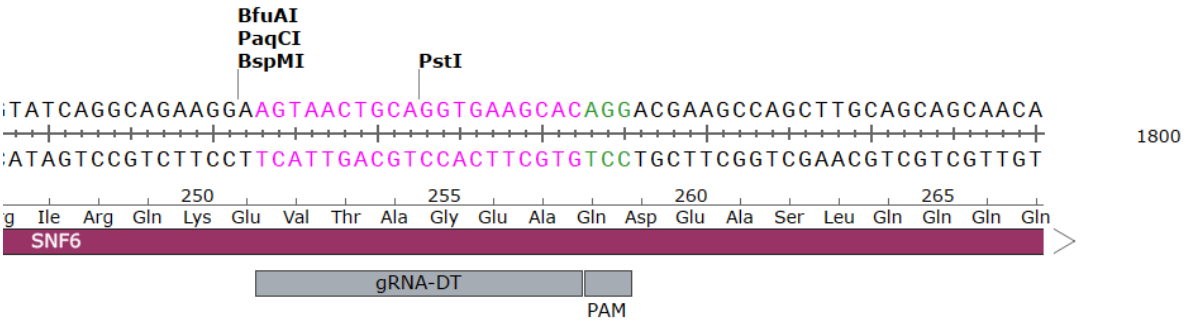
a. In the downloaded DNA sequence file, the open reading frame of *SNF6* spans 1000 -- 1999bp. You can see the DNA sequence including restriction enzyme digest sequences and its translated sequence, as depicted below.



2) Select a PAM sequence located in the region of DNA you would like to target Cas9 to. For demonstration purposes, the sequence encoding Gln-258 was selected. Use your cursor to highlight it.
3) Right click your selection, then press “Set DNA Color”, and choose a color other than black to recolor the text for your PAM sequence.
4) Next, highlight 20bp immediately upstream of your PAM sequence, and recolor this sequence as well, but with a different color from your PAM sequence.



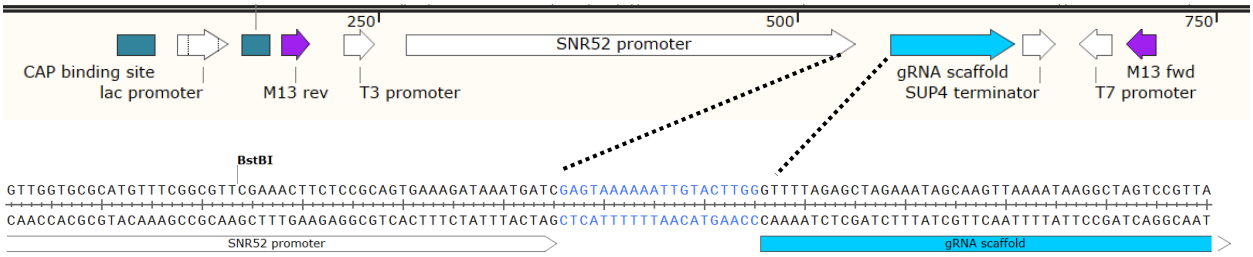
5) **Tip:** You can also highlight your sequences separately and click Feature > Add Feature, then name it “gRNA”, or “PAM” to be able to find these sequences more easily in your document.



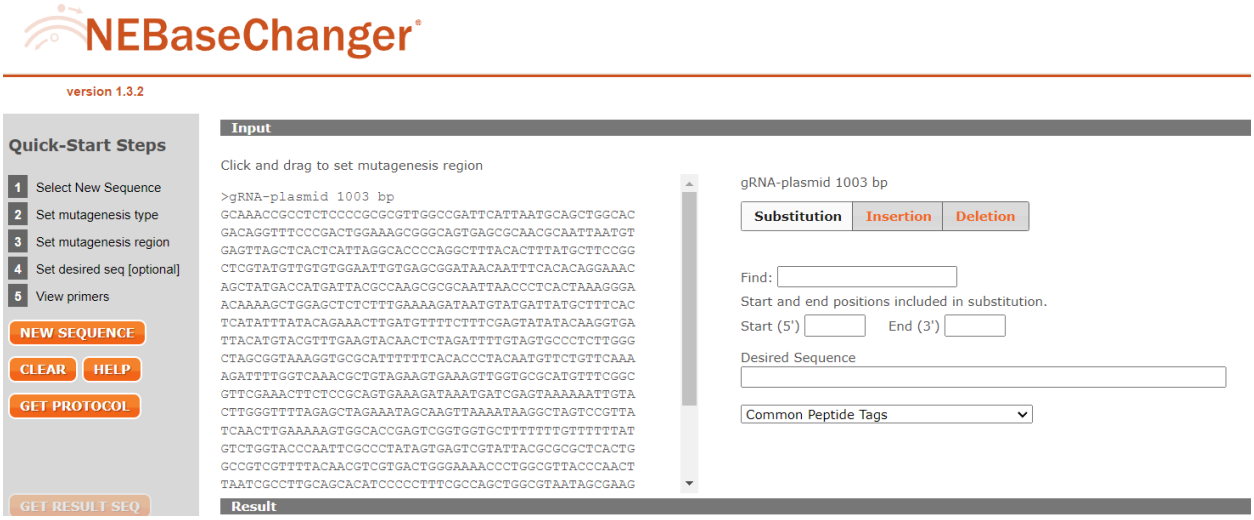
6) Obtain this gRNA sequence from Snapgene by copying and pasting it elsewhere.

Step 3: Design primers to replace the guide RNA sequence in the gRNA plasmid with your guide RNA sequence.

1) Open the gRNA-ura-HYB sequence in the Snapgene Viewer and locate the 20bp region between the SNR52 promoter and the gRNA scaffold. This is the existing guide RNA target sequence encoded in the gRNA-ura-HYB plasmid.



2) This is the sequence that you will replace with the gRNA sequence you created in the previous step.
3) We will now use **NEBaseChanger** to design primers that will bind the gRNA-ura-HYB plasmid and replace this 20bp sequence with your gRNA target sequence located in the gene of interest.
4) Navigate to https://nebasechangerv1.neb.com/
5) Click on the button titled "**Please enter a new sequence to begin.**"
6) Copy and paste the entirety of the gRNA-ura-HYB sequence into the text box that appears on your screen, and name it.
    a. You should see something that looks like this:



7) Now copy and paste the 20bp sequence you obtained from the **gRNA-ura-HYB** sequence into "Find". You should then see your sequence highlighted in blue to the left.

8) Bring your cursor on top of the first and last base pair highlighted in blue, and type what positions they are located at. They should be at 536 and 555.
9) Type those numbers into the Start (5') and End (3') regions and verify that the orange highlighted sequence matches what was highlighted in blue.
10) Next, paste your desired gRNA target sequence into "Desired Sequence".
    a. You should now have content under "Result":

Result

```
   S  E  R  *  M  I  S  N  C  R  *  S  T  F  *  S  *  K  *
  Q  *  K  I  N  D  Q  *  L  Q  V  K  H  V  L  E  L  E  I
 A  V  K  D  K  *  S  V  T  A  G  E  A  R  F  R  A  R  N  S
GCAGTGAAAGATAAATGATCagtaactgcaggtgaagcacGTTTTAGAGCTAGAAATAGC
CGTCACTTTCTATTTACTAGtcattgacgtccacttcgtgCAAAATCTCGATCTTTATCG
```

Required Primers

Name (F/R)	Oligo (Uppercase = target-specific primer)	Len	% GC	Tm	Ta *
Q5SDM_2/6/2024_F	ggtgaagcacGTTTTAGAGCTAGAAATAGC	30	43	56°C	56°C
Q5SDM_2/6/2024_R	tgcagttactGATCATTTATCTTTCACTGC	30	37	55°C	

* Ta (recommended annealing temperature)

11) These primers can be used to engineer the gRNA-ura-HYB plasmid to express a guide RNA targeting Cas9 to induce a double-stranded break at your gene of interest.

Design of donor DNA sequence

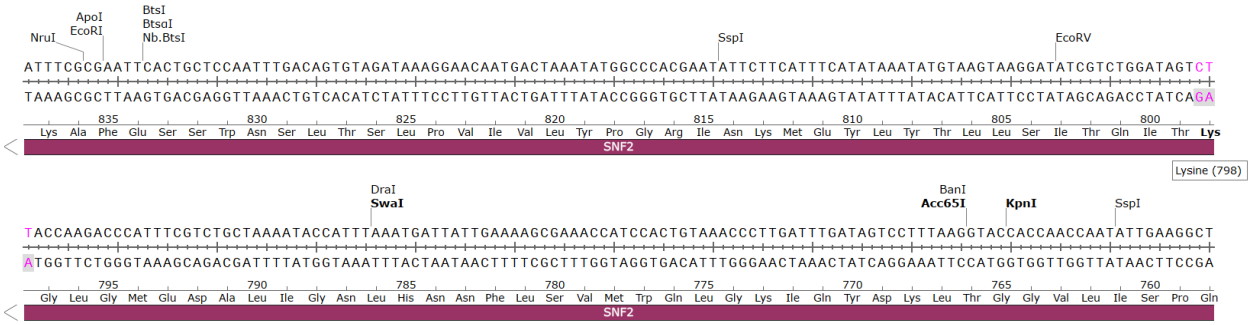
The donor DNA sequence design resembles a ~1kb portion of the yeast genome at your gene of interest with a few key mutations:

1) Introduce your desired mutation to your gene of interest.
2) The gRNA target sequence contains silent mutations to not be cleaved by Cas9.
3) Silent mutations to eliminate an endogenous restriction enzyme cut site.
4) Silent mutations to introduce a new restriction enzyme cut site.

The third and fourth mutations are optional, as incorporation of the donor DNA into the genome can be confirmed by sequencing alone. If your desired mutation produces a significantly different length of DNA than the starting sequence, this step is not required as PCR amplification alone will allow you to distinguish wild type from mutant DNA sequences. However, if your desired mutation does not change the length of your genomic DNA sequence, inclusion of these mutations allows for a diagnostic test to reduce the number of samples submitted for sequencing. Use these instructions to design a donor DNA sequence for CRISPR-Cas9 genome editing of a gene of interest. For demonstration purposes, these instructions outline the design of the *snf2-K798A* mutation strategy.

Step 1: Introduce your desired mutation to your gene of interest.

1) Open Snapgene Viewer and locate your desired site(s) of mutation. **This should be within ~500bp of your selected gRNA target sequence!**



Note that the SNF2 gene is encoded on the Crick strand of DNA and the highlighted sequence "5'-AAG-3'" is the codon for lysine.

2) If you are introducing point mutations into the genome, obtain the codon sequences for your desired amino acid changes. **Ensure that the codons are utilized frequently in yeast!**
    a. Use this tool from the Codon Usage Database to view the codon usage in *S. cerevisiae*: https://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4932

***Saccharomyces cerevisiae* [gbpln]: 14411 CDS's (6534504 codons)**

fields: [triplet] [amino acid] [fraction] [frequency: **per thousand**] ([number])

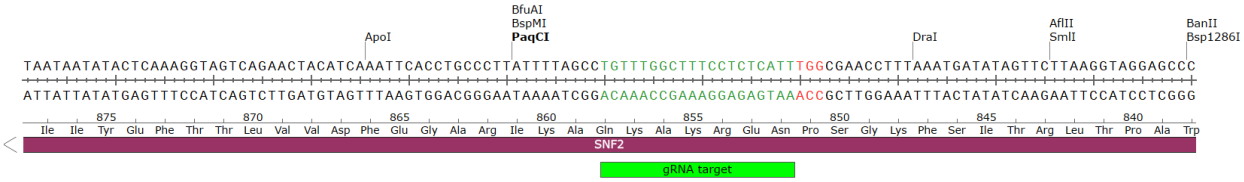
```
UUU F 0.59 26.1 (170666)  UCU S 0.26 23.5 (153557)  UAU Y 0.56 18.8 (122728)  UGU C 0.63  8.1 ( 52903)
UUC F 0.41 18.4 (120510)  UCC S 0.16 14.2 ( 92923)  UAC Y 0.44 14.8 ( 96596)  UGC C 0.37  4.8 ( 31095)
UUA L 0.28 26.2 (170884)  UCA S 0.21 18.7 (122028)  UAA * 0.47  1.1 (  6913)  UGA * 0.30  0.7 (  4447)
UUG L 0.29 27.2 (177573)  UCG S 0.10  8.6 ( 55951)  UAG * 0.23  0.5 (  3312)  UGG W 1.00 10.4 ( 67789)

CUU L 0.13 12.3 ( 80076)  CCU P 0.31 13.5 ( 88263)  CAU H 0.64 13.6 ( 89007)  CGU R 0.14  6.4 ( 41791)
CUC L 0.06  5.4 ( 35545)  CCC P 0.15  6.8 ( 44309)  CAC H 0.36  7.8 ( 50785)  CGC R 0.06  2.6 ( 16993)
CUA L 0.14 13.4 ( 87619)  CCA P 0.42 18.3 (119641)  CAA Q 0.69 27.3 (178251)  CGA R 0.07  3.0 ( 19562)
CUG L 0.11 10.5 ( 68494)  CCG P 0.12  5.3 ( 34597)  CAG Q 0.31 12.1 ( 79121)  CGG R 0.04  1.7 ( 11351)

AUU I 0.46 30.1 (196893)  ACU T 0.35 20.3 (132522)  AAU N 0.59 35.7 (233124)  AGU S 0.16 14.2 ( 92466)
AUC I 0.26 17.2 (112176)  ACC T 0.22 12.7 ( 83207)  AAC N 0.41 24.8 (162199)  AGC S 0.11  9.8 ( 63726)
AUA I 0.27 17.8 (116254)  ACA T 0.30 17.8 (116084)  AAA K 0.58 41.9 (273618)  AGA R 0.48 21.3 (139081)
AUG M 1.00 20.9 (136805)  ACG T 0.14  8.0 ( 52045)  AAG K 0.42 30.8 (201361)  AGG R 0.21  9.2 ( 60289)

GUU V 0.39 22.1 (144243)  GCU A 0.38 21.2 (138358)  GAU D 0.65 37.6 (245641)  GGU G 0.47 23.9 (156109)
GUC V 0.21 11.8 ( 76947)  GCC A 0.22 12.6 ( 82357)  GAC D 0.35 20.2 (132048)  GGC G 0.19  9.8 ( 63903)
GUA V 0.21 11.8 ( 76927)  GCA A 0.29 16.2 (105910)  GAA E 0.70 45.6 (297944)  GGA G 0.22 10.9 ( 71216)
GUG V 0.19 10.8 ( 70337)  GCG A 0.11  6.2 ( 40358)  GAG E 0.30 19.2 (125717)  GGG G 0.12  6.0 ( 39359)
```

GCU (GCT in the DNA sequence) and GCA are preferred codons for Alanine

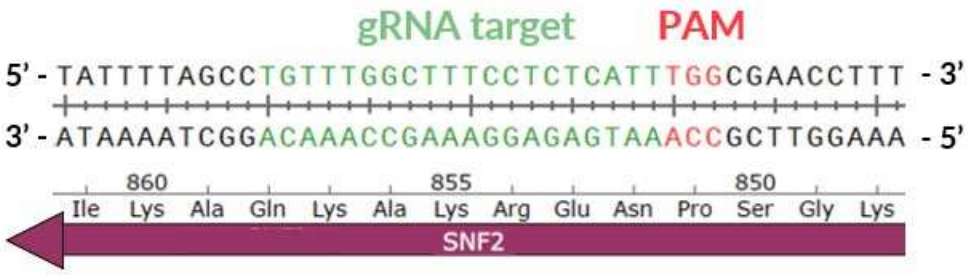
3) In this example, AAG was changed to GCT to encode the lysine-798 to alanine-798 mutation.

Step 2: Design silent mutations in the donor DNA to prevent Cas9 targeting and cleavage of donor DNA.

1) Locate your guide RNA target sequence in your gene of interest.



2) Snapgene Viewer provides the encoded amino acid sequence contained within the gRNA target. **Our objective is to mutate the gRNA target without changing this amino acid sequence.**



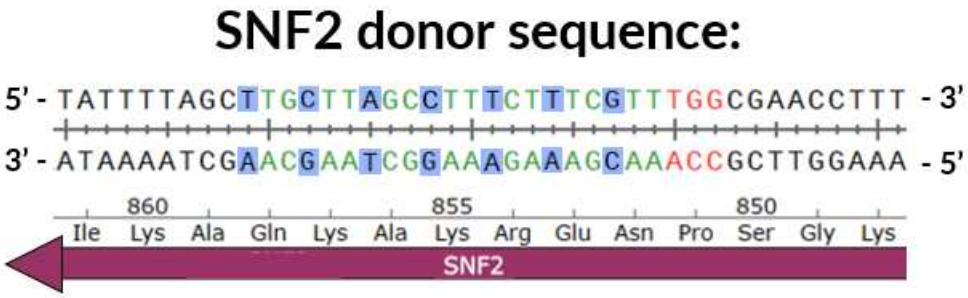
Translated protein sequence for *SNF2* is from **right to left** as depicted here:

852	853	854	855	856	857	858
Asn	Glu	Arg	Lys	Ala	Lys	Gln
AAT	GAG	AGG	AAA	GCC	AAA	CAG

3) In this example, the encoded sequence is on the Crick strand and read from right to left. To produce silent mutations in this sequence, use the codon usage table for *S. cerevisiae* and select alternate codon sequences for each codon within the gRNA. (https://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4932)
   a. ***Note**: we introduced a silent mutation in each codon for the snf2-K798A strategy. We have not tested experimentally the number of mutations required to prevent Cas9 cleavage of the donor DNA.*
4) The new sequence for the *SNF2* example is depicted below:

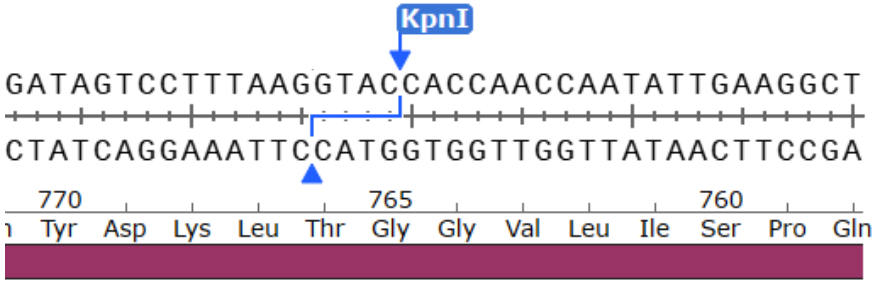
AAC	GAA	AGA	AAG	GCT	AAG	CAA
Asn	Glu	Arg	Lys	Ala	Lys	Gln

5) Replace the existing target sequence in your gene of interest with your new sequence.

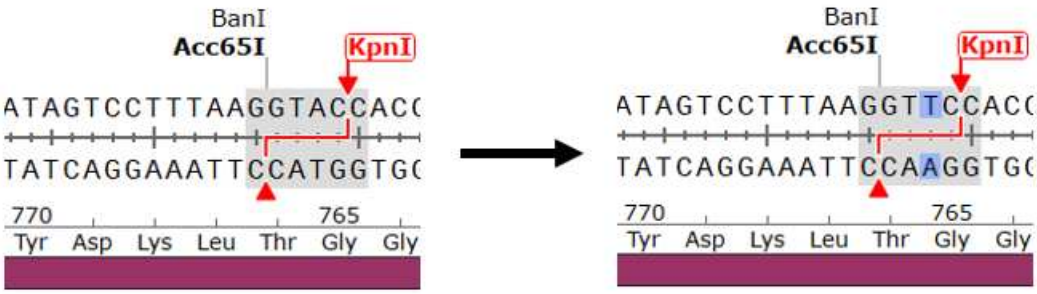


Step 3: Introduce silent mutations to eliminate an endogenous restriction enzyme cut site.

1) Identify a restriction enzyme cut site within your gene of interest. **This also should be within 500bp of your gRNA target.**
    a. *Note: Data from the diagnostic test using these cut sites will be easiest to interpret if the selected restriction enzyme cut site is **unique** within the region contained within the donor DNA.*



2) In this example, KpnI recognizes the palindromic sequence 5'-GGTACC-3'. In this case, the reading frame reads "GGT", "ACC", which is Gly-765, Thr-764. Introduce a silent mutation using an alternate codon sequence with high usage in *S. cerevisiae*. In this example, we mutated to 5'-GG**A**ACC-3' on the Crick strand.



Step 4: Introduce silent mutations to introduce a new restriction enzyme cut site.

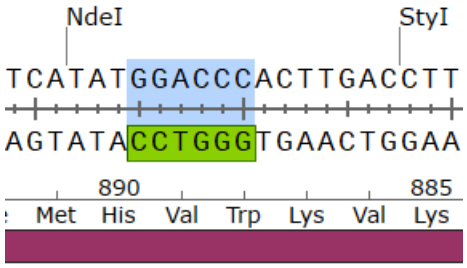
1) This step will likely be the most cumbersome to design, as it requires your sequence to not have your desired enzyme's recognition sequence, as well as for it to be possible to

make **silent** mutations to introduce the recognition sequence within your region of interest.

2) Search using CTRL + F and type in your desired restriction enzyme recognition sequence in the search bar.

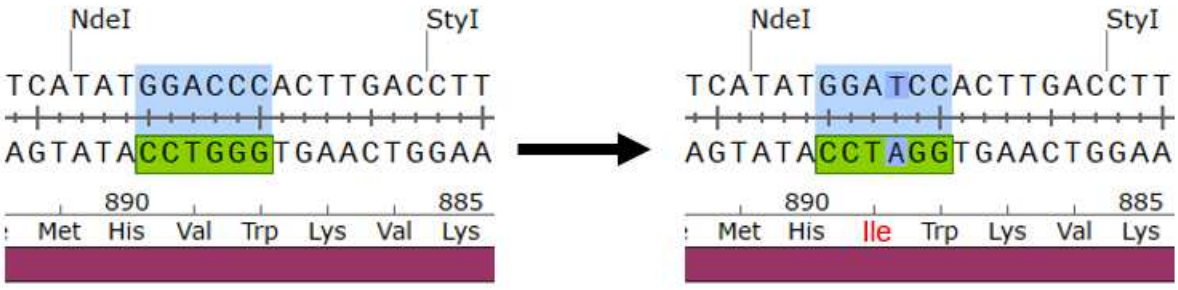
3) In the case of *SNF2*, there are no existing BamHI (5'-GGATCC-3') cut sites.
    a. *Note: The cut site should be unique within the donor DNA for ease of interpretation during the diagnostic test. However, the sequence can be within the gene if it is not represented in the donor DNA, as the PCR reaction performed for the diagnostic test will only amplify the region replaced by the donor DNA.*

4) To search for a sequence that does exist within your region of interest that could be mutated to your desired sequence (5'-GGATCC-3' in our case), search again but replace characters in the search with "N" to allow for any nucleotide to be in the search. (i.e. NGATCC, GNATCC, GGNTCC, etc.)
5) Ensure that any search results fall within the desired donor DNA region (**within 500bp of the gRNA sequence**). If no results fit the criteria, repeat the search with a different letter replaced with N.



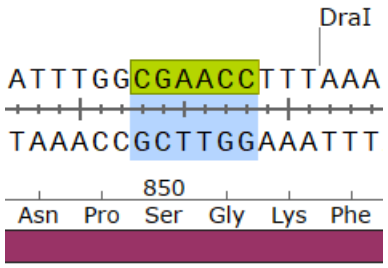
Depicted is an example result for "GGNTCC".

6) Next, test to see if introducing the change from the generic sequence (GGNTCC) to the desired sequence (GGATCC) will change the coding sequence.

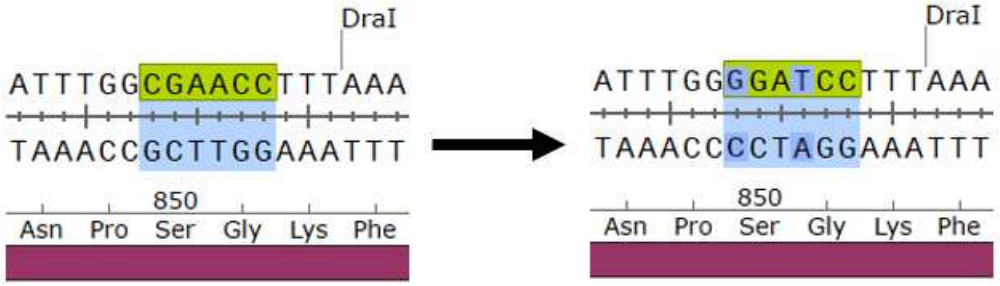


7) In this case, the change in sequence does change the encoded amino acid sequence. Repeat this process until you obtain a suitable candidate. If needed, the sequence can be made to be more generic, but it will require more nucleotide changes to be changed to the consensus sequence.

8) For this example, we identified a candidate using the generic sequence “NGANCC”.



9) In this instance, the existing sequence from 5’ to 3’ on the Crick strand is “GGTTCT”, which encodes Gly-851 and Ser-850. Changing this sequence to the desired recognition sequence of GGATCC does not change the encoded amino acid sequence.



Step 5: Assemble the donor DNA sequence incorporating each mutation in the design.

1) Obtain the entire region of your gene of interest and highlight the regions to be mutated using your previously designed mutations. For our design, we selected roughly ~500bp upstream and downstream of the gRNA target.
    a. *Note: For this example, shown is the sequence from 5’ to 3’ of the **Watson** strand, since this is the sequence that would be purchased.*

GGTACATAATTTGTTGTAAAGCGCTCATTTTGCACTTCACTACCTTCTCAACTTTATCCGGTAATTCTTTTTCTA
CATCCTTTTTCAAACGACGCAATAAAAAGGGTCTCAAGACTTTATGCAATCTCCTGATAACCAAAAGCGTCT
CTTCTTCACTCAACTCAATTTTATCTTGCCCACCGGTGTTGGCAAAGGGTGTATTGAACCATTCATCAAAAG
ATTTCACGGAATTGAAAATCTTGGGTAACACAAAATTCAATAAGGCCCATAATTCTGGCAAGTTGTTTTGAAG
TGGTGTACCTGTCAAAATTAATCTATAATCTGCATGGTAATGTGTGTTCAAGGTTAAAGAAAGCTTGGATTGCG
CATTTTTCATTCTATGGCCTTCATCAATAATCATATGGACCCACTTGACCTTGGATAAAAGTGCTCTTTCTTTAA
TAATATACTCAAAGGTAGTCAGAACTACATCAAATTCACCTGCCCTTATTTTAGCCTGTTTGGCTTTCCTCTCA
TTTGGCGAACCTTTAAATGATATAGTTCTTAAGGTAGGAGCCCATTTCGCGAATTCACTGCTCCAATTTGACA
GTGTAGATAAAGGAACAATGACTAAATATGGCCCACGAATATTCTTCATTTCATATAAATATGTAAGTAAGGAT
ATCGTCTGGATAGTCTTACCAAGACCCATTTCGTCTGCTAAAATACCATTTAAATGATTATTGAAAAGCGAAA
CCATCCACTGTAAACCCTTGATTTGATAGTCCTTTAAGGTACCACCAACCAATATTGAAGGCTGCTTCTTGAT
ATCTTCTTTAATTCTATGTGCGACATTGTAATAATCGACGTTAGAATTGTCGTCGTCGTCATCATATTCTTCATC
CTTCATCTTGGGGACCATGGACAAATCATCCACTTCCTCCGATGCTTCCTTTATGTGGGAATCAATCATTTCT
TTAGTGTACTTTTGTTGATCTTTCACGGCTCTTGTTAAAGAATCTAAAAATGCGTTTGTTTG

Red: Primers (snf2-K798A-F and snf2-K798A-R) bind here to amplify donor DNA sequence.

Green: gRNA Target for Cas9 cleavage

Red: PAM sequence

Blue: Lysine 798 to be mutated to an Alanine

Yellow: KpnI site in residues 765-766 to mutate in donor DNA

Purple: Gly 849 Ser 850 to be mutated to a BamHI cut site

2) Modify the sequence to contain your designed mutations.

GGTACATAATTTGTTGTAAAGCGCTCATTTTGCACTTCACTACCTTCTCAACTTTATCCGGTAATTCTTTTTCTA
CATCCTTTTTCAAACGACGCAATAAAAAGGGTCTCAAGACTTTATGCAATCTCCTGATAACCAAAAGCGTCT
CTTCTTCACTCAACTCAATTTTATCTTGCCCACCGGTGTTGGCAAAGGGTGTATTGAACCATTCATCAAAAG
ATTTCACGGAATTGAAAATCTTGGGTAACACAAAATTCAATAAGGCCCATAATTCTGGCAAGTTGTTTTGAAG
TGGTGTACCTGTCAAAATTAATCTATAATCTGCATGGTAATGTGTGTTCAAGGTTAAAGAAAGCTTGGATTGCG
CATTTTTCATTCTATGGCCTTCATCAATAATCATATGGACCCACTTGACCTTGGATAAAAGTGCTCTTTCTTTAA
TAATATACTCAAAGGTAGTCAGAACTACATCAAATTCACCTGCCCTTATTTTAGCCTG**C**TT**A**GC**C**TT**T**CTTC
GTTTGG**G**GA**T**CCTTTAAATGATATAGTTCTTAAGGTAGGAGCCCATTTCGCGAATTCACTGCTCCAATTTGA
CAGTGTAGATAAAGGAACAATGACTAAATATGGCCCACGAATATTCTTCATTTCATATAAATATGTAAGTAAGG
ATATCGTCTGGATAGT**AGC**ACCAAGACCCATTTCGTCTGCTAAAATACCATTTAAATGATTATTGAAAAGCGA
AACCATCCACTGTAAACCCTTGATTTGATAGTCCTTTAAGGT**T**CCACCAACCAATATTGAAGGCTGCTTCTT
GATATCTTCTTTAATTCTATGTGCGACATTGTAATAATCGACGTTAGAATTGTCGTCGTCGTCATCATATTCTTC
ATCCTTCATCTTGGGGACCATGGACAAATCATCCACTTCCTCCGATGCTTCCTTTATGTGGGAATCAATCAT
TTCTTTAGTGTACTTTTGTTGATCTTTCACGGCTCTTGTTAAAGAATCTAAAAATGCGTTTGTTTG

Bolded letters indicate single base changes to incorporate the mutations necessary for this donor DNA.

3) This sequence can be synthesized as desired. For the *SNF2* donor DNA, this was purchased from Twist Bioscience as a “Gene Fragment with Adapters” (Product Number: 103211).

Daily Experimental Procedures with Instructor Comments

Day 1: PCR and Pouring an Agarose Gel

In today's lab we will be doing two things:

1) A polymerase chain reaction (PCR)
2) Pouring an agarose gel

The agarose gel will be used the next time we meet.

Reagents/Supplies:

- Ice bucket
- 5X Phusion GC Buffer – *the GC buffer and dNTPs should be thawed on ice prior to class time, and aliquoted into Eppendorf tubes for students to pipette from.*
- 10 mM dNTPs
- Milli-Q water
- 100μM Forward primer (F-HPH) – *provide students with an aliquot of each primer rather than have them take from the stock tubes. These primers are used to amplify the hphMX6 cassette from the pHPH-13myc plasmid with homology to delete a gene of interest. Name these using your desired gene of interest.*
- 100μM Reverse primer (R-HPH)
- 5ng/μl DNA template (pHPH-13myc)
- Phusion DNA Polymerase
- 0.5x TAE
- SYBR safe stain
- Agarose
- Tupperware for gel storage

Tips:

- Be very careful in your pipetting; small errors can lead to failed PCR amplification due to an imbalanced template:primer ratio.
- Check off each reagent added to each tube with a checkmark to keep track of your progress in the protocol. It is very easy to forget which reagents you have/haven't added if you aren't keeping track!
- Handle all reagents wearing gloves!

Experimental Procedure (PCR Reaction):

1) Obtain your primers, pHPH plasmid, dNTPs, and buffer, and thaw each on ice.
2) Label two 1.5 mL Eppendorf tubes and label with the name of each primer. (F-HPH and R-HPH)
3) Prepare 100μL of 10μM working concentration of each primer in these new tubes from your 100μM stock solution. *(How many μL of your stock do you need for this dilution? ____ μL)*
4) Obtain three 0.2μL PCR tubes and label as follows:
    i. Label the name of your gene-HPH & your initials

ii. Positive (+) control & your initials
iii. Negative (-) control & your initials

5) Prepare a (4X) master mix containing the following:

Reagent:	Volume
Milli-Q water	65µL
5X Phusion GC Buffer	20µL
10mM dNTPs	2µL
Phusion DNA Pol.	1µL
Total:	**88µL**

Keep all reagents on ice and add Phusion DNA Polymerase **LAST.

***Ensure that the students write a check next to each reagent added in their notebooks AFTER it is pipetted into the master mix.*

6) Next, pipette the following into your labelled 0.2µL PCR tubes:

Reagent:	Your gene-HPH	+ control	- control
pHPH (5ng/µL)	1µL	1µL	--
Milli-Q water	--	--	1µL
F-HPH (10µM)	1µL	--	1µL
R-HPH (10µM)	1µL	--	1µL
F-HPH-control (10µM)	--	1µL	--
R-HPH-control (10µM)	--	1µL	--
Total:	**3µL**	**3µL**	**3µL**

**Make sure your initials are somewhere on the tubes!

7) Vortex your master mix to ensure solution is mixed well, then pipette 22µL of the mix directly into the liquid in each one of your PCR tubes.
8) Place the tubes in the thermocycler using the following program:
    Thermocycler program: (Hotstart)
        i. 98°C – 50s
        ii. 98°C – 10s
        iii. 52°C – 20s
        iv. 72°C – 30s (minimum of 15s per kb)
        v. 72°C – 3min
            1. Steps 2 – 4 will be run 35x
9) Store the PCR reactions in the 4°C refrigerator in an Eppendorf rack labelled with your name on a piece of tape.

Experimental Procedure (Pouring agarose gel):

1) Obtain 0.35g of agarose and place in a 250mL Erlenmeyer flask.
2) Add 35 mL of 0.5x TAE to the agarose.
3) Place the solution in the microwave for 20 – 30 seconds.
4) Take the solution out of the microwave and swirl it to ensure all the agarose has dissolved. If this is not the case, place it back in the microwave for some additional time. (Usually another 20 – 30 seconds is sufficient)
5) Let this solution cool for ~10 minutes.
6) During this time, you can assemble your gel casting tray (we will help demonstrate this in class).
7) Following the cooling period, add 3.5 µL of SYBR safe stain and gently swirl the solution.
8) Pour the molten agarose into the casting tray to cover the entire bottom of the tray.
9) Place the comb in the tray.
10) Let the gel solidify for ~15 minutes.
11) Carefully remove the comb and transfer the gel to a plastic Tupperware containing a sufficient volume of 0.5X TAE to cover the entire gel.

Clean up and storage:

1. Clean up all relevant glassware and bench tops.
2. Store your stock and diluted primers in the designated freezer box in the -20°C freezer.

Day 2: Gel electrophoresis and guide RNA plasmid engineering

In today's lab we will be doing two things:

1) Visually assess the success of our PCR reaction using agarose gel electrophoresis. *If our PCR reactions were successful, we should see a single band running approximately 1750bp in size, which we will crudely quantify by comparing the PCR products motility in the gel to a DNA ladder containing known sizes of DNA.*
2) Perform site-directed mutagenesis on a plasmid that expresses guide RNA to engineer the plasmid to target Cas9 to our gene of interest. We will be following the standard protocol found at: (https://www.neb.com/protocols/2013/01/26/q5-site-directed-mutagenesis-kit-protocol-e0554)

We will run the agarose gel from the site-directed mutagenic PCR reaction during off-class hours if possible, or at the start of Day 3. *The procedure for Day 3 is long for a three-hour class period, and while not required, it is advantageous to test the students' PCR results prior to continuing with the KLD treatment and NEB 5-alpha bacterial transformation.*

Reagents/Supplies:

- PCR samples (from Day 1)
- 6X DNA loading dye
- DNA ladder (1kb Gene Ruler)
- Parafilm
- 0.5X TAE
- 5X Phusion GC Buffer *-- the GC buffer and dNTPs should be thawed on ice prior to class time, and aliquoted into Eppendorf tubes for students to pipette from.*
- 10mM dNTPs
- Milli-Q water
- 5ng/µl DNA template (gRNA-ura-HYB)
- 10µM of each primer *– provide students with an aliquot of each primer rather than have them take from the stock tubes. We suggest naming these primers with the targeted gene of interest.*
    - F-gRNA
    - R-gRNA
- SYBR safe stain
- Agarose *– if the agarose gels produced on Day 1 have enough wells, students can use the same gel for their Day 2 PCR products.*
- Tupperware for gel storage

Tips:

- Be very careful in your pipetting; small errors can lead to failed PCR amplification due to an imbalanced template:primer ratio.
- Check off each reagent added to each tube with a checkmark to keep track of your progress in the protocol. It is very easy to forget which reagents you have/haven't added if you aren't keeping track!
- Handle all reagents wearing gloves!

Experimental Procedure (Agarose Gel Electrophoresis):

1) Ensure your gel from Day 1 is oriented in the casting tray with the wells toward the negative electrode (black).
2) Fill the chamber with sufficient 0.5X TAE to fully cover each well in the gel.
3) Obtain a strip of Parafilm, which will act as a "mixing station" for each of your samples.
4) On this strip, pipet three 1µL drops of 6X DNA loading dye, with the drops sufficiently far away from each other.
5) Take 5µL of each of your three PCR samples and mix each with a separate drop of dye. **Be sure to make note of which PCR sample is mixed with which drop!**
6) After mixing, load 5µL of each sample into your gel. **Make sure you write in your notebook which lane you load each sample in!**
7) Load 5µL of the DNA ladder into one of the lanes of your gel. (I recommend loading the first well with your ladder.)
8) Place the lid of the electrophoresis apparatus in the correct orientation on the gel running tray.
9) Set the voltage of the power box to 100V (ensure the constant voltage setting is selected). The gel will take approximately 45 minutes to run.
10) After the run is completed, bring your gel to your laboratory's gel imaging instrument to obtain a digital image of your gel.
    i. Place your gel directly on the tray and ensure there are no bubbles residing between the gel and the tray.
    ii. Close the door and image your gel using parameters to excite the SYBR Safe dye in the gel.
    iii. Save an image of your gel with your name and date, then label the contents of each lane in PowerPoint and print a copy for your notebook.

Experimental Procedure (Site-directed mutagenesis):

1) Obtain your primers, gRNA plasmid, dNTPs, and buffer, and thaw each on ice.
2) Obtain two 0.2µL PCR tubes and label as follows:
    a. The name of your gene-gRNA & your initials
    b. Negative (-) control & your initials

3) Prepare a (4X) master mix containing the following:

Reagent:	Volume
Milli-Q water	65µL
5X Phusion GC Buffer	20µL
10mM dNTPs	2µL
Phusion DNA Pol.	1µL
Total:	**88µL**

Keep all reagents on ice and add Phusion DNA Polymerase **LAST.

4) Next, pipette the following into your labelled 0.2µL PCR tubes:

Reagent:	Your gene-gRNA	- control
gRNA plasmid (5ng/µL)	1µL	--
Milli-Q water	--	1µL
F-gRNA (10µM)	1µL	1µL
R-gRNA (10µM)	1µL	1µL
Total:	**3µL**	**3µL**

**Make sure your initials are somewhere on the tubes!

5) Vortex your master mix to ensure solution is mixed well, then pipette 22µL of the mix directly into the liquid in each one of your PCR tubes.
6) Place the tubes in the thermocycler using the following program:
   Thermocycler program: (Hotstart)
   i. 98°C – 50s
   ii. 98°C – 10s
   iii. 52°C – 20s
   iv. 72°C – 1m 30s (minimum of 15s per kb)
   v. 72°C – 3min
- Steps 2 – 4 will be run 35x

7) Store the PCR reactions in the 4°C refrigerator in an Eppendorf rack labelled with your name on a piece of tape.

Clean up and storage:

1. Clean up all relevant glassware and bench tops.
2. Store your primers in your labeled Eppendorf tube rack in the -20°C freezer.
3. Store your other reagents for the site-directed mutagenesis PCR in the designated freezer box in the -20°C freezer.

Day 2.5: Precipitating linear PCR products

Today's lab is a brief procedure, precipitating the PCR product from the Day 1 procedure. This protocol can be done at any time prior to yeast transformation but is best to perform Part 1 immediately following the confirmation of PCR success on Day 2. This procedure is also reused after PCR amplification of the linear donor DNA used in the yeast transformations using CRISPR. *This procedure is simple to perform but has significant downtime. It may be most efficient to have the instructor(s) perform this approach outside of class time.*

Reagents/Supplies:

- 7.5M ammonium acetate (NH_4OAc)
- 95% ethanol (EtOH)
- 70% ethanol (EtOH)
- TE buffer
    - 10mM Tris-HCl, pH 8.0
    - 1mM EDTA, pH 8.0
- Tabletop centrifuge for 1.5mL Eppendorf tubes
- Instrument to measure DNA concentration (Nanodrop)

Tips:

- Handle all reagents wearing gloves!
- Ethanol easily removes your labels written with Sharpie markers. Ensure your labels do not become smeared; if they do, use 95% ethanol and a paper towel to wipe the label completely off, then re-write it.

Experimental Procedure (Part 1):

1) Transfer the HPH PCR product amplified by primers specific to your gene (not your controls) into a 1.5ml Eppendorf tube. *– Pool PCR products from students whose reactions successfully generated a PCR product to produce a highly concentrated volume of DNA for the HPH DNA transformation later in the course.*
2) Add 0.4 volumes of 7.5M ammonium acetate and 2 volumes of 95% ethanol to your PCR product
    a. You should know roughly what volume of PCR product you have in your PCR tubes (Total amount used for PCR – amount used for gel electrophoresis)
    b. How much ammonium acetate do you need to add? _____ µl
    c. How much ethanol do you need to add? _____ µl
3) Leave your sample in the -20°C freezer for minimally 2 hours, or overnight.

Experimental Procedure (Part 2):

1) Centrifuge your sample for 10 minutes at 13K RPM in a tabletop centrifuge.
    a. Ensure you have a balance in the centrifuge in the position directly across your tube!
2) Decant the supernatant into a waste container. You should be able to see a white pellet at the bottom of your tube; this contains your DNA amplicon!

3) Add 200µl of 70% ethanol to your pellet and mix gently by pipetting the ethanol around the pellet to dislodge it from the bottom of the tube.
    a. This step will wash your DNA pellet, but the DNA will be insoluble in the ethanol, so it will not resuspend in the solution.
4) Centrifuge your sample for 10 minutes at 13K RPM in a tabletop centrifuge.
5) Decant the supernatant into a waste container and use a pipette to remove excess ethanol remaining in your tube.
    a. **Be careful not to disturb the pellet when pipetting off the ethanol!**
6) Allow the pellet to dry thoroughly by leaving the Eppendorf tube open for 30 minutes – 1 hour until the ethanol has fully evaporated.
7) Resuspend your DNA pellet in 50µl of TE buffer and quantify the concentration of DNA in your sample using the Nanodrop. **Write this concentration in your notebook and on the Eppendorf tube!**

Clean up and storage:

1. Clean-up your bench top and dispose of any tips/empty tubes in the biohazard waste.
2. Store your purified PCR product in the -20°C freezer in your labelled Eppendorf rack.

Day 3: Kinase, Ligase, DpnI treatment and NEB 5-alpha transformation

In today's lab we will treat the PCR products from Day 2 with a reagent containing a kinase, ligase, and restriction enzyme DpnI to circularize your products and digest the template plasmid. Afterwards, we will transform your engineered plasmids into competent *E. coli* cells to express the plasmids. This protocol is written with the assumption that the PCR was confirmed to be successful by gel electrophoresis, performed as described in Day 1 and 2 during an out of class time prior to this lab. *Save lecture portions for down time during lengthy incubation steps, as the protocol can take an entire three-hour class period.*

Reagents/Supplies:

- PCR samples (from Day 2)
- 2X KLD Reaction Buffer
- 10X KLD Enzyme Mix *– keep in the freezer until students are ready to use.*
- Milli-Q water
- SOC (rich media) *– aliquot by pouring into a sterile container, never pipet from the stock solution.*
- NEB 5-alpha competent *E. coli* cells
- LB + 100µg/mL ampicillin plates
- Bunsen burner
- 70% ethanol
- Metal spreader

Tips:

- Keep all reagents on ice for the duration of the experiment. The most critical are the NEB 5-alpha competent *E. coli* cells, which must remain on ice unless specified otherwise by the protocol.
- Keep all flammable materials away from the Bunsen burner.
- Handle all reagents wearing gloves!

Experimental Procedure: KLD Treatment

1) Label A 1.7mL Eppendorf tubes for your KLD reaction with "KLD", the name of your PCR sample, and your initials. (We only want the gRNA PCR product specific to your gene of interest, not your other controls)
2) Fill each tube with the following:

Reagent:	Volume:
gRNA PCR product	1µL
2X KLD Reaction Buffer	5µL
10X KLD Enzyme Mix	1µL
Milli-Q water	3µL
Total:	**10µL**

3) Mix well by gently pipetting up and down and incubate at room temperature for 5 minutes.

Experimental Procedure: NEB 5-alpha transformation

1) Thaw a tube of NEB 5-alpha Competent E. coli cells on ice.
2) Add 5 µl of the KLD mix from your KLD treatment to the tube of thawed cells. Carefully flick the tube 4-5 times to mix. Do not vortex.
3) Place the mixture on ice for 30 minutes.
4) During this time, label three LB + ampicillin plates with the names of each of your plasmid samples, the dilution (undiluted, 10X, 40X), your initials, and the date.
5) Heat shock at 42°C for 30 seconds by placing your tubes in the heat block.
6) Place on ice for 5 minutes.
7) Pipette 950µL of room temperature SOC into the mixture.
8) Incubate at 37°C for 60 minutes with shaking (250 RPM). We will tape the tubes into the incubator/shaker.
9) In a final volume of 100µL, prepare 10X and 40X dilutions of your cells using SOC, then pipet each sample on their labelled LB + ampicillin plates.
    a. **Be sure to record in your notebook the volumes of cells and SOC required to make these dilutions!**
10) Spread your cells in order from highest to lowest dilution using a metal spreader.
11) Sterilize your spreader with a Bunsen burner and ethanol (70%) between each plate. – *Keep the plates with the agar side down for about 15-20 minutes after plating to allow the transformation mix to soak into the media.*
12) Incubate overnight at 37°C, with the agar side of your plates facing up.

Clean up and storage:

1. Clean up your bench top and wipe down your station with 70% ethanol after your Bunsen burner has been turned off.
2. Save your remaining PCR sample and KLD reaction in your labelled tube rack in the -20°C freezer.
3. Dispose of any materials in contact with bacteria in the biohazard waste. You can also save your transformation tubes for a day in case you need to plate more cells.

Day 3.5: Overnight bacterial cultures

Today's lab is a brief procedure, preparing overnight bacterial cultures for plasmid isolation on Day 4. The best time to perform this procedure is in the late afternoon on the day before Day 4.

Reagents/Supplies:

- 100mg/mL ampicillin
- LB media
- LB + ampicillin plates used on Day 3
- Plastic culture tubes
- p10 pipette with tips

Tips:

- Handle all reagents wearing gloves!
- Sterile technique is incredibly important when working with live cells. Do not leave lids of containers on benchtops or culture tubes on their sides.
- Use only sterile plastic containers when working with media.
- Grabbing single colonies with a pipet tip can be tricky at first; remember to **only grab one colony per culture** and try not to gouge the media!

Experimental Procedure:

4) Obtain two sterile culture tubes for your new bacterial strain you generated during the Day 3 transformation.
5) Select the plate with isolated colonies for each strain produced.
    a. If the transformation efficiency was low, the undiluted plate will be best, but if the transformation efficiency is sufficiently high, it will be hard to obtain a single colony from that plate. In that situation, use the 10X or 40X dilution plate.
6) Using a 15mL sterile conical tube, obtain 8mL of LB.
7) Pipet a sufficient volume of 100mg/mL ampicillin to obtain a final concentration of 100µg/mL ampicillin, then mix the solution well.
8) Transfer 4mL of your LB + ampicillin media to each of your culture tubes.
9) Inoculate each tube with a single colony. To do this, obtain your p10 pipette and equip it with a disposable tip. Scrape a single colony off your plate, then eject the tip into your LB + ampicillin solution. Repeat this process for your other overnight culture.
10) Once your overnight cultures are ready, place the culture tubes in a 37°C shaker.

Clean up and storage:

1. Clean up your bench top and wipe down your station with 70% ethanol to disinfect.
2. Dispose of any materials in contact with bacteria in the biohazard waste.
3. Wrap your LB + ampicillin plates from Day 3 with Parafilm, and store in the 4°C refrigerator.

Day 4: Plasmid isolation and DNA sequencing

In today's lab we will isolate plasmid DNA from the overnight cultures set up on Day 3.5. The procedure we will use employs a kit from Qiagen. There are other ways to do this, but most research/industrial labs use some sort of kit due to efficiency and reproducibility. We will then send an aliquot of your plasmids to Azenta (Genewiz) to sequence the region containing your engineered guide RNA sequence.

Reagents/Supplies:

- Bacterial overnight cultures from Day 3.5 *– these should be cloudy by the morning after Day 3.5. If the course is taught in the afternoon, remove the cultures from the shaker in the morning and store in a 4°C refrigerator before class.*
- 50% glycerol
- Sterile 1.7mL Eppendorf tubes
- Tabletop microcentrifuge
- P1 buffer* *-- Aliquot the buffers from the plasmid isolation kit for each student. Ensure you provide students with a small excess of each solution.*
- P2 buffer**
- N3 buffer**
- PB buffer**
- PE buffer**
- Spin columns
- MQ water

*The P1 buffer needs to remain on ice or in the refrigerator.
**The other buffers can remain at room temperature on your bench top.

Tips:

- Ensure that the microcentrifuge is always balanced whenever you use it.
- Handle all reagents wearing gloves!

Experimental Procedure: Plasmid isolation

1) You will need to start by obtaining each of your overnight cultures. You will be doing one DNA extraction from each culture. These cultures should be exceedingly cloudy.
    a. Before proceeding, thoroughly mix this solution as your cells have settled at the bottom of the culture tube.
2) First, transfer 700µL of each solution into a sterile 1.7mL tube labelled with the name of the bacterial strain, your initials, and the date. Then pipet 300µL of 50% glycerol into each tube and mix thoroughly.
    a. This step is performed to save a portion of your culture as a frozen cell stock for future use. Store these tubes in the -80°C freezer.
3) Next, transfer 1.5mL from each overnight culture to a sterile 1.7mL tube and spin the solution down at 13K RPM for 1 minute in your tabletop centrifuge. *– Ensure students have balanced the microcentrifuge! Students can share instruments and place their samples across from each other.*

4) After the spin, discard the supernatant and place an additional 1.5mL of your culture on top of the pellet. Spin for 13K for another minute and discard the supernatant. Repeat this step so that you have spun down the entire culture.
5) Resuspend the pellet in 250µL of buffer P1.
    a. **TIP:** This solution contains EDTA (enzyme inhibitor) and RNase (destroys RNA).
6) Add 250µL of buffer P2 and carefully invert the tube 4-5 times and let it sit for no longer than 5 minutes.
    a. **TIP:** The solution should turn clear at this point. This solution lyses the cell and is comprised of SDS and NaOH. SDS disrupts cell membranes and the NaOH denatures the DNA.
7) Now add 350µL of buffer N3 and again carefully invert the tube 4-5 times.
    a. **TIP:** The solution should immediately form a white precipitate. This solution contains potassium acetate and leads to the precipitation of SDS/lipids/proteins.
8) Pellet the precipitate by spinning the tube at 13K for 10 minutes.
9) After the spin carefully remove the supernatant and place it onto the Qiaprep® spin column. Spin this sample down at 13K for 1 min and discard the flow-through.
10) Apply 500µL of Buffer PB to the column and spin it at 13K for another minute. Again, discard the flow-through.
11) Finally, wash the column with 750µL of Buffer PE and spin at 13K for 1 minute. Discard the flow through and spin the column for an additional 3 minutes at 13K.
12) You will now need to place the column into a labeled 1.5mL tube. This tube should be labeled precisely in the same manner that you labeled your LB + Amp plates and your overnight cultures. Place 40µL of MQ water directly in the center of the column and let it sit for 2 minutes. Spin the column at 13K for 1 minute. You can now discard the column; your DNA should be in the collection tube.
13) Assessing your DNA extraction success:
    a. To determine if we were successful in extracting DNA using the Qiagen protocol we will be quantitating our DNA using the Nanodrop.
        - Before measuring the concentration of 1µL of each plasmid, use 1µL of MQ water to blank the instrument.
14) After confirming the presence of DNA in each sample you will need to prepare at least 20µL of your plasmid DNA sample at 50ng/µL.
15) Bring this to your instructor and be prepared to pipet 10µL of your sample for the forward sequencing reaction.
    a. These samples will be submitted to Genewiz for sequencing. *– Have the students pipet their sample into an 8-well strip labelled in accordance with Genewiz submission guidelines. See more at: https://www.genewiz.com/Public/Resources/Sample-Submission-Guidelines/Sanger-Sequencing-Sample-Submission-Guidelines*

Clean up and storage:

1. Clean-up your bench top and wipe down your station with 70% ethanol.
2. Save your remaining DNA samples in your labelled tube rack in the -20°C freezer.
3. Dispose of any materials in contact with bacteria in the biohazard waste.

Day 5: Plasmid sequencing analysis

In today's lab we will be analyzing the sequencing results from Genewiz for your plasmids to determine if the PCR to engineer each guide RNA sequence was successful.

Reagents/Supplies:

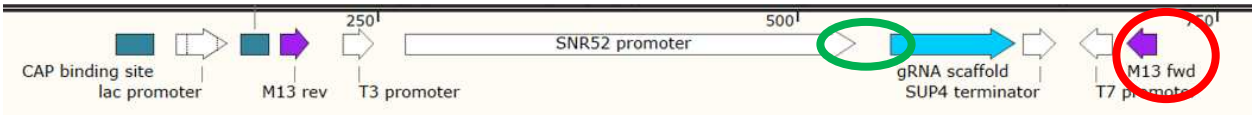
- Sequence Scanner 2 software
- Sequence (.fasta) and trace (.ab1) files from Genewiz*

*The files from Genewiz will be downloaded to your folder by your instructor. *– Create a folder in a shared drive for each student. The files are accessed by an e-mail link sent to the Azenta (Genewiz) account holder who submits the sequencing reactions.*

This class period can easily be coupled with Day 6, if this procedure is completed during the autoclave time from Day 6.

Experimental Procedure: Sequence analysis

1) Launch the program "Sequence Scanner 2" on your benchtop computer.
2) In the software your file should appear in "Details". Click on your file.
    a. Now click on "Open Traces" in the left-hand menu.
    b. The file will now open at the bottom of the page in the software. Tabs just below the file allow you to view different aspects of the data such as the text of the sequence and the chromatogram trace.
    c. Using the chromatogram trace, determine the region of the sequencing read with the highest quality sequencing data. This region should begin roughly 40-50bp after the start of the sequencing read.
    d. Go to the text of the sequence and highlight the sequence. Copy it by hitting "Ctrl-C". You should now be able to paste it into a Microsoft Word document.
    e. Repeat this step for each sequencing reaction performed. In our case, you had 2 different plasmids sequenced. **TIP:** Make sure you label which sample the text came from on the Word document!
3) To answer the question of if our guide RNA plasmid engineering was successful, we need to understand where our sequencing results came from.
    a. The primer used for sequencing was **M13 fwd**, circled in **red**.
    b. Our primary region of interest resides between the SNR52 promoter and the gRNA scaffold. This region is circled in **green**.



4) Note that this primer binds the coding strand (non-template) and will produce a sequence of the **template strand**, and **in the reverse direction** relative to our guide RNA sequence.
    a. To interpret the sequences you copied into a Word document, we first need to take the reverse complement of this sequence. This will generate a sequence of the non-template strand in the forward direction, which we can directly compare to our known sequence of the gRNA plasmid that we started with during Day 2.

   b. You should use this website to obtain the reverse complement of your sequences: http://www.bioinformatics.org/SMS/rev_comp.html
   c. Copy and paste the resulting sequences into your Word document, and label them with the sample name and “Reverse complement”.
5) Now that you have the reverse complement of each sequence, we now need to compare these sequences to the portion of the gRNA plasmid containing the gRNA sequence.
   a. Green: SNF52 promoter
   b. Red: gRNA sequence that targets Cas9
   c. Blue: gRNA scaffold
   d. Orange: SUP4 terminator
6) TCTTTGAAAAGATAATGTATGATTATGCTTTCACTCATATTTATACAGAAACTTGATGTT
TTCTTTCGAGTATATACAAGGTGATTACATGTACGTTTGAAGTACAACTCTAGATTTTG
TAGTGCCCTCTTGGGCTAGCGGTAAAGGTGCGCATTTTTTCACACCCTACAATGTTC
TGTTCAAAAGATTTTGGTCAAACGCTGTAGAAGTGAAAGTTGGTGCGCATGTTTCGG
CGTTCGAAACTTCTCCGCAGTGAAAGATAAATGATCGAGTAAAAAATTGTACTTGGGT
TTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT
GGCACCGAGTCGGTGGTGCTTTTTTTGTTTTTTATGTCTGGTACCCAATTCGCCCTAT
AGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTAC
7) To compare these sequences, use BLAST to align the sequences to each other: https://blast.ncbi.nlm.nih.gov/Blast.cgi
   a. Select “Nucleotide BLAST”, then check “Align two or more sequences” in the checkbox at the bottom of the section where you enter your query sequence.
   b. Copy and paste one of your reverse complement sequences into the “Query” section, and the above sequence from the gRNA plasmid into the “Subject” section, then click “BLAST” at the bottom of the page.
8) The resulting BLAST result should show a region of 100% identity between the two sequences **except for the gRNA sequence** directly upstream of the scaffold.
9) The gRNA sequence you are looking for with each sequencing reaction will be provided by your instructor.
10) Ensure that this sequence has replaced the existing gRNA sequence for each sample, and that no other mutations are present in your sequence.
11) For each of your plasmids whose sequencing reactions confirmed that the gRNA engineering was successful, ensure you label both your plasmid stock tubes and glycerol stocks with more detail!

Day 6: Yeast media preparation and reviving yeast from glycerol stocks

In today's lab we will be performing two experiments:

1) We will learn about yeast and prepare media to grow yeast in.
2) We will also revive yeast strains from frozen glycerol stocks.

Reagents/Supplies:

- Yeast extract
- Peptone
- Bacto-Tryptone
- NaCl
- 40% glucose (dextrose) *– This solution needs to be made and autoclaved ahead of class. If making 500ml of 40% glucose, 200g of dextrose is required. Slowly add the dextrose to 200ml of Milli-Q water with heat and a stir bar because the dextrose will comprise a significant portion of the volume of the solution. Bring the volume up to 500ml after the glucose is in solution. Then autoclave using a 45min sterilization.*
- Agar
- MQ water
- Aluminum foil
- Autoclave tape
- 250mg/ml hygromycin *– the final concentration in the plates containing hygromycin should be 500µg/ml to ensure as little background growth as possible. To save on cost, the concentration should be brought down to no lower than ~200µg/ml in the plates.*
- 100mg/ml nourseothricin *– nourseothricin is much more expensive than hygromycin. An instructor could make these plates instead of a student if desired.*
- Wild-type yeast glycerol frozen stock *– this strain should be whatever laboratory strain you want to transform the Cas9 and guide RNA plasmids into with the donor DNA.*
- YPD plate

Tips:

- When working with YPD, sterile technique is **incredibly important** because the media can be easily contaminated. *– It is critical for an instructor to demonstrate proper technique for preparing media.*
- Keep lids on media containers and throw any excess ingredients in the trash; never return them to their containers!

Experimental Procedure: Yeast media preparation

YPD (**Y**east Extract, **P**eptone, **D**extrose) is a rich medium that contains everything needed for yeast to grow. In some circumstances, we grow yeast on solid YPD plates that contain agar. In other cases, we need YPD without agar that will remain a liquid for preparing cultures of yeast. We will need both for our experiments this semester, but today we will just prepare solid YPD plates.

1) For today's lab, we will be preparing plates containing media for yeast growth including media with antibiotics that are critical for our future experiments.

a. YPD + Agar + **nourseothricin**
    i. **Nourseothricin (NTC)** is an antibiotic that is a mixture of Streptothricin C, D, E and F, and it inhibits protein synthesis by interfering with the mRNA translocation step, causing the misreading of the RNA molecule. The presence of this antibiotic is lethal to yeast cells. Our plasmid expressing the *CAS9* gene contains a cassette (natMX6) containing the gene NrsR which confers resistance to nourseothricin. Thus, we can use media containing nourseothricin to positively select for successful transformation of the Cas9 plasmid in our yeast strains.

b. YPD + Agar + **nourseothricin** + **hygromycin**
    i. **Hygromycin (Hygro)** is an antibiotic that also disrupts translation and is lethal to yeast cells. Our guide RNA plasmid contains a cassette (hphMX6) that has a gene called HPH (or Hyg^R) which confers resistance to hygromycin. Thus, we can use media containing hygromycin to positively select for successful transformation of the guide RNA plasmid in our yeast strains.

2) Obtain three clean 250mL Erlenmeyer flasks and label it with your initials, and the media you will prepare in each flask as shown below:
    a. YPD
    b. YPD + NTC
    c. YPD + NTC + Hygro

Depending on the size of the class, it may be more efficient for each student to only make one kind of media but produce a sufficient volume for their classmates. Students could also make liquid YP (no agar or dextrose) as the liquid media will be required for later experiments. However, every student should have the opportunity to pour plates.

3) Add the following reagents to each flask:

Reagent:	YPD plates
Yeast Extract	1g
Peptone	2g
Agar	2g
Milli-Q water	Raise final volume to 95mL

4) Obtain a sheet of aluminum foil, folded over on itself, for each flask. Ensure each sufficiently covers each Erlenmeyer flask.
5) Place a stir bar in the flasks. This step is important to ensure the stir bar is also sterilized for stirring the remaining ingredients for the YPD plates.
6) Obtain autoclave tape and place a strip on the foil of each flask. When the autoclave reaches a sufficient internal temperature to sterilize the media, segments of the autoclave tape will change color.
7) Autoclave the media with your classmates, using a program that sterilizes for 45 minutes. The run will take approximately an hour and 10 minutes.
8) After autoclaving, the flasks containing the media are extremely hot and are not ready to pour.

9) We still need to add dextrose (glucose) to our flasks. This gets autoclaved separately because it caramelizes when autoclaved with yeast extract and peptone. This can be added while the media is still hot.
10) Add 15mL of 40% glucose to your flask and stir the solution gently on a stir plate.
11) When the media is cool enough to hold the flask relatively comfortably on the inside of your forearm (also known as the “baby bottle test”), pipet your designated antibiotic to the solution as indicated below:

    a. YPD + NTC: 100µl of 100mg/ml nourseothricin

    b. YPD + NTC + Hyg: 100µl of 100mg/ml nourseothricin,
    200µl of 250mg/ml hygromycin

12) Allow the media to mix on a stir plate for at least a minute.
13) Use a magnet to remove the stir bar from each flask and prepare the Petri dishes by removing them from the plastic sleeve they are contained in.
14) When you’re ready to pour, grab your flask with one hand, grab a plate with your other hand and slide it towards the edge of the bench, while keeping it closed.
15) Once the plate is at the edge, open the lid as if there is an imaginary hinge at one end, so the plate opens like a clamshell.
16) Pour the media into the bottom of the plate until it covers the surface. Our target is to pour **four** plates from each flask.
17) Without disturbing the plates, allow them to solidify on your benchtop overnight.

Experimental procedure: Reviving yeast glycerol frozen stocks

1) We will now revive our wild-type yeast strain which we will transform our plasmids into.
2) To do this, take a YPD plate and label the agar side of the plate “WT” with your initials, the date, and “YPD”.
3) Obtain the stocks of yeast from the -80°C freezer. **TIP:** Only keep these tubes of frozen yeast on your bench for **as short of a time as possible**, as they will thaw quickly!
4) Using a toothpick with a flat end, scrape some yeast onto the toothpick and dab it near the edge of one end of the plate.
5) Flip the toothpick over and pull it across the top of the plate to dilute the cells across your streak. Dispose of the toothpick.
6) Grab a new toothpick and pull from the end of your previous streak, then streak back and forth across the rest of the plate for the strain.
7) We will place these plates agar side up in a 30°C incubator.

Clean up and storage:

1. Clean up your bench top and wipe down your station with 70% ethanol.
2. Be extra careful not to disturb your plates while they solidify.
3. Return any excess antibiotic to the -20°C freezer.

Day 6.5: Overnight yeast starter cultures

Today's lab is a brief procedure, preparing overnight yeast cultures for transformation on Day 7. The best timing for this procedure is in the late afternoon/evening on the day before Day 7.

Reagents/Supplies:

- YPD liquid media *– some students can prepare this media during the Day 6 procedure if desired. This medium contains all ingredients of YPD plates excluding agar. It is advised to keep the 40% glucose separate from the YP until needed for preparing the starter cultures.*
- Starting yeast strain revived on Day 6
- Glass culture tubes
- Sterile plastic conical tubes
- Bunsen burner
- Metal inoculating loop
- Incubator with rotator or shaker *– our lab is equipped with a rotator to maintain cell suspension during incubation. If unavailable, an incubator with a shaker works also.*

Tips:

- Handle all reagents wearing gloves!
- Sterile technique is incredibly important when working with live cells. Do not leave lids of containers on benchtops or culture tubes on their sides.
- Use only sterile plastic containers when working with media.

Experimental Procedure:

1) Obtain four glass culture tubes and label their caps with "Und", "1:2", "1:5", and "1:10" using a Sharpie.
    a. For this procedure, we will be diluting the undiluted culture **before** growing at 30°C overnight. This is because we need a culture to start with few enough cells that they aren't overgrown by the morning!

    If possible, it would be best to experimentally determine the appropriate dilution for obtaining a log phase culture by the next class period ***if the course is taught in the morning.*** *If taught in the afternoon, don't prepare dilutions of the culture now; instead, dilute the overnight culture to an* $OD_{600} = 0.2$ *in the morning, ensuring you have 3-5 hours to wait for the cultures to reach log phase* ($OD_{600} = 0.7 – 1.0$) *by class time.*

2) Turn on your Bunsen burner by twisting the gas valve to the open position and use the lighter directly over the burner.
3) Fill your glass culture tubes with the following volumes of YPD:

	Und.	**1:2**	**1:5**	**1:10**
YPD Volume	6ml	2.5ml	4ml	4.5ml

TIP: YPD is **very** easy to contaminate. To prevent contamination of the stock container of YPD, always pour into a sterile conical tube prior to distributing the YPD to the glass culture tubes.

4) Sterilize your metal inoculating loop by holding it over the burner; you can remove it from the heat once the metal begins to glow orange.
5) Allow the loop to cool for 5-10 seconds, then touch the agar on your yeast plate at a region far away from the colonies to allow remaining heat to dissipate into the media.
6) Scoop up a **single** yeast colony, then transfer it to the Und. culture tube and ensure the colony is off the loop by tapping the loop back and forth in the media in the tube.
7) Vortex the suspension to ensure the cells are well distributed in the culture tube. **Make sure the vortexer is on a low enough setting that the media doesn't come out over the top of the tube!**
8) Pipette the suspended yeast from the Und. culture tube as follows:

	1:2	**1:5**	**1:10**
Culture Volume	2.5ml	1ml	0.5ml

9) Place the glass culture tubes in the rotator located in a 30°C incubator.

Clean up and storage:

1. Clean up your bench top and wipe down your station with 70% ethanol to disinfect the surface.
2. Dispose of any materials in contact with yeast media in the biohazard waste.
3. Wrap your plates from Day 6 with Parafilm, and store in the 4°C refrigerator.

Day 7: Yeast transformation with Cas9 plasmid DNA

In today's lab we will start getting our CRISPR reagents into our wild-type yeast. The first step is to transform a plasmid that expresses the Cas9 enzyme into yeast.

Reagents/Supplies:

- Yeast cultures from Day 6.5 *– These cultures need to be at log phase (0.7 – 1.0) by the start of class. Follow the Day 6.5 procedure to ensure your cultures are at the proper growth phase.*
- Lithium acetate / Tris EDTA (LiAc/TE) solution
    - 0.1M LiAc
    - 10mM Tris pH 8.0
    - 1mM EDTA
- Lithium acetate/ Polyethylene glycol (LiAc/PEG) solution
    - 0.1M LiAc
    - 10mM Tris pH 8.0
    - 1mM EDTA
    - 400g/L PEG 3350
- 10mg/mL salmon sperm DNA (ssDNA)
- 500ng/µL Cas9 plasmid (Cas9-NAT)
- Liquid YPD
- YPD + 100µg/ml nourseothricin plates

Tips:

- Ensure that the microcentrifuge is always balanced whenever you use it.
- When working with YPD, sterile technique is **incredibly important** because the media is rich and can be easily contaminated.

Experimental procedure:

1) Obtain the overnight wildtype cultures prepared on Day 6.5.
2) Take a 1mL aliquot of each culture and pipette them into cuvettes. Also prepare a 1mL blank for the spectrophotometer by pipetting 1mL of YPD into a cuvette.
    a. **TIP: ALWAYS** pour YPD from the stock container into another container (conical tube) to obtain your aliquots from; this helps prevent contamination of the YPD stock.
3) Measure the absorbance at 600nm (OD_{600}) in the tabletop spectrophotometer, and determine which culture is at an OD_{600} of 0.7 – 1.0. If the OD_{600} of one of the cultures is above 1.0, pipette a smaller volume of culture into your cuvette and dilute with a volume of water to reach a total volume of 1mL. Multiply the OD_{600} of this solution by the dilution factor used in preparing your diluted solution.
4) Select the wildtype culture that has an OD_{600} between 0.7 – 1.0 and transfer 1.2mL of it to a sterile Eppendorf tube.
    a. Label your tubes as "WT" & your initials.
5) Harvest the cells by centrifugation at 5,000 x g for 3 minutes.
6) Decant the supernatant (pour it off) and resuspend the cell pellet in 1mL of sterile MQ ddH_2O.

7) Pellet the cells by centrifugation at max speed for 1 minute.
8) Pull off the supernatant with your pipette, thoroughly suspend the cells in 1mL of the LiAc/TE solution and repeat step 7.
9) Pull off the supernatant and suspend the cells in 100µL of LiAc/TE.
10) Obtain a tube of salmon sperm DNA (10 mg/mL) and denature it by incubating at 95°C for 5 minutes, then quickly chill on ice.
    a. **TIP:** The salmon sperm does not need to be boiled after each freeze/thaw cycle; transformation efficiency will start to drop after 3 or 4 freeze/thaw cycles. Ask your instructor if it is necessary to boil your aliquot of salmon sperm DNA.
11) Add 10µL of the salmon sperm DNA to the LiAc/TE cell suspension and mix.
12) Obtain sterile 1.7mL Eppendorf tubes, label them with your initials and prepare transformation mixes as shown below:

Reagent:	**WT +DNA**	**WT -DNA**
Cas9 plasmid (500ng/µL)	2µL	--
Milli-Q water	--	2µL
WT cell suspension	50µL	50µL

13) Add 300µL of the LiAc/PEG solution to each of the transformation tubes and mix.
14) Incubate the transformations at 30°C for 30 minutes by taping the tubes to a shaker.
15) Incubate the transformations at 42°C for 20 minutes in a heat block.
16) Add 750µL of YPD to the transformation samples and transfer the entire sample to a sterile glass culture tube. Label these culture tubes as either “WT + DNA” or “WT – DNA”.
17) Prepare dilutions of the **WT + DNA** transformation sample into new glass culture tubes as described below:
    a. Take 100µL of each undiluted culture and transfer it to glass sterile culture tubes containing 900µL of YPD.
    b. Label these tubes as **WT + DNA 1:10**
18) Incubate your WT + DNA and WT – DNA glass culture tubes at 30°C for 1.5 hours on the rotator.
    a. Your instructor will then perform the remainder of the protocol after class time.
    b. Transfer the samples to sterile Eppendorf tubes and pellet by centrifuging at max speed for 1 minute.
    c. Pull off the supernatant and resuspend in 250µL of YPD and plate half on a YPD plate containing 100µg/ml nourseothricin.
    d. Incubate plates for 2 – 4 days at 30°C to allow the cells to grow.

Clean up and storage:

1. Clean up your bench top and wipe down your station with 70% ethanol.

Day 8: Donor DNA PCR Amplification

In today's lab we will use PCR to amplify large quantities of donor DNA required for our upcoming transformation into our yeast expressing the Cas9 gene.

Reagents/Supplies:

- 5X Phusion GC Buffer
- Phusion DNA Polymerase
- 10mM dNTPs
- Milli-Q water
- 10ng/µl DNA template (donor DNA) *– This DNA template can be purchased from a variety of places, such as Twist Biosciences. Always maintain a stock of this linear DNA for future experiments or ensure that you have a yeast strain containing the mutation so that a source of the DNA can be obtained by PCR amplification. See the "Design of donor DNA sequence" in the "How to design each reagent required for this course" section of the instructor manual to design the donor DNA.*
- 10µM of each primer *– These primers should amplify the entire donor DNA sequence as described in the "Design of donor DNA sequence" section.*
    - F-donor
    - R-donor

Tips:

- Be very careful in your pipetting; small errors can lead to failed PCR amplification due to an imbalanced template:primer ratio.
- Check off each reagent added to each tube with a checkmark to keep track of your progress in the protocol. It is very easy to forget which reagents you have/haven't added if you aren't keeping track!
- Handle all reagents wearing gloves!

This procedure is simple and can be incorporated earlier in the course if desired. It is a nice opportunity to walk students through the procedure more closely if they are struggling with PCR.

Experimental Procedure (Donor DNA PCR amplification):

1) Obtain your primers, donor DNA, dNTPs, and buffer, and thaw each on ice.
2) Obtain a 0.2µL PCR tube and label with your initials.
3) Pipet the following into the PCR tube:

Reagent:	Volume
Milli-Q water	32.5µl
5X Phusion GC Buffer	10µl
10mM dNTPs	1µl
Donor DNA (10ng/µl)	2µl
F-donor	2µl
R-donor	2µl
Phusion DNA Pol.	0.5µL
Total:	**50µL**

***Keep all reagents on ice and add Phusion DNA Polymerase **LAST**.*

4) Place your tube in the thermocycler using the following program:
   Thermocycler program: (Hotstart)
   i. 98°C – 50s
   ii. 98°C – 10s
   iii. 52°C – 20s
   iv. 72°C – 30s
   v. 72°C – 3min

   ***Steps 2 – 4 will be run 35x*

5) We will run an agarose gel as described in Day 1 and then precipitate the PCR product as described in Day 2.5. We need 5µg per CRISPR-Cas9 transformation, and your instructor will amplify more donor DNA if we don't produce enough from these reactions.

Clean up and storage:

1. Clean up all bench tops.
2. Store your PCR reagents in the -20°C freezer.

Day 9: High efficiency guide RNA and donor DNA transformation

In today's class, we will transform our yeast with the guide RNA plasmid and donor DNA required to engineer a mutation in your gene of interest. Remember that our cells from Day 7 carry a plasmid that expresses the Cas9 enzyme!

Reagents/Supplies:

- Overnight cultures of yeast from Day 8.5 *– generate these cultures in the same manner as the Day 6.5 cultures, with the following changes:*
    - *For the starting strain transformed with the Cas9 plasmid, ensure that these cultures are grown with nourseothricin in the medium to continue selecting for the Cas9 plasmid. Students need ~11ml for each transformation, so prepare larger volumes as needed if students perform more transformations.*
    - *If performing the HPH transformation, transform this into your starting strain.*
- 50% glycerol
- 100mM LiAc
- 1M LiAc
- 50% PEG 3350
- 10mg/ml salmon sperm DNA
- Your gRNA plasmid DNA (>1µg required for each reaction)
- Your donor DNA (>5µg required for each reaction)
- Your HPH DNA (>1µg required for each reaction) *– This is required only if you plan to make a deletion using the one-step homologous recombination of a cassette as described previously.*
- Liquid YPD
- Bunsen burner
- YPD + nourseothricin + hygromycin plates
- YPD + hygromycin plates *– These are required only if the HPH transformation is done.*

Tips:

- Ensure that the microcentrifuge is always balanced whenever you use it.
- When working with YPD, sterile technique is **incredibly important** because the media is rich and can be easily contaminated.
- Take your time with the protocol, ensuring that each of your tubes is well labelled. We're doing multiple transformations simultaneously today, be vigilant to not mix any samples with each other!

Experimental procedure: Preparing DNA mixes for transformation

1) Fill out the tables on the next page with the concentration of each DNA reagent we have (guide RNA plasmid, donor DNA, and HPH DNA) and the name of the reagents you are using. Calculate the number of µl needed to create each DNA mix. We will use these mixes later in the protocol.

Each DNA mix consists of DNA and water; for the CRISPR reaction, this will comprise of the guide RNA plasmid, donor DNA, and water. For the deletion using the one-step homologous recombination approach, this will comprise of only HPH DNA and water.

CRISPR-Cas9 edit for your gene:

gRNA plasmid:	
Donor DNA:	
gRNA plasmid concentration[1]	
Donor DNA concentration[1]	
Volume for >1 µg plasmid[2]	
Volume for >5µg of donor[2]	
Total DNA volume:[3]	
Total water volume:[4]	

One-step deletion:

HPH DNA:	
HPH DNA concentration[1]	
Volume for >1µg HPH DNA[2]	
Total DNA volume:	
Total water volume:[4]	

[1]Write in the table above the concentration of each reagent indicated in the rows above.

[2]Calculate how much of each reagent is required to reach the desired amount of the indicated DNA.

[3]Calculate the total DNA volume for the mix.

[4]Now, take 74µL – your total DNA volume, and enter this here. This is the volume of water to add to your cocktail.

Change this table as needed depending on the strains desired from each student. If students are producing multiple mutations, have them prepare different DNA mixtures for each. If students are attempting to generate the mutation in multiple yeast strains, adjust the total volume of the DNA mix to be sufficient for each transformation.

Experimental procedure: High efficiency transformation

1) Obtain the overnight cultures prepared on Day 8.5 by your instructor. (wild-type [to be transformed with HPH DNA] and wild-type + Cas9 [to be transformed with gRNA plasmid and donor DNA]) prepared on Day 8.5 by your instructor.
    a. **Two days before lab**: A small (3ml) overnight culture was inoculated from your strains.
    b. **One day before lab** (Day 8.5): The small culture was used to inoculate a larger culture. The starting OD_{600} of the larger culture was calculated to ensure that the culture used today is at approximately log phase growth.
2) Take a 1mL aliquot of each culture and pipette them into cuvettes. Also prepare a 1mL blank for the spectrophotometer by pipetting 1mL of YPD into a cuvette.
    a. **TIP: ALWAYS** pour YPD from the stock container into another container (conical tube) to obtain your aliquots from; this helps prevent contamination of the YPD stock.

3) Measure the absorbance at 600nm (OD_{600}) in the tabletop spectrophotometer.
4) Transfer 10mL of each culture to sterile 15mL conical tubes.
    a. Label these tubes as WT and WT + Cas9; the WT strain is our starting strain for the HPH DNA transformation, while the WT + Cas9 strain is our starting strain for the guide RNA and donor DNA transformation.
5) Harvest the cells by centrifugation at 3000 rpm for 5min.
6) Pour off the supernatant, suspend the cells in 5mL of sterile water and centrifuge again.
7) Pour off the supernatant, suspend the cells in 1.0mL of 100mM LiAc and transfer the suspension to a 1.7mL Eppendorf tube.
8) Pellet the cells by centrifuging at max rpm for 1min and then pull-off and discard the supernatant.
9) Suspend the cells in 100µL 100mM LiAc.
10) Incubate salmon sperm DNA (ssDNA) at 95°C for 5min.
    a. **TIP:** This is the same reagent used in the previous transformation: you don't have to boil it after each freeze-thaw, but after every 3-4. Keep in ice when not in the freezer.
11) Vortex the cell suspension and transfer 50µL another Eppendorf tube. Label the tubes you started with "+ DNA", and the tubes you transferred to "- DNA". **You will need four tubes, each with 50µL of cell suspension.**
12) Label the add the following reagents to each labelled cell pellet:

Reagent:	WT cells:	WT + Cas9 cells:
PEG (50% w/v)	240µL	240µL
1M LiAc	36µL	36µL
ssDNA (10mg/mL)	10µL	10µL
CRISPR DNA or ddH_2O*	--	74 µL
HPH DNA or ddH_2O*	74 µL	--
Total volume:	360µL	360µL

***TIP:** Add the appropriate DNA mix to your tubes labelled "+ DNA" and add ddH_2O instead to the tubes labelled "- DNA".

13) Suspend the cell pellets with the transformation mix by vortexing vigorously.
14) Incubate at 42°C for 40min.
15) Add 1mL of YPD to the transformation samples and transfer the entire sample to a sterile glass culture tube.
16) Incubate your glass culture tubes at 30°C for 3 hours on the rotator. Your instructor will complete the remaining steps.
    a. Transfer the samples to sterile Eppendorf tubes and pellet by centrifuging at max speed for 1 minute.
    b. Pull off the supernatant and resuspend in 500µL of YPD and plate half on a YPD plate containing the appropriate antibiotic(s).
    c. Plate the cells transformed with the CRISPR machinery on YPD + nourseothricin + hygromycin.
    d. Plate the cells transformed with HPH DNA on YPD + hygromycin.
    e. Incubate plates for 2 – 4 days to allow the cells to grow.

Clean up and storage:

a. Clean up your bench top and wipe down your station with 70% ethanol.
b. Dispose of any tubes and tips in contact with yeast in the biohazard waste.
c. The remainder of each cell suspension can be stored at 4°C overnight, in case more needs to be plated the next day.

Day 10: Rapid isolation of yeast chromosomal DNA and diagnostic PCR

In today's lab we will test the success of our genome editing. We will harvest chromosomal DNA from our transformants and perform PCR to obtain an amplicon that spans where our planned mutations are located.

Reagents/Supplies:

- Yeast cultures from Day 9.5 – *depending on the size of the class and how many students are testing for the same mutation, prepare overnight cultures (**undiluted**) for multiple colonies per student as described in Day 6.5. Students should test no more than eight colonies at a time to ensure they can complete the procedure in a timely manner.*
- Breaking buffer
    - 2% Triton X-100
    - 1% SDS
    - 100mM NaCl
    - 10mM Tris-HCl, pH 8.0
    - 1mM EDTA, pH 8.0
- Glass beads – (0.1mm or 0.5mm diameter) *It may be helpful to mark an Eppendorf tube at the 0.2ml mark so students can pour the beads into this this Eppendorf to measure the amount needed per sample.*
- Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v)
- TE buffer
    - 10mM Tris-HCl, pH 8.0
    - 1mM EDTA, pH 8.0
- 100% ethanol
- 7.5M ammonium acetate
- RNase A

Tips:

- Ensure that the microcentrifuge is always balanced whenever you use it.
- The phenol:chloroform:isoamyl alcohol (PCI) solution is considered **hazardous**, and very volatile. Follow your instructors demonstration to pipette phenol properly.

Experimental procedure: Rapid isolation of yeast chromosomal DNA

1) Obtain your overnight yeast cultures that were prepared on Day 9.5.
2) Take 700µL of each culture and mix it with 300µL of 50% glycerol in a sterile 1.7mL Eppendorf tube. Label these tubes with the name designated by your instructor. Store these glycerol stocks in the -80°C freezer.
3) Centrifuge your cultures for 5 minutes in a tabletop centrifuge at 3000rpm.
4) Remove the supernatant, resuspend in 1mL of ddH_2O, then transfer the resuspended cells to a 1.7mL Eppendorf tube and spin 30s at max speed.
5) Remove the supernatant then disrupt the cell pellets by vortexing briefly.

6) Resuspend cells in 200µL breaking buffer. Add 0.3g glass beads (~200µL volume) to each tube.
    a. **TIP:** You can measure out these beads in an Eppendorf tube marked at the 200µL mark. Be careful not to spill these!
7) Bring your samples to the fume hood and pipet 200µL of the phenol/chloroform/isoamyl alcohol mixture (PCI).
    a. **TIP:** Phenol can quickly cause burns if spilled on your skin, so ensure you are being careful when pipetting the PCI mixture into each tube!
    b. **Ensure you're pipetting from the organic layer (bottom)!**

It is worth demonstrating to the students how to pipet this solution into their Eppendorf tubes without splashing outside of the tube. Phenol is hazardous, very volatile, and easily removes marker labels, so make sure students are pipetting the PCI mixture carefully.

8) Vortex your tubes at the highest speed for 3 minutes.
    *Ensure the lids are closed **tightly** prior to vortexing!*
9) Add 200µL TE buffer and vortex briefly.
10) Centrifuge for 5min at max speed at room temperature.
    a. After centrifugation, notice how two layers are present within your samples. The denser organic layer residues in the bottom half while the less dense aqueous layer remains on top.
        i. **TIP:** Chloroform is significantly denser than water, which helps for a defined separation between the organic and aqueous layers. Non-polar, hydrophobic molecules like lipids will be pulled into this layer while our desired polar, hydrophilic nucleic acids will remain in the aqueous layer.
        ii. Proteins are denatured in this solution and end up in either the organic phase or at the interface between the two layers.
11) Carefully transfer the aqueous layer (~400µL) to a clean microcentrifuge tube.
12) Add 1mL of 100% ethanol and mix by inversion.
13) Centrifuge for 3min at max speed, room temperature, and remove the supernatant.
    a. **TIP:** You should see a small, white pellet after this centrifugation. Be careful not to disturb these pellets as you are removing the ethanol!
14) Resuspend your pellets in 400µL TE buffer.
15) Add 30µL of 1mg/mL DNase-free RNase A, mix, and incubate 5min at 37°C.
    a. **TIP**: This enzyme will degrade RNA present within the samples. Keep the RNase on ice when not in use!
16) Add 5.3µL of 7.5M ammonium acetate and 1mL of 100% ethanol, then mix by inversion.
17) Centrifuge for 3 minutes at max speed at room temperature.
18) Add 1mL 75% ethanol, mix the tube by inversion, and centrifuge again for 3 minutes at room temperature.
19) Discard the supernatant, then dry the pellets by leaving the tubes with their lids open and inverted. *– The instructor may need to finish the procedure if pellets are not dry by the end of class.*
20) Resuspend in 100µL TE buffer and use 1µL for a 20µL PCR reaction.

Clean up and storage:

1. Clean up your bench top and wipe down your station with 70% ethanol.
2. Eject tips used to pipette solutions with phenol into the designated container in the fume hood

Day 10.5: PCR amplification from genomic DNA

This procedure is performed to amplify the region of the genomic DNA containing the mutation to your gene of interest. *This procedure can be done during class time if prior experiments have worked well, and the class is not running behind.*

Reagents/Supplies:

- Ice bucket
- 5X Phusion GC Buffer
- 10 mM dNTPs
- Milli-Q water
- 10μM Forward upstream primer (F-upstream) *– These primers should amplify a genomic region including the gene replaced by homologous recombination.*
- 10μM Reverse downstream primer (R-downstream)
- 10μM F-donor *– These primers are the same as the ones used on Day 8.*
- 10μM R-donor
- Phusion DNA Polymerase
- 0.5x TAE
- SYBR safe stain
- Agarose
- Tupperware for gel storage

Tips:

- Be very careful in your pipetting; small errors can lead to failed PCR amplification due to an imbalanced template:primer ratio.
- Check off each reagent added to each tube with a checkmark to keep track of your progress in the protocol. It is very easy to forget which reagents you have/haven't added if you aren't keeping track!
- Handle all reagents wearing gloves!

Experimental Procedure (PCR Reaction):

1) Obtain your primers, donor DNA, dNTPs, and buffer, and thaw each on ice.
2) Obtain a 0.2μL PCR tube and label with your initials.
3) Prepare PCR reactions as indicated in the table below:

Reagent:	**1X**	**____ X**
Milli-Q water	16.25μl	
5X Phusion GC Buffer	5μl	
10mM dNTPs	0.5μl	
Donor DNA (10ng/μl)	1μl	--
Forward primer	1μl	
Reverse primer	1μl	
Phusion DNA Pol.	0.25μL	
Total:	**25μL**	

***Keep all reagents on ice and add Phusion DNA Polymerase **LAST**.*

It is best to prepare a master mix for testing multiple genomic DNAs for the presence of your desired mutation. Use the F-donor and R-donor primers for testing for the CRISPR-Cas9 mediated mutation and use the F-upstream and R-downstream primers for testing for deletion of your gene with HPH.

4) Place your tube in the thermocycler using the following program:
   Thermocycler program: (Hotstart)
   1) 98°C – 50s
   2) 98°C – 10s
   3) 52°C – 20s
   4) 72°C – 30s
   5) 72°C – 3min

   Steps 2 – 4 will be run 35x

5) Prepare a 1% agarose gel as described in Day 1 and perform gel agarose electrophoresis as described in Day 2.

Clean up and storage:

1. Clean up all bench tops.
2. Store your PCR reagents in the -20°C freezer.

Day 11: Restriction enzyme digest diagnostic test

For this procedure, we will use restriction enzymes to test for the presence of integrated donor DNA in the genomic DNA we isolated from our yeast colonies previously. This procedure is only required for a subset of our samples, as indicated below.

Reagents/Supplies:

- PCR reactions from Day 10.5
- CutSmart buffer
- Restriction enzymes
    - KpnI-HF *– this enzyme cuts only the wild-type sequence amplified from the SNF2 gene; the enzyme used will change depending on which cut site was mutated in the donor DNA sequence.*
    - BamHI-HF *– this enzyme cuts only the mutant sequence amplified from the SNF2 gene; the enzyme used will change depending on which cut site was added in the donor DNA sequence.*
- Agarose *– depending on the size of the digestion products obtained from the digest, it may be beneficial to use a 1.5% agarose gel for electrophoresis to better resolve small fragments.*
- 0.5% TAE

Tips:

- It is critical to always keep the restriction enzymes in the -20°C freezer except when pipetting for the digestion reactions.

Experimental procedure: Restriction enzyme digestion

1) Today we are digesting samples transformed with donor DNA containing the CRISPR-Cas9 mediated mutation.

    a. Today you will be digesting **separately** with *KpnI-HF* and *BamHI-HF*.

    Replace these with whichever enzymes are used for the diagnostic test.

2) Fill in the table below to create a master mix for your restriction enzyme digests. Set up your digests using the table below.

    a. TIP: Your master mix should contain everything except for the PCR product and ensure that the volume in your tubes will be sufficient for at least your number of reactions + 1!

    b. For example, if you have 4 samples to digest, you will make a **5X** master mix for *KpnI-HF*, and a **5X** master mix for *BamHI-HF*.

Reagent:	1X *KpnI-HF*	1X *BamHI-HF*	____X *KpnI-HF**	____X *BamHI-HF**
PCR product	8µL	8µL	--	--
10X CutSmart buffer	2.5µL	2.5µL		
KpnI-HF	0.5µL	--		--
BamHI-HF	--	0.5µL	--	
ddH_2O	14µL	14µL		
Total:	**25µL**	**25µL**		

***Calculate the volumes needed to make a master mix for each restriction enzyme, excluding the PCR product! Fill in the numbers in the table.**

3) Grab two Eppendorf tubes for each PCR product you have, and label each with your initials and the sample number. Designate which master mix will go in the tube with a "*K*", or an "*H*".
4) Pipette 17µL of the master mix into their respective tubes, then add 8µL of your PCR product to the designated tubes.
5) Incubate your samples at 37°C for 1 hour.
6) Mix 5µL of each digest with 1µL of 6X DNA loading dye and assay the success of your digestions by gel electrophoresis. (See Day 1 protocol).

Clean up and storage:

1. Clean up your bench top and wipe down your station with 70% ethanol.
2. Save your cell pellets in the 4°C refrigerator, and your extra genomic DNA in the -20°C freezer.

Day 12: Spot assay for yeast growth phenotyping

Today, we will perform our final experiment for the semester, assaying the growth of our new yeast strains on different media to test for changes in phenotype caused by the mutations we've generated.

Reagents/Supplies:

- Yeast cultures from Day 11.5 *– These cultures must be at log phase growth (OD_{600} = 0.7 – 1.0), and should be prepared as described in the Day 6.5 protocol.*
- YPD plates
- Sterile dd_2O *– this water should be autoclaved prior to use for the spot assay.*
- Glass culture tubes

Tips:

- Use all you've learned during the semester to perform this experiment with sterile technique. We will be spotting onto rich media today, which is very easy to contaminate!
- When spotting your plates, be careful not to puncture the agar.
- Ensure your plates remain as still as possible during your spotting to generate a uniform array of spots.
- When finished, allow your plates to sit on your benchtop for a sufficient period of time for them to completely soak into the media.

Experimental procedure:

1) Your TA prepared overnight cultures yesterday for today's experiment. Measure the OD_{600} of each of your cultures. **We want them to be at around an OD_{600} of 0.7 – 1.0.**
2) Transfer 1mL of your cultures to sterile 1.7mL Eppendorf tubes and pellet by centrifugation at max rpm for 2 minutes.
3) Remove and discard the supernatant.
4) Wash the cell pellet by suspending cells in 1mL of sterile ddH_2O followed by centrifugation at max rpm for 2 minutes and removal of the supernatant.
5) Suspend the cells in sterile ddH_2O to an approximate OD_{600} of one. For example, if one of your samples had an OD_{600} of 0.843, suspend the pellet in 843µL of ddH_2O.
6) Prepare **four** 10-fold serial dilutions of the cells into the provided glass culture tubes.
7) This can be done by pipetting 100µL of your cells into a glass culture tube containing 900µL of sterile ddH_2O. Then pipet 100µL from this culture tube into another culture tube containing 900µL of ddH_2O to generate another serial dilution. Repeat this process to get each of your dilutions.

8) Label three YPD plates as shown below:

   i. YPD 30°C

   ii. YPD 37°C

   iii. YPD 39°C

9) Place each plate agar side up onto the grids given to you and write above the row in which each of the spots will be placed.

The grid is pictured at the end of this experimental procedure. Ensure that your printed protocol prints the grid to be the same size as your petri dishes.

10) Turn the plate over, place it on the grid, and remove the lid.

11) Starting with the most dilute sample (10^{-4}) obtain 5µL with your pipet and drop the cells into the middle of the first box in its designated row.

Demonstrate how to perform the spots for the students. After drawing up the sample in the pipet, depress the pipet to the first stop and gently bring the liquid at the end of the tip to the surface of the YPD plate.

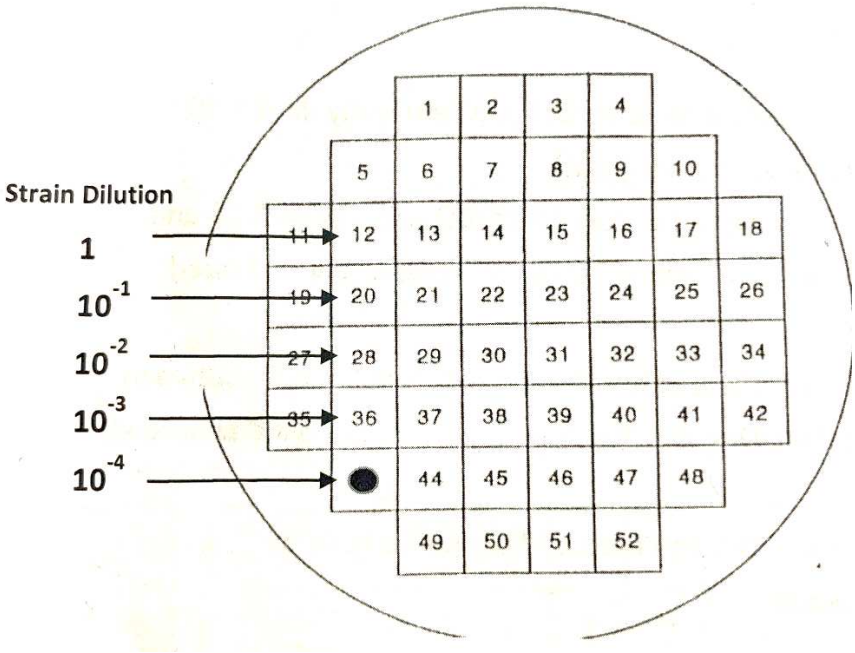
12) Repeat this process with the other dilutions. You do not need to change tips until you are spotting a different strain into a different row.

13) Let the plates sit at room temperature until the spots have all soaked into the media.

14) Incubate the YPD 30°C and 39°C plates in the designated incubators in the Stargell lab, and the YPD 37°C plate in the incubator in the teaching lab.

15) Your TA will photograph these plates daily until colonies are 0.5 – 1.0mm in diameter.

Ensure the plates remain as still as possible during spotting to generate a uniform array of spots. Also, let them soak for at least 30 minutes after the students have finished to make sure they are all dry before putting the plates in the incubators. Make sure you don't see any liquid on top of the plates prior to moving them.



Clean up and storage:

1. Combine any unused cells into a glass culture tube and add bleach to it before mixing and dumping it down the sink.
2. Wipe down your benchtop with 70% ethanol.

Daily Check In 1 (Key): Name: _____

Q1: What is the function of Cas9 in CRISPR mediated genome editing?

Cas9 is an endonuclease which creates a double-stranded break in DNA. The location of the double-stranded break is determined by the guide RNA.

Q2: What is the function of guide RNA in CRISPR mediated genome editing?

The guide RNA (gRNA) targets the Cas9 enzyme to the desired location of a double-stranded break by containing a region of homology to the host genome.

Q3: What do you dissolve agarose in when making a gel for electrophoresis?

We dissolve agarose in TAE buffer (Tris base, acetic acid and EDTA).

Q4: Which reagent in the PCR reaction below would you **exclude** to test for DNA contamination in your master mix? (**Negative control**)

Reagent:	Volume:
Milli-Q water	16.25µL
5X Phusion HF Buffer	5µL
10mM dNTPs	0.5µL
Plasmid (5ng/µL)	1µL
Forward primer	1µL
Reverse primer	1µL
Phusion DNA Pol.	0.25µL
Total:	**25µL**

We can exclude the plasmid DNA to test for DNA contamination. If amplification occurs without the plasmid DNA, then we know there was contamination.

Daily Check In 2(Key): Name: _____

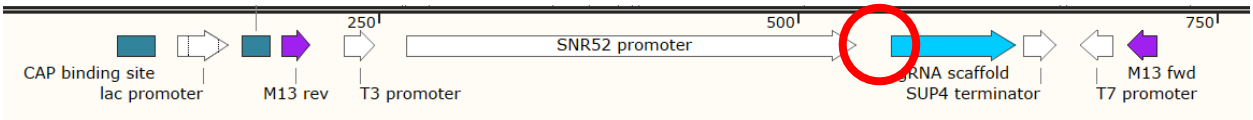
Q1: What is the paper that demonstrates that the SNF2 protein (Snf2p) is necessary for the structural integrity of the SWI/SNF complex?

Richmond E and Peterson CL (1996) Functional analysis of the DNA-stimulated ATPase domain of yeast SWI2/SNF2. Nucleic Acids Res 24(19):3685-92

Q2: What does a kinase do?

Kinases are enzymes which catalyzes the transfer of a phosphate group from ATP to another molecule.

Q3: Diagrammed below is a portion of the plasmid that expresses guide RNA used for CRISPR. **Circle the part of the plasmid that contains the guide RNA sequence** (that we engineered in Day 2's PCR).



The guide RNA sequence resides between the SNR52 promoter and the scaffold.

Q4: What type of organism are we transforming our guide RNA plasmids into today?

Chemically competent E. coli cells.

Daily Check In 3 (Key): Name: _____

Q1+Q2: What is the function of (1) **Ligase** and (2) **DpnI** in the KLD treatment we use prior to transforming our plasmid into *E. coli?*

The Ligase (L) will ligate the linear PCR product back into a circular plasmid after it has been phosphorylated by the Kinase (K).

DpnI (D) is a restriction enzyme which specifically recognizes a methylated recognition sequence, and will cleave the template plasmid DNA which is methylated since it came from bacterial cells. Our PCR reaction did not add methyl groups to our PCR product.

Now that we're two weeks into our course, I'd love to get some feedback from you!

Q3: What is a concept from our course that you feel you understand well?

Q4: What is a concept from our course that is still unclear to you?

Daily Check In 4 (*Key*): Name: _____

Q1: The P1 resuspension buffer is kept in the refrigerator once RNase is added to the reagent. Why in general do we keep enzymes in cold conditions when not in use?

Heat can denature our enzymes, rending them inactive. At temperatures near freezing, enzyme activity is minimized, and protein stability is maximized.

Q2: When performing plasmid isolation, how does the QIAGEN silica column bind DNA?

The QIAGEN silica column is positively charged and attracts negatively charged DNA while allowing for elution of other contaminants in the sample.

Q3: Why does the DNA stay bound to the column during the PB wash (contains a high ethanol concentration) but it elutes when Milli-Q water is added?

The PB wash contains a high concentration of salt and ethanol. The salt helps neutralize the charge of DNA from the sugar phosphate backbone, resulting in less hydrophilicity, and in turn the DNA is less soluble. Milli-Q water (or elution buffer from QIAGEN) has a low salt concentration and higher pH, rendering the silica membrane negatively charged. DNA is very soluble in this solution and is also negatively charged, so it can elute through silica.

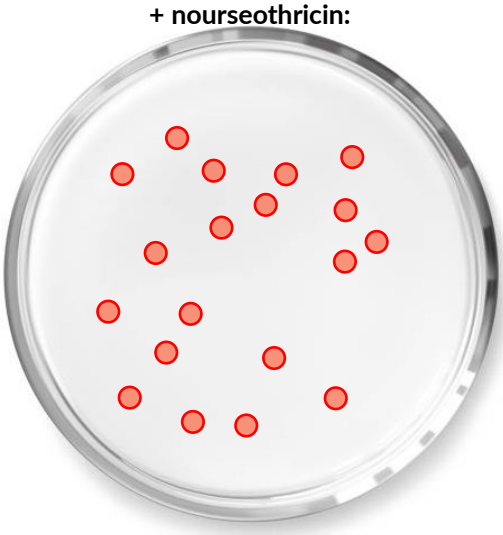
Q4: Why does a ddNTP terminate a Sanger sequencing reaction?

The extension of the polynucleotide in a Sanger sequencing reaction will stop if a dideoxy nucleotide (ddNTP) is included in the chain in place of a deoxynucleotide (dNTP) as ddNTPs lack a 3′ OH group which is required for formation of a phosphodiester bond with the next nucleotide.

Daily Check In 5 (Key): Name: _____

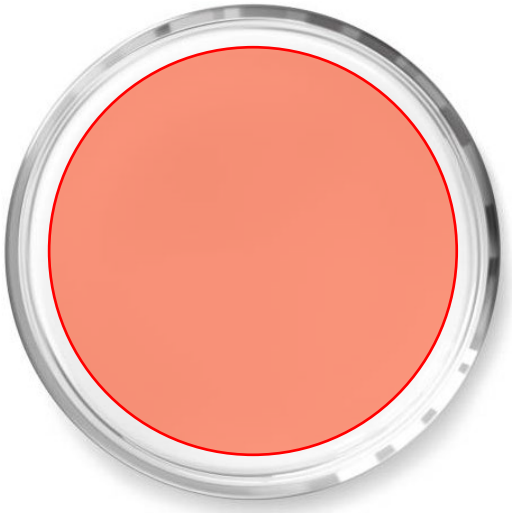
Q1-Q4: Today we are transforming the Cas9 plasmid into our wild-type yeast. **Draw and explain what you would expect to see on the plates below in the following situations:**

(1) Successful transformation on YPD + nourseothricin:



(2) Your plasmid was omitted from the transformation:

(3) Nourseothricin is missing from the media:



(4) You plated on YPD + hyg plates:

(1) *Only yeast successfully transformed with the Cas9 plasmid will grow. Transformation efficiency is inherently low, and we see a countable number of isolatable colonies.*

(2) *The Cas9 plasmid contains a nourseothricin resistance cassette. If the plasmid was omitted, we would expect no growth on the media.*

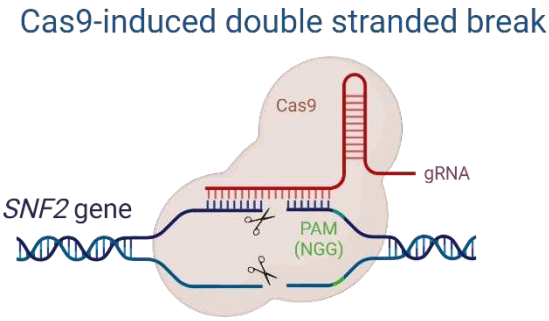
(3) *Without nourseothricin, all yeast can grow on the media. Since we're plating many cells knowing the transformation efficiency is low, we'll see a lawn growth.*

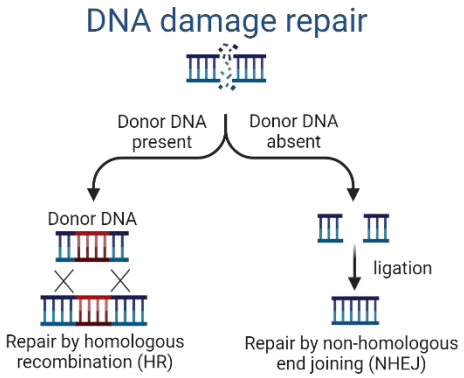
(4) *Hygromycin will kill all the yeast since our plasmid does not contain a hygromycin resistance cassette, and our starting yeast is not resistant to hygromycin.*

Daily Check In 6 (Key):

Name: _____

Q1: To the best of your ability, draw the mechanism of CRISPR-Cas9 mediated genome editing. Make sure you include what **Cas9**, **gRNA**, **PAM** performs, and the two options for repair after the genome edit.





The guide RNA targets the Cas9 endonuclease to our gene of interest by homology to its DNA sequence. This sequence must contain a PAM sequence (NGG) directly adjacent to our guide RNA target sequence. Cas9 induces a double stranded break in our gene of interest, which can be repaired through two pathways. Since our cells are haploid and have only a single copy of each chromosome, cells can only use homologous recombination (HR) if donor DNA is present. This donor DNA is highly homologous to the region containing the double-stranded break. Alternatively, if donor DNA is not present, the double stranded break is repaired by non-homologous end joining (HR), where the two ends are ligated back together. However, exonuclease activity results in loss of some sequence surrounding the double-stranded break.

Daily Check In 7 (Key): Name: _____

Q1: What is the purpose of Lithium cations when transforming DNA into yeast cells?

The lithium cations help to neutralize the charge of DNA (which is highly negatively charged), as well as neutralize negative charges on the surface of the yeast to help facilitate passage of the DNA into the cell.

Q2: Do colonies on our YPD + hygromycin + nourseothricin plates guarantee that we successfully generated the mutation we wanted through CRISPR?

No – Our media selected for cells which were successfully transformed with the guide RNA and Cas9 plasmids, but our donor DNA does not contain a selectable marker. Thus, we don't know if our cells have our desired mutation from growth on YPD + hygromycin + nourseothricin plates alone.

Q3: What are each of the steps we will perform to confirm if our mutation is present in the yeast genome?

We will isolate genomic DNA from our yeast colonies, perform PCR to amplify the region of SNF2 that we're trying to edit, and then perform a restriction enzyme digestion as a quick test to see if our donor DNA was recombined into the gene. Finally, we'll perform Sanger sequencing to confirm that all of our mutations in SNF2 are present without other undesired mutations.

Q4: If your transformation did not work last class period, propose a hypothesis why it didn't yield colonies, but others were successful. (If it did work, feel free to leave blank.)

Students can have a variety of reasonable hypotheses for a failed transformation. Most commonly we observed students propose that they forgot a reagent in their transformation mix or that they missed a step of the protocol.

Daily Check In 8 (Key): Name: _____

Today we will listen to each other's presentations on CRISPR clinical trials. For your check in, please write a question you have for each of your classmates:

Q1: Speaker _____

Our class sizes were very small which allowed us to have each student give feedback to each other. This activity may need to be revised depending on the size of the class.

Q2: Speaker _____

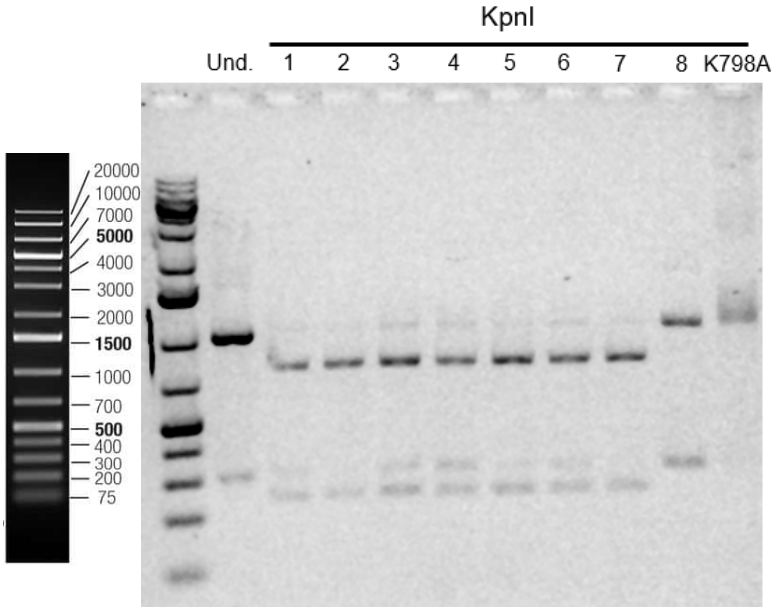
Daily Check In 9 (Key):

Name: _____

Q1: Drew performed a PCI extraction and unfortunately failed to get PCR amplification. However, he clearly saw a white pellet after ethanol precipitation. What else could be in this pellet?

RNA is also in this pellet.

Q2-4: Our donor DNA repairs the double-stranded break produced by Cas9 at the *SNF2* gene. It removes the only KpnI cutsite in the gene. With this information and the image below, answer the following questions:



What size fragments does KpnI digestion yield? **(Und. indicates the undigested PCR product)**

SNF2 cells digested with KpnI will yield 243bp and 775bp products.

Which (if any) of the candidates look like they successfully obtained the donor DNA in their genomic DNA?

Sample 8

What's the next step in confirming the presence of our mutation in our PCR product?

We need to digest the DNA with BamHI to see if the PCR product from Sample 8 is digested into 523bp and 493bp products.

Daily Check In 10 (Key): Name: _____

Q1: To the best of your ability, list out the proper steps for pipetting yeast from a glass culture tube into an Eppendorf with sterile technique.

1. *While wearing gloves, pick up the glass culture tube from its test tube rack and gently vortex to resuspend and yeast that have settled to the bottom.*
2. *Remove the cap and hold it in one hand, while using the other to briefly flame the opening of the glass tube with a lit Bunsen burner.*
3. *Pipette your volume of culture from the culture tube using only sterile tips.*
4. *Re-flame the glass culture tube briefly and replace the lid on the tube. Set it in its test tube rack.*
5. *Dispense your culture into the Eppendorf tube and dispose of your pipette tip in a waste container. Always use a new tip for each culture!*

Q2: Why is it important for each culture to be resuspended to **the same optical density** (OD_{600} = 1) prior to spotting the yeast onto your plates?

We want our strains to be at the same initial optical density to ensure that our spotted yeast begin at the same base level of growth, so that we can best compare their growth rates. Differences in growth can then be attributed to differences in yeast strain rather than differences in the starting amount of yeast.

Q3-4: Which class period/experiment did you enjoy the most? Which was the most helpful for you?

Thanks for a fantastic semester!

Student Assignments

DNA Design Assignment

25 points

The purpose of this assignment is to give you the experience of engineering a guide RNA plasmid that can be used for CRISPR-Cas9 mediated genome editing.

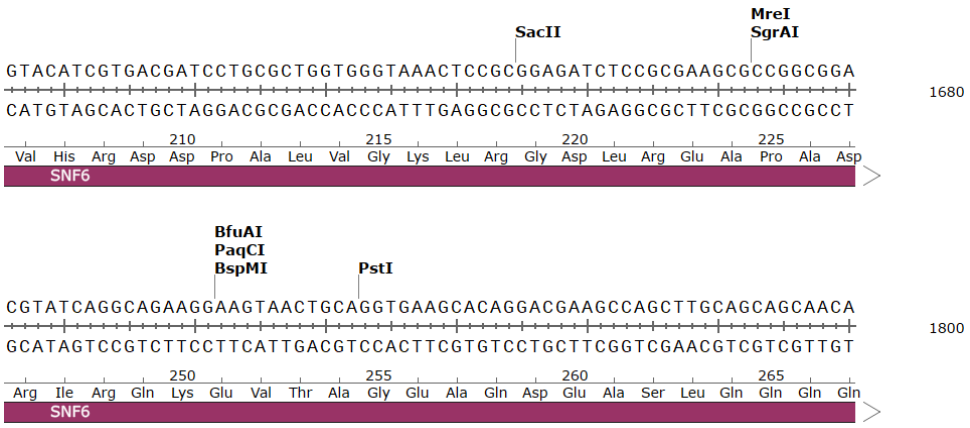
This semester, we are targeting Cas9 to *SNF5*, whose gene product is part of the SWI/SNF complex. Our lab is also interested in creating mutations in *SNF6*, another gene whose product is in the complex. Through this assignment, you will design primers to target Cas9 to the *SNF6* gene.

Step 1: Download Snapgene Viewer and the DNA sequence files for *SNF6* and the gRNA plasmid (See the assignment page on Canvas)

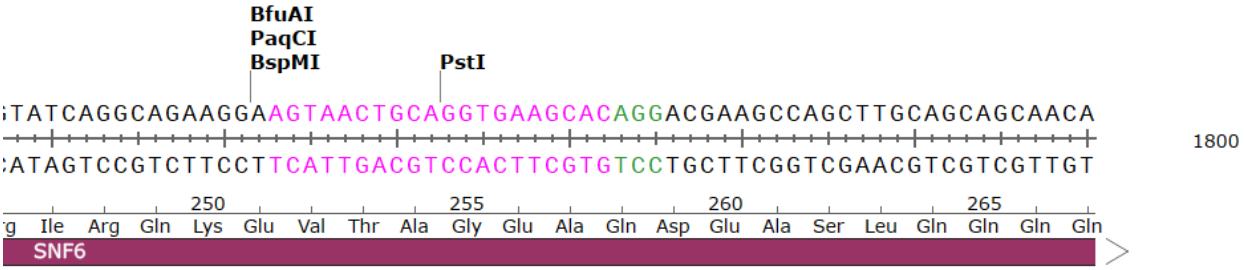
3) Navigate to https://www.snapgene.com/snapgene-viewer to download Snapgene Viewer (if you don't already have this software on your computer)
    a. This program will allow you to work with the DNA sequences required for engineering a guide RNA for targeting Cas9 to generate a double-stranded break at the *SNF6* gene.
4) Obtain the gRNA-ura-HYB.dna and SNF6.dna files from your instructor. *These files contain the DNA sequence for the portion of the gRNA plasmid that encodes the guide RNA and the genomic sequence +/- 1kb for the SNF6 gene.*

Step 2: Locate a sequence of *SNF6* adjacent to a PAM sequence.

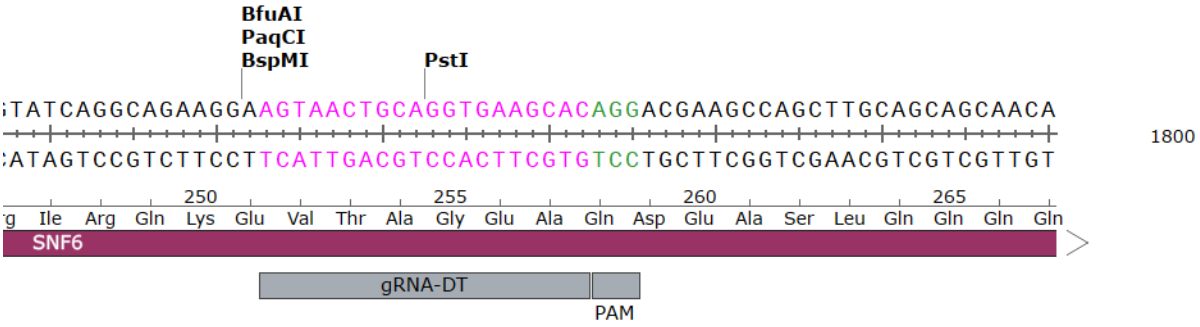
7) Your goal is to find a PAM sequence within the SNF6 gene near the middle of the gene. (The sequence for Cas9 is **NGG**.)
    a. In this file, the open reading frame of SNF6 spans 1000 -- 1999bp. You can see the DNA sequence including restriction enzyme digest sequences and its translated sequence, as depicted below.



8) Select a PAM sequence between position **1450 - 1550** and use your cursor to highlight it.
9) Right click your selection, then press “Set DNA Color”, and choose a color other than black to recolor the text for your PAM sequence.
10) Next, highlight 20bp **immediately upstream** of your PAM sequence, and recolor this sequence as well, but with a different color from your PAM sequence.
11) Your SNF6 file should now have your gRNA target and PAM sequences colored like this below:



12) You can also highlight your sequences separately and click Feature > Add Feature, then name it “gRNA-xx” (where xx are your initials), or “PAM” to be able to find these sequences more easily in your document. You can change the color of these features as well if you’d like!



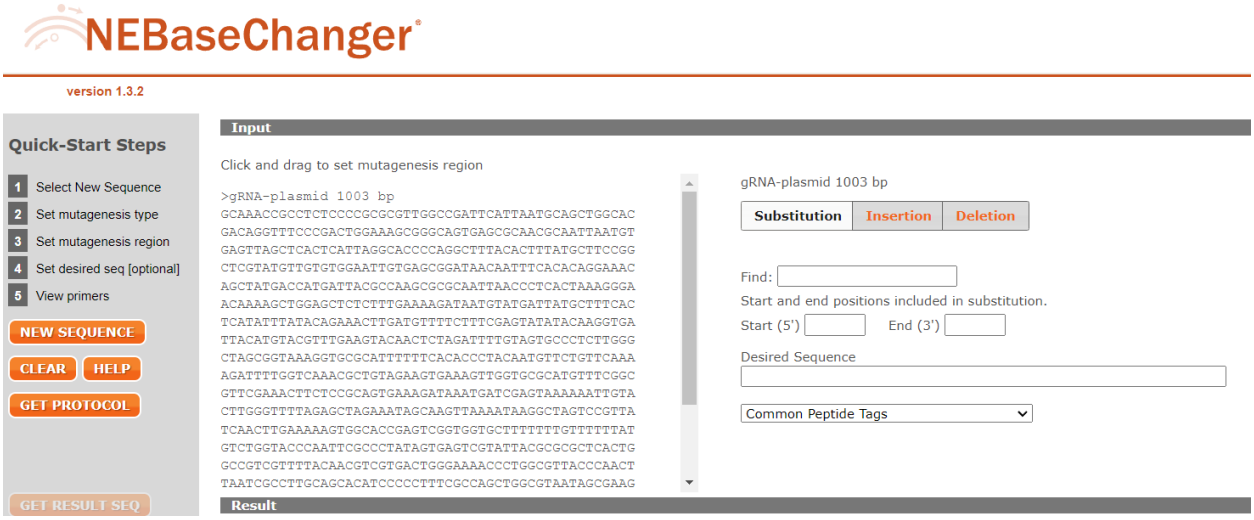
13) **What is your gRNA sequence?** (You can copy and paste this from the SNF6 document.)

14) What is your adjacent PAM sequence?

Step 3: Design primers to replace the guide RNA sequence in the gRNA-HYB plasmid with your guide RNA sequence.

12) Open the gRNA-ura-HYB sequence and locate the 20bp between the SNR52 promoter and the gRNA scaffold.
13) **What is this sequence in this document?**

14) This is the sequence that you will replace with the gRNA sequence you created in the previous step.
15) We will now use **NEBaseChanger** to design primers that will bind the gRNA-ura-HYB plasmid and replace the 20bp sequence you just found, with your sequence you located in the SNF6.dna file.
16) Navigate to https://nebasechanger.neb.com/ .
17) Click on the button titled "**Please enter a new sequence to begin.**"
18) Copy and paste the entirety of the gRNA-ura-HYB sequence into the text box that appears on your screen, and name it.
    a. You should see something that looks like this:



19) Now copy and paste the 20bp sequence you obtained from the **gRNA-ura-HYB** sequence into "Find". You should then see your sequence highlighted in blue to the left.
20) Bring your cursor on top of the first and last base pair highlighted in blue, and type what positions they are located at.
21) **What is the base pair number for the start of the highlighted sequence?**

22) **What is the base pair number for the end of the highlighted sequence?**

23) Type those numbers into the Start (5') and End (3') regions and verify that the orange highlighted sequence matches what was highlighted in blue.
24) Next, paste your desired gRNA sequence that you copied from the SNF6 document into "Desired Sequence"
    a. You should now have content under "Result": The primer sequences, the melting temperature, etc. **Take a screenshot of this result and paste it below.**

25) Congratulations, you've now designed the primers required to engineer your plasmid that will express a guide RNA targeting Cas9 to cleave the *SNF6* gene!

Data Quantification Assignment
25 points

The purpose of this assignment is to quantify our results from this semester's experiments for reporting them in your notebook and your final presentation. We will quantify transformation efficiency from the yeast transformations we've performed, which is an important metric we use in designing future CRISPR-Cas9 experiments. We will also score the growth of our yeast spots.

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (mL)}}{\text{vol plated (mL)}} \times \text{Dilution factor} = \text{Number of transformants per } \mu\text{g}$$

Count the colonies (transformants) from the images taken of your plates and use the protocols from Day 7 and Day 9 to fill in the tables below. **Include the data from these tables in your final presentation!**

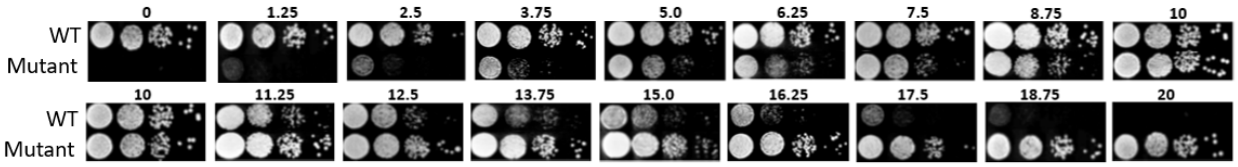
Cas9 plasmid transformation: transformation:

	WT + Cas9
Number of colonies on plate	
Mass of plasmid (µg)	
Final volume at recovery	250µL
Volume plated	125µL
Dilution factor	1
Number of transformants per µg	

Guide RNA plasmid

gRNA plasmid:	
Donor DNA:	
Number of colonies on plate	
Mass of plasmid (µg)	
Final volume at recovery	500µL
Volume plated	250µL
Dilution factor	1
Number of transformants per µg	

Use the diagram below to score the growth on each of your plates containing your yeast spots. Keep in mind that the scoring is **relative to your starting strain**.Fill in the table below with your results, and present both the images of the spots and the scoring in your lab notebook and final presentation.



Courtesy of Dr. Catherine Radebaugh

Fill out this table below with your scores from your plates.

Strain 2			**Strain 3**			**Strain 4**		
30°C	37°C	39°C	30°C	37°C	39°C	30°C	37°C	39°C

Adjust this table as necessary depending on the number of mutant strains the students are using in their spot assay. Additionally, the table and experimental procedure can be modified if testing other growth conditions is desired.