Developmental Control Throughout the Budding Yeast Life Cycle by Jhd2, a JARID family Histone H3 Lysine-4 Demethylase

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Abstract

Histone H3 lysine-4 methylation (H3K4me) is a well-studied chromatin modification that is associated with active transcription; however, its physiological roles are not well understood. The importance of maintaining proper H3K4 methylation dynamics is underlined by the fact that H3K4 methyl transferases and demethylases control the balance between pluripotency and differentiation, and their misregulation is associated with oncogenesis. My research contributed to the conclusion that the budding yeast JARID1 family H3K4 demethylase, encoded by $JHD2$, is required during yeast gametogenesis (sporulation) to sustain the duration of global gene expression in opposition to developmentally programmed quiescence.

To elucidate the underlying mechanisms of Jhd2 function during sporulation, I quantified mRNA transcript accumulation and mapped genome-wide nucleosome positioning using high resolution tiling microarrays over time courses. I also carried out analysis of a pre-existing H3K4me ChIP-chip (chromatin immuno-precipitation followed by microarray) data set. Integrating data analysis from these experiments provided evidence to support the model that $JHD2$ broadly represses interfering non-coding transcription during post-meiotic stages of sporulation. Moreover, my analysis suggests that $JHD2$-repression of non-coding transcription permitted the sustained
transcription of their proximal protein-coding genes as developing spores transitioned into quiescence. I went on to characterize 3’ NDRs (3’ nucleosome depleted regions) and found that both transcription activity and DNA-histone interactions influence 3’ NDR structure.

Furthermore, I found that Jhd2 promotes expression of genes involved in mitochondrial function and positively regulates mitochondrial respiration during sporulation and germination. My studies support the idea that spores utilize a maternal load of cellular resources to drive the earliest stages of germination prior to commencement of de novo transcription.

Together these studies show that Jhd2/JARID1 plays important roles during development and differentiation in budding yeast, and genomic data sets support a model whereby Jhd2 regulates gene expression via modulating levels of gene-associated non-coding transcription.
Acknowledgments

First of all, I would like to thank my supervisor Marc Meneghini for his guidance and support over the years. Marc’s boundless enthusiasm for research and scientific discovery has been a constant source of inspiration and encouragement through my graduate studies. In his own words:

“Marc is such an awesome boss and brilliant scientist. How Science could have ever happened before he became a professor is beyond my capacity to understand. We are all truly blessed to live in world that has Marc in it.”

To my supervisory committee members: Thanks to Alan Davidson for trying (often in vain) to keep my committee meetings from lasting over three hours. Special thanks to Jacqueline Segall, for putting in the extra effort and care to help me edit my thesis. Also special thanks to Corey Nislow for his technical expertise and help.

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<th>Definition</th>
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<tbody>
<tr>
<td>ARID</td>
<td>AT-rich interaction domain</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide 3-chlorophenylhydrazone</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CDS</td>
<td>coding sequence</td>
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<tr>
<td>Ch</td>
<td>chromosome</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>ChIP chip</td>
<td>chromatin immunoprecipitation followed by microarray</td>
</tr>
<tr>
<td>ChIP seq</td>
<td>chromatin immunoprecipitation followed by high-throughput sequencing</td>
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<tr>
<td>COMPASS</td>
<td>Complex Proteins Associated with Set1</td>
</tr>
<tr>
<td>C-term</td>
<td>Carboxyl-terminal</td>
</tr>
<tr>
<td>CUT</td>
<td>cryptic untranslated transcripts</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>Flavin adenine dinucleotide hydroquinone</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
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<tr>
<td>GO term</td>
<td>Gene Ontology term</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyl transferase</td>
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<td>Histone H2A</td>
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<tr>
<td>H2B</td>
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<td>histone deacetylase</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HOX</td>
<td>Homeobox</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
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<td>JARID</td>
<td>Jumonji/ARID domain</td>
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<td>jhd2Δ</td>
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<td>JmjC</td>
<td>Jumonji C-terminal domain</td>
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<td>Jumonji N-terminal domain</td>
</tr>
<tr>
<td>Kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>KDM</td>
<td>histone lysine demethylase protein</td>
</tr>
<tr>
<td>KMT</td>
<td>histone lysine methyl transferase protein</td>
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lincRNA: long intergenic non-coding RNA
MAP: mitogen-activated protein
MLL: multiple-lineage leukemia
MNase I: Monococcal nuclease I
mRNA: messenger RNA
MUT: meiotic untranslated transcripts
NADH: Nicotinamide adenine dinucleotide
NaDOC: Sodium deoxycholate
ncRNA: non-coding RNA
Nat: nourseothricin
NDR: nucleosome depleted region
NET-seq: Nascent elongating transcript sequencing
NFR: nucleosome-free region
NP40: nonidet 40
N-term: Amino-terminus
NCBI: National Center for Biotechnology Information
NGS: next-generation sequencing
O.D.: optical density
OXPHOS: oxidative phosphorylation
PAGE: polyacrylamide gel electrophoresis
PCR: polymerase chain reaction
PHD: plant homeo domain
PKA: protein kinase A
pol II: RNA polymerase II
qPCR: quantitative PCR
RDN: ribosomal DNA
ROS: reactive oxygen species
RNAP II: RNA polymerase II
RNAP II CTD: RNA polymerase II carboxyl-terminal domain
RP(L/S): ribosomal protein (large/small) subunit
RPM: revolutions per minute
RT-qPCR: reverse transcriptase quantitative polymerase chain reaction
SC: synthetic complete
SDS: sodium dodecyl sulfate
SET: Su(var)3-9, Enhancer-of-zeste, Trithorax
SNP: single nucleotide polymorphism
SUT: Stable untranslated transcript
TBS: tris-buffered saline
TBST: tris-buffered saline with Tween 20
TCA: trichloroacetic acid
TE: tris ethylenediaminetetraacetic acid
tRNA: transfer RNA
TSS: transcriptional start site
TTS: transcriptional termination site
USB: universal serial bus
WT: wild type
XUT: Xrn1-sensitive unstable transcript
YPA: yeast peptone acetate (1% yeast extract, 2% peptone, 2% potassium acetate)
YPD: yeast peptone adenine dextrose (1% yeast extract, 2% peptone, 2% dextrose, 0.3mM adenine)
YPAGE: yeast peptone adenine glycerol ethanol (1% yeast extract, 2% peptone, 2% ethanol, 2% glycerol, 0.3mM adenine)
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Chapter 1

INTRODUCTION
1.1 Chromatin

1.1.1 Nucleosomes and their role in gene regulation

Eukaryotic genomic DNA is compacted and organized through packaging into chromatin. The basic unit of chromatin is the nucleosome, which is composed of an octameric complex containing two copies each of the highly conserved histone proteins H2A, H2B, H3, and H4 bound to 147 bp of DNA and distributed across DNA in a formation that is often conceptualized as “beads on a string” (Figure 1). This structure functions to stabilize and package genomic DNA and also serves as the primary template upon which all manner of DNA regulation, including gene transcription, acts (Jenuwein and Allis 2001). Transcriptional regulation can be accomplished in two ways. First, the positioning of nucleosomes relative to cis-acting regulatory sequences and the stability of these histone-DNA interactions can modulate access of trans-acting, sequence-specific binding factors to DNA. Second, post-translational modifications on histone N-terminal tails or substitution of canonical histones with histone variants can affect chromatin structure or control recruitment of trans-acting factors that potentiate additional layers of control. These two general mechanisms are not distinct from each other; for example, histone modifications can affect nucleosome stability or recruit trans-acting factors that reposition nucleosomes relative to DNA. Given the profound influence nucleosome positioning and histone modifications have on gene expression, studies in these fields are bringing us a better understanding of mechanisms that govern gene regulation.

Figure 1. Nucleosomes form the basic organizational unit of chromatin
Shown is a cartoon representation of nucleosomes in a “beads on a string” configuration. The boxed nucleosome shows detail of the eight modifiable N-terminal tails of the histones.
1.1.2 Chromatin, nucleosomes, histone modifications and their role in transcription

In general, nucleosomes impede transcription factors and RNA polymerase from gaining access to DNA, and require remodeling for gene expression to occur. The accessibility of regulatory DNA sequences to trans-acting factors is determined in part by post-translational modifications on histone N-terminal tails, and by the positioning of nucleosomes relative to cis-elements.

Histone N-terminal tail modifications can alter biophysical properties of the nucleosome and also serve as signals for other chromatin-associated factors (Figure 1). Histone tails can be covalently modified on specific residues by methylation, acetylation, sumoylation, ubiquitination, or phosphorylation (STRAHL and ALLIS 2000). The “histone code” hypothesis, first proposed in 2000, posits that histone modifications can exert biological control alone or in combination to directly gene expression states in the cell via factors that can specifically distinguish between histone N-terminal tail modification states (STRAHL and ALLIS 2000). While this has been shown to be the case for certain specific modifications, it appears that other modifications, such as acetylations, have overlapping functions and are often interchangeable (LIU et al. 2005). Furthermore, individual modifications can cause differing consequences depending on the downstream factors that are present to recognize it (BERGER 2007). In other words, molecular interpretation of histone marks is context-dependent, with the same modification exerting different consequences depending on the biological state of the cell (CAMPOS and REINBERG 2009).

1.1.3 Co-transcriptional deposition of histone marks

Histone methylation on histone H3 lysine 4 (H3K4), Histone H3 lysine 36 (H3K36), and histone H3 lysine 79 (H3K79), are accomplished by histone methyl transferases Set1, Set2 and Dot1, respectively, and these residues can become mono-, di-, or tri-methylated (me1, me2, me3) (MILLER et al. 2001; STRAHLM et al. 2002; VAN LEEUWEN et al. 2002; KROGAN et al. 2003). H3K4me, H3K36me, and H3K79me, as well as numerous histone acetylation marks, are associated with actively transcribed genes (BRIGGS et al. 2001; SANTOS-ROSA et al. 2002; HAMPSEY and REINBERG 2003; KROGAN et al. 2003; DION et al. 2005; LIU et al. 2005). The biological functions of these marks are far from being thoroughly understood. Some compelling mechanistic insights suggest that their functions are not necessarily to promote gene expression.
per se, but to repress spurious transcription events which can impact the transcription of protein coding genes through a variety of mechanisms (HAMPSEY and REINBERG 2003; BURATOWSKI and KIM 2010).

Amongst histone modifications, methylation on H3K4 (H3K4me) is one of the most prominent and well-studied. H3K4me is one of the first epigenetic marks to be deposited on chromatin during transcription (Ng et al. 2003). The histone H3K4 methyl transferase enzyme, Set1, is recruited specifically to the initiating form of RNAP II that is phosphorylated on the serine 5 of the Rbp1 subunit C-terminal domain (CTD) heptad repeat, and deposits H3K4me marks over the transcriptional unit (MCCracken et al. 1997; Ng et al. 2003) (Figure 2A). 5’ ends of transcription units are enriched for H3K4 trimethylation (H3K4me3) while domains of H3K4 dimethylation and monomethylation (H3K4me2/me1) are found 3’ to H3K4me3 (SANTOS-ROSA et al. 2002; LIU et al. 2005) (Figure 2B). Although these distinct domains are a hallmark of transcriptional activity over a gene in diverse species, their downstream functions are not well understood. It used to be a commonly held view that H3K4me is an activating histone mark, but accumulated evidence shows that transcription activation is often not dependent on H3K4me (reviewed in PINSKAYA and MORILLON 2009).
Figure 2. H3K4 methylation is a marker of transcription events
A) Histone H3 lysine 4 can be post-translationally modified with addition of one, two or three methyl groups. B) H3K4me species are laid down in characteristic domains across transcribed regions.

1.1.4 Deposition of H3K4 methylation marks by Set1-family proteins

The H3K4 methylation mark, along with the enzymes responsible for its deposition and removal, are involved in development and differentiation in diverse organisms (Benevolenskaya et al. 2005; Dey et al. 2008; Eissenberg and Shilatifard 2010; Xu et al. 2012). The gene encoding the first histone H3K4 methyl transferase enzyme was initially identified on the basis of a mutation that altered thoracic and abdominal development in fruit fly Drosophila melanogaster, and was named trithorax (trx) (Ingham and Whittle 1980). Trithorax was shown to regulate the expression of homeobox family transcription factors that control segmentation in Drosophila, which explains its roles in development (Ingham and Whittle 1980). At the time, Trithorax was not known to have histone methyl transferase activity; however, it shared a conserved domain with two other fruit fly proteins called Su(var)3-9 (Suppressor of variegation) and enhancer of zeste; thus this domain was named SET (Su(var)3-9, Enhancer of zeste, and Trithorax) (Tschiersch et al. 1994). The enzymatic function of the SET domain was discovered in the budding yeast Trithorax homolog Set1, which was identified based on protein sequence similarity to metazoan SET domains (Nislow et al. 1997). Biochemical studies showed that Set1 was the catalytic subunit of a conserved protein complex called COMPASS (Complex proteins associated with Set1) that exhibited H3K4-specific methylation activity (Miller et al. 2001). Set1/COMPASS can mono-, di-, or tri-
methylate H3K4 (Miller et al. 2001; Roguev et al. 2001; Krokan et al. 2002) (Figure 2A). While Set1 is the sole H3K4-specific methyl transferase in budding yeast, in mammals the SET-domain H3K4-specific methyl transferases comprise an extended family of homologs that include MLL1, MLL2, MLL3, MLL4, SET1A, and SET1B (Reviewed in Eissenberg and Shilatifard 2010). The MLL (mixed lineage leukemia) genes were all isolated as translocation mutations in various types of human cancers (Djabali et al. 1992). In mice, MLL1 and MLL2 are both essential genes that cause embryotic lethality in knockouts mutants and anterior-posterior patterning defects in partial loss-of-function mutants (Yu et al. 1995; Glaser et al. 2006). MLL3 and MLL4 play roles in expression of genes that respond to nuclear receptor signalling (Goo et al. 2003; Lee et al. 2007a). Little is known about the function of SET1A and SET1B, although they seem to be widely expressed in human tissues (Lee and Skalnik 2005; Lee et al. 2007a).

In yeast, Set1/COMPASS is non-essential for viability in laboratory trains, but plays roles in silencing at the ribosomal DNA, silent mating type loci, and at telomeres (Nislow et al. 1997; Briggs et al. 2001; Krokan et al. 2002). Even though H3K4me is a mark of transcription, a growing body of evidence shows that the function of H3K4me and Set1 is not necessarily to promote gene expression (reviewed in Pinskaya and Morillon 2009). For example, loss of H3K4me in yeast grown under standard laboratory conditions has exceedingly modest effects on transcript abundance of protein-coding genes (Briggs et al. 2001; Bryk et al. 2002, Meneghini lab unpublished data; Venkatasubrahmanyam et al. 2007; Margaritis et al. 2012). Even more incongruent with the idea of its role in gene activation, H3K4me2/3 marks have been shown to promote transcription repression via two different mechanisms that involve recruitment of repressive histone deacetylases (HDACs). A study by Pinskaya et al. showed that H3K4me2/3 marks deposited by cryptic transcription that initiates upstream of GAL1 recruits the RPD3s HDAC complex to the promoter region of GAL1, which serves to suppress GAL1 induction (Pinskaya et al. 2009a). Another study from the Buratowski lab showed that H3K4me2 marks promote histone deacylation by recruiting the HDAC Set3 to the body of genes. This resulted in repression of intragenic transcription initiation (Kim and Buratowski 2009). A common feature of these two examples of H3K4me-mediated gene regulation is the involvement of non-coding transcription. In keeping with this trend, a recent insight into Set1 function revealed that Set1/COMPASS promotes Nab1-Nrd3-Sen1-mediated termination
activity (TERZI et al. 2011). Nab1-Nrd3-Sen1 interacts with the initiating RNAPII complex and mediates termination and degradation of cryptic unstable transcripts (CUTs) and snoRNAs. Set1 was found to positively influence this process by promoting recruitment of deacetylases to sites of transcription (TERZI et al. 2011). The impact of termination and breakdown of abortive and non-coding transcripts on gene expression is not yet well understood, but evidence from studies at specific gene loci indicate that in many cases, repressing non-coding and abortive transcripts can promote gene expression (Reviewed in SHEARWIN et al. 2005; PRUNESKI and MARTENS 2011; VAN WERVEN et al. 2012).

Studies of SET1 have revealed surprisingly few fitness consequences for loss of H3K4 methylation in yeast. Although set1Δ cells exhibit slow-growth (NISLOW et al. 1997 and Meneghini lab unpublished data), this phenotype is not due to loss of H3K4me, since cells that have the H3 lysine 4 residue replaced with the non-methylatable alanine (H3K4A) do not exhibit this phenotype (Meneghini lab, unpublished data). I will discuss the apparent lack of fitness consequences for loss of H3K4 methylation later in the chapter, and will provide a possible explanation.

1.1.5 Histone H3K4 demethylation is carried out by JARID1/Jhd2

Because histone methylation is a remarkably long-lived modification with a half-life in the range of hours (NG et al. 2003), it was long thought to be an irreversible modification. It was thought that histone methylation was only removed passively during genome replication when newly synthesized (unmethylated) histones are assembled onto replicated DNA strands (reviewed in BANNISTER et al. 2002). The first definitive H3K4 demethylase enzyme LSD1 was discovered in humans and in Schizosaccharomyces pombe (SHI et al. 2004). However, LSD1 is not a widely expanded protein family (it is not found in Saccharomyces) and it cannot demethylate trimethylated H3K4 (SHI et al. 2004).

The Jumonji-domain containing family of histone lysine demethylases was discovered in 2006 (TSUKADA et al. 2006). Jumonjis are an ancient and conserved family of proteins whose members span a large range of organisms from bacteria to yeast to mammals (TSUKADA et al. 2006; SMART). A key characteristic of Jumonji family members is the presence of a JmjC (Jumonji C terminal) domain, a catalytic domain with the ability to demethylate histone lysine
residues. The conserved catalytic JmjC domain contains an iron-binding pocket and requires Fe$^{2+}$ and alpha-ketoglutarate for enzymatic activity (Schneider and Shilatifard 2006; Tsukada et al. 2006). The name Jumonji comes from the Japanese word meaning ‘cruciform’, which was used by researchers to describe and name the gene mutation that causes a cross-shaped malformation in the neural crest of embryonic mice (Takeuchi et al. 1995).

The H3K4-specific sub-family, called JARID1 (Jumonji and ARID domain-containing), was identified phylogenetically in mammals, C. elegans and S. cerevisiae (Klose et al. 2007; Lee et al. 2007a; Secombe et al. 2007; Tahiliani et al. 2007; Yamane et al. 2007). The budding yeast genome encodes a single H3K4-specific demethylase called Jhd2 (JmjC domain-containing histone demethylase 2) (Liang et al. 2007; Seward et al. 2007; Tu et al. 2007). Jhd2 contains three conserved domains: the catalytic JmjC domain, a JmjN (Jumonji N-terminal) domain that is thought to contribute to JmjC domain activity, and a methyl-lysine binding PHD domain (Klose et al. 2006; Shi et al. 2007; Tu et al. 2007; Huang et al. 2010). Jhd2 acts as a monomer to demethylate mono-, di- or tri-methylated H3K4, and together with Set1/COMPASS, Jhd2 modulates the dynamic H3K4 methylation landscape in the yeast genome (Figure 2).

1.1.6 Jumonji family proteins in yeast

Jhd2 is one of five JARID proteins found in yeast (Tu et al. 2007); the other JmjC domain-containing proteins are Jhd1, Rph1, Gis1 and Ecm5. With the exception of Ecm5, which has a catalytically non-functional JmjC domain, these proteins all exhibit varying degrees of histone demethylase activity (Tu et al. 2007). Jhd1 demethylates H3K36me2 and me1 while Rph1 demethylates H3K36me3 and me2. Gis1 is a paralog of Rph1 that has modest demethylase activity and functions as a transcription factor during response to nutrient limitation (Zhang et al. 2009a).

1.1.7 JARID1s in higher eukaryotes

Mammals encode four JARID1 family H3K4 demethylases: JARID1A, B, C, and D (Shi and Whetstone 2007; Shukla et al. 2009). Like the Set1/MLLs, JARID1 proteins function during development and are misregulated in many cancers. JARID1A represses genes involved in differentiation and mitochondrial function in human cell lines (Benevolenskaya 2007; Lopez-Bigas et al. 2008). Similarly, JARID1B negatively regulates cellular differentiation and
promotes the pluripotent state in embryonic stem cells (DEY et al. 2008). Perhaps related to their role in promoting the proliferative pluripotent state, both JARID1A and JARID1B act as oncogenes, and are overexpressed in a large number of cancers (LU et al. 1999; VAN ZUTVEN et al. 2006; XIANG et al. 2007; HAYAMI et al. 2010; ZENG et al. 2010). JARID1C/SMCX and JARID1D/SMCY are encoded on the X and Y chromosomes, respectively, and both are believed to have tumour-suppression activity (PERINCHERY et al. 2000; SMITH et al. 2010).

1.1.8 Biological roles of Jhd2/JARID1

There has been a paucity of insights into Jhd2 function in yeast since its discovery. The earliest studies that identified Jhd2 as the yeast H3K4me demethylase did not uncover any overt phenotypic consequences in jhd2Δ cells (LIANG et al. 2007; SEWARD et al. 2007). Liang et al. reported that jhd2Δ exhibits a modest increase in telomere silencing. There have been several attempts to identify gene expression consequences of jhd2Δ by microarray RNA profiling. jhd2Δ exhibits essentially no measurable gene expression consequences in cells grown under standard laboratory conditions (LENSTRA et al. 2011; MALTBY et al. 2012; XU et al. 2012).

The lack of phenotypic consequence for deletion of SET1 or JHD2 in yeast has been perplexing and seems to suggest that the biological contexts in which the functions of these genes have been interrogated may not be relevant. Experiments that address chromatin function in yeast have been predominantly carried out in mitotically dividing yeast grown in nutritionally complete media containing a surplus of nutrients. Studies have revealed that yeast are capable of employing various developmental programs to adapt to changing and non-ideal environmental conditions (HERSKOWITZ 1988). In fact, as I discussed above, metazoan JHD2/JARID1 homologs are active during differentiation and play important roles in developmental programs.

Yeast cells differentiate from their proliferative state to a resting state as specialized gametes (spores) through a developmental program called sporulation. Sporulation involves meiotic division of diploid genomes into four haploid progeny, which are then packaged into spores in a process that is analogous to gametogenesis in higher eukaryotes. Previous studies in my lab addressed the hypothesis that sporulation might be a relevant context during which Jhd2 functions. In support of this hypothesis, these studies showed that JHD2 controls global H3K4me dynamics and global transcriptional induction during sporulation, and that
misregulation of these processes in \textit{jhd2Δ} is associated with precocious differentiation and production of stress-sensitive spores (Xu \textit{et al.} 2012). A large part of my Ph.D. thesis work advanced these findings to elucidate the underlying mechanisms by which \textit{JHD2} controls the gene expression program of yeast sporulation.

Here I will introduce the yeast life cycle from vegetative growth through developmental stages including sporulation and germination. Most of my studies of \textit{JHD2} have been done under the latter contexts, so more a detailed description of these developmental programs is necessary for contextualizing \textit{JHD2} functions.

1.2 The budding yeast life cycle

1.2.1 Vegetative growth

\textit{Saccharomyces cerevisiae} can exist in either diploid or haploid states that reproduce by asexual budding (Figure 3). During asexual reproduction, the ‘mother’ cell develops a bud which enlarges into a ‘daughter’ cell that is genetically identical to the ‘mother’ cell. These morphological changes are tied to the cell division cycle, which consists of \textit{G}_1 (gap 1), \textit{S} (DNA synthesis), \textit{G}_2 (gap 2) and \textit{M} (mitosis) stages (Hartwell \textit{et al.} 1970; Hartwell \textit{et al.} 1974; Reed \textit{et al.} 1985). During \textit{G}_1, the cell grows in size; upon commitment to cell division, the cell enters \textit{S} phase and carries out DNA synthesis to replicate the genome. The daughter bud begins to develop at this stage, but is devoid of organelles or nuclear material. After a short rest in \textit{G}_2 phase, the cell undergoes mitosis in \textit{M} phase, during which homologous chromosomes segregate, the nucleus divides, and cytoplasmic material is divided between the mother and daughter cell, and the daughter cell buds off (Hartwell \textit{et al.} 1970).

Interestingly, the choice of the site of budding is non-random, and distinguishes haploid and diploid cells (reviewed in Casamayor and Snyder 2002). In haploids, bud sites originate from a single pole of the cell, adjacent to previous bud sites in what is called “axial budding”. In contrast, budding alternates between poles in diploid cells in what is termed “bipolar” budding. More specifically, virgin diploid daughter cells bud from the pole opposite to their “birth pole”, and mothers can bud from either pole (reviewed in Sheu \textit{et al.} 2000). It is thought that haploids carry out axial budding so that cells of opposite mating types can remain adjacently placed to mate and form diploids (for example Madhani 2007). Diploidy is the preferred state in wild
yeast apparently because it is more developmentally adaptable to the changing environment by sporulation or pseudohyphal growth (TANNENBAUM 2009). Diploid cells are thought to benefit from bipolar budding because it allows formation of extended branching structures in pseudohyphal states for foraging in the environment (reviewed in MADHANI and FINK 1998; MADHANI 2007).

1.2.2 Vegetative yeast developmental programs: pseudohyphal growth, invasive growth, and stationary phase

Vegetatively growing Saccharomyces cerevisiae can undergo three major developmental programs, each of which is initiated in response to severe limitations in environmental nutrients and involve major transcriptional reprogramming of the genome. One of these developmental programs, termed pseudohyphal growth in diploids and invasive growth in haploids, is initiated when diploid cells are starved for nitrogen or haploid cells are starved for glucose (Figure 3 and Figure 5) (reviewed in MADHANI and FINK 1998; DICKINSON 2008; ZAMAN et al. 2008). When this occurs in certain Saccharomyces cerevisiae lab strains, the cells undergo filamentation and form pseudohyphae in diploids and filaments in haploids. Despite sensitivity to different nutrient limitations, differences between filamentation programs in diploids and haploids appear to be minor (GAGIANO et al. 2002; ZAMAN et al. 2008). In both cases, cells that initiate filamentation become elongated, form long chains of unseparated cells and develop thicker cell walls, which are all properties that enable yeast to expand into the environment in order to forage for nutrients (GANCEDO 2001; PALECEK et al. 2002). These morphological modifications are controlled by activation or deactivation of major nutrient signaling pathways such as the PKA pathway and a MAP kinase pathway which promote major transcriptional changes in genes involved in processes such as cell adhesion and uptake of amino acids (LIU et al. 1993; ROBERTSON and FINK 1998; PAN and HEITMAN 1999; RUPP et al. 1999). While filamentous growth is important for pathogenic organisms such as Candida albicans and Cryptococcus neoformans, many lab strains of Saccharomyces cerevisiae have lost the ability to filament (LO et al. 1997; D’SOUZA and HEITMAN 2001; DICKINSON 2008).

Stationary phase, another major development program of Saccharomyces cerevisiae, commonly occurs in cultures that are incubated in rich glucose media for 5 to 7 days. When yeast cells in culture begin to exhaust their glucose, a developmental program is initiated where the cells
reprogram to: (1) aerobically process available non-fermentable carbon and (2) shut down the activity of the cell once the non-fermentable carbon source has been exhausted (HERMAN 2002; ZAMAN et al. 2008). This shut-down, termed stationary phase, involves exit from the cell cycle at the G₁ phase and entry into a G₀-like quiescent phase that is characterized by the condensation of chromosomes, thickening of cell walls and attenuation of transcription and translation (HERMAN 2002). Much like with filamentous growth, these modifications to the cell structure and activity reflect changes in nutrient signaling pathways such as the PKA and TOR pathways which regulate the transcription of genes involved in processes such as stress response and ribosome biogenesis (BROACH 1991; SCHMELZLE and HALL 2000; GRAY et al. 2004). While stationary phase occurs in most Saccharomyces cerevisiae lab strains, it likely does not represent an evolutionarily significant developmental response because in the absence of genetic engineering, wild yeast (which are predominantly diploid) undergo sporulation in response to starvation (HICKS and HERSKOWITZ 1976). Stationary phase is discussed in more detail in Chapter 4.

Figure 3. The budding yeast life cycle

1.2.3 Sporulation

When a/α diploid cells encounter conditions of nitrogen starvation in the presence of a non-fermentable carbon source, they exit vegetative growth at G₁ and undergo meiosis and spore
formation to produce four haploid gametes called spores (Figure 3). Sporulation is an evolutionary survival mechanism that prepares the cell for dormancy during unfavourable growth conditions for extended periods of time (Gerke et al. 2006) (Figure 3 and Figure 5). When exposed to favourable nutrient conditions, spores undergo germination and return to vegetative growth as haploids (Figure 3) (Savarese 1974; Coluccio et al. 2008). Germinated haploid cells typically mate to reform diploids. Sporulation requires response to environmental cues, successful execution of sporulation programming and finally rendering the genome into a state appropriate for quiescence. Genes involved in sporulation are expressed in groups corresponding to major stages of the program. This is accomplished by transcriptional cascades where expression of later genes is dependent on activation of earlier ones (Chu and Herskowitz 1998; Clancy 1998; Neiman 2005).

Sporulation is initiated by nutrient deprivation-activated transcriptional induction of a gene called IME1, the master regulator of meiosis. Once expressed, Ime1 activates the initial processes of meiosis, namely DNA replication, homologous chromosome pairing and the formation of DNA crossovers (Matsuura et al. 1990; Chu et al. 1998; Sagee et al. 1998). Upon completion of these processes, the cell moves on to nuclear division by expressing a transcription factor called Ndt80, which activates the expression of numerous genes required for meiosis I and II. (Xu and Kleckner 1995; Ozsarac et al. 1997; Chu et al. 1998; Hepworth et al. 1998). Following meiosis, the newly formed haploid meiotic progeny engage in spore differentiation in a two-stage process that culminates in acquisition of stress resistance through the formation of the spore coat. The first step involves recruitment of vesicles to the four spindle pole bodies. Here, the vesicles initiate the formation of the prosopore double membrane, which surrounds each haploid nucleus and becomes the new plasma membrane (Coluccio et al. 2004; Neiman 2005) (Figure 5). During the second stage, the spore coat is formed between the lipid bilayers of the prosopore membrane. Successive layers composed of mannan, glucan, chitosan and dityrosine are laid down in a step-wise manner on the outside of preceding layers (Figure 4 and Figure 5) (Kreger-Van Rij 1978; Pammer et al. 1992; Briza et al. 1994; Coluccio et al. 2004; Neiman 2005). Upon terminal differentiation, spores acquire an impressive resistance to numerous environmental stresses including high osmolarity, desiccation, exposure to noxious substances and temperature extremes.
Figure 4. *The cell wall in yeast cells and spores*

Many standard laboratory strains of *S. cerevisiae* suffer from low sporulation efficiency (defined as the proportion of cells that sporulate in culture) and delayed initiation of sporulation, seemingly due to domestication and a consequential relaxation of selective pressures to maintain robust sporulation capacity (Gerke et al. 2006). I utilized the SK1 strain background for most of my studies. SK1 cells undergo highly efficient and relatively synchronous sporulation (Wan et al. 2006). Note that differences in sporulation efficiency between strains arise from varying propensity to initiate sporulation; once initiated, it is thought that sporulation proceeds with stereotypical kinetics across different strains (Gerke et al. 2006).

<table>
<thead>
<tr>
<th>Cell</th>
<th>Spore</th>
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<tbody>
<tr>
<td>Plasma membrane</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>1-3 β glucan</td>
<td>Mannan</td>
</tr>
<tr>
<td>Mannan</td>
<td>1-3 β glucan</td>
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<tr>
<td></td>
<td>Chitosan</td>
</tr>
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<td></td>
<td>Dityrosine</td>
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Figure 5. *Yeast sporulation*

Top: Stages of sporulation. Bottom: WT spores attain developmentally programmed transcriptional quiescence within a few hours following the completion of meiosis. JHD2 delays both the onset of global transcriptional quiescence, and the terminal differentiation of spores.
1.3 Global Demethylation of Histone H3 lysine-4 Modulates the Gene Expression Program of Yeast Gametogenesis

Here I describe key findings of Jhd2 studies from the Meneghini lab which served as the groundwork for my research. These findings were published together with my thesis work described in Chapter 2 in a paper entitled “Timing of Transcriptional Quiescence During Gametogenesis is Controlled by Global Histone H3K4 Demethylation” in Developmental Cell (Xu et al. 2012). People who contributed to data described in this section were my PhD supervisor Marc Meneghini, fellow graduate students Maria Soloveychik, Mathieu Ranger, and Mike Schertzberg (laboratory technician), Zarna Shah (undergraduate student), Ryan Raisner and Shiv Venkatasubrahmanyan (both from Hiten Madhani lab).

1.3.1 Jhd2 controls H3K4 methylation dynamics during sporulation

While in vitro assays have demonstrated that Jhd2 protein isolated from vegetative cells is enzymatically capable of demethylating H3K4me (Liang et al. 2007), vegetative jhd2Δ cells do not exhibit perturbations in levels of H3K4 mono-, di- or tri-methylation (Xu et al. 2012). Consistent with the absence of a molecular phenotype, vegetative jhd2Δ cells do not exhibit growth or survival defects across an extensive range of growth conditions (Liang et al. 2007; Mersman et al. 2009; Huang et al. 2010; Xu et al. 2012). Therefore, it was a significant advancement when studies in the Meneghini lab revealed that Jhd2 is required for maintaining the balance of H3K4me3, me2, and me1 species during sporulation and in spores (Xu et al. 2012). In sporulating jhd2Δ cells, the abundance of H3K4me3 is significantly increased at the expense of H3K4me2 and me1 species (Xu et al. 2012). This is likely because Jhd2 is normally required to generate H3K4me2 and me1 species from H3K4me3 that is laid down by Set1.

Note that for the rest of this thesis, when I refer to jhd2Δ cells, I am referring to diploid cells that have the genotype jhd2Δ/jhd2Δ, shortened to jhd2Δ for simplicity.

1.3.2 JHD2 sustains the duration of global transcription in opposition to programmed onset of quiescence

During the later stages of sporulation programming, cells shut down cellular activities and become metabolically and transcriptionally quiescent (Hopper et al. 1974; Brengues et al. 2002). The timing of transcriptional shut-down during sporulation as measured by quantitative
western blot for elongation-licensed pol II (CTD heptad repeat Serine 2 phosphorylation) showed dramatically decreased transcription elongation activity commencing between 10 hours to 12 hours into sporulation (Kim et al. 2010; Xu et al. 2012). Meiosis is completed between 8 to 9 hours into sporulation (Figure 5) (Xu et al. 2012), so the time window of productive transcription is sustained for approximately 2 hours past completion of meiosis. In contrast, jhd2Δ exhibits transcription shut-down 2 hours earlier, between 8 hours and 10 hours into sporulation (Xu et al. 2012). This result was corroborated by measurements of transcript abundance by microarray; jhd2Δ began to exhibit reduced mRNA signal in transcripts that were induced in WT starting at 8 to 10 hours into sporulation, and this defect in mRNA accumulation was still observable in terminally differentiated spores at 24 hours into sporulation (schematic shown on the bottom of Figure 5.) (Xu et al. 2012). This supports a model where Jhd2 is required to sustain the duration of global transcription for approximately 2 hours after completion of meiosis. Without this period of transcription activity, the abundance of mRNA in mature spores is reduced.

1.3.3 Jhd2 contributes to gamete fitness under ecologically relevant conditions

Studies from the Meneghini lab have shown that robust sporulation occurs in the presence of environmental carbon concentrations that are greatly reduced from those used in standard laboratory protocols (Eastwood et al. 2012). Under these low carbon conditions, jhd2Δ spores exhibited increased sensitivity to stresses such as heat or to treatment with zymolyase, an enzyme that digests yeast cell walls. Thus, Jhd2 plays a role in the production of robust gametes.

1.3.4 Jhd2 modulates H3K4 methylation at intergenic regions

As I mentioned before, sporulating jhd2Δ cells have increased levels of H3K4me3 and decreased levels of H3K4me2 and me1. In order to investigate how these perturbations are distributed over the genome, the landscape of H3K4 methylation marks was mapped across yeast chromosome III at 10 hours into sporulation in WT and jhd2Δ cells by ChIP tiling microarray (Figure 6).

Interestingly, most of the excess H3K4me3 signal in jhd2Δ accumulated within intergenic regions, and not in coding regions (Figure 6). More intriguingly, despite a decrease in total
H3K4me2 abundance, H3K4me2 signals were increased within intergenic regions in \textit{jhd2Δ}. Since H3K4me3 and me2 marks are laid down during transcription initiation and elongation, respectively, this strongly suggests that \textit{jhd2Δ} causes increased transcription activity in intergenic regions. Another notable feature of the H3K4me landscape in \textit{jhd2Δ} was that both H3K4me3 and me2 signal accumulated in the 5’ NDR (nucleosome depleted region) upstream of genes. 5’ NDRs exhibit low nucleosome occupancy by definition (DION \textit{et al.} 2005; RAISNER \textit{et al.} 2005), so the increase in ChIP signal suggested that Jhd2 could regulate nucleosome occupancy in 5’ NDRs. Studies in yeast have shown that increased intergenic transcription and nucleosome occupancy in promoter regions of genes can negatively influence gene expression (WHITEHOUSE \textit{et al.} 2007; HAINER \textit{et al.} 2010; YADON \textit{et al.} 2010).

![Figure 6. ChIP H3K4me3 and H3K4me2 mapping across Chromosome III](image)

Figure 6. ChIP H3K4me3 and H3K4me2 mapping across Chromosome III

Average profiles of \textbf{A}) H3K4me3 and \textbf{B}) H3K4me2 in WT and \textit{jhd2Δ} (MMY718, MMY1879) cells sporulated for 10 hours were calculated and graphed. Average signal for \textasciitilde{}100 genes on chromosome III are shown centered at the TSS, displaying 500 bp of upstream and 1000 bp downstream signal. Error “clouds” represents one standard error.

The role that nucleosomes and chromatin play in gene regulation is currently an active area of research. Nucleosome architecture at a gene can influence its expression and in some cases, even determine its ON/OFF state (MARTINEZ-CAMPA \textit{et al.} 2004; PINSKAYA \textit{et al.} 2009b); this is especially true for genes that are induced under specific conditions (BASEHOAR \textit{et al.} 2004). Therefore it is important to consider nucleosome occupancy in order to understand how genes are regulated. Nucleosome deposition or repositioning can be accomplished by non-coding
intergenic transcription (THEBAULT et al. 2011). In other cases, non-coding transcription can act through mechanisms that do not involve nucleosomes to influence coding transcription. Here I will describe the current state of knowledge in these topics, with an emphasis on findings in budding yeast.

1.3.4.1 The Nucleosome landscape in budding yeast

In budding yeast, nucleosomes are typically well positioned (or phased), such that the position of many nucleosomes appear to be relatively consistent across a population of cells (YUAN et al. 2005; LEE et al. 2007b). Nucleosome positioning data from a variety of yeast strains show that positioning is fairly conserved across strain backgrounds, different nutrient environments and developmental states (SHIVASWAMY et al. 2008; ZHANG et al. 2011a; Xu et al. 2012). Around 80% of the yeast genome is nucleosome-bound, and the most well-positioned nucleosomes are found near gene promoters (YUAN et al. 2005). Regardless of the expression state of the gene, virtually all gene promoters (~95%) in budding yeast contain a 5’ nucleosome-depleted region (NDR, alternatively called a nucleosome-free-region, or NFR), averaging at around 140 bp in length (Figure 7). Many genes also have a 3’ NDR located near the transcriptional termination site (TTS). The NDRs are flanked by two well-positioned nucleosomes, referred to as the -1 nucleosome and +1 nucleosome (LIU et al. 2005; YUAN et al. 2005; LEE et al. 2007b; MAVRICH et al. 2008a) (Figure 7). The average position of the +1 nucleosome at 5’ NDRs overlaps the transcriptional start site (TSS) of genes in budding yeast. Studies suggest that the precise positioning of the 5’ NDR +1 nucleosome can critically influence gene expression (LIU et al. 2005; MELLOR 2005; HARTLEY and MADHANI 2009). Nucleosomes downstream of the 5’ NDR +1 nucleosome are typically arranged at regular intervals, with their spacing becoming more disordered farther away from the gene promoter.

![Figure 7. Nucleosome architecture of a typical transcription unit](image-url)
Typically, genes that are highly transcribed tend to exhibit more canonical nucleosome architecture, with clearly defined NDRs and well positioned +1 nucleosomes (Tirosh and Barkai 2008). Also, perhaps counter-intuitively, more highly transcribed regions contain high-occupancy and well positioned nucleosomes. For example, ribosomal protein genes, which account for 50% of all RNA Pol II transcription, contain very well positioned nucleosomes (Basehoar et al. 2004; Shivaswamy et al. 2008). Current models posit that RNA pol II deposits nucleosome during transcription by associating with elongation factors that replace nucleosomes that are displaced by the Pol II complex behind the advancing transcription machinery (Dion et al. 2007). There is also evidence that RNA polymerase can position nucleosomes based on the fact that nucleosome spacing is compromised in certain RNA polymerase II mutants (Zhang et al. 2009b). Regulated genes in yeast, i.e. genes that are activated under specific conditions, tend to have more non-canonical structures, often with no clearly positioned +1 nucleosome or NDR upstream of the TSS. Yeast TATA-box containing genes are enriched for this type of promoter architecture; they are highly regulated, and are typically more reliant on chromatin remodeling complexes for expression (Basehoar et al. 2004).

1.3.4.2 Nucleosome positioning in higher eukaryotes

It appears that the nucleosome landscape in higher eukaryotes is much less phased and less consistent across cell types compared to budding yeast. Nucleosome mapping studies in mouse liver cells as well as human T cells and HeLa cells show that most nucleosomes are generally not well-positioned, with only 20% of nucleosomes showing distinctly definable occupancy in one study (Jin et al. 2009; Li et al. 2011; Valouev et al. 2011). Similar to observations in yeast cells, coding regions tend to show more nucleosome occupancy compared to non-coding or silenced regions (Valouev et al. 2011). The nucleosome spacing (i.e. the distance between positioned nucleosomes measured in basepairs) in mammalian cells is highly variable and depends on the cell type and extent of differentiation. The fraction of the genome that is associated with nucleosomes ranges from 60% in hepatocytes to 99% reported in granulocytes (Celona et al. 2011; Li et al. 2011; Valouev et al. 2011). When mammalian genes become activated, they acquire characteristics that are analogous to those observed in yeast genes; active genes acquire an NDR upstream of the TSS, and a +1 nucleosome becomes positioned with
adjacent phased nucleosomes that become more disordered further away from the NDR (Ozsolak et al. 2007; Schones et al. 2008; Valouev et al. 2011). The level of nucleosome phasing around the promoter correlates with the level of transcription from the gene locus (Ozsolak et al. 2007; Valouev et al. 2011). This arrangement of nucleosomes around the average active gene is conserved in diverse eukaryotes, including C. elegans, D. melanogaster, A. thaliana and mammals (Bannister et al. 2001; Lachner et al. 2001; Chodavarapu et al. 2010).

1.3.4.3 Cis and trans-acting determinants that specify chromatin architecture in yeast

How are the positions of nucleosomes specified across the genome? Kornberg and colleagues proposed in 1981 that certain fixed nucleosomes are specified by DNA sequences that favour DNA-histone interactions, and these serve as barriers against which other nucleosomes line up; this is known as the ‘statistical stacking’ model (Kornberg 1981). This model is consistent with observations of nucleosomes displaying increasingly random positioning farther away from the anchoring nucleosomes found at the +1 position of genes. Consistent with this model, it has been known for a long time that certain DNA sequences confer structural properties that either promote or impede wrapping around a nucleosome. AA/TT dinucleotides distributed at 10 bp intervals confer bendability to DNA that favours nucleosome association (Simpson and Stafford 1983; Drew and Travers 1985). Such sequences are most enriched at -1 and +1 nucleosome positions near gene promoters (Ioshikhes et al. 2006). Conversely, poly-dA:dT tracts, which are rigid, specify NDRs and may promote transcription by allowing access by transcription factors (Iyer and Struhl 1995).

A seminal study from 2009 comprehensively addressed the contribution of DNA sequence to nucleosome positioning (Kaplan et al. 2009). In this study, purified yeast DNA was reconstituted with nucleosomes in vitro and their positions were determined using high-resolution microarray methods. The in vitro DNA-histone interaction-mediated nucleosome positioning was similar to chromatin structure observed in vivo, suggesting that DNA sequence is a major factor in determining nucleosome occupancy. The major differences between in vivo and in vitro nucleosome positioning were found at 5′ NDRs and at regions proximal to certain transcription factor binding sites (e.g. Reb1 and Abf1-bound regions). These regions exhibit
greater levels of nucleosome depletion in vivo, indicating nucleosome exclusion is actively maintained at these regions in live cells (KAPLAN et al. 2009). A shortcoming of the statistical stacking model is the fact that in vitro nucleosomes do not exhibit decreasing phasing away from anchoring nucleosomes or the level of regular spacing observed in vivo (ZHANG et al. 2011b). Instead, in vitro reconstituted chromatin acquires phasing and architecture that resembles statistical stacking when chromatin factors are provided (as whole cell lysate) along with ATP (ZHANG et al. 2011b). Therefore, while in vivo nucleosome positioning resembles statistical stacking, it is evident that in addition to intrinsic DNA sequence, other factors such as histone chaperones, chromatin remodelers, histone modifying enzymes and transcription machinery all contribute to the observed chromatin architecture.

Nucleosome remodeling factors such as ATPase-dependent complexes can move nucleosomes along DNA. Such remodelers include the evolutionarily conserved protein complexes SWI/SNF, RSC, ISW1, ISW2, CHD1 and INO80 (CLAPIER and CAIRNS 2009). Histone chaperones and histone assembly factors (FACT, CAF1, NRD, Rtt106) regulate nucleosome assembly and maintenance (LENSTRA et al. 2011). The abundance and variety of chromatin regulating factors may reflect the profound impact DNA packaging exerts on transcription and other aspects of DNA biology (NARLIKAR et al. 2002; CAMPOS and REINBERG 2009).

1.3.4.4 The role of chromatin in transcription

The primary roles of chromatin are to compact, organize and stabilize vast lengths of DNA into the nucleus while still allowing efficient access for biological processes such as replication and transcription (KORNBERG and KLUG 1981). In regards to gene expression, nucleosomes are generally viewed as impediments to transcription due to their ability to occlude access to DNA in vitro. However, the in vivo consequences of nucleosome binding to DNA for transcription may be more complex (WYRICK et al. 1999; Li et al. 2011). When the abundance of nucleosomes is depleted (up to 75%) in yeast cells using genetic manipulations (WYRICK et al. 1999; CELONA et al. 2011), expression of only 10-25% of genes is affected. Most of these involve de-repression of telomere-proximal genes, but surprisingly, a significant number of genes are down-regulated. While these gene repression events may be indirect, these findings suggest that the role of the nucleosome with respect to transcription is complex, gene-specific and not necessarily repressive in general (WYRICK et al. 1999). In support of this view, we know
that many transcription factors in yeast and mammals have no difficulty accessing DNA, as they can bind to DNA that is occupied by a nucleosome or displace a nucleosome to bind DNA. This class of effectors, which includes Reb1, Rsc3 and Mcm1 in yeast (WANG et al. 2011), and FoxA, GATA, PU.1 and TF33 in mammals, are known as pioneering transcription factors because they can initiate binding and facilitate binding by other factors (IYER 2012). Alternatively, nucleosomes may promote gene expression by preventing repressor protein binding (CHAROENSAWAN et al. 2012).

1.3.5 Non-coding transcription

With the advent of genome-wide sequencing technologies within the last decade, it has become clear that a large proportion of RNA pol. II transcription produces non-coding RNAs in organisms ranging from budding yeast to humans (XU et al. 2009; DJEBALI et al. 2012; HANGAUER et al. 2013). This is especially evident in the human genome, as exons account for less than 3% of the genome, but recent whole genome sequencing efforts by the ENCODE project found that 83.7% of the non-repetitive genome produces long intergenic non-coding RNAs (lincRNAs) (DJEBALI et al. 2012). It appears that these lincRNAs play important biological roles because disease association studies find that half of known disease-associated SNPs (single nucleotide polymorphisms) are intergenic (HINDORFF et al. 2009). While some of these regions act from the level of DNA, at least a significant proportion are due to production of lincRNAs, since disease-associated SNPs are five times more likely to occur in regions encoding known lincRNAs compared to other intergenic regions (HANGAUER et al. 2013).

Similarly, even in the yeast genome, which is very compact and the average distance between genes is only a few hundred base-pairs, non-coding RNAs (ncRNAs) are pervasive (XU et al. 2009; LARDENOIS et al. 2011). In the last few years, there has been significant effort from several groups to systematically characterize non-coding transcripts in yeast. This resulted in identification of SUTs (stable untranslated transcripts), CUTs (cryptic/unstable UTs), MUTs (meiosis-specific UTs), and XUTs (Xrn1-sensitive unstable transcripts) (NEIL et al. 2009; XU et al. 2009; YADON et al. 2010; LARDENOIS et al. 2011; VAN DIJK et al. 2011). So far, around 2500 SUTs and CUTs, 700 MUTs and almost 1000 XUTs have been annotated. ncRNAs in yeast can overlap both intergenic regions and coding regions in sense and antisense orientations.
There have been numerous studies that describe non-coding transcription regulating the expression of nearby or overlapping coding genes in yeast (Whitehouse et al. 2007; Neil et al. 2009; Xu et al. 2009; Yadon et al. 2010; Xu et al. 2011). In most cases, non-coding transcription acts antagonistically on coding transcription. At certain loci, repressive nucleosomes are deposited co-transcriptionally over NDRs of coding genes; which has been described at SER3, ADH1, and ADH3 (Martens et al. 2004; Hainer et al. 2010; Pruneski and Martens 2011; Thebault et al. 2011). Non-coding transcriptional events can also recruit or abolish chromatin modifiers to repress transcription at nearby sites (e.g. at PHO84, GAL1-10 and IME1) (Camblong et al. 2007; Houseley et al. 2008; Camblong et al. 2009; Kim and Buratowski 2009; van Werven et al. 2012). The process of non-coding transcription alone can also interfere with expression of an adjacent gene (e.g. at FLO11) (Bumgarner et al. 2009).

Nucleosome positioning is involved in many of the known examples of non-coding transcription-mediated gene regulation. A large proportion of ncRNA transcripts originate from NDRs of coding genes and many overlap coding regions (Lardenois et al. 2011). It is becoming clear that in addition to being able to regulate protein coding genes via the NDR, nucleosome architecture also controls cryptic/non-coding transcription. In some cases, nucleosome positioning around a promoter can promote coding transcription whilst repressing non-coding transcription, or vice versa, e.g. at the SER3, ADH1/ADH3 and IME1 loci mentioned above. The expression outcome of competing coding and non-coding transcription represents another layer of gene expression control (Martens et al. 2004; Hongay et al. 2006; Camblong et al. 2007; Whitehouse et al. 2007; Xu et al. 2009; Yadon et al. 2010; Pruneski and Martens 2011; van Werven et al. 2012).

Nucleosome remodeling describes a variety of structural changes that can occur between histones and DNA. Some of these include sliding nucleosomes along DNA, disruption or strengthening DNA-histone interactions or changes in DNA superhelical torsional strain (Bruno et al. 2003; Kassabov et al. 2003). So how is nucleosome remodeling accomplished? Many ATPase-dependent chromatin factors can reposition nucleosomes in yeast; among these, Ino80, Isw1, Isw2, and Chd1 complexes are known to slide +1 nucleosomes upstream into the NDR (Whitehouse et al. 2007; Quan and Hartzog 2010; van Bakel et al. 2013). Studies
have found that inactivation of these factors causes nucleosome remodeling and correspondingly increased non-coding transcription from cryptic sites (WHITEHOUSE et al. 2007; YADON et al. 2010; VAN BAKEL et al. 2013).

Next, I will introduce the subject of mitochondrial respiration. In studies related to Jhd2, I uncovered a physiological phenotype associated with \( jhd2\Delta \) by performing gene enrichment analyses on microarray data sets. I found that genes which are involved in mitochondrial function are particularly affected by Jhd2 function. Upon further investigation, I found that \( jhd2\Delta \) cells are defective in mitochondrial respiration during sporulation and germination.
1.3.6 Mitochondria and oxidative phosphorylation

Mitochondria are evolved from prokaryotic endosymbionts of ancient eukaryotic cells over two billion years ago (SAGAN 1967). Over the course of evolution, the mitochondrial genome has undergone reductive selection, accompanied by an almost total transfer of its genes into the host nuclear genome. This process occurred independently in various lineages, so that the subset of genes remaining in contemporary mitochondria genomes differs slightly across species. In budding yeast, the mitochondrial genome is around 86Kb long, and contains 17 genes encoding components of the electron transport chain, the full complement of 24 mitochondrial tRNA genes, and the 37S mitochondrial ribosomal protein gene (SGD as of June 2013). The human mitochondrial genome contains largely the same complement of genes (13 genes, 22 tRNAs, 2 rRNAs), in a much more compact genome of 16.6 Kb due to the absence of most intergenic sequences and introns (BARRELL et al. 1979).

There are around 800 genes annotated as encoding mitochondrial proteins in the yeast nuclear genome (SGD). These genes are involved in energy maintenance functions such as oxidative phosphorylation (OXPHOS), the electron transport chain (ETC), various carbohydrate and fatty acid metabolic pathways, as well as mitochondrial genome maintenance, iron-sulfur cluster production, autophagy and apoptosis.

Oxidative phosphorylation is a complex process that requires coordinated regulation of both the mitochondrial and nuclear transcription and genomes. Furthermore, the mitochondrial translation machinery components are themselves encoded in the nuclear genome. Perhaps surprisingly, nuclear mitochondrial genes do not have any apparent shared transcriptional regulatory elements (LAI et al. 2006); instead, it is thought that mitochondrial gene expression is regulated post-transcriptionally. Most mitochondrial transcripts are transported and tethered to the outside of mitochondria, where they are stabilized, translated and the proteins are transported into mitochondria. The mechanisms and regulation of this process are not well understood (KELLEMS et al. 1975; SAINT-GEORGES et al. 2008). A large proportion of mitochondrial transcripts have long 3’ UTRs that contain recognition motifs for the RNA-binding Puf3 protein (SAINT-GEORGES et al. 2008; DEVAUX et al. 2010). The role of Puf3 in regulating mitochondrial biology is complex. It promotes mitochondrial function by binding and
delivering transcripts to mitochondria, but also targets them for rapid turn-over (Chatenay-Lapointe and Shadel 2011).

1.3.6.1 Petite mutants

Yeast have an unusual ability to retain viability when the mitochondrial genome is damaged (rho⁻) or completely absent (rho⁰) (Ephrussi and Slonimski 1955; Faye et al. 1973). These mutant strains are called petites because they grow slowly and form small colonies on fermentable carbon sources. Since they lack genes that encode components of the electron transport chain, petite cells cannot perform oxidative phosphorylation, and thus cannot grow on non-fermentable carbon sources. Mitochondria of petite cells retain the ability to perform other essential functions, such as iron-sulfur cluster synthesis.

1.3.6.2 The mitochondrial electron transport chain

Oxidative phosphorylation, or OXPHOS, is the process during which energy from NADH and FADH₂ (produced during glycolysis, TCA cycle or fatty acid oxidation) are transferred to ATP through a series of redox reactions in the mitochondria, with O₂ acting as the final electron acceptor (Stryer 1988). OXPHOS takes place at the electron transport chain of the inner mitochondrial membrane, which is comprised of five multi-subunit complexes (Figure 8). The energy generated from the flow of high-energy electrons from NADH and FADH₂ through the components of the ETC is used to pump H⁺ ions into the mitochondrial intermembrane space, creating membrane potential. Complex I (NADH dehydrogenase) accepts electrons from NADH, and Complex II (Succinate dehydrogenase) accepts electrons from FADH₂. Budding yeast, along with some other lineages of fungi, have lost Complex I function. Instead, reducing potential from NADH is transferred to the ETC via three alternate proteins Nde1, Nde2 and Nde1 (Fang and Beattie 2003). Unlike Complex I, these proteins do not pump protons across the inner mitochondrial membrane (Rea et al. 2010). Electrons from both Complex I and II pass through Ubiquinone (Q), then Complex III (Cytochrome bc₁), Cytochrome C, and Complex IV (Cytochrome C oxidase). At Complex IV, four electrons are passed onto the final electron acceptor, O₂, to produce two molecules of H₂O. ATP synthase (or F0F1-ATPase, Complex V) harnesses the release of energy from H⁺ flowing back into the mitochondrial matrix to convert ADP to ATP (Figure 8). Oxidative phosphorylation generates 32 of the 36 ATP molecules
available from one molecule of glucose that is completely oxidized to CO\textsubscript{2} and H\textsubscript{2}O. Since O\textsubscript{2} is converted to H\textsubscript{2}O in the last step of oxidative phosphorylation, the rate of oxygen consumed by a culture of cells can be used as a proxy for the rate of respiration in a cell.

Figure 8. The mitochondrial electron transport chain. (Figure adapted from Ow et al. 2008)
IMM (inner mitochondrial membrane), I (Complex I, NADH dehydrogenase), II (Complex II, Succinate dehydrogenase), Q (uniquinone), III (Complex III, Cytochrome bc\textsubscript{1}), C (cytochrome C), IV (Complex IV, Cytochrome C oxidase), V (Complex V, F\textsubscript{0}F\textsubscript{1}-ATPase / ATP synthase).

1.3.7 Rationale of Thesis Research
Experiments to characterize Jhd2-mediated regulation of gene expression through regulation of nucleosome occupancy and intergenic transcription constitute a large portion of my thesis research. In Chapter 2, I describe studies to map nucleosome occupancy and also the non-coding and coding transcriptomes in WT and jhd2\textDelta cells. In Chapter 3, I describe analyses utilizing genome-wide positioning data combined with RNA microarray data to investigate the role that transcription plays in determining 3’ NDR structure. In Chapter 4, I describe analysis of RNA abundance data from sporulation time courses that revealed a role for Jhd2 in promoting expression of genes that are involved in mitochondrial function. This insight led to studies concerning mitochondrial respiration during sporulation and germination.
Chapter 2

Jhd2 represses intergenic non-coding transcription and modulates nucleosome occupancy dynamics during sporulation

This work is published in


Maria Soloveychik performed RNA tiling microarrays for cultures at 20 hours of sporulation.

Harm van Bakel performed normalization and data smoothing for the nucleosome occupancy and RNA abundance microarrays.

Marc Meneghini performed the H3K4me2 and me3 ChIP microarrays.

All other data collection and data analysis was performed by me.
2.1 Abstract

Studies from the Meneghini lab confirmed that gamete differentiation (sporulation) in budding yeast is accompanied by metabolic shut-off following completion of meiosis; Jhd2 promotes protein coding gene expression in opposition to this programmed shut-off, sustaining the period of productive transcription during spore differentiation (Xu et al. 2012). In order to investigate how Jhd2 accomplishes this task, a survey of the H3K4me landscape in jhd2Δ was performed, which suggested that Jhd2 could modulate non-coding transcription and/or nucleosome occupancy. Using genome-wide and locus-specific approaches, I found that this is indeed the case. My findings are consistent with a model whereby Jhd2 promotes productive transcription by repressing interfering non-coding transcription. Furthermore, Jhd2 opposes a program of globally increased nucleosome occupancy in 5’ and 3’ NDRs during sporulation. While numerous gene-specific examples of such regulation have been described, my findings show an instance where a single chromatin factor modulates non-coding and coding transcription at a large number of loci to regulate a developmental program.

2.2 Introduction

2.2.1 Nucleosome positioning studies

The earliest global nucleosome mapping efforts were carried out in Saccharomyces cerevisiae starting in 2004 (Bernstein et al. 2004; Yuan et al. 2005). These studies illuminated some fundamental properties of nucleosome organization. The nucleosome landscapes of higher eukaryotes have proven to be more challenging to map due to their much larger genomes, but recent advances in deep sequencing technology have enabled robust genome-wide nucleosome mapping in Drosophila melanogaster, Caenorhabditis elegans, Arabidopsis thaliana, and various human cell types (Mavrich et al. 2008b; Kaplan et al. 2009; Chodavarapu et al. 2010; Valouev et al. 2011). However, the S. cerevisiae nucleosome landscape remains the best studied to date.

Genome-wide analysis of nucleosome positions typically involves micrococcal nuclease I (MNase I) digestion to eliminate linker DNA regions that are not bound by nucleosomes, followed by size selection for mono-nucleosomal fragments, and microarray hybridization or deep sequencing to identify the relative abundance of nucleosome-protected DNA (Iyer 2012).
Some studies also incorporate an immuno-precipitation (IP) step to selectively purify histone-associated DNA. The earliest nucleosome mapping studies in yeast were carried out using microarrays that “tiled” Chromosome III (the shortest chromosome in budding yeast) at 20 bp resolution. This was followed by high resolution genome-wide studies using Affymetrix tiling arrays (at 4 bp resolution). Currently, studies utilizing next generation sequencing (NGS) technologies can generate hundreds of millions of sequencing reads and yield single base pair resolution occupancy data (Tolstorukov et al. 2010; Zhang et al. 2011a).

Nucleosomes are described by several important properties which I will define here: ‘occupancy’ describes a dynamic property of the degree to which a nucleosome occupies a genome position in a population of cells; ‘positioning’ refers to the preferential location of a nucleosome relative to a DNA coordinate in a population of cells. ‘Phasing’ is similar to ‘positioning’, and was first used to describe fragment “laddering” observed in DNase I digestion of eukaryotic chromatin which indicated the presence of regularly spaced nucleosomes (Lohr et al. 1977). Nucleosomes across the genome fall on a spectrum from those that are very well positioned to those that are delocalized (fuzzy); “fragile” nucleosomes that are loosely associated with DNA are hence less capable of protecting the bound DNA from MNase digestion (Raisner et al. 2005; Yuan et al. 2005; Lee et al. 2007b; Xi et al. 2011).

2.3 Materials and Methods

2.3.1 Yeast strains

Standard yeast genetic methods were used for construction of all strains (Xu et al. 2012).

Unless otherwise stated, “WT” and “jhd2Δ” cells refer to diploid strains MMY718 and MMY1879, respectively.

Table 1. Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Background</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMY718</td>
<td>SK1</td>
<td>MAT$a$/a his3/his3 leu2/leu2 ura3/ura3 trp1/trp1</td>
</tr>
<tr>
<td>MMY1879</td>
<td>SK1</td>
<td>MAT$a$/a his3/his3 leu2/leu2 ura3/ura3 trp1/trp1 jhd2Δ::NAT/jhd2Δ::NAT</td>
</tr>
</tbody>
</table>
2.3.2  Sporulation protocol

Strains were patched onto YPAGE plates (1% yeast extract, 2% bacto peptone, 0.3 mM adenine, 2% glycerol, 2% ethanol) from -80°C freezer glycerol stocks and grown overnight. Cells were then streaked out from YPAGE plates for single colonies on YPD plates (1% yeast extract, 2% bacto peptone, 0.3 mM adenine, 2% glucose). Single colonies from the YPD plates were grown overnight to saturation in liquid YPD media and then diluted into YP-Acetate (YPA, 1% yeast extract, 2% bacto peptone, 1% potassium acetate) at an O.D. \(_{600}\) of 0.3. Cultures were grown for 12-14 hours at 30°C with shaking at 200 (revolutions per minute) RPM to an O.D. of ~1.5. Cells were pelleted, washed with double distilled water (ddH\(_2\)O) and resuspended into SPO media (1% potassium acetate, 0.02% raffinose) and sporulated at 30°C with shaking at 200 RPM. Meiotic progression was quantified microscopically using 4′,6-amidino-2-phenylindole (DAPI) staining of formaldehyde-fixed cells.

When referring to sporulation time points, I use the label ‘Vegetative’ or ‘Veg’ or ‘YPD’ to refer to cells growing in YPD media. Sporulation T=0 refer to cells that have been grown in YPA for 12-14 hours, washed, resuspended in SPO medium, and harvested immediately after resuspension in SPO.

2.3.3  Histone H3K4me ChIP-chip on Chromosome III

H3K4me ChIP-chip experiments and data normalization were performed by my PhD supervisor Marc Meneghini while he was a post-doctoral fellow in Dr. Hiten Madhani’s lab. I performed additional data analyses, including aligning gene signals at the TSS and TTS, as well as statistical analyses.

MMY718 (wild type) and MMY1879 (jhd2Δ) were sporulated as described above. At the 10 hour time point, 100 O.D. of cells were harvested and pelleted at 3,000g for 10 min at 4°C. Cells were resuspended in 3 mL buffer Z (1 M sorbitol, 50 mM Tris-Cl pH 7.4, 10 mM β-mercapto ethanol) and treated with 500 mg of zymolyase for 4 hours at 30°C on a platform shaker. Completeness of spheroplasting was assessed by visual inspection of cells using phase contrast microscopy. Spheroplasts were pelleted and resuspended in 500 µL buffer NPS (0.075% NP-40, 50 mM NaCl, 10 mM Tris-Cl pH 7.4, 5 mM MgCl\(_2\), 1 mM CaCl\(_2\)) and treated with MNase (Worthington) for 20 minutes at 37°C to obtain > 90% mononucleosomes. MNase
digestion was confirmed by running samples on a 1% ethidium bromide agarose gel. Nucleosomes were immunoprecipitated using 2 µL of αH3K4me3 antibody (Abcam, ab8580) and 6 µL of αH3K4me2 antibody (Abcam, ab7766), respectively. Chromatin IP and array hybridizations were performed as described previously using tiling microarrays that span chromosome III (Bernstein et al. 2002; Yuan et al. 2005; Hartley and Madhani 2009). Dye flipping was incorporated into the experimental design to minimize any systematic bias arising from choice of fluorescent dyes. For each array, the ratios of DNA associated with H3K4me3/2 histones to total mononucleosomal DNA were log2-transformed, mean-centered and scaled such that mean and standard deviation of the normalized ratios were 0 and 1 respectively. Quadruplicate data were then averaged on a probe-wise basis.

To reveal the global average profiles of H3K4me3 and me2 deposition around gene promoters and coding regions, I produced an aggregate data profile that represented all of the genes in each sample. To do this, for each gene, the values for probes representing the 1.5 Kb region between -500 and +1000 base pairs with respect to their transcription start sites (TSS) were extracted. Signals for all genes were aligned based on the positions of the TSS in wild-type cells. TSS positions were based on published data (Xu et al. 2009). I then calculated the mean signal profile for the 119 genes in 20 bp bins (Figure 6 and Figure 9). This was done for both WT and jhd2Δ cells for H3K4me3 ChIP and H3K4me2 ChIP data. To interrogate the 3’ ends of genes, the same aggregate data profile method was used, except 500 bp of signal were aligned by the annotated transcriptional termination site (TTS) (Figure 9 and Figure 10). Standard error was calculated and shown as an error cloud around the aggregate data profiles.

2.3.4 Global nucleosome positioning using high resolution Affymetrix tiling array

Vegetative cells were cultured using standard laboratory techniques in YPD media. WT and jhd2Δ cell cultures were sporulated in pairs under identical conditions using standard laboratory techniques. Sporulation synchrony during meiosis was verified by DAPI staining nuclei (0 to 9 hours into sporulation). Beyond 9 hours into sporulation, sporulation progression was monitored by appearance of the spore wall under brightfield microscopy. Crosslinked cell pellets from YPD grown cells and sporulation time points corresponding to 0, 4, 6, 8, 10, 12, and 16 hours were lysed by sonication using a Diagenode Bioruptor followed by bead beating for 7 cycles of
1 min with 2 min rests on ice. Mononucleosomal DNA was prepared from these extracts and processed for Affymetrix microarray hybridization as described (Lee et al. 2007b), with the following modifications adapted for processing spores. At each time point, 400 O.D.\textsubscript{600} equivalents of cells (200 mL of 2.0 O.D.\textsubscript{600} culture) were crosslinked at room temperature using methanol-free formaldehyde at a final concentration of 1%. The crosslinking reaction was quenched by addition of glycine to a final concentration of 0.125 M and incubating for 5 min at room temperature. Crosslinked cells were washed twice in ice-cold PBS and snap frozen in liquid nitrogen as cell pellets. Crosslinked cell pellets were resuspended in 1 mL FA lysis buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% NaDOC, Protease inhibitor cocktail (Sigma P8215, 96 µL/ 25 mL buffer)), and lysed by sonication using a Diagenode Bioruptor for 30 s on high power, 30 s resting, for a total of 30 min. followed by addition of 1mL of glass beads and bead beating for 7 cycles of 1 min with 2 min rests on ice. The chromatin fraction was pelleted by centrifugation at 8000 rpm for 10 min at 4°C. The pellet was washed and resuspended in 15 mL NPS buffer (0.5 mM spermidine, 0.075% IGEPAL, 50 mM NaCl, 10 mM Tris-Cl pH 7.5, 5 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 1 mM β-mercaptoethanol, Protease inhibitor cocktail). This suspension was split into 7.5 mL portions, and digested with 80 units of MNase (Worthington) at 37°C for two different time periods ranging from 15 to 18.5 min. Following reversal of crosslinks (65°C in 10 mM EDTA, 0.1% SDS, 10 mM Tris-Cl, Proteinase K 0.7 µg / mL, overnight) and phenol:chloroform extraction followed by ethanol precipitation, the nucleosomal DNA was run on a 2% / 0.8% agarose gel, and the mono-nucleosomal gel band was excised and purified using a QiaQuick gel extraction kit. More specifically for agarose gel electrophoresis, the upper 2% portion of the 2% / 0.8% agarose gel was used to resolve mono-, di-, tri- nucleosomal bands. The mono-nucleosomal band was then allowed to run into the 0.8% agarose portion of the gel for more efficient gel excision and extraction. Mono-nucleosomal DNA was DNase I-digested, biotin-labelled and hybridized onto high-resolution Affymetrix microarrays as described (Lee et al. 2007b). Custom Affymetrix Array probes were tiled at 8 bp resolution with 25 bp long probes in both forward and reverse strand orientation, with a 4 bp off-set in the reverse strand, giving 4 bp resolution data. Data normalization was performed by Harm van Bakel as described (Lee et al. 2007b). Raw data was cross array quantile normalized to equalize variation across Affymetrix microarray chips, then normalized to WT genomic DNA hybridization signal to yield a ratio that was finally log\textsubscript{2}
transformed. All subsequent data analysis was performed by me. The raw data, as well as normalized TSS/TTS centered nucleosome occupancy data have been uploaded to NCBI Gene expression omnibus under the accession number GSE40874. They are also linked to this thesis as electronic files (APPENDIX A).

2.3.5 Global transcription analysis using high resolution Affymetrix tiling array

Yeast culturing, RNA extraction and processing, and Affymetrix hybridizations were performed by Maria Soloveychik and myself. Data normalization was done by Harm van Bakel, and subsequent data analysis was done by me. MMY718 and MMY1879 were sporulated and aliquots of culture were harvested at 20 hours into sporulation and flash-frozen in liquid nitrogen. RNA was prepared by bead beating cell pellets (Mini Bead beater, Biospec Products) for 4 x 2.5 minutes in Trizol (Invitrogen) with 5 min incubations on ice between cycles. After addition of chloroform and phase separation, RNA was further purified by extraction with acidic phenol at 65°C for 30 min. The complete Affymetrix transcript microarray data set is linked to this thesis as an electronic file (APPENDIX B). It can also be downloaded at NCBI Gene expression omnibus under the accession number GSE41002.

2.3.6 Reverse Transcription Quantitative real time PCR (RT-qPCR)

RNA was extracted from MMY718 and MMY1879 cells using the Trizol bead beating method described in section 2.3.5. The resuspended RNA samples were then treated with DNase I and further purified using RNeasy columns (Qiagen). Five µg of total RNA was reverse-transcribed using either random nonamers or site-specific primers and Superscript III RT (Invitrogen) according to the manufacturer’s instructions. 1/200th of the resulting cDNA was used per reaction for qPCR using 2X SYBR Green/PCR buffer (APARICIO et al. 2005) with the MyIQ thermocycler (BioRad). Reactions were quantified relative to a common genomic DNA standard and transcript abundances normalized to a RT qPCR of RDN25-I rRNA transcript.
Table 2. RT-qPCR Primers

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIT1</td>
<td>-446 F/-363 R</td>
<td>ATTTAAGGGGCAAGTAAGCAT/GATCAATTCTCTGAGATTTGTT</td>
</tr>
<tr>
<td></td>
<td>chip2_F/chip2_R</td>
<td>GACATTCTAGCAACTTTGC/TAGTGTGGATAAAGTGAGC</td>
</tr>
<tr>
<td>RPS6A</td>
<td>RT primer -25 R</td>
<td>TTGTCACTTCTCTGAAGC</td>
</tr>
<tr>
<td></td>
<td>qPCR primers -222_F/-56_R</td>
<td>GAAAGTTATCTCTCTCGTAAGCG/CAATGACGATAAGCATTAACCAC</td>
</tr>
<tr>
<td></td>
<td>RT primer 601_R</td>
<td>GAGCTTGAGCGTTTCTGACC</td>
</tr>
<tr>
<td></td>
<td>qPCR primers 30_F/209_R</td>
<td>CGGGTTCTCAAAGGACT/CGGAACAAAAACACCTCTGCTTC</td>
</tr>
<tr>
<td>RPL10</td>
<td>RT primer/qPCR primer -166to-186R</td>
<td>GTCACATATTATGTGCTAAC</td>
</tr>
<tr>
<td></td>
<td>qPCR primers -304to-284R</td>
<td>TATAGTAGCGGTTATTTCCG</td>
</tr>
<tr>
<td></td>
<td>RT primer 642R</td>
<td>TCCAAAGAACCCTTTGAGGACA</td>
</tr>
<tr>
<td></td>
<td>qPCR primers 116-135F/321-302R</td>
<td>AGAAGGCTACCAGTGATGAA/ACCGGCACAAGACACATC</td>
</tr>
<tr>
<td>RPS1B</td>
<td>RT primer -190to-210R</td>
<td>TCACACCGCCACCACCTGATT</td>
</tr>
<tr>
<td></td>
<td>qPCR primers -300to-280F/-220to-240R</td>
<td>AAGAGCAGCAGGGATCGTCCG/ACACCTGTGGATCCACACCTGGA</td>
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<tr>
<td>RPL11B</td>
<td>RT primer 76to-97R</td>
<td>CCTCTCTTAACAGTATACCT</td>
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<td></td>
<td>qPCR primers -169to-149F/-96to-116R</td>
<td>TCTTTTGGAAACCCTCTGTC/CTTTAATGATAGTTATGTCC</td>
</tr>
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2.4 Results

2.4.1 JHD2 represses the accumulation of H3K4me2/3 at intergenic regions near the 5' and 3' transcription boundaries of protein coding genes

As discussed in Chapter 1, the H3K4me3/2 ChIP-chip data collected by my supervisor Marc Meneghini revealed that H3K4me3 signals across coding regions did not differ significantly between WT and jhd2Δ cells during sporulation (Figure 6A and Figure 9A). Instead, there was increased accumulation of both H3K4me3 and me2 signal upstream of coding regions in jhd2Δ cells (Figure 6, left panels and Figure 9). The location of these signals away from coding regions, upstream of where CDS-associated 5' H3K4me3 signal is typically found, suggested that they may be deposited by intergenic non-coding transcription. To more comprehensively determine whether jhd2Δ caused increased H3K4me accumulation at intergenic regions, I interrogated the 3' ends of genes by re-analyzing the H3K4me ChIP-chip data and centering
signals at the transcriptional termination site (TTS) (Figure 9, right panels). Strikingly, I found that jhd2Δ cells exhibited a pronounced accumulation of H3K4me3 and me2 signal at intergenic regions directly downstream of protein coding genes. These peaks overlapped with the regions that are typically associated with the 3’ NDR. Since the deposition of H3K4me peaks is mechanistically coupled with transcription (Santos-Rosa et al. 2002), the presence of H3K4me3 and me2 signal peaks in 3’ NDRs in jhd2Δ cells suggested the presence of non-coding transcription initiation and elongation in these regions. To rule out the possibility that this TTS-proximal H3K4me signal in jhd2Δ cells was due to overlap with TSSs of downstream tandemly transcribed coding genes, I confirmed that H3K4me2/3 also accumulated at TTSs of convergently oriented genes in jhd2Δ cells (Figure 10). At convergent genes, the 3’ region does not border any 5’ transcription initiation sites of coding genes, so that the presence of any peaks of H3K4me3/2 signal is most consistent with non-coding transcription-coupled H3K4me deposition.
**Figure 9.** *jhd2Δ* cells accumulate H3K4me signals in 5’ and 3’ NDRs that are not associated with coding genes.

Chromatin immunoprecipitation followed by high-resolution microarray hybridization (ChIP chip) was performed using chromatin prepared from WT and *jhd2Δ* cells at the 10 hour sporulation time point. Shown are average gene profiles of H3K4me3 **A)** and H3K4me2 **B)** ChIP signal of 119 genes on Ch III in WT and *jhd2Δ* cells. Average profiles were centered at the TSSs (left panels) or TTSs (right panels). Error-“clouds” represent one standard error.
Figure 10. Accumulation of H3K4me signal at 3’ ends of genes in jhd2Δ cells is not due to H3K4me deposition from downstream coding gene transcription. Shown are TTS-centered average profiles of H3K4 me3 A) and me2 B) ChIP signal from genes in Figure 9 that have converging 3’ ends (54 genes). Error—“clouds” represents one standard error.

2.4.2 JHD2 globally represses accumulation of intergenic non-coding RNAs (ncRNA)

Since H3K4me deposition is dependent on transcription (Hampsey and Reinberg 2003), accumulation of H3K4me at regions flanking both TSSs and TTSs in sporulating jhd2Δ cells suggested that JHD2 represses noncoding transcription within these regions. Together with Maria Soloveychik, I measured strand-specific RNA signal from WT and jhd2Δ cells across the genome at high resolution using Affymetrix microarrays. This experiment measured RNA from terminally sporulated cells at the 20 hour time point, which is later than post-meiotic time points at which we have observed Jhd2-related phenotypes. However, since mRNA stability is dramatically up-regulated during sporulation (Bregues et al. 2002), it nevertheless afforded us a means to evaluate this hypothesis. When I reassembled these data to construct a globally averaged protein-coding gene transcript profile, I found, as expected, that the abundance of mRNA from the averaged protein coding gene was decreased in jhd2Δ (Figure 11). I found that jhd2Δ spores exhibited increased accumulation of RNA signal on the sense strand immediately upstream to the TSS, as well as increased antisense RNA signal throughout the entire body of the averaged protein coding gene (Figure 11).
Collectively, these results support the model that *JHD2* functions globally to promote protein coding gene transcription and to repress the transcription of associated noncoding regions on both the sense and antisense strands in post-meiotic cells. Noncoding transcription can negatively impact the transcription of associated protein coding genes (Shearwin et al. 2005). One such mechanism is transcriptional interference. For example, inhibition of protein coding gene transcription can be caused by a noncoding transcription unit situated 5′ to the target gene and expressed on the same DNA strand. This type of transcriptional interference has been described previously at the *SER3, ADH1, ADH3*, and *FLO11* loci in yeast (Martens et al. 2004; Bird et al. 2006; Bumgarner et al. 2009).

To study the mechanism of Jhd2-mediated transcription interference in more detail, I characterized levels of mRNA and associated ncRNA expression at specific loci in WT and *jhd2Δ*. I noticed that the well-studied sporulation specific gene *DIT1* is flanked on its 5′ end by an intergenic “stable untranslated transcript” (SUT) called *SUT1036* that is transcribed in the sense orientation with respect to *DIT1* (Lardenois et al. 2011) (Figure 12 Top). I used RT-qPCR to measure the abundance of *SUT1306* RNA at the 10 hour time point of sporulation. In *jhd2Δ*, I found that *SUT1306* transcript levels were significantly increased while *DIT1* transcript levels were correspondingly significantly decreased (Figure 12A, B), consistent with a mechanism whereby *JHD2* represses *SUT1306* transcription, which in turn represses *DIT1* transcription (Figure 12C). This result was observed in multiple biological replicates, where RNA signal was normalized to either total RNA input (Figure 12A) or to *rDNA* transcript *RDN25-1* (Figure 12B). *RDN25-1* rRNA is a standard input control because it is transcribed by RNA pol I, an enzyme that is not known to be affected by H3K4me.
**Figure 11. JHD2 globally represses noncoding transcription during sporulation**

Affymetrix RNA expression microarray data from 20 h sporulating cells. Normalized RNA signals from 5147 protein coding genes were scaled into 20 bins for each coding region and averaged. Shown are the binned average transcript and antisense signals and an additional 500 bp of flanking signal on either side.
Figure 12. Increased abundance of ncRNA SUT1306 correlates with decreased abundance of the flanking DIT1 mRNA

A) RT q-PCR was performed with transcript-specific primers to measure levels of SUT1306 and DIT1 RNA in WT and jhd2Δ cells at the 10-h sporulation time point. RNA signals were normalized to input RNA quantity and shown as relative abundance compared to WT SUT1306 transcript levels. Error bars represent one standard deviation of technical replicates. * P < 0.05, **P < 0.005. Negative control reaction “no RT” was identical to RT, except no reverse transcriptase enzyme was used. — represents the reverse transcription primer and —— represents a qPCR amplicon. B) RT q-PCR was performed as in A), RNA signals were normalized to RDN25-1 rRNA, and then normalized to WT SUT1306 transcript levels. * P < 0.01 by two-variable t-test. C) Proposed mechanism of transcriptional interference at DIT1.
2.4.3 Sporulation is accompanied by a programmed increase in global nucleosome occupancy at 5' and 3' NDRs

As introduced in detail in Chapter 1, TSSs are typically located within nucleosome-depleted regions (NDRs) immediately upstream to the well positioned +1 nucleosome. Increased nucleosome occupancies spanning these TSS regions typically inhibit transcription, presumably by interfering with the recruitment of trans-activating factors (BADIS et al. 2008; HARTLEY and MADHANI 2009; BAI et al. 2010). Since it is known that nucleosome deposition occurs in the wake of elongating RNA Pol II during both coding and noncoding transcription (BELOTSEKOVSKAYA et al. 2003; PRUNESKI and MARTENS 2011; HUGHES et al. 2012), I hypothesized that high levels of intergenic noncoding transcription might be accompanied by an increase in global nucleosome occupancy near these TSSs (Figure 13A). Sporulation may comprise an unexplored biological context in yeast that involves global nucleosome occupancy changes near TSSs and TTSs, and JHD2-mediated repression of this program may illuminate a mechanism whereby JHD2 regulates the expression of protein coding genes during sporulation.

To test this model, I profiled nucleosome occupancies across the genomes of sporulating cells using high-resolution microarray hybridizations of mono-nucleosomal DNA. A recent study found that WT cells progressing through meiosis (0-7 hour sporulation time points) exhibited surprisingly limited global dynamics in nucleosome occupancies (ZHANG et al. 2011b), our data suggested a role for JHD2 in global transcriptional control in post-meiotic cells. Therefore, for my study, I designed my time course to evaluate nucleosome occupancies throughout the 0-16 hour time points of sporulation with particular focus on post-meiotic (8 - 12 hour) time points. From cross-sample normalized data, I extracted genomic regions flanking 5147 protein coding genes that were represented on a custom Affymetrix tiling array. I plotted the averaged nucleosome occupancy at positions surrounding the TSSs (600 bp upstream to 900 bp downstream) and TTSs (400 bp upstream to 400 bp downstream) for each sporulation time point (Figure 13B). As expected, and in agreement with Zhang et al. (2011), I observed prominent 5’ and 3’ NDRs directly upstream of TSSs and downstream of TTSs, respectively, with well positioned nucleosomes moving into the bodies of transcript units in both vegetative cells and throughout sporulation (Figure 13B).
When I examined the global TSS-proximal nucleosome occupancy signals in temporal order throughout sporulation in more detail, I found that the depth of the globally averaged NDR decreased significantly as WT cells progressed through the program (Figure 13C shows data from different time points offset from each other, for clarity). Beginning at the 6 hour time point and continuing through the 10 hour time point, confidence interval analysis (BONN et al. 2012) and two-tailed t-tests showed that this effect was both highly statistically significant and progressively increasing in magnitude (Figure 15A). Intriguingly, at the 12 hour time point (and continuing until my last examined time point at 16-hours), the averaged NDR depth reverted to the level found in vegetative cells (Figure 13C & Figure 15A). Coincident with these 5' NDR dynamics, the globally averaged +1 nucleosome underwent an apparent decrease in occupancy signal starting at the 4 hour time point that persisted until the 10 hour time point before reverting to a level close to the basal state. Statistical analysis showed that this effect was highly significant (Figure 15A).
Figure 13. *JHD2* represses a program of increasing nucleosome binding to TSSs during sporulation.

A) Working model: Jhd2 promotes protein coding gene expression by negatively regulating interfering non-coding transcription. B) Genome-wide high-resolution nucleosome occupancy data was collected from sporulating WT cultures. TSS centered and log2 transformed mean occupancy signal of 5147 coding genes is shown with the time point of sporulation represented by color, as indicated in the legend. C) Globally averaged regions from -250 bp to +150 bp with respect to the TSS are displayed. For clarity, data from different sporulation time points are shown offset from each other, with the x-axes for each time point displayed underneath. * P value = 4x10^-8, ** P value = 4x10^-10 by two-variable t-test. D) Same as C), globally averaged regions from -250 bp to +350 bp with respect to the TSS are displayed.

By analyzing nucleosome occupancy changes of individual genes, I found that increased nucleosome occupancy in 5’ NDRs is not limited to a small subset of genes, but is exhibited by the majority of genes. It becomes pronounced in magnitude and affects increasing number of genes starting at 4 hours into sporulation and becomes most apparent at 10 h into sporulation. At 10 h into sporulation, most genes (83%, or ~4300/5147) exhibit some degree of increased nucleosome occupancy in the 5’ NDR. This is shown in Figure 14, where the change in nucleosome occupancy (compared to vegetative cells) surrounding the 5’ NDR is represented by
heat maps for 5147 genes. The genes are ordered based on amount of change in occupancy in the 5’ NDR. The yellow regions indicate increased occupancy and they are found in the regions within 300 bp upstream of the TSS. Interestingly, increased nucleosome occupancy in the 5’ NDR is accompanied by decreased occupancy in the +1 and -1 nucleosomes. Since histone production is tightly regulated and only occurs during S phase (MEEKS-WAGNER and HARTWELL 1986; GUNJAN and VERREAULT 2003), the pool of free histones in the cell (outside of S phase) is extremely small, and in fact, toxic to cells (MORILLO-HUESCA et al. 2010; MAYA et al. 2013), so it is not surprising that increased nucleosome occupancy in some regions was accompanied by decreased occupancy in other regions. Due to their coincidence and proximity, the filling-in at the 5’ NDR and decreased occupancy at the +1 nucleosome might be related; developmentally regulated +1 nucleosome shifts into the 5’ NDR may cause the increase in occupancy signal of averaged NDRs.
Figure 14. Nucleosome occupancy increases at almost all 5’ NDRs to some extent during post-meiotic sporulation

Differential analysis of global nucleosome positioning changes was performed by calculating the change in Log₂ signal at each time point in sporulation compared to the signal in vegetative cells (Log₂(tSPO/tYPD)). Each row represents 1 Kb of signal from a TSS-centered gene, divided into 10 bp bins. Genes are ordered by the magnitude of change in nucleosome occupancy in the 5’ NDR. At 10 h into sporulation, ~4300, or 83% of 5’ NDRs exhibit increased nucleosome occupancy. ‘Veg’ vegetative time point represent cells grown in glucose-containing YPD medium.

Similarly to 5’ NDRs, the presence of 3’ NDRs near TTSs of coding regions has been well described (Yuan et al. 2005; Lee et al. 2007b; Fan et al. 2010); however, the functional significance of 3’ NDRs is less well understood. Analogous to what I observed at 5’ NDRs, the global 3’ NDR also undergoes dynamic occupancy changes throughout sporulation (Figure 13D). Again, when I examined the global TTS-proximal nucleosomal occupancies in temporal order throughout sporulation in more detail, I found that the depth of the globally averaged NDR decreased significantly as WT cells progressed through the program. Following an initial increase in averaged 3’ NDR depth as cells progressed to the 4 hour time point, the averaged 3’ NDR depth significantly decreased as cells progressed to the 10 hour time point (Figure 13D, Figure 15B). By 12 h of sporulation, the averaged 3’ NDR depth reverted to a level similar to the basal state observed in vegetative cells.
The magnitude of change that I observed in global nucleosome occupancy during sporulation was very dramatic when compared to those observed in other studies. A recent nucleosome occupancy study of a compendium of 52 chromatin factor mutants involved in chromatin, histone, and transcription biology found that most mutants had modest effects on global average nucleosome occupancy (Van Bakel et al. 2013). That study utilized Affymetrix microarray platforms and data analysis methods that were identical to those used in my study, which enabled more meaningful comparison of the two data sets. The levels of global nucleosome remodeling that I observed during sporulation were comparable to those observed in mutants with the most dramatic nucleosome occupancy phenotypes in the recent study (Xu et al. 2012; Van Bakel et al. 2013). Examples include factors such as Rsc3 (remodel the structure of chromatin), which is the catalytic component of an ATPase-dependent chromatin remodeler complex, and Rap1 (repressor activator protein), a transcription factor that has DNA-binding and nucleosome exclusion activity (Badis et al. 2008; Pinskaya et al. 2009b). Conditional inactivation of these factors causes dramatic nucleosome accumulation at 5' NDRs of a large subset of genes. In comparison to these chromatin remodeler mutants, developmentally programmed nucleosome remodeling during sporulation affects even greater numbers of genes with comparable magnitude. This underscores the apparent fact that many chromatin-associated factors are very active during response to stress or during development, and here I show that sporulation is one such context during which nucleosomes are actively and dramatically remodeled.

Figure 15. Global mean 5' and 3' NDR and +1 nucleosome occupancy signals change significantly over the course of sporulation.
Global average signals from 5147 genes are shown with 95% confidence intervals (±97.5%) represented by coloured clouds. $P$ values from two-component t-tests are shown. (A) During sporulation in WT cells, global mean nucleosome occupancy at the 5’ NDR (TSS) and +1 nucleosome becomes significantly different from the basal state observed in YPD. (B) Global mean nucleosome occupancy at the 3’ NDR (TTS) becomes significantly different from the basal state observed in YPD at 4 h and 10 h of sporulation.

2.4.4 JHD2 opposes the program of globally increased nucleosome accumulation at 5’ and 3’ NDRs during sporulation

Having characterized nucleosome architecture dynamics during sporulation in WT cells, I moved on to analyze Jhd2’s contribution to sporulation nucleosome occupancy by comparing experimentally paired WT and jhd2Δ data sets. While the globally averaged nucleosome profiles of WT and jhd2Δ were nearly indistinguishable near the TSS throughout most of sporulation, at the 8 h time point confidence interval analyses and two-tailed t-tests showed that jhd2Δ cells exhibited a highly significant increase in nucleosome occupancy at the globally averaged 5’ NDR (Figure 13C and Figure 16A. $P$ value = 4x10$^{-10}$). The same affect was also observed at the 3’ NDR at the 6 h and 8 h time points of sporulation (Figure 13D and Figure 16B).

My results show that sporulation is accompanied by developmentally programmed global increase in nucleosome occupancy at TSS- and TTS-associated sites. This program initiates at 6 hours into sporulation, peaks at the 10 hour time point, and reverts to a state that is similar to the basal state of vegetative cells, at 12-hours, coincident with the acquisition of transcriptional quiescence. Interestingly, JHD2 repressed this program, with jhd2Δ resulting in a further increase in nucleosome occupancy at TSSs at the 8 hour time point and TTSs at the 6 h and 8 h time points. Consistent with the hypothesis that the programmed increase in nucleosome occupancy is a consequence of non-coding transcription-coupled nucleosome deposition, the reversion to the “baseline” nucleosome occupancy state occurs concomitant with dramatically reduced global transcription rates. Nucleosomes presumably return to their more energetically favorable states during this time period (KAPLAN et al. 2009). As the timing of the nucleosome positioning phenotype coincides with Jhd2-mediated effects on noncoding and coding transcription, these results are consistent with my model shown in Figure 13A. Indeed, other researchers have also found that increased NDR occupancy that is due to depletion of transcription factors or ATPase-dependent chromatin remodelers is correlated with increased

Figure 16. TSS-proximal NDR nucleosome occupancy is significantly increased in jhd2Δ cells during sporulation.
Global average signals from 5147 genes are shown with 95% confidence intervals (± 97.5%) represented by colored clouds. P values from two-component t-tests are shown. A) TSS-proximal NDR occupancy is significantly increased in jhd2Δ cells at 8 h into sporulation. B) TTS-proximal NDR occupancy is significantly increased in jhd2Δ cells at 6 & 8 h into sporulation.

2.4.5 JHD2 acts broadly at ribosomal protein-encoding and Rap1-bound genes in post-meiotic cells and appears to inhibit upstream non-coding transcription and nucleosome deposition

To further test my model for JHD2 function, I examined specific classes of genes that are known to play important roles during sporulation. During sporulation, ribosomal protein (RP) gene transcription is repressed throughout meiosis and de-repressed following its completion. The later wave of RP expression presumably provides spores with a “maternal” load of RP mRNAs and/or ribosomes to be utilized upon germination (Pearson and Haber 1980; Chu et al. 1998). In yeast, coordinated transcription of the 137 RP genes represents a substantial energetic investment, accounting for approximately 50% of all RNA Pol II transcription events (Warner 1999). Given the role of JHD2 in promoting post-meiotic transcription, this post-
meiotic de-repression phase of RP gene transcription therefore seemed likely to be of significance. After first ordering the protein coding genes alphabetically so that all of the RP genes (named “RPL-” and “RPS-”) were adjacent to each other, I visualized the sporulation dynamics in nucleosome occupancies surrounding TSSs using heat maps (Figure 17A). This analysis showed that RP genes exhibited a robust “filling in” of TSS-associated NDRs during sporulation that stood out from the rest of the genome (Figure 17A).

To see whether RP genes exhibit uniform chromatin remodeling or segregate into distinct subclasses, I performed K-means clustering, which revealed two classes of RP genes (Cluster 3.0 software). The first class exhibited dramatic increases in nucleosomal occupancies near TSSs at the 8 hour sporulation time point, while the other class did not (Figure 17B, clusters 1 and 2). I next identified two classes of RP genes in jhd2Δ mutants: one that hyper accumulated nucleosomes near TSSs at the 8 hour time point of sporulation compared with WT and one that did not (Figure 17C, clusters A and B). The overlap between cluster 1 and cluster A was very significant (Figure 17D), suggesting that JHD2 opposes a sporulation program that promotes the accumulation of nucleosomes near the TSSs of a large class (almost half) of RP genes. Confidence interval analysis and two-tailed t-tests confirmed that the differences in nucleosome occupancy between WT and jhd2Δ were highly significant for cluster A (Figure 17E). If the RP genes in cluster A accumulated nucleosomes as a consequence of increased non-coding transcription, then they could also exhibit increased RNA signal upstream of their TSSs in jhd2Δ compared with cluster B genes. In agreement with this, I found that cluster A was associated with increased noncoding RNA in jhd2Δ compared with cluster B (Figure 17F). Many classes of RNAs (such as CUTs and XUTs) are highly unstable, so the actual levels of noncoding transcription could be higher than was measured.
Figure 17. Jhd2 acts at ribosomal protein-encoding genes in post-meiotic cells.
A) Differential analysis of global nucleosome positioning changes was performed by calculating the change in log$_2$ signal at each time point in sporulation compared to the signal in vegetative cells (log$_2$(t$_{SPO}$/t$_{YPD}$)). Each row depicts 1Kb of signal from a TSS-centered gene. Genes are ordered alphabetically by their standard name. Data from each time point of sporulation is labeled. Small brackets demarcate the ribosomal protein (RP) encoding genes (RPL/RPS).

B) Log$_2$(t$_{SPO}$/t$_{YPD}$) signal from 129 rProtein genes was K-means clustered into two groups (Cluster 1 and 2) using a Euclidian distance matrix. C) Log$_2$(jhd2∆/WT) signal of 129 RP genes were independently K-means clustered into two groups (Cluster A and B). D) There is significant overlap between Cluster 1 and Cluster A. The P-value from the hypergeometric distribution test is shown. * P value <0.05.

E) The average nucleosome occupancy signals from -500 to +150 with respect to the TSS of the RPs in Cluster A and Cluster B. For clarity, data from different sporulation time points are shown offset from each other, with the x-axes for each time point shown underneath.

RP gene transcriptional control involves the general regulator factor Rap1 (LIEB et al. 2001). To extend my analysis of RP genes, I interrogated the nucleosomal occupancies of the 421 known Rap1-bound genes (RHEE and PUGH 2011), after first removing all of the RP genes from the list. I again identified a cluster of Rap1-bound genes that exhibited increased nucleosome binding at the 8 hour time point of sporulation in WT cells and one that did not (Figure 18A, clusters 1 and 2). In striking recapitulation of my RP gene analysis, jhd2∆ caused a highly significant hyper accumulation of nucleosomes in a cluster of genes that overlapped significantly with cluster 1 (Figure 18B-D). Like my observation in RP genes, this cluster was associated with increased JHD2-repressed noncoding transcription upstream of the TSS (Figure 18E).

2.4.6 Jhd2 appears to inhibit nucleosome occupancy in large NDRs

One commonality of the genes that exhibit strong evidence of JHD2-repressed nucleosome occupancy is that their NDRs are significantly larger than genome average size of ~140 bp (compare cluster A with cluster B in both Figure 17E and Figure 18D) (YUAN et al. 2005). As these NDRs possess more exposed DNA to accommodate nucleosome deposition, they stand out dramatically in my analysis of JHD2-mediated nucleosome occupancy changes. These genes with large NDRs may be regarded as diagnostic of mechanisms that act broadly at most other genes, even those with smaller NDRs. This interpretation is supported by the fact that both clusters A and clusters B from the RP and Rap1-bound genes exhibit equivalent defects in protein coding mRNA expression (Figure 17F and Figure 18E). Perhaps relatedly, repressive noncoding transcription can act through a variety of mechanisms in addition to nucleosome
occlusion, such as the accumulation of stalled polymerases, or the displacement of transcription factors and pre-initiation complexes (PALMER et al. 2011).

Figure 18. JHD2 acts at Rap1-bound genes in post-meiotic cells. 
A) Log₂ (tSPO/tYPD) signal from 327 Rap1-bound genes was K-means clustered into two groups (Cluster 1 and 2) using a Euclidian distance matrix. B) Log₂(jhd2Δ/WT) signal of 327 Rap1-bound non-RP genes were independently K-means clustered into two groups (Cluster A and B). C) There is significant overlap between Cluster 1 and Cluster A. The P-value from the hypergeometric distribution test is shown. D) The average nucleosome occupancy signals from -500 to +100 with respect to the TSS of the genes in Cluster A and B are shown. For clarity, data from different sporulation time points are shown offset from each other, with the x-axes for each time point shown underneath. * P value < 0.05. E) The average normalized Affymetrix RNA transcript signal at 20 h of sporulation for clusters in D) are shown. Signal associated with coding region are scaled into 20 bins. An additional 500 bp of flanking signals are shown on each side.
2.4.7 Increased abundance of upstream ncRNAs in \textit{jhd2Δ} cells correlates with decreased abundance of transcripts from their associated coding genes

Cluster A and B RP genes \textit{RPS6A} and \textit{RPL10} are flanked on their immediate 5' ends by the annotated noncoding transcripts \textit{SUT2593} and \textit{SUT258}, respectively (Lardenois et al. 2011) (Figure 19A and B). In agreement with the conclusion that RP genes from both clusters are generally targeted by \textit{JHD2}-repressed noncoding transcription, using RT-qPCR, I found that both \textit{SUT2593} and \textit{SUT258} were upregulated in \textit{jhd2Δ} while their associated RP genes were down-regulated (Figure 19A, 8 hour sporulation time point). While most RP genes do not have annotated noncoding transcripts in their 5' regions (only 17 RPs have annotated 5' sense ncRNAs to date), my analysis suggested that noncoding transcripts are widespread upstream of RP genes. I again used RT-qPCR to interrogate the region directly upstream of two such genes from cluster A and cluster B for the existence of \textit{JHD2}-regulated noncoding transcripts on the sense strand. In addition to confirming my observations of \textit{SUT258} and \textit{SUT2593} in this biological replicate, I found that \textit{RPS1B} and \textit{RPL11B} are both flanked on their 5' ends by previously un-annotated \textit{JHD2}-repressed noncoding transcripts (Figure 19C, right side). In contrast, vegetative cells did not show any differences in the abundance of these transcripts between WT and \textit{jhd2Δ}, consistent with a sporulation-specific role for \textit{JHD2} (Figure 19C, left side).
Figure 19. Jhd2 negatively regulates abundance of non-coding transcripts during sporulation.
Transcript-specific RT qPCR was performed to measure levels of non-coding transcripts associated with coding genes. Signals were normalized to RDN25-1 rRNA levels, and expressed as fold difference from WT ncRNA signal. Error bars represent one standard deviation from technical replicates. A) Relative levels of SUT2593 and RPS6A transcript are shown. B) Relative levels of SUT258 and RPL10 transcript are shown. C) Relative levels of ncRNA were measured in vegetatively growing cultures and at 10 h of sporulation for WT and jhd2Δ.

2.5 Summary and Discussion

Work from the Meneghini lab has shown that yeast sporulation is accompanied by a programmed onset of global transcriptional quiescence following the completion of meiosis, and that the highly conserved H3K4 demethylase Jhd2 promotes protein coding gene transcription in opposition to this programmed transcriptional quiescence, and functions to specify the proper duration of global transcriptional activity during post-meiotic sporulation. My results also show
that Jhd2 represses 5′ sense-strand oriented intergenic noncoding transcription in post-meiotic cells and an associated program of globally increased nucleosomal occupancies near TSSs, suggesting an explanation for at least part of JHD2’s mechanism of action. Although I have not investigated the underlying mechanism of action, I found that JHD2 globally represses antisense RNA that overlaps with gene bodies and appears to originate from the TTSs of genes. It seems likely that this accumulation of antisense RNA may act to repress gene transcription and/or mRNA stability of their associated protein coding genes in post-meiotic cells. In addition to setting the time interval of post-meiotic transcriptional activity, JHD2 correspondingly defines the time of completion for sporulation. These results are summarized in Figure 20. As JARID1A/B acts antagonistically to the terminal differentiation of mammalian stem cells (DEY et al. 2008; XIE et al. 2011), my results suggest that the JARID1 family serves analogous developmental functions in yeast and humans.

Figure 20. JHD2 sustains the proper duration of global transcriptional activity during post-meiotic sporulation by negatively regulating interfering non-coding transcription
2.6 Future Directions

2.6.1 Global RNA profiling of \( jhd2\Delta \) and WT cells by RNA-seq

The genetic and molecular mechanisms of Jhd2-mediated control of non-coding and coding transcription still remain to be elucidated. Existing global RNA abundance data sets that I collected from 20 hour sporulated cells have revealed insights on a global level; however, to make observations on the level of individual genes, RNA profiling should be performed at the relevant post-meiotic time points (8-10 hours of sporulation). These experiments will reveal relative abundances of coding and non-coding transcripts in WT and \( jhd2\Delta \) in the resolution of specific genes, as well as revealing global trends. Furthermore, RNA abundance data can be combined with nucleosome occupancy data, ChIP datasets and other published genomic datasets to answer specific questions regarding mechanisms of gene regulation. For example, 1) what types of nucleosome occupancy changes occur over intergenic regions when non-coding transcripts become induced, or repressed? Do these changes appear to follow a paradigm, or do they behave in a locus-specific manner? 2) Does the presence of chromatin regulatory factors influence nucleosome occupancy or non-coding transcription?

2.6.2 What causes nucleosome occupancy changes in \( jhd2\Delta \) cells?

In my model of Jhd2-mediated gene expression, (see Figure 20), I proposed that Jhd2 demethylates H3K4me within intergenic regions, and this represses non-coding transcription. I further proposed that increased nucleosome occupancy in intergenic regions is caused by non-coding transcription-mediated deposition; this is a phenomenon that has had precedence in the literature, and is consistent with my data (PrunESKi and Martens 2011). My model can be tested using genomic data sets by determining whether sites of increased non-coding transcript abundance in \( jhd2\Delta \) are correlated with increased nucleosome occupancy. A more mechanistic test for transcription-directed nucleosome deposition and its effect on coding gene transcription can be done at specific loci by attenuating expression of a non-coding transcript. For example, transcription of the non-coding RNA \( SUT1306 \) upstream of \( DIT1 \) can be disrupted using a premature transcription termination signal (Bird et al. 2006). I can then determine whether \( SUT1306 \) transcription deposits nucleosomes in the \( DIT1 \) promoter by comparing nucleosome
occupancy in sporulating cells with normal *SUT1306* transcription to cells with attenuated *SUT1306* transcription.

### 2.6.3 Technical and normalization considerations for RNA seq

Recently, the molecular genetics field has advanced from high-resolution tiling array-based methods to next generation sequencing (NGS), or RNA-seq, for genome-wide RNA quantification studies. Future RNA profiling studies should be performed using these new RNA-seq platforms. Currently, RNA-seq platforms can yield up to hundreds of millions of short reads of RNA sequence per sample, which enables very deep coverage (i.e. each RNA species is sequenced many times on average) (Valouev et al. 2011). Deep sequencing coverage enables detection of low-abundance RNA species, which is an important consideration when studying the non-coding transcriptome since many non-coding RNAs can be 100-fold lower in abundance than coding transcripts (Lardinois et al. 2011; Xu et al. 2012).

A critically important issue to consider for RNA-seq studies in *jhd2Δ* cells is global normalization of mRNA signal levels between *jhd2Δ* and WT samples. *jhd2Δ* cells exhibit a global defect in mRNA abundance; therefore, in order to make meaningful comparisons of relative mRNA abundance, WT and *jhd2Δ* data cannot be normalized based on the commonly used method of equalizing total bulk signal. Instead, a method involving spike-in controls should be used. For this method, the amount of input cells for each sample (WT and *jhd2Δ*) should be precisely measured and equal. This can be done by counting out exact numbers of cells using FACs or by careful input of equal amounts of cells based on optical density (O.D. 600) of yeast cultures. An equal amount of spike-in RNA is added to each sequencing sample during the RNA extraction process. During data processing all RNA signals are normalized to those of the spike-in RNA (Yang 2006; Loven et al. 2012). The spike-in RNA corrects for discrepancies in RNA extraction efficiency, RNA degradation and differences in sequencing coverage (Loven et al. 2012). To differentiate spike-in RNA from RNA in the experimental sample, RNA from a sufficiently evolutionarily distant species with distinguishable mRNA sequence is typically used (Sun et al. 2012). Alternative, artificial RNA that has no sequence homology to *S. cerevisiae* can also be used (Loven et al. 2012).
2.6.4 Targeted mechanistic studies of Jhd2-mediated ncRNA regulation

To interrogate molecular mechanisms that underlie Jhd2 regulation of coding and non-coding transcription, many experiments can be done at specific gene loci. A good candidate locus is at DIT1, which I have previously characterized and described in this chapter. DIT1 and its associated upstream non-coding transcript SUT1306 are temporally co-expressed. I found that DIT1 is significantly (>2 fold) down-regulated in jhd2Δ cells during post-meiotic sporulation (Figure 12A), while levels of SUT1306 RNA were significantly increased in jhd2Δ cells at the same time points (Figure 12). This data is consistent with a model where SUT1306 negatively influences DIT1 transcription through a transcription interference mechanism. To directly test the effect of SUT1306 on DIT1 expression, SUT1306 transcription can be attenuated to observe the resulting effect on DIT1 transcription. The following strain constructs would be useful for these types of experiments: 1) a construct with a transcription termination site inserted into the ncRNA coding region, or 2) a promoter deletion construct. These constructs can be used to determine whether decreased non-coding transcription can de-repress the adjacent coding transcript, or rescue the coding transcription defect in jhd2Δ cells. Whether these ncRNAs can act in trans can be determined by expressing them ectopically from a plasmid and measuring whether the corresponding coding transcript abundance is affected. These experiments would help determine which types of ncRNA interference mechanisms govern gene regulation at Jhd2-controlled loci.
Chapter 3

Characterizing the nucleosome architecture of 3' Nucleosome Depleted Regions

The global nucleosome data sets used in analyses in this chapter have been published.

The RNA Affymetrix microarray data set from cells grown in YPA is unpublished and collected by Maria Soloveychik

The data analysis presented here is unpublished.
ABSTRACT

3’ NDRs have long been described in global nucleosome occupancy mapping studies; however, their function is not well understood (YUAN et al. 2005; LEE et al. 2007b; ZHANG et al. 2011a). It is not clear if nucleosome depletion in the 3’ NDR is shaped by DNA-histone interactions or maintained actively by chromatin factors, and furthermore, whether it serves a regulatory function in gene expression or represents a consequence of transcription (FAN et al. 2010). Due to relatively high gene density and short intergenic distances in the Saccharomyces cerevisiae genome, approximately one half of all 3’ NDRs are also 5’ NDRs of downstream genes. I found that at shared 5’ and 3’ NDRs, most of the nucleosome depletion is attributable to the 5’ NDR. Furthermore, non-coding transcription initiation (which is pervasive in 3’ NDRs) also contributes modestly to 3’ NDR structure. When I restricted analysis to sites that are only associated with 3’ ends of coding transcripts, I found that higher levels of transcription (as measured by RNA abundance) correlated with more nucleosome depletion in 3’ NDRs.

INTRODUCTION

3.1 3’ nucleosome-depleted regions

3’ NDRs are on average much less nucleosome depleted compared to 5’ NDRs (YUAN et al. 2005; LEE et al. 2007b; FAN et al. 2010; ZHANG et al. 2011a; XU et al. 2012). Positioning of the terminal nucleosomes of genes (the last nucleosome upstream of the TTS) can be highly variable (MAVRICH et al. 2008a); at loci where the terminal nucleosome is well positioned, there is a correlation with DNA-sequence mediated positioning. The statistical stacking model (described in Chapter 1), wherein a well-positioned nucleosome acts as an anchor against which neighboring nucleosomes stack up, is also observed at terminal nucleosomes, with positioning effects extending upstream from the terminal nucleosome (MAVRICH et al. 2008a).

Some 3’ NDRs in yeast can mediate an interesting phenomenon known as transcriptional looping. A study by Singh and Hampsey found that RNA pol II can loop back to the promoter of genes from its TTS though a process involving the general transcription factor TFIIB (SINGH and HAMPSEY 2007). These “looping” transcription units are characterized by the presence of TFIID at both the TSS and TTS. Some 120 yeast genes have this characteristic (MAVRICH et al. 2008a).
Transcriptional looping appears to promote efficient re-initiation and high transcriptional output (Singh and Hampsey 2007).

### 3.1.1 Analyses of 3' NDRs are confounded by overlap with 5' NDRs

Several models have been proposed for mechanisms that specify 3’ NDR formation; it has been suggested that cleavage and poly-adenylation signals (AATAAA), which resemble nucleosome formation-occluding poly-dA:dT tracts and often overlap the 3’ NDR, could be antagonistic to nucleosome occupancy (Iyer and Struhl 1995; Mavrich et al. 2008a; Iyer 2012). However, a caveat to these observations stems from the fact that the yeast genome is very compact and a large proportion of 3’ NDRs are also the 5’ NDRs of downstream genes. Therefore, analyses of 3’ NDRs that do not take this into account could have been describing nucleosome properties of downstream 5’ NDRs. A study by Fan et al., which addressed this issue by analyzing only 3’ NDRs that contained convergent 3’ ends of coding transcripts found that 3’ NDR depth is correlated with pol II transcription termination activity (Fan et al. 2010). This study supports a model whereby both inherent DNA-histone affinity and transcription termination contribute to 3’ NDR formation.

There is an important additional consideration for 3’ NDR structure which has not been addressed by published studies. That is, many 3’ NDRs are sites of non-coding transcription initiation. In yeasts, thousands of non-coding transcripts have been identified in recent global surveys (Granovskaia et al. 2010; Lardenois et al. 2011; Van Dijk et al. 2011; Xu et al. 2011). In fact, bidirectional transcription initiation appears to be the default state of promoter and NDRs, meaning that 3’ NDRs of many coding genes also serve as initiation sites of antisense and sense orientation non-coding transcription (Neil et al. 2009; Xu et al. 2009; Wei et al. 2011).

### 3.1.2 Are 3’ NDRS actually just 5’ NDRs?

Given the pervasiveness of non-coding transcription initiation at 3’ NDRs, and the fact that many 3’ NDRs also act as the 5’ NDRs of downstream coding genes, I hypothesized that nucleosome depletion at most 3’ NDRs are actually attributable to overlap with 5’ NDRs of
coding and non-coding transcripts. As such, I wanted to determine 1) whether there are any 3’ NDRs that are completely unassociated with 5’ initiation, and whether they still exhibit nucleosome depletion 2) if so, whether transcriptional termination correlates with 3’ NDR depth.

One difficulty associated with this analysis lies with identifying regions that do not have any transcriptional initiation, since many ncRNAs are very unstable and may therefore be undetectable (LARDENOIS et al. 2011; VAN DIJK et al. 2011).

For the analyses described in this chapter, I used global nucleosome occupancy Affymetrix microarray data sets from logarithmic phase vegetative cells grown in YPA. These data were collected by Mike Schertzberg and normalized by Harm van Bakel. To correlate nucleosome occupancy to transcription, I used high resolution Affymetrix microarray RNA abundance data collected from vegetative cells grown under the same conditions. The custom Affymetrix microarray used single stranded-probes that yield strand-specific RNA signal, so I could analyze sense and antisense RNA signal independently. The RNA data set was collected by Maria Soloveychik.

3.2 Results

3.2.1 The depth of the 3’ NDR of a transcript is strongly correlated with levels of downstream transcription initiation

To survey 3’ NDRs of coding regions globally, I aligned the nucleosome occupancy signals of 5147 yeast genes at their TTS with 1 Kb of signal upstream and downstream. To visualize the range of 3’ NDR depths across the genome, I ranked genes based on average occupancy signal in the TTS-proximal region, from the lowest to the highest. Figure 21A shows this analysis visualized on a heat map, with deeper blue representing lower signal at more nucleosome-depleted 3’ NDRs. Most genes have a 3’ nucleosome depleted region near the TTS, with the depth of the 3’ NDR falling on a gradient from very distinct (i.e. very nucleosome-depleted) to not detectable. When genes are binned into groups comprising the top ¼, bottom ¼, bottom 500 genes, and middle 1/2 of genes based on their ranked 3’ NDR depth, their average nucleosome occupancy signal (plotted in Figure 21F) shows that roughly the top quarter of the genes displayed a distinct 3’ NDR, approximately half of the genes in the genome have a very modest NDR, and ~500 genes do not have any detectable nucleosome depletion around the TTS.
Figure 21. The depth of the 3’ NDR correlates with transcription levels from the overlapping downstream 5’ NDR

A) Genome-wide nucleosome occupancy data from log phase vegetative yeast was normalized and log transformed. Signals centered on annotated TTSs of 5147 genes are presented with 10 bp binned signals as shown in the colour key. 1Kb of signal is shown upstream and downstream of the TTS. Genes are ranked by increasing signal in the TTS-proximal region. Sense strand B) and antisense strand C) RNA signal from the same cell cultures as A) are shown. Signal along each gene was divided into 20 bins regardless of gene length, with 500 bp of signal shown upstream and downstream of the coding region. The coding region is indicated by the blue and yellow arrows. Gene ordering in B) and C) are the same as in A). Coloured bars (blue, green, olive green and purple) indicate the ‘top ¼’, ‘middle ½’, ‘bottom ¼’ and ‘bottom 500’ bins, respectively. D) & E) Average RNA signals of the bins shown in B) & C). F) Average nucleosome occupancy signals of bins shown in A).

To determine whether the depth of the 3’ NDR correlates with transcription levels of a gene, I displayed RNA signals associated with the 5147 genes in the same order as the 3’ NDR-ranked nucleosome data in Figure 21A. The sense and antisense RNA data are shown in Figure 21B and Figure 21C, respectively. For each gene, the signal associated with the coding region is divided into 20 equally sized bins, regardless of the size of the gene, with the average signal of
each bin represented on a colour-scale. An additional 500 bp of flanking upstream and downstream RNA signal is also displayed. Figure 21B shows that transcript signal levels do not correlate with 3’ NDR depth. Instead, genes with deeper 3’ NDRs are associated with higher levels of downstream sense RNA signal (Figure 21C). This is very apparent in Figure 21D, which shows the average transcript signal of genes binned into the same groups as shown in Figure 21F. Genes in the top ¼ bin have the deepest 3’ NDRs and the highest downstream sense RNA signal, and the bottom 1/4 bin and bottom 500 genes have very little to no detectable downstream sense RNA signal or 3’ NDR. This means that the predominant factor that influences 3’ NDR depth is the presence and level of transcription at the shared 5’ NDR of downstream transcripts. In other words, genes with tandemly arranged downstream genes have deeper 3’ NDRs. In Figure 21B and C, genes that have downstream antisense RNA signal have correspondingly shallower 3’ NDRs. These are probably enriched for genes that converge onto the 3’ end of another gene. I will refer to these as “convergent genes” from here on.

3.2.2 Transcriptional initiation sites are associated with a deeper NDR than transcriptional termination sites

My initial analysis confirmed the established observation that transcription initiation correlates strongly with 5’ NDR depth. When 3’ and 5’ NDRs overlap, the determinants of 5’ NDR structure are much stronger and mask those that shape 3’ NDRs. Therefore, to study mechanisms that regulate the 3’ NDR, I chose to analyze loci that have convergent 3’ transcription termination sites. Within the 5147 genes in my data set there are 2501 ‘tandem’ genes and 2646 ‘convergent’ genes that are useful for my 3’ NDR analysis (cartoons illustrating these gene orientations are shown in the bottom of Figure 22). The average nucleosome occupancy signals of ‘tandem’ and ‘convergent’ genes showed that they are dramatically different (Figure 22A). The average 3’ NDR of ‘tandem’ genes is very well defined and resembles 5’ NDR architecture, whereas the average ‘convergent’ gene 3’ NDR is much more shallow, with the bottom of the NDR trough localized more upstream. The average RNA signal of these two classes of genes is shown in Figure 28B. The average RNA signal from ‘convergent’ genes is slightly lower than ‘tandem’ genes. This might be due to run-on transcription induced convergent transcriptional interference; RNA pol II has a tendency to sometimes transcribe past the conventionally defined TTS into neighboring genes (PELECHANO et al. 2013). Since RNA polymerases cannot pass
each other, head-on collisions between pol II complexes result in transcriptional termination on both sides (HOBSON et al. 2012).

![Figure 22. Transcriptional initiation is associated with higher levels of nucleosome depletion than transcriptional termination](image)

A) Nucleosome occupancy signal of all ‘convergent’ and ‘tandem’ genes. Signal is centered on the annotated TTS. B) Average RNA signals associated with ‘convergent’ and ‘tandem’ genes. Signal along each gene was divided into 20 bins regardless of gene length, with 500 bp of signal shown upstream and downstream of the coding region. Bottom of figure shows a schematic of the orientation of ‘convergent’ and ‘tandem’ genes.

3.2.3 3ʹ NDR depth correlates modestly with detected levels of non-coding transcription at convergent genes

I next asked whether the 3ʹ NDR at convergent genes can be attributed to 1) non-coding transcription initiation. That is, are 3ʹ NDRs that are associated with initiating non-coding transcripts more defined than 3ʹ NDRs that lack any transcription initiation events? Or alternatively, 2) does transcriptional termination promote nucleosome depletion? Or 3) does the DNA sequence at 3ʹ intergenic regions inhibit nucleosome deposition intrinsically? Regarding the first hypothesis, my analysis is confounded by the fact that I have to assume that RNA signal levels accurately represent levels of transcription initiation. Of course this is often not the case since many non-coding transcription products (e.g. CUTs and XUTs) are very unstable (XU et al. 2009; VAN DIJK et al. 2011).
I ranked the 2646 ‘convergent’ genes based on decreasing levels of RNA signal emanating from the TTS region (downstream sense signal and coding region antisense signal) (Figure 23A). The genes at the bottom of Figure 23A have the lowest levels of RNA signal emanating from their TTS. TTS-centered nucleosome occupancy signals were ordered according to this ranking, and binned into the top ¼, middle ½, bottom ¼, and bottom 1/10 groups. Nucleosome signals from these bins were plotted and shown in Figure 23B. There is a positive correlation between non-coding transcription initiation and 3’ NDR depth, but the effect is very subtle. A 3’ NDR is present even at the ~250 genes loci with the lowest ranked levels of non-coding DNA signal, with a trough depth that is comparable to that of the average ‘convergent’ gene 3’ NDR (Figure 23B and Figure 22A).
3.2.4 Transcription termination activity impacts the depth of the 3' NDR

To determine whether levels of transcription termination contribute to the depth of the 3’ NDR, I ranked convergent genes based on transcript RNA signal levels (Figure 24A). The TTS-centered nucleosome occupancy signal was ordered according to this ranking (Figure 24B), and then binned and averaged as before (Figure 24C). The averaged bins show that the top ¼ most transcribed ‘convergent’ genes have deeper 3’ NDRs than less transcribed genes; however, the correlation does not exist for genes with modest (½ middle) or lowest (1/4 bottom) transcript accumulation levels. This result is consistent with transcription termination having some
influence on 3’ NDR depth, but it is clear that other factors also contribute to 3’ NDR architecture.

Figure 24. The depth of the 3' NDR is correlated with the amount of transcription

A) 2646 convergent genes were ranked by level of RNA signal. Signal along each gene was divided into 20 bins, with 500 bp of signal shown upstream and downstream of the coding region. B) TTS-centered nucleosome occupancy data was ordered according to the ranking of RNA signal in A). Coloured lines indicate bins shown in C). C) TTS-centered nucleosome occupancy data, showing 1Kb up and downstream. Shown are averages of bins indicated by colour-coded lines in B).

3.2.5 Summary

Analysis in this chapter explored the mechanisms that contribute to 3’ NDR architecture and function. I showed that 5’ NDRs are inherently more well-defined, i.e. more nucleosome depleted than 3’ NDRs. I also show data suggesting that nucleosome depletion at 3’ NDRs is specified in part by transcription (possibly more specifically by transcription termination) and very modestly by non-coding transcription initiation. My initial hypothesis that NDR architecture is shaped solely by transcription initiation was not supported, as 3’ NDRs are present at loci without any detectable transcription initiation, albeit it is possible that the experimental techniques that were used might not be able to detect transcription that is actually present. Therefore while transcription initiation is a strong determinant of 3’ NDR depth, it seems that DNA-histone interactions also determine 3’ NDR architecture.
3.2.6 Future Directions

3.2.6.1 Improving detection of non-coding and unstable transcripts

Since many ncRNAs are unstable (Xu et al. 2009), my RNA data set probably underrepresented their transcription levels, which would lead to underestimation of their contribution to NDR structure. To more reliably detect transcription levels, a nascent elongating transcript sequencing (NET-seq) experiment (which specifically measures elongating transcripts) could be performed (CHURCHMAN and WEISSMAN 2012). NET-seq involves native IP of FLAG-tagged pol II subunit Rpb3, followed by purification of associated transcript RNA, and sequencing; it is strand-specific, quantitative, and able to detect unstable transcripts (CHURCHMAN and WEISSMAN 2012). With an improved data set, I could better address my hypothesis that proposed that nucleosome depletion at 3’ NDRs is partly attributable to transcription initiation from the same region.

3.2.6.2 Intrinsic DNA-histone interactions that specify 3’ NDRs

My analysis showed that 3’ NDRs are observable at many genes that do not have 1) overlapping 5’ NDRs or 2) observable non-transcription initiation, or 3) high levels of gene transcription. This suggested that DNA sequences at 3’ NDRs might inherently disfavor nucleosome association. To address the contribution of DNA-histone interactions to nucleosome depletion at 3’ NDRs, DNA sequences could be analyzed at this above-mentioned class of 3’ NDRs. There are known nucleosome-favoring and nucleosome-excluding DNA sequence motifs. For example, as discussed in Chapter 1, poly-dA:dT tracts are rigid and disfavor nucleosome-association (IYER and STRUHL 1995). Analyses should be performed to determine whether the frequency at which these motifs occur in 3’ NDRs correlates with the magnitude of nucleosome depletion found in these regions.
Chapter 4

JHD2 controls mitochondrial function during yeast sporulation and germination
4.1 Abstract

As I described in previous Chapters, *JHD2* promotes accumulation of transcribed mRNA globally during the post-meiotic stages of sporulation, with the most highly induced genes exhibiting strong dependence on *JHD2* for their expression (Xu et al. 2012). To determine whether genes that are most prominently regulated by *JHD2* belonged to any specific categories, I performed gene ontology enrichment analysis and found that nuclear genes encoding mitochondrial proteins were significant targets of *JHD2* activation. In accordance with this analysis, using an oxygen consumption assay, I found that *JHD2* promotes respiration during sporulation. Interestingly, this respiration defect in *jhd2Δ* is also exhibited during the early stages of spore germination prior to re-entry into the cell cycle. Interestingly, *jhd2Δ* spores produced by heterozygous WT/ *jhd2Δ* cells (which exhibit normal respiration), do not display respiration defects during germination, suggesting that respiration activity in germinating spores is dependent on the maternal contribution of mitochondria, proteins, and/or mRNAs, and not on spore-autonomous processes. These studies showed that Jhd2 promotes mitochondrial respiration during sporulation and germination by positively regulating expression of genes involved in mitochondrial function during sporulation. Moreover, my findings suggest that spores may generally utilize inherited maternal material during the earliest steps of germination.

4.2 Introduction

4.2.1 Quiescence in budding yeast

Quiescence is a prominent but lesser-studied state of both prokaryotes and eukaryotes. Since multicellular organisms are largely composed of terminally differentiated somatic cells, research concerning regulatory pathways that govern entry as well as maintenance and exit from quiescence is highly relevant to better understanding of immune responses, oncogenesis, ageing, and cellular degenerative diseases (Gray et al. 2004).

Yeast cells exist in two types of quiescent states, as 1) quiescent spores, or 2) quiescent stationary phase cells. Stationary phase cultures are obtained by growing haploid yeast in glucose-containing media for 5-7 days until the culture becomes saturated. The culture depletes glucose and alternative carbon sources within the first few days and arrest at a G0-like state. Many characteristics of stationary phase quiescent cells are also shared by spores. Quiescent
cells are non-proliferative, and do not increase in mass or volume (Hartwell et al. 1974). It was also customarily thought that quiescent cells are also metabolically inactive, however studies of stationary-phase cells show that there is ongoing transcription and translation, although at rates that are at 5% and <1% of those found in logarithmically growing cells, respectively (Fuge et al. 1994). Transcription and translation from long-lived stores of mRNA is possible due to the presence of nucleoprotein complexes such as processing bodies (P bodies) and stress granules, which sequester and protect mRNA from degradation (Jona et al. 2000). Likely as adaptations to their environment, quiescent cells repress transcription of genes involved in glycolytic processes and ribosome biogenesis (Werner-Washburne et al. 1996) and induce expression of genes involved in nutrient salvaging through autophagy (Noda and Ohsumi 1998). To protect cellular structures from environmental stresses, quiescent cells possess condensed chromatin and thicker cell walls. They exhibit increased resistance to thermal and osmotic stress, exposure to toxic compounds, and desiccation (Pinon 1978; Plesset et al. 1987; de Nobel et al. 2000).

4.2.2 Mitochondrial respiration function contributes to fitness during quiescence

Studies have found that mitochondrial respiration is active in quiescent cells and is necessary for maintaining long term viability. This is contrary to the idea that reactive oxygen species (ROS) produced by higher respiration activity causes damage in the cell, and therefore quiescent cells would be best served by shutting off mitochondrial activity. In fact, studies from Werner-Washburne lab found that cells in stationary phase cultures belong to two biologically distinct populations: 1) quiescent cells which are unbudded, metabolically active, contain low levels of ROS, are viable upon return to favourable conditions, and 2) non-quiescent cells which are budded, possess low metabolic activity but high levels of ROS, and are largely unable to return to growth (Allen et al. 2006; Aragon et al. 2008). Significant proportions (up to 40%) of non-quiescent cells that do return to growth tend to be petites. The high incidence of petite progeny amongst non-quiescent cells is consistent with higher incidence of damage-induced instability in mitochondria and in mitochondrial genomes.

The somewhat surprising observation that higher levels of mitochondrial activity correlate with improved cellular viability and lower ROS levels, suggests that respiration could actually
promote viability by activating mechanisms that counteract ROS (ARAGON et al. 2008). Indeed, researchers have found that mitochondrial activity upregulates expression of oxidative stress response factors (ARAGON et al. 2006; AUESUKAREE et al. 2009; KURIHARA et al. 2012) and positively regulates autophagy (a process that prevents over-production of ROS by targeting damaged and excess mitochondria and proteins for destruction) in quiescent cells (NODA and OHSUMI 1998). Therefore, while mitochondrial activity is responsible for the production of harmful ROS species, it may also promote activation of cellular programs that counteract ROS levels in such a way that there is a net benefit to the fitness of the cell.

4.2.2.1 Mitochondrial activity is essential for sporulation

Respiration is required for entry into sporulation and for meiosis (JAMBHEKAR and AMON 2008). Respiration deficient yeast cells like petites and cells treated with respiration-inhibiting drugs cannot sporulate (PUGLISI and ZENNARO 1971). Indeed, a landmark study of the global gene expression landscape of sporulating cells by the Herskowitz and Brown labs found that the earliest genes induced upon initiation of sporulation are enriched for those involved in metabolism and response to nutrient limitation (CHU et al. 1998). Results from my oxygen consumption measurements in vegetative and sporulating cells showed that mitochondrial respiration activity is very high through meiosis and early sporogenesis (Figure 26), in agreement with earlier studies (HOPPER et al. 1974). Respiration begins to decrease during late sporogenesis when spores start to enter quiescence, however, even terminally differentiated spores (24 h and beyond) respire at levels that are 5% of those found in vegetative cells (Figure 26). These values are in good agreement with $^{3}$H Uracil and $^{35}$S Methionine incorporation studies in spores, which showed that transcription and translation also occur at 5% of the rate measured in vegetative cells (BRENGUES et al. 2002).

4.2.3 Spore germination and return to growth from stationary phase

Upon exposure to the right nutrient conditions, spores return to the vegetative cell state through a developmental program, called germination. While stationary phase cells do not have spore coats, they also develop a thickened cell wall that is more resistant to zymolyase treatment (DE NOBEL et al. 2000). Quiescent stationary phase cells go through a process that is similar to germination, called ‘return-to-growth’. The first stage of exit from quiescence involves...
detection of the nutrient signal. In both spores and stationary phase cells, this signal is a carbon source (SAVARESE 1974; GRANOT and SNYDER 1991; GRANOT and SNYDER 1993). Spores and stationary phase quiescent cells respond to a carbon source (such as glucose) and commit to germination/return-to-growth by shedding the spore coat (called uncoating) and the stress-resistant cell wall, respectively. Without the additional presence of an utilizable nitrogen source, uncoated cells do not enter the cell cycle and become vulnerable to death from exposure (HERMAN and RINE 1997). It has been suggested that this apparent “risky behavior” can be explained by the fact that quiescent cells are not likely to encounter a carbon nutrient source that is completely devoid of nitrogen in nature (HERMAN and RINE 1997; GRAY et al. 2004). Neither spores nor stationary phase cells exit from quiescence in response to a nitrogen source alone (HERMAN and RINE 1997; GRAY et al. 2004).

Following uncoating, germinating cells enlarge in size, lose characteristics that define quiescent cells and acquire traits of vegetative cells. The final step of germination involves re-entry into the cell cycle, which is observable as formation of a bud on the germinated cell (see Figure 25).

**Figure 25. Schematic of germination events**
The timing of germination events are based on spores germinating in glucose media. Germination in non-fermentable carbon sources takes a longer period of time.
4.2.3.1 Transcription and translation during germination

When spores emerge from quiescence, do they first initiate transcription or translation, or both? Various germination studies have monitored $[^{14}\text{C}]$ adenine / $[^{3}\text{H}]$ uracil and $[^{35}\text{S}]$ phenylalanine/methionine incorporation to measure the kinetics of de novo transcription and translation, respectively (ROUSSEAU and HALVORSON 1973b; XU and WEST 1992). While transcription is detectable within minutes of germination initiation, the levels were extremely low until more than 90 minutes after germination initiation. In contrast, translation attains levels found in vegetative cells within 30 minutes after germination initiation. These studies suggest that the initial germination gene expression program relies on protein synthesis from pre-existing stores of mRNA, and that contribution from de novo transcription does not become significant until at least 1.5 hours after initiation of germination, after commitment to uncoating.

4.2.3.2 Stores of long-lived mRNA in quiescent cells

The existence of stores of long-lived mRNAs is well documented (FUGE et al. 1994; BRENGUES et al. 2002; ARAGON et al. 2006; GEIJER et al. 2012). During times of starvation or stress, evolutionarily conserved ribonucleoprotein structures form in the cytoplasm of cells in organisms ranging from yeasts, worms, flies, and mammals (ARRIBAS-LAYTON et al. 2013). In general, mRNAs in these structures are stabilized but translationally-repressed (FRANKS and LYKKE-ANDERSEN 2008). Two well characterized ribonucleoprotein complexes include processing bodies (or P-bodies) which are assembled under starvation conditions, and stress granules, which form after entry into quiescence (SHAH et al. 2013).

4.2.4 Chapter rationale

The experiments described in this chapter fall into two categories, those that were designed to investigate $JHD2$’s role in modulating mitochondrial biology, which was the original impetus of these studies, and those that were designed to better understand processes that underlie germination and stationary phase exit in general. The latter category of experiments is important in itself, as spore germination remains a poorly studied field. Better understanding of the mechanisms that underlie germination in yeast could also be applicable to those involved in oncogenic transformation in otherwise non-proliferative somatic cells in higher organisms.
4.3 Materials and Methods

4.3.1 Yeast strains

Table 3. Yeast strains

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<td>S288c</td>
<td>MATa MET15 HIS3 LEU2 URA3 LYS2 jhd2Δ::Htg</td>
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</tbody>
</table>

4.3.2 Yeast culturing media

Synthetically defined media:

YNB synthetically defined media was used for germination experiments. YNB (yeast nitrogen base) medium contains vitamins, minerals and salts; its name is somewhat misleading since it does not contain a nitrogen source. To support yeast growth, a nitrogen source of a cocktail of amino acids and a carbon source is added to YNB medium. The following media were used for my germination experiments:

- YNB a.a. 2% glucose media (YNB with amino acid cocktail and 2% glucose),
- YNB a.a. (YNB with amino acid cocktail and no glucose), and
- YNB 2% glucose (YNB with 2% glucose and no amino acids).

Complex media:

YP (containing yeast extract and peptone) complex media was used with addition of either acetate as a non-fermentable carbon source (YPA), or glucose as a fermentable carbon source (YPD).

4.3.3 Expression microarray

Sporulation expression microarray profiling using ORF arrays was performed by my supervisor Marc Meneghini and the protocol is described in (Xu et al. 2012).
4.3.4 Oxygen consumption assay

Oxygen consumption of sporulating cells in suspension was measured using a platinum oxygen electrode (also called a Clark oxygen electrode) (Strathkelvin 782 oxygen meter) at 30°C. For maximal oxygen consumption measurements, mitochondrial membrane potential was decoupled by addition of CCCP (carbonyl cyanide m-chlorophenyl hydrazone) to a final concentration of 8 µM. To assess whether mitochondrial respiration was responsible for oxygen consumption, cyanide was used to inhibit electron transport chain function at a final concentration of 10 µM. Oxygen consumption rate was measured in units of nmol atoms (natoms) of oxygen per minute per one million cells (natom/min/M).

4.3.5 Yeast culture growth assay

Liquid culture time course growth assays were performed using an automated temperature-controlled microplate spectrophotometer (TECAN infinite 200). Cultures were grown overnight to saturation in test tubes, and then inoculated for growth assays at 0.2 O.D.₆₀₀ in 200 µL of fresh growth media in 96-well microplates (Sarstedt, round bottom). Microplates were sealed with an air-permeable membrane (E&K Scientific) and incubated at 30°C with shaking at 15 min intervals for 48 hours. O.D.₆₀₀ readings were collected every 15 minutes.

4.3.6 Germination growth methods

Spores for germination experiments were made using standard sporulation protocols that have been described in previous sections. To eliminate unsporulated vegetative cells from spore samples, spore cultures were pelleted, resuspended in 3 mg/mL zymolyase (Seikagaku) and incubated for 1 h at room temperature, followed by washing and resuspension in 0.5% Triton X-100 and vortexing. Lysis of unsporulated cells was confirmed by visual inspection under a light microscope. Zymolyase-treated spores are extremely sticky, so Triton X-100 was added to any liquid media containing germinating spores to a final concentration of 0.5% in order to keep spores well suspended.
4.4 Results

4.4.1 JHD2 promotes expression of genes involved in mitochondrial respiration functions during sporulation

JHD2 promotes protein coding gene transcription in opposition to programmed onset of global transcriptional quiescent following the completion of meiosis, and jhd2Δ causes precocious global transcriptional shut-down (Xu et al. 2012). In order to better understand Jhd2’s role during sporulation, I asked whether any specific cellular processes were particularly affected in jhd2Δ. To do this, I performed differential analysis on transcript accumulation signals at time points through sporulation (Transcript signal\textsubscript{WT} - Transcript signal\textsubscript{jhd2Δ}). First, I ranked the genes by magnitude of defect in jhd2Δ, and then I determined gene ontology (GO) term enrichments in the top 10% (500) transcripts at each sporulation time point. Strikingly, this analysis revealed enrichment for genes involved in mitochondrial function (see Table 4). In particular, a significant number of components of the mitochondrial respiratory chain were enriched at nearly all time points. Based on this, I posited that jhd2Δ cells might exhibit compromised mitochondrial respiration function during sporulation.

4.4.2 Does JHD2 promote mitochondrial respiration during germination?

As discussed earlier, studies suggest that the initial steps of germination/stationary phase exit rely on translation of pre-existing long-lived mRNA stores (Herman and Rine 1997; Brenagues et al. 2002; Aragon et al. 2006). Therefore, jhd2Δ spores might retain the defect of reduced abundance of mitochondrial transcripts from sporulation. Based on this reasoning, I hypothesized that any mitochondrial respiration phenotypes exhibited by sporulating jhd2Δ cells might be inherited and exhibited by germinating spores as well. In other words, if jhd2Δ cells exhibit a respiration defect during sporulation, then germinating jhd2Δ spores are likely to exhibit the same phenotype. Furthermore, since spores germinate with high levels of synchrony (Brenagues et al. 2002), germination may serve as a more accessible context than sporulation to measure jhd2Δ phenotypes.
Table 4. GO term enrichment terms of the 500 genes with the greatest (WT-jhd2Δ) differential mRNA signal through sporulation

<table>
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4.4.3 Respiration kinetics of cells during sporulation and germination

Before investigating the contribution of Jhd2 function to respiration during sporulation and germination, I first characterized respiration kinetics of sporulation and germination in wild type SK1 cells. While one early study describes respiration during sporulation, it utilized a strain background that exhibits reduced sporulation efficiency compared to the SK1 background that I use for my studies (HOPPER et al. 1974).
I measured the rate of dissolved O$_2$ consumption as an indicator of the metabolic status of sporulating cells. O$_2$ consumption rates throughout sporulation showed that cells began exhibiting reduced mitochondrial respiration rates during meiosis and reached a minimum metabolic rate by the 16 hour time point. This was in agreement with earlier studies (Figure 26) (HOPPER et al. 1974; KUENZI et al. 1974). I found that terminally differentiated spores (36 hours post induction) consume oxygen at approximately 4% the rate of vegetative cells (Figure 26). I confirmed that the observed oxygen consumption reflects mitochondrial respiration by showing that it can be enhanced by the addition of CCCP (carbonyl cyanide m-chlorophenyl hydrazine). CCCP causes decoupling of the proton gradient across the mitochondrial inner membrane, which results in maximal activity of the electron transport chain (ETC) and oxygen uptake at the maximal capacity. I also showed that addition of cyanide, an inhibitor of the mitochondrial ETC, completely inhibited oxygen consumption (Figure 26, red line).

Some early studies that describe exit from metabolic quiescence during germination showed that oxygen consumption rates increase steadily over the course of hours as cells exit from quiescence and enter the cell cycle (SEIGEL and MILLER 1971; DONNINI et al. 1986). To characterize respiration kinetics of SK1 WT germinating spores, I treated sporulated cultures (which contained more than 95% spores) with zymolyase and Triton-X100 to remove unsporulated cells which might confound germination measurements, as described in earlier studies (HERMAN and RINE 1997). Next, I germinated the spores in glucose-containing media. I found that spores responded to the germination media by increasing their respiration rate within minutes, and respiration rates continued to increase over the course of the next 5-7 hours as the germinating spores progressed (Figure 26 and Figure 25), finally reaching pre-sporulation levels as spores re-entered the cell cycle as vegetative cells (Figure 26). I found that germination takes a much longer time in media containing non-fermentable carbon sources, with cells re-entering the cell cycle after more than 24-30 hours in YPA (acetate) (Figure 32). Despite the much longer germination time in non-fermentable carbon, spores still responded to incubation in growth media by increasing respiration rate within minutes, and respiration rates continued to increase steadily over time from 1-30 hours after addition of acetate medium (Figure 30 A and B, black line). It is interesting to note that spores do not uncoat until after more than 24 hours in acetate-containing (YPA) germination medium, despite exhibiting high levels of respiration at that time point (Figure 30B). These data suggest that the induction of mitochondrial metabolic
activity is one of the earliest cellular responses during initiation of germination, and that restoration of mitochondrial activity back to vegetative levels is an ongoing process during germination.

Figure 26. Mitochondrial respiration throughout yeast sporulation and germination
Mitochondrial respiration rates of sporulating cells were assessed by measurement of rate of dissolved oxygen consumption in units of natoms (molar atoms) per minute per 1 million cells (natom/min/M). Error bars represent one standard deviation of technical replicates. Maximal respiration rate (O$_2$ MAX) was measured after addition of the respiratory chain uncoupler carbonyl cyanide $m$-chlorophenyl hydrazine (CCCP). Cyanide was added to inhibit the mitochondrial electron transport chain (specifically, cytochrome C oxidase). ‘Vegetative’ represents yeast grown in YPD media (complex media with glucose).
4.4.4  **JHD2 promotes mitochondrial respiration during sporulation**

Having established the wild type kinetics of respiration during sporulation, I moved on to address my first hypothesis: that a reduction in the abundance of mitochondrial transcripts in \( jhd2\Delta \) cells during sporulation would manifest as reduced respiration rate. To test this, I sporulated WT and \( jhd2\Delta \) cells under identical culturing conditions and verified that the cultures were synchronous by monitoring meiotic progression by microscopic examination of DAPI stained cells. Using oxygen consumption assays, I found that \( jhd2\Delta \) cells exhibited reduced respiration during sporulation (Figure 27), in good agreement with my analysis of the \( jhd2\Delta \) transcription defects (Table 4).

![Graph showing oxygen consumption rates](image)

**Figure 27. JHD2 promotes mitochondrial respiration during yeast sporulation**

Oxygen consumption rates were measured in WT and \( jhd2\Delta \) cultures over the course of sporulation. Meiotic synchrony between the two cultures was monitored microscopically using DAPI staining of meiotic nuclei. Error bars indicate one standard deviation of technical replicates. ‘Vegetative’ represents yeast grown in YPD media (complex media with glucose).

4.4.5  **JHD2 promotes mitochondrial respiration during early germination**

To test the theory that germinating \( jhd2\Delta \) spores exhibit mitochondrial activity defects inherited during sporulation, I germinated WT and \( jhd2\Delta \) spores under identical growth conditions in media that contained glucose as the carbon source, and found that \( jhd2\Delta \) spores respire at significantly lower levels compared to WT cells (Figure 28). This was true for germinating spores that range in age from 24 hours to 2 weeks. These respiration results have been
confirmed with many biological replicates, using both complex (YPD) and synthetically defined (YNB 2% glucose) glucose-containing media, Figure 28 displays some representative results. I will sometimes refer to this phenotype as jhd2Δ exhibiting 'hypo-respiration'. The defect in jhd2Δ is restricted to germination; by the time cells re-enter the cell cycle and return to vegetative growth, respiration levels in jhd2Δ cells catch up to those in WT cells (6-7 hours germination) (Figure 28C). This was expected since I knew from earlier experiments that jhd2Δ cells had no respiration defect in the vegetative state when grown in glucose medium (Figure 27, vegetative time point).
Figure 28. jhd2Δ spores exhibit reduced mitochondrial respiration rates during early germination compared to WT

Sporulated cultures were treated with zymolyase and triton-X100 to remove un-sporulated cells, and spores were resuspended in various glucose-containing media (2%) at an O.D.₆₀₀ of 0.5 and incubated at 30°C with shaking at 200 rpm. Oxygen consumption and maximal oxygen consumption (CCCP treatment) were measured at the indicated time points. Error bars represent one standard deviation of technical replicates. A) 24 h old spores germinated in YPD B) 7-day old spores germinated in synthetically defined YNB a.a. 2% glucose media C) 6-day old spores germinated in synthetically defined YNB a.a. 2% glucose media.

4.4.6 Reduced respiration capacity exhibited by jhd2Δ germinating spores likely reflects a defect in maternal contribution

To determine whether the respiration defect in jhd2Δ germinating spores was due to: 1) a defect in the maternal-load of mitochondria/mRNA as I first hypothesized, or 2) to spore-autonomous
defects in $jhd2\Delta$ spores, I utilized a heterozygous $jhd2\Delta/JHD2$ strain to test for maternal effect. First, I made the assumption that $jhd2\Delta/JHD2$ cells behave like WT cells for respiration phenotypes, due to the presence of a functional copy of $JHD2$ (this assumption proved to be valid, see Figure 29). When a heterozygous cell sporulates, it produces two WT and two $jhd2\Delta$ spore progeny. If maternal effects dominate respiration functions during early germination, then both WT and $jhd2\Delta$ progeny should respire like WT cells; conversely, if respiration phenotypes are spore-autonomous, then $jhd2\Delta$ progeny would exhibit a respiration defect that should be detectable in a germinating culture.

I found that the germination culture of spores from $jhd2\Delta/JHD2$ heterozygotes (consisting of one half WT and one half $jhd2\Delta$ spores), respire at WT levels (Figure 29). This means that $jhd2\Delta$ spores of phenotypically WT mother cells respire at WT levels, which is consistent with the maternal-load model. Maternal contribution of cellular components and/or mRNA might play a role in early germination in general, preceding any de novo transcription in germinating spores, making early germination an useful context in which to study sporulation-related phenotypes.

![Figure 29. Heterozygous $jhd2\Delta/JHD2$ spores germinate with WT levels of respiration.](image)

6-day old cultures of isogenic WT, heterozygous $jhd2\Delta/JHD2$, and homozygous $jhd2\Delta/jhd2\Delta$ spores were germinated in YNB a.a. 2% glucose media. The $jhd2\Delta/JHD2$ sporulation culture does not exhibit any oxygen consumption defects. Error bars represent one standard deviation of three technical replicates. *Note that the same WT and $jhd2\Delta$ respiration data is also shown in Figure 28C.
4.4.7 In vegetative respiratory conditions, *JHD2* repress mitochondrial gene expression and mitochondrial function

Although Jhd2 does not impact gene expression in non-respiring vegetative cells, studies from the Meneghini lab have found that Jhd2 acts as a global regulator of H3K4me dynamics and gene expression in respiring cells utilizing non-fermentable carbon sources during vegetative growth (Meneghini lab unpublished data) and sporulation (*Xu et al.* 2012). In sporulating cells, Jhd2 acts to promote respiration. In contrast, fellow PhD student Maria Soloveychik discovered that Jhd2 represses mitochondrial function in vegetative cells grown in non-fermentable carbon sources (e.g. acetate) (Meneghini lab unpublished data). Correspondingly, *jhd2Δ* cells are hyper-respiratory and exhibit up-regulation of genes encoding proteins involved in mitochondrial respiration function.

I was able to document the predicted transition from hypo-respiration to hyper-respiration in *jhd2Δ* by germinating *jhd2Δ* and WT spores in non-fermentable (YPA) media. *jhd2Δ* spores are initially hypo-respiratory compared to WT, but become hyper-respiratory at some point between 5 hours and 21 hours incubation in non-fermentable media (Figure 30). As mentioned before, spore cultures in YPA only start to re-enter the cell cycle around 24 to 30 h after incubation. Interestingly, even though the germination cultures incubated in acetate-containing medium were morphologically indistinguishable from spores, they exhibited high levels of mitochondrial activity that are comparable to those of vegetative cells. These later germination time points (around 20-24 hours) in acetate medium present a good context to study *JHD2* and mitochondrial respiration phenotypes in general; because the germinating cells have not re-entered the cell cycle, there are no confounding effects that arise from differences in cultures densities and growth. In addition, the magnitude of the *jhd2Δ* phenotype during germination (1.4 to 1.5 fold hyper-respiration over WT) is much more pronounced than those observed during vegetative growth.
Figure 30. *JHD2* exhibits reversal of activity in regulation of mitochondrial activity in sporulation/germination cells versus resiping vegetative cells

**A)** Sporulation cultures incubated in SPO media for 12 days were treated to remove non-sporulated cells as described before and germinated in YPA media. At 30 h of incubation in germination media, spores are still morphologically identical to un-germinated counterparts. **B)** 10-day old sporulated cultures were germinated in YPA as in A) in an independent biological replicate. Error bars represent one standard deviation of technical replicates.

4.4.8 Mitochondrial activity is not required for germination in fermentable carbon sources, but contributes greatly to timely germination

I next asked whether the respiration defect in jhd2Δ cells affects cell growth during germination, specifically, whether jhd2Δ cells are delayed in exit from dormancy and re-entry into active cell division. Published data from the Meneghini lab show there is no detectable germination growth phenotype for jhd2Δ cells by spot assay on solid YPD media (Xu et al. 2012). I performed more sensitive growth curve measurements using an automated spectrophotometer taking O.D. measurements of liquid cultures in microplates every 15 minutes for over 48 hours. This also failed to show any difference in growth dynamics between germinating jhd2Δ and WT cells (Figure 31). Many studies have shown that mitochondrial activity is not required for germination in fermentable carbon sources (Savarese 1974; Tingle et al. 1974; Donnini et al. 1986); however, since I have found that mitochondrial respiration is one of the earliest cellular responses to germination signal, I hypothesized that it might nevertheless contribute to germination in an important way. To evaluate the contribution of mitochondrial respiration...
activity to germination dynamics in wild type cells germinating in glucose media (YPD), I compared germination in normal YPD medium and in YPD medium containing the mitochondrial ETC inhibitor cyanide. Very interestingly, germination in YPD media (measured as re-entry into the cell cycle) was delayed by at least 10 hours when mitochondrial respiration activity was inhibited (Figure 32). This showed that while respiration is not required for germination, it clearly contributes to processes that prepare cells for exit from quiescence. To show that the cyanide was able to completely block respiration, I carried out germinations using YPA media containing cyanide as a negative control. YPA contains the non-fermentable carbon source acetate, which cannot be metabolized without mitochondrial respiration. These cultures failed to germinate and re-enter the cycle, as expected (Figure 32).

Figure 31. WT and jhd2∆ spores germinate with the same growth kinetics in liquid culture using standard YPD media. Sporulated cultures were treated with zymolyase and triton-X100 to remove un-sporulated cells, and spores were inoculated in YPD at an O.D.₆₀₀ of 0.2 and incubated at 30°C. Optical density was measured using a TECAN microplate reader. Graphed data represents one technical replicate of three. All technical replicates showed identical growth kinetics.
Figure 32. Mitochondrial ETC activity is not required for germination, but very important for timely germination in media containing fermentable carbon
Sporulated wild type culture was treated with zymolyase and triton-X100 to remove non-sporulated cells, and spores were inoculated in YPD or YPA at an O.D.\textsubscript{600} of 0.2 and incubated at 30°C. Optical density was measured using an automated microplate spectrophotometer (Tecan). Cyanide was added to a final concentration of 10 µM. An equal volume of carrier solvent DMSO was added to the control culture.

4.4.9 Protein synthesis is required starting from the early stages of germination to drive continued respiration response

Studies in yeast germination have established that protein synthesis is required to accomplish early stages of germination such as uncoating. Spore uncoating occurs around 2 h into the germination program and it is the earliest known morphological landmark that fails to occur in the absence of protein synthesis (ROUSSEAU and HALVORSON 1973a; CHOIH \textit{et al.} 1977; HERMAN and RINE 1997). I investigated the role of protein synthesis in germination respiration response. To inhibit translation, I incubated spores in media containing protein synthesis inhibitor cycloheximide, and measured oxygen consumption in time course experiments. These cultures failed to germinate and re-enter the cell cycle, as expected. I found that even in the absence of protein synthesis, spores responded to the presence of germination media within minutes by increasing their rate of oxygen consumption, as observed before. However, oxygen consumption rates failed to increase past one hour into incubation (Figure 33). This suggests that protein synthesis is involved in restoring mitochondrial activity back to levels found in metabolically active cells.
Figure 33. Protein synthesis is required for increases in respiration response beyond 1h of germination

Sporulated wild type culture was treated with zymolyase and triton-X100 to remove non-sporulated cells, and pre-incubated in 30 µg/mL cycloheximide and 0.5% triton-X for 30 min. Spores were then inoculated in YPD with 30 µg/mL cycloheximide at a density of 0.5 O.D.600 at 30°C. Control cultures were incubated with equivalent amounts of DMSO (the solvent for cycloheximide). Error bars represent one standard deviation of technical replicates.

The rapid initial response in respiration within the first hour of germination likely comes from oxygen consumption by the existing spore mitochondria that metabolize the influx of nutrients in the germination media. However, further increases in mitochondrial activity past one hour appear to be dependent on de novo synthesis of protein and cellular components that contribute to mitochondrial function and mitochondrial biogenesis.

4.4.10 Germination requires the presence of both a nitrogen source and a carbon source; however, introducing either nutrient source on its own is sufficient to induce a respiration response

Previously studies investigating the nutritional requirements for spore germination have found that a metabolizable carbon source alone is sufficient for spore uncoating to occur during early germination (2 hours into germination in YPD) (HERMAN and RINE 1997). In the presence of a nitrogen source alone, spores do not uncoat. The presence of both a carbon and a nitrogen source are required for germinated spores to re-enter the cell cycle. I investigated the contribution of carbon and nitrogen sources on triggering the respiration response in spores.
When I incubated spores in synthetically defined medium containing only glucose, I detected the initial respiration response in spores, but no further respiration increases beyond the first hour (Figure 34A). I observed the same type of response in spores incubated in synthetically defined medium containing only a nitrogen source (amino acid cocktail) but lacking glucose (Figure 34B). The respiration responses from incubating spores with only a carbon source or only a nitrogen source are very modest compared to rates observed when both carbon and nitrogen sources are provided (compare ‘Carbon and Nitrogen’ to ‘Carbon only’ or ‘Nitrogen only’ in Figure 34 A and B, respectively). There is no measureable difference in the respiration response of WT and jhd2Δ spores in these media conditions (Figure 34C and D). Therefore, spores respond to the presence of either carbon (glucose) or nitrogen (amino acid cocktail) by increasing rates of mitochondrial respiration, but do not upregulate respiration response beyond one hour of incubation.
Figure 34. A carbon or nitrogen source alone are sufficient to trigger an early mitochondrial respiration response, but not beyond the first hour of incubation.

Four-day old spores were incubated in A) ‘Carbon source only’: YNB 2% Glucose without a nitrogen source, B) ‘Nitrogen source only’: YNB (a.a.) amino acid cocktail without glucose. Both cultures in A) and B) were paired with a culture in ‘Carbon and Nitrogen’: YNB a.a. 2% glucose for comparison. C) & D) 24 h cultures of WT and jhd2∆ spores were incubated in media as in A) and B), respectively. Note that the same respiration data for WT are also shown in A) and B). Error bars represent one standard deviation of technical replicates.

4.4.11 Stationary phase yeast cultures exhibit respiratory quiescence and restoration of respiration upon return-to-growth that phenotypically parallel sporulation and germination.

Considering the physiological and molecular similarities between stationary phase and sporulation, I asked whether stationary phase cells also exhibit similar mitochondrial respiration activity, and whether JHD2 plays a role in return-to-growth respiration phenotypes. Following established protocols to make stationary phase cells, I cultured WT and jhd2∆ cells in glucose containing media (YNB a.a. 2% glucose) for six or more days until the culture had reached saturation and ceased to increase in optical density (ARAGON et al. 2008). I found that stationary phase cells respire at very low rates, even when compared to terminal spores (1-2 natoms/min/M
in stationary phase cells compared to 4-5 natoms/min/M in spores). Similar to spores, I found that stationary phase cells respond very quickly to nutrient availability, with oxygen consumption rates increasing a small but significant amount within minutes of incubation in growth media (data not shown). Stationary phase cells return to growth within 5 hours of incubation in rich media. Analogous to germination, jhd2Δ cells exhibit reduced mitochondrial respiration during stationary phase exit (Figure 35). Also paralleling germination, once cells resumed vegetative growth in a fermentable carbon source, the respiration defect in jhd2Δ cells disappeared and respiration rates became indistinguishable from those of WT cells (Figure 35, 5 h).

**Figure 35.** jhd2Δ cells exhibit reduced rates of oxygen consumption upon exit from stationary phase
To obtain a population of stationary phase cells, WT and jhd2Δ cultures were grown to saturation for 6 days in YNB a.a. 2% glucose medium at 30°C. Cells were washed and incubated in fresh YNB a.a.2% glucose media at an O.D.₆₀₀ of 0.5 for stationary phase exit oxygen consumption measurements. Error bars represent one standard deviation of technical replicates.

### 4.5 Summary
Studies described in this chapter were focused on investigating the role of JHD2 in regulating mitochondrial respiration activity. By analyzing sporulation expression microarray data, I found that nuclear-encoded mitochondrial genes, which are one of the first classes of genes to be induced during sporulation (Chu et al. 1998), are particularly dependent on Jhd2 for transcript accumulation. Genes that encode components of the mitochondrial respiratory chain were especially affected throughout sporulation time points (Table 4). I hypothesized that JHD2
could promote mitochondrial activity during sporulation, and that \textit{jhd2}\textdagger cells would exhibit compromised mitochondrial respiration function. By measuring rates of oxygen consumption in sporulating cultures, I found that \textit{jhd2}\textdagger does indeed exhibit reduced respiration activity.

Studies of transcription and translation activity in spores have suggested that during early stages of germination (within 1.5 hours of initiation of germination), spores utilize maternal transcripts for protein synthesis. I hypothesized that if this is the case, then the mitochondrial transcript accumulation defect of \textit{jhd2}\textdagger during sporulation could be carried over to germination as well. I performed oxygen consumption assays of germinating \textit{jhd2}\textdagger and WT to test this idea, and found that \textit{JHD2} does indeed promote mitochondrial respiration during germination, prior to time points that correspond to commencement of spore-autonomous transcription programs.

In the process of investigating \textit{jhd2}\textdagger-related germination phenotypes, I also began to study the contribution of mitochondrial respiration to germination biology in general. I found that spores, which are considered to be completely metabolically quiescent, possess mitochondria which are poised for respiration activity. Spores exhibit increased mitochondrial respiration activity in the presence of carbon sources such as glucose, ethanol, and acetate (data not shown). This respiration response is very rapid, as oxygen consumption rates increase within minutes of introduction of nutrients. Quite surprisingly, spores responded with increased respiration activity even to introduction of a nitrogen source alone (Figure 34). During germination and re-entry into the cell cycle, spores exhibited progressively increased levels of oxygen consumption. These rates finally reached levels that were comparable to those observed for vegetative cells as germinating cells re-entered the cell cycle.

My studies suggest that there are two stages of respiration response; in the first stage, spores respond to the availability of nutrients almost immediately with a low level of oxygen consumption activity. This respiration activity likely reflects metabolic response of existing spore mitochondria to the influx of nutrients. In nutritionally complete rich media, this stage lasts for one hour. In media that contain only carbon, or only nitrogen, or in the absence of protein synthesis, spore respiration does not progress past the initial level of activity. In the presence of both carbon and nitrogen, spores are able to move on to the second stage of respiration response. They exhibit progressively increased rates of mitochondrial respiration until the completion of germination. The secondary respiration response of germinating spores
is dependent on *de novo* translation, which likely contributes to biosynthetic processes that return germinating spores to a metabolically active state.

### 4.6 Discussion and Future directions

Studies of the global transcription program of germinating cells show that thousands of mRNAs are significantly upregulated within minutes of exposure to a germinant (Martínez *et al.* 2004; Geijer *et al.* 2012). Since levels of *de novo* transcription are very modest during early germination (Rousseau and Halvorson 1973a; Xu and West 1992; Brengues *et al.* 2002), the rapid and prominent transcript upregulation during germination could reflect mRNA released from ribonucleoprotein complexes. This idea has been proposed previously by Grier *et al.* (2012). I think it is likely that transcript profile data reflect a combination of *de novo* transcription and release of extraction-resistant mRNAs from nucleoprotein complexes. However, no study to date has attempted to differentiate between these processes.

In this chapter of my thesis, I presented an interesting phenomenon in *jhd2Δ* cells that supports this theory. Sporulating *jhd2Δ* cells exhibit a hypo-respiration phenotype due to a global defect in gene expression that is especially pronounced in genes that encode mitochondrial respiration proteins. Work by fellow Ph.D. student Maria Soloveychik shows that vegetative *jhd2Δ* cells grown in respiring conditions (on non-fermentable carbon) over-express mitochondrial respiration genes and exhibit a hyper-respiration phenotype, which is the reverse of its phenotype during sporulation. I was able to show that *jhd2Δ* spores germinating in non-fermentable carbon exhibit the hypo-respiration phenotype of sporulation for the duration of germination (0-5 h), but switch to the hyper-respiration phenotype upon return to growth (past 6-7 h germination). This is consistent with the idea that early germination utilizes stored mRNA that retain the expression profile of respiration deficiency, which is replaced by the vegetative expression profile of hyper-respiration as spore mRNAs are degraded and *de novo* transcription increases during later germination time points.

Interestingly, Geijer *et al.* found that transcripts involved in protein folding and transport are detected at higher abundance at the very onset of germination. This supports the idea that translation and protein synthesis dominates the initial germination response. Considering that gene expression is the combined outcome of transcription, mRNA stability, the rate of protein
synthesis, it is very difficult to infer gene expression from mRNA microarray data alone, especially during a biological context where it is known that translation is much more active than transcription. As such, global proteomic profiling studies of germination and stationary phase exit may prove to be very informative.

An interesting question that remains to be addressed is whether de novo transcription is required for early stages of germination, and more specifically for the respiration response. To address this question, respiration experiments should be performed on germinating cells that lack transcription activity. Transcription can be inhibited using multiple approaches. For example, cells expressing temperature sensitive alleles of RNA pol II can be sporulated under permissive temperatures, and shifted to the non-permissive temperature for germination. Alternatively, drugs that inhibit transcription could be added to germinating cultures (e.g. thiolutin) (GRIGULL et al. 2004). There could be caveats to each of these methods, e.g. germination at a higher temperature might have confounding effects on respiration, and transcription inhibitors might have off-target effects. Therefore it will be important to try both approaches. If transcription is dispensable during early stages of germination, I expect that a secondary respiration response would occur despite pol II inhibition. The maternal genetic contribution in germinating spores could prove to be analogous to maternal to zygotic transition processes in metazoans. If so, it could have potential utility as a model system to study such mechanisms in a powerful and genetically facile system.

I was unable to find a growth defect associated with the germination respiration deficiency associated with loss of Jhd2 function. In light of the importance of mitochondrial activity for timing of germination, I propose that JHD2’s contribution to germination should be more apparent under germination conditions that are more dependent on the respiration capabilities of the germinating cell. For instance, the respiration defect in jhd2Δ could cause germination and cell cycle re-entry phenotypes when germination occurs in suboptimal media that challenge the metabolic capacity of spore mitochondria.
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