Topic Introduction

Analysis of Heterochromatin in *Schizosaccharomyces pombe*

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This introduction briefly describes the biology of heterochromatin in the fission yeast *Schizosaccharomyces pombe*. We highlight some of the salient features of fission yeast that render it an excellent unicellular eukaryote for studying heterochromatin. We then discuss key aspects of heterochromatin that are of interest to those in the field, and last we introduce experimental approaches often used to investigate heterochromatin.

INTRODUCTION

The genetic content of the nucleus has long been known to partition into active and inactive regions termed euchromatin and heterochromatin, respectively (Passarge 1979). The study of heterochromatin has yielded fundamental insights into the factors and processes that control gene silencing and genome organization (Weiler and Wakimoto 1995). Even though initial observations and studies of heterochromatin were made in plants and metazoans, fission yeast has proven to be an invaluable model for elucidating the conserved pathways governing heterochromatin formation (Klar 2007). Compared with the budding yeast *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* possesses several features that make its heterochromatin more similar to that of metazoans. These include possessing a SUV39H1 homolog (Clr4p) responsible for histone H3 lysine 9 methylation (H3K9me) and Swi6p, a heterochromatin protein 1 (HP1) ortholog (Grewal and Jia 2007). Furthermore, similar to metazoans, the *S. pombe* genome encodes large tracts of repetitive elements that are assembled into heterochromatin (Cam and Grewal 2004). The discovery of RNA interference (RNAi)-mediated heterochromatin assembly coupled with the growing interest in epigenetics has further cemented fission yeast as a popular single-cell organism for studying heterochromatin (Volpe et al. 2002). In addition to the availability of sophisticated molecular, biochemical, and genomic tools, a wealth of information can be obtained from simple reporter gene assays. These attractive features of *S. pombe*, combined with low maintenance and ease of genetic manipulation, result in a somewhat low barrier to entry for those wishing to adopt fission yeast for heterochromatin research. This introduction covers the aspects of fission yeast heterochromatin that are most often studied: (1) nuclear organization of heterochromatin; (2) heterochromatic silencing of reporter genes; (3) heterochromatin-associated transcripts and short interfering RNAs (siRNAs); (4) nucleosome organization within heterochromatin; and (5) molecular factors and processes controlling heterochromatin assembly. Below is a brief overview of the techniques that span these areas.

The original discovery of heterochromatin by cytogenetic staining techniques in plants was possible because heterochromatin has a different DNA sequence composition compared with that of

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euchromatin. In addition, because heterochromatic loci tend to cluster and occupy a distinct nuclear area, microscopic and cytological staining techniques can be used to visualize heterochromatic foci (for further discussion of this approach, see Introduction: Fixed Cell Imaging of *Schizosaccharomyces pombe* [Hagan and Bagley 2016]).

One of the simplest yet most powerful tools to monitor heterochromatin function is the use of reporter genes. Reporter genes inserted into, or adjacent to, heterochromatin in *Drosophila* are subject to clonally inherited transcriptional silencing, a process known as position-effect variegation (Elgin and Reuter 2007). A similar phenomenon occurs in *S. pombe* (Thon and Klar 1992; Allshire et al. 1994; Nimmo et al. 1994), and so by using reporters that encode auxotrophic markers, one can exploit simple growth assays on selective media as reliable readouts of heterochromatic silencing. These assays take advantage of two features of heterochromatin. First, heterochromatin often spans large blocks of DNA, allowing an inserted reporter gene to be silenced (Henikoff 2000). Second, heterochromatin can spread in a manner that is DNA sequence-independent. Therefore, a reporter located outside heterochromatin can be silenced if the spreading of heterochromatin is enhanced (e.g., by HP1/Swi6 overexpression). By designing genetic screens for suppressors and enhancers of silencing, one can identify heterochromatin components. Reporter assays can also be used to distinguish between those factors required for heterochromatin establishment and those required for maintenance (Sadaie et al. 2004; Buscaino et al. 2013).

Despite their utility, reporter genes cannot fully capture the status of heterochromatic transcription for several reasons. First, a reporter gene is not sufficiently sensitive to detect subtle changes in heterochromatic transcription in certain mutants (Noma et al. 2004). Second, transcription of heterochromatic repeats occurs on both strands (Volpe et al. 2002). Third, heterochromatin contains hot-spots of siRNAs that act as nucleation sites of heterochromatin assembly (Cam et al. 2005). In the following section of this introduction we, therefore, discuss the biology of heterochromatin-associated bidirectional transcripts and siRNAs and describe techniques for their detection (see Protocol: Analysis of *Schizosaccharomyces pombe* Heterochromatin-Associated Short Interfering RNAs [Zhang 2016]).

Proper nucleosome positioning and occupancy is central to chromatin function. This can be probed using micrococcal nuclease (MNase) and, accordingly, we describe below the use of this tool to study silent regions of the *S. pombe* genome (a method is provided in Protocol: Micrococcal Nuclease Digestion of *Schizosaccharomyces pombe* Chromatin [Cam and Whitehall 2016a]). Ultimately, much of the recent headway in understanding heterochromatin is due to our ability to identify its molecular constituents. This is possible with the use of chromatin immunoprecipitation (ChIP) assays to detect proteins that localize to heterochromatin in vivo. By performing ChIP in various mutants, one can define steps that govern the assembly of heterochromatin. The use of ChIP to study heterochromatin is discussed below, and a ChIP method is presented in Protocol: Chromatin Immunoprecipitation (ChIP) in *Schizosaccharomyces pombe* (Cam and Whitehall 2016b).

Great strides in genomics have led to genome-scale approaches for the study of heterochromatin and many techniques, including Protocol: Analysis of *Schizosaccharomyces pombe* Heterochromatin-associated Short Interfering RNAs (Zhang 2016); Protocol: Micrococcal Nuclease Digestion of *Schizosaccharomyces pombe* Chromatin (Cam and Whitehall 2016a); and Protocol: Chromatin Immunoprecipitation (ChIP) in *Schizosaccharomyces pombe* (Cam and Whitehall 2016b), are readily adaptable to genome-scale analysis. However, because the fission yeast genome is well-annotated and most of the major domains are well-studied with many reporter strains already available (see Table 1), a researcher can address many questions about heterochromatin without resorting to still somewhat costly genome-scale analysis.

STUDYING HETEROCHROMATIN

Analysis of Heterochromatin Reporter Gene Silencing

Numerous reporter strains harboring insertions at a variety of heterochromatic sites have been described (Table 1). Although reporter strains using *GFP* or *kanMX* (G418 resistance) markers have recently become available (Keller et al. 2012; Castonguay et al. 2015), the majority of reporter

TABLE 1. *Schizosaccharomyces pombe* reporter genes

Insertion site	Reporter genes	Notes	References
Pericentromeric heterochromatin			
<i>otr1R</i> (SphI)	<i>ura4⁺</i> <i>ade6⁺</i>	Commonly used for assay of <i>otr</i> silencing	Allshire et al. 1995 Ekwall et al. 1997
<i>otr1L</i> (<i>dh</i> BglII)	<i>ura4⁺</i>		Allshire et al. 1995
<i>otr1L</i> (<i>dh</i> HindIII)	<i>ura4⁺</i>		Allshire et al. 1995
<i>otr1L</i> (<i>dg1a</i> HindIII)	<i>ura4⁺</i>		Allshire et al. 1995
<i>imr1L</i> (NcoI)	<i>ura4⁺</i>		Allshire et al. 1995
<i>imr1R</i> (NcoI)	<i>ura4⁺</i> <i>CBR68</i>	Click beetle luciferase	Allshire et al. 1995 Shimada and Buhler 2012
	<i>kanMX</i>	Resistance to G418	Castonguay et al. 2015
	<i>GFP</i> <i>ura4⁺</i>	<i>GFP</i> driven by the <i>ura4⁺</i> promoter	Keller et al. 2012 Pidoux et al. 2003
<i>otr2</i>			
Mating type			
<i>mat3-M</i> (EcoRV)	<i>ura4⁺</i> <i>ade6⁺</i> <i>CBR68</i>	Click beetle luciferase	Thon and Klar 1992 Thon et al. 1999 Shimada and Buhler 2012
	<i>GFP</i>	<i>GFP</i> driven by the <i>ura4⁺</i> promoter	Keller et al. 2012
<i>mat2-P</i> (XbaI)	<i>ura4⁺</i>	Insertion into the <i>cenH</i> repeat	Thon et al. 1994
<i>mat2-P</i> (BamHI)	<i>ade6⁺</i>	7.5 kb of the <i>K</i> region (including <i>cenH</i>) replaced by the reporter	Ayoub et al. 1999
<i>K</i> region - <i>Kint1</i>	<i>ura4⁺</i>		Grewal and Klar 1997
<i>K</i> region - <i>Kint2</i>	<i>ura4⁺</i>		Grewal and Klar 1997
<i>K</i> region - <i>KΔ</i>	<i>ura4⁺</i> <i>ade6⁺</i>		Grewal and Klar 1996 Ayoub et al. 1999
<i>L</i> region (SacI)	<i>ade6⁺</i>		Ayoub et al. 1999
<i>L</i> region (BglII)	<i>ade6⁺</i>		Ayoub et al. 1999
<i>L</i> region (BssHII)	<i>ade6⁺</i>	Insertion at the junction between <i>mat2-P</i> and the <i>L</i> region	Ayoub et al. 1999
<i>L</i> region (EcoRI)	<i>ade6⁺</i>	Insertion on the centromere proximal side of Repressive Element II (REII)	Ayoub et al. 1999
<i>L</i> region - <i>LΔ</i>	<i>ade6⁺</i>	REII element (BssHII-EcoRI) replaced by the reporter	Ayoub et al. 1999
Telomere and subtelomere			
<i>tel1</i> (L)	<i>his3⁺</i>	Reporter inserted ~300 bp from the left telomere of chromosome I	Nimmo et al. 1998
<i>tel2</i> (L)	<i>ura4⁺</i>	Reporter inserted ~300 bp from the left telomere of chromosome II	Nimmo et al. 1998
Ch16 M23-Tel	<i>ura4⁺</i> <i>ade6⁺</i>	Markers inserted adjacent to the telomere of a 350-kb minichromosome derived from chromosome III. The telomere lacks telomere-associated sequences (TASs).	Nimmo et al. 1994
Subtelomere Chr IIR	<i>ura4⁺</i>	Insertions at ~7, 11, 33, 52, and 56 kb from the telomere	Kanoh et al. 2005
Centromere central core			
<i>cnt1</i> TM1 (NcoI)	<i>ura4⁺</i> <i>ade6⁺</i> <i>arg3⁺</i>	Central core of centromere I	Allshire et al. 1994 Allshire et al. 1994 Pidoux et al. 2003
<i>cnt2</i> CC2 (SphI)	<i>ura4⁺</i> <i>ade6⁺</i>	Central core of centromere II	Allshire et al. 1994 Allshire et al. 1994
<i>cnt3</i> TM3	<i>ura4⁺</i>	Central core of centromere III	Allshire et al. 1994
<i>imrR/L</i> (<i>iHindIII</i>)	<i>ura4⁺</i>	The inner regions of the <i>imr</i> repeats are associated with CENP-A containing chromatin.	Allshire et al. 1995
rDNA			
Chr III rDNA	<i>ura4⁺</i> <i>LEU2</i>		Thon and Verhein-Hansen 2000
Ectopic loci			
<i>ade6::L5</i>	<i>ura4⁺</i>	1.6 kb fragment of <i>otr</i> repeats inserted at the <i>ade6⁺</i> locus	Partridge et al. 2002
<i>ura4::K</i> region	<i>ade6⁺</i>	Various fragments of the <i>K</i> region and the REII element from the <i>mat</i> locus	Ayoub et al. 2000
<i>ura4::tlh</i>	<i>ade6⁺</i> <i>ura4⁺</i>	<i>tlh1</i> or -2 inserted at <i>ura4⁺</i> ; <i>tlh</i> genes are subtelomeric helicase genes that contain regions of homology with <i>dg-dh</i> repeats.	Hansen et al. 2006
<i>ura4::3xgbs-ade6⁺</i>	<i>ade6⁺</i>	Expression of Clr4 as a fusion with a Gal4 DNA binding domain (GBD) directs RNAi-independent heterochromatin to a Gal4 binding site (<i>gbs</i>)- <i>ade6⁺</i> reporter.	Kagansky et al. 2009
<i>ura4::4xTetO-ade6⁺</i>	<i>ade6⁺</i>	Four TetO sites adjacent to an <i>ade6⁺</i> reporter	Buscaino et al. 2013
Minichromosome plasmids	<i>sup3-5/ade-704</i>	Plasmids containing various fragments of a <i>dg</i> repeat adjacent to a central core (CC). Plasmids contain the <i>sup3-5</i> tRNA gene, which suppresses the <i>ade-704</i> allele. Red/white colony color assays are used to monitor plasmid retention resulting from heterochromatin establishment	Buscaino et al. 2013

(continued)

TABLE 1. Continued

Insertion site	Reporter genes	Notes	References
Barrier elements			
<i>IR-L IR-R</i>	<i>ade6⁺ ura4⁺</i>	Marker genes placed adjacent to the barrier elements <i>IR-L</i> and <i>IR-R</i> that flank the <i>mat</i> locus	Singh and Klar 2008
<i>IR-R</i>	<i>ura4⁺</i>	Marker adjacent to <i>IR-R</i>	Noma et al. 2006
<i>L5-tRNA</i>	<i>ura4⁺</i>	730-bp barrier sequence containing the tRNA ^{Ala} genes from the <i>imr</i> repeats inserted between the <i>L5</i> element and a <i>ura4⁺</i> marker	Scott et al. 2006
<i>IRC1R, IRC3L-tRNAASP.05</i>	<i>ura4⁺</i>	Marker genes placed adjacent to barrier elements flanking the right side of centromere I (IRC1R) or left side of centromere III (IRC3L)	Wang et al. 2013

strains use either the *ade6⁺* or *ura4⁺* auxotrophic markers, which can be assayed by monitoring cell growth on minimal medium lacking adenine or uracil, respectively. Silencing of *ura4⁺* can also be assessed by the addition of 5-fluorouracil (5-FOA) to the medium. The *ura4⁺* gene encodes an enzyme that converts 5-FOA into the toxic compound, 5-fluorouracil (Grimm et al. 1988). Hence, loss of *ura4⁺* silencing leads to 5-FOA sensitivity. Colony color can be used to monitor *ade6⁺*, because a silenced copy of this reporter leads to red colonies on agar containing limiting adenine due to the accumulation of biosynthetic intermediates (Fisher 1969). Partial disruption of silencing results in pink colonies, whereas its abolition results in white colonies; thus, colony color provides a semi-quantitative assessment of silencing (for further discussion on reporter gene readout see Introduction: **Growth and the Environment of *Schizosaccharomyces pombe*** [Petersen and Russell 2016]).

A fully quantitative measurement of *ade6⁺* and *ura4⁺* reporters requires the determination of mRNA levels. This is straightforward for *ura4⁺*, because in most reporter backgrounds the endogenous gene is either deleted (*ura4-D18*) or is a minigene (*ura4-DS/E*) (Grimm et al. 1988; Allshire et al. 1994). Thus, it is easy to distinguish between transcripts derived from the reporter and the endogenous locus (Allshire et al. 1994). Many *ade6⁺* reporter strains have the point mutant *ade6-M210* at the endogenous locus, which confounds measurement of reporter mRNA. However, an *ade6-DN/N* minigene is available that allows transcripts from the reporter and endogenous locus to be distinguished (Ekwall et al. 1997). Recently, quantitative silencing assays based upon luciferase reporters have also been described (Shimada and Buhler 2012). These assays can be performed without prior cell lysis and in a multiwell format and so can be easily adapted to high-throughput screens.

The expression of artificial hairpin RNAs can induce silencing of reporter genes (Sigova et al. 2004; Iida et al. 2008; Simmer et al. 2010), whereas tethering of heterochromatin components to Gal4 or TetO sites induces RNAi-independent heterochromatin (Kagansky et al. 2009; Buscaino et al. 2013). Reporter genes have also been used to study *S. pombe* barrier (boundary) elements that prevent the inappropriate spread of heterochromatin. Here the reporter gene is inserted into the euchromatin adjacent to the barrier. When barrier function is impaired, heterochromatin can spread into nearby euchromatin and silence the reporter gene. This approach has been used to identify factors and sequences that are required for barriers associated with centromeres (Scott et al. 2006; Wang et al. 2013) and the *mat* locus (Noma et al. 2006; Singh and Klar 2008).

Reporter genes are repressed in H3K9me-dependent heterochromatic domains and at several other sites in the *S. pombe* genome, such as the rDNA repeats (Thon and Verhein-Hansen 2000) and the CENP-A containing chromatin-associated with the centromeric central core (Allshire et al. 1994; Partridge et al. 2000). In the latter, silencing results from kinetochore assembly; therefore, analysis of central core silencing has identified factors required for the assembly of CENP-A and the kinetochore (Pidoux et al. 2003).

Detecting Bidirectional Transcription and siRNAs

Analysis of heterochromatic mRNA transcripts is challenging because of their high content of repetitive sequences and the lack of clearly defined 5' and 3' ends. In addition, transcription occurs on both

strands, requiring modified methods to detect strand-specific transcription. Central to the study of RNAi-mediated heterochromatin formation is the ability to detect siRNAs. Because siRNAs are only ~22–24 nt, it is not possible to detect their levels using the conventional method of reverse transcription coupled with polymerase chain reaction (PCR). Fortunately, most of the heterochromatin-associated siRNAs originate from the pericentromeric repeats, *dg* and *dh* (Cam et al. 2005), enabling one to detect the majority of the siRNA population with only a few probes. In addition to their small sizes, siRNAs in wild-type cells are expressed at very low levels, making a radiolabeled probe the only means to detect these small molecules reliably. siRNAs can be detected from either total RNA or from affinity-copurified RNAi complexes (e.g., RITS) (Verdel et al. 2004). The latter approach, although more laborious, tends to give cleaner background signals (Noma et al. 2004; Cam et al. 2005; Buhler et al. 2008).

Analysis of Heterochromatin Using Micrococcal Nuclease

It is now established that proper nucleosome positioning is critical for the regulation of gene expression (Jiang and Pugh 2009). Similarly, nucleosome positioning is also important for the function of heterochromatic domains (Shim et al. 2012). A tool that is widely used to map nucleosomes is MNase, which preferentially digests linker DNA and so facilitates the isolation of nucleosome-associated sequences. MNase digestion has been used in several ways to analyze silent regions of the *S. pombe* genome. Southern blotting of MNase digests with probes specific to different centromeric regions has been used to analyze overall nucleosome spacing at centromeres (Takahashi et al. 1992). Nucleosome mapping has been achieved by indirect end-labeling techniques. Here the locus of interest is defined by restriction digestion of MNase-treated DNA, followed by Southern blotting with a probe specific to one end of the restriction fragment (Nedospasov et al. 1989). Heterochromatin is commonly enriched in repeat sequences, making the production of a unique probe difficult. Nonetheless, indirect end labeling has been used to map nucleosomes in the *mat* locus (Sugiyama et al. 2007). More recently, it has become feasible to determine nucleosome positions on a genomic scale (Yuan et al. 2005). This requires the generation of mononucleosomal DNA from total chromatin, which is then analyzed using high-density tiling arrays or next-generation sequencing. These techniques were established using *S. cerevisiae* but have since been applied to *S. pombe* (Lantermann et al. 2009). Both sequencing and array-based approaches have been used to study nucleosome organization in heterochromatin (Garcia et al. 2010; Yamane et al. 2011; Shim et al. 2012; Gal et al. 2015).

MNase digestion of *S. pombe* chromatin can be achieved using isolated nuclei (Godde and Widom 1992; Mason and Mellor 1997; Hirota et al. 2009). Protocols for nuclei isolation often involve the enzymatic removal of the cell wall followed by lysis of the resulting spheroplasts to yield a nucleus-enriched fraction. Recently, a protocol for isolating nuclei using cryo-grinding that yielded excellent results with MNase digests has been published (Givens et al. 2011). An alternative method relies on the permeabilization of spheroplasts, which can then be treated with MNase (Pidoux et al. 2004). This method is relatively rapid and requires no specialized equipment (see Protocol: **Micrococcal Nuclease Digestion of *Schizosaccharomyces pombe* Chromatin** [Cam and Whitehall 2016a]).

Detecting Heterochromatin-Associated Proteins and Their Modifications Using ChIP

ChIP has become the gold standard for detecting in vivo protein localization at heterochromatin. It provides a direct establishment of function between an observed effect (i.e., loss of heterochromatic silencing) and association with target loci. ChIP detection of proteins that primarily localize at heterochromatin can be less challenging than for those proteins that bind to many sites of the genome. This is because heterochromatin tends to exist in blocks spanning many kilobases yet constitutes only a small fraction of the genome. In addition, at the beginning of the protocol, cells are treated with a fixative such as formaldehyde that prevents protein degradation. Thus, although the histone methyltransferase Clr4 and its methylated substrate (H3K9me) are difficult to detect by

western blotting, their enrichment at heterochromatin can be detected by ChIP (Nakayama et al. 2001; Zhang et al. 2008).

ChIP is one of the more technically demanding techniques for heterochromatin analysis. Several critical parameters govern the success of a ChIP experiment. First, a chemical such as formaldehyde is used to rapidly establish a covalent cross-link between the protein and its localized target DNA. Formaldehyde also provides cross-linking between proteins and is thus effective for detecting proteins that do not associate directly with DNA. However, formaldehyde is a small molecule with a relatively short spacer arm. For some proteins, the addition of a molecule with a longer spacer arm (e.g., dimethyl adipimidate) can enhance the detection of proteins (Kurdistan and Grunstein 2003; Cam et al. 2005). Second, after cell lysis (usually with a BeadBeater), chromatin is fragmented into segments of < 500 bp by either sonication or MNase digestion. This achieves two things: solubilization of chromatin and prevention of nonspecific enrichment of protein–DNA interactions. Third, good antibodies are critical for successful enrichment of chromatin-associated factors. This can only be determined experimentally for it is no guarantee that antibodies that work for other assays (i.e., western blotting, immunofluorescence, etc.) will also work well under more stringent ChIP conditions. Fortunately, because most *S. pombe* proteins can be tagged with epitopes recognized by antibodies that work well under ChIP conditions, this last parameter is not generally a large hurdle (for further discussion on protein tagging in *S. pombe*, see Introduction: **Molecular Genetics of Fission Yeast** [Murray et al. 2016]).

Some variations of ChIP (Furey 2012) are worth mentioning in this context. First, the combined use of MNase with chromatin immunoprecipitation (MNase-ChIP) can be used to study the composition and modifications of specific nucleosomes (Infante et al. 2012). Second, tagged histone subunits can be used with ChIP to investigate the dynamics of histone turnover (Aygun et al. 2013; Audergon et al. 2015; Svensson et al. 2015). Third, “ChIP-exo,” in which exonuclease is used to trim immunoprecipitated chromatin fragments, has recently been reported by Ekwall and colleagues (Svensson et al. 2015). Compared with conventional ChIP, “ChIP-exo” offers much lower background and subnucleosomal resolution.

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