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Chapter 10

Methods to Study the Atypical Roles of DNA Repair and SMC Proteins in Gene Silencing

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Abstract

Silenced heterochromatin influences all nuclear processes including chromosome structure, nuclear organization, transcription, replication, and repair. Proteins that mediate silencing affect all of these nuclear processes. Similarly proteins involved in replication, repair, and chromosome structure play a role in the formation and maintenance of silenced heterochromatin. In this chapter we describe a handful of simple tools and methods that can be used to study the atypical role of proteins in gene silencing.

Key words *S. cerevisiae*, Heterochromatin, Silencing, Nuclear organization, DNA repair, Cohesin, Condensin

1 Introduction

1.1 Gene Silencing

Silencing in *Saccharomyces cerevisiae* is a chromatin-based mechanism that leads to specific loci being replicated late in S-phase and also becoming resistant to recombination and transcription (reviewed in [1–3]). The loci adopt a chromatin structure that partially inhibits access of the DNA in these loci by various molecular probes.

There are three classes of loci that undergo slightly variable forms of silencing: the cryptic mating type loci *HML* and *HMR*, the thirty two subtelomeric regions of the sixteen chromosomes and approximately half of the rDNA repeats in the nucleolus.

Silencing at *HML* and *HMR* initiate at specific sites called silencers. The silencers contain binding sites for the Origin Recognition Complex (ORC) and the transcriptional regulatory proteins Rap1 and Abf1 [4]. Binding of any two of these three proteins to the silencer is necessary to initiate silencing. The silencer bound proteins recruit an accessory protein Sir1 to the silencer. Sir1 in turn interacts with Sir4 and recruits the Sir4/Sir2 complex to the silencer while Rap1 has the ability to interact with and recruit Sir4 as well as Sir3 to the silenced domain.

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At telomeres multiple tandem binding sites for the Rap1 protein function as a silencer [5]. Telomere bound Rap1 helps recruit Sir3 and Sir4 leading to the formation of silenced chromatin in subtelomeric domains. Silencing at telomeres is weaker than silencing at *HML* and *HMR*. In addition to Rap1, the telomere binding protein Ku plays a role in silencing. Loss of these proteins from the silencers results in a significant loss of silencing [6–8]. Ku interacts with Sir4 and is thought to initiate an alternative pathway to recruit the Sir proteins to subtelomeric heterochromatin. Additional proteins that bind telomeres such as the telomerase complex and repair proteins MRX and Tel1 also aid in silencing to varying degrees.

Sir2 is an NAD-dependent histone deacetylase [9] that interacts with Sir4 [10, 11] and the recruitment of Sir2 (via Sir4) to the *HML* and *HMR* silencers as well as sub-telomeric chromatin leads to the deacetylation of the N-terminal tails of the histone H3 and H4 in nucleosomes flanking the silencer [4, 5]. Following histone deacetylation, Sir3 interacts with Sir4 as well as the unacetylated histone tails. Nucleosome bound Sir3 in turn recruits additional Sir4/Sir2 complexes leading to the propagation of Sir proteins along the chromatin fiber, thereby forming a domain of inaccessible silent chromatin. Silencing at telomeres is weaker than silencing at *HML* and *HMR*. It is dependent on the Sir proteins, Rap1 as well as telomere binding proteins and other factors.

1.2 Gene Insulation

Specific *cis*-acting DNA sequences block the spread of the silenced chromatin state. While gene silencing leads to the transcriptional repression of many gene promoters, it appears that strong promoters, which have the ability to resist silencing, function as gene insulators [12–14]. Studies on sub-telomeric silenced chromatin identify barriers to silenced chromatin, which are mediated by strong binding sites for Tbf1 and Reb1 as well as Rap1. The promoter of the *CHA1* gene via its transcriptional activator Cha4 mediates the centromere proximal boundary at *HML*. The telomere proximal boundary of *HMR* has been intensively studied. A tRNA gene (tDNA) along with its flanking sequence is the key *cis*-acting element required to block the spread of silenced chromatin at this locus [12]. While transcription of the tDNA is not required, the TFIIIC and TFIIIB transcription factors that bind the promoter and flanking sequences of the tDNA are necessary for this activity [14, 15]. Additional chromatin modifying and remodeling proteins play a role in blocking the spread of silenced chromatin [16, 17]. The data collectively suggest that two independent pathways function to restrict silencing. One pathway relies on a competition between histone deacetylation (mediated by the Sir proteins) and nucleome-wide histone acetylation. The second pathway occurs at the tDNA and stable transcription promoters and consists of nucleosome eviction and stable transcription factor binding to DNA. This functions both as a roadblock and by eliminating the

histone substrate necessary for Sir protein binding and spreading. In addition mutations in replication and repair proteins as well as in cohesin proteins affect boundary activity mediated by the tDNA promoter and thus affect the organization of the silenced heterochromatin.

1.3 Silencing and Nuclear Organization

The *S. cerevisiae* nucleus is spatially organized such that the 16 centromeres cluster together adjacent to the spindle pole body [18]. The telomeres interact with one another at a few loci based on the length of the chromosome arms tethered at the nuclear periphery [19–22]. The rDNA repeats form the nucleolar compartment at the nuclear periphery [23–27]. Rap1 and the Sir silencing proteins are enriched at these loci adjacent to the nuclear envelope [23, 28, 29]. As a result of this organization, the nuclear periphery exists as a nuclear subcompartment rich in silencing proteins [19]. A consequence of this organization is to sequester the repressor proteins away from most of the active genes in the nuclear interior [30] while the nuclear periphery adjacent to the nuclear membrane is a zone where transcriptionally silenced genes reside (though some active genes do reside at the periphery clustered near the nuclear pore). While localization to the periphery is not necessary for transcriptional silencing of a locus, it improves the efficiency with which one is silenced [31–35].

Nuclear organization also affects recombination and can facilitate the efficient repression of recombination within the silenced compartment, thereby blocking illegitimate recombination between various telomeric repeats. The homologous recombination protein Rad52 is present in the nuclear interior and sequestration of the repeats occurs at the periphery. This suggests that compartmentalization may prevent Rad52-mediated repeat recombination [36, 37].

The formation of the silencing compartment in the nucleus depends primarily on the proper clustering and anchoring of telomeres to the nuclear envelope. Clustering is dependent in part on the Sir proteins. This is independent of their function in transcriptional silencing [38]. Anchoring of the telomeres to the periphery utilizes the Sir proteins as well as other proteins. This is mediated by two pathways, one utilizing the protein Esc1 and the second the repair protein Ku [6, 32, 39–41]. Both Esc1 and Ku anchor silenced chromatin to the periphery via their direct interactions with Sir4. Esc1 is exclusively found at the inner nuclear envelope and does not co-localize with nuclear pores, and telomere anchoring to the periphery requires Esc1 primarily during the S-phase of the cell cycle. Ku is a chromatin bound protein that does not have a membrane-binding domain and likely mediates telomere tethering to the nuclear membrane via accessory proteins. Ku functions to tether telomeres to the membrane in the G1 phase of the cell cycle [42]. The domains of Ku involved in gene silencing and those

involved in perinuclear anchoring are distinct and separable [41, 43]. The Ku protein also localizes to the silent *HML* and *HMR* domains [44, 45] and participates in the perinuclear anchoring of *HML* though not *HMR*, which utilizes a different mechanism to anchor to the periphery [46–48].

Another protein that plays a role in anchoring telomeres to the periphery is the telomerase complex which functions via the SUN domain protein Mps3. Data suggest that to tether telomeres to the periphery Ku functions via this pathway [37, 49]. Specific nuclear pore sub-complexes are also involved in this process but the exact mechanism by which the pore proteins mediate tethering is not clear [49–53]. It seems though it seems that Mps3 mediated tethering is distinct from the Nup84/Slx5/Slx8 mediated pathway [50, 54].

1.4 SMC Proteins and Silencing

The SMC proteins are found in multiple complexes: the cohesin complex, the condensin complex, and the repairsin complex [55–59]. The cohesin complex is composed of the Smc proteins Smc1 and Smc3 as well as the non-Smc proteins Mcd1/Scc1 and Scc3 [60–63]. The condensin complex is composed of Smc2, Smc4 and the non-Smc proteins Brn1, Ycg4, and Ycs1 [57, 58]. The repairsin complex proteins Smc5 and Smc6 are primarily involved in DNA repair along with additional non-Smc proteins Nse1-6 [56, 64, 65].

Mapping studies show that the cohesins localize to the centromere where they play a critical role in the bi-orientation of the kinetochores. They also localize to discrete A-T rich sites along the chromosome arms mediating arm cohesion [66–70]. An early study showed that the condensins localize to and affect the condensation of the rDNA repeats [71]. However, subsequent genome-wide analysis revealed that these proteins also function at pericentromeric chromatin [72–74] as well as tRNA genes [75].

The cohesin complex is loaded onto chromatin by the Scc2/Scc4 proteins [76] with the aid of the chromatin remodeler Rsc [77–79] during the G1 phase of the cell cycle [76, 80]. Cohesins are also loaded at origins of replication [81] to help origins cluster together [82] and these proteins spread along the DNA as forks progress. The establishment of cohesion between sister chromatids occurs only in S phase concomitant with replication [83, 84] and this process is dependent on the protein acetyltransferase Eco1 as well as numerous replication proteins [63, 85–88] (reviewed in [89]). Certain DNA sequences are enriched for cohesin binding such as convergently transcribed genes [90], silenced loci [91], tRNA genes [92] and origins of replication [88]. Various models have been proposed to explain the distribution of these proteins. The enrichment of cohesins at convergently transcribed genes is thought to be a function of the cohesin proteins sliding along the chromatin as genes are transcribed and eventually come to rest at sites of convergent transcription [90, 93] though alternative scenarios are possible. At other

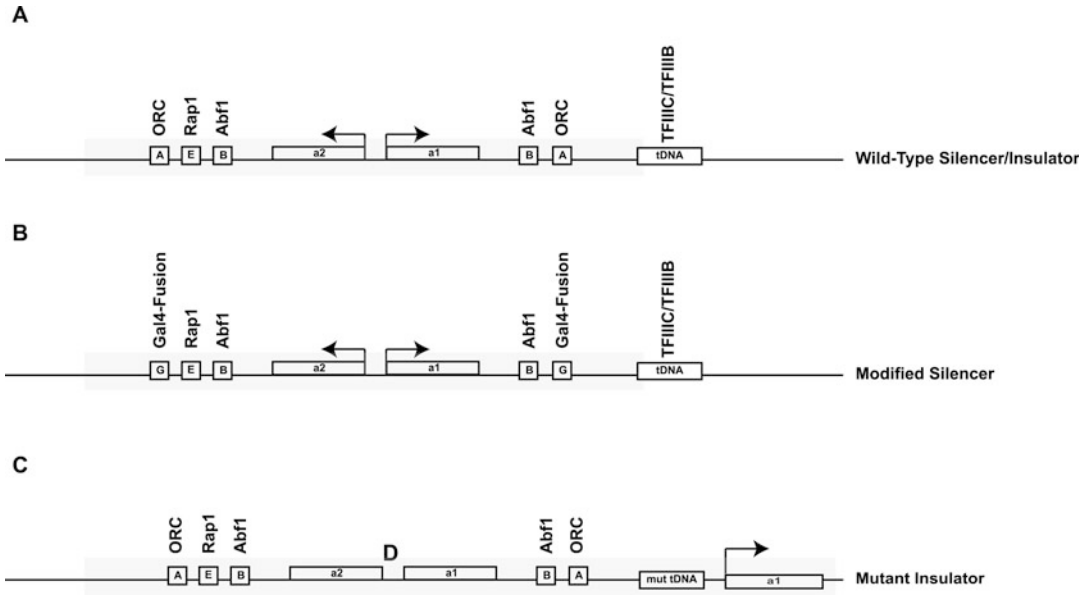

Fig. 1 (**a**) Schematic of the wild type silenced *HMR* locus. The *grey box* reflects the extent of the silenced heterochromatic domain. The ability of α cells to mate is used as an indication of silencing at this locus. (**b**) Schematic of the modified *HMR* locus where the ORC binding site is replaced with binding sites for Gal4. Proteins fused to the Gal4 DNA binding domain are expressed in α cells containing this modified locus. The proteins are recruited to these binding sites and are assayed for their ability to silence the *a1* gene in a Sir protein dependent manner. (**c**) Schematic of a modified locus where the promoter of the *a2* and *a1* genes at *HMR* is deleted and a wild type *a1* gene is inserted outside the locus. Expression of the *a1* gene in an α is monitored to investigate the extent of tDNA-mediated insulation of the silenced *HMR* domain

sites the mechanism of loading these proteins is believed to be more direct.

Cohesins are recruited to the tDNA via direct interactions between the promoter bound transcription factor TFIIIC and Scc2/Scc4 [75]. The binding of cohesins to tDNAs plays a role in the cell cycle regulated transcription of the tDNAs [94] and also helps maintain a nucleosome free region at these loci [59]. Whether the tDNA-mediated recruitment occurs in G1 and this results in cohesion coordinated with replication is currently not known. Cohesin loading at the silenced *HMR* locus requires an adjacent tDNA insulator [48, 92] (Fig. 1). Silenced telomeric domains are also enriched for SMC proteins [67, 68] and the binding of these proteins to silenced chromatin is dependent on the Sir proteins [91, 95] but the mechanism of their recruitment to the telomeres is not known.

Various Smc proteins including cohesins and condensins localize to the rDNA repeats at the nucleolus [71, 96]. Loss of cohesins result in reduced rRNA production with an increase in unequal sister chromatid exchange between the rDNA repeats [97] while loss of condensins results in the decondensation of the repeats

[71]. It is speculated that these proteins suppress illegitimate recombination by condensing the chromatin and also help pair repetitive sequences allowing equal but not unequal sister chromatid exchange.

In addition to coordinating cohesion and condensation, the Smc proteins affect other processes in the cell including gene regulation. In metazoans cohesins play an important role in mediating long-range interactions between distal enhancers and proximal promoters during gene activation [98–101]. In yeast there is no documented role for the Smc proteins in gene activation but the Smc proteins play a role in restricting the spread of silenced chromatin [12]. Conditional mutants in Smc1 lead to loss of tDNA-mediated insulation of the silent *HMR* domain, which results in the spread of this domain into adjacent euchromatin. The Smc proteins also play a role in the long-range clustering of silent loci [48]. *HML* and *HMR* are located at opposite ends of chromosome III but interact with one another in the nucleus [48, 102]. Mutations in homologous recombination (HR) repair proteins, including the phosphorylation of H2A, disrupt this long-range association [48]. Phosphorylated H2A is constitutively present at the silent loci and this modification is required to recruit/stabilize the Smc proteins to silenced chromatin. Importantly, mutations in Scc2 lead to the loss of the long-range interaction between *HML* and *HMR* [48]. Whether the loss of this interaction affects the establishment or inheritance of the silent state at these loci is currently not known and neither is the reason for the clustering of *HML* with *HMR* though it is possible that this is a mechanism to sequester the loci away from the active *MAT* locus, thereby preventing ectopic recombination.

Approximately a third of all tRNA genes cluster around the centromeres while another third cluster on the outer surface of the nucleolus [103, 104]. This long range clustering of the tDNAs in the nucleus is also dependent on Scc2/Scc4 and the condensins [75, 105]. The 3D clustering results in the transcriptional repression of neighboring RNA pol II transcribed genes in a phenomenon termed tRNA gene mediated (TGM) silencing [106] which is distinct from the silencing of *HML*, *HMR*, and the telomeres.

1.5 Repair and Silencing

Physical and chemical agents as well as errors in basic biological processes induce a vast assortment of DNA damage and arguably the most lethal is a double strand break. Double-strand breaks occur via physical or chemical mutagens in different stages of the cell cycle [107–110]. They also form as spontaneous lesions during replication when the replication forks collide with DNA-protein adducts leading to fork collapse [111, 112].

Cells have developed multiple mechanisms to sense and repair double strand breaks. The two major pathways for repair of these breaks in yeast are nonhomologous end joining (NHEJ) which is

used primarily in the G1 phase of the cell cycle and homologous recombination (HR) mediated repair employed in the S/G2 phase of the cell cycle. Specific proteins signal the damage, choose the repair pathway and finally mediate repair [113].

NHEJ involves numerous proteins including the Ku heterodimer complex, the MRX complex and DNA ligase [114, 115]. Ku binds the ends of double strand breaks, which then leads to the recruitment of Mre11/Rad50/Xrs2 complex and the bridging and ligation of the broken ends by the Lig4/Lif1 proteins [114, 116].

HR repair occurs in the S/G2 phase of the cell cycle where the intact sister chromatid is used as a template for repair as it is an identical copy of the damaged template [117, 118]. If a sister chromatid is unavailable, then a search for homologous sequences is initiated via mobilization of the damaged locus [119, 120]. Since chromosomes occupy specific territories and are tethered to specific structures, the search mechanism is influenced by territory organization [121]. In yeast the Rad52 epistasis group is critical for HR mediated repair [116, 122–125]. The MRX complex binds the broken ends of the chromosomes [116, 124] and one of the first steps involves the phosphorylation of histone H2A on Serine-129 (γ-H2A) by Tel1 (ATM) or Mec1 (ATR) [126, 127]. Phosphorylated H2A allows the recruitment/tethering of the resection machinery followed by homology search, sequence recognition, strand invasion, and repair [123–125, 128–130]. Additionally, the chromatin remodelers and histone acetyltransferase [131] as well as the Smc proteins function during this process [55, 56, 64, 132–134].

Cohesins are found in a domain flanking DNA double strand breaks [133, 135] and play a crucial role in the efficient repair of these breaks via the HR repair pathway [64]. The recruitment of cohesins to the break site requires Scc2/Scc4 and the MRX complex as well as histone H2A serine129 phosphorylation indicating that checkpoint signaling is important [133, 135]. Studies with conditional mutants show that sister chromatid cohesion is established in S-phase and cannot be established de novo in the G2/M phase of the cell cycle unless the cohesion complex is activated [90]. Surprisingly, induction of double strand breaks reactivates cohesins genome-wide. This reactivation during repair of double strand breaks requires Eco1, the MRX complex, Rsc and the checkpoint kinases Mec1, Tel1 in addition to phosphorylated H2A [133, 135, 136].

Cohesins are also involved in repair of double strand breaks generated after replication fork collapse. Cohesins are loaded at origins of replication and spread along the DNA as forks progress. When forks pause at impediments, cohesins accumulate at the pause sites and are critical for recovery of the paused/stalled forks. Cohesin localization at these sites requires Rad50 as well as the checkpoint kinases Mec1 and Tel1 [81, 137]. Since tDNAs are known to induce fork pausing and stalling [138, 139] and are

also enriched for cohesins it is tempting to suggest that this may be a mechanism by which cohesins accumulate at tDNAs [48] though it is currently unknown if tDNA mediated fork stalling leads to the deposition/accumulation of cohesins.

There are additional links between specific repair proteins and gene silencing [140, 141]. Telomeres are composed of $C_{1-3}A$ repeats which are maintained via the enzyme telomerase as well as via the binding of Cdc13 and Ku to the ends. The Ku heterodimer [115] is directly involved in both DNA repair and gene silencing. In addition to Ku, the MRX repair complex and the checkpoint kinase Tel1 also localize to telomeric ends [142–148]. While Ku is required for telomeric silencing, other proteins involved in repair such as Lig4 have no effect on silencing [149]. Mutations in the MRX complex and Tel1 have only minor effects on telomeric silencing [8] although they do have the ability to interact with silencing proteins [150].

Another link between silencing and repair is the observation that the Sir proteins are mobilized from telomeres in response to DNA damage [151–153] and following mobilization, Sir3 and Sir2 localize to double strand breaks present in euchromatin [151, 152, 154]. During HR repair, histone acetylation at the site of damage is followed by repair and histone deacetylation [133, 154–158]. Mutant analyses suggest that Sir2 may in part mediate this deacetylation. However besides histone deacetylation by Sir2, the exact role of the other Sir proteins at the site of damage is not known. It is also unclear how the Sir proteins are recruited to sites of double strand breaks or which specific repair proteins play a role in this process. Recently it was shown that the ATM kinase Tel1 has the ability to interact with the Sir proteins and recruit Sir proteins to chromatin whenTel1 is tethered to a specific DNA sequence [150]. To understand if this is how Sir proteins are recruited to sites of DNA damage, whether this recruitment is necessary for repair or if the dispersal of Sir proteins from telomeres is simply a consequence of the mobilization of Tel1 and MRX repair proteins from telomeres all await further investigation.

Breaks that are repaired with slower kinetics or are unrepairable are recruited to the nuclear periphery in a Mps3 [159] and the nuclear pore protein Nup84 [50, 54] dependent manner requiring robust checkpoint signaling. The damaged sites are repaired via alternative adaptation pathways, which utilize Mps3 and Nup84 [37, 159–162]. In addition, breaks that occur in silenced heterochromatin require tethering to the nuclear pore to be repaired [50] and this process depends on specific chromatin remodelers [163]. Furthermore, other telomere specific factors are also recruited to persistent breaks and it has been suggested that the tethering of a persistent break to the periphery enables telomere bound repair proteins (and possibly Sir proteins) to aid in alternative repair pathways that are distinct [164] such as de novo telomere formation as

a part of adaptation. It is therefore apparent that repair and silencing processes utilize the same proteins and affect one another.

1.6 Introduction to the Methods

Different methods that have been developed to study silencing in yeast [165]. To study the role of specific proteins in silencing, various mutant and wild type strains are utilized. One family of methods investigates the effects of mutants on the expression of variously configured reporter constructs—often using growth of cells on selective media as readout for silencing. A second set of methods investigate the binding of specific proteins to specific DNA sequences using quantitative chromatin immunoprecipitations while the third group of methods investigate changes in the three dimensional organization of silenced loci in the nucleus using microscopy. Each method generates a specific view of the chromatin state and it is advisable to use a combination of these different methods to gain a more complete view of silencing.

To study silencing a variety of different reporters (*MATa1*, *MATα2*, *TRP1*, *HIS3*, *URA3*, *ADE2*, amongst others) are used [165]. One of the most sensitive is a mating assay. Wild type haploid yeast cells have two different mating types-**a** and α and cell identity is determined by the genes present at the *MAT* locus on chromosome III. Haploid **a** cells have *MATa* genes at the *MAT* locus and express the **a** genes while haploid α cells have *MAT*α genes at the *MAT* locus and express the α genes. Haploid **a** cells mate with haploid α cells to form diploid cells. Both **a** and α genes are also present in haploid cells at *HMR* and *HML* but these copies of the genes are not expressed due to silencing. If however silencing is disrupted (in specific mutants or under specific conditions) then these genes are expressed in haploid cells and this cell now acts as a diploid cell and consequently will not mate with the opposite mating type to form actual diploid cells. The change in the ability to mate can be measured and used as a sensitive quantitative assay for silencing.

A modified version of this assay involves replacing the native silencers flanking the *MATa* genes at *HMR* with binding sites for the Gal4 DNA binding domain. Recruitment of specific proteins to the Gal4 binding sites (via Gal4 DNA binding domains fused to the protein of interest) can be used to determine if recruitment of a specific protein can generate a silenced domain dependent on the Sir proteins. This can be used as an indicator of the ability of this protein to interact with Sir proteins and silenced chromatin.

The silent domain initiates at a specific silencer and spreads along the DNA. Often the domain is delimited by specific barrier insulator sequences such as tDNAs [12]. tDNA insulators are conserved from yeast to mammals and play important roles in the organization of the nucleus. To study the mechanism of tDNA-mediated insulation, reporter genes such as *MATa1*, *ADE2*, or *URA3* are placed immediately outside the silent domain. Mutations that compromise the barrier insulator result in the ectopic spread

of silencing proteins, the consequence of which is to silence the reporter located immediately outside the silent domain and this silencing can be measured quantitatively.

Chromatin immunoprecipitation is also a very informative tool for studies on gene silencing. Using specific antibodies against silencing proteins such as the Sir proteins one can ascertain whether the binding of these repressor proteins is altered in various mutant backgrounds. These alterations in the binding of specific proteins can be measured by quantitative ChIP. The ChIP assay can also be used to study disruptions in the barrier insulator since loss of insulation leads to increased spread of silencing proteins, which can be measured directly by chromatin immunoprecipitations. The assay can also be used to investigate the binding of other proteins to the insulator and silenced domain. This method therefore provides insight into the structure of the silent state as a function of various mutations.

Silenced domains cluster at the nuclear periphery. Clustering of these loci improves the efficiency of silencing. Microscopic visualization of these loci is another assay for monitoring the movement of silenced domains. Genetic mutations and environmental perturbations that cause this organization to be disrupted can be studied using this assay. To observe changes at the silent loci in live cells, typically a LacO array with between 64 and 256 binding sites for LacI is integrated near (within 1–5 kb) the silenced domain. LacI-GFP expressed in these cells binds the LacO array and marks the location of the silenced domain in the nucleus. The location of the silenced locus is measured with respect to either the nuclear envelope or with respect to another locus (active or silent) in the cell. The change in localization of the silent domain in specific genetic backgrounds is informative with regards to the nature of the defect caused by that mutant. In a related assay, the Sir proteins can be fused to GFP allowing the visualization of all silent loci simultaneously in the cell. The dispersal of the fluorescent signal is a good indicator of perturbations to the silent state. Taken together, these approaches provide a clear picture of the molecular mechanisms underlying silencing and repair and the role of specific proteins such as the Smc proteins in these processes.

2 Materials

2.1 Serial Dilution Assay for Silencing

Sterile 96-well flat bottom microtiter plate with lid.

Stainless steel stamp (Sigma R2383).

2.1.1 Hardware

16 mm diameter test tubes.

1.5 ml microcentrifuge tubes.

2.1.2 Media

YPD: 1 % yeast extract, 2 % peptone, 2 % glucose.

YMD: 0.67% yeast nitrogen base without amino acids but with ammonium sulfate, 2% glucose.

YMD plates: YMD with 2% agar.

100× supplement mixes are made and filter sterilized such that the final concentration of each supplement is 20 mg/lit adenine, 20 mg/lit uracil, 20 mg/lit tryptophan, 20 mg/lit histidine, 30 mg/lit leucine, 30 mg/lit lysine.

2.2 Chromatin Immunoprecipitation

Corbett Life Science RotorGene 6000 machine Rotorgene-Q.

Bioruptor sonicator (Diagenode, Belgium).

2.2.1 Hardware

Cup-horn Sonicator (Branson, USA).

Eppendorf Benchtop Microcentrifuge.

Sorvall Benchtop Centrifuge.

Perkin Elmer Viktor^3 Spectrophotometer.

Nutator (Adams).

Aspiration device.

Sterile 500 ml graduated cylinder.

500 ml conical flask.

Dolphin microcentrifuge tubes (helpful when aspirating).

4 × 2 ml screw cap tubes.

Sterile qPCR tubes.

5 ml snap-top polypropylene tubes.

Needles (25 G).

Clip tops for tubes (for use during boiling).

Chilled glass beads.

Sterile aspiration tips.

Bead Beat (MP Fast Prep 24).

2.2.2 Media

Formaldehyde (37.5% w/v). Store at 37 °C.

1.25 M Glycine Stock.

10% w/v Chelex 100 Resin (Bio-Rad, USA).

30 mg/ml DNase free RNAase A (Sigma).

Proteinase K (Roche at 20 mg/ml).

SYBR Green (Use at manufacturer's recommendation) (Invitrogen, USA) (S-7536).

SYBR Gold (Invitrogen, S-11494).

Bovine Serum Albumin – BSA (NEB at 1% w/v).

Sodium Azide: 0.1% w/v.

Lambda DNA: 0.5 mg/ml.

Pico Green (Use at manufacturer's recommendation) (Invitrogen, USA).

PCR Reaction Mix (ImmoMix, Bioline, USA).

PCR Cleanup Kit. (Bioline)

Sodium bicarbonate: 1 M made fresh the day of use.

10% SDS (w/v).

5 M NaCl.

1× PBS: 10 mM KH_2PO_4 (Monobasic), 40 mM K_2HPO_4 (Dibasic), 150 mM NaCl.

FA-140: 50 mM HEPES–KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 7.6, 1% w/v Triton X-100, 0.1% w/v deoxycholic acid. Supplement before use with 0.01 μg/ml leupeptin, 0.01 μg/ml pepstatin, 1 mM PMSF (0.2 M stock in isopropanol). Vortex buffer immediately after adding PMSF.

FA-500: 50 mM HEPE–SKOH, pH 7.5, 0.5 M NaCl, 1 mM EDTA, pH 7.6, 1% w/v Triton-X-100, 0.1% w/v deoxycholic acid.

LiDWash: 250 mM LiCl, 1% w/v NP-40, 1% w/v deoxycholic acid, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1.

1× TE: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 7.

TE-R: 0.015 mg/ml DNase free RNAase A (stock = 30 μg/μl) in TE.

Real Time PCR Primers: All primer pairs used were initially screened for the absence of any primer- dimers or cross-hybridization. Furthermore only primer pairs with similar amplification efficiencies were used. On average we test three pairs of primers for each PCR fragment and chose one for the experiment. Primer pairs located at sites distant from the locus being investigated within euchromatic and other heterochromatic regions are often included as positive and negative controls. In general primers are located at sites so that the PCR product is 100–150 bp in length.

2.3 Live-Cell Fluorescence Microscopy

Teflon Printed Microscope Slide Well (EMS cat# 63422-06).

Plain slides.

Coverslips.

2.3.1 Hardware

Clear nail polish.

Kimwipes.

Microcentrifuge tubes.

2.3.2 Media

YPD.

YMD.

1% w/v agarose in YMD.

3 Methods

3.1 Mating Assay for Silencing and Insulation Using Serial Dilution

1. In test tubes, inoculate 5 ml YPD (or YMD liquid media with appropriate supplements) with cells from a single colony for test strains and mating type strains (we usually use *ahis4* and α*his4* testers for W-303 based strains).
2. Grow O/N at 30 °C with vigorous rotation/shaking.
3. Prepare labeled tubes for serial dilutions and YMD media plates; top each plate with amino acid drop in mixes as required.
4. Take the mating lawns *ahis4* and α*his4* that were grown overnight, spin down the cells from the overnight culture in sterile tubes in a Sorvall refrigerated benchtop centrifuge at 1300 × *g* for 3 min. Resuspend cells in fresh YPD at a concentration of 4.4 OD A_{600}/ml. Spread 1.1 OD A_{600} onto a YMD plate (250 μl) so that the cells are distributed evenly across the plate. Let the plate dry.
5. Measure the cell density of each strain being tested using a spectrophotometer. Dilute each culture to 1 OD A_{600}/ml with sterile YMD. Serially dilute this 1:10 (0.1 OD A_{600}/ml), 1:100 (0.01 OD A_{600}/ml), 1:1000 (0.001 OD A_{600}/ml) and 1:10,000 (0.0001 OD A_{600}/ml) with sterile YMD. Make sure to mix thoroughly between each dilution and also to change the pipet tip between each dilution.
6. Place 100% ethanol in a tray for the sterilization of the metal stamp.
7. Transfer 200 μl of each dilution to a sterile 96 well flat bottom microtiter plate. Note the wells used for each strain.
8. Arrange the plates in order with mating lawn plates last. Flame the metal stamper after dipping into the ethanol to sterilize. Dip the stamper into the diluted samples present in the microtiter plates and transfer 1–3 μl of each sample onto the plates (*see* **Note 1**).
9. Repeat #8 for all of the test plates and the first mating lawn plate. After stamping the first mating lawn plate return the stamp to the ethanol soaking tray and flame to sterilize before continuing to the remaining mating lawn plate.
10. Transfer plates to 30 °C incubator and allow to grow 2–3 days. Take photographs to image the plates as a record of the growth.

3.2 Chromatin Immunoprecipitation

3.2.1 Growth and Crosslinking (*See* **Note 2**)

1. Inoculate the test strain into 10 ml YPD in a sterile 50 ml conical flask with and grow 6 h at 30 °C with shaking at 125 rpm.
2. Inoculate 0.0015OD/ml into 300 ml YPD in a sterile 1-l conical flask and grow overnight at 30 °C with shaking (grow to A_{600} of ~2.2–2.4 OD).

3. Next morning, measure volume and add YPD to restore the volume to 300 ml using the sterile graduated cylinder and shake at 125 rpm in a shaker at room temperature.
4. Add 37.5% formaldehyde stock to a final concentration of 1% (8.2 ml HCHO in 300 ml YPD) (*see* **Note 3**). Add the formaldehyde slowly as the flask is shaking. Allow formaldehyde to crosslink proteins to proteins and proteins to DNA for between 10 and 60 min at room temperature.
5. Stop the crosslinking by adding 1.25 M glycine to a final concentration of 0.125 M (31 ml of stock solution to ~310 ml culture).
6. Allow glycine to quench the formaldehyde by shaking the culture for 15 min at room temperature (*see* **Note 4**). Place culture on ice for 5 min, transfer culture to centrifuge tubes and spin down the cross-linked cells (2500×g for 2 min. in a GS3 rotor in a Sorvall centrifuge); pour off supernatant and keep cell pellet on ice.
7. Resuspend cell pellet with chilled 25 ml of 1× PBS and transfer to 50 ml polypropylene tubes. Spin the cells in a Sorvall benchtop centrifuge at 1300×g for 4 min at 4 °C (swinging bucket rotor). Remove supernatant by aspiration. Repeat the cell wash step.
8. Resuspend cells in 3 ml chilled FA-140 without protease inhibitors. Divide equally into four 2 ml screw cap tubes (without a skirt). Spin at 13,000×g in benchtop microcentrifuge for 10 s. at 4 °C; Aspirate off supernatant.
9. Freeze in dry ice and store at −80 °C.

3.2.2 Cell Lysis

1. To the cell pellet add additional 0.2 ml chilled FA-140 with protease inhibitors to each of the four tubes of cells and allow cell pellet to thaw on ice.
2. Thoroughly resuspend cells in the FA-140. To each tube add chilled glass-beads till the beads cover all of the cells in that tube. Chill for a further 15 min on ice.
3. Bead beat using the MP Fast-Prep24 bead beater for 40 s. Place the cells on ice 5 min and repeat the bead beating for an additional 40 s.
4. Using a with a 25 G needle punch a hole in the bottom of the 2 ml tube containing the lysed cells and glass beads (*see* **Note 5**). Place the punctured tube onto a clean 2 ml tube and place these two tubes in a 5 ml snap-top polypropylene tube. Spin in a benchtop centrifuge at 1300×g for 30 s to separate the cell lysate from the glass beads, collecting the cell lysate in the clean 2 ml tube.
5. Add and additional 0.2 ml FA-140 with protease inhibitors to the glass beads and spin again collecting this wash as well.
6. Pool all of the lysates (from the four tubes) into a clean 14 ml screw-top polystyrene tubes (~1.6 ml in each tube) (*see* **Note 6**).

3.2.3 Sonication

1. Sonicate lysed cells using the Bioruptor sonicator, programmed for 30 s. sonication followed by 1′ off ×5 on the highest setting (2.5 min of sonication). Replenish the ice water in the sonication chamber and repeat the sonication three more times for a total sonication time of 10 min per lysate (*see* **Note 7**).
2. Transfer lysate into labeled 2 ml skirted screw-cap tubes (~0.4 ml per tube; i.e., four tubes) and sonicate using the cup-horn sonicator, sonicating for 80 s in ice cold water and cooling the lysate for 1 min. Repeat this four times for each lysate (*see* **Note 7**).
3. Pool sonicated chromatin (~1.6 ml), mix and remove 40 μl of lysate into 50 μl chilled FA-140 for size analysis (*see* **Note 8**).
4. Freeze the remainder of the sonicated lysate in dry ice and transfer to −80 °C (*see* **Note 9**).

3.2.4 Size Analysis

1. Thaw the sonicated lysate at room temperature and spin for 5 min. in the benchtop microcentrifuge at 13,000×g to remove insoluble cell debris. Transfer supernatant to new tube.
2. Add 4 volumes of 50 mM sodium bicarbonate/1% w/v SDS (160 μl). Mix. Add 1/25 volume of 5 M NaCl (8 μl) (**Note 10**).
3. Heat sample 100 °C for 20 min. Cool at room temperature for 10 min.
4. Add 2 μl of 30 mg/ml DNase free RNaseA. Incubate at 37 °C for 30 min (*see* **Note 11**).
5. Heat to 50 °C for 5 min.
6. Add 400 μl buffer CB (Bioline PCR cleanup kit).
7. Transfer solution to a Bioline PCR cleanup column. Spin 30 s. 13,000×g in a benchtop microcentrifuge. Discard liquid.
8. Wash column with 700 μl buffer CW (Bioline PCR cleanup kit). Spin 30 s 13,000×g in a benchtop microcentrifuge. Discard liquid. Spin the column dry by spinning for 1 min 13,000×g in the benchtop microcentrifuge.
9. Add 50 μl sterile water to the column. Place column in a clean 1.5 ml microcentrifuge tube. Let column stand for 2 min at room temperature. Spin 30 s 13,000×g in a benchtop microcentrifuge collecting the DNA.
10. To the DNA add 6 μl 10× loading dye and 6 μl SYBR gold stock solution.
11. Analyze the size of the sonicated DNA using a 1.25% agarose gel without ethidium bromide. Sonicated DNA should have an average size of 300–500 bp. For yeast DNA, sequential sonication is necessary to obtain an average length of 300–500 bp (*see* **Note 12**).

3.2.5 ProtA/G Equilibration

1. Place Protein A/G slurry (50 µl/sample) (*see* **Note 13**) in a microcentrifuge tube.
2. To the 50 µl slurry add 2 volumes (100 µl) of FA-140 with protease inhibitors. Mix.
3. Transfer the150 µl of slurry into a clean 2 ml dolphin microcentrifuge tube.
4. Spin for 3 s. at 2000×g, in the benchtop microcentrifuge. Aspirate off the supernatant.
5. Add 400 µl FA-140 with protease inhibitors to beads and leave on ice for 30 min (*see* **Note 14**).
6. Spin for 10 s at 2000×g the benchtop microcentrifuge and aspirate off the supernatant.
7. To each tube add 100 µl of FA-140 + 0.1 % BSA.
8. Leave on ice for 2 h or more (*see* **Note 15**).
9. Spin for 15 s at 2000×g in the benchtop microcentrifuge and aspirate off the supernatant. Resuspend protein A/G beads in 35 µl FA-140 + BSA (0.1 %) + NaN_3 (0.05 %).
10. To the slurry add 2 µg sonicated Lambda DNA (100–2000 bp long) (Stock = 0.5 µg/ul).

3.2.6 Immunoprecipitation

1. Thaw samples on ice and spin for 15 min at 4 °C.
2. Transfer sonicated chromatin to a clean tube. Measure volume and transfer chromatin into 10 ml snap-top polypropylene tubes. If necessary, dilute with FA-140 + PIs. to a final sonicated chromatin volume of 2 ml.
3. Remove 100 µl of chromatin as the input sample and freeze at −80 °C.
4. Transfer remaining chromatin into 2×2 ml skirted centrifuge tube with caps (~1 ml sonicated chromatin/tube).
5. Add 5 µg of antibody into each tube and mix.
6. Incubate for 2–3 h on ice.
7. To the 35 µl equilibrated Protein A/G bead slurry add the ~1 ml chromatin–antibody mixture.
8. Incubate overnight at 4 °C on the Nutator.
9. Spin chromatin–antibody–protein A/G beads in the benchtop microcentrifuge (1500×g, 10 s at room temperature). Remove aliquots of supernatants (~0.3 ml) (*see* **Note 16**). Discard remaining supernatant.
10. Resuspend beads with 1 ml of buffer. Incubate slurry on the Nutator for 5 min. Spin at 1500×g for 10 s in the benchtop microcentrifuge and aspirate off supernatant. Keep on ice between washes.

11. Sequentially wash the beads using 1 ml of the following buffers:
    (a) FA-140.
    (b) FA-500 Buffer.
    (c) LiCl/Det (LiDW).
    (d) TE.
    (e) TE**

**Transfer beads to a new tube with the second TE wash.

3.2.7 Elute with Chelex (See **Note 17**)

1. To the beads add 100 µl of the premade Chelex-100 suspension (*see* **Note 9**, above).
2. Treat the input sample similarly. To the 100 µl of input sample add 100 µl of the premade Chelex-100 suspension.
3. Incubate at 100 °C for 10 min (*see* **Note 18**).
4. Spin at 1500 × g in the benchtop microcentrifuge for 10 s at room temperature), allow to cool to room temperature for 5 min.
5. Add 1 µl of Proteinase K. Mix and incubate at 55 °C for 30 min with mixing.
6. Incubate at 100 °C for 10 min.
7. Spin at room temperature for 5 min at 13,000 × g in the benchtop microcentrifuge and remove the supernatant into a freshly labeled tube.
8. To the Chelex resin add 50 µl of sterile water, leave at room temperature for 5 min.
9. Spin as above; remove the supernatant into the tube with the first elution.
10. Freeze the eluted DNA (both Input and IP) at −80 °C.

3.2.8 Measurement of IP and Input DNA

1. Thaw both the Input and IP DNA at room temperature.
2. Prepare a RNAse TE-R solution.
3. To 10 µl immunoprecipitated DNA add 90 µl TE-R.
4. Dilute the Input DNA 1:100 with sterile water. To 20 µl diluted input DNA, add 80 µl of TE-R (this will be 0.2 µl of input per 100 µl).
5. Incubate at 37 °C for 40–50 min.

Preparation of Standards: measured in duplicate.

6. Heat Lambda DNA stock (500 µg/ml) at 65 °C for 5 min. Dilute the stock 1:100 to 5 µg/ml.
7. Dilute 6 µl of Lambda DNA Stock (5 µg/ml) into 228 µl of TE-R.

8. Prepare twofold serial dilutions (200 µl DNA into 200 µl diluent) to achieve the following concentrations: 6400 pg/100 µl; 3200 pg/100 µl; 1600 pg/100 µl; 800 pg/100 µl; 400 pg/100 µl; 200 pg/100 µl; 0 pg/100 µl (diluent only) (*see* **Note 19**).
9. Dilute PicoGreen stock solution 1:200 in TE (15 µl: 3 ml).

Keep in dark from here through the end of the procedure.

10. Add 200 µl of diluted PicoGreen to each standard (200 µl), vortex, and spin.
11. Add 100 µl of diluted PicoGreen to each IP DNA and input DNA, vortex.
12. Transfer 195 µl of each standard and unknown DNA (IP and input) into a 96-well fluorescent plate (*see* **Note 20**).
13. Measure concentrations using a Fluorescence spectrophotometer with Fluorescein detection 485/535 nm 1.0 NA (*see* **Note 21**).

3.2.9 Real Time PCR Setup

Each chromatin immunoprecipitation experiment requires at least two independently cross-linked samples and typically each sample is checked twice with each antibody.

1. Prepare tubes for quantitative PCR in metal rack on ice (*see* **Note 22**).
2. Compose reactions of 20 µl final volume for each input in triplicate and IP sample (equal amounts) in duplicate, use the following two Master Mixes (MM) (*see* **Note 23**):
   (a) DNA MM [enough for Inputs ×3 and IPs ×2]: PCR Mix (10 µl), DNA (5 µl) and ultrapure water (1.8 µl).
   (b) Primer MM: [for each set of primers]: Primers (0.6 µl each) and 1:10 SYBR Green (2 µl).

   First add 16.8 µl DNA MM per tube and next add 3.2 µl per tube of the Primer MM (*see* **Note 24**).
3. Real time PCR was carried out as with the following program: 95 °C for 5 min (1 cycle); 95 °C for 15 s, 53–58 °C for 20 s, 68–70 °C for 20 s (45 cycles) (*see* **Note 25**).
4. Analyze data as follows: The fold difference between the immunoprecipitated material and total input sample for each primer pair is calculated using the formula (IP/input = $2^{\text{Input Ct—IP Ct}}$) (*see* **Note 26**). The data are presented as mean values with standard error.

3.3 Microscopy Preparation for Yeast

3.3.1 Preparation of Yeast Cells for Slide Plating

1. Grow 2 ml cultures overnight in YMD + all six supplements.
2. Measure density of cells at A_{600}, and if over grown dilute to ~1 OD/ml.
3. Spin down and resuspend cells in 100 µl fresh YMD/all.

3.3.2 Preparation of Slides for Yeast Microscopy

1. Prepare fresh 1.5% agarose in YMD/all mix.
2. After microwaving to dissolve, transfer ~400 μl into a 1.5 ml microcentrifuge tube and place in heat block at 50 °C to equilibrate the temperature. You can also autoclave agarose/YMD and aliquot into 1.5 ml microcentrifuge tubes to store.
3. Allow equilibration for ~15 min.
4. Put 7.5 μl of the 1.5% agarose solution into a Teflon Printed Microscope Slide with wells and count out to 15 s.
5. Place a regular microscope slide firmly on top of the wells to flatten the agarose.
6. Let sit 2–5 min.
7. Remove by slowly twisting top slide off the Teflon-coated slide.
8. Check to see that the wells are smoothly coated with agarose. If not wipe with Kim wipes and repeat **steps 4**–7.
9. Place 0.8 μl of yeast cells into each agarose filled well.
10. Wait ~15 s and place square glass coverslip on top.
11. Fix coverslip to slide with nail polish.

3.3.3 Alternative Microscopy Prep for Yeast

1. Grow yeast overnight in 5 ml of YPD.
2. Next morning measure density of cells at A_{600}, and reinoculate in YMD + all mix at 0.5 OD.
3. Allow to grow 2 h at room temperature (cells should be in log phase for imaging).
4. Resuspend 1 ml of cells in ~20 μl of YMD (*see* **Note 27**).
5. Plate ~2 μl of cells on a slide and place coverslip on top (*see* **Note 28**).
6. Use a Kimwipe to wipe of the side of the slide and coverslip that will be in contact with the cells.
7. Seal all around each edge of the coverslip with nail polish.

4 Notes

1. Remove the metal stamp from the ethanol filled soaking tray and flame (tilting away). Take care that all of the ethanol is burned from the stamp. Touch the stamp to the inside lid of the petri dish to cool. Place stamp into the microtiter plate wells to collect a drop of the diluted liquid. Remove the stamp from the microtiter being careful not to cross-contaminate the liquid in neighboring wells. Place the stamp straight down onto the agar surface and let the stamp sit for several seconds on the agar before lifting straight back up and returning to the

same place in the 96 well plate. Check that equal sized drops were placed onto the agar.

2. Timing of Procedures—Day 1 Inoculation; Day 2 Crosslinking, Lysis and Sonication; Day 3 Size Analysis and IP; Day 4 Protein A/G Equilibration; Day 5 Wash and Elute DNA; Day 6 Quantification and real-time PCR.
3. Formaldehyde is toxic. Remove in a fume hood and wear gloves during use. Necessary fixing times vary by protein (some requiring up to 1 h). Wrap the lid with Parafilm to avoid evaporation. Store at 30–37 °C.
4. Prepare refrigerated centrifuge: set to 4 °C.
5. Use heated 25 G needle to poke hole in bottom of tube; loosen top during spin to allow liquid to spin out.
6. Polystyrene tubes are essential for sonication; glass beads may be recycled.
7. It is crucial to keep the sonication water cool by adding fresh ice-cold water with a small amount of ice after each round of sonication.
8. FA-140 can be added next day if desired.
9. No need to spin down, just freeze; may prepare Chelex for size analysis: 10% solution (100 mg Chelex 100 Resin in 1 ml MQ) and store in refrigerator overnight the day IPs are set up.
10. Mix (do *not* vortex) Chelex so that it is homogeneous; use pipet with cut tip to pipet chelex.
11. Prepare 1.2% agarose gel without Ethidium bromide.
12. If sonicated DNA is larger than 1000 bp then additional sonication may be necessary.
13. Shake and resuspend before each use; use pipet with cut tip; wash the tip well so there are no beads stuck to it.
14. During incubation make up FA-140 + BSA 0.1%. Also make up mixture of FA-140, 0.1% BSA, and 0.05% sodium azide (NaN3); NaN3 is toxic, take appropriate precautions.
15. Do IP here; this can stay on ice for longer than 2 h.
16. These aliquots can be used to perform Western analysis for presence of protein if necessary.
17. Steps can be performed at room temperature from here on; keep reagents on ice as needed.
18. Preheat oven to 55 °C.
19. Diluent = TE-R. Dilute 200 µl down removing 200 µl from final 200 pg/100 µl and adding none to 0 pg/100 µl.
20. Place filled microtiter plate in a black box to keep it in the dark.
21. May arrange rack low to high 0–6400.

22. When handling qPCR tubes wear gloves/tweezers as fingers will leave oil and prints may affect measurements.
23. Always make at least 10% extra for master mixes.
24. If a new box of tips is used it is easy to keep track of the addition of primers to the samples.
25. Choose the appropriate annealing temperature and extension time based on composition of the reaction primers.
26. For accuracy, record/calculate values in an Excel sheet with preset formulas.
27. Microscopy Preparation for Yeast: Use your judgment, if cells look a little denser, then dilute further.
28. Use a Kim wipe to wipe of the side of the slide and coverslip that will be in contact with the cells.

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