Chromatin Remodeling Factors Isw2 and Ino80 Regulate Checkpoint Activity and Chromatin Structure in S Phase

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ABSTRACT When cells undergo replication stress, proper checkpoint activation and deactivation are critical for genomic stability and cell survival and therefore must be highly regulated. Although mechanisms of checkpoint activation are well studied, mechanisms of checkpoint deactivation are far less understood. Previously, we reported that chromatin remodeling factors *Is*w2 and *Ino*80 attenuate the S-phase checkpoint activity in *Saccharomyces cerevisiae*, especially during recovery from hydroxyurea. In this study, we found that *Is*w2 and *Ino*80 have a more pronounced role in attenuating checkpoint activity during late S phase in the presence of methyl methanesulfonate (MMS). We therefore screened for checkpoint factors required for *Is*w2 and *Ino*80 checkpoint attenuation in the presence of MMS. Here we demonstrate that *Is*w2 and *Ino*80 antagonize checkpoint activators and attenuate checkpoint activity in S phase in MMS either through a currently unknown pathway or through RPA. Unexpectedly, we found that *Is*w2 and *Ino*80 increase chromatin accessibility around replicating regions in the presence of MMS through a novel mechanism. Furthermore, through growth assays, we provide additional evidence that *Is*w2 and *Ino*80 partially counteract checkpoint activators specifically in the presence of MMS. Based on these results, we propose that *Is*w2 and *Ino*80 attenuate S-phase checkpoint activity through a novel mechanism.

KEYWORDS *Is*w2; *Ino*80; chromatin accessibility; S-phase checkpoint

FOR proper propagation of cells, DNA must be faithfully copied during replication in S phase. However, during replication, cells are prone to encountering nucleotide depletion or DNA damage, which generates replication stress and induces replication fork stalling. Upon replication stress, cells trigger a mechanism called the S-phase checkpoint to allow for proper completion of replication before exit out of S phase. Because this pathway is essential for genomic stability, as evidenced by the development of cancer or cell death in checkpoint mutants (Zeman and Cimprich 2014) and strict conservation in eukaryotic cells, mechanisms of S-phase checkpoint activation have been studied extensively (Branzei and Foiani 2009).

When replication forks stall in budding yeast cells, excess single-stranded DNA (ssDNA) is produced that is bound by replication protein A (RPA). Accumulation of the ssDNA-RPA

complex recruits a central kinase *Mec*1 [ataxia telangiectasia and Rad3-related kinase (ATR) in mammalian cells] with its interacting partner *Ddc*2 [ATR interacting protein (ATRIP)], which activates the downstream effector kinases *Rad*53 (*Chk*2) and *Chk*1 (*Chk*1) to stabilize replication forks, up-regulate damage-inducible genes, and slow S-phase progression through the delay of late origin firing until DNA is replicated correctly (Rouse and Jackson 2002; Zou and Elledge 2003; Friedel *et al.* 2009). The S-phase checkpoint can be activated via two different pathways, the DNA damage checkpoint (DDC) and/or the DNA replication checkpoint (DRC), depending on the type of damage recognized by the cell (Crabbe *et al.* 2010). In the DDC of budding yeast, the clamp loader RFC^{Rad24} (RFC^{Rad17}) and subsequently the PCNA-like 9-1-1 clamp Rad17-Ddc1-Mec3 (Rad9-Hus1-Rad1) are recruited to ssDNA–double-stranded DNA (dsDNA) junctions to facilitate activation of the checkpoint along with *Dpb*11 (TopBP1) and *Rad*9 (Gilbert *et al.* 2001; Kondo *et al.* 2001; Majka *et al.* 2006; Navadgi-Patil and Burgers 2008). In the DRC, *Tof*1 (TIM), *Mrc*1 (claspin), and *Dna*2 are recruited to replication forks to facilitate activation of the checkpoint (Katou *et al.* 2003; Kumar and Burgers 2013).

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doi: 10.1534/genetics.115.174730

Manuscript received January 19, 2015; accepted for publication February 13, 2015; published Early Online February 19, 2015.

Supporting information is available online at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.174730/-DC1>

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In contrast to S-phase checkpoint activation mechanisms, mechanisms that regulate checkpoint deactivation are far less understood. The best-characterized mechanism of checkpoint deactivation is via *Rad53* phosphatases *Pph3* and *Ptc2*. Deletion of *Pph3* and *Ptc2* results in elevated sensitivity to replication inhibitors and complete replication fork arrest (Szyjka *et al.* 2008). This result demonstrates that proper control of the amplitude and inactivation of the S-phase checkpoint is also essential for DNA replication control in the presence of replication stress. It also has been shown that other modes of checkpoint deactivation exist that have yet to be identified, highlighting the need for more studies on checkpoint deactivation mechanisms (Travesa *et al.* 2008).

We have recently proposed that the highly conserved chromatin remodeling factors *Isw2* and *Ino80* play roles in S-phase checkpoint attenuation (Au *et al.* 2011). Chromatin remodeling factors use the energy of ATP hydrolysis to alter chromatin structure and have been implicated in many DNA-dependent processes, including transcription, DNA replication, DNA repair, and checkpoint regulation (Morrison and Shen 2009). *Isw2* has been demonstrated to slide nucleosomes *in vivo* (Fazzio and Tsukiyama 2003). Functionally, *Isw2* has been shown to repress transcription of genes by sliding nucleosomes over transcriptional start sites (Goldmark *et al.* 2000; Whitehouse *et al.* 2007). In the mean time, *Ino80* can replace the histone variant *Htz1* within nucleosomes with canonical histone H2A (Papamichos-Chronakis *et al.* 2011). *Ino80* is required for proper DNA damage response after DNA double-strand breaks (Shen *et al.* 2000; van Attikum *et al.* 2004), as well as replication stress responses such as maintaining fork stability and promoting recovery of stalled forks (Papamichos-Chronakis and Peterson 2008; Shimada *et al.* 2008). However, how this complex performs these functions remains largely unknown.

We previously reported that *Isw2* and *Nhp10*, a subunit of the *Ino80* complex, function together to attenuate the S-phase checkpoint after transient exposure to the DNA replication inhibitor hydroxyurea (HU) (Au *et al.* 2011). In this study, we show that *Isw2* and *Nhp10* exhibit a much stronger effect in attenuating checkpoint activity in the presence of the DNA alkylating agent methyl methanesulfonate (MMS) throughout late S phase. Through systematic genetic tests, we show that *Isw2* and *Nhp10* antagonize checkpoint activators and facilitate checkpoint attenuation in MMS either through a currently unknown pathway or through the ssDNA binding protein RPA. In investigating changes in chromatin structure in *isw2 nhp10* cells during replication stress, we unexpectedly found that *Isw2* and *Ino80* increase chromatin accessibility around replicating regions through a mechanism unrelated to their known biochemical activities. Consistent with checkpoint activity control, growth assays in the presence of MMS reveal that *Isw2* and *Ino80* partially oppose checkpoint activators specifically in the presence of MMS. Based on these results, we propose that *Isw2* and *Ino80* alter chromatin structure at replicating regions and attenuate the S-phase checkpoint through currently unknown mechanism(s).

Materials and Methods

Yeast strains and culture

All yeast strains are *MATa* and congenic to W303-1a with a correction for the weak *rad5* allele in the original W303 (Thomas and Rothstein 1989; Zhao *et al.* 1998). Strains used for stable isotope labeling by amino acids in cell culture (SILAC) mass spectrometry were in a Δ *lys2* Δ *arg4* *CAN1* + background. Strains were constructed using standard gene knockout protocols and genetic crosses (Table 1).

Cell synchronization and flow cytometry (FACS)

All yeast strains were grown at 30° to early log phase to a density of OD₆₆₀ = 0.2–0.25 and then arrested in G₁ phase with a final concentration of 5 μ g/ml α factor. Cells were filtered on a 0.45-mm nitrocellulose membrane, washed twice with YPD, and released into half the volume of prewarmed YPD containing 0.02% MMS. Cells were harvested at each time point, stored in a final concentration of 66.7% (v/v) ethanol, and then processed for flow cytometry as described previously (Vincent *et al.* 2008).

Rad53 in situ autophosphorylation assay (ISA) and Rad53 Western blotting

Cells were arrested in G₁ by α factor (5 μ g/ml) and released into rich medium (YPD) containing 0.02% MMS, as described earlier. In the initial protein preparation method, ~25 ml of cells was harvested for each time point. However, in the modified method accounting for cell density, 10⁸ cells were harvested at each time point. Cells were washed once with water and then with 20% trichloroacetic acid (TCA). Protein samples were prepared as described previously and run on an 8% SDS–polyacrylamide gel (Pelliccioli *et al.* 1999). *Rad53* autophosphorylation was normalized by autophosphorylation of nonspecific proteins within the same sample that served as a loading control. For *Rad53* Western blotting, goat polyclonal *Rad53* (yC-19) antibody (sc-6749, Santa Cruz Biotech, Santa Cruz, CA) was used.

Chromatin fractionation and SILAC

Strains were grown in yeast culture (YC) medium with either 15 mg/liter L-arginine and 30 mg/liter L-lysine or 15 mg/liter [¹³C₆]L-arginine and 30 mg/liter [¹³C₆, ¹⁵N₂]L-lysine. Then 4 × 10⁹ cells were harvested at mid-S phase in 0.02% MMS, and chromatin was prepared as described previously (Kubota *et al.* 2012). The chromatin pellet was resuspended in 1.5× Tris-glycine SDS sample buffer. An H2B Western blot using the H2B C-term antibody (Active Motif, Carlsbad, CA) was used for normalization. RPA polyclonal antibody (AS07 214, Agrisera, Vännäs, Sweden) was used to detect *Rfa1*. Equivalent amounts of protein were loaded onto an 8–16% Tris-glycine gel (Thermo Fisher Scientific, Rockford, IL), washed in sterile water, stained with GelCode Blue Safe Protein Stain (Thermo Fisher Scientific), and then destained in water. The lane was cut into 10 slices and subjected to mass spectrometry (MS).

Table 1 Summary of Yeast Strains Used in This Study

Strain	Genotype	Source
W1588-4c	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5+</i>	Zhao <i>et al.</i> 1998; Thomas and Rothstein 1989
YTT3777	<i>MATa Δisw2::NatMX Δnhp10::Hyg</i>	Au <i>et al.</i> 2011
YTT4960	<i>MATa Pol1-3FLAG-KanMX</i>	Rodriguez and Tsukiyama 2013
YTT5031	<i>MATa Pol1-3FLAG-KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT5787	<i>MATa Δexo1::KanMX</i>	This study
YTT5805	<i>MATa Δexo1::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT5822	<i>MATa Δsgs1::KanMX</i>	This study
YTT5852	<i>MATa Δsgs1::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT2616	<i>MATa Δrad50::KanMX</i>	Vincent <i>et al.</i> 2008
YTT2653	<i>MATa Δrad50::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	Vincent <i>et al.</i> 2008
YTT5969	<i>MATa Δslx4::KanMX</i>	This study
YTT5931	<i>MATa Δslx4::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT5277	<i>MATa Δelg1::KanMX</i>	This study
YTT5279	<i>MATa Δelg1::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT3203	<i>MATa Δmrc1::KanMX</i>	Vincent <i>et al.</i> 2008
YTT3224	<i>MATa Δmrc1::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	Vincent <i>et al.</i> 2008
Ssy072	<i>MATa Δmrc1::HIS5 pRS405-mrc1aq::LEU2</i>	Szyjka <i>et al.</i> 2005
YTT5860	<i>MATa Δmrc1::HIS5 pRS405-mrc1aq::LEU2 Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT5952	<i>MATa Δrad9::KanMX Δmrc1::HIS5 pRS405-mrc1aq::LEU2</i>	This study
YTT5956	<i>MATa Δrad9::KanMX Δmrc1::HIS5 pRS405-mrc1aq::LEU2 Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT3394	<i>MATa Δtof1::KanMX</i>	Vincent <i>et al.</i> 2008
YTT3409	<i>MATa Δtof1::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	Vincent <i>et al.</i> 2008
YTT5986	<i>MATa dna2-WY-AA</i>	Modified from Kumar and Burgers 2013
YTT5990	<i>MATa dna2-WY-AA Δddc1::KanMX</i>	This study
YTT5994	<i>MATa dna2-WY-AA Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT5996	<i>MATa dna2-WY-AA Δddc1::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT3759	<i>MATa Δctf18::KanMX</i>	This study
YTT5275	<i>MATa Δctf18::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT2883	<i>MATa Δddc1::KanMX</i>	Vincent <i>et al.</i> 2008
YTT2904	<i>MATa Δddc1::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	Vincent <i>et al.</i> 2008
YBP82	<i>MATa dpb11-600::Hyg</i>	Pfander and Diffley 2011
YTT5831	<i>MATa dpb11-600::Hyg Δisw2::NatMX Δnhp10::KanMX</i>	This study
YTT5943	<i>MATa dpb11-600::Hyg Δddc1::URA3</i>	This study
YTT5948	<i>MATa dpb11-600::Hyg Δddc1::URA3 Δisw2::NatMX Δnhp10::KanMX</i>	This study
YTT2953	<i>MATa Δrad17::KanMX</i>	Vincent <i>et al.</i> 2008
YTT2958	<i>MATa Δrad17::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	Vincent <i>et al.</i> 2008
YTT2938	<i>MATa Δrad24::KanMX</i>	Vincent <i>et al.</i> 2008
YTT2943	<i>MATa Δrad24::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	Vincent <i>et al.</i> 2008
YTT2885	<i>MATa Δrad9::KanMX</i>	Vincent <i>et al.</i> 2008
YTT2909	<i>MATa Δrad9::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	Vincent <i>et al.</i> 2008
YTT4976	<i>MATa Δmec1::KanMX Δsml1::NatMX</i>	Au <i>et al.</i> 2011
YTT4967	<i>MATa Δmec1::KanMX Δsml1::NatMX Δisw2::LEU2 Δnhp10::Hyg</i>	Au <i>et al.</i> 2011
YTT4560	<i>MATa Δtel1::KanMX</i>	This study
YTT4584	<i>MATa Δtel1::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT5327	<i>MATa Δchk1::KanMX</i>	This study
YTT5329	<i>MATa Δchk1::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
RDKY2218	<i>MATa rfa1-t11</i>	Umezū <i>et al.</i> 1998
YTT6113	<i>MATa rfa1-t11 Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT5293	<i>MATa ADE2 URA3::CUP1-Ub-Rfa1-8ala-CFP</i>	Modified from Lisby <i>et al.</i> 2004
YTT5683	<i>MATa ADE2 URA3::CUP1-Ub-Rfa1-8ala-CFP Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT3621	<i>MATa Δhtz1::KanMX</i>	This study
YTT3636	<i>MATa Δhtz1::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT6083	<i>MATa Δlys2 Δarg4::HIS3 CAN1+ Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT6080	<i>MATa Δlys2 Δarg4::HIS3 CAN1+</i>	This study
YTT6184	<i>MATa Δrad51::KanMX</i>	This study
YTT6192	<i>MATa Δrad51::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT6188	<i>MATa Δrad52::KanMX</i>	This study
YTT6196	<i>MATa Δrad52::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT5323	<i>MATa dpb11-1</i>	Masumoto <i>et al.</i> 2000
YTT5325	<i>MATa dpb11-1 Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT6277	<i>MATa yku70::KanMX</i>	This study

(continued)

Table 1, continued

Strain	Genotype	Source
YTT6282	<i>MATa yku70::KanMX Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT6115	<i>MATa rad52-21</i>	Miles et al. 2013
YTT6117	<i>MATa rad52-21 Δisw2::NatMX Δnhp10::Hyg</i>	This study
YTT6355	<i>MATa orc2-1</i>	This study
YTT6357	<i>MATa orc2-1 Δisw2::NatMX Δnhp10::KanMX</i>	This study
YTT2612	<i>MATa Δswr1::Hyg</i>	This study
YTT6363	<i>MATa Δswr1::Hyg Δnhp10::KanMX</i>	This study
YTT6365	<i>MATa Δswr1::Hyg Δisw2::NatMX</i>	This study
YTT6367	<i>MATa Δswr1::Hyg Δisw2::NatMX Δnhp10::KanMX</i>	This study
YTT3122	<i>MATa Δhtz1::Hyg</i>	This study
YTT2189	<i>MATa Δisw2::NatMX Δies5::KanMX</i>	This study
YTT6371	<i>MATa Δisw2::NatMX Δies5::KanMX Δhtz1::Hyg</i>	This study
06328	<i>MATa pol2-12</i>	Li et al. 2008

All strains are congenic to W1588-4c with a correction for the weak *rad5* allele.

Liquid chromatography–tandem mass spectrometry and data analysis

The 10 gel pieces were destained with 50% methanol, 5% acetic acid and washed consecutively with water twice and 50% acetonitrile. After rinsing once more with 100 mM ammonium bicarbonate in water, the gel pieces were dehydrated using acetonitrile. The protein was digested overnight at 37° with 12.5 ng/μl trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate. After transferring excess solutions to separate vials, the peptides were first extracted using 0.1% trifluoroacetic acid in water for 30 min, and then acetonitrile was added to make 50% acetonitrile, 0.1% trifluoroacetic acid. The pooled extracts were dried in a speed vacuum and cleaned using ZipTip C18 (Millipore, Bedford, MA) before the subsequent MS analysis following the manufacture's protocols. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was performed with an Easy-nLC 1000 (Thermo Fisher Scientific) coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific). The LC system configured in a vented format (Licklider et al. 2002) consisted of a fused-silica nanospray needle (PicoTip emitter, 50 μm ID, New Objective, Woburn, MA) packed in-house with Magic C18 AQ 100 Å reverse-phase medium (25 cm, Michrom BioResources, Auburn, CA) and a trap (IntegraFrit Capillary, 100 μm ID, New Objective) containing Magic C18 AQ 200 Å (2 cm). The peptide sample was diluted in 10 μl of 2% acetonitrile and 0.1% formic acid in water, and 8 μl was loaded onto the column and separated using a two-mobile-phase system consisting of 0.1% formic acid in water (A) and 0.1% acetic acid in acetonitrile (B). A 90-min gradient from 7 to 35% acetonitrile in 0.1% formic acid at a flow rate of 400 nl/min was used for chromatographic separations. The mass spectrometer was operated in a data-dependent MS/MS mode over the m/z range of 400–1800. The mass resolution was set at 120,000. For each cycle, the 15 most abundant ions from the scan were selected for MS/MS analysis using 35% normalized collision energy. Selected ions were dynamically excluded for 30 sec. Data analysis was performed using Proteome Discoverer 1.4 (Thermo Fisher Scientific). The data were searched against *Saccharomyces*

cerevisiae strain S288C protein sequences that were updated on February 3, 2011, from the Saccharomyces Genome Database (SGD, <http://www.yeastgenome.org/>), which included common contaminants. Trypsin was set as the enzyme, with maximum missed cleavages set to 2. The precursor ion tolerance was set to 10 ppm, and the fragment ion tolerance was set to 0.6 Da. Variable modifications were set to methionine oxidation (+15.995 Da), 6 × ¹³C (+6.020 Da) on arginine, and 6 × ¹³C and 2 × ¹⁵N on lysine. Sequest (Eng et al. 1994) was used for search, and search results were run through Percolator (Kall et al. 2007) for scoring. The ratio between heavy and light amino acids was normalized to an average of all histone proteins detected by MS.

Genomic DNA profiles, microarray hybridization, and data analysis

DNA samples were amplified, fragmented, and labeled as described previously (Rodriguez and Tsukiyama 2013). S-phase DNA samples were competitively hybridized to G₁ DNA for replication profiling to custom-tiled arrays, as described previously (Rodriguez and Tsukiyama 2013). The data were smoothed using a previously established pseudomedian method (Royce et al. 2007) with a 50-bp moving window. Relative RPA levels were ranked based on increasing amounts of genomic DNA content for both wild-type (WT) and *isw2 nhp10* samples, where the average amounts of RPA were further smoothed with a 100-bp sliding window. The ribosomal DNA (rDNA) locus and outliers from either extremely low or saturated DNA signals were left out of the analysis.

Normalized chromatin accessibility to MNase (NCAM) assay

Cells were harvested from G₁ and at an early time point S phase in 0.02% MMS, where FACS profiles were similar between WT and *isw2 nhp10* cells. Sample harvest, preparation, and analysis were performed as described previously (Rodriguez and Tsukiyama 2013; Rodriguez et al. 2014). Z-score normalization was performed as described previously (Rodriguez and Tsukiyama 2013) but based on digestion patterns at the +2 to +6 nucleosomes within ORFs genome-wide rather

than transcriptional start sites (TSSs) because promoter regions tend to be targets for *Isw2* and *Ino80* remodeling (Whitehouse *et al.* 2007; Papamichos-Chronakis *et al.* 2011). The NCAM graphs were generated by integrating the signal over 40-kb regions surrounding three classes of origin: early high ($n = 11$), early low ($n = 12$), and late ($n = 37$). To determine S-phase-specific changes, NCAM from G₁ was subtracted from S phase. NCAM was averaged over a 40-kb region surrounding the origin or a 3-kb region surrounding TSSs to quantitate relative NCAM signal.

Results

Isw2 and *Ino80* attenuate the checkpoint on MMS treatment in middle-late S phase

S. cerevisiae *Isw2p* is the ATPase subunit of the *Isw2* chromatin remodeling factor involved in gene repression (Goldmark *et al.* 2000; Whitehouse *et al.* 2007). *Nhp10p* is a subunit of *Ino80* that is known to exist exclusively within the *Ino80* complex and facilitates interactions with chromatin (Morrison *et al.* 2004). We previously reported that the *isw2 nhp10* mutant exhibits two phenotypes in the presence of replication inhibitors: (1) slow growth due to a prolonged S phase without an increase in cell death and (2) overactivation of the checkpoint during S phase (Vincent *et al.* 2008; Au *et al.* 2011). Previously, we reported that *isw2 nhp10* elevates and prolongs checkpoint activity during S phase in the presence of replication inhibitors, especially after transient exposure to HU (Au *et al.* 2011). Our *Rad53* ISA revealed rapid loss of checkpoint activity in WT cells but not in *isw2 nhp10* cells after transient treatment with HU (Au *et al.* 2011). However, on examining *Rad53* checkpoint activity by Western blot, we noticed that the *Rad53* protein could no longer be detected in later time points when samples were prepared according to the protocol used in our previous report (Au *et al.* 2011) (Figure 1A). Importantly, *Rad53* protein was lost at much earlier time points in WT than in *isw2 nhp10* cells (Figure 1A). We found that the loss of *Rad53* protein was attributed to an increase in cell density as the time course went on, which we did not adjust for previously, causing inefficient TCA precipitation of proteins. Because of the slower S-phase progression in *isw2 nhp10* cells, *Rad53* is lost later in *isw2 nhp10* compared with WT cells (Figure 1A). Thus the apparent checkpoint hyperactivity in *isw2 nhp10* cells after HU treatment detected by ISA could be attributed to inefficient recovery of *Rad53* protein in WT cells. We therefore prepared protein samples adjusting for the differences in cell numbers between WT and *isw2 nhp10* cells (see *Materials and Methods* for details) and repeated the ISA. This revealed that although *Rad53* protein is still slightly more phosphorylated based on Western blotting in *isw2 nhp10* compared with WT cells after HU treatment (Figure 1B), the difference in phosphorylation is not to the same degree as what we previously reported using ISA (Figure 1A).

These results prompted us to reexamine the checkpoint activity of WT and *isw2 nhp10* cells under various conditions

using both Western blotting and *Rad53* ISA while accounting for cell density. These tests revealed that the difference in *Rad53* checkpoint activity between WT and *isw2 nhp10* cells is much more robust upon MMS treatment than HU treatment (Figure 1, B and C). To investigate how *Isw2* and *Ino80* attenuate checkpoint activity in MMS, we sought to determine when, during S phase, *Isw2* and *Ino80* perform their roles by defining the kinetics of checkpoint activity. To this end, we performed a time-course experiment in which cells were arrested in G₁ and released into S phase in rich medium containing MMS, and we monitored cell cycle progression and checkpoint activity by FACS and *Rad53* ISA, respectively (Figure 1D and Supporting Information, Figure S1). Consistent with our previous report (Vincent *et al.* 2008), *isw2 nhp10* cells have a delay in S-phase progression that is not a result of incomplete replication (Figure 1D and Figure S1B). The delay is associated with stronger and prolonged checkpoint activity in the presence of MMS (Figure 1E). This experiment revealed that the biggest difference in checkpoint activity occurs during middle-late S phase (Figure 1E). Consistent with the fact that early origin firing is not greatly affected in *isw2 nhp10* cells (Vincent *et al.* 2008), these results suggest that *Isw2* and *Ino80* function after replication has already been initiated. Once checkpoint activity is more strongly triggered in S phase, activation remains elevated via transition into G₂/M phase, which coincides with the presence of a subpopulation of cells that continue to remain in S phase in *isw2 nhp10* cells (Figure 1, C and D). Taken together, our results suggest that *Isw2* and *Ino80* prevent hyperactivation of the checkpoint activity in middle to late S phase in the presence of MMS to allow for proper replication progression and transition into G₂/M phase.

Isw2 and *Ino80* function outside of DNA repair or fork protection pathways

Because checkpoint activity is much more strongly activated in *isw2 nhp10* cells by MMS than by HU, we next tested whether *ISW2* and *NHP10* could function through DNA repair or replication fork protection pathways. We have shown previously, by genetic tests, that *ISW2* and *NHP10* do not function through several genes involved in replication fork protection and DNA repair pathways (Vincent *et al.* 2008), leading to the conclusion that they do not function via these pathways (Au *et al.* 2011). However, these tests were not very quantitative, and additional genes involved in DNA damage response and replication fork protection were identified after our previous tests (Crabbe *et al.* 2010; Engels *et al.* 2011; Hegnauer *et al.* 2012; Gonzalez-Prieto *et al.* 2013; Kubota *et al.* 2013). We therefore performed more comprehensive genetic tests to determine whether *ISW2* and *NHP10* function in these pathways. *ISW2* and *NHP10* were deleted in a given mutant to create a triple mutant, and the single- and triple-mutant growth phenotypes were compared on gradient plates of rich medium with increasing concentrations of MMS (Figure 2A). If *ISW2* and *NHP10* function through a pathway in which a DNA damage response or replication fork protection gene plays a central role, the single and triple mutants should exhibit similar MMS

Figure 1 *isw2 nhp10* cells display stronger checkpoint activity throughout S phase and into G₂/M phase in the presence of MMS. (A) Rad53 Western blot of WT and *isw2 nhp10* cells using the protein preparation methods in our previous report (Au *et al.* 2011). Cells were arrested in G₁ with α factor for 90 min and released into 200 mM HU for 90 min, and then HU was washed out. rec, recovery time in minutes. (B) Rad53 Western blot of protein preparations that used the amount of TCA normalized for cell volume. (C) A Western blot (W) and *in situ* autophosphorylation assay (ISA) of Rad53 of samples taken from indicated time points. Time points indicated in purple represent when cells are in S phase. (D) Cell cycle profiles of WT and *isw2 nhp10* cells after release from α factor into medium containing MMS. Asynchronous profiles are shown in gray. FACS profiles shown in purple indicate time points in S phase. (E) Quantitation of Rad53 ISA time course. WT checkpoint activity was measured at 60 min for middle S, 90 min for late S, 120 min for early G₂/M, and 150 min for late G₂/M. *isw2 nhp10* checkpoint activity was measured at 120 min for middle S, 180 min for late S, 240 min for early G₂/M, and 300 min for late G₂/M. The error bars denote the standard errors of the mean from two experiments using independently created strains.

sensitivity. In contrast, if *ISW2* and *NHP10* function independently of a DNA damage response or replication fork protection gene, the phenotypes should be additive, and the triple mutant should be more sensitive than the single mutant. *CTF18*, *ELG1*, and *SGS1* all influence checkpoint activity (Kanellis *et al.* 2003; Crabbe *et al.* 2010; Hegnauer *et al.* 2012). All the triple mutants within these mutant backgrounds show additive MMS sensitivity, suggesting that these genes are not required for *ISW2* and *NHP10* function (Figure 2A). Other genes involved in fork protection (lavender), replication (gray), and different DNA damage response pathways (teal) also show that *Isw2* and *Ino80* act independently of these pathways (Figure 2B). DNA polymerase ϵ (Pol ϵ) associates with *Dpb11* to facilitate DNA replication and the S-phase checkpoint (Masumoto *et al.* 2000), and the *Dpb4* subunit of Pol ϵ also exists in the *Isw2* complex (Iida and Araki 2004; McConnell *et al.* 2004). Therefore, to test genetic interactions of Pol ϵ with *Isw2* and *Nhp10*, we used a *pol2-12* mutant that attenuates S-phase checkpoint activity (Li *et al.* 2008). However, the *pol2-12*

isw2 nhp10 mutants exhibited extreme growth defects and readily picked up suppressor mutations (data not shown). While we were not able to measure MMS sensitivity of this mutant, the additive effect even in the absence of replication stress suggests that *Isw2* and *Nhp10* have functions independent of Pol ϵ . Together these results reinforce the conclusion that *Isw2* and *Ino80* do not function through DNA repair or replication fork protection pathways.

***Isw2* and *Ino80* antagonize checkpoint activators and attenuate checkpoint activity independent of any single checkpoint factor downstream of RPA**

The preceding results are consistent with our model that *Isw2* and *Ino80* play a direct role in attenuating the S-phase checkpoint activity. Thus, to gain insight into the mechanism of checkpoint attenuation by *Isw2* and *Ino80*, we performed systematic genetic tests to screen for checkpoint factors required for *Isw2* and *Ino80* checkpoint function using the Rad53 ISA. Based on a similar reasoning as earlier, if a checkpoint protein

Figure 2 *Isw2* and *Ino80* function is independent of DNA damage response or fork protection pathways. (A) Representative results of the spot tests on 0.02% MMS gradient plates performed as described previously (Au *et al.* 2011). The top panel shows that deletion of replication components such as *ELG1* or *CTF18* results in additive MMS sensitivity with *isw2 nhp10*. The bottom panel shows that deletion of a DNA resection factor *SGS1* is additive with *isw2 nhp10* as well. *mec1 sml1* serves as a control to determine the severity of sensitivity on MMS. (B) A summary of the spot-test results with genes involved in DNA repair and replication fork protection. MMS sensitivity is ranked on a scale of

1–6, with 1 being the least sensitive (WT level) and 6 being the most sensitive. Repair genes are shown in teal, replication fork protection genes are shown in lavender, and other genes involved in replication are shown in gray.

is required for *Isw2* and *Ino80* to attenuate checkpoint activity, we would expect deletion of *ISW2* and *NHP10* to not increase checkpoint activity in the relevant mutant background. Alternatively, if a given checkpoint protein is not required for *Isw2* and *Ino80* function, then deletion of *ISW2* and *NHP10* still would increase the deficient checkpoint activity in the checkpoint mutants. Through these analyses, we aimed to identify the most upstream checkpoint factor by which *Isw2* and *Ino80* attenuate checkpoint activity. We screened all major checkpoint factors known to be required for robust S-phase checkpoint activity (Figure 3A). Unless otherwise indicated, the checkpoint mutants tested were null mutations. The S-phase checkpoint cascade has two partially overlapping pathways (Figure 3A), the DRC and the DDC, that both function to activate the S-phase checkpoint. Although the DDC (blue) is activated primarily in the presence of MMS, the DRC (pink) can redundantly function to partially activate the S-phase checkpoint in the absence of DDC factors (Branzei and Foiani 2009). Some Checkpoint components (yellow) function in both the DDC and the DRC and thus are most critical for proper activation. Complete deletion of a DRC component resulted in severe MMS sensitivity because such components are also involved in DNA replication, in addition to the DRC (Figure 2B). Therefore, for checkpoint factors that also have roles in DNA replication, we used *mrc1-aq* (Szyjka *et al.* 2005), *dpb11-600* (Pfander and Diffley 2011), and *dna2-WY-AA* (Kumar and Burgers 2013), which are alleles that were shown previously to be specifically deficient in checkpoint activation. Unexpectedly, we found that the *isw2 nhp10* mutation partially rescued checkpoint activity in all the checkpoint-deficient mutants tested, for both DRC and DDC mutants, as indicated by a checkpoint activity ratio that was significantly higher than one on *ISW2* and *NHP10* deletion (Figure 3B). These results suggest that *Isw2* and *Ino80* antagonize checkpoint activators in affecting S-phase checkpoint activity. *Mec1* plays a central role in S-phase checkpoint activation, and the absence of the *Mec1* kinase severely abrogates the S-phase checkpoint (Branzei and Foiani 2010). Perhaps most surprisingly, even in *mec1 sml1*

cells, where checkpoint activity is mostly depleted, deletion of *ISW2* and *NHP10* still results in higher checkpoint activity during S phase, indicating that *Isw2* and *Ino80* function independent of *Mec1* (Figure 3, C and D). Residual checkpoint activity in the *mec1 sml1* mutant is due to the partially redundant function of *Tel1* (Mantiero *et al.* 2007). The mode of *Tel1* activation in S phase is not well understood, but in the absence of *Mec1*, *Tel1* can activate the checkpoint to a much lesser degree (Zou 2013). We attempted to examine the effects of *Isw2* and *Ino80* when the S-phase checkpoint is completely abolished by making a *mec1 tel1 sml1 isw2 nhp10* mutant. However, *tel1* deletion alone causes strong synthetic growth defects with the *isw2 nhp10* mutation independent of S-phase checkpoint function (Figure 6A). Because of this genetic interaction, the quintuple mutant had extreme growth defects and readily picked up suppressors (data not shown), making phenotype analyses impossible. The *dna2-WY-AA ddc1* mutant, which abolishes *Mec1* activation through both the DRC and DDC pathways (Kumar and Burgers 2013), also displayed higher checkpoint activity on *ISW2* and *NHP10* deletion (Figure 3B). This result is consistent with the partial rescue of the checkpoint activity of the *mec1* mutant by *isw2 nhp10*. These findings suggest that *Isw2* and *Ino80* deactivate checkpoint activity independent of both the DRC and DDC pathways. We were unable to test RPA because of the lack of an *rpa* mutant allele that solely affects checkpoint activity. Because RPA is essential for viability and has various roles in DNA replication, checkpoint activation, DNA damage response, and recombination, deleting *ISW2* and *NHP10* in *rpa* mutant backgrounds *rfa1-deg* and *rfa1-t11* (Umezaki *et al.* 1998; Lisby *et al.* 2004) causes severe growth defects (data not shown). While the strong synthetic growth defects of the *rpa isw2 nhp10* mutant were not unexpected, they made measuring the checkpoint activity in the mutant impossible. Nevertheless, our data demonstrate that no known S-phase checkpoint factor downstream of RPA is required for *Isw2* and *Ino80* to attenuate checkpoint activity. These results collectively suggest that *Isw2* and *Ino80* function either through currently unidentified checkpoint

Figure 3 *Isw2* and *Ino80* function is independent of any single checkpoint factor downstream of RPA. (A) Schematic drawing of S-phase checkpoint pathway, which is divided into the DNA replication checkpoint (DRC) in pink, DNA damage checkpoint (DDC) in blue, and factors present in both pathways in yellow. (B) Bar graph showing the ratio of S-phase checkpoint activity in checkpoint mutants in the absence of *ISW2* and *NHP10* compared with the checkpoint mutant alone. The dashed red line indicates the expected S-phase checkpoint ratio if a given checkpoint protein is required for *Isw2* and *Ino80* function. (C) Rad53 ISA of *mec1 sml1* and *mec1 sml1 isw2 nhp10* cells released from G₁ into S phase containing 0.02% MMS. Time points in S phase are denoted in purple. (D) Corresponding FACS profiles of time points from the ISA, with profiles in purple denoting time points in S phase.

activator(s) or through RPA, which feeds into multiple independent checkpoint activation pathways. For *MRC1*, *DPB11*, and *DNA2*, we cannot rule out the possibility that the alleles we used did not inactivate their relevant checkpoint activation function.

Isw2 and *Ino80* do not affect the abundance of checkpoint factors

We envision that *Isw2* and *Ino80* attenuate checkpoint activity via at least two possible mechanisms. One possibility is that *Isw2* and *Ino80* may facilitate removal of a checkpoint protein from replication forks. Alternatively, they may down-regulate the activity of a checkpoint protein. We first tested whether *Isw2* and *Ino80* alter the abundance of chromatin-bound RPA by chromatin fractionation from cells in G₁ and S phase with 0.02% MMS (Figure 4A), followed by Western blotting. This analysis revealed that relative levels of chromatin-bound RPA were similar in both WT and *isw2 nhp10* cells in G₁ and S phase, indicating that higher checkpoint activity in the mutant is not a result of RPA accumulation on bulk chromatin (Figure 4B). Consistent with this result, overexpression of *Rad51* and *Rad52*, proteins involved in displacement of RPA from double-strand break (DSB) sites (Sugiyama and Kowalczykowski

2002), did not affect MMS sensitivity of *isw2 nhp10* cells (data not shown). In order to test whether *Isw2* and *Ino80* affect the abundance of other checkpoint proteins bound to chromatin, we performed stable SILAC mass spectrometry to compare differences in global levels of chromatin-bound proteins in WT and *isw2 nhp10* cells. This method was used successfully to find an abnormal increase in chromatin-bound replisome components in Δ *ctf18* cells (Kubota *et al.* 2011). This analysis revealed no notable difference in the abundance of the vast majority of chromatin-bound proteins between WT and *isw2 nhp10* cells in the presence of MMS ($r^2 = 0.92$), including DNA replication, replication checkpoint, and DNA repair proteins (Figure 4C). The outliers that increase the most in abundance in *isw2 nhp10* cells are *Sen34p* (~20-fold), a subunit of the tRNA splicing endonuclease; *Hsp26p* (~20-fold), a small heat-shock protein; and *Gpx2* (~7-fold), a phospholipid hydroperoxide glutathione peroxidase that is normally induced on replication stress (Tkach *et al.* 2012). Outliers that correspond to the most reduced abundance in *isw2 nhp10* cells include *Sts1p* (~1/8-fold), which is involved in protein transport and ribosomal RNA stability; *Ptc2p* (~1/5-fold), a *Rad53* phosphatase; and not surprisingly, other *Ino80* subunits such as *Ies1* (~1/4-fold). Other than *Ptc2p*, no outliers have been described

Figure 4 Bulk levels of chromatin-bound checkpoint proteins are largely unchanged between WT and *isw2 nhp10* cells. (A) FACS profiles of samples used for chromatin preparation in B during G₁ and S phase in 0.02% MMS. (B) Western blot of RPA and H2B levels from whole-cell extracts and fractionated chromatin of WT and *isw2 nhp10* cells. Levels of RPA were normalized to H2B, and the quantitation is shown. (C) Chart graphing the SILAC mass spectrometry data of nuclear proteins from WT vs. *isw2 nhp10* cells in middle S phase in 0.02% MMS. Total protein was normalized based on the total amount of histones.

previously to have a function in the S-phase checkpoint. No other Rad53 phosphatases were found to have different protein abundances in the SILAC data, and because of the redundancy of the Rad53 phosphatases, Ptc2 alone plays only a minor role in checkpoint recovery after MMS treatment (Szyjka *et al.* 2008). Furthermore, we have shown previously that *Isw2* and *Ino80* clearly function independent of the Rad53 phosphatases (Au *et al.* 2011). Taken together, our results show that *Isw2*- and *Ino80*-dependent checkpoint attenuation cannot be explained by changes in the abundance of chromatin-bound proteins, at least at the bulk level.

Chromatin accessibility around replication forks is decreased in *isw2 nhp10* cells in the presence of MMS

Previously, we (Vincent *et al.* 2008) and others (Papamichos-Chronakis and Peterson 2008; Shimada *et al.* 2008) have shown that both *Isw2* and *Ino80* are enriched at stalled replication forks. Therefore, we considered the possibility that *Isw2* and *Ino80* may affect chromatin structure around replication forks, thereby altering checkpoint activity. *Ino80* functions in opposition to *Swr1* and exchanges nucleosomal H2A.Z/H2B dimers for H2A/H2B dimers (Papamichos-Chronakis *et al.* 2006, 2011). Meanwhile, *Isw2* alters chromatin structure by sliding nucleosomes away from coding regions or toward gene promoters (Fazzio and Tsukiyama 2003; Yadon *et al.* 2010). We sought to determine whether these known biochemical activities might be required for the S-phase functions of *Isw2* and *Ino80*. If the important role of *Nhp10* is in *Htz1* exchange, we can expect to see partial rescue of the MMS sensitivity of *isw2 nhp10* cells by deleting *HTZ1*. However, *htz1* mutation not only showed no rescue but also increased the MMS sensitivity of *isw2 nhp10* cells, suggesting that *Htz1* exchange is not the relevant activity of *Isw2* and *Nhp10* in MMS (Figure 5A). To confirm that the genetic interaction with *Htz1* was representative of the *Ino80*

complex rather than *Nhp10* alone, we tested another subunit of *Ino80*, *Ies5*. The *isw2 ies5 htz1* cells displayed an even stronger additive growth defect in the presence of MMS (Figure S2A). Deleting *swr1* in *isw2 nhp10* cells also resulted in a stronger growth defect in the presence of MMS compared with either *swr1* or *isw2 nhp10* (Figure S2B). These results collectively suggest that *Htz1* deposition is not the relevant biochemical activity for growth in MMS.

We also mapped nucleosome positions around replicating regions in WT and *isw2 nhp10* cells in both G₁ and S phase in MMS. For this experiment, harvesting cells in early S phase was necessary to achieve the best possible synchrony of replication forks. We first confirmed that nucleosome positions at known *Isw2* targets shift in *isw2 nhp10* cells, as anticipated. However, we did not detect significant changes in nucleosome positioning at replicating regions in either G₁ or early S phase in MMS (data not shown). Although this negative result has caveats that replication forks are not completely synchronous within cell population and that the resolution of our nucleosome mapping may not be high enough, it does not support the idea that a model that regulates nucleosome positions accounts for the mechanism behind checkpoint regulation by *Isw2* and *Ino80*.

We next tested whether chromatin accessibility might be altered in *isw2 nhp10* cells around replication forks. We have recently developed a method to measure chromatin accessibility to micrococcal nuclease (MNase) in a way normalized for nucleosome occupancy called *normalized chromatin accessibility to MNase* (NCAM) (Rodriguez and Tsukiyama 2013; Rodriguez *et al.* 2014). To measure NCAM, we digest chromatin by MNase and purify mononucleosomal DNA, followed by hybridization of nucleosomal DNA to high-density tiled microarrays. We then quantify nucleosome signals, which can be affected by both nucleosome occupancy and the sensitivity of

Figure 5 Isw2 and Ino80 increase chromatin accessibility at replicating regions. (A) Spot assay of *htz1* and *htz1 isw2 nhp10* cells grown at 30°, as described previously (Au *et al.* 2011), on YPD and YPD with 0.01% MMS. (B) Graphs represent NCAM and DNA profiles over a 40-kb window from three classes of origin on chromosomes III, VI, and XII: early high ($n = 11$), early low ($n = 12$), and late ($n = 37$). The dashed line indicates the origin midpoint. (C) Measurement of NCAM around replication origins in WT and *isw2 nhp10* cells. WT cells are depicted in blue, while *isw2 nhp10* cells are in red. NCAM has been Z-score normalized by the +2 to +6 ORF nucleosomes. To visualize S-phase-specific changes, S-phase NCAM was subtracted from G₁ NCAM. The bar graph quantitates the average S-phase-specific differences in NCAM from 40 kb around origins in C and 3 kb around TSSs in D. (D) NCAM signal from all TSSs ($n = 780$) at G₁ and S phase in MMS in a 3-kb window. The dashed line indicates the TSS midpoint.

nucleosomes to MNase at any given site (Weiner *et al.* 2010; Rodriguez and Tsukiyama 2013). At the same time, we perform chromatin immunoprecipitation using anti-histone H3 antibody to measure nucleosome occupancy. By normalizing the strength of MNase signals to nucleosome occupancy, we can calculate NCAM, which measures the relative amount of MNase digestion per nucleosome (Rodriguez and Tsukiyama 2013). As expected for both WT and *isw2 nhp10* cells, NCAM increases at regions of

active replication, which is depicted near early efficient and early inefficient origins that have fired in most cells at this time point. However, NCAM is significantly reduced at these regions in *isw2 nhp10* cells (Figure 5, B and C). It is unlikely that the lower NCAM at this time point is caused by inefficient replication initiation in the mutant because we have shown previously that *isw2 nhp10* cells do indeed efficiently fire early origins similarly to WT cells (Vincent *et al.* 2008). Indeed, WT and

isw2 nhp10 cells exhibit similar levels of DNA synthesis around early-firing origins at this time point (Figure 5B). In contrast, nonreplicating regions, which are represented by chromatin surrounding late origins that have not fired at this time point, are overall less accessible than the rest of the genome, as expected (Figure 5, B and C). We did detect minor decreases in NCAM in *isw2 nhp10* compared with WT cells at these non-replicating regions, which is most likely attributed to passive replication occurring at these sites (Figure 5C). Taken together, these results suggest that *Isw2* and *Ino80* complexes affect NCAM around actively replicating regions. Furthermore, the reduction of NCAM in *isw2 nhp10* cells is not a result of global reduction of the NCAM signal because NCAM is very similar or even slightly increased in the mutant cells at transcriptional start sites in both G₁ and S phase in MMS (Figure 5D). These results collectively show that *Isw2* and *Ino80* increase chromatin accessibility specifically around replication forks in the presence of MMS. Although the decrease in NCAM in *isw2 nhp10* cells at actively replicating regions can be attributed in part to differences in nucleosome occupancy, the changes are mainly driven by a decrease in nucleosome signal (Figure S2, C and D). We have confirmed the genome-wide decrease in nucleosome signals around replication forks in *isw2 nhp10* cells by deep sequencing nucleosomal DNA (data not shown).

***isw2 nhp10* partially rescues MMS sensitivity of severe checkpoint-deficient mutants**

Because *isw2 nhp10* can stimulate checkpoint activity even in the absence of the known checkpoint activators, we sought to determine whether deletion of *isw2 nhp10* in checkpoint-deficient mutants also could rescue cell growth in the presence of MMS. The DRC checkpoint-specific mutations yielded WT-like MMS sensitivity, consistent with previous reports that the DRC checkpoint components are dispensable for the checkpoint in MMS (Alcasabas *et al.* 2001; Crabbe *et al.* 2010) (Figure 6A). Deletion of *ISW2* and *NHP10* in these backgrounds did not cause detectable genetic interactions. However, the DDC mutants were more sensitive to MMS than the WT cells, as expected (Hustedt *et al.* 2013), and unexpectedly, deletion of *ISW2* and *NHP10* did not yield a rescue in growth despite a rescue in checkpoint activation, as shown earlier (Figure 6, A and B). These results show, for the first time, that checkpoint overactivation and growth in MMS are genetically separable because checkpoint overactivation does take place without affecting MMS sensitivity in DDC mutants (Figure 6, A and B). However, in the mutants that could not activate either the DRC or the DDC in S phase, *isw2 nhp10* partially rescued MMS sensitivity, which is consistent with the partial rescue in checkpoint activity (Figure 6, A and C). We previously failed to detect the rescue of MMS sensitivity of *mec1* mutants by *isw2 nhp10* mutation (Au *et al.* 2011) because the rescue is slight and can be detected only after an extended period of time owing to very slow growth of the mutant. While the rescue of the *mec1* phenotype by *isw2 nhp10* was modest, we saw a more prominent rescue in *ddc1 dna2*-WY-AA cells (Figure 6, A and C). Because sensitivity of *mec1* to MMS is

a result of both an abrogated checkpoint and a replication fork protection defect, it is not surprising that *isw2 nhp10* only slightly rescues MMS sensitivity. In contrast, *isw2 nhp10* dramatically rescues *ddc1 dna2*-WY-AA cells, which have been shown to be sensitive to replication inhibitors solely because of their defect in activating the checkpoint. To determine whether the rescue was specific to MMS, we also tested genetic interactions on other replication inhibitors, including HU and camptothecin (CPT) (Figure S3, A and B). Both CPT and HU exposure yielded either minimal or additive sensitivity when *ISW2* and *NHP10* were deleted in the checkpoint-deficient mutants (Figure S3, A and B). These results are consistent with the fact that *Isw2* and *Ino80* only minimally affect checkpoint activity on HU treatment (Figure 1B). While growth sensitivity to MMS does not always directly reflect the changes in checkpoint regulation, a partial rescue of the severe MMS sensitivity of these checkpoint-deficient mutants by *isw2 nhp10* is consistent with the notion that *Isw2* and *Ino80* oppose checkpoint activators in the presence of MMS.

Discussion

We have shown previously that *Isw2* and *Ino80* are important for attenuation of the S-phase checkpoint, especially after transient HU treatment (Au *et al.* 2011). However, in this study, we discovered that the previous method of protein preparation resulted in the loss of *Rad53* protein. Using a method that can efficiently retain *Rad53* protein, we found that *Isw2* and *Ino80* play much bigger roles in attenuating S-phase checkpoint activity in the presence of MMS than in the presence of HU. Consistent with this finding, growth assays also show that *Isw2* and *Ino80* antagonize checkpoint activators more profoundly in the presence of MMS compared with other replication inhibitors. Although *isw2 nhp10* cells are sensitive to other replication inhibitors (Vincent *et al.* 2008), our results suggest that *Isw2* and *Ino80* may play distinct roles in the presence of different replication inhibitors.

Based on previous findings (Vincent *et al.* 2008; Au *et al.* 2011) and this study, we believe that *isw2 nhp10* cells do not have increased replication fork problems or DNA damage response defects, leading to our model that *Isw2* and *Ino80* directly attenuate checkpoint activity. We cannot completely exclude the possibility that there is an increased uncoupling of the replicative helicase to DNA polymerase (Byun *et al.* 2005) in *isw2 nhp10* cells in MMS, which could be the cause of stronger checkpoint activation and slower S-phase progression. However, we do not favor this model. As we have shown previously (Au *et al.* 2011) and here, the dissociation of replicative helicase and DNA polymerases in the presence of MMS is detrimental in the absence of replication fork protection by the S-phase checkpoint. For example, we previously reported that deletion of *CTF4* or *TOF1*, which are implicated in coupling MCM helicase with DNA polymerase during replication (Katou *et al.* 2003; Tanaka *et al.* 2009), in the *mec1* background leads to strongly additive MMS sensitivity (Au *et al.* 2011). In contrast, *isw2 nhp10* mutations cause no increase but

Figure 6 *Isw2* and *Nhp10* antagonize checkpoint activators. (A) A summary of the spot-test results with checkpoint proteins. MMS sensitivity is ranked on a scale of 1–6, with 1 being the least sensitive (WT level) and 6 being the most sensitive. Mutated genes are grouped into colored boxes, with pink representing the DRC genes, blue representing the DDC genes, and yellow presenting components of both DRC and DDC genes. Results are color-coded, with blue indicating additive sensitivity, red indicating similar sensitivity, and green indicating a partial rescue in sensitivity with the deletion of *ISW2* and *NHP10*. The mutations in black did not show detectable MMS sensitivity as single mutants and exhibited no genetic interactions with *isw2 nhp10*. (B) The gradient plate shows the lack of genetic interactions between *ISW2*, *NHP10*, and *RAD24*. (C) Both gradient plates show a partial rescue of varying degrees to MMS sensitivity of either the *mec1 sml1* mutant (top panel) or the *ddc1 dna2-WY-2A* mutant (bottom panel) by *isw2 nhp10* mutation.

partially rescue the MMS sensitivity of severe checkpoint mutants such as *mec1* and *ddc1 dna2-WY-AA*.

How do *Isw2* and *Ino80* complexes function? We do not believe that *Isw2* and *Ino80* function by affecting transcription because there were no significant differences in transcription between MMS-treated WT and *isw2 nhp10* cells (Vincent *et al.*, 2008, data not shown). Our checkpoint activity assays suggest that *Isw2* and *Ino80* attenuate checkpoint activity independent of all major checkpoint activators except RPA. Therefore, we propose that they function either through a currently unidentified checkpoint regulator(s) or through RPA. We show here that *Isw2* and *Ino80* do not affect the amounts of chromatin-bound checkpoint proteins including RPA at the bulk level, but it is possible that they affect the location of checkpoint proteins on chromatin. *Isw2* has the ability to translocate on ssDNA (Fischer *et al.* 2009). Furthermore, chromatin remodeling factors have been shown to interact with nonhistone proteins, and *Mot1*, an ATPase that is highly related to chromatin remodeling factors, can displace *TBP*, a nonhistone protein, from DNA (Auble *et al.* 1994; Moreau *et al.* 2003; Au *et al.* 2011; Sugimoto *et al.* 2011). Because *Isw2* and *Ino80* physically interact with RPA (Au *et al.* 2011), we initially considered the possibility that they may be able to directly remove RPA from the ssDNA template using DNA translocase activity. However, the results of the

direct test for this possibility *in vitro* have been negative so far (data not shown). We also have attempted RPA chromatin immunoprecipitation with DNA microarray (ChIP-chip) and chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-seq), which have yet to yield any conclusive results because of the difficulty of capturing RPA peaks on moving replication forks with imperfect synchrony (data not shown). While it is still possible that *Isw2* and *Ino80* could be affecting RPA localization at sites of stalled replication, we currently do not have adequate tools to address this scenario. Although it seems unlikely based on biochemical activities of chromatin remodeling factors, it is also possible that *Isw2* and *Ino80* directly affect the activity of a checkpoint kinase rather than localization of checkpoint proteins.

Most intriguingly, we demonstrate for the first time that *Isw2* and *Ino80* promote chromatin accessibility around actively replicating regions. Together with the fact that both *Isw2* and *Ino80* are enriched at stalled replication forks (Vincent *et al.* 2008), this is consistent with a model suggesting that the chromatin remodeling factors function at stalled replication forks. Multiple reports have suggested that chromatin remodeling factors can alter chromatin structure to facilitate replication fork progression (Fyodorov *et al.* 2004; Poot *et al.* 2004; Shimada *et al.* 2008; Vincent *et al.* 2008). Moreover, we showed

previously that chromatin accessibility increases around replication forks to promote replication (Rodriguez and Tsukiyama 2013). Therefore, one possibility is that *Isw2* and *Ino80* may attenuate checkpoint activity by changing the way checkpoint protein(s) interact with chromatin at stalled forks, which, in turn, affects replication progression and checkpoint dynamics. Because we found that *Isw2* and *Ino80* likely do not function through their known biochemical activities, we speculate that *Isw2* and *Ino80* function in a currently unknown manner. This can be either by modifying how DNA and core histones interact or by changing histone turnover rate, which may influence other factors to bind and potentially affect checkpoint factor localization or activity. For example, weakening histone-DNA interactions around stalled replication forks could cause an increase in accessibility to recruit checkpoint repressors to facilitate removal of checkpoint factor(s) and promote resumption of DNA replication. Indeed, another ATP-dependent chromatin remodeling factor, *Fun30*, and its mammalian counterpart, *SMARCD1*, were both found to be recruited to sites of DSBs to promote resection presumably by removing *Rad9* from DSB ends (Chen *et al.* 2012; Costelloe *et al.* 2012). Alternatively, checkpoint hyperactivation in the chromatin-remodeling-factor mutant may be the cause of the observed decrease in chromatin accessibility. Interestingly, we have shown previously that a mutation in *mec1* also results in decreased chromatin accessibility at replicating regions in S phase in HU (Rodriguez and Tsukiyama 2013). However, because *Mec1* facilitates chromatin accessibility around stalled replication forks (Rodriguez and Tsukiyama 2013), it is difficult to explain the increased checkpoint activity in *isw2 nhp10* cells as the direct cause of the decreased chromatin accessibility in the mutant. Whether these changes in chromatin structure are related to the phenotypes of *isw2 nhp10* cells in MMS remains to be explored and will be investigated in future work. Finally, it is also possible that the changes in chromatin accessibility are separable from checkpoint activation.

Our findings suggest that *Isw2* and *Ino80* likely function through currently unknown mechanisms to regulate both chromatin accessibility and S-phase checkpoint attenuation. In the future, understanding the molecular mechanisms underlying *Isw2*- and *Ino80*-dependent chromatin accessibility at replication forks will be crucial in defining new biochemical activities of chromatin remodeling factors as well as elucidating a currently unknown mechanism of checkpoint control.

Acknowledgments

We thank Steve Elledge, Peter Burgers, Dave Toczyski, John Diffley, Hiroyuki Araki, Jac Nickoloff, Sue Biggins, Linda Breeden, Keiko Umez, Rodney Rothstein, and Oscar Aparicio for plasmids and strains used in this study. We also thank Anne Donaldson for her advice on SILAC protocols and Phil Gafken for his help with mass spectrometry and data analysis. We are grateful to Tsukiyama Laboratory members and Sue Biggins for discussion and comments on this manuscript. This work is supported by Molecular and Cellular Biology Training Grant GM-07270 to L.L. and Grant R-01GM058465 to T.T.

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Communicating editor: J. A. Nickoloff

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Supporting Information

<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.174730/-/DC1>

Chromatin Remodeling Factors Isw2 and Ino80 Regulate Checkpoint Activity and Chromatin Structure in S Phase

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Figure S1 Checkpoint hyperactivation is not a result of insufficient replication in *isw2 nhp10*. (A) The Rad53 ISA shown in Figure 1C with nonspecific bands. WT and *isw2 nhp10* were arrested in G1 and released into S phase in the presence of 0.02% MMS. Phosphorylation of Rad53 is depicted by the arrow and phosphorylation of two nonspecific bands are indicated by the asterisks. The nonspecific bands were used as loading controls to normalize and quantitate Rad53 phosphorylation. The times in purple denote times in S phase. (B) A snap shot of DNA profiles from Chromosome XII for WT (bottom) and *isw2 nhp10* (top) in S phase as shown in blue. Cells with similar FACS profiles were taken at a late time point in S phase, 90 min. in WT cells and 150 min. in *isw2 nhp10* cells, but at this time point the checkpoint was more highly activated in *isw2 nhp10* as shown in A. The green boxes indicate origins of replication (ORIs) and in black depicts the chromosomal coordinates.

Figure S2 Isw2 and Ino80 function in S phase using novel mechanisms. (A) A spot assay of *htz1* and *htz1 isw2 ies5* on YPD and YPD with 0.015% MMS. (B) Spot assay of *swr1* and *isw2 nhp10 swr1* on YPD and YPD with 0.01% MMS. (C) Graphs represent Z-score normalized MNase and H3 ChIP profiles from cells in G1 over a 40kb window from three classes of origins on chromosomes III, VI and XII: early high (n=11), early low (n=12) and late (n=37). WT is shown in blue and *isw2 nhp10* is shown in red, with the dashed line indicating the origin midpoint. (D) Z-score normalized MNase and H3 ChIP profiles from cells in early S phase.

Figure S3 Checkpoint deficient mutants show additive MMS sensitivity with *isw2 nhp10*. (A) Gradient plates comparing *mec1 sml1* and *mec1 sml1 isw2 nhp10* with increasing amounts of 10mg/mL CPT and 5mM HU. (B) Gradient plates comparing *ddc1 dna2-WY-AA* and *ddc1 dna2-WY-AA isw2 nhp10* with increasing amounts of 10mg/mL CPT and 50mM HU.