Distinct roles for SWR1 and INO80 chromatin remodeling complexes at chromosomal double-strand breaks

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INO80 and SWR1 are two closely related ATP-dependent chromatin remodeling complexes that share several subunits. Ino80 was reported to be recruited to the HO endonuclease-induced double-strand break (DSB) at the budding yeast mating-type locus, *MAT*. We find Swr1 similarly recruited in a manner dependent on the phosphorylation of H2A (γ H2AX). This is not unique to cleavage at *MAT*; both Swr1 and Ino80 bind near an induced DSB on chromosome XV. Whereas Swr1 incorporates the histone variant H2A.Z into chromatin at promoters, H2A.Z levels do not increase at DSBs. Instead, H2A.Z, γ H2AX and core histones are coordinately removed near the break in an INO80-dependent, but SWR1-independent, manner. Mutations in INO80-specific subunits Arp8 or Nhp10 impair the binding of Mre11 nuclease, yKu80 and ATR-related Mec1 kinase at the DSB, resulting in defective end-processing and checkpoint activation. In contrast, Mre11 binding, end-resection and checkpoint activation were normal in the *swr1* strain, but yKu80 loading and error-free end-joining were impaired. Thus, these two related chromatin remodelers have distinct roles in DSB repair and checkpoint activation.

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Introduction

Cells constantly encounter potentially harmful DNA lesions. DNA double-strand breaks (DSBs) pose the most severe risks for cell viability, since their inefficient or inaccurate repair can result in deleterious mutations, chromosomal translocations, cancer or cell death. Cells respond to DNA breaks by

rapidly deploying a host of proteins to the site of damage. Some of these factors engage in DNA repair, while others trigger signaling pathways known as DNA damage checkpoints, which delay cell cycle progression until repair is complete. Two evolutionary conserved pathways mediate DSB repair: homologous recombination (HR) and non-homologous end-joining (NHEJ). HR starts with the processing of the DNA ends into single-stranded DNA (ssDNA), which is bound by proteins that invade and copy information from a homologous DNA duplex to repair the break in an error-free manner. In contrast, the alternative pathway of repair, NHEJ, requires little or no processing and involves either an error-free or error-prone mechanism for re-ligation of the ends (Khanna and Jackson, 2001; van Gent *et al*, 2001).

In eukaryotes, genomic DNA is organized into a nucleosome structure called chromatin, whose basic unit, the nucleosome, consists of ~ 147 bp of DNA wrapped around a protein octamer of histones H2A, H2B, H3 and H4. This structure generally reduces accessibility for enzymes involved in DNA-based cellular processes. To overcome this nucleosome barrier, cells possess histone-modifying enzymes and chromatin remodeling complexes (Marmorstein, 2001). These latter complexes use the energy of ATP hydrolysis to disrupt contacts between DNA and histones in order to reposition or remove nucleosomes, or exchange histone variants (Lusser and Kadonaga, 2003). A number of reports have demonstrated that both histone modifications and chromatin remodeling play important roles in controlling transcription. While histone modifications have long been recognized to be part of the cellular response to DNA damage, the importance of chromatin remodeling in DNA repair has only recently been addressed (Peterson and Cote, 2004; van Attikum and Gasser, 2005).

One of the most rapid events that occurs in yeast in response to a DSB is the phosphorylation of a conserved serine residue (S129) near the C-terminus of histone H2A by ATM and ATR checkpoint kinases (scTel1 and scMec1, respectively; (Downs *et al*, 2000; Burma *et al*, 2001; Shroff *et al*, 2004)). In mammalian cells, their target is a variant histone, H2AX, producing γ H2AX. While γ H2AX occurs over megabases of chromatin surrounding DSBs (Rogakou *et al*, 1999), in yeast, H2A phosphorylation spreads over ~ 50 kb (Shroff *et al*, 2004). For simplicity we will use the mammalian nomenclature γ H2AX to describe the analogously modified yeast histone H2A. In budding yeast, mutation of the H2A S129 acceptor site leads to mildly elevated sensitivity to DNA damaging agents, and defects in DSB repair by NHEJ (Downs *et al*, 2000; Moore *et al*, 2006). Moreover, yeast γ H2AX is required for the recruitment and loading of two types of protein complexes: Cohesin, which aids repair of DSBs through sister chromatid recombination (Strom *et al*, 2004; Unal *et al*, 2004), and chromatin remodeling complexes from

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the Snf2 superfamily (Downs *et al*, 2004; Morrison *et al*, 2004; van Attikum *et al*, 2004; Chai *et al*, 2005; Shim *et al*, 2005).

Mutations in four members of the Snf2 superfamily of ATPases, Snf2, Sth1, Ino80 and Swr1, which are present in large multi-protein complexes called SWI/SNF, RSC, INO80 and SWR1, respectively, have been shown to result in hypersensitivity to a wide range of DNA damaging agents (Shen *et al*, 2000; Kobor *et al*, 2004; Mizuguchi *et al*, 2004; van Attikum *et al*, 2004; Chai *et al*, 2005; Shim *et al*, 2005). Although all four complexes also alter nucleosome structure and control transcription of large sets of genes (Ebbert *et al*, 1999; Shen *et al*, 2000; Saha *et al*, 2006), it is unlikely that their involvement in the DNA damage response stems solely from their role in transcription. Chromatin immunoprecipitation (ChIP) experiments revealed the direct binding of Snf2, Sth1 and Ino80 ATPases, along with other subunits, at an HO endonuclease-induced DSB at the yeast mating-type locus (Downs *et al*, 2004; Morrison *et al*, 2004; van Attikum *et al*, 2004; Chai *et al*, 2005; Shim *et al*, 2005). Amongst the proteins recruited to the DSB were unique subunits of the SWI/SNF2 (Snf5) and INO80 (Arp5 and Arp8) complexes, as well as shared subunits such as Arp4 and Rvb1 (Bird *et al*, 2002; Downs *et al*, 2004; Chai *et al*, 2005). Given that the yeast SWR1 complex shares Act1, Arp4, Rvb1 and Rvb2 subunits with the INO80 complex, it was unclear whether the SWR1 complex was recruited.

Several laboratories have examined how chromatin remodeling alters nucleosomal structure at the site of damage. Mutations in *RSC2* or *STH1* of the RSC complex, or *ARP8* of the INO80 complex, impair core histone loss near the HO-induced DSB (Tsukuda *et al*, 2005; Shim *et al*, 2007). Consistently, some authors detected a delayed conversion of double-stranded (ds) DNA into ssDNA (van Attikum *et al*, 2004; Shim *et al*, 2005, 2007). This suggested that chromatin remodeling by RSC and/or INO80 facilitates access to DNA ends for enzymes involved in end-processing and DSB repair. Indeed, downregulation of Sth1 was then shown to impair loading of yKu70, Mre11 and RPA and to delay the loading of Rad51 at DSBs (Shim *et al*, 2007). Arp8, on the other hand, was reported to facilitate Rad51, but not RPA loading (Tsukuda *et al*, 2005). It was not addressed whether INO80 regulates the binding of other repair proteins, such as yKu70 or Mre11.

Despite strong similarities in the catalytic subunits, and the presence of shared components, the two yeast remodeling complexes, INO80 and SWR1, show distinct affinities for histone H2A variants: INO80 binds γ H2AX uniquely, while SWR1 binds both γ H2AX and H2A.Z, with a preference for H2A.Z (encoded by *HTZ1* in budding yeast) (Morrison *et al*, 2004). SWR1 catalyzes the incorporation of H2A.Z into chromatin at promoters, centromeres and subtelomeric regions, with resulting effects on gene expression (Krogan *et al*, 2003; Kobor *et al*, 2004; Mizuguchi *et al*, 2004; Guillemette *et al*, 2005; Raisner *et al*, 2005). A recent report suggested that Swr1 could incorporate H2A.Z into chromatin proximal to an HO-induced DSB at *MAT*, yet only in the absence of Ino80 and when γ H2AX levels were reduced (Papamichos-Chronakis *et al*, 2006).

Here we have carefully examined the roles of INO80 and SWR1 with respect to H2A.Z deposition, nucleosome removal and downstream events at a DSB. We have established that

both the Ino80 and Swr1 ATPases are recruited to DSBs at two different loci (*MAT* and *PDR10*). Surprisingly, Swr1 is not recruited to incorporate or remove H2A.Z. Rather, at DSBs H2A.Z, γ H2AX and histone H3 are removed in an INO80-dependent, but SWR1-independent, manner. Efficient binding of the Mre11 nuclease, processing of the ends into ssDNA and recruitment of Mec1 kinase are all INO80-dependent. Loss of Swr1, on the other hand, affects yKu80 binding, but not Mre11 association or end-resection. Consistent with these phenotypes, we find that *swr1* deletion selectively impairs error-free NHEJ events, while mutations in the INO80 complex reduce Mec1-dependent checkpoint activation.

Results

Ino80 and Swr1 are recruited to chromosomal DSBs

Mating-type switching in budding yeast provides a well-controlled system to study events that occur at a specific DSB. During mating-type switching the HO endonuclease cleaves uniquely at the *MAT* locus, an event followed by efficient gene conversion requiring donor sequences at *HML* or *HMR*. In the absence of these donors, cells must repair the break by NHEJ to survive (Figure 1A). As mentioned above, ChIP studies have shown that the INO80 chromatin remodeling complex is recruited to the DSB at *MAT*, while the situation for SWR1 remained unclear (Downs *et al*, 2004; Morrison *et al*, 2004; van Attikum *et al*, 2004; Tsukuda *et al*, 2005). Equally unclear was whether or not the recruitment of nucleosome remodelers to breaks is locus-specific.

To test this, we generated a second yeast strain lacking the HO consensus at *MAT*, but with an HO cleavage site at the 3' end of a non-essential gene, *PDR10*, located on chromosome XV. Real-time (rt)PCR probes were designed to monitor protein binding up to 23 kb from the cleavage site at *MAT*, and up to 4.4 kb from the cut site at *PDR10* (Figure 1A and B). Importantly, ChIP experiments for Swr1-Myc and Ino80-Myc detected no significant binding of either remodeler at *MAT* or *PDR10* when the galactose-inducible HO endonuclease in these strains was repressed by growth on glucose (Figure 1C), in contrast to an earlier report (Tsukuda *et al*, 2005). Following HO induction by the addition of galactose, cleavage is equally efficient at *PDR10* and *MAT* (Figure 1B), and both remodelers are efficiently recruited to the induced DSBs (Figure 1D and E). Their binding increases over 4 h, yet the kinetics and efficiency of recruitment were locus-dependent. At *MAT* Ino80 peaked very close to the cut site, spreading weakly by 2–4 h after HO induction, whereas at *PDR10* Ino80 was bound equally over the 5-kb region analyzed. Swr1, on the other hand, rapidly accumulated close to the cut site at both *MAT* and *PDR10* (Figure 1D and E). We conclude that both INO80 and SWR1 are recruited to DSBs.

INO80 and SWR1 recruitment to DSBs requires γ H2AX

Several studies examined the prerequisites for recruiting different chromatin remodeling complexes to DSBs. In the case of RSC, two rapidly recruited repair proteins, yKu70 and Mre11, were found necessary for the association of RSC with the break (Shim *et al*, 2005). Recruitment of Ino80, on the other hand, was reduced in the absence of H2A phosphorylation (Morrison *et al*, 2004; van Attikum *et al*, 2004).

We next checked whether there was a similar effect on the recruitment of Swr1 in an H2A phospho-acceptor mutant.

Figure 1 Ino80 and Swr1 are recruited to chromosomal DNA DSBs. **(A, B)** Schematic representations of genes and rt-PCR primer sets on chromosome III (A) and XV (B) in the haploid yeast strain JKM179 and its derivative GA3632. Potential donor loci *HML* and *HMR* are deleted. The cleavage efficiency at *MAT* and *PDR10* upon induction of HO is shown over time. **(C)** Absolute fold enrichment of Ino80-Myc and Swr1-HA ChIPs at the indicated distances from the HO site at *MAT* and *PDR10* for strains without a DSB. Cells were grown in the presence of glucose to repress HO. **(D)** Relative fold enrichment of Ino80-Myc and Swr1-HA ChIPs at the indicated distances from the HO cut site at *MAT*, and the indicated times on galactose. The value at 0 h is arbitrarily set as 1. Cleavage efficiencies were 96, 98 and 99% (Ino80-Myc ChIP), and 89, 98, 99% (Swr1-HA ChIP) at 1, 2 and 4 h after HO induction, respectively. **(E)** As panel D, except that this shows Ino80-Myc and Swr1-HA ChIPs at the DSB in the *PDR10* locus. Cleavage efficiencies were 93, 97 and 99% (Ino80-Myc ChIP), and 89, 98, 99% (Swr1-Myc ChIP).

Using a double S129-to-stop mutation of the H2A phospho-acceptor residue, the efficiency of both Ino80 and Swr1 recruitment near the *MAT* DSB was reduced by 75–80% (*hta1/2^{S129*}* mutant; Figure 2). Our results argue that these chromatin remodeling complexes are recruited following modification of γ H2AX by either Tel1 or Mec1 kinase (Morrison *et al*, 2004; Shroff *et al*, 2004). Arp4, a subunit shared by INO80, SWR1 and NuA4 complexes, was shown to interact with yeast γ H2AX, and is likely to be involved in the recruitment of these complexes (Downs *et al*, 2004), although Nhp10 was also implicated in a stable INO80– γ H2AX interaction (Morrison *et al*, 2004). The kinetics of accumulation and distribution of SWR1 and INO80 near the DSBs at *MAT* and *PDR10* differ, suggesting that chromatin context may modulate both binding and distribution. Nonetheless, γ H2AX seems to function as a common recognition signal that triggers the recruitment of both SWR1 and INO80 to DSBs.

The SWR1 complex does not incorporate H2A.Z. near DSBs

The SWR1 complex catalyzes the incorporation of the histone variant H2A.Z into chromatin at promoters, centromeres and telomeres (Krogan *et al*, 2003; Kobor *et al*, 2004; Mizuguchi *et al*, 2004; Guillemette *et al*, 2005; Raisner *et al*, 2005). We therefore examined whether SWR1 was recruited to a chromosomal DSB to insert H2A.Z. We performed ChIP using an antibody against H2A.Z and probed for its presence near the HO site at *MAT* under conditions that either repress or induce

the HO endonuclease. In the absence of cleavage, we detected a significant enrichment for H2A.Z at the *MAT α 2* and *BUD5* promoter regions (+0.6 and +1.6 kb, respectively), whereas little or no H2A.Z was detected in coding regions on the centromere proximal side of the HO consensus (+4.5, +9.6 and +23 kb, respectively; Figures 1A and 3A). Similarly, and consistent with earlier reports showing that promoters are generally enriched for H2A.Z (Krogan *et al*, 2003; Kobor *et al*, 2004; Mizuguchi *et al*, 2004; Guillemette *et al*, 2005; Raisner *et al*, 2005), we detected a significant enrichment for H2A.Z at the telomere-proximal *PER1* promoter (+7.2 kb), but not in the *TAF2* coding region (+0.9 and +1.9 kb) (Supplementary Figure 1). The presence of H2A.Z at these sites is SWR1-dependent: we found no detectable H2A.Z at *MAT* in the *swr1* mutant (Figure 3A, and data not shown). Importantly, however, after 4 h of HO cleavage, there was no increase in H2A.Z levels at the DSB. Instead, the H2A.Z signal decreased to background level seen in the *swr1* strain, suggesting that histone removal occurred (Figure 3A and Supplementary Figure 1). Thus, rather than H2A.Z recruitment, we scored an eviction of H2A.Z on both centromere- and telomere-proximal sides of the HO cut.

In order to examine whether the break-induced eviction of H2A.Z was *MAT*-specific, we probed for H2A.Z near the HO consensus at the *PDR10* locus. Again under conditions that repress HO cleavage, we scored a significant enrichment for H2A.Z at the *SNC2* promoter (+4.5 kb), but not in the coding regions (+0.9 and +1.9 kb; Figure 3B). However, upon induction of the DSB, the H2A.Z signal at *PDR10* dropped

Figure 2 H2AX phosphorylation at sites near a DSB at *MAT* is required for recruitment of INO80 and SWR1. (A) Relative fold enrichment of Ino80-Myc ChIP for WT and *hta1/2^{S129*}* strains at the indicated distances from the HO cut site, and the indicated times on galactose. The enrichment is corrected for cleavage efficiencies, which were 96, 98 and 99% (WT), and 89, 98, 99% (*hta1/2^{S129*}*). (B) As panel A, except that this shows ChIP for Swr1-HA. Cleavage efficiencies were 89, 98 and 99% (WT), and 78, 92, 95% (*hta1/2^{S129*}*).

Figure 3 H2A.Z is evicted from sites near a DSB at *MAT* and *PDR10*. (A) Absolute fold enrichment of H2A.Z ChIP at the indicated distances from the HO site at *MAT* for a WT strain with and without DSB, and a *swr1* mutant without DSB is shown. Cells were grown in the presence of glucose to repress HO, or in the presence of galactose for 4 h to induce HO. Cleavage efficiency was 99% at 4 h after HO induction. (B) As panel A, except that this shows H2A.Z ChIP at the DSB in the *PDR10* locus for a WT strain. Cleavage efficiency was 97%. (C) As panel A, except that this shows H2A.Z ChIP at *MATinc* for a WT strain. Cells were grown in the presence of glucose to repress HO or in the presence of galactose for 4 h to induce HO. On galactose no DSB induction at *MATinc* occurs. (D) As panel C, except that this shows histone H3 ChIP.

to near background levels (Figure 3B). We conclude that H2A.Z eviction is a general phenomenon at a DSB.

To confirm that H2A.Z eviction is a direct consequence of HO cleavage, and not a reflection of transcriptional changes provoked by galactose, we monitored H2A.Z and histone H3 levels in a strain carrying an uncleavable HO site (*MATinc*). Indeed, when cells with *MATinc* were placed on galactose, H2A.Z levels increased slightly at +0.6 kb, whereas histone H3 levels remain unchanged (Figure 3C and D). The H2A.Z increase is found at a promoter, and may reflect transcriptional potentiation by galactose. We conclude that the observed loss of H2A.Z at *MAT* and *PDR10* occurs exclusively in response to a DSB. Thus, Swr1 is not recruited to incorporate H2A.Z at promoters near a break. Instead, when present, H2A.Z disappears from chromatin with kinetics that mirror the recruitment of both SWR1 and INO80 remodelers.

INO80, but not SWR1, is required for variant histone eviction at a DSB

If SWR1 does not insert H2A.Z. at a DSB, then why is it recruited? The Swr1 complex is often considered equivalent to the mammalian and fly TIP60 complexes, which are implicated in the repair of DNA damage largely due to their ability to acetylate histone tails. However, unlike SWR1, TIP60 possesses both acetylation and ATPase activities. It is thought that the *Drosophila* TIP60 complex first binds to and acetylates phosphorylated histone H2Av, and then exchanges it for the unmodified form. Consequently, cells lacking a

functional TIP60 complex accumulate phospho-H2Av following ionizing radiation (Kusch *et al*, 2004).

We therefore examined whether either the INO80 or the SWR1 complex exchanges yeast γ H2AX for unmodified H2A at sites of DNA damage. To do this, we tested the presence of γ H2AX near the HO-induced DSB at *MAT*, comparing the wild-type (WT) situation with mutants lacking either Swr1 or the INO80 subunits Arp8 or Nhp10. By 1 h of HO induction in WT and *swr1* strains, γ H2AX has accumulated and spread from the DSB at *MAT*, peaking at +9.6 kb from the DSB (Figure 4A). This accumulation is slower in *nhp10* and *arp8* mutants, where it peaks at 2 h (Figure 4A and Supplementary Figure 2), suggesting that INO80 actually facilitates H2A phosphorylation (Papamichos-Chronakis *et al*, 2006). However, by 2–4 h of HO induction, the levels of γ H2AX decreased significantly in WT and *swr1* cells, but not in the *nhp10* and *arp8* mutants, indicating that γ H2AX is removed from chromatin surrounding the DSB in an INO80-dependent, but SWR1-independent manner (Figure 4A and Supplementary Figure 2).

We showed in Figure 2A and Supplementary Figure 1 that H2A.Z is also evicted around the HO-induced cut at *MAT* in a WT strain. We next examined whether the INO80 complex is required for H2A.Z eviction, even though no association between INO80 and H2A.Z had been reported. Indeed, by 2–4 h after cleavage, H2A.Z was lost within a 5-kb region of *MAT* in WT, but not in *nhp10* cells. Moreover, the *hta1/2*^{S129*} phospho-acceptor mutant, which recruits INO80 inefficiently

Figure 4 INO80 is involved in γ H2AX, H2A.Z and histone H3 eviction from sites near a DSB at *MAT*. (A) Relative fold enrichment of γ H2AX ChIP at the indicated distances from the HO cut site at *MAT*, and the indicated times on galactose for WT, *nhp10*, and *swr1* strains is shown as in Figure 1. Cleavage efficiencies were 90, 98 and 99% (WT), 44, 75 and 95% (*nhp10*), and 76, 91 and 96% (*swr1*). (B) As panel A, except that this shows H2A.Z ChIP for WT, *nhp10* and *hta1/2^{S129*}* strains. Cleavage efficiencies were 92, 97 and 99% (WT), 62, 83 and 94% (*nhp10*), and 88, 96 and 97% (*hta1/2^{S129*}*). (C) As panel A, except that this shows histone H3 ChIP for WT, *nhp10* and *swr1* strains. Cleavage efficiencies were 89, 96 and 98% (WT), 46, 79 and 94% (*nhp10*), and 89, 96 and 98% (*swr1*).

(Figure 2A), is similarly impaired for H2A.Z eviction (Figure 4B). While there is detectable H2A.Z at *MAT* in the *nhp10* and *hta1/2^{S129*}* phospho-acceptor mutants, there is none in a *swr1* mutant (Figure 3A and Supplementary Figure 2), and HO-mediated cleavage does not alter this in *swr1* cells (Supplementary Figure 2). We conclude that SWR1 neither inserts nor removes H2A.Z at a DSB, and that H2A.Z and γ H2AX are both evicted near a DSB in an INO80-dependent manner.

Loss of H2A.Z. and γ H2AX reflects general nucleosome eviction

To test whether the loss of H2A.Z and γ H2AX near a DSB reflects general nucleosome eviction, we performed ChIP under similar conditions using an antibody that recognizes both modified and unmodified forms of histone H3. Upon HO

induction, histone H3 levels decreased significantly by 2–4 h in both WT and *swr1* cells, but not in the *nhp10* mutant (Figure 4C). This mirrors results reported earlier for an *arp8* mutant (Tsukuda *et al*, 2005). A significant decrease in histone H3 levels was also detected on the other side of the break in the *TAF2* coding region (+0.9 and +1.6 kb), but not at the *PER1* promoter region (+7.2 kb; Supplementary Figure 1). Despite the relative stability of histone H3 at the *PER1* promoter, we scored a significant drop in H2A.Z levels, which may indicate that H2A.Z is selectively removed from this promoter to regulate a transcriptional response to the DSB (Supplementary Figure 1). In contrast, H2A.Z and histone H3 loss at *TAF2* and *MAT* is cleavage-specific (Figure 3D).

To examine whether the mechanisms and kinetics of histone eviction occur similarly at other cleavage sites, we induced the HO DSB at *PDR10* and monitored H2A.Z and

histone H3 levels by ChIP. H2A.Z levels significantly decreased within 4.5 kb of the DSB at *PDR10*, as early as 1 h after HO induction, and maximal displacement occurred by 4 h (Figure 5A). Histone H3 levels also decreased within a 4.5-kb region near this DSB, although with slightly slower kinetics (Figure 5B). Surprisingly, in both the *nhp10* and *swr1* mutants the H3 levels first significantly increased after 1 h on galactose, and only later decreased to reach WT levels of loss (Figure 5C). This increase was not seen in WT cells. Importantly, the absolute levels of histone H3 at *PDR10*, as at *MAT*, are not reduced in the *nhp10* or *swr1* mutant when compared with WT on glucose (Figure 5D), ruling out the possibility that the increase at 1 h in these mutants reflects a return to WT levels. We conclude that INO80, but not SWR1, is needed to evict both core histones and histone variants at *MAT*. In contrast, at *PDR10* the absence of either complex results in a transient increase in histones near the break, although eviction eventually succeeds.

INO80 and SWR1 differently affect the binding of Mre11 and yKu80 to DSBs

What is the outcome of nucleosome eviction on other events at DSBs? It has been proposed that the removal of histones may facilitate the binding of repair proteins, such as Mre11 and yKu80, to broken ends (Shim *et al*, 2007). To test the impact of histone removal on their binding, we monitored their presence at the HO-induced DSB at *MAT* in mutants deficient for either INO80 or SWR1 function.

Consistent with published ChIP experiments, we monitored significant recruitment of Myc-tagged Mre11 and yKu80 by 1 h of HO induction, with signals peaking at 0.6 kb from the cleavage site (Figure 6A and B). If a similar ChIP was performed on a strain with no Myc epitope tag, we saw no precipitation of the induced DSB at *MAT*. If we monitored this in the *arp8* mutant, we scored a 65% reduction in the efficiency of Mre11 recruitment, whereas it

Figure 5 H2A.Z and histone H3 are evicted from sites near a DSB at *PDR10*. (A) Relative fold enrichment of H2A.Z ChIP at the indicated distances from the HO cut site at *PDR10*, and the indicated times on galactose for a WT strain is shown as in Figure 1. Cleavage efficiencies were 84, 95 and 97%. (B) As panel A, except that this shows histone H3 ChIP. Cleavage efficiencies were 87, 92 and 94%. (C) As panel B, except that this shows histone H3 ChIP for *nhp10* and *swr1* strains. Cleavage efficiencies were 72, 88 and 91% (*nhp10*), and 89, 96 and 98% (*swr1*). (D) Absolute fold enrichment of histone H3 ChIP at the indicated distances from the HO site at *MAT* and *PDR10* for WT, *nhp10* and *swr1* strains in the absence of a DSB. Cells were grown in the presence of glucose to repress HO.

Figure 6 Effect of *ARP8*, *NHP10* and *SWR1* deletion on Mre11 and yKu80 recruitment, end-resection and non-homologous end-joining. (A) Absolute fold enrichment of Mre11-Myc ChIP at the indicated distances from the HO cut site at *MAT* at 0 and 1 h on galactose. The enrichment is corrected for cleavage efficiencies, which were 86% (WT), 84% (*arp8*), 86% (*swr1*) and 96% (WT no tag). (B) As panel A, except that this shows ChIP for yKu80-Myc. Cleavage efficiencies were 98% (WT), 85% (*arp8*), 90% (*swr1*), and 96% (WT no tag). (C) Schematic representation of the QAOS assay (see text). DNA template molecule, generated using primer HO1-ss, is quantified by rt-PCR using primers HO1-f and Tag. (D) QAOS assay for ssDNA formed at the HO1 site at the indicated times on galactose in WT, *arp8*, *nhp10* and *swr1* strains. The amount of ssDNA is corrected for cleavage efficiencies, which were 92, 97, 99% (WT), 56, 78, 85% (*arp8*), 48, 79, 93% (*nhp10*) and 77, 90, 96% (*swr1*). (E) Error-prone NHEJ repair of a DSB at *MAT* is slightly reduced in mutants in *INO80*, but not *SWR1*. Repair of the DSB at *MAT* can only occur by NHEJ. Error-prone NHEJ was monitored by scoring survival of the indicated strains after plating serial 10-fold dilutions on medium with either 2% glucose or galactose. (F) Error-free NHEJ repair of a DSB at *MAT* is reduced in *swr1*, but not in *arp8* or *nhp10* mutants. NHEJ proficiency was determined by rt-PCR, using primers that span the DSB at *MAT* or anneal to the *SMC2* control locus. HO was repressed ($t = 0$) and induced for 1 h ($t = 1$). After 1 h, cells expressing HO were shifted to a glucose medium and incubated for 2 h ($t = 3$) to allow error-free NHEJ. Error-free end-joining efficiency was calculated from PCR cycle threshold values for *MAT* (ct_{MAT}) normalized to *SMC2* (ct_{SMC2}) from $t = 0$, $t = 1$ and $t = 3$, using the following formula: $((2^{ct_{MAT}-ct_{SMC2}})_{t=2} - (2^{ct_{MAT}-ct_{SMC2}})_{t=1}) / ((2^{ct_{MAT}-ct_{SMC2}})_{t=0} - (2^{ct_{MAT}-ct_{SMC2}})_{t=1}) * 100\%$. Each point represents the average of two independent experiments. Standard deviation is shown.

retained WT levels in the *swr1* background (Figure 6A). In contrast, the efficiency of yKu80 recruitment was significantly reduced in both the *arp8* and *swr1* strains (by 70–85%; Figure 6B). Thus, both *INO80* and *SWR1* facilitate the binding

of yKu80, yet only *INO80* affects the binding of Mre11. Since *INO80*, but not *SWR1*, removes nucleosomes at the DSB, we propose that *INO80*-mediated histone eviction specifically facilitates the binding of Mre11.

INO80, but not SWR1, facilitates ssDNA formation at DSB ends

The Mre11–Rad50–Xrs2 complex and the exonuclease Exo1 have been implicated in the 5′–3′ resection of HO-induced DSBs (Lee *et al*, 1998; Nakada *et al*, 2004). As efficient binding of Mre11 to a DSB requires INO80 chromatin remodeling, we next scored end-resection after HO cleavage at *MAT*. The amount of ssDNA accumulated at a DSB can be measured at the HO1 site (+1.6 kb from the cut) by an rt-PCR method termed QAOS (quantitative amplification of ssDNA) (Figure 6C). Previously we have detected ssDNA at HO1, as early as 1 h after HO induction in WT cells, reaching maximal levels (85%) of ssDNA after 4 h (van Attikum *et al*, 2004). At this time point, both the *arp8* and H2A phospho-acceptor mutants showed two-fold less ssDNA than WT cells (van Attikum *et al*, 2004).

We confirmed this result by performing the analogous assay in *nhp10* and *swr1* mutant strains. In the case of *nhp10* ssDNA levels were low, as in the *arp8* mutant, whereas the loss of *swr1* had little or no effect (Figure 6D). This confirms a similar result reported for the *nhp10* mutant and is consistent with the Mre11 recruitment profiles in *arp8* and *swr1* mutants (A Morrison and X Shen, personal communication, and Figure 6A). Impaired end-resection was not found, however, in an *arp8* mutant monitored by Southern hybridization (Tsukuda *et al*, 2005). To see if this discrepancy reflected differences in strain background, we compared the *arp8* deletion strain of Tsukuda and co-workers, with our independently derived *arp8* deletion. Both strains were similarly sensitive to the DNA damaging agent hydroxyurea and both showed a 2- to 3-fold reduction in ssDNA formation as detected by QAOS (Supplementary Figure 3). To see if detection by Southern might have obscured the *arp8* effect, we reproduced their Southern analyses and again could confirm that the generation of the ssDNA overhang at a DSB is impaired in the *arp8* mutant (data not shown). We conclude that chromatin remodeling driven by INO80, but not SWR1, favors end-processing, which is mediated at least in part by recruitment of the Mre11 endonuclease.

SWR1 facilitates DSB repair by error-free NHEJ

Mre11 and yKu are both necessary for the repair of HO-induced DSBs by NHEJ. In addition, Mre11 is also involved in DSB repair by HR (Khanna and Jackson, 2001; van Gent *et al*, 2001). The reduced binding of Mre11 and yKu80 in the *arp8* mutant and the reduced binding of yKu80 in *swr1* prompted us to test the efficiency of HO-induced DSB repair at *MAT* by NHEJ or HR in cells deleted for *ARP5*, *ARP8*, *NHP10*, *SWR1* and *YKU70* or *RAD52*.

We used a variety of assays that score for different types of resistance to the expression of the HO endonuclease. By providing the potential donor sequence or not, it is possible to differentiate and score for repair by HR (Supplementary Figure 3), or error-prone end-joining (Figure 6E) and error-free end-joining (Figure 6F), respectively. Growth of donor-proficient (*MATinc*) strains on galactose requires repair of the DSB by HR (Supplementary Figure 3). In this assay only an HR-deficient *rad52* mutant showed a reduced survival (Supplementary Figure 3). This suggests that neither INO80 nor SWR1 plays a role in mating type-specific HR, in agreement with an earlier report (Papamichos-Chronakis *et al*, 2006).

Growth of donorless strains on galactose-containing medium requires error-prone repair of the DSB, in order to mutate the HO site and prevent subsequent rounds of cleavage. Using a similar assay we have previously reported that *arp5* and *arp8* mutants display minor defects in error-prone end-joining of an HO-induced DSB (van Attikum *et al*, 2004). While we confirm this defect, neither the *nhp10* nor the *swr1* mutant shows similar sensitivity. This suggests that if INO80 has any role in error-prone NHEJ, it is minor, whereas SWR1 has none.

Using an alternative method we could monitor the efficiency of error-free end-joining at the HO cut site. HO was expressed for 1 h on galactose and then was rapidly shut off by the addition of glucose to allow repair. The rate of end-joining was determined by rt-PCR, using primers that span the DSB site, 2 h after HO repression. We found WT rates of error-free end-joining in *arp8* or *nhp10* mutants, whereas the *swr1* deletion had a significant drop in error-free end-joining efficiency (Figure 6F). The defect was, as expected, less pronounced than in *yku70* or *mre11* mutants. Nonetheless, our functional assays indicate that SWR1 specifically facilitates error-free NHEJ, that INO80 plays a minor role in error-prone NHEJ, and that neither complex is necessary for mating-type HR.

INO80, but not SWR1, affects Mec1 binding and checkpoint activation at DSBs

The generation of ssDNA at a DSB is a prerequisite for both HR and checkpoint activation. As impaired ssDNA formation in *arp8* or *nhp10* mutants did not affect the efficiency of HR, we wondered whether such mutants would be defective in the checkpoint response. ssDNA at DSBs recruits the ssDNA binding complex RPA, which in turn recruits the Ddc2–Mec1 complex (Harrison and Haber, 2006). Thus, Mec1 recruitment to a DSB is dependent on the formation of ssDNA, an event that requires INO80 activity at the break. To test whether Mec1 recruitment to the DSB requires INO80 activity, we performed ChIP in appropriate strains bearing Myc-tagged Mec1. We detected a significant recruitment of Mec1 as early as 1 h after HO induction, reaching maximum levels at 4 h of HO induction in the WT. Mec1 preferentially accumulated at sites close to the DSB (within 5 kb from cut) and spreads away from the DSB site at later time points (2–4 h), reaching sites 10–23 kb from the DSB end (Figure 7A, and data not shown). However, ChIP showed a significant reduction in the efficiency of Mec1 recruitment in *arp8* (five-fold) and *nhp10* (three-fold) mutants, but not in the *swr1* mutant, as compared with WT cells (Figure 7A and Supplementary Figure 4). As Mec1 is thought to bind ssDNA at DSBs, the reduction of Mec1 in mutants lacking INO80 strongly reinforces our finding that this complex facilitates end-resection.

The checkpoint kinase Mec1 activates the central checkpoint kinase Rad53 upon induction of a DSB at *MAT*, which results in cell cycle arrest at G2/M phase (Harrison and Haber, 2006). Rad53 becomes hyper-phosphorylated, which is seen as a mobility shift after Western blot analysis. We tested whether impaired Mec1 recruitment to DSBs in mutants in INO80 would reduce Rad53 activation. Western analysis after HO induction demonstrated that Rad53 activation is seen as early as 1–2 h after HO induction, reaching full induction at 4–6 h in WT cells (Figure 7B and Supplementary Figure 4). As expected, Rad53 activation was abolished in the

Figure 7 Deletion of *ARP8*, but not *SWR1*, reduces Mec1 recruitment and checkpoint activation in response to a DSB at *MAT*. (A) Absolute fold enrichment of Myc-Mec1 ChIP for WT and *arp8* strains (left panel) and WT and *swr1* strains (right panel) is shown as in Figure 1. Cleavage efficiencies were 89, 97 and 99% (WT, left panel), 56, 78 and 85% (*arp8*), 95, 98 and 99% (WT, right panel), and 85, 95 and 97% (*swr1*). (B) Rad53 kinase activation was determined by Western blot analysis in WT, *arp8*, *swr1* and *rad9rad24* strains. (C) Representation of INO80- and SWR1-dependent events at a DSB. INO80 removes histone H2A variants (γ H2AX and H2A.Z) and core histones, facilitating Mre11 binding, end-processing, Mec1 recruitment and checkpoint activation. In contrast, SWR1 does not affect histone loss, Mre11 binding or end-processing, but is required for yKu80 binding, as is INO80. Whereas SWR1 facilitates error-free NHEJ, INO80 only plays a minor role in error-prone NHEJ.

checkpoint-defective *rad9rad24* mutant. Interestingly, we found that Rad53 activation was delayed in an *nhp10* mutant and strongly reduced in an *arp8* mutant, whereas the *swr1* mutant was comparable to WT (Figure 7B and Supplementary Figure 4). Consistently, the *arp8* mutant recruited ~2-fold less Mec1 to the DSB when compared with the *nhp10* mutant, a difference reflected in the efficiency of Rad53 activation. From these results we conclude that INO80, but not SWR1, affects checkpoint activation in response to the induction of a DSB.

Discussion

The ATP-dependent chromatin remodeling complexes INO80 and SWR1 define a highly conserved subclass of the Snf2 family of ATPase-containing complexes and are involved in both transcriptional regulation and DNA damage repair (Shen *et al*, 2000; Krogan *et al*, 2003; Kobor *et al*, 2004; Mizuguchi *et al*, 2004; van Attikum *et al*, 2004). The Ino80 ATPase and a number of subunits of the INO80 complex, including the Arp4 and Rvb1 subunits, are recruited to an HO-induced DSB at the *MAT* locus in yeast (Downs *et al*, 2004; Morrison *et al*, 2004; van Attikum *et al*, 2004). Resolving previous ambi-

guities that arose from the analyses of shared components, we show here that the Swr1 ATPase itself is recruited to an induced DSB at the mating type locus on Chr III.

The *MAT* locus is a specialized locus with unusual recombination properties that favor directional intrachromosomal repair by HR. Although the recruitment of both Ino80 and Swr1 to the *MAT* DSB was monitored in a donorless strain, one cannot rule out that this recruitment is linked to specialized features of the locus. Therefore, we examined the recruitment of the INO80 and SWR1 remodeling complexes at a cleavage site near the 3' end of an unrelated gene on Chr XV. Both Ino80 and Swr1 were again recruited, suggesting that their binding occurs generally at DNA breaks. Nonetheless, the efficiency of Ino80 and Swr1 recruitment at *PDR10* differs significantly from that at *MAT*. Further experiments may explain whether this reflects variations in transcription and/or chromatin modifications.

It was previously reported that INO80 is present at the *MAT* locus before HO induction, possibly to regulate transcription of this locus (Tsukuda *et al*, 2005). We detect no significant levels of either Ino80 or Swr1 before cleavage at either locus. This agrees with genome-wide localization studies that also failed to detect Ino80 or Swr1 at *MAT*, *PDR10* or at our

control locus *SMC2* (M Harata, personal communication). Differences in growth conditions may be responsible for the result by Tsukuda and co-workers, since incomplete repression of the HO endonuclease gene can lead to a background of cleavage and low level Ino80 recruitment.

SWR1 incorporates histone variant H2A.Z into chromatin at telomeres, centromeres and promoters (Krogan *et al*, 2003; Kobor *et al*, 2004; Mizuguchi *et al*, 2004; Guillemette *et al*, 2005; Raisner *et al*, 2005). A recent report by Papamichos-Chronakis *et al* (2006) suggests that Swr1 actually inserts H2A.Z into chromatin near a DSB at *MAT*, yet only in a mutant lacking the first 900 bp of the *INO80* gene. We find that Swr1 does not incorporate H2A.Z near a DSB at *MAT*, in either the presence or absence of INO80 subunits Arp8 or Nhp10, which are required for INO80 remodeling activity (Shen *et al*, 2003; Morrison *et al*, 2004). We also found that, in contrast to the aforementioned *ino80* mutant (Papamichos-Chronakis *et al*, 2006), mutants in *arp8* and *nhp10* are impaired for end-resection, Mec1 recruitment and checkpoint activation.

The reasons for these discrepancies are not known, but can probably be attributed to the type of mutation studied. In the universally used JKM179 background, *ino80* deletion is lethal; surprisingly, the partial deletion of *ino80* used by Papamichos-Chronakis and co-workers, does not show HU sensitivity like other *INO80*- or *arp8*-deficient strains (Supplementary Figure 3; Shen *et al*, 2000; van Attikum *et al*, 2004). This may suggest the presence of a suppressor or gain-of-function mutation in this strain. Here we demonstrate that the phenotypes attributed to our *arp8* deletion indeed result from loss of Arp8, because expression of a LexA-Arp8 fusion protein, but not of LexA alone, rescued both the growth defect and the HU sensitivity of the *arp8*-deficient strain (Supplementary Figure 3). The two studies agree, nonetheless, that SWR1, although recruited, does not insert H2A.Z into chromatin near a DSB in WT cells.

We have shown that SWR1 is recruited to an HO-induced DSB in a γ H2AX-dependent manner, to facilitate binding of yKu80 and repair by error-free NHEJ (Figure 7C). This provides the first evidence that SWR1 has a role in DSB repair. Likewise, RSC is recruited to facilitate yKu70 binding and error-free NHEJ (Shim *et al*, 2007), suggesting that SWR1 and RSC may have overlapping roles in NHEJ. Impaired SWR1 recruitment may explain a previous report that the *hta1/2*^{S129*} mutation impaired NHEJ (Downs *et al*, 2000). On the other hand, RSC and INO80 both facilitate histone eviction, end-resection and Mre11 binding at the HO-induced DSB (Figure 7C; Shim *et al*, 2007), which SWR1 does not do. Interestingly, mutants in RSC and INO80 have different outcomes on repair processes: loss of INO80 impairs Mec1 recruitment and checkpoint activation (Figure 7C), while mutants in RSC have defects in repair by HR and NHEJ (Chai *et al*, 2005; Shim *et al*, 2005, 2007). This suggests that INO80 and RSC have both overlapping and non-overlapping functions. Future studies may address whether the joint inactivation of INO80 and RSC produces synergistic effects on histone eviction, end-resection, Mre11 loading and checkpoint activation. It is at present unclear which interactions determine the unique functions of these remodelers.

At a DSB, ssDNA becomes rapidly coated by RPA, which then recruits both Rad51 and the checkpoint kinase complex

Mec1-Ddc2 (Harrison and Haber, 2006). Consistently, the defects in end-processing detected in mutants in INO80 impair not only Rad51, but also Mec1 binding at DSBs. Correlated with the reduced levels of Mec1 binding, *arp8* and *nhp10* mutants show reduced activation of the G2/M checkpoint. This *arp8* effect is not observed when DNA damage is induced by MMS, probably because end-resection is not required for intra-S phase checkpoint activation (van Attikum *et al*, 2004). Tsukuda *et al* (2005) suggested that the reduced binding of Rad51 detected in an *arp8* mutant might impair HR. However, normal levels of repair by HR were detected at *MAT* in cells lacking Ino80, Arp5, Arp8 or Nhp10 (Papamichos-Chronakis *et al*, 2006). There may still be a subset of HR events that require INO80, since defects in DNA damage-induced sister chromatid and interchromosomal recombination were reported for an *arp8* mutant (Kawashima *et al*, 2007), and recombination mediated transposition events in *Arabidopsis* require INO80 (Fritsch *et al*, 2004). Here we show that SWR1 affects neither HR nor checkpoint activation, but uniquely facilitates error-free NHEJ, arguing that INO80 and SWR1 have distinct roles in the response to DSBs (Figure 7C). Given the high degree of conservation among yeast and human INO80 remodelers, it will be important to examine whether INO80, like the mammalian SWI/SNF complex, influences mammalian DSB repair (Park *et al*, 2006).

Materials and methods

Yeast strains and media

Yeast strains are listed in Supplementary Table 1. All yeast strains were grown at 30°C on YPAD, YPLGg (2% lactic acid, 3% glycerol, 0.05% glucose) or selective SC media, as indicated. Complete null, HA- and Myc-tagged alleles were made by standard methods (Wach *et al*, 1994; Goldstein and McCusker, 1999), except for the Myc-Mec1 allele, which was described earlier (Paciotti *et al*, 2000). Expression and complementation by tagged proteins was verified by Western blot and MMS sensitivity assays. The HO-URA-HO construct was used to amplify and integrate the HO cleavage site at the *PDR10* locus (SGD coordinate 936199; primer sequences available on request) (Strom *et al*, 2004). The presence of a single copy of the HO cleavage site was verified by PCR.

Chromatin immunoprecipitation

ChIP was performed as described (<http://www.epigenome-noe.net/researchtools/protocol.php?protid=27>). An aliquot of each extract was not immunoprecipitated and was used as Input. The following antibodies were used: Mab anti-Myc (9E10), Mab anti-HA (Santa Cruz), rabbit anti-H2A.Z (Abcam), rabbit anti-histone H3 (Abcam) and rabbit anti- γ H2AX (W Bonner). Input and immunoprecipitated DNA were purified and analyzed by rt-PCR, using the Perkin-Elmer ABI Prism 7700 Sequence Detector System. For each ChIP, real-time (rt) PCR was performed two to four times. Absolute fold enrichment was calculated as follows. For each time point, the signal from a site near the HO DSB at *MAT* or *PDR10* was normalized to that from the non-cleaved *SMC2* locus in ChIP and input DNA samples. For each time point and site, the normalized ChIP signals were normalized to input DNA signals, since end-resection can reduce the available DNA template. Finally, relative fold enrichment was calculated by dividing the absolute fold enrichment from induced cells to that of uninduced cells. For histone loss log₂ values of the relative enrichment were calculated. ChIP results are presented as the mean of two to four experiments $\pm$ the standard error of mean (s.e.m.).

The efficiency of DSB induction was determined as described (van Attikum *et al*, 2004), except that rt-PCR was used. Absolute fold enrichment was normalized to the efficiency of DSB induction, as indicated in the figure legends, using the following formula:

absolute fold enrichment = fold enrichment/(% cut efficiency/100).
Primer and probe sequences are available on request.

Quantitative amplification of ssDNA

The rt-PCR-based method, QAOS, was previously described (Booth *et al*, 2001; van Attikum *et al*, 2004), and made use of primers at the HO1 site (Figures 1A and 6C).

NHEJ assays and Western analysis of Rad53

Yeast strains were grown overnight to $\sim 5 \times 10^6$ cells/ml in YPLGg. To monitor error-prone NHEJ, aliquots of 10-fold serial dilutions were plated on YPLGg supplemented with 2% of either galactose or glucose. Colonies were scored 3–4 days after plating to monitor the efficiency of error-prone NHEJ. To monitor error-free NHEJ, 2% of either galactose or glucose was added to the overnight cultures ($t=0$). Cells in which HO was induced for 1 h ($t=1$) were rapidly shifted to glucose to shut off HO expression and incubated 2 h ($t=2$). End-joining efficiency was determined by quantitative rt-PCR at $t=2$, using primers that span the DSB site at *MAT* or anneal to the *SMC2*. To monitor Rad53 activation by Western

analysis, 2% of either galactose or glucose was added to the overnight culture. Protein extracts were prepared using TCA precipitation. Western analysis was performed as described (van Attikum *et al*, 2004).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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