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INO80 and γ-H2AX Interaction Links ATP-Dependent Chromatin Remodeling to DNA Damage Repair

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Summary

While the role of ATP-dependent chromatin remodeling in transcription is well established, a link between chromatin remodeling and DNA repair has remained elusive. We have found that the evolutionarily conserved INO80 chromatin remodeling complex directly participates in the repair of a double-strand break (DSB) in yeast. The INO80 complex is recruited to a HO endonuclease-induced DSB through a specific interaction with the DNA damage-induced phosphorylated histone H2A (γ-H2AX). This interaction requires Nhp10, an HMG-like subunit of the INO80 complex. The loss of Nhp10 or γ-H2AX results in reduced INO80 recruitment to the DSB. Finally, components of the INO80 complex show synthetic genetic interactions with the *RAD52* DNA repair pathway, the main pathway for DSB repair in yeast. Our findings reveal a new role of ATP-dependent chromatin remodeling in nuclear processes and suggest that an ATP-dependent chromatin remodeling complex can read a DNA repair histone code.

Introduction

The packaging of the eukaryotic genome into chromatin restricts the access of DNA processing enzymes in various nuclear processes. To overcome these barriers, cells use two major ways to modify chromatin structure, either by posttranslational modifications of histones or by ATP-dependent chromatin remodeling, both of which are clearly implicated in transcription (Fyodorov and Kadonaga, 2001; Kornberg and Lorch, 1999; Roth et al., 2001). Recently, it has been shown that histone acetylation and/or deacetylation are required for efficient DSB repair (Bird et al., 2002; Jazayeri et al., 2004). However, little is known about the role of ATP-dependent chromatin remodeling in DNA damage repair. The INO80 class of complexes represents an evolutionarily conserved class of ATP-dependent chromatin remodeling complexes in the SWI/SNF superfamily (Mizuguchi et al., 2004; Shen et al., 2000). The founding member of this class is the yeast INO80 complex, which contains actin and several actin-related proteins (Arps) in addition to the Ino80 core ATPase (Shen et al., 2000). The INO80 complex is implicated in both transcription and DNA repair. Mutants of INO80 subunits, such as the *ino80*-, *arp5*-, and *arp8*-null mutants, show hypersensitivities to DNA-damaging agents, such as methylmethanesulfonate (MMS) and ionizing radiation (IR) (Shen et al., 2000, 2003a). In addition, the INO80 complex contains RuvB-like DNA helicases, and RuvB is involved in DNA recombination and repair in bacteria. These findings suggest a coupling between chromatin remodeling and DNA repair (Shen et al., 2000). However, it is not known whether the INO80 complex functions in DNA damage repair directly or indirectly through other mechanisms. Here, we show biochemical and genetic evidence that the INO80 complex is directly involved in DNA repair. The key mechanism is through a specific interaction between the INO80 complex and the DNA damage-induced γ-H2AX. These results provide a direct link between ATP-dependent chromatin remodeling and DNA damage repair.

Results

Transcriptional and Checkpoint Responses to DNA Damage Are Normal in *ino80* Mutant

We first investigated whether the DNA repair defects of the *ino80* mutant could be attributed to defects in transcriptional response to DNA damage or defects in DNA damage checkpoint functions. We found that the major DNA damage response pathway, the Mec1 kinase pathway, appeared to be normal in the *ino80*-null mutant. In response to DNA damage, the transcription of ribonucleotide reductase genes *RNR1* and *RNR3*, which are the downstream targets of the DNA damage checkpoint gene *MEC1* (Huang and Elledge, 1997), is induced. We found that after treatment of *ino80* cells with hydroxyurea (HU), the induction of *RNR1* and *RNR3* was normal (Figure 1A). In addition, *ino80* mutant cells showed normal cell cycle arrest in the presence of HU, consistent with functioning DNA damage checkpoints (Figure 1B). Moreover, a survey of the global transcription profile of the *ino80* mutant identified no major changes in genes annotated as being involved in DNA repair in the *Saccharomyces* genome database (SGD; www.yeastgenome.org), and most genes involved in homologous recombination (HR) and nonhomologous end joining (NHEJ) were not affected in the *ino80* mutant (Mizuguchi et al., 2004) (Experimental Procedures). These findings are consistent with a direct role of *INO80* in DNA repair.

INO80 Is Recruited to HO-Induced DSB

Since the *ino80* mutant is hypersensitive to IR, which produces DSBs (Shen et al., 2000), we investigated whether the INO80 complex might directly participate

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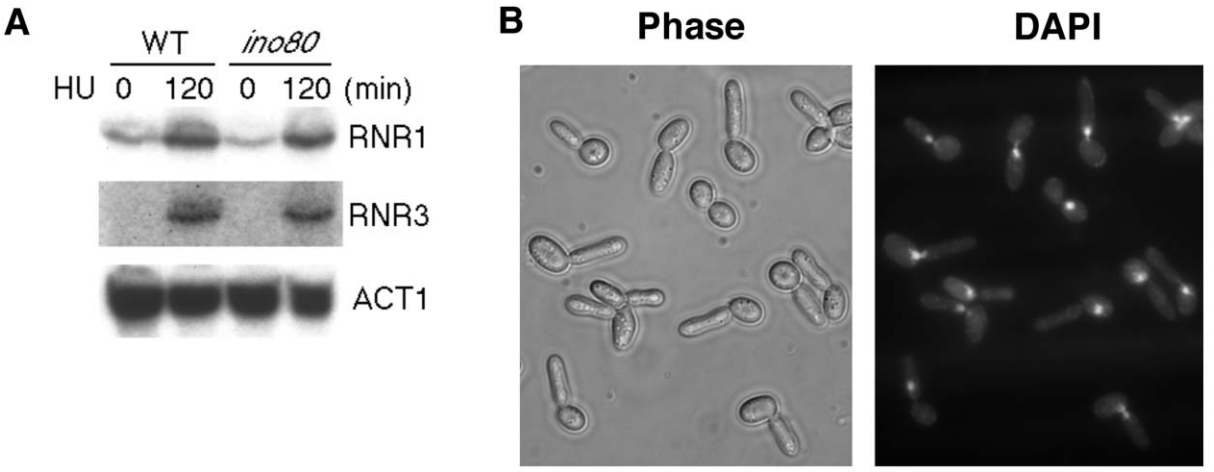

Figure 1. Transcriptional Response to DNA Damage and DNA Damage Checkpoints Are Normal in *ino80* Mutant

(A) Northern analysis of *RNR1* and *RNR3* induction before and after hydroxyurea treatment (HU). *ACT1* is a loading control.

(B) Phase contrast and DAPI staining images of *ino80* mutant cells treated with 100 mM HU (5 hr). Large buds and nuclei at the bud neck indicate cell cycle arrest in response to DNA damage checkpoints. *ino80* mutant cells show normal arrest pattern, despite having abnormally elongated buds.

in DSB repair by determining the potential recruitment of the INO80 complex to a DSB. We used the well-established technique of introducing a DSB in the yeast genome by expressing the HO endonuclease under the control of a *GAL*-inducible promoter. HO endonuclease cuts at the *MAT* locus and produces a DSB (Haber, 2000). This DSB is mainly repaired through HR. In our experiments, we used an HO-DSB strain in which the recombination donors have been deleted; therefore, the HO-induced DSB cannot be efficiently repaired (Moore and Haber, 1996). This system has allowed for the characterization of recruited DNA repair factors at the DSB without the complication of subsequent repair steps (Figure 2A). At 2 hr postinduction, chromatin immunoprecipitation (ChIP) studies have shown that proteins involved in both HR and NHEJ, such as Rad51, Rad52, Rad54, Rad55, and Yku80, and checkpoint proteins, such as Dcd1 and Dcd2, are clearly recruited to the DSB (Kondo et al., 2001; Martin et al., 1999; Wolner et al., 2003). Therefore, we tested INO80 recruitment at this time point. We transformed the HO-DSB strain with a FLAG-tagged Ino80 plasmid (Shen et al., 2003a) and determined the in vivo distribution of Ino80 using ChIP assays with a monoclonal FLAG-antibody (Mizuguchi et al., 2004). Induction of DSB was monitored by real-time PCR. At 2 hr postinduction, about 80%–90% of the cells contained a DSB (Figure 2B). By using a primer set close to the DSB and a control primer set from another chromosome, we were able to detect a 3- to 4-fold enrichment of Ino80 near the site of DSB after HO induction (Figure 2B). We also tested another subunit of the INO80 complex, Arp8. Arp8 was also recruited in a manner similar to that of Ino80 (Figure 2B). Since Ino80 and Arp8 are unique subunits of the INO80 complex (Shen et al., 2000; Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/6/767/DC1/), these findings indicate that the INO80 complex is recruited to a DSB in vivo.

Subsequent testing of another subunit of the INO80 complex, Arp5, further confirmed the recruitment of the whole complex (Supplemental Figure S2 on the *Cell* website). We also examined the distribution of Ino80 around the HO-induced DSB and found that recruitment can be detected up to 9 Kb on each side of the DSB (Supplemental Figure S2 on the *Cell* website). We then determined the kinetics of INO80 recruitment and found that recruitment could be detected as early as 30 min after HO induction and continued to increase (Figure 2C). This recruitment is similar to that of many proteins involved in DSB repair (Kondo et al., 2001; Martin et al., 1999; Wolner et al., 2003), suggesting a coordinated function of the INO80 complex with other DNA repair factors.

INO80 Interacts with γ-H2AX

To address the mechanism of INO80 recruitment to DSBs, we reasoned that the INO80 complex might be recruited by physical interaction with DNA repair proteins. One of the earliest responses to the formation of DSBs in mammals is the rapid phosphorylation of histone H2AX adjacent to the break site (Rogakou et al., 1998). The phosphorylated derivative, γ-H2AX, forms foci and is necessary for subsequent recruitment or retention of many DNA repair factors (Paull et al., 2000). The yeast histone species that are orthologous to H2AX are the major H2A proteins encoded by *HTA1* and *HTA2*. Similarly, yeast H2As are also phosphorylated on the homologous serine that is located four residues from the carboxyl terminus in response to DNA damage, and the phosphorylated species was referred to as γ-H2AX (Redon et al., 2003). We will also refer to the phosphorylated yeast histone H2As as γ-H2AX for simplicity. It has been shown that like mammalian γ-H2AX, the yeast γ-H2AX is also involved in DNA repair, although the mechanism remains unknown (Downs et al., 2000; Redon

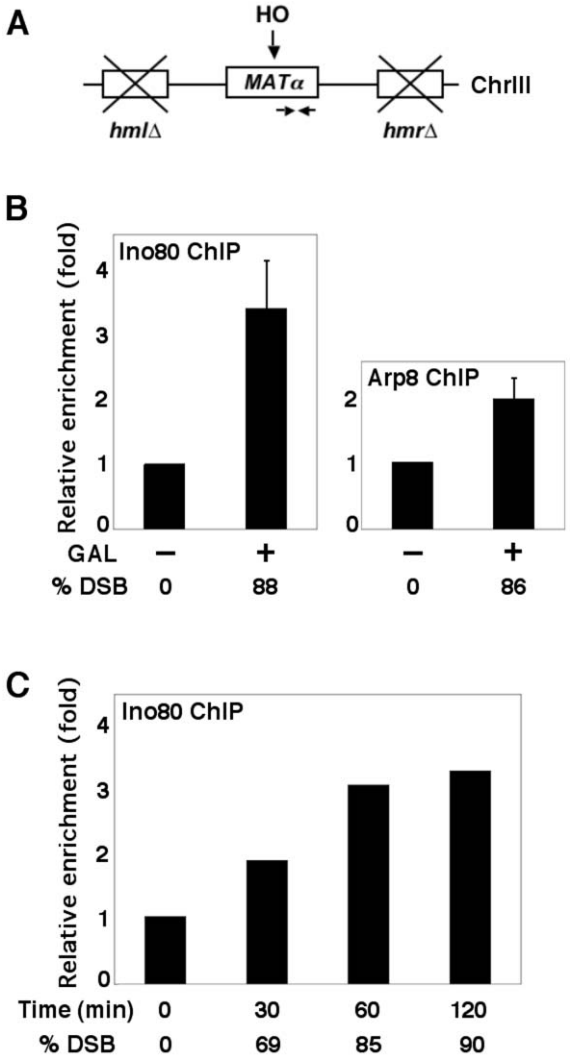

Figure 2. The INO80 Complex Is Recruited to the HO-Induced DSB

(A) Schematic representation of the HO-induced DSB at the *MAT* locus on ChrIII. The two donors on each side of the DSB have been deleted. Arrows indicate primers near the DSB for ChIP analysis; control primers are from ChrXII (not shown).

(B) Graphs of ChIP analyses showing the relative enrichment (fold) of Ino80 (left, n = 6) and Arp8 (right, n = 3) near the DSB in the *MAT* locus. Fold enrichment 2 hr after HO induction (+GAL) is normalized using signals before the induction of DSB (−GAL) as 1; error bars denote the standard deviation among experiments. Cutting efficiency (% DSB) is shown at the bottom and is calculated based on the real-time PCR quantified decrease in abundance of a fragment spanning the DSB after HO induction compared to a control fragment, which is not cut by HO, assuming that cells contain no DSBs before induction.

(C) Graph of ChIP analyses showing the time course in min of Ino80 recruitment to HO-induced DSB. Results are averages of two experiments. Cutting efficiency (% DSB) is shown below graph and is calculated as described in (B).

et al., 2003). In response to an HO-induced DSB, γ-H2AX is detected within 15 min of the time when the DSB is detected in the region surrounding the DSB (Shroff et al., 2004). Therefore, yeast and mammals share similarities in γ-H2AX induction at DSBs, and the timing of γ-H2AX induction at the HO-induced DSB is consistent with a role of γ-H2AX in the recruitment of DNA repair factors, including the INO80 complex (Figure 2).

Previously, it has been shown that the INO80 complex is associated with histones under physiological salt conditions (Mizuguchi et al., 2004). These findings prompted us to investigate whether INO80 might be associated with γ-H2AX under DNA-damaging conditions. We used MMS to induce DNA damage. MMS is an alkylating agent and results in DNA single- and double-strand breaks. After MMS treatment, the DNA damage-induced γ-H2AX could be detected from whole-cell extract by using an antibody specific to γ-H2AX, which does not recognize unphosphorylated H2AX (Redon et al., 2003). We then purified the INO80 complex under moderate salt conditions (0.25 M KCl) using the whole-cell extract made after MMS treatment. Interestingly, we detected a strong interaction between the INO80 complex and γ-H2AX (Figure 3A). The association of histones with the INO80 complex might be due to nonspecific DNA/chromatin binding; however, we found that the INO80 and γ-H2AX interaction was unlikely to be dependent on the presence of DNA or RNA since it persisted after extensive DNase I and RNase A digestion or ethidium bromide treatment (Figure 3B and data not shown). This interaction was also stable under higher salt conditions (up to 0.35 M KCl) (Figure 3B). To determine whether this interaction is specific, we compared INO80 with other ATP-dependent chromatin remodeling complexes. Under the same MMS treatment, the SWI/SNF complex, which belongs to another class of the SWI/SNF superfamily (Eisen et al., 1995), failed to interact with γ-H2AX (Figure 3C), even when 5-fold more SWI/SNF was analyzed (data not shown). We also investigated the SWR1 complex, which belongs to the INO80 class (Mizuguchi et al., 2004). Both native INO80 and SWR1 complexes associate with all histones under physiological salt conditions, although SWR1 shows a preferential association with a histone variant, Htz1, under higher salt conditions (0.5 M KCl) (Mizuguchi et al., 2004). When normalized using either histone bands or the ATPase subunits, Ino80 and Swr1, we consistently detected a stronger association of γ-H2AX to the INO80 complex compared to the SWR1 complex (Figure 3C). These results indicate that the INO80 complex preferentially interacts with the DNA damage-induced γ-H2AX and suggest that an ATP-dependent chromatin remodeling complex recognizes a specific DNA repair histone code (Fernandez-Capetillo and Nussenzweig, 2004; Strahl and Allis, 2000). This interaction provides a potential mechanism for the recruitment of the INO80 complex to DSBs.

The Nhp10 Subunit Is Required for INO80 and γ-H2AX Interaction

To gain insights into this interaction, we investigated the subunits of the INO80 complex, which might be involved in the interaction with γ-H2AX. We used deletion strains of various INO80 subunits and purified the mutant INO80 complexes after MMS treatment. The deletions strains of INO80 subunits showed normal induction of γ-H2AX after MMS treatment (Figure 4A). Therefore, the INO80 complex is not required for the induction

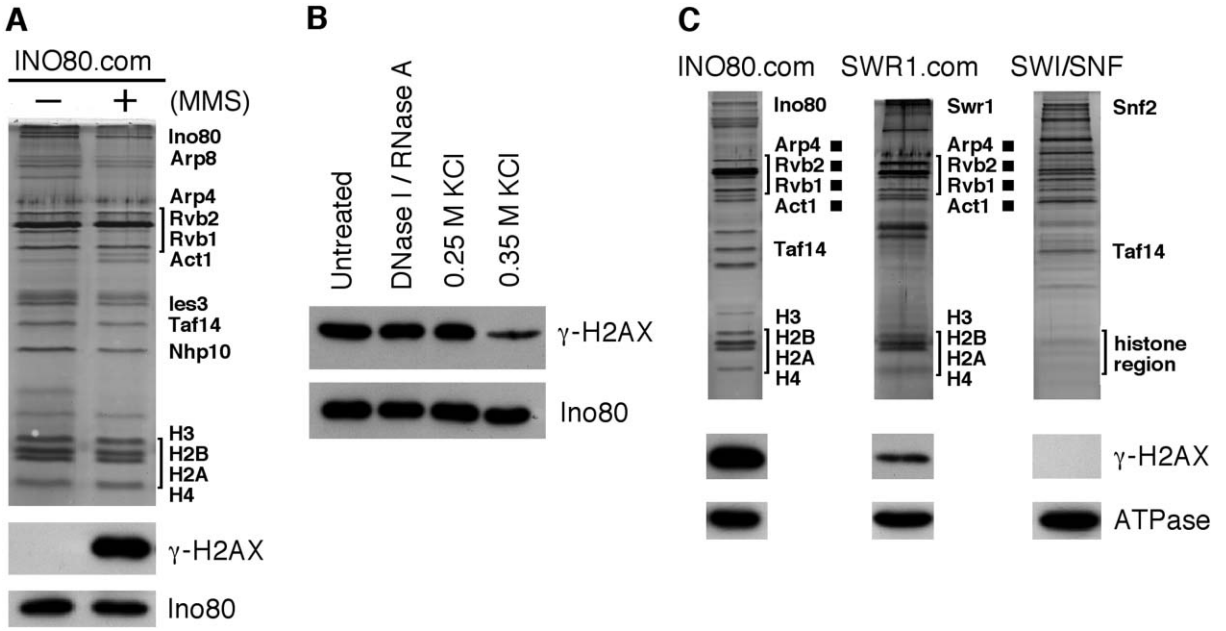

Figure 3. The INO80 Complex Interacts with γ-H2AX Specifically

(A) SDS-PAGE and Western analyses of INO80 complexes (INO80.com) purified under 0.25 M KCl salt conditions before and after MMS treatment. Top panel is a silver-stained SDS-PAGE gel showing purified INO80 complex with relevant subunits labeled. Middle panel shows a Western blot of the same INO80 complexes as shown in top panel, using an antibody specific for γ-H2AX. Equal loading of the complexes is shown by a Western blot of the epitope-tagged Ino80 ATPase subunit with a FLAG-antibody (bottom panel).

(B) Western analyses using the γ-H2AX antibody showing that γ-H2AX associates with the INO80 complex when the INO80 complex is purified under different conditions, such as DNase I and RNase A treatment, as well as varying salt concentrations (top panel). Ino80 is used as a loading control to show equal loading of the INO80 complex as described in (A) (bottom panel).

(C) SDS-PAGE and Western analyses of interactions between γ-H2AX and other remodeling complexes (SWR1.com and SWI/SNF) purified after MMS treatment. Top panels are silver-stained SDS-PAGE gels showing three different purified chromatin remodeling complexes with relevant subunits labeled. Solid squares indicate shared subunits between INO80 and SWR1 complexes. Taf14 is shared by INO80 and SWI/SNF. Middle panels show Western analysis of the same three complexes as shown in top panels using the γ-H2AX antibody, indicating that the INO80 complex preferentially associates with γ-H2AX. Equal molar loading of three complexes is shown by Western analysis of the epitope-tagged core ATPase subunit of each complex (Ino80, Swr1, and Snf2, respectively) with an FLAG-antibody (bottom panel).

of γ-H2AX. This result is consistent with an intact Mec1 kinase pathway in the *ino80* mutant (Figure 1A), since the Mec1 kinase pathway has been proposed to be the main pathway for γ-H2AX induction (Downs et al., 2000; Redon et al., 2003; Shroff et al., 2004). Interestingly, the INO80 complex contains actin and three Arps, Arp4, Arp5, and Arp8 (Shen et al., 2000). Previous studies have suggested that these Arps might interact with histones (Harata et al., 1999; Shen et al., 2003a). Notably, Arp4 has been shown to interact with histone H2A (Harata et al., 1999). We have shown that the deletion of Arp8 resulted in the loss of actin and Arp4 in the INO80 complex (Shen et al., 2003a). Surprisingly, in the absence of actin, Arp4, and Arp8, the INO80 mutant complex showed the same level of interaction with γ-H2AX as the wild-type complex (Figure 4B). Similarly, Arp5 was found to be dispensable for γ-H2AX interaction (data not shown). These findings suggest that actin and the Arps are not required for the interaction between INO80 and γ-H2AX. The SWR1 complex also contains actin and Arp4 (Mizuguchi et al., 2004; Figure 3C); therefore the lack of a strong interaction between SWR1 and γ-H2AX is consistent with the dispensable role of these proteins in γ-H2AX interactions.

We next deleted the Nhp10 subunit of the INO80 complex. Nhp10 is an HMG-like protein, which potentially can bind to structured DNA. We have shown previously that the deletion of Nhp10 also resulted in the loss of the Ies3 subunit, indicating that Ies3 association with the INO80 complex is dependent on Nhp10 (Shen et al., 2003a; Figure 4B). Although the loss of Nhp10 slightly reduced the level of bulk histone associated with the INO80 complex, when normalized using either histone bands or the Ino80 ATPase subunit, we found a significant reduction of INO80 and γ-H2AX interaction in the absence of Nhp10 (Figure 4B). This result indicates that the Nhp10 subunit is involved in the specific interaction between INO80 and γ-H2AX. Nhp10 mainly functions through the INO80 complex and is not present in the SWR1 or other known chromatin remodeling complexes (Supplemental Table S1 on the *Cell* website). This observation is consistent with a unique role of Nhp10 in specifying the γ-H2AX interaction for the INO80 complex. Moreover, unlike the loss of actin and Arps, the chromatin remodeling activity of the INO80 complex lacking Nhp10 is not significantly affected (Shen et al., 2003a), consistent with a role of Nhp10 in generating specific interactions with other factors, rather than playing an essential role in chromatin remodeling per se.

INO80 and γ-H2AX Interaction Provides a Key Mechanism for INO80 Recruitment to DSB

To investigate the biological relevance of INO80 and γ-H2AX interaction, we determined the effect of Nhp10

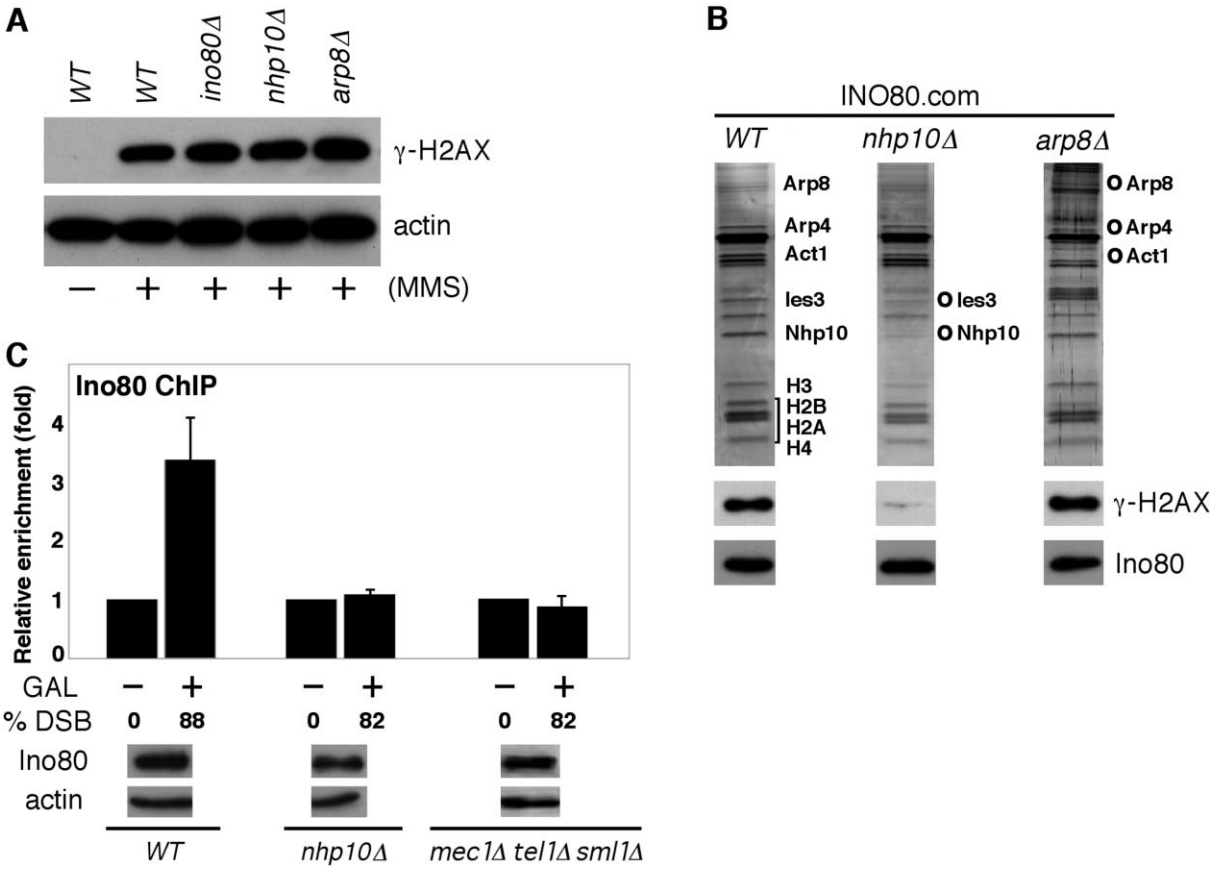

Figure 4. Nhp10 Is Involved in the Interaction between INO80 and γ-H2AX

(A) Western analyses of γ-H2AX induction in mutants of INO80 subunits using whole-cell extracts, indicating that γ-H2AX induction is normal in mutants of INO80 subunits (top panel). Actin is used as a loading control (bottom panel).

(B) SDS-PAGE and Western analyses of interactions between γ-H2AX and INO80 complexes purified from mutant strains after MMS treatment. Top panels are silver-stained SDS-PAGE gels showing purified wild-type (wt) as well as mutant INO80 complexes lacking Nhp10 or Arp8 subunits (*nhp10*Δ and *arp8*Δ) with relevant subunits labeled. Open circles indicate subunits lost as a result of specified subunit deletion. The residual bands at the positions of Ies3 and Nhp10 in the *nhp10*Δ mutant are due to unrelated contaminations. The loss of Arp8 in the *arp8*Δ mutant is not obvious in this 15% SDS-PAGE gel due to its large size, but it is clear in a 10% SDS-PAGE gel (data not shown). Middle panels show Western analysis of the same INO80 complexes as shown in the top panels using the γ-H2AX antibody. Equal loading of the complexes is shown by Western analysis of the epitope-tagged Ino80 ATPase subunit with a FLAG-antibody (bottom panel).

(C) Graphs of ChIP analyses showing the relative enrichment of Ino80 near the DSB at the *MAT* locus 2 hr after HO induction in wild-type (wt, n = 6), *nhp10* deletion (*nhp10*Δ, n = 5), and *mecl tel1 sml1* deletion (*mecl*Δ *tel1*Δ *sml1*Δ, n = 4) strains. Error bars denote the standard deviation among experiments. Percent DSB is calculated as described in Figure 2 (B). Flag-tagged Ino80 protein levels revealed by Western analysis are shown at the bottom. Actin is used as a loading control (bottom panel).

loss on INO80 recruitment to a DSB using the HO-DSB strain containing an *nhp10* deletion. In this strain, γ-H2AX was normally induced (data not shown); however, the INO80 recruitment was significantly reduced compared to wild-type (Figure 4C). This decrease in recruitment was not due to a reduction of HO-cutting efficiency, or a reduction of the Ino80 protein in the *nhp10* mutant, since both were near wild-type levels (Figure 4C). As an alternative approach, we wished to investigate the effect of γ-H2AX loss on INO80 recruitment to the HO-DSB. It has been shown that Mec1 and Tel1 kinases are responsible for the induction of γ-H2AX (Downs et al., 2000; Redon et al., 2003). We therefore engineered the HO-DSB strain and deleted both Mec1 and Tel1 kinases, eliminating the induction of γ-H2AX at the HO-DSB (Shroff et al., 2004). This strain showed normal HO-cutting efficiency and normal expression of the Ino80 protein; however, INO80 recruitment was significantly reduced compared to wild-type (Figure 4C). These results establish that interaction with γ-H2AX provides a key mechanism for recruiting the INO80 ATP-dependent chromatin-remodeling complex to a DSB.

INO80 Subunits Genetically Interact with DNA Repair Pathways

To complement the biochemical studies, we examined the genetic interactions between subunits of the INO80 complex and DNA repair pathways. Synthetic genetic interactions can reveal parallel pathways or factors involved in the same pathway (Tong et al., 2001). Given that the HO-induced DSB is mainly repaired through HR, we focused on the *RAD52* pathway, which is the main HR pathway (Haber, 2000). We analyzed the genetic interactions between genes in the *RAD52* pathway (*RAD52*, *RAD55*, and *RAD59*) and genes encoding two subunits of the INO80 complex (*ARP8* and *NHP10*). We

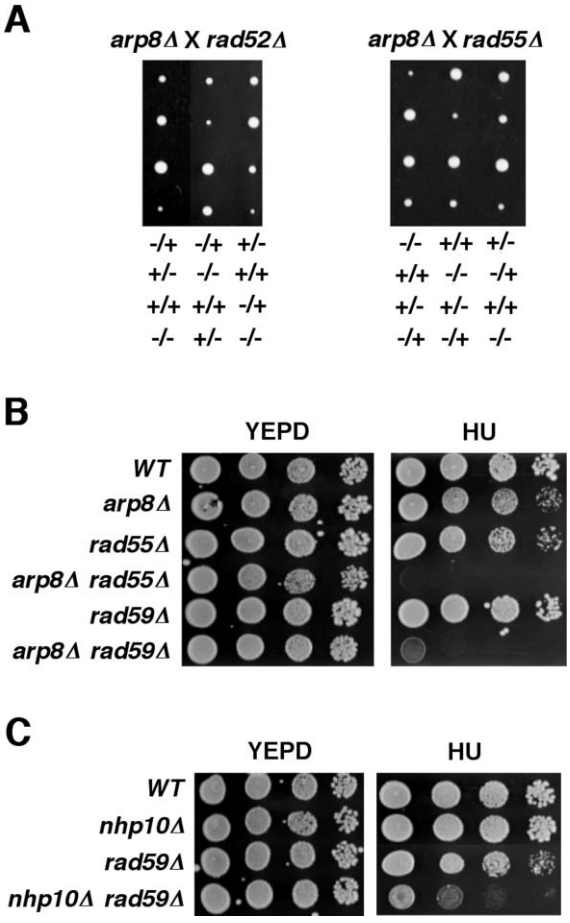

Figure 5. Genetic Interactions Implicate the INO80 Complex in DSB Repair

(A) Mutations in *ARP8* show synthetic growth defects when combined with mutations in genes involved in HR (*RAD52, RAD55*). The smallest tetrads are double mutants (−/−).

(B and C) Double mutants of either *arp8* or *nhp10* with genes involved in HR are more sensitive than single mutants to DNA-damaging agents (50 mM HU).

individually crossed *arp8*Δ or *nhp10*Δ strain to *rad52*Δ, *rad55*Δ, or *rad59*Δ strains and performed tetrad analyses using the resulting heterozygous strains to obtain double deletion haploid strains. Synthetic sick phenotypes were observed in *arp8*Δ *rad52*Δ, *arp8*Δ *rad55*Δ, and *arp8*Δ *rad59*Δ double deletion strains (Figure 5A and data not shown). Furthermore, the double deletion strains were more sensitive than single deletion strains to the DNA-damaging agents HU (Figures 5B and 5C) and camptothecin (data not shown). In addition, genes encoding several other subunits of the INO80 complex, such *as IES1, IES2, IES3, IES4, IES5*, and *IES6* also showed synthetic genetic interactions with genes in the *RAD52* pathway (Supplemental Table S2 on the *Cell* website). These genetic interactions suggest that the INO80 complex is either directly involved in the *RAD52* DNA repair pathway, or it might be part of a parallel DNA repair pathway, such as the NHEJ pathway. Similarly, the induction of γ-H2AX has been implicated in both NHEJ and HR pathways (Bassing et al., 2003; Celeste et al., 2003; Rothkamm et al., 2003). Together, our results establish that the INO80 ATP-dependent chromatin-remodeling complex is directly involved in the repair of a DSB.

Discussion

Our findings provide strong evidence for a previously elusive role of ATP-dependent chromatin remodeling in DNA repair. Although reasonably expected, the involvement of ATP-dependent chromatin remodeling in nuclear processes other than transcription, such as DNA repair, was poorly defined, largely due to the lack of evidence in physical interactions between remodeling complexes and DNA damage sites or DNA repair proteins (Fyodorov and Kadonaga, 2001). Here, we show that the INO80 complex is recruited to a DSB, and the key mechanism of recruitment is through a specific interaction with the DNA damage-induced γ-H2AX. Disruption of this interaction, either by deleting the INO80 subunit required for the interaction or by deleting the kinases responsible for the induction of γ-H2AX, resulted in reduction of INO80 recruitment to the DSB. Moreover, blocking γ-H2AX induction by mutating the serine residue, which is phosphorylated in γ-H2AX, also resulted in reduction of INO80 recruitment to the DSB (van Attikum et al., 2004 [this issue of *Cell*]). These lines of evidence indicate a key role of γ-H2AX in recruiting the INO80 ATP-dependent chromatin remodeling complex to DSBs, consistent with the important roles of γ-H2AX in recruiting many other DNA repair factors (Paull et al., 2000). Importantly, both the INO80 complex and γ-H2AX are evolutionarily conserved (Downs et al., 2000; Redon et al., 2003; Shen et al., 2000). Therefore, we speculate that in higher organisms, orthologous INO80 complexes might be recruited to DSBs through interaction with γ-H2AX as well. Our findings have implications that link chromatin remodeling to carcinogenesis through DNA repair since deficiency in H2AX results in genome instability and cancer predisposition in mice (Bassing et al., 2003; Celeste et al., 2003). Interestingly, in addition to hypersensitivities to many DNA-damaging agents (Shen et al., 2000), we also observed evidence of genomic instability in the *ino80* mutant, such as chromosome duplications in haploid cells and defects in plasmid maintenance (unpublished data).

In addition to γ-H2AX, other factors, such as DNA repair proteins, or damaged DNA itself, may help to recruit or retain the INO80 complex to different types of DNA damage. Interestingly, the Rad52 protein was recently found to interact with the INO80 complex (T.A. Motycka et al., submitted). Additional factors may be uncovered through biochemical and genetic analyses. Among ATP-dependent chromatin remodeling complexes, the INO80 complex preferentially interacts with γ-H2AX (Figure 3C). The same preference of INO80 to γ-H2AX was also observed in Carl Wu's laboratory (personal communication). Aside from the interaction between INO80 and γ-H2AX, it is possible that ATP-dependent chromatin remodeling complexes could be recruited to DNA damage sites through interactions with other factors. Therefore, it remains to be seen to what extent other chromatin

remodeling complexes might participate in DNA repair, and whether multiple chromatin remodeling complexes are required for the repair of a single type of DNA damage.

Several lines of evidence suggest that INO80 chromatin remodeling activity is required for the repair of a DSB. First, mutations of INO80 subunits result in DSB repair defects, as indicated by the hypersensitivities of mutants to IR (Shen et al., 2000). Here, we show that the INO80 complex directly targets a DSB and is unlikely to affect DSB repair indirectly through transcription. Second, a point mutation that inactivates the ATPase activity of the Ino80 protein without affecting the composition of the complex abolishes INO80 chromatin remodeling activity in vitro, and the same mutant is defective in DSB repair (Shen et al., 2000), indicating that the INO80 chromatin remodeling activity is indeed required for DSB repair. Third, subunits of the INO80 complex play distinct roles in chromatin remodeling and DSB repair. For instance, the Nhp10 subunit is critical for the recruitment of the INO80 complex to a DSB (Figure 4), but it is not essential for INO80 chromatin remodeling activity per se (Shen et al., 2003a). By contrast, the Arp8 subunit does not significantly contribute to the INO80 and γ-H2AX interaction, which is important for INO80 recruitment to a DSB (Figure 4); however, Arp8 is essential for the INO80 chromatin remodeling activity per se (Shen et al., 2003a). Since Arp8 is also recruited to the HO-induced DSB (Figure 2), it is likely that Arp8 contributes directly to INO80 chromatin remodeling activity at the DSB. Last, it can be argued that activities of the INO80 complex other than chromatin remodeling, such as the potential helicase activities of the Rvb1/2 subunits, might be responsible for the function of the INO80 complex in DSB repair. However, our finding that INO80 interacts with γ-H2AX and the fact that induction of γ-H2AX is exclusively linked to DSB repair clearly implicate a chromatin-related function of the INO80 complex at a DSB. Taken together, these evidence suggest that the INO80 complex is not only recruited to a DSB, but also the ATP-dependent chromatin remodeling activity driven by the INO80 complex is directly involved in the repair of a DSB.

Since γ-H2AX induction is an early event in DSB repair, INO80 chromatin remodeling might be required for the processing of newly broken DNA ends in a nucleosomal context to facilitate HR or NHEJ. However, the persistence of INO80 recruitment at the HO-DSB suggests involvement of INO80 chromatin remodeling in subsequent repair steps as well. How might chromatin remodeling facilitate DNA repair? Based on lessons learned from transcription, these remodeling complexes might be used to generate nucleosome-free regions around the DNA damage sites in order to facilitate the access of large DNA repair machinery or to create specific chromatin structures suitable for DNA repair. These changes in chromatin can be achieved by the two known mechanisms of ATP-dependent chromatin remodeling—nucleosome sliding or histone depletion/replacement (Korber and Horz, 2004). The precise steps and contributions of INO80 chromatin remodeling in DSB repair will need to be determined. Our studies also dovetail the emerging theme that ATP-dependent chromatin remodeling and histone modifications/histone variants are intimately connected (Korber and Horz, 2004). The finding that both histone acetylation/deacetylation and phosphorylation, as well as ATP-dependent chromatin remodeling, are required for DSB repair suggests that remodeling complexes, such as INO80, might be able to read specific DNA repair histone codes (Fernandez-Capetillo and Nussenzweig, 2004; Strahl and Allis, 2000). Recognition of these codes may provide one mechanism to target chromatin remodeling complexes to specific types of DNA damage. It remains to be seen whether other histone modifications, such as acetylation and methylation, are also involved in recruiting the INO80 complex to a DSB. Further biochemical and genetic dissections will likely provide insights into the interplay between ATP-dependent chromatin remodeling and histone modification/histone variants in DNA repair.

Experimental Procedures

Microarray Expression Analyses

Microarray analyses of *INO80*-regulated genes revealed that 446 genes were *INO80* activated, and 779 genes were *INO80* repressed (Mizuguchi et al., 2004). Among 1225 *INO80*-regulated genes, only nine genes (*MMS21, UNG1, DDR48, NTG1, RRD1, IMP2, APN1, NEJ1, PSO2*) were DNA repair related genes based on the annotations in the SGD. None of these genes had greater than 2-fold changes (repressed or activated by *INO80*).

Protein Purification and Analysis

Standard protein techniques such as SDS-PAGE, Western blotting, and silver staining were followed. γ-H2AX antibody was a gift from Dr. William M. Bonner (NIH). For MMS treatment, cells were subjected to 0.25% MMS for 2 hr at 30°C. Preparation of whole-cell extracts and FLAG-immunoaffinity purification were described in detail previously (Shen, 2004). INO80.com and SWR1.com were purified using chromosomally FLAG-tagged *INO80* and *SWR1* strains (Mizuguchi et al., 2004; Shen et al., 2000). SWI/SNF was purified using an *snf2* deletion strain transformed with a FLAG-tagged Snf2 plasmid (Shen et al., 2003b). INO80 mutant complexes were purified from mutants of INO80 subunits as described (Shen et al., 2003a). For DNase I and RNase A treatment, a standard purification using 5 ml of whole-cell extract was performed; just prior to elution of the complex from FLAG beads, 50 μg of DNase I and 50 μg of RNase A were added to the beads in 200 μl of buffer H-0.1 and incubated for 30 min at 30°C. After digestion, the beads were washed three times with buffer H-0.1, and the complex was eluted as described (Shen et al., 2000).

Chromatin Immunoprecipitation

ChIP analyses were performed as described (Mizuguchi et al., 2004). To examine the Ino80 subunit, donorless *JKM179* strain (*MAT*α) (Moore and Haber, 1996) was transformed with a double-FLAG-tagged Ino80 plasmid, pINO80-2F (Shen et al., 2003a). To examine the Arp8 subunit, *JKM179* was transformed with p2F-ARP8. p2F-ARP8 was made by cloning a PCR fragment of *ARP8* containing the entire ORF and its native promoter and terminator into pRS416 (Brachmann et al., 1998); a double-FLAG sequence was inserted after the start codon. Standard PCR-mediated gene disruption was used to generate the *nhp10* deletion, as well as the *mec1 tel1 sml1* triple deletions in the *JKM179* strain containing pINO80-2F. Deletion of the ribonucleotide reductase inhibitor gene, *SML1*, suppresses the lethality of the *mec1* deletion (Zhao et al., 1998). ChIP was performed using FLAG-agarose (M2) (Sigma). To determine the HO cutting efficiency, a primer set (5′-CAGGATAGCGTCTGGAAGTCA AAA-3′, and 5′-GAGCAAGACGATGGGGAGTTTCAA-3′), which amplifies DNA that spans the HO cut site was used. To monitor protein recruitment to the DSB, a primer set near the DSB (184 bp away from the HO cut) (5′-TCCCCATCGTCTTGCTCTTGTTCC-3′, and 5′-GCATGGGCAGTTTACCTTTACGGT-3′) was used. A control primer set from ChrXII (in the middle of the longest yeast ORF *YLR106C*) (5′-TATACAAACCTGCAAACCTAAAATTCC-3′ and 5′-GATAATAAGTA

TAACGACTCTGGAGAT-3′) was used. Immunoprecipiated DNA was analyzed using a Bio-Rad iCycler iQ real-time PCR detection system. Relative fold of enrichment was calculated by normalizing samples using values from uninduced cells (−GAL) as 1. Therefore, these results reflect the additional recruitment of factors upon induction (+GAL) and do not reflect any prebinding of factors prior to induction.

Synthetic Genetic Analysis

Standard yeast culture and transformation techniques were followed. Heterozygous diploid strains containing two deletion mutations were generated using synthetic genetic analysis (SGA) technology as previously described (Tong et al., 2001) then subjected to sporulation and tetrad dissection. To determine sensitivity to hydroxyurea or camptothecin, yeast strains were grown to an absorbance at 600 nm of 0.5 before being plated at 5-fold serial dilutions on YEPD medium with or without the DNA-damaging agents. The plates were incubated for 2–3 days at 30°C then photographed.

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