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Proficient repair in chromatin remodeling defective *ino80* mutants of *Saccharomyces cerevisiae* highlights replication defects as the main contributor to DNA damage sensitivity

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Abstract

Ino80 is an evolutionarily conserved member of the *SWI2/SNF2*-family of ATPases in *Saccharomyces cerevisiae*. It resides in a multiprotein helicase/chromatin remodeling complex, and has been shown to play a key role in the stability of replication forks during replication stress. Though yeast with defects in *ino80* show sensitivity to killing by a variety of DNA-damaging agents, a role for the INO80 protein complex in the repair of DNA has only been assessed for double-strand breaks, and the results are contradictory and inconclusive. We report that *ino80Δ* cells are hypersensitive to DNA base lesions induced by ultraviolet (UV) radiation and methyl methanesulfonate (MMS), but show little (or no) increased sensitivity to the DNA double-strand break (DSB)-inducing agents ionizing radiation and camptothecin. Importantly, *ino80Δ* cells display efficient removal of UV-induced cyclobutane pyrimidine dimers, and show a normal rate of removal of DNA methylation damage after MMS exposure. In addition, *ino80Δ* cells have an overall normal rate of repair of DSBs induced by ionizing radiation. Altogether, our data support a model of INO80 as an important suppressor of genome instability in yeast involved in DNA damage tolerance through a role in stability and recovery of broken replication forks, but *not* in the repair of lesions leading to such events. This conclusion is in contrast to strong evidence for the DNA repair-promoting role of the corresponding INO80 complexes in higher eukaryotes. Thus, our results provide insight into the specialized roles of the INO80 subunits and the differential needs of different species for chromatin remodeling complexes in genome maintenance.

Keywords

DNA repair; INO80; Ultraviolet light; Ionizing radiation; Methyl methanesulfonate

1. Introduction

The packaging of DNA in chromatin can present a repressive barrier for protein factors involved in DNA processing. To control access to DNA, the degree of chromatin condensation has to be dynamically regulated by a number of chromatin-modifying and remodeling enzymes. Histone proteins can be covalently modified by enzyme complexes entailing, for example, methyl- and acetyl-transferases, leading to alterations which effect nucleosome mobility and/or play a role in signaling, to facilitate transcription and DNA

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

repair [1]. In addition, there are a number of multi-subunit chromatin remodeling complexes that use ATP hydrolysis to alter DNA–protein contacts allowing the sliding of nucleosomes along DNA or the removal of histones from the DNA for complete nucleosome eviction [2,3]. To date, the ATP-dependent chromatin remodeling complexes have been strongly implicated in regulation of gene expression or nucleosome assembly, but their involvement in other DNA processing events such as DNA repair remains unclear (e.g. [4]).

The ATP-dependant remodeling complex INO80 plays a key role in regulating gene expression [5,6], with microarray evidence revealing a contribution of this complex to the transcriptional regulation of up to 20% of genes in yeast [7-9]. Like other ATP-dependant chromatin remodeling complexes, the INO80 complex contains a member of the *SWI2/SNF2*-family of ATPases, the Ino80 subunit [5,10], which provides the complex with the catalytic activity for chromatin remodeling and nucleosome sliding [10]. The purified complex has DNA-dependent ATPase activity, remodels chromatin, facilitates transcription *in vitro*, and displays 3' → 5' helicase activity [10,11].

The INO80 complex contains 11–14 different polypeptide sub-units in addition to the Ino80 ATPase (varying among different species and summarized in [12]). Some of these subunits are highly conserved among all species, including actin, three actin-related proteins (Arp4, Arp5, and Arp8) [10,13], and two hexameric helicases (Rvb1 and Rvb2) [14]. Though the role of actin and the actin-related proteins in the INO80 complex are not well understood, their absence from the complex disrupts its chromatin remodeling activities [11]. Studies have also shown that these proteins play a role primarily in nucleosome or histone binding, as the yeast Arp4p and Arp8p have been shown to bind core histones [11,15]. The Rvb helicases are AAA⁺ATPases, which generally function in the cell to promote conformational change, remodeling and/or disassembly of protein complexes [16]. Rvb1 and 2 are highly conserved among eukaryotes [17], and are related to the bacterial RuvB ATPase, a protein necessary for Holliday junction resolution during the process of homologous recombination (HR) [18]. The function of these helicases in the *INO80* has not been determined, however the uncommon helicase activity possessed by this complex might be attributed to the presence of Rvb1 and Rvb2. Studies with yeast lacking Rvb2 also indicate a possible role for these AAA⁺ ATPases in assembly of other subunits, such as Arp5p, into the complex [8].

The INO80 complex has been implicated in a general role in DNA repair that may be independent of its role in transcriptional control. Yeast null mutants in *ino80*, *arp5*, or *arp8* are viable but show a lack of chromatin remodeling activity that correlates well with increased sensitivity to a broad range of genotoxic agents, including methyl methanesulfonate (MMS), 254 nm ultraviolet light (UV), and ionizing radiation (IR) [10,11], each inducing DNA lesions repaired primarily by distinct, independent cellular repair pathways. This effect appears to be unrelated to a defect in cellular transcriptional activities, as the INO80 complex is not required for DNA damage checkpoint activation [19] and does not affect DNA repair or cell cycle checkpoints indirectly through alteration in gene expression [9,10].

Assessment of a potential role for *INO80* activity in DNA repair has focused primarily on repair of DSBs, for which there is conflicting results. The complex shows specific recruitment to HO endonuclease-catalyzed DSBs, likely through the interactions of Arp4p and another subunit, Nhp10, with phosphorylated histone H2A (γ -H2AX, a posttranslational modification which occurs rapidly in nucleosomes around sites of DSBs [20]) [9,21,22]. This recruitment correlates well with nucleosome loss from the region of DNA around DSBs (a process which is blocked or delayed in cells with mutations in the INO80 complex [23,24]), and requires the activity of the DNA damage sensor complex MRX (MRE11-

Rad50-Xrs2) [24]. In addition, a functional INO80 complex facilitates formation of single strand DNA (ssDNA) at the sites of DSBs, a necessary DNA resection step catalyzed by the MRX complex for HR-directed repair [9]. Consistent with this, delayed recruitment of the key HR-recombinase protein Rad51 also directly results from the defects of *INO80* histone eviction [24].

On the other hand, additional studies have found no defect in DNA resection at DSBs in *ino80* mutants, which showed proficiency in repair of HO-induced DSBs by both HR and non-homologous end joining (NHEJ) [23,25], suggesting that *INO80* does not play an essential role in DSB repair. Though an *ino80* mutant was shown to be deficient in DNA damage checkpoint adaptation to unrepaired DSB [25], the exact role of *INO80* in the DNA damage response remains unclear as this model does not account for the observed pattern of DNA damage sensitivity of *ino80* mutants.

Independent of a potential role in DNA repair, the INO80 complex may be protecting cells from the deleterious effect of DNA damage by acting during replication, when DNA lesions cause replication fork breaks and subsequent cell cycle arrest. Indeed, recent reports strongly suggest a key role for the yeast INO80 complex in replication fork stability during replication stress [26-29]. The INO80 complex is recruited to sites of stalled replication forks and to unfired origins of replication in cells after exposure to the replication inhibitor hydroxyurea (HU) [26-29]. Moreover, the *ino80* mutant cells showed a general reduction in the rate of DNA replication, and increased dissociation of replication machinery components at stalled forks [26]. In addition, after HU exposure, cells deficient in the Ino80p ATPase were unable to resume DNA synthesis and appeared to accumulate DSBs as they attempted to restart replication [26,27]. Thus, the role for the INO80 complex in replication progression during stress, and replication restart, may account for earlier studies showing general DNA damage sensitivity in *ino80* mutants.

In this study, we wished to determine the role of Ino80 in promoting repair of DNA damage, induced by a variety of DNA-damaging agents, by directly measuring removal of lesions in a previously characterized *ino80Δ* mutant strain. Our data strongly suggest that the involvement of the INO80 complex in DNA damage tolerance is primarily limited to its role in replication fork function and stability, as we find it is not necessary for the repair of DNA lesions.

2. Materials and methods

2.1. Strains and growth conditions

Wild-type *S. cerevisiae* strain BY4733 [30] and its isogenic *ino80Δ* derivative [10] were used in this study, with additional control strains including wt-BY4741, *rad51Δ*, *rad4Δ*, *mag1Δ* (Open Biosystems). Cells were grown at 30 °C in YEPD containing 2% glucose. For all experiments with exponential-phase cells the yeast were grown to OD₆₀₀ of (0.4–0.6). Stationary cultures were grown for 130–140 h until the density was more than 5×10^7 cells/ml. Cell titers were determined by hemacytometry.

2.2. Cytotoxicity assays

Quantitative tests for colony forming ability were performed by plating treated cells on solid YEPD and counting colonies produced by surviving cells. Untreated cells were also plated and scored as 100% survival. Assays were performed in triplicate, and three plates were used for each data point. Plates were incubated for 3–4 days at 30 °C before counting colonies. For analysis of UV survival, cells were spread on YEPD agar plates and subjected in the dark to 254 nm UV-light, with doses measured with a Spectroline DM-254N short wave UV meter (Spectronics Corp.). MMS sensitivity was analyzed on YEPD agar plates

supplemented with varying concentrations of MMS. To analyze the effect of ionizing radiation, for minimization of oxygen radical damage and increased proportion of direct DSB lesions, stationary-phase cells (~130 h) were washed once in water, resuspended to 2×10^7 cells/ml in water, and irradiated on ice with X-rays from a SL15 linear accelerator (Philips) at an emission rate of 10 rad/min. Camptothecin sensitivity was analyzed in a quantitative test described previously [6]. The relative growth inhibition was measured as a ratio of the growth curve slopes of the drug treated culture to the untreated control.

2.3. Treatment of cells for DNA repair experiments

MMS (Sigma–Aldrich) was added to exponential-phase cultures to final concentration of 0.035%, and the cultures were incubated at 30°C for 3 h in an orbital shaker. Cells were then harvested by centrifugation, washed twice in ice-cold PBS, resuspended in pre-warmed YEPD and incubated at 30°C with continuous shaking for different repair times. For UV damage and repair studies, a UV dose of 80 J/m², measured with a Spectroline DM-254N short wave ultraviolet meter (Spectronics Corp.), was used to irradiate the cells as previously described [31]. Cells were then harvested, resuspended in pre-warmed YEPD, and incubated in the dark at 30 °C with continuous shaking for sampling over different repair times. Prior to X-ray irradiation, the exponentially growing cells were arrested in G₂/M with 15 µg/ml nocodazole for 3 h, then harvested, resuspended in ice-cold water and irradiated on ice with X-rays emitted from a SL15 linear accelerator (Philips) at a rate of 10rad/min. Following irradiation, cells were harvested, resuspended in pre-warmed YEPD with 15 µg/ml nocodazole, and incubated at 30 °C with continuous shaking for sampling over different repair times.

2.4. Analysis of repair following MMS treatment

High molecular weight genomic DNA from yeast cells was isolated following cellular membrane disruption with glass beads as described previously [31]. MMS-induced sites of methylation damage; 3-methyladenine and 7-methylguanine were revealed by the digestion of DNA samples (5–10 µg) with enzymes Aag (kindly provided by Dr. Leona Samson) and Ape1 (NEB) at 37 °C for 1 h. DNA samples (5–10 µg) were incubated with Aag and Ape1 in the reaction buffer (70 mM MOPS, pH 7.5, 1 mM dithiothreitol (DTT), 1 mM EDTA, 5% glycerol) at 37 °C for 1 h in a total reaction volume of 20 µl. Methylated bases were cleaved by Aag glycosylase and the resulting abasic sites were cleaved by Ape1 endonuclease. The reactions were stopped by adding DNA loading buffer (50 mM NaOH, 1 mM EDTA, 3% ficoll, 0.025% bromocresol green, 0.041% xylene cyanol) [32]. Following enzyme treatment, DNA cleavage products (single and double strand breaks were resolved on 1.2% alkaline agarose gels and stained with SYBR Gold). The internal control for DNA repair assay was performed with *mag1Δ* strain, which is defective in recognition and removal of MMS-induced DNA methylation. Quantification of methylated bases in genomic DNA was performed on phosphorimage data using ImageQuant software (Molecular Dynamics) and number-average DNA length analysis [31].

2.5. Measurement of total genome repair following UV irradiation

Genomic DNA from yeast cells was isolated using glass beads as described previously [31]. DNA samples (5–10 µg) were treated or mock-treated with T4 endonuclease V and separated on 1% alkaline agarose gels [31]. Following electrophoresis, Southern blot analysis was performed on the genomic DNA by hybridized with random hybridization probes as described [31,33]. Assay control experiment was performed with *rad4Δ* strain defective in NER. DNA damage repair of the control experiment was revealed on 1.2% agarose gel, stained with SYBR gold. Quantification of cyclobutane pyrimidine dimers (CPDs) in DNA was performed on blots using ImageQuant software (Molecular Dynamics) and number-average DNA length analysis [31,34].

2.6. Measurement of chromosome restoration using contour-clamped homogeneous electric field (CHEF) gel electrophoresis and Southern blotting

Chromosomes embedded in agarose plugs were prepared using a Yeast Genomic Kit (Bio-Rad) as instructed by the manufacturer. For analysis of chromosomal DSBs, CHEF gel electrophoresis was performed as previously described [35], on a Bio-Rad CHEF-DR11 apparatus. On completion of, gels were stained with ethidium bromide for 1 h, destained overnight, and photographed. Chromosomal products were qualitatively compared using ImageQuant software (Molecular Dynamics). The control experiment demonstrating deficiency in DSB repair was performed with *rad51Δ* strain and isogenic wt-BY4741.

3. Results

3.1. Cells lacking INO80 are hypersensitive to certain classes of DNA-damaging agents

Exposure to either UV-light (254 nm) or MMS had significant deleterious effects on *ino80Δ* cells (Fig. 1a and b). UV-light induces bulky adducts in DNA which obstruct replication fork progression [36]. MMS is a monofunctional DNA alkylating agent that induces primarily methylated purines [37], some of which inhibit progression of replication forks [38]. In contrast to the enhanced sensitivity to UV and MMS, deletion of *INO80* had no significant effect on the sensitivity to X-rays as compared to wild-type strain. The difference in the survival between *INO80* and WT is $\leq 10\%$ (Fig. 1c). This data is consistent with our previous experiments, where the sensitivity of *INO80* homozygous diploid mutant to X-ray radiation was assessed (data not shown). It is important to note that ionizing radiation induces direct DSBs as well as oxidative damage to DNA bases, where the yield of the latter depends on both media composition and oxygen concentration [39]. In this study, we analyzed sensitivity to X-rays under conditions that minimize oxidative base damage and its interaction with replication machinery (see Section 2). This procedural difference may in part explain the lack of X-ray sensitivity we observe compared to the increased sensitivity of the same mutant reported previously [10]. Increased sensitivity was also not observed in *ino80Δ* cells treated with the topoisomerase I inhibitor camptothecin (Fig. 1d), which leads to replication-associated DSBs as the polymerases effectively “run off” single strand ends held open by drug–topoisomerase I complexes bound covalently to the DNA. Cell survival during camptothecin treatment requires a heavy reliance on HR repair for restart of replication forks [40], and the treatment dose used, 5 $\mu\text{g/ml}$, is a concentration shown to be deleterious to HR-deficient [40] and other susceptible strains [6]. Previous studies have shown a drop in viability of *ino80Δ* cells after treatment with hydroxyurea (HU) [26,27], sensitivity to which relies primarily on the inability of cells to prevent or mitigate aberrant DNA structures formed as a result of inappropriate recombination at stalled forks [41]. Thus, *ino80Δ* cells show a *selective* increase in sensitivity only to those agents that cause replication fork-stalling, and show little sensitivity to agents, such as X-rays or camptothecin, that cause direct or “non-fork-disrupting” replication-associated DSBs, respectively.

3.2. Efficient repair of MMS damage in cells lacking INO80

Surprisingly, replication-blocking lesions induced by MMS treatment (3-methyladenine in particular [37]) in yeast do not induce detectable replication-associated DBSs [42]. Thus, the MMS hypersensitivity of the *ino80Δ* mutant cells for survival might be due to their inability to repair the methylated bases associated with treatment of this drug. Such a result would highlight the impact of nucleosomal rearrangements on the completion of the base excision repair (BER) pathway responsible for recognition and repair of these lesions [43]. To test this possibility, we measured the repair of DNA methylation adducts by quantifying cleavage of the genomic DNA of *ino80Δ* and *wt* control cells with the purified human proteins Aag and Ape1 in at different time intervals after MMS treatment. Methylated DNA

bases in DNA are recognized by the glycosylase Aag, which cleaves the N'-glycosidic bond of a wide range of methylated bases, leaving abasic sites and the DNA which are subsequently cleaved into single strand breaks by Ape1 [44]. SYBR Gold was used to detect fragmented DNA after separation of DNA products (Fig. 2a and b). Sites of methylation in DNA were revealed by the enzymatic formation of single strand breaks which were subsequently resolved by agarose gel electrophoresis (Fig. 2a and b). DNA fragmentation is evident from the increased migration of DNA molecules following treatment with MMS for 1–3h (Fig. 2a and b compare C, 1 2 and 3 h). Repair of methylated bases is observed by the gradual return of these fragments to nearly full length following increased incubation after MMS exposure (Fig. 2a and b compare 4, 5 6, and 9 h). Quantitation of these gel patterns (see Section 2) shows that the kinetics of removal of methylated bases from genomic DNA is very similar between the *ino80Δ* and *wt* control (Fig. 2c, note that MMS exposure occurs during the first 3 h of the experiment), with the damage returning to levels near background within 6 h after conclusion of treatment.

In yeast, Mag1 (ortholog of the Human Aag) is the a glycosylase necessary for removal of the primary MMS-induced lesions, including 3-methyl- and 7-methy-purines [45], and strains deficient in *mag1* are highly sensitive to the toxicity of this agent [46]. To verify the ability of the assay to detect a defect in repair of MMS-induced methylation damage, a cell strain deficient in *mag1* was also assessed for DNA cleavage after MMS exposure. Unlike the *ino80Δ* strain, the *mag1* strain shows little repair of MMS-associated DNA cleavage compared to the wild-type control (Fig. 2b and c).

3.3. Cells lacking INO80 are proficient in repair of CPDs induced by UV radiation

Irradiation at 254 nm induces DNA photoproducts, primarily CPDs, which are removed via nucleotide excision repair (NER) [47]. To examine a possible defect in NER associated with *INO80* deficiency, we compared the efficiency of CPD repair between the *ino80Δ* and *wt* control cells. The number of CPDs/kb was determined at different time intervals, after exposure to 80 kJ/m², by assessing the T4 endonuclease V (T4 endo V) digestion pattern of genomic DNA on alkaline agarose gels [31]. The CPD-specific nuclease activity of T4 endo V allows for the quantification of CPDs in genomic DNA following denaturing gel electrophoresis by Southern analysis with random-primer probes (Fig. 3a and b). The shift in the number-average length of DNA molecules is used to calculate the average number of CPDs/kb at each repair time [31,34]. As shown in Fig. 3c, no significant difference in repair of CPDs in the genome overall was observed between *wt* and *ino80Δ* cells. The small lag in repair of CPDs seen in *ino80Δ* mutants (Fig. 3c) may be attributed to the difference in replication rate between *wt* and *ino80Δ* mutant (as mentioned in the methods above) as unsynchronized exponential cultures were used for these experiments. The protein Rad4 plays a key role in the recognition of UV-induced DNA lesions for the process of NER [48], and the UV-induced DNA damage and repair in *rad4Δ* mutant and isogenic wt-BY4741 were assessed as a positive controls for use of T4 endo V as a means of detecting reduced repair of UV DNA damage. As shown in Fig. 3d and c there is a dramatic difference in the repair of CPDs between *rad4Δ* mutant and wild-type strains. Like the *ino80Δ* strain, the wild-type strain (BY4741) demonstrated progressive repair of DNA damage over the time course and nearly 100% CPDs were repaired at 4 hr after UV damage. By contrast, the *rad4Δ* strain showed little DNA repair over the course of the experiment (<10% of CPDs removed by 4 h after UV exposure, Fig. 3f).

3.4. Cells lacking INO80 are proficient in repair of chromosomal DSBs induced by ionizing radiation

Ionizing radiation induces chromosomal DSBs requiring NHEJ and, as cells progress through the S and G₂ phases of the cell cycle, HR for repair (reviewed in [49]). Though

INO80 has been implicated in HRrepair (as discussed in Section 1), the lack of *ino80Δ* cell sensitivity to X-rays (Fig. 1c) suggests a normal repair response to breaks caused by this agent. To determine a potential difference in repair of X-ray-induced DSBs between the *ino80Δ* and *wt* cells, we monitored the repair of these lesions over a 2 h period in G₂-arrested cells, during which yeast cells show fast and efficient repair of DSBs by HR [50]. Chromosomal preparations were isolated after different incubation times and analyzed by CHEF gel electrophoresis. Immediately following irradiation with a dose of 300 Gy the largest chromosomes (upper bands in control lanes of Fig. 4a, asterisk) almost completely disappeared and form a smear due to introduction of randomly distributed DSBs (Fig. 4a and b, 0 h). Importantly, scans of these gels show that almost all chromosomes are restored to full length within the 2 h repair time in both *wt* and *ino80Δ* cells (Fig. 4b) demonstrating a normal (or near normal) rate of repair of X-ray-induced DSBs in *ino80Δ* mutant cells. To verify the detection of reduced HR repair after IR by CHEF gel electrophoresis, the assay was performed with the *rad51Δ* strain, deficient in the key recombinase enzyme used to repair IR-induced lesions in cells via the process of homologous recombination, Rad51 [51], and an isogenic *wt* (BY4741). As expected, the HR repair-defective *rad51Δ* mutant strain showed little to no repair of DSB over the 2 h repair period as compared to its *wt* control strain (Fig. 4c–e).

4. Discussion

Evidence for the participation of the INO80 complex in repair of DNA damage has been intriguing but indirect. This reasoning evolved primarily from studies showing hypersensitivity of *ino80* mutants to various DNA-damaging agents including MMS, UV, and IR (reviewed in [52]). Though it is unlikely that the INO80 complex plays a *direct role* in the repair of lesions that span distinct repair pathways, such as NER (repair of CPDs), BER (repair of methylated bases), and NHEJ and HR (repair of DSBs), we examined the possibility that Ino80 may be necessary for general DNA processing at sites of damage, independent of the type of lesion or repair system activated.

We have shown that *ino80Δ* cells are hypersensitive to both 254 nm UV-light and MMS relative to *wt* cells (Fig. 1a and b), results similar to those seen previously [10]. However, our results showing similar rates of methylation damage repair between *wt* and *ino80* cells argues against the possibility that *INO80*-dependant chromosomal rearrangements are necessary for efficient BER (Fig. 2). The same conclusion can be drawn for a requirement of *INO80* activity in the cell's ability to perform NER on UV-induced CPDs (Fig. 3). It is important to note, however, that the presence of other chromatin remodeling factors in the cell may provide redundant activities during DNA repair, accounting for the lack of necessity for functional INO80.

All DNA lesions, regardless of their source, have the potential to cause disruption of the replication machinery during DNA synthesis, and therefore potentially lead to DSB formation. Indeed, HR-defective cells are generally sensitive to MMS and UV-light [53,54]. Thus, *INO80* could play a role only in DSB mitigation, and *ino80* defective strains would be expected to show sensitivity to different types of DNA damage. However, the evidence of a role for *INO80* in DSB repair remains controversial. Perhaps the strongest arguments for a role of *INO80* in HR repair comes from evidence of its recruitment to sites of damage via localization of γ -H2AX [9,21,22] and a defect in processing of the ends of DSBs, necessary for initiation of HR repair [9]. However, these early steps in HR are not likely needed in the requirement of HR factors for survival after MMS treatment, since MMS does not actually lead to detectable DSBs during replication in yeast [42]. Rather, the proposed processes of HR associated with assisting in the reversal of stalled replication forks [55] or resolution of Holliday junctions or Holliday junction-like structures associated with MMS damage

[56,57] may be hampered by defects in *INO80*. In agreement with this, the presence of the Rvb1 and 2 AAA+ ATPases in the INO80 complex may be assisting these activities with chromatin rearrangements at Holliday Junctions during the process of HR. However, we find that *ino80Δ* cells are not more sensitive to the direct DSBs produced by ionizing radiation compared to *wt* cells (Fig. 1c). In agreement with previous studies showing no evidence for the necessity of *INO80* in rejoining endonuclease-induced breaks by either NHEJ or HR [23,25], the near *wt* sensitivity to ionizing radiation of *ino80Δ* cells (Fig. 1c) and the direct demonstration of the ability of *ino80Δ* cells to repair X-ray induced DSB with similar efficiency as *wt* cells (Fig. 4), implies that the INO80 complex is largely dispensable for DSB repair. Our results also rule out a defect in repair of replication-dependent DSBs in *ino80Δ* cells, showing a very modest effect of the *INO80* deletion on sensitivity to camptothecin (Fig. 1d), known to cause DSBs during DNA synthesis [58].

The different subunits of a chromatin remodeling complex have distinct functions in a given process, some factors playing roles in multiple complexes. Other chromatin remodeling complexes have been implicated in direct participation of DNA repair. For example, the multiprotein ATP-dependent chromatin remodeling complex SWI/SNF was genetically and physically linked to the NER pathway in chromatin of yeast [59]. The primary function of *INO80* in yeast appears to be the contribution to efficient replication fork progression and replication restart after DNA damage. However, in higher eukaryotes, *INO80*-dependant remodeling of nucleosomes at the sites of DSBs seems to play a role in assuring rapid HR repair, possibly through direct participation in the process. Human cells depleted of Ino80 are hypersensitive to topoisomerase inhibitors and UV, and the frequency of HR-based repair of DSBs induced by the I-Sce endonuclease is substantially reduced after depletion of Ino80 [60]. Furthermore, in *Arabidopsis thaliana*, the frequency of HR-based DSB repair is severely reduced in mutants lacking a functional Ino80 ATPase [61]. Thus, the different subunits associated with the INO80 complexes of yeast and higher eukaryotes likely hold the key to the functional differences each complex has in its role in genomic stability.

Finally, human orthologs of the Rvb1 and Rvb2 helicases of the yeast INO80 complex (TIP49a and TIP49b) have an intriguing connection to c-Myc-promoted oncogenic transformation. Both helicases physically interact with c-Myc *in vivo* [17], apparently as a part of the human INO80-like complex. Moreover, TIP49b is an essential mediator of oncogenic transformation by c-Myc [17]. In light of our present results, it is possible that overexpression of c-Myc promotes tumorigenesis via increased genome instability mediated by the chromatin remodeling activity of INO80.

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Fig. 1.

Sensitivity of *ino80Δ* mutant to genotoxic agents. (a) Colony forming ability following UV-light irradiation. Exponentially growing cultures of *wt* and *ino80Δ* cells were appropriately diluted, spread on YEPD plates, and subjected to 10–200 J/m² of 254 nm UV, after which their survival was monitored. (b) Colony forming ability on MMS-containing agar plates. Exponential-phase cells were appropriately diluted, spread on solid YEPD plates containing from 0 to 0.025% of MMS, and their survival was monitored. (c) Sensitivity of stationary-phase cells (grown ~130 h) of *wt* and *ino80Δ* mutant to ionizing irradiation. Cells were exposed to X-ray doses equivalent to 200–600 Gy. Data represents the mean $\pm$ 1 SD of three replicates of the sample. (d) Sensitivity of exponentially growing cells to camptothecin. Concentrations of camptothecin from 0 to 10mg/ml were analyzed. On all plots, filled circles (●) represent *wt* (BY4733) and open circles (○) represent *ino80Δ* mutant. Data are the mean $\pm$ 1 SD of three independent experiments.

Fig. 2. Induction and repair of MMS-induced methylated bases in genomic DNA of *ino80Δ* cells. Exponentially growing *wt* and *ino80Δ* cells were treated with 0.035% MMS for 3 h, after which MMS was washed out and the cells were allowed to repair in rich medium for the next 6 h. The cells were harvested at the indicated times after initiation of MMS treatment and genomic DNA was purified. Methylated DNA damage was converted to single and double DNA strand breaks upon the treatment with glycosylase Aag and endonuclease Ape1. Treated DNA was resolved in alkaline agarose gel and stained with SYBRgold. The approximate positions of the ensemble average size of DNA in each lane are denoted with asterisks (*) and connected by a dotted line. Representative gel scans demonstrate time course of induction and repair of MMS-induced methylated bases in genomic DNA from *wt*-BY4733 and *ino80Δ* cells (a) and *wt*-BY4741 and *mag1Δ* cells (b). Incubation times (in hours, after start of MMS treatment) are indicated above the lanes. Other labels are: C, DNA from untreated cells; λ DNA markers (Fermentas, Inc.). (c) Induction and repair of MMS-induced methylated bases in DNA as a function of repair time.

Fig. 3.

Repair of UV-induced CPDs in genomic DNA of *ino80Δ* cells. Exponentially growing *wt* and *ino80Δ* cells were irradiated with 80 J/m² UV and allowed to repair in pre-warmed YEPD. Genomic DNA was isolated and aliquots were digested with (+) or without (–) T4 endo V. Samples were run on a 1.2% alkaline agarose gel, blotted and probed with labeled random-primer yeast genomic DNA and λ DNA or stained with SYBR gold nucleic acid stain. Representative Southern blots (a and b) and gel scans (c and d) demonstrate the time course of repair of UV-induced CPDs in genomic DNA from *wt* (a), *ino80Δ* cells (b), control strain *rad4Δ* (e) and the isogenic *wt*-BY4741 (d). Incubation times (in hours) are indicated above the lanes and the approximate positions of the ensemble average size of DNA in each lane are denoted with *asterisks* (*) and connected by a *dotted line*. Other labels denote: C, DNA from untreated (control) cells; and M, DNA/*Eco*RI markers. (c) Repair of UV-induced CPDs in genomic DNA of *wt* and *ino80Δ* cells. Data for each time point are expressed as a percentage of CPDs/kb for undamaged cells to yield percent repair. Filled circles (●) or filled squares (■) represent *wt* cells, open circles (○) represent *ino80Δ* and open diamonds (◇) denote *rad4Δ* cells. Values represent the mean $\pm$ 1 SD of three independent experiments.

Fig. 4.

Repair of X-ray-induced chromosomal DSBs in *ino80Δ* mutant cells. Exponentially growing *wt*, *ino80Δ* and *rad51Δ* cells were arrested with 15 $\mu\text{g/ml}$ nocodazole for 3 h, after which cells were collected and resuspended in ice-cold water, irradiated with 300 Gy on ice, then collected and resuspended in pre-warmed YEPD containing 15 $\mu\text{g/ml}$ nocodazole. Aliquots were taken at the times indicated. Genomic DNA was extracted in agarose plugs and separated by CHEF gel electrophoresis to analyze chromosomal DSBs of *wt*-BY4733 and *ino80Δ* (a). (W) indicates position of the well and asterisk (*) denotes approximate positions of the longest chromosomes (VII and XII). (b) Scans of selected *wt*-BY4733 (blue) and *ino80Δ* (red) lanes in gel shown in panel (a). (c) CHEF gel electrophoresis of *rad51Δ* and isogenic *wt*-BY4741. Chromosome size and position are indicated. Standard yeast chromosomal marker was used (M). Chromosomes (VII, XV) of *wt* (blue frame), *rad51Δ* (red frame) are indicated. Gel scans of select lanes from panel c for comparison (d), and quantitation of DSB repair in chromosomes VII, XV (e), illustrate efficient repair of DSB in *wt*-BY4741 and significant deficiency in DNA repair in *rad51Δ* mutant.