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YNK1, the yeast homolog of human metastasis suppressor NM23, is required for repair of UV radiation- and etoposide-induced DNA damage

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Abstract

In humans, NM23-H1 is a metastasis suppressor whose expression is reduced in metastatic melanoma and breast carcinoma cells, and which possesses the ability to inhibit metastatic growth without significant impact on the transformed phenotype. NM23-H1 exhibits three enzymatic activities *in vitro*, each with potential to maintain genomic stability, a 3′–5′exonuclease and two kinases, nucleoside diphosphate kinase (NDPK), and protein histidine kinase. Herein we have investigated the potential contributions of NM23 proteins to DNA repair in the yeast, *Saccharomyces cerevisiae*, which contains a single NM23 homolog, *YNK1*. Ablation of *YNK1* delayed repair of UV- and etoposide-induced nuclear DNA damage by 3–6 hrs. However, *YNK1* had no impact upon the kinetics of MMS-induced DNA repair. Furthermore, *YNK1* was not required for repair of mitochondrial DNA damage. To determine whether the nuclear DNA repair deficit manifested as an increase in mutation frequency, the *CAN1* forward assay was employed. An *YNK1* deletion was associated with increased mutation rates following treatment with either UV (2.6x) or MMS (1.6x). Mutation spectral analysis further revealed significantly increased rates of base substitution and frameshift mutations following UV treatment in the *ynk1*Δ strain. This study indicates a novel role for Y*NK1* in DNA repair in yeast, and suggests an anti-mutator function that may contribute to the metastasis suppressor function of NM23-H1 in humans.

1. Introduction

NM23-H1 was first identified by virtue of its reduced expression in highly metastatic melanoma and breast carcinoma cells, and the ability of forced NM23-H1 expression to inhibit metastatic potential without significant impact on the transformed phenotype [1]. The metastatic process requires the accumulation of mutations and high levels of genomic instability to permit tumor cells to overcome the barriers to metastatic growth [2–4]. Despite the fact that NM23-H1 has been recognized to play a pivotal role in the development of metastasis, the underlying mechanisms by which NM23-H1 exhibits its anti-metastastic effect remains unknown.

Consistent with a role in DNA repair the NM23 molecule possesses at least three distinct enzymatic activities that could participate in genomic maintenance and antimutator activity [5]. NM23-H1 possess significant 3′–5′ exonuclease (3′–5′ EXO) activity [6,7] and these DNA cleaving molecules are predominantly involved with maintaining genomic fidelity during DNA

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synthesis and repair [8]. Accordingly, deficiencies in 3′–5′ EXO activity have been shown to be associated with the mutator phenotype [8–11]. NM23-H1 also exhibits a nucleoside diphosphate kinase (NDPK) activity that maintains homeostasis of nuclear nucleotide pools which may limit pro-mutagenic mismatches during DNA repair [12,13]. Furthermore, a protein histidine kinase (hisK) activity has been described for NM23-H1, implicated as an inhibitor of signaling pathways underlying cell motility [14], but which could also initiate signaling to DNA repair pathways. Moreover, despite the repair-relevant enzymatic activities of NM23-H1 *in vitro*, its contribution to maintenance of genomic integrity *in vivo* is poorly understood.

Previous studies strongly suggest that NM23 proteins exhibit functions consistent with DNA repair. DNA damage has been reported to induce nuclear localization of NM23-H1, consistent with a role in the DNA damage response [7]. In addition, co-incubation of NM23-H1 with the base excision repair (BER) enzyme uracil-DNA glycosylase (UDG) results in enhanced 3′–5′ EXO activity against single-stranded oligodeoxynucleotide substrates *in vitro*, suggesting the potential for functional cooperativity between these proteins [7]. A function in genomic stability was also suggested earlier by the marked mutator phenotype of *ndk*-null *E. coli*, which exhibit elevated rates of base substitutions and frameshifts [15]. While a recent study suggested that NDK possesses an intrinsic UDG function, subsequent studies show that *NDK* exhibits little if any intrinsic UDG activity [16–18], but does enhance that of the prototypical UDG, *UNG*, upon physical association between the proteins [17]. Consistent with this function, a very recent study has attributed the mutator phenotype of the *ndk*-null strain of *E. coli* to excess misincorporation of uracil, as well as a defect in the uracil base excision pathway [19].

To explore the potential function of NM23 proteins in maintenance of genomic integrity, we have employed the yeast *S. cerevisiae*, which harbors a single NM23 homologue, *YNK1*. Despite of its phylogenetic distance from the eight human NM23 isoforms, ynk1p shares approximately 60% amino acid sequence identity and structural similarities with human NM23-H1 and NM23-H2 [20], including conservation of glutamic acid-5 and lysine-12 residues which are critical for the 3′–5′ EXO function [6], histidine-118, which is essential for NDPK activity, and proline-96, which has been implicated in the histidine kinase function [21]. Our results demonstrate that an *YNK1*-null strain exhibits significantly reduced kinetics of nuclear DNA repair in response to damage induced by UV irradiation, and etoposide, as well as increased rates of UV-induced mutations.

2. Materials and methods

2.1 Saccharomyces cerevisiae strains and media

S. cerevisiae strains harboring single genetic lesions were obtained commercially (Open Biosystems) and were derived from BY4741 wild-type strains and listed in Table 1 (Supplemental Table 1). Open reading frames for the gene of interest were replaced with a *KanMX* marker by a PCR-based strategy. Yeast strains were grown in standard media consisting of yeast extract/peptone/dextrose (YPD) medium (Fisher Scientific).

2.2 MMS, etoposide and UV treatment

S. cerevisiae strains (1×10^7 cells/ml) were treated with MMS (0.1%; Sigma) or etoposide (1 mM; Sigma) for 1 h at 30°C shaking at 250 rpm, followed by centrifugation at 5,000 × *g* for 5 min. The pellet was washed in 50 mM potassium phosphate, pH 7.0, aspirated, centrifuged at 5,000 × *g* for 5 min, and YPD added. The repair time course was 0.5, 1, 3 and 6 h for MMS and 0.5, 3, 6, 24 and 48 h for etoposide at 30°C. For UVB exposure (Model XX-15M, UVP Products), *S. cerevisiae* were grown on YPD plates for 48 h at 30°C, exposed to UV (192 J/m^2) and maintained at 30°C and for a repair time course of 0.5, 1, 3 and 6 h.

2.3 Quantitative extended-length PCR (QXL-PCR)

QXL-PCR measures the average lesion frequency and works on the premise that damage on the DNA template will block a thermostable polymerase, resulting in reduced amplification of the DNA fragment. Thus, only DNA templates devoid of polymerase blocking lesions will be amplified. DNA lesion frequencies were calculated as the amplification of damaged (treated) samples (A_d) relative to the amplification of non-damaged fragment controls (A_0) resulting in the ratio (A_d/A_0). To determine average lesion frequency, a random distribution of lesions was assumed, and the following equation was used, $\lambda = -\ln A_d/A_0$ [22,23]. The DNA lesion frequencies were used to determine percentage repair of the initial DNA damage caused by the DNA damaging agent. The PCR conditions and primer sequences used are shown in supplemental information.

2.4 CAN1 forward mutation assay and sequence analysis

The standard *CAN1* forward mutation assay was performed as previously described [24,25]. Independent *CAN1* colonies were isolated and the *CAN1* gene sequenced at University of Kentucky Genetic Technologies Center.

2.5 Statistical analyses

A two-tailed *t* test was used for comparison between two treatments and for comparison between three or more experimental groups, one way ANOVA with the Bonferroni *post hoc* test was used. Values of $p<0.05$ were considered statistically significant.

3. Results

3.1 A ynk1Δ strain exhibits attenuated repair of etoposide- and UV radiation--induced nuclear DNA damage

To determine whether *YNK1* has a functional role in DNA repair, we compared the repair rates of *ynk1*Δ versus wild-type cells following MMS (0.1%), etoposide (1 mM) and UV (192 J/m^2) treatment (Figure 1). Repair of MMS-induced nDNA damage did not significantly differ throughout the repair period between *ynk1*Δ and wild-type strains. In contrast, *ynk1*Δ mutants demonstrated a significantly reduced capacity to repair etoposide- and UV-induced nuclear DNA damage compared to wild-type up to 6 hr and 3 hr post-treatment, respectively ($p < 0.05$).

3.2 YNK1 does not have a role in the repair of MMS-, etoposide-, or UV-induced mitochondrial DNA damage

The mitochondrial genome is frequently challenged by DNA damaging agents, and mitochondrial genomic instability is associated with impaired nucleotide metabolism and development of the mutator phenotype [26,27]. Intriguingly, a fraction of the total cellular ynk1p has been localized to the mitochondrion [28]. However, its function within this compartment is not fully understood. Therefore, we aimed to examine whether *YNK1* has a functional role in mitochondrial DNA repair following DNA damage caused by MMS (0.1%), etoposide (1mM) and UV (192 J/m^2) (Supplemental Figure 1). The repair of MMS-, etoposide- and UV-induced mtDNA damage did not significantly differ between the *ynk1*Δ and wild-type strains, throughout the respective repair periods. Furthermore, only ~25% of the initial lesions were repaired at 6hr post-treatment for all DNA damaging agents.

3.3 The ynk1Δ strain exhibits a mutator phenotype following MMS and UV exposure

The *ynk1Δ* strain displayed a significantly slower repair of DNA damage induced by UV irradiation. To determine whether this impairment was manifested as an increase in mutation frequency, the *CAN1* forward mutation assay was employed under spontaneous conditions (no treatment), and following exposure to MMS and UV irradiation (Figure 2). Under spontaneous

conditions, no significant difference in mutation rate between *ynk1*Δ and wild-type strains occurred. In contrast, treatment with UV (192J/m^2) and MMS (0.1%) generated 2.6-fold and 1.6-fold increases in the mutation rate of *ynk1*Δ compared to wild-type, respectively ($p < 0.05$).

3.4 YNK1 mutants display a greater rate of base substitutions and frameshift mutations

Mutation spectra for *ynk1*Δ and wild-type strains were obtained following UV exposure (192 J/m^2) by sequencing the *CAN1* gene (Table 1). The *ynk1*Δ strain displayed a greater rate of base substitutions compared to the wild-type strain (70% vs. 59%), and a lower rate of complex alterations (12% vs. 26%). Following UV irradiation, there was no significant change in base substitutions or more complex alterations in either the *ynk1*Δ or wild-type strains. However, only the *ynk1*Δ mutants underwent frameshift events following UV-induced damage (15% vs. 0%), with mutations found at higher frequencies in simple repeat tracts of thymines in *ynk1*Δ mutants (Supplemental Figure 2).

4. Discussion

This investigation provides novel information regarding the role of *YNK1* in the maintenance of genomic integrity. Firstly, *YNK1* is required for the repair of UV- and etoposide-induced damage of nuclear DNA. However, *YNK1* has no measurable impact upon the kinetics of repair of nuclear DNA damage caused by MMS. Secondly, *YNK1* is not required for the repair of mitochondrial DNA damage. Thirdly, *YNK1* ablation promotes a mutator phenotype following UV and MMS exposure. This is the first study to our knowledge demonstrating a direct involvement of *YNK1* in DNA repair and in providing an anti-mutator function *in vivo*.

To test the hypothesis that *YNK1* has a role in DNA repair, we used QXL-PCR to study DNA repair kinetics [22,23]. This approach demonstrated that ablation of *YNK1* attenuated the repair of a 9.3-kb fragment in the nuclear phosphofructokinase-2 gene (a key enzyme involved in glycolysis) following UV and etoposide treatment. In the case of UV-induced DNA damage, which is repaired primarily by nucleotide excision repair (NER) [29], *YNK*1 could be involved in the NER pathway by functioning as a redundant 3′–5′ EXO. Furthermore, this function is also a plausible candidate for repair of etoposide-induced topoisomerase II-mediated DNA strand breaks [30]. In contrast, *YNK1* did not appear to facilitate the repair of DNA damage caused by MMS, as both wild-type and *ynk1*Δ displayed similar repair rates. The DNA damage caused by MMS is largely repaired by the BER [31]. Thus, this suggests *YNK1* does not have a functional involvement in BER, at least not against MMS-induced lesions (e.g., 7-methylguanine, O^6-methylguanine and, 3-methyladenine) [32,33].

The mitochondrial DNA comprises approximately 15% of the DNA content of *S. cerevisiae*, and its stability is crucial for cell viability [34]. A fraction of cellular ynk1p is localized to the mitochondria's intermembrane [28], yet its function within this compartment in yeast is not fully understood. *YNK1* exhibited no beneficial effect in the repair of MMS, etoposide or UV-induced mtDNA lesions in a 6.9-kb fragment of the mitochondrial *COX1* gene. However, this data has revealed important differences with respect to the repair of DNA damage in the nucleus and mitochondria in *S. cerevisiae*, and is consistent with the general notion, that mtDNA in higher eukaryotes is highly susceptible to genomic injury with limited repair potential [35].

To determine whether the deficit in DNA repair manifests to an increased incidence of mutations in S. *cerevisiae*, mutation rates were measured and sequenced following DNA damage. Indeed, mutation rates significantly increased in the *ynk1Δ* strain following treatment with UV and MMS. Intriguingly, NM23-H1 is an autonomous 3′–5′ EXO [5,6,8], an enzymatic function often implicated in enhancing the proofreading capacities of error-prone DNA polymerases during translesion repair (TLS). Thus, it is possible NM23 may have a direct proofreading function during TLS or as a facilitory role in protein complexes associated with

DNA during TLS [8,36,37]. Mutation spectral analysis of the *CAN1* gene revealed increased frameshift mutations in *ynk1*Δ after UV treatment. In particular, homonucleotide tracts of thymines were hot spots for these UV-induced mutations, which is consistent with the primary lesions generated by UV exposure (i.e. bulky lesions such as pyrimidine dimers and 6-4 photoproducts) [38]. These observed mutational events further support a role of *YNK1* in mutation avoidance, and possibly a function in TLS, as this mutation spectra is consistent with both 3′-5 EXO deficiency and impaired TLS following UV damage [36,38–40].

While DNA repair is an obvious potential function of *YNK1*, an alternative and complementary role in maintaining genomic stability must also be considered, via its NDPK activity [41]. NDPK has a central role in the maintenance of dNTP pools with perturbations in the dNTP balance associated with increased base substitutions, frameshift mutations as well as strong mutator phenotypes [42–46]. A previous study has provided evidence that *YNK1* possesses NDPK activity [28], and *E. coli* deficient in *NDK* suffer chronic perturbations in dNTP pools and increased mutagenesis [47,48]. These mutator phenotypes have been attributed to an imbalance in nucleotide pool sizes as a consequence of NDPK deficiencies, of particular note elevation in intracellular concentrations of dCTP [47]. Furthermore, of relevance to the present study, alterations in the intracellular dNTP pool may also compromise DNA replicative synthesis, and enhance fragile sites where chromosomes are susceptible to breakage and inhibit DNA repair [26,49–51].

Together, these observations suggest novel mechanisms underlying the metastasis suppressor activity of NM23-H1. We postulate that *YNK1* is an important factor in the mutation avoidance machinery by facilitating efficient DNA repair and limiting the generation of DNA mutations, primarily by virtue of its 3′–5′ EXO and/or NDPK activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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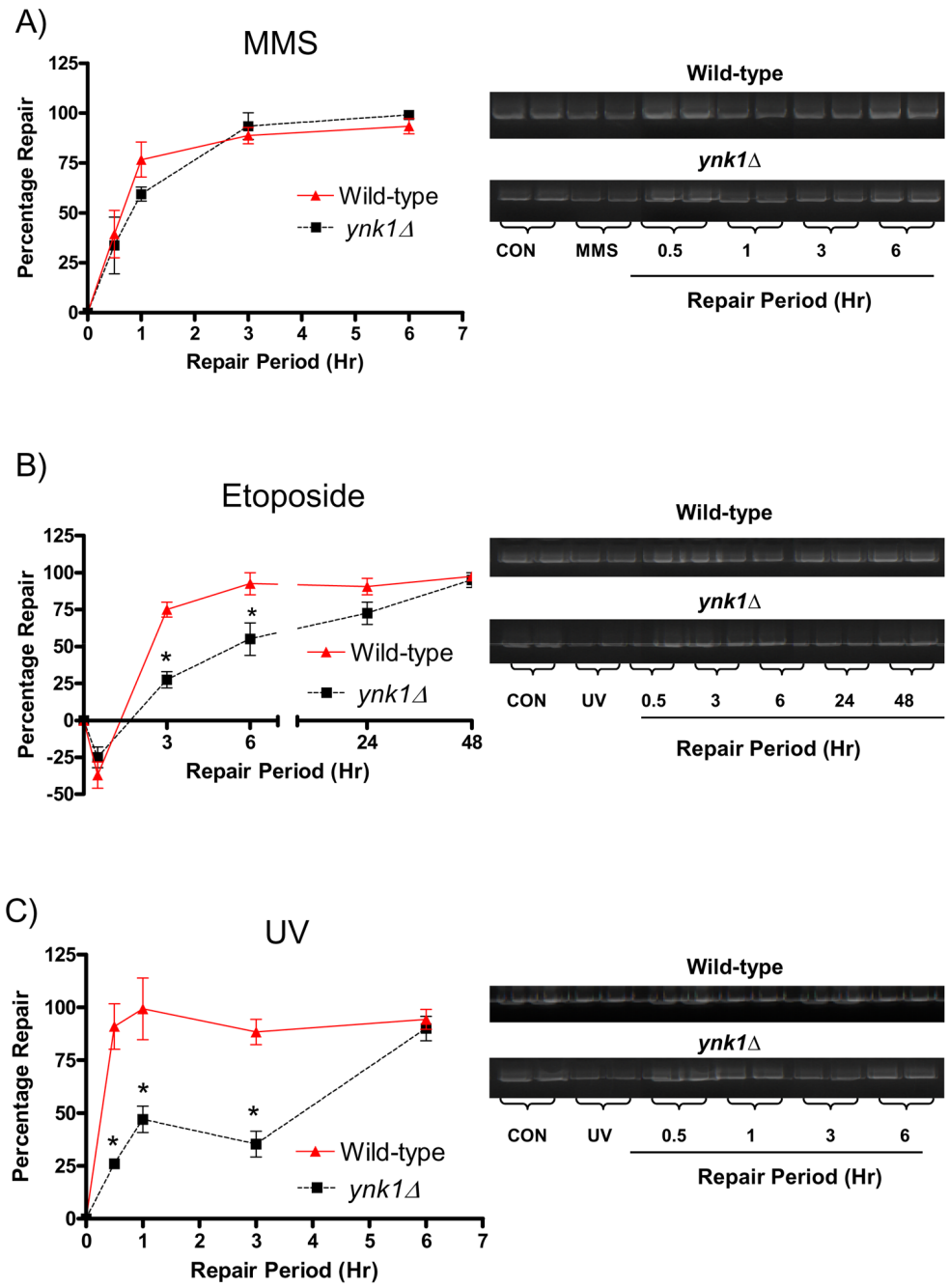

Figure 1. ynk1Δ attenuates nDNA repair of etoposide-and UV-induced damage but not MMS

Cells were harvested at the indicated time points, DNA extracted and QXL-PCR performed. The lesion frequencies were determined as described in Materials and Methods and data expressed as percentage repair of the initial DNA damage. A) MMS-induced DNA damage and repair at 0.5, 1, 3, and 6 h post-treatment; B) etoposide-induced DNA damage and repair at 0.5, 3, 6, 24 and 48 h post-treatment and C) UV-induced DNA damage and repair at 0.5, 1, 3 and 6 h post-treatment. Bars represent mean ± SEM. **P* < 0.05, *ynk1*Δ vs. wild-type, one way ANOVA, *n* = 3 per group.

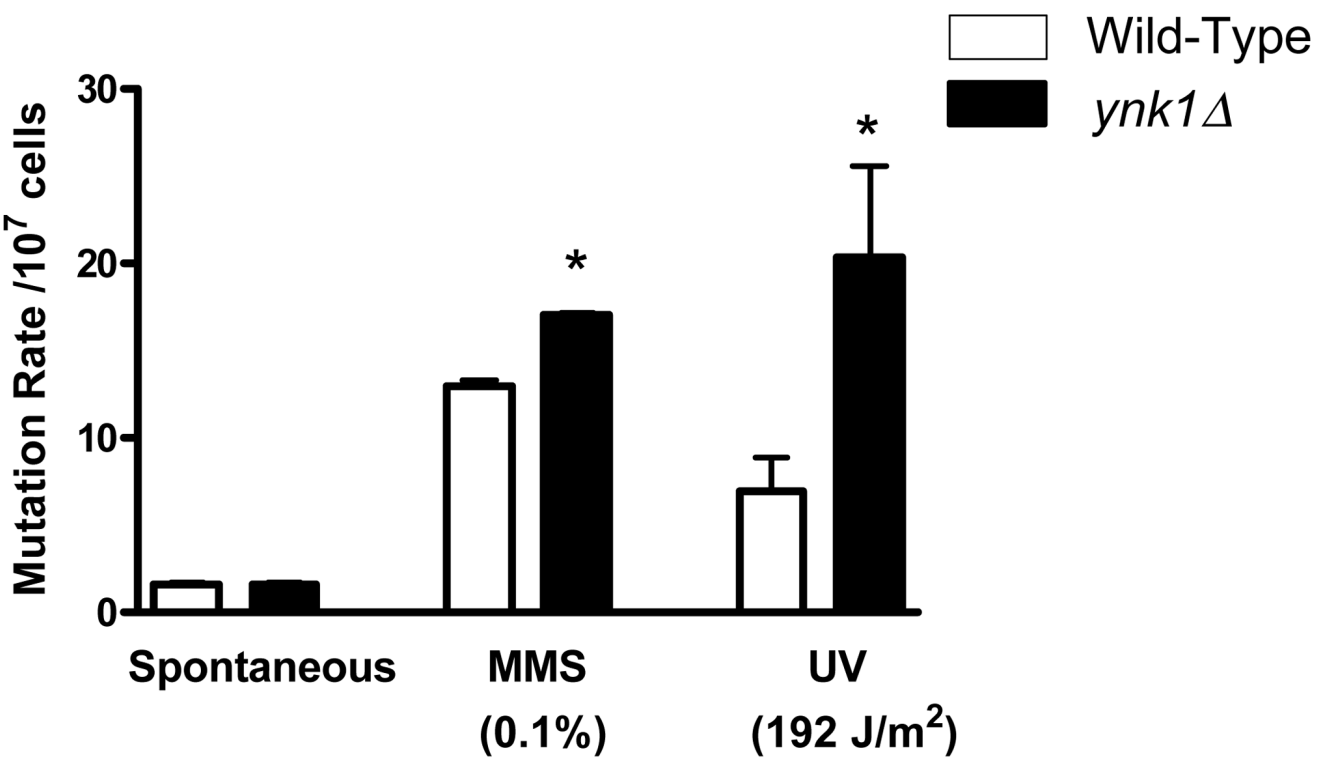

Figure 2. ynk1Δ causes a mutator phenotype following MMS and UV exposure

The *CAN1* forward mutation rate was measured in *ynk1*Δ and wild-type strains under spontaneous conditions or following treatment with either MMS (0.1%) or UV (192 J/m^2). The *CAN1* mutation rate was determined from the median in a fluctuation test of more than 10 independent cultures. **P* < 0.05, *ynk1*Δ vs. wild-type.

Table 1

The percentage of specific mutation types in wild-type and ynk1Δ strains

	−UV Exposure				+UV Exposure			
	Base substitution	**Frameshift**	**Complex**	**No mutation**	**Base substitution**	**Frameshift**	**Complex**	**No mutation**
Wild-type	59	0	26	15	53	0	21	26
ynk1Δ	70	0	12	18	70	15	10	5

Data is expressed as the percentage of mutation type i.e. base substitution, frameshift, complex or no mutation of the total number of mutational events in wild-type and *ynk1*Δ strains.