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Role of polymerase η in mitochondrial mutagenesis of *Saccharomyces cerevisiae*

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ABSTRACT

DNA polymerase η mostly catalyzes an error-free bypass of the most frequent UV lesions, pyrimidine dimers of the cyclobutane-type. In addition to its nuclear localization, we show here for the first time its mitochondrial localization in budding yeast. In mitochondria, this polymerase improves bypass replication fidelity opposite UV damage as shown in base pair substitution and frameshift assays. For base pair substitutions, polymerase η appears to be related in function and epistatic to DNA polymerase ζ which, however, plays the opposite role in the nucleus.

1. Introduction

Prokaryotic and eukaryotic cells have developed various mechanisms to repair or tolerate spontaneously occurring or externally introduced DNA damage [1]. Our knowledge of mitochondrial DNA repair pathways is still incomplete [2,3]. However, given the relevance of mitochondrial mutations in disease and aging [4–6], this topic is of considerable importance. Mitochondria harbor various enzymes of the base excision repair pathway, such as DNA glycosylases for uracil, 8-oxo-G, thymine glycol, AP endonucleases as well as DNA ligase, and mismatch repair may also occur [2,3]. With regard to UV damage, mitochondria were shown to be unable to excise UV-induced pyrimidine dimers [7] whereas budding yeast mitochondria are at least capable of photoreactivation [8]. Yeast and mammalian mitochondrial DNA also actively undergo homologous recombination [9,10] which may lessen the impact of unrepaired UV damage.

Since nucleotide excision repair is absent in mitochondria, the presence of efficient replicative bypass mechanisms can easily be rationalized. Polymerase η represents an example of the evolutionary conserved Y-type family of polymerases that is capable of bypassing the most frequent UV photoproducts, pyrimidine dimers of the cyclobutane-type, in a largely error-free manner [1,11,12]. In humans, this polymerase contributes significantly to UV resistance and genetic stability. Inactivation of Pol η was identified as the underlying cause of Xeroderma pigmentosum variant type (XP-V) [13].

In *Saccharomyces cerevisiae*, there is evidence that error-prone Pol ζ (complex of Rev3/Rev7) and Rev1 localize to mitochondria and, unlike in the nucleus, reduce the frequency of mitochondrial point mutations [14–16]. However, nothing was known about Pol η (Rad30). Here, we show its mitochondrial presence and a physiological role in reducing UV-induced base pair substitutions similar to Pol ζ.

2. Materials and methods

2.1. Yeast strains

All yeast strains used are listed in Table 1. The standard haploid strain BY4741 was originally purchased from OpenBiosystems and construction of 13xMYC tagged Rad30 (Pol η) has been described previously [17]. BY4741 carrying a *RAD30*-GFP fusion was from Invitrogen [18] and a *GAL1* promoter was constructed upstream of the fusion by recombination-mediated insertion, using PCR cassettes described in [19]. Modules were also used for deletion by transplacement of *REV3* or *RAD30* in strain Y100 (originally from Stephen Elledge) and TF236 (from Bill Copeland). Strain TF236 contains an *arg8* frameshift allele placed within the mitochondrial genome [20].

2.2. Pol η localization

Unless indicated otherwise, standard yeast media and growth conditions were used [21]. Logarithmic-phase cells of BY4741

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; PBS, phosphate-buffered saline; YPD, yeast extract/peptone/dextrose medium; YPG, yeast extract/peptone/glycerol medium.

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Table 1
S. cerevisiae strains used in this study.

BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RAD30–13xMYC::KanMX6*
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pGAL::KanMX6 RAD30-GFP::HIS3*
Y100	*MATa ade2–1 ura3–1 trp1–1 his3–11,15 leu2–3,112 can1–100*
Y100	*MATa ade2–1 ura3–1 trp1–1 his3–11,15 leu2–3,112 can1–100 rad30Δ::KanMX6*
Y100	*MATa ade2–1 ura3–1 trp1–1 his3–11,15 leu2–3,112 can1–100 rev3Δ::HIS3*
Y100	*MATa ade2–1 ura3–1 trp1–1 his3–11,15 leu2–3,112 can1–100 rev3Δ::HIS3 rad30Δ::KanMX6*
TF236	*MATa ino1::HIS3 arg8::hisG pet9(op1) ura3–52 cox3::arg8 m*
TF236	*MATa ino1::HIS3 arg8::hisG pet9(op1) ura3–52 cox3::arg8 m rad30Δ::KanMX6*

GAL1-RAD30-GFP were induced for Rad30-GFP expression in YPG medium (3% glycerol, 1% yeast extract, 2% Bacto peptone) containing 0.5–2% galactose for up to 3 h at 30 °C. Cell samples were spun down, incubated for 10 min with 1 μg/ml DAPI (Research Organics) and resuspended in PBS. Cells were then examined with an Olympus AX70 upright microscope, equipped with filters optimized for GFP and DAPI fluorescence. Extranuclear DAPI signals indicated mitochondrial DNA, as verified by examination of an isogenic strain void of mitochondrial DNA (ρ^0) (not shown).

2.3. Purification of mitochondria

First, a crude mitochondrial extract was prepared from a 5 l culture of BY4741 RAD30–13xMYC by spheroblasting with zymolyase (US Biological), homogenization and differential centrifugation as described in [22]. Next, the mitochondrial pellet was resuspended in 20 ml 40% w/v iodixanol (OptiPrep™) and purified according to manufacturer's instructions (Axis-Shield). Briefly, the suspension was overlayered by 14 ml each of OptiPrep solutions of ρ = 1.16 g/ml and ρ = 1.10 g/ml in a 38 ml centrifuge tube. The discontinuous gradient was centrifuged for 3 h at 80,000*g* in a Beckman SW28 rotor. The mitochondrial band formed at the interface of ρ = 1.16 g/ml and ρ = 1.10 g/ml solutions was collected and mitochondria harvested by centrifugation (10 min, 10,000*g*). SDS–PAGE electrophoresis and Western blotting was performed according to standard techniques, using primary antibodies for porin (monoclonal, Molecular Probes) and MYC (Covance), both at 1:1000 dilution.

2.4. Mitochondrial mutation assays

Mitochondrial point mutations conferring resistance to erythromycin were detected as described [14], except that a two-step protocol proved to be more reliable at higher UV doses. Strains of Y100 background were grown for 4 days to saturation in YPG, appropriate dilutions were spread on plates containing 3% glycerol, 2% peptone, 1% yeast extract, 50 mM phosphate buffer (pH 6.5), 1 g/l erythromycin, added from a stock of 40 g erythromycin per liter ethanol. After irradiation with a germicidal UV lamp at 254 nm, plates were incubated for 6 days at 30 °C and replica-plated onto the same medium, except that the erythromycin concentration was raised to 4 g/l. Resistant colonies were counted after additional 3 days of incubation. Colony survival was determined using the same medium without added erythromycin.

Following UV treatment of Y100 strains in logarithmic phase, petite clones were identified on YPD medium as small, white colonies and respiration deficiency was verified on medium containing glycerol instead of dextrose (YPG).

Arg8 mitochondrial frameshift reversion frequencies in TF236 [20] were measured in a two-step procedure. Cells from a 48 h culture in YPD were initially plated on arginine-dropout plates, containing 2% dextrose, 6.7 g/l yeast nitrogen base w/o amino acids, 740 mg/l CSM–Arg drop-out mix (Sunrise Scientific Products), supplemented with 500 mg/l yeast extract. Following seven days of incubation at 30 °C, cells were replica-plated onto plates of the identical medium but lacking any yeast extract supplement. Arginine-prototrophic revertant colonies were scored after an additional five days of incubation.

3. Results and discussion

When yeast strains containing a chromosomal Rad30-GFP fusion were investigated, we found indications for mitochondrial localization of Rad30 in addition to its expected nuclear localization. Its detection was facilitated if a *GAL1* promoter was integrated in front of the fusion gene, and increasing amounts of galactose were added to induce overexpression of Rad30-GFP (Fig. 1A and B). GFP foci colocalized with cytoplasmic DAPI signals which in yeast exclusively originate from mitochondrial DNA. Previous UV treatment or cell cycle position did not appear to influence the relative distribution of Rad30-GFP (not shown); this issue, however, awaits more detailed investigation. Interestingly, elevating overexpression by increasing the galactose concentration above 1.5% resulted in an exclusion of Rad30-GFP from mitochondria (Fig. 1B). Increased self-aggregation of the Rad30-GFP fusion protein may be responsible for this observation; if this reflects a propensity of Rad30 to specifically self-interact, remains to be studied.

Importantly, even without any overexpression, we found evidence for mitochondrial localization of Rad30 by purifying mitochondria through OptiPrep gradient centrifugation from a strain expressing Rad30-Myc downstream of the native promoter. A significant Rad30-Myc level was associated with a strong mitochondria-specific porin signal.

Further confirming localization to mitochondria, we demonstrate a physiological role of Pol η in mitochondrial UV mutagenesis without overexpression. The well-established assay of UV-induced mutations conferring resistance to erythromycin was used. Erythromycin resistance following UV radiation is mostly due to base pair substitutions at residues 1951 and 1952 of 21S rRNA, indicating TT dimers as the underlying lesions [14]. If a

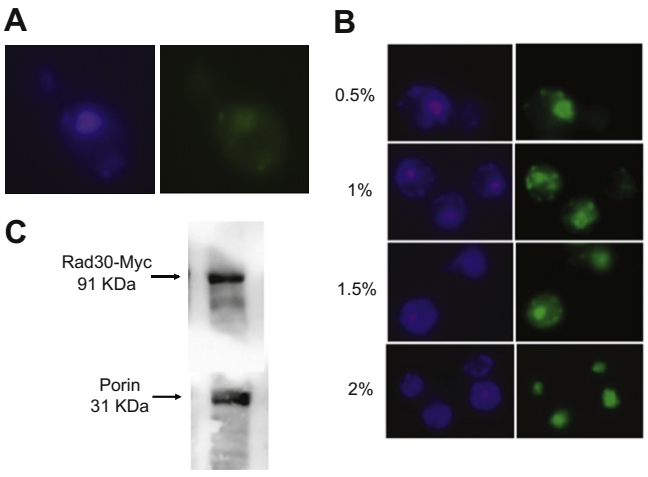

Fig. 1. Subcellular localization of yeast Rad30. (A) A chromosomal Rad30-GFP fusion was overexpressed from a *GAL1* promoter. When GFP fluorescence (right) is compared to DAPI staining of nucleus and mitochondria (left), nuclear as well as mitochondrial localization of Rad30 is observed. (B) Rad30 localization is affected by the degree of its overexpression, depending on the amount of galactose added (0.5–2%). If >1.5% galactose was used, Rad30 appears to be nuclear only. (C) Normally expressed Rad30–13xMyc was detectable in mitochondria that had been highly purified through an OptiPrep density gradient, as indicated by a strong mitochondria-specific signal (porin) following reprobing of the same membrane.

haploid deletion mutant of *RAD30* and its isogenic wild-type strain were compared, elevated mutation frequencies were found over a broad range of UV doses (Fig. 2A and B). Thus, as in the nucleus, Pol η functions to reduce the frequency of UV-induced base pair substitutions in mitochondria opposite TT damage. Interestingly, unlike its role in the nucleus, Pol ζ was described to also reduce UV-induced base pair substitution frequencies [14]. We confirmed this observation and addressed the mutability of a double-deletion strain (*rad30Δ rev3Δ*) (Fig. 2A and B). As expected, survival of colony-forming cells is reduced non-epistatically in the double mutant (Fig. 2A). However, induced mutation frequencies indicate an epistatic interaction in UV-induced base pair substitution leading to erythromycin resistance (Fig. 2B).

Although more subtle, a similar mutation-reducing effect of Pol η was found for mitochondrial UV-induced frameshift reversion, as detected in the *arg8* system [20] (Fig. 2C). In this system, however, an opposing, error-promoting effect of Pol ζ has been described [15].

However, no significant influence of Pol η was found for UV-induced petite mutation leading to respiration deficiency (Fig. 2D), indicating no role in generating non-functional mitochondria due to major deletions or complete loss of DNA. Here, in contrast to the base substitution system of erythromycin resistance, Pol ζ acts in its usual error-promoting fashion (Fig. 2D), confirming previously published data over a range of UV doses [14]. Additional deletion of *RAD30* did not influence this effect (Fig. 2D).

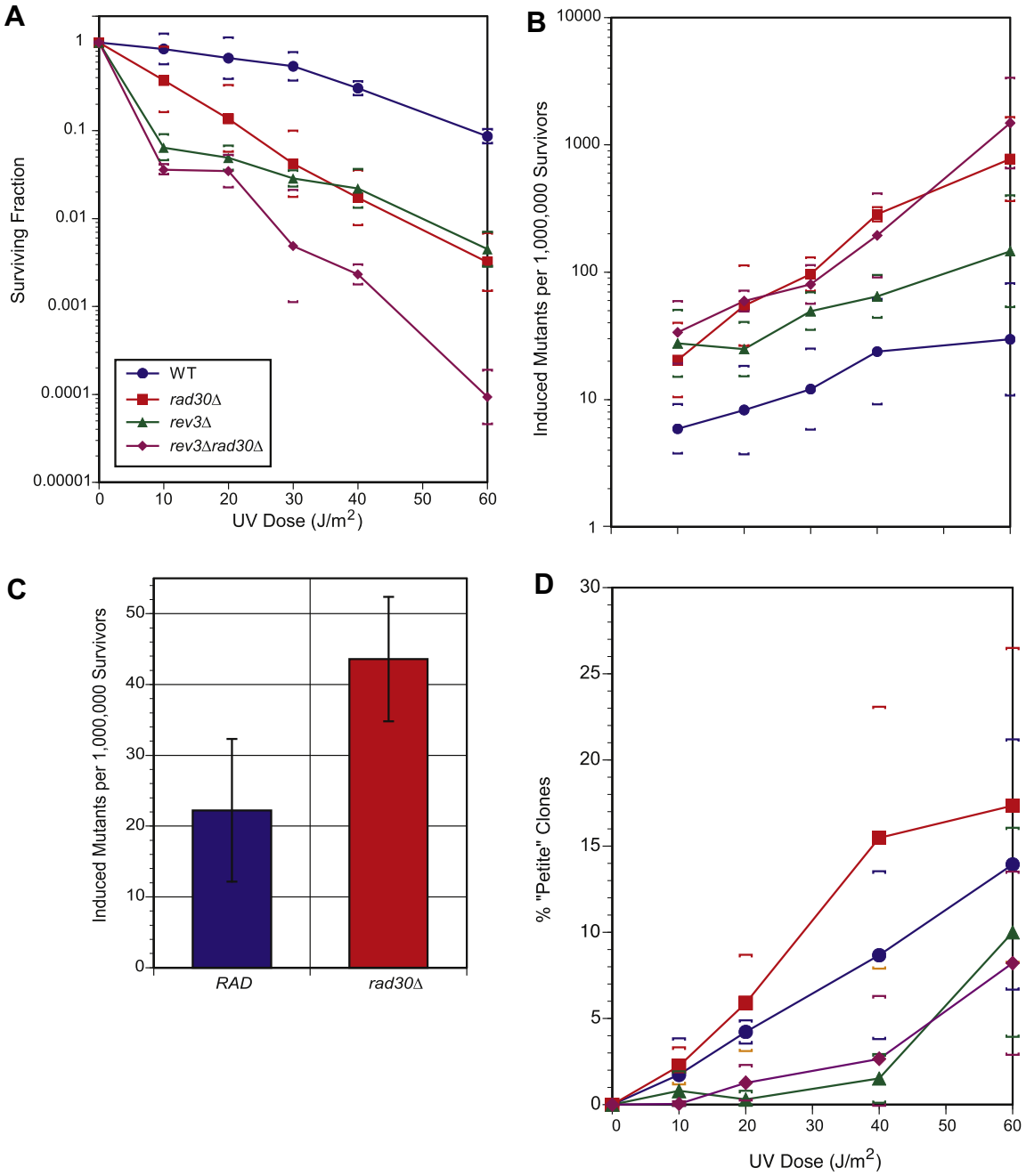

Fig. 2. Influence of Rad30 and Rev3 on UV survival and on the probability of UV-induced mitochondrial base pair substitution, frameshift reversion and "petite" mutations. (A) Survival of colony-forming haploid yeast cells following UV irradiation of strain Y100 (wild type, or deleted for *RAD30*, *REV3*, or both) is shown. (B) Frequency of UV-induced erythromycin-resistant mutants among surviving cells. (C) Frequency of UV-induced mitochondrial frameshift reversions (*arg8*) among surviving cells in strain TF236 (wild-type and *rad30* deleted) at 20 J/m^2. The difference is significant at $p = 0.01$. (D) Percentage of respiration-deficient colonies ("petites") among colony-forming survivors as a function of UV dose (Y100 strain background). All data shown represent the means of 3–5 independent experiments, with standard deviations indicated.

The most important result of this study is the localization of Rad30 to mitochondria and confirmation of its expected role of improving replication fidelity of a UV-damaged template. In the prevention of UV-induced base pair substitutions (but not frameshift mutations), Pol η cooperates epistatically with Pol ζ. Although Pol η is capable of translesion synthesis on its own [11], Pol ζ may assist in a two-step bypass using its well-characterized role of extension of imperfectly matched terminal base pairs [23].

These activities of Pol η and Pol ζ reduce error frequency in the erythromycin system. Thus, the polymerase that introduces base pair substitutions by competing with Pol η and ζ for UV-lesion bypass remains to be identified. An obvious candidate is Pol γ whose UV damage bypass capabilities have only recently been evaluated [24]. The human enzyme is severely inhibited by a cyclobutane TT dimer in the template but the rare bypass appears to be highly mutagenic. The possibility of Pol γ modifications in response to DNA damage, increasing its translesion synthesis activity, perhaps in conjunction with elevated dNTP levels, should also be explored.

It needs to be elucidated if lesion bypass in mitochondria is executed by a coordinated handover of different DNA polymerases. A main nuclear signaling pathway involving PCNA and its monoubiquitinated form [25–28] cannot be responsible due to the apparent absence of PCNA from mitochondria. (However, the Rev1 protein interacting with ubiquitinated PCNA and activating Pol ζ [29,30], is present.) Polymerase ζ clearly plays a role in mitochondrial DNA replication since its overexpression suppresses the mutagenic consequences of certain Pol γ mutations, even without any obvious connection to spontaneous or induced DNA damage [16]. Possibly, DNA polymerases present in mitochondria act in a largely uncoordinated fashion on a damaged template, enhancing or reducing genome stability depending on the nature of the DNA damage and its location. Given the importance of mitochondrial UV damage for aging and cancer [5,6], these are important issues to be addressed in the future.

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