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Deletion of the *DEF1* gene does not confer UV-immutability and frequently causes self-diploidization in yeast *Saccharomyces cerevisiae*

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Abstract

In yeast *Saccharomyces cerevisiae*, the *DEF1* gene is responsible for regulation of many cellular processes including ubiquitin-dependent degradation of DNA metabolism proteins. Recently it has been proposed that Def1 promotes degradation of the catalytic subunit of DNA polymerase δ at sites of DNA damage and regulates a switch to specialized polymerases and, as a consequence, DNA-damage induced mutagenesis. The idea was based substantially on the severe defects in induced mutagenesis observed in the *def1* mutants. We describe that UV mutability of *def1* strains is actually only moderately affected, while the virtual absence of UV mutagenesis in many *def1* clones is caused by a novel phenotype of the *def1* mutants, proneness to self-diploidization. Diploids are extremely frequent (90%) after transformation of wild-type haploids with *def1::kanMX* disruption cassette and are frequent (2.3%) in vegetative haploid *def1* cultures. Such diploids look “UV immutable” when assayed for recessive forward mutations but have normal UV

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Author Contributions

YP conceived and designed the study. ES and AS conducted the experiments and analysed the data. ES wrote the first draft. YP, ES and AS worked on the follow-up versions of the manuscript.

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mutability when assayed for dominant reverse mutations. The propensity for frequent self-diploidization in *def1* mutants should be taken into account in studies of the *def1* effect on mutagenesis. The true haploids with *def1* mutation are moderately UV sensitive but retain substantial UV mutagenesis for forward mutations: they are fully proficient at lower doses and only partially defective at higher doses of UV. We conclude that Def1 does not play a critical role in damage-induced mutagenesis.

Keywords

DEF1; ploidy; induced mutagenesis; DNA polymerase switch; ubiquitin

1 Introduction

The *DEF1* gene in *S. cerevisiae* encodes for a protein with yet-to-be-found biochemical activity and multiple biological functions. The *DEF1* gene has been shown to promote ubiquitination and proteolysis of RNAPII [1]. Null *def1* mutants exhibit diverse phenotypes, including slow growth [2], defective cytokinesis and meiosis [3], abnormal cell size [4] and increased sensitivity to mutagens [3]. *DEF1* is involved in genome stability control: *def1* is synthetically sick with mutations in the *PIF1* gene encoding for DNA helicase that participates in DNA maintenance and replication associated with DNA breaks [5, 6]; Def1 is found at sites of double strand breaks [7]; Def1 assists repair of abasic sites on the transcribed strands [8].

It has been proposed that Def1 participates in DNA damage induced mutagenesis in yeast, by promoting degradation of the catalytic subunit of DNA polymerase (pol) δ (encoded by the *POL3* gene) at stalled forks, which subsequently leads to a switch to translesion (TLS) DNA polymerases, ultimately responsible for mutagenesis [9]. Biochemical evidence in favor of the hypothesis was Def1-dependent Pol3 ubiquitylation leading to subsequent degradation of Pol3 in proteasomes [9]. Genetic support was a complete absence of UV-induced mutagenesis in *def1* strains [9]. Here we re-investigated the effect of *def1* mutation on mutagenesis. Our initial experiments revealed amazing heterogeneity of mutability of newly generated *def1* *::kanMX* strains. We show that, in fact, the deletion of *DEF1* leads to only a relatively small reduction of UV-mutagenesis. The apparent complete loss of induced mutagenesis seen in many strains with the deletion of the *DEF1* (including the strain present in the collection of deletions of all yeast reading frames in BY4742 background created in Genome Deletion Project, “The Yeast Knockout Collection” (YKO)) can be explained by a novel finding of frequent self-diploidization of *def1* haploids. Diploids have sharply reduced recovery of forward recessive mutations in reporters commonly used for mutagenesis assays [10–13]. Our study extends the knowledge on pleiotropic functions of the *DEF1*. The results suggest that the propensity for ploidy changes in strains with *def1* should be taken into account when interpreting the *DEF1* role in the control of genome stability.

2 Materials and Methods

We used *S. cerevisiae* strains LAN201-ura3 (*MATa ade5-1 ura3 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112*) [14], its *def1::kanMX* and corresponding diploid derivatives. Deletion of

the *DEF1* gene in haploid strain was made by one-step gene replacement using PCR product amplified from genomic DNA of the BY4742-*def1::kanMX* strain from yeast YKO collection (Dharmacon, U.S.A.). To amplify *def1::kanMX* allele, we used primers DEF1-F (5' GCAGCTCTCGTCAAACAAGG) and DEF1-R (5' AGTGGCACCTGTACTATCGC), Fig. 1A. Diploid derivative of the LAN201-ura3 strain was obtained by HO-endonuclease expression in transformants by HO-LEU plasmid, followed by selection for diploids. Diploid *def1* strain was a result of spontaneous self-diploidization in haploid *def1* mutant. Yeast strains were cultivated in standard conditions and media (YPDAU, standard YPD supplemented with extra adenine and uracil, YPDAU with G418 (200 µg/mL) and various selective synthetic media, SD) [15]. Qualitative and quantitative mutagenesis tests on induction of canavanine-resistant (Canr) mutants and His⁺ and Trp⁺ reversions were done as described before [14, 16, 17]. Ploidy of yeast strains was determined by flow cytometry as described in [18], but we used SYBR GREEN I instead of SYTOX dye. Data were analyzed using FlowJo software. To estimate the frequency of self-diploidization during vegetative growth, colonies of 3–6 independent cultures of LAN201-ura3 or its *def1::kanMX* derivative where cultivated in YPDAU broth overnight, then 4×10⁴-fold dilutions where plated on YPDAU agar. Individual colonies where picked up (500–1400 for each culture) and streaked as small patches on new YPDAU plates (72 patches per plate). After 2 days of growth, the patches were replica-plated on minimal complete medium with canavanine and UV irradiated (20 J/m²). UV mutability was evaluated after 5–7 days of incubation. To find how integrative transformation affects the recovery of UV-immutable clones, we used integrative plasmid pRS306-TRP1, with *URA3* and *TRP1* genes (*TRP1* gene was amplified by PCR using Trp1-BamHI (5'-AAGCCCAAGGATCCGATTGTACTGAGAGTGCACC) and Trp1-XhoI (5'-TTCGGGAACCTCGAGTTTACAATTCCTGATGCGG) and cloned using BamHI and XhoI sites into pRS306 vector). Wild-type and *def1* strains were transformed by the plasmid cut inside the *TRP1* gene by HindIII. Transformants were selected on SC-ura media. Individual transformants were re-cloned on selective medium and then the proportion of UV-immutable clones was then determined as described above.

3 Results

3.1 De novo creation of *def1::kanMX* strains results in clones with highly variable UV mutability.

To obtain deletion of the *DEF1* gene in a wild-type *S. cerevisiae* strain that is extensively used in our laboratories [12, 14] we transformed the LAN201-ura3 strain with PCR fragment with selectable *kanMX* marker flanked by long up- and downstream regions of the *DEF1* (Fig. 1A). We selected 20 independent transformants on YPDAU media with G418. We named different *def1* isolates “T1–T20”. Five of them (T2, T5, T9, T10 and T17) had the correct *def1::kanMX* disruption in their genomes (Fig. 1B). Transformant T2 gave two PCR fragments: the long fragment corresponding to the wild-type allele of the *DEF1* gene and the shorter fragment corresponding to the deletion *def1::kanMX* (Fig. 1AB). Yeast with *def1::kanMX* were analyzed for Canr UV mutability in qualitative patch test (Fig. 1C). Two of five transformants (T2 and T9) showed near-normal number of UV-induced Canr clones. Transformants T5, T10 and T17 were completely defective in UV mutagenesis, essentially

as described before [9]. Our subclone of BY4742-*def1* from the YKO was also completely UV- mutagenesis defective.

The results suggest that deletion of the *DEF1* gene does not always lead to the loss of UV-induced mutagenesis and additional genetic events might have transpired to generate a variety of UV mutability levels.

3.2 Ploidy changes in *def1* mutants

It is known that high ploidy precludes recovery of induced *can1* or other recessive mutants [10, 13]. One reason for the UV-immutability of most *def1* clones could be self-diploidization. To check the ploidy of *def1* clones, we measured the amount of DNA in all five *def1* mutants obtained *de novo*, BY4742-*def1* and controls by flow cytometry (Fig. 2). According to the DNA content analysis, the *def1* mutant clone present in our copy of YKO (BY4742-*def1*) is tetraploid. Transformants that were UV-immutable (T5, T10 and T17 in Fig. 1C), are diploids (Fig. 2). UV-mutable transformants T2 and T9 are haploids. Transformant T2 is unusual haploid because it has two alleles of the *DEF1* gene – wild-type and disruption *def1::kanMX* (Fig. 1B). It is likely that the transformant T2 may have chromosome 11 or its short region duplicated with wild-type and deletion *DEF1* alleles and therefore is UV mutable by default. For next experiments, we used T9, which was proved to be haploid with *def1::kanMX* allele. We measured the frequency of appearance of UV-immutable, presumably diploid clones, in vegetative cultures (as described in Methods). Clones with increased ploidy accumulated during cultivation of the T9 *def1* haploid strains with a frequency 2.3 ± 0.4 % (here and below Mean $\pm$ SEM). This is much higher, than in wild-type haploid strain (our estimate is 0.07 ± 0.04 %, consistent with the published data [19]). We next found that integrative transformation with the linear DNA fragment derived by cutting integrative plasmid inside the *TRP1* gene with selection for different marker (Methods) increased the proportion of UV-immutable clones to 2.4 ± 1.5 % in wild-type haploid strains and to 25.1 ± 2.1 % in the *def1* T9 strain.

3.3 *def1* strains are UV-mutable

Next, we quantitatively compared effects of UV irradiation in the haploid and diploid control and *def1::kanMX* strains (Fig. 3). Wild-type diploid is more resistant to UV than haploid (Fig. 3A). The *def1* mutation leads to a moderate increase of sensitivity to UV. The difference in Canr mutability of diploids and haploids is dramatic, two orders of magnitude, for either wild-type or *def1* strains (Fig. 3B). This is what expected for two-hit (mutation plus loss of heterozygosity) mechanism of appearance of mutants with recessive phenotype in diploids [10]. The deletion of *DEF1* in haploid did not lead to a severe decrease of frequency of UV-induced forward mutations in comparison to wild-type haploid. At low doses of UV, *def1* mutant was as UV-mutagenesis proficient as wild-type strain; at high doses of UV mutant frequency in *def1* strain was moderately lower than in the wild-type strain. Consistent with the finding that the *def1* mutation *per se* does not affect UV mutability, both haploid and diploid *def1* mutants were fully proficient in UV mutagenesis when assayed for dominant reversions of nonsense (selected for Trp⁺) or frameshift mutations (His⁺), (Fig. 3CD).

4 Discussion

Temporary switch from replicative pol δ to specialized polymerases and eventually to pol ζ during replication on damaged DNA is a part of essential mechanism preventing replication blocks, chromosome rearrangements and cell death induced by DNA damage [20, 21]. This pathway is mutagenic because pol ζ has a propensity to efficiently extend aberrant primer/template termini. The mechanism of switching between replicative and TLS DNA pols on damaged template that results in mutagenesis is far from complete understanding. The important elements of this complex pathway in yeast are ubiquitin ligases, monoubiquitinated PCNA, specialized DNA polymerases, pol η , Rev1 and pol ζ , shared subunit of pol δ and pol ζ (Pol32), and [Fe-S] clusters associated with C-terminuses of catalytic subunits of pol δ (Pol3) and pol ζ (Rev3) [22–24]. According to models for the switch between pol δ and pol ζ , undamaged DNA is replicated by processive and highly-efficient pol δ , a complex of three subunits (Pol3, Pol31 and Pol32), associated with processivity factor PCNA. Lesions in template block the replisome movement along the DNA and lead to PCNA monoubiquitination. This signal is an effector of proposed exchange of pol δ to pol ζ [23, 25]. Despite the fact that many individual participants of the process of pol switches are established, not much is known about coordination of all participants DNA damage response. For example, it is not known yet if Pol3 leaves at the stalling point alone or in complex with Pol31 and Pol32 [21]. According to one model for the switch between pol δ and pol ζ [25, 26], monoubiquitinated PCNA is an effector of exchange of Pol3 subunit to Rev3 [23, 25]. In a recent model for DNA damage tolerance pathway, the *DEF1* gene emerged as a new key participant [9]. Def1 was shown to genetically interact with Rad6, E2 ubiquitin ligase. Def1 stimulated polyubiquitylation-mediated degradation of Pol3, thus allowing for the proposed exchange to Rev3. Therefore, it has been proposed that the absence of Def1 prevents Pol3 degradation and makes pol switching impossible. This suggestion was supported by the observation that *def1* strains were completely defective in UV-induced mutagenesis. The defect of switch to polymerase pol ζ in *def1* is predicted to abolish UV-induced mutagenesis. Our study documents that *def1* mutants are in fact UV mutable. We describe a new phenotype of *def1* mutants, which explains why most *def1* strains look immutable.

One typical but frequently overlooked cause of the absence of induced mutagenesis is altered ploidy. In common mutagenesis assays that are based on selection of recessive forward mutations are used, in order for a mutant phenotype to appear in diploids both alleles must be either mutated or single mutant allele uncovered by loss of heterozygosity ([13] and references therein). Therefore, mutant frequency in diploids is 100 times less than in haploid strains [10, 12], Fig. 3B. Under normal conditions of growth of haploids, this is a rare event ($\sim 10^{-4}$). The proportion of diploids is elevated under harsh conditions or during integrative transformation [19, 27, 28]. It appears that *def1* mutation favors diploid formation. By DNA content analysis, most *def1* strains (present in the deletion library or made *de novo*) have ploidy higher than 1, which perfectly explains the severe lack of induced forward mutants in such clones. Our results are consistent with earlier data where *def1* mutant was ascribed as a strain with complex DNA content [29]. The novel phenotype of the *def1* mutant, propensity to increase ploidy levels, should be taken into account in

studies on the Def1 role in mutagenesis, because the high chances of inadvertently working with frequently arising diploid clones that would mimic “immutability” phenotype. We recommend to check the ploidy of the *def1* strains on regular basis. This is especially important when constructing strains combining *def1* mutation with mutations conferring damage-induced mutagenesis by one-step gene disruption. For *def1* single mutants, an express genetic method can be used, which is based on qualitative patch-test for induction of Canr mutations after UV-irradiation (Fig. 1C).

It appears that the double strand breaks required for the integration of cassettes predispose for polyploidization as evidenced by hyper-high frequency of diploid *def1* transformants during *DEF1*-disruption experiments in comparison to vegetative *def1* cells. We found that, consistent with the literature [28], integrative transformation for an unrelated marker also increases the rate of diploid formation, but it is much higher in *def1* than in wild-type strains. It is possible that the slow growth of *def1* mutants, along with other features of *def1* strains, contribute to this elevation.

Our findings add to the knowledge of the *DEF1* role in the control of genome stability. The *def1* mutant is not defective in UV-induced mutagenesis. We have shown that, in forward mutation assay, haploid *def1* strains are normally UV-mutable at low and moderate UV doses and have modestly reduced mutagenesis at high doses of UV. The mechanism of this reduction is currently not clear. The effects of the *def1* resemble the effects of the deletion of the C-terminal part of *REV3* [17]. In addition, we have shown that diploids *def1*, despite immutable for Canr, are fully UV mutable in reversion assays detecting dominant mutations (Fig.2C,D). The refinement of the *def1* phenotype does not necessarily disprove the model proposed in [9], but suggests that there are much more efficient mechanisms of exchange of replicative and specialized DNA polymerases on damaged DNA besides Def1.

It appears that Def1 might contribute to chromosome replication/segregation during mitosis. This role is more pronounced in cells undergoing mitotic recombination. It is also possible that Def1 pleiotropic functions helps maintain ploidy or the slower growth of *def1* haploids may help for selection of diploid variants [30]. Thus, Def1 is involved in the control of the frequency of both point mutations and chromosome copy number changes, which arise through different mechanisms. The last feature of Def1 is relevant to cancer etiology, as copy number changes are pretty common in tumors [31]. In future, the mechanism of ploidy changes in the *def1* strains can be found by additional studies of phenotypes and genetic interactions of the *def1*. We are planning to test a few ideas on the molecular mechanism of self-diploidization in *def1* strains that are relevant to ongoing studies in our laboratory, connected to the altered regulation of pol ζ , which has a role in control chromosome stability and cell cycle arrest [32]; to Def1 role in chromatin maintenance [33]; or the ability of Def1 to regulate the formation of amyloid aggregates and stimulate aggregation of other proteins [34, 35].

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Highlights

- The *def1* mutants are prone to self-diploidization
- Diploid *def1* mutants look UV-immutable when assayed for canavanine-resistance
- Diploid *def1* mutants are UV-mutable when assayed for dominant reversions
- The true haploids with *def1* mutation retain substantial UV mutagenesis

Figure 1. PCR-based generation, verification and mutability of the *DEF1* knockouts.

(A). Expected length of amplicon in PCR with genomic DNA of *DEF1* and *def1::kanMX4* strains.

(B). Agarose gel electrophoresis of PCR for presence of the *def1::kanMX* disruption in different strains. GR – GeneRuler 1 kb DNA Ladder; wt – LAN201-ura3 strain; T2 – T17 – transformants by *def1::kanMX* cassette; *def1* – BY4742-*def1* strain from YKO.

(C). Variable UV-mutability of *def1* mutants in qualitative test. Single colonies of wild-type (wt), *rev3* and *def1* strains were plated on YPDAU media as wide patches. After 24 hours of incubation at 30°C, yeast patches were replica plated on selective media with L-canavanine (3 plates). Two of Can-plates were irradiated by different doses of UV. One (left) plate of three is a negative control without irradiation. Can-plates were then incubated at 30°C for 5 days. Relative mutability was scored.

Figure 2. Most yeast *def1* isolates tend to have ploidy higher than 1n. Ploidy was estimated by DNA content comparison in stationary cultures of *def1* mutants and control strains (haploid and diploid strains isogenic to LAN201-ura3).

Figure 3. The *def1* strains are sensitive to UV and UV mutable.

(A). Effect of UV irradiation in *wild-type* and *def1* haploid and diploids on survival. Frequency of different genetic endpoints wild-type and *def1* haploids and diploids with or without UV: (B) Can^r mutants, Trp⁺ reversions (C) and His⁺ reversions (D). Mean and standard error of the mean are shown. All experiments were done in triplicate with six independent measurements for each UV dose.