HHS Public Access

Author manuscript

Curr Genet. Author manuscript; available in PMC 2022 June 01.

Published in final edited form as:
Curr Genet. 2021 June ; 67(3): 439–445. doi:10.1007/s00294-021-01162-7.

DNA damage response of major fungal pathogen *Candida glabrata* offers clues to explain its genetic diversity

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Abstract

How cells respond to DNA damage is key to maintaining genome integrity or facilitating genetic change. In fungi, DNA damage responses have been extensively characterized in the model budding yeast *Saccharomyces cerevisiae*, which is generally not pathogenic. However, it is not clear how closely these responses resemble those in fungal pathogens, in which genetic change plays an important role in the evolutionary arms race between pathogen and host and the evolution of antifungal drug resistance. A close relative of *S. cerevisiae*, *Candida glabrata*, is an opportunistic pathogen that displays high variability in chromosome structure among clinical isolates and rapidly evolves antifungal drug resistance. The mechanisms facilitating such genomic flexibility and evolvability in this organism are unknown. Recently we characterized the DNA damage response of *C. glabrata* and identified several features that distinguish it from the well characterized DNA damage response of *S. cerevisiae*. First, we discovered that, in contrast to the established paradigm, *C. glabrata* effector kinase Rad53 is not hyperphosphorylated upon DNA damage. We also uncovered evidence of an attenuated DNA damage checkpoint response, wherein in the presence of DNA damage *C. glabrata* cells did not accumulate in S-phase and proceeded with cell division, leading to aberrant mitoses and cell death. Finally, we identified evidence of transcriptional rewiring of the DNA damage response of *C. glabrata* relative to *S. cerevisiae*, including an upregulation of genes involved in mating and meiosis – processes that have not been reported in *C. glabrata*. Together, these results open new possibilities and raise tantalizing questions of how this major fungal pathogen facilitates genetic change.

Keywords

Candida glabrata; genome stability; DNA damage response; DNA damage checkpoint; Rad53; HO endonuclease; mating; meiosis

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Conflicts of interest/Competing interests. D.S.P. has received funding from the U.S. National Institutes of Health and contracts with The Centers for Disease Control and Prevention, Amplyx, Astellas, Cidara, and Scynexis. He serves on advisory boards for Amplyx, Astellas, Cidara, Matinas, N8 Medical, and Scynexis.

Availability of data and material. The RNAseq data are available at the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155701) and have been previously described (Shor, et al. 2020).

Code availability. The heatmaps were generated using the R studio gplots package, and the code is available upon request.

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Candida glabrata – a major fungal pathogen with high chromosomal variability and rapid evolution of drug resistance

An ability to regulate the capacity for genetic change, or evolvability, is especially important for pathogenic microbes that exist in the frequently changing environment of the host. Fungal pathogens are well known for their capacity to rapidly adapt to environmental pressures, such as cytotoxic antifungal drugs, by inducing genetic variants (e.g. aneuploidies and segmental duplications) with increased drug tolerance (Coste, et al. 2007, Selmecki, et al. 2006, Selmecki, et al. 2009). Genetic variation is likewise induced by passaging fungi through a mammalian host, likely in response to the host's immune defense mechanisms (Ene, et al. 2018, Forche, et al. 2018). These phenomena have been largely described in diploid and polyploid fungi, such as *Candida albicans* and *Cryptococcus neoformans* (Bennett, et al. 2014). However, haploid fungi also show high capacity for generating genetic variation. *Candida (Nakaseomyces) glabrata* is a haploid commensal and opportunistic pathogen fungus closely related to baker's yeast *Saccharomyces cerevisiae* (Shen, et al. 2018). *C. glabrata* is currently the second most prevalent cause of invasive candidiasis in North America and Europe, and its incidence is rising due to its reduced intrinsic susceptibility to azole class antifungals, which have been in wide clinical use for the last 40 years, and its ability to rapidly evolve drug resistance (Barber, et al. 2019, Bizerra, et al. 2014, Bordallo-Cardona, et al. 2017, Perlin, et al. 2017). Interestingly, *C. glabrata* clinical isolates display highly variable chromosome structures (karyotypes) due to translocations and copy number variation (Carrete, et al. 2018, Healey, et al. 2016, Lopez-Fuentes, et al. 2018, Muller, et al. 2009, Polakova, et al. 2009, Shin, et al. 2007). It is thought that the propensity of *C. glabrata* to rapidly evolve drug resistance is related to its ability to generate and tolerate extensive genetic variation; however, the mechanisms enabling this variation are so far unknown.

Attenuated DNA damage checkpoint signaling in *C. glabrata*

How cells respond to DNA damage is at the crux of maintenance of genome integrity. Eukaryotic cells have evolved intricate DNA damage checkpoint programs, which, among other things, slow down S-phase and prevent cell division in the presence of damaged DNA (Branzei and Foiani 2010, Friedel, et al. 2009). Defective checkpoint responses, for example in cancer cells, are associated with increased genome instability (Lobrich and Jeggo 2007), and in *S. cerevisiae* mutations in checkpoint genes greatly increase chromosome rearrangements, producing the variable karyotypes reminiscent of *C. glabrata* clinical isolates (Myung, et al. 2001, Serero, et al. 2014).

To investigate how *C. glabrata* responds to DNA damage, we examined the phosphorylation state of Rad53 (CHK2 in higher eukaryotes), a conserved effector kinase that is extensively phosphorylated upon DNA damage in *S. cerevisiae* and other organisms (Chen, et al. 2014, Jung, et al. 2019, Lee, et al. 2003, Pellicioli and Foiani 2005, Sanchez, et al. 1996, Sweeney, et al. 2005, Wang, et al. 2012). This hyper-phosphorylation, carried out by upstream DNA damage sensing kinases as well as by autophosphorylation, is a key signal transduction event necessary for multiple aspects of a functional DNA damage response (Lee, et al. 2003).

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Rad53 is also hyperphosphorylated upon genotoxic stress in *C. albicans* (Sun, et al. 2011, Wang, et al. 2012), where it is required for the DNA damage-induced switch from the yeast to the filamentous form (Shi, et al. 2007). In *S. cerevisiae*, dozens of Rad53 serines and threonines are phosphorylated upon DNA damage (Chen, et al. 2014), such that its mobility in acrylamide gels is significantly slowed to produce a characteristic band shift on a Western blot, which has become one of the hallmarks of a functional DNA damage response. Interestingly, unlike *S. cerevisiae* Rad53, *C. glabrata* Rad53 (CgRad53) did not show a size shift in the presence of heavy DNA damage – either 0.1% MMS (alkylating damage) or 10 mM H_2O_2 (oxidative damage) (Shor, et al. 2020). Importantly, both of these treatments produced robust phosphorylation of Ser129 of histone H2A (a.k.a. γH2A.X), indicating that cells were experiencing DNA damage but that CgRad53 was nevertheless not hyperphosphorylated (Shor, et al. 2020). We also confirmed the lack of DNA damage-induced CgRad53 hyperphosphorylation by mass spectrometry of immunoprecipitated endogenous CgRad53 (Shor, et al. 2020). These results were the first to indicate that the DNA damage checkpoint response in *C. glabrata* deviates from the paradigmatic response elucidated by studies of *S. cerevisiae*.

A key function of the DNA damage checkpoint is to slow down DNA replication, stabilize replication forks, and prevent cell division in the presence of DNA damage; failure to do this, for example in checkpoint mutants, results in chromosomal instability and cell death (Myung, et al. 2001, Segurado and Diffley 2008). Thus, we investigated these aspects of the DNA damage response in *C. glabrata*. We found that, unlike *S. cerevisiae* cells, *C. glabrata* cells did not accumulate in S phase in the presence of MMS (Shor, et al. 2020). Furthermore, time-lapse microscopy showed that some *C. glabrata* cells proceed with cell division even in the presence of MMS, and marking the nucleoplasm with nuclear-localized RFP allowed us to detect aberrant mitoses (i.e. those marked by unequal nuclear division), which were followed by cell death (Shor, et al. 2020). In accordance with these results, we also observed that *C. glabrata* cells exhibited higher lethality in the presence of moderate and high doses of MMS than *S. cerevisiae* (Shor, et al. 2020). These results are consistent with a lack of DNA damage-induced CgRad53 phosphorylation in *C. glabrata* and indicate that its DNA damage checkpoint response is attenuated relative to that of *S. cerevisiae*, suggesting that *C. glabrata* is more "permissive" of DNA replication and cell division in the presence of damaged DNA. This "permissiveness" may be one mechanism contributing to the high karyotype diversity of *C. glabrata* clinical isolates. Thus, it is possible that the obligate commensal and opportunistic pathogenic lifestyle of *C. glabrata* has been accompanied by relaxed DNA damage checkpoint activity and increased genetic flexibility, allowing chromosomal rearrangements at the cost of higher DNA damage-induced lethality.

Transcriptional response to DNA damage response in *C. glabrata*

In *S. cerevisiae*, DNA damage-activated Rad53 in turn phosphorylates multiple transcription factors that activate or repress hundreds of genes, resulting in the stereotypical transcriptional reprogramming in response to DNA damage (Edenberg, et al. 2014, Gasch, et al. 2001, Jaehnig, et al. 2013, Smolka, et al. 2007). We compared the transcriptional response to 0.1% MMS in *S. cerevisiae* and *C. glabrata* and found that, with a few intriguing differences highlighted below, the global transcriptional responses to DNA damage are quite

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similar in the two organisms (Shor, et al. 2020). Indeed, most of the genes whose orthologs' up- or downregulation is dependent on Rad53 in *S. cerevisiae* were still up- or downregulated by DNA damage in *C. glabrata* within the timeframe (1 hour) where no CgRad53 hyper-phosphorylation was occurring (Shor, et al. 2020). This result suggests that some transcriptional rewiring has occurred in *C. glabrata*, wherein genes that are Rad53-dependent in *S. cerevisiae* are regulated by Rad53-independent means in *C. glabrata*. Alternatively, it is possible that CgRad53 is mildly phosphorylated upon DNA damage (which our results do not exclude), and that this is sufficient for downstream transcriptional activation.

Interestingly, several genes involved in the maintenance of genome stability (DNA repair, replication integrity, chromosome segregation) were differentially regulated by DNA damage in *S. cerevisiae* and *C. glabrata*, most notably *POL30*, which encodes the proliferating cell nuclear antigen (PCNA). Consistent with previous studies, *ScPOL30* was strongly induced by DNA damage whereas, in contrast, *CgPOL30* was repressed by DNA damage both at RNA and protein levels (Shor, et al. 2020). The reasons for these differences are not yet clear, yet they are likely to have profound implications for how the two organisms maintain chromosome integrity in the presence of DNA damage because of the multiple roles of PCNA in preserving the integrity of the replisome and mediating its interactions with DNA repair complexes (Amin and Holm 1996, Ayyagari, et al. 1995, Brothers and Rine 2019, Chen, et al. 1999).

DNA damage induces orthologs of mating and sporulation genes in *C. glabrata*

One of the more mysterious aspects of the transcriptional response of *C. glabrata* to MMS was revealed by gene ontology (GO) analysis of downregulated and upregulated genes. Consistent with previous studies, the major functional classes downregulated by DNA damage in both *S. cerevisiae* and *C. glabrata* were involved in growth and protein synthesis, whereas genes upregulated by DNA damage were involved in stress and damage responses (Shor, et al. 2020). However, an additional functional category was found for MMS-induced genes only in *C. glabrata*: genes whose orthologs in *S. cerevisiae* functioned in meiosis. This was surprising and intriguing because, as mentioned above, sexual reproduction has not been observed in *C. glabrata* in the lab, and only genome analyses of clinical isolates have provided indirect evidence of rare sex and recombination in this species (Carrete, et al. 2018, Lott, et al. 2010). Nevertheless, *C. glabrata* possesses the full complement of genes whose orthologs in *S. cerevisiae* function in mating, meiosis, and sporulation, suggesting either that sexual reproduction does occur in *C. glabrata* under as yet unknown conditions, or that these genes have perhaps been coopted for another function in this fungus (Muller, et al. 2008, Wong, et al. 2003). To begin to understand the connection between DNA damage and mating and meiosis, we more closely scrutinized the transcriptional changes in mating and meiosis-related genes in *C. glabrata*.

The process of sexual reproduction in *S. cerevisiae* can be broken down into several distinct stages. First, two cells of the opposite mating type signal their presence to each other by

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secreting pheromones. Mating type identity is determined by the MAT locus, which contains either MAT**a**- or MATα-specific transcription factors that regulate pheromone production and other mating factors (Heitman, et al. 2013). Yeast cells can switch their mating type by expressing HO endonuclease, which creates a DNA double strand break at the MAT locus, triggering a gene conversion event from a transcriptionally silenced locus carrying MAT genes of the opposite mating type (HMR**a** or HMLα) (Haber 2012). Most lab strains of *S. cerevisiae* contain point mutations in HO and are therefore incapable of mating type switching. Interestingly, our transcriptome analysis showed that the HO gene was downregulated by DNA damage in *S. cerevisiae* but significantly upregulated in *C. glabrata* (Figure 1, "Mating type determination, pheromone production"), suggesting that DNA damage may promote mating type switching in *C. glabrata* or perhaps other reconfiguring of the MAT locus. Whether this is indeed true remains to be experimentally determined.

Because the focus of our study had been on the DNA damage checkpoint, with no anticipation of the effects of DNA damage on mating-related processes, the *S. cerevisiae* and *C. glabrata* strains exposed to MMS and used for RNAseq happened to be of different mating types: the *C. glabrata* strain was MATα whereas the *S. cerevisiae* strains was MAT**a**. Interestingly, *MF(alpha)2* gene, which encodes the MATα-specific pheromone α-factor, and STE3, which encodes the receptor for the MAT**a** pheromone **a**-factor, were both strongly induced by MMS in *C. glabrata*, as if in preparation for mating (Figure 1, "Mating type determination, pheromone production"). Furthermore, all genes involved in the pheromone-signaling mitogen-activated protein kinase (MAPK) cascade (Wang and Dohlman 2004) were also upregulated by MMS in *C. glabrata* relative to *S. cerevisiae*, with the exception of *STE4*, which was downregulated in *S. cerevisiae* but unchanged in *C. glabrata* (Figure 1, "Pheromone MAPK cascade"). However, genes involved in subsequent steps of mating (cell shape change known as "shmooing", cell fusion, karyogamy) were not, as a whole, more likely to be induced in *C. glabrata* than in *S. cerevisiae* (Figure 1, "Shmooing, cell fusion, karyogamy"). Together, these data suggest several possibilities. First, it is possible that DNA damage, or perhaps another kind of damage caused by MMS, truly triggers mating type switching and mating in *C. glabrata*. Consistent with this hypothesis, the early genes involved in pheromone signaling were induced within the timeframe of our experiment (1 hour MMS exposure), whereas the later genes involved in cell fusion were not. An alternative explanation is also possible, however: that the orthologs of mating genes in *S. cerevisiae* have been coopted for another process in *C. glabrata*, one that is induced by MMS. There is precedent for non-mating functions of the mating gene cascade: for example, in *C. albicans* pheromone signaling also promotes cell adhesion and biofilm formation (Alby and Bennett 2011).

The second stage of sexual reproduction is meiosis, upon which a diploid cell forms four haploid spores. In *S. cerevisiae* mating and meiosis are two distinct processes occurring in haploid or diploid cells, respectively, and triggered by different stimuli: presence of the opposite mating type and nutrient limitation, respectively. Likewise, mating and meiosis are associated with distinct transcriptional programs. In contrast, in the haploid pathogenic yeast, *C. lusitaniae*, mating and meiosis occur together in quick succession, and the two gene sets are coordinately upregulated by the same set of stimuli and are controlled of the same

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transcription factor (Ste12) (Sherwood, et al. 2014). We examined the transcription changes in orthologs of meiosis-related genes in *C. glabrata*, including the early regulators of meiosis, genes involved in meiosis-specific chromosomal transactions, and genes involved in spore development. None of the orthologs of well-defined early meiotic regulators (e.g. *IME1*, *IME2*, *NDT80*) were induced more strongly in *C. glabrata* than in *S. cerevisiae* (Figure 1, "Early meiotic regulators"). Furthermore, although a few genes involved in regulating meiosis-specific chromosomal transactions (*MAM1*, *SPO16*, *MSH4*, *RED1*) were strongly induced by MMS in *C. glabrata* relative to *S. cerevisiae*, most such genes were as likely to be repressed as induced in *C. glabrata* (Figure 1, "Synaptonemal complex, chromosome cohesion, recombination"). Finally, genes involved in spore development (mostly those promoting the formation of the spore membrane or cell wall) did tend to be upregulated in *C. glabrata* relative to *S. cerevisiae*, with several showing very strong induction by MMS (log2ratio > 4, Figure 1, "Ascospore membrane & cell wall formation"). Given the importance of the cell wall in *C. glabrata* (Lopez-Fuentes, et al. 2018), it is possible that the upregulation of these genes reflects not their meiotic functions but perhaps the fact that in *C. glabrata* they serve to maintain cell wall integrity in the face of stress, such as MMS exposure. In sum, these data do not provide strong evidence of induction of the meiotic program within the timeframe of the experiment, and further experiments, with longer DNA damage exposures, are required to clarify this issue.

Summary and future directions

Our study of the DNA damage response in *C. glabrata* revealed several unique features, some easier to interpret than others, which likely impact genetic flexibility and karyotype diversity in this organism. The absence of Rad53 hyper-phosphorylation, the attenuated checkpoint response, and downregulation of PCNA expression upon DNA damage are all expected to affect chromosome stability and may contribute to the variable karyotypes that characterize *C. glabrata* clinical isolates (Figure 2). The observation that HO endonuclease and pheromone signaling genes are strongly induced by MMS in *C. glabrata* but not *S. cerevisiae* suggests that mating in *C. glabrata* – which has never been observed as yet – may be connected to its DNA damage response or another stress response triggered by MMS. If true, this would fit with the paradigm of generating genetic diversity – via sexual reproduction – in the presence of stress (Ram and Hadany 2016). Alternatively, DNA damage may induce other functions mediated by the orthologs of mating and sporulation genes, e.g. cell adhesion, biofilm formation, or cell wall integrity. If this is the case, it would support the conclusion that *C. glabrata* is largely asexual, and perhaps its relaxed DNA damage checkpoint function helps generate genetic diversity in the absence of sex. In any case, what these data show is that *C. glabrata* responds to DNA damage differently than its close non-pathogenic relative *S. cerevisiae*, and understanding these differences will help explain how *C. glabrata* regulates genetic change and evolvability, and may identify routes towards inhibiting rapid emergence of antifungal drug resistance.

Funding.

This work was supported by NIH 5R01AI109025 to D.S.P.

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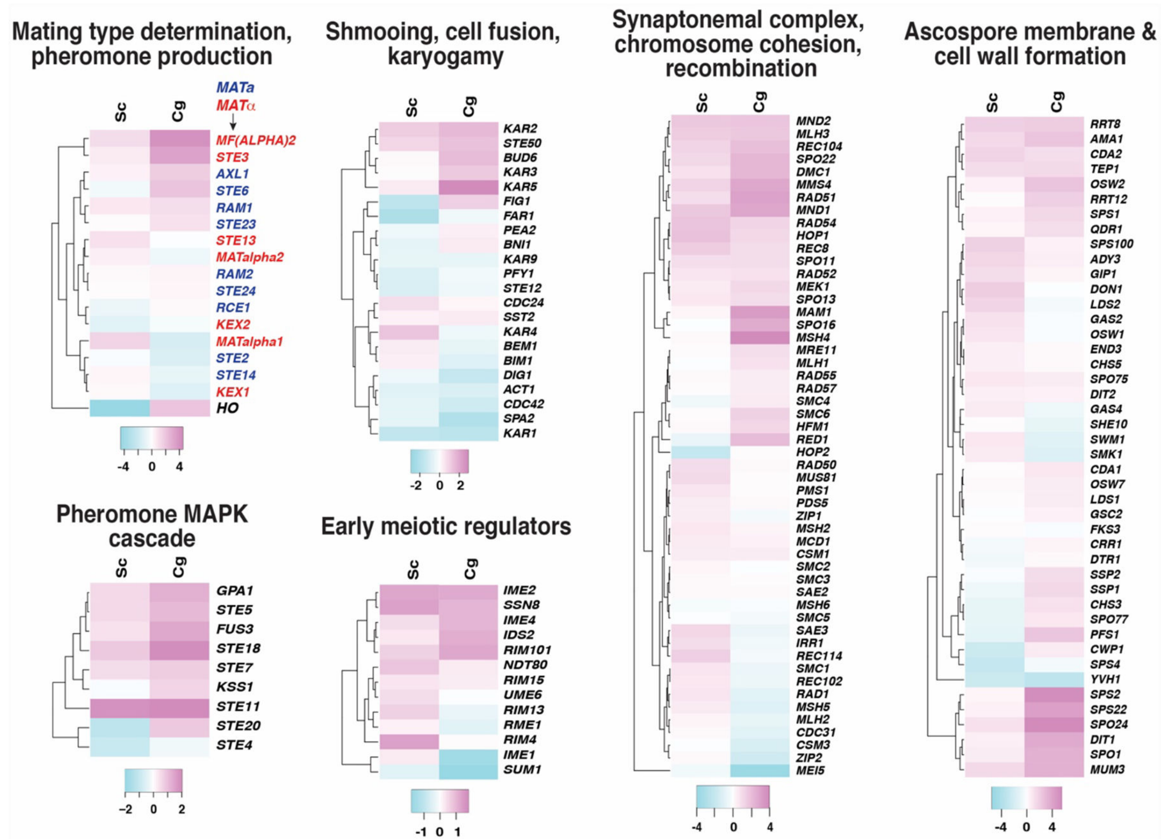

Figure 1. DNA damage-induced transcriptional changes of *C. glabrata* orthologs of genes involved in sexual reproduction.

The color scheme represents transcript abundance log2 ratios (MMS/no MMS). The RNAseq data are available at the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155701) and have been previously described (Shor, et al. 2020). The heatmaps were generated using the R studio gplots package.

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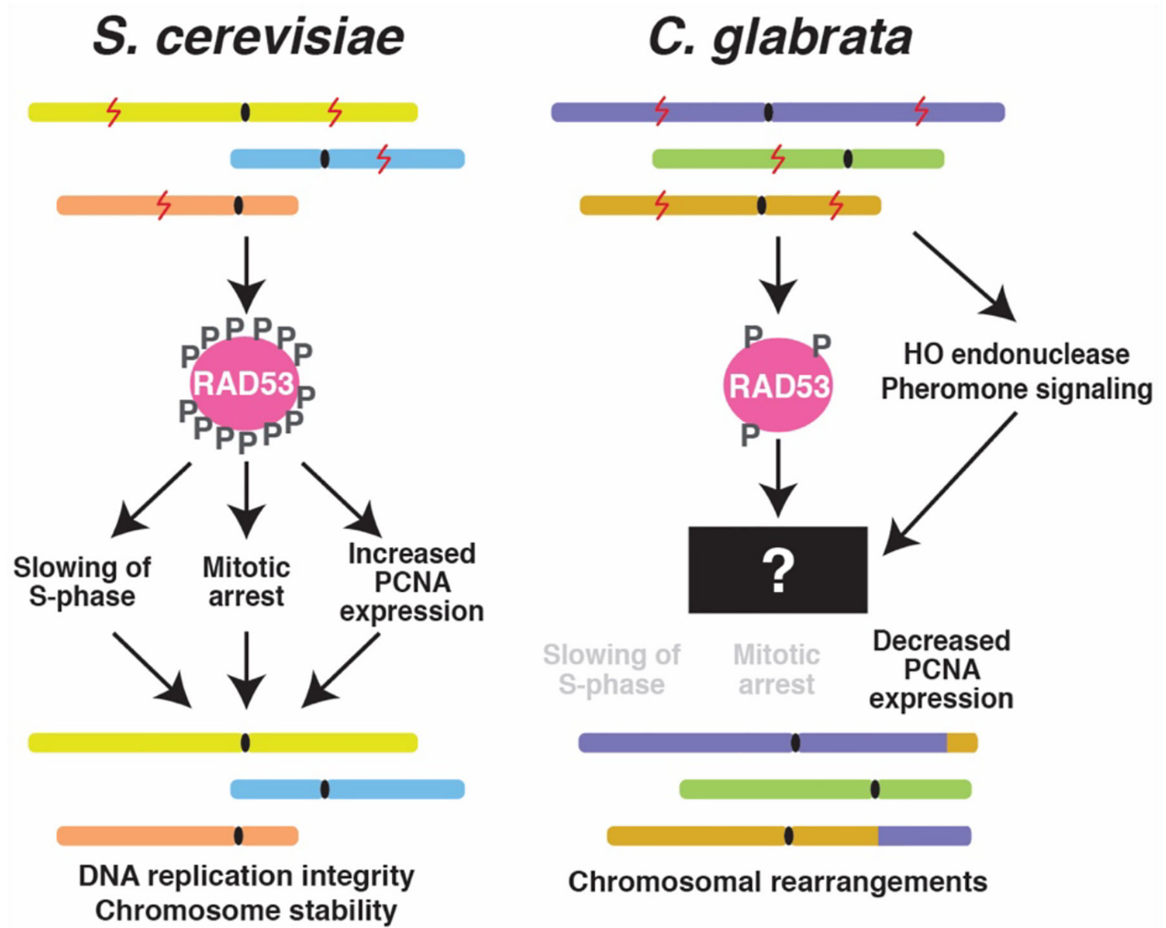

Figure 2. Schematic representation of the differences between the responses to DNA damage (ϟ) in *C. glabrata* and *S. cerevisiae*.