A single Ho-induced double-strand break at the MAT locus is lethal in Candida glabrata

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Abstract

Mating-type switching is a complex mechanism that promotes sexual reproduction in Saccharomycotina. In the model species Saccharomyces cerevisiae, mating-type switching is initiated by the Ho endonuclease that performs a site-specific double-strand break (DSB) at MAT, repaired by homologous recombination (HR) using one of the two silent mating-type loci, HMLα and HMRα. The reasons why all the elements of the mating-type switching system have been conserved in some Saccharomycotina, that do not show a sexual cycle nor mating-type switching, remain unknown. To gain insight on this phenomenon, we used the yeast Candida glabrata, phylogenetically close to S. cerevisiae, and for which no spontaneous and efficient mating-type switching has been observed. We have previously shown that expression of S. cerevisiae’s Ho (ScHo) gene triggers mating-type switching in C. glabrata, but this leads to massive cell death. In addition, we unexpectedly found, that not only MAT but also HML was cut in this species, suggesting the formation of multiple chromosomal DSBs upon HO induction. We now report that HMR is also cut by ScHo in wild-type strains of C. glabrata. To understand the link between mating-type switching and cell death in C. glabrata, we constructed strains mutated precisely at the Ho recognition sites. We find that even when HML and HMR are protected from the Ho-cut, introducing a DSB at MAT is sufficient to induce cell death, whereas one DSB at HML or HMR is not. We demonstrate that mating-type switching in C. glabrata can be triggered using CRISPR-Cas9, without high lethality. We also show that switching is Rad51-dependent, as in S. cerevisiae, but that donor preference is not conserved in C. glabrata. Altogether, these results suggest that a DSB at MAT can be repaired by HR in C. glabrata, but that repair is prevented by ScHo.

Author summary

Mating-type switching is one of the strategies developed by fungi to promote sexual reproduction and propagation. This mechanism enables one haploid cell to give rise to a cell of the opposite mating-type so that they can mate. It has been extensively studied in the yeast S. cerevisiae in which it relies on a programmed double-strand break performed by the Ho endonuclease at the MAT locus which determines sexual identity. Little is
known about why the mating-type switching components have been conserved in species like \textit{C. glabrata}, in which neither sexual reproduction nor mating-type switching is observed. We have previously shown that mating-type switching can be triggered, in \textit{C. glabrata}, by expression of the \textit{HO} gene from \textit{S. cerevisiae} but this leads to massive cell death. In this work, we show that mating-type switching in \textit{C. glabrata} can be triggered by CRISPR-Cas9 and without any high lethality. We demonstrate that the cut at \textit{MAT} is only lethal when the Ho endonuclease performs the break, a situation unique to \textit{C. glabrata}. Our work points to a degeneration of the mating-type switching system in \textit{C. glabrata}. Further studies of this phenomenon should shed light on the evolution of mating systems in asexual yeasts.

\section*{Introduction}

In eukaryotes, sexual reproduction is a nearly ubiquitous feature and implies fundamental conserved processes such as gamete fusion, zygote formation and meiosis [1]. Sexual reproduction leads to genetic recombination between organisms and thus enables them to purge their genomes from deleterious mutations, as well as to increase their genetic diversity. It is in the fungal kingdom that the greatest diversity of sexual reproduction is found [1]. Particularly, sexual reproduction in fungal pathogens of human exhibits a considerable plasticity between species [2,3]. While many were thought to be asexual, several atypical sexual or parasexual cycles have been discovered. It has been shown that the yeast \textit{Candida albicans} can perform a parasexual cycle by mating of two diploid cells, forming a tetraploid, that can undergo chromosome loss [4]. The more distant filamentous opportunistic pathogen, \textit{Aspergillus fumigatus} exhibits a sexual cycle but only mates after spending 6–12 months in the dark [5]. Altogether, this suggests that, in most fungi, performing genetic exchange is crucial, even in well-adapted human pathogens.

In fungi, sexual reproduction can occur through three mechanisms [1]: heterothallism (requiring two compatible partners for mating to occur), homothallism (self-fertility), and pseudo-homothallism (where a single individual can go through a complete sexual cycle but mating only occurs between two compatible partners). Pseudo-homothallism has mainly been described in ascomycete yeasts where it occurs through a programmed differentiation process called mating-type switching [6]. This mechanism enables one haploid cell to give rise to a cell of the opposite mating-type so that they can mate. In all cases studied so far, it implies a genomic DNA rearrangement of the mating-type locus (\textit{MAT}, encoding the key regulators of sexual identity) and species have evolved very different molecular pathways for the same aim. In the fission yeast \textit{Schizosaccharomyces pombe}, an imprint at \textit{mat1} (it is unknown whether the imprint is an epigenetic mark or a single nick) is introduced, that leads to a DSB during DNA replication [7,8]. Repair occurs with one of the two silent copies of \textit{mat1}, called \textit{mat2} and \textit{mat3}. In the ascomycete \textit{Kluveromyces lactis}, mating-type switching involves a DSB at \textit{MAT} but it is performed by two specific nucleases depending on the mating-type of the cell [9,10]. Mating-type switching has been extensively studied in the model yeast \textit{S. cerevisiae} and has notably allowed a better understanding of cell identity, DSB repair and silencing mechanisms [11].

In \textit{S. cerevisiae}, haploid cells can be of either mating-type, \textit{MATa} or \textit{MATa}, which encodes “alpha” or “a” information, respectively, at the Y sequence of the \textit{MAT} locus [12] (Fig 1). Mating-type switching relies on a programmed DSB at the \textit{MAT} locus performed by the Ho endonuclease at its 24-bp recognition site (Fig 1). DSBs are highly toxic DNA lesions, and
thus have to be efficiently repaired to ensure cell viability. This can be achieved through two major pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR) in the presence of a repair template. The DSB at MAT is repaired ~90% of the time by HR [11], probably because of efficient resection of the DSB that has been shown to prevent NHEJ [13].

The Ho cut at the MAT locus generates 4 bp, 3' -overhanging ends and its repair involves the following steps: the DSB ends are processed by several 5' to 3' exonucleases to create long 3' -ended tails [14]; single-strand tails are then converted to Rad51-coated nucleoprotein filaments, which search for homology and promote homologous template invasion [11]; once the homologous donor is found, the MAT locus is repaired by gene conversion. The homologous donor is one of the two silent loci located on the same chromosome as MAT: HML carrying the "alpha" information or HMR carrying the "a" information. The "alpha" or "a" sequence from HML or HMR respectively, replaces the original Y MAT sequence whereas HML and HMR remain unchanged. Despite the fact that HML and HMR contain the Ho recognition site, both are resistant to Ho cleavage, being located in heterochromatic regions [15]. It must be noted that the Ho recognition site is quite different between the "a" and "alpha" versions, one side is unchanged since it is located in the identical Z sequence, while the other side, corresponding to the end of the Y fragment, is different between the two mating-types (Fig 1).

There is no measurable difference in the efficacy of the cut between the two sequences, nor between efficiency of mating-type switching from one to another [16,17]. This illustrates the fact that Ho is part of the family of meganucleases, that do not function like type II restriction endonucleases, but recognize large (larger than 12bp), degenerate, non-palindromic cut-sites.
In *S. cerevisiae*, a "donor preference" mechanism ensures an efficient mating-type switching at MAT by promoting the use of the silent locus from the opposite mating-type (MATa is preferentially repaired by HMLalpha and MATalpha by HMRa). This donor preference depends on both the "a" or "alpha" information at the MAT locus and the presence of a specific sequence, the recombination enhancer (RE), located between HML and MAT [18]. It must be noted that mating-type switching occurs only once per cell-cycle, in G1, and that this is thought to be regulated through the control of the expression of HO [12], but experiments of overexpression of HO under the control of a galactose-inducible promoter have shown that the switch can be induced in any part of the cell cycle [19]. Intriguingly, there is no report of switching back and forth between the two mating-types in such overexpression experiments, leaving open the possibility that another mechanism than HO gene expression, is responsible for the "unswitchability" of newly (i.e., unrelicated) switched cells.

*C. glabrata* is an opportunistic pathogenic yeast, phylogenetically close to *S. cerevisiae* [20]. Its genome has retained the three-locus system, with homologs of HML, MATa/alpha, and HMR, called Mating-Type Like (MTL) loci (Fig 1). The three loci display a structure comparable to *S. cerevisiae*'s, the main difference being that HMR is located on a different chromosome from HML and MAT [20]. Despite these similarities, added to the fact that both MATa and MATalpha cells are found naturally and that they maintain some mating-type identity [21–23], *C. glabrata* is unable to switch mating-type spontaneously at an efficient level, even though rare signs of mating-type switching are observed in culture [24] and in populations [25]. We have previously shown that the expression of the HO gene from *S. cerevisiae* can trigger mating-type switching in *C. glabrata*, and that over 99% of *C. glabrata* cells are unable to survive the expression of *S. cerevisiae*’s Ho (ScHo) [26]. We have also observed gene conversion events at the HML locus in survivors, revealing that, unlike *S. cerevisiae*, HMR is not protected from the Ho cut. We suggested that the lethality was due to multiple chromosomal DSBs, which would prevent homologous recombination with an intact template in most cells.

In this work, we investigate the reasons for the lethality associated with mating-type switching induced by ScHo. For this purpose, we constructed a series of inconvertible (Inc) *C. glabrata* strains, mutated precisely at the Ho recognition site, allowing us to control the number and position of DNA breaks during induction of ScHo, as well as to track which donor sequence is used as template. We analyzed two aspects: viability, that reflects both the efficiency of the cut and the success of repair; and molecular structure of repaired loci, in order to reveal which repair pathways were used. We now show that HMR is also cut by Ho in wild-type strains of *C. glabrata*. In addition, by mimicking *S. cerevisiae*’s situation, in which HML and HMR are protected from the cut, we unexpectedly find that one DSB at the MAT locus is sufficient to induce cell death, whereas one at HML or HMR is not. Finally, the use of the CRISPR-Cas9 technology enables us to show that mating-type switching can be induced independently of the Ho protein in *C. glabrata*, and that such switching is efficient and not lethal. Thus, we show for the first time that a chromosomal DSB is repaired by HR efficiently in *C. glabrata*, at HML and HMR (Ho-cut) and at MAT (Cas9-cut), indicating that, in principle, MAT switching could occur in this species. The fact that an Ho endonuclease, able to induce switching, also induces cell death may be evidence for degeneration of the three MTLs/Ho system in this species, in accordance with the observed asexuality.

**Results**

**All three sites are cut by Ho in *C. glabrata*, including the one at HMR**

We expressed *S. cerevisiae*’s HO gene (ScHO) using the URA3 selectable plasmid p7.1 in which ScHO is under control of the inducible MET3 promoter [26]. As previously described,
Table 1. Strains used in this work.

| Strains                  | Parent  | Genotype                        | Reference |
|--------------------------|---------|---------------------------------|-----------|
| CBS138                   |         | HMLα/MATA α HMRα               | [27]      |
| BG2                      | CBS138  | HMLα/MATA α HMRα               | [28]      |
| BG14                     | BG2     | HMLα/MATA α HMRα ura3Δ::Tn903 G418 Δ | [28]      |
| BG87                     | BG14    | HMLα/MATA α HMRα ura3Δ::NeoR his3Δ | [29]      |
| HM100                    | CBS138  | HMLα/MATA α HMRα ura3Δ::KANMX   | [21]      |
| HM100 Δrad51             | HM100   | HMLα/MATA α HMRα Δrad51 ura3Δ::KANMX | This work.|

C. glabrata strains with mutated Ho sites

| Strains                  | Parent  | Genotype                        | Reference |
|--------------------------|---------|---------------------------------|-----------|
| YL01                     | HM100   | HMLα/MATA α HMRα ura3Δ::KANMX   | This work.|
| YL02                     | HM100   | HMLα/MATA α HMRα ura3Δ::KANMX   | This work.|
| YL03-MATα                | YL02    | HMLα/MATA α HMRα ura3Δ::KANMX   | This work.|
| YL04                     | YL07    | HMLα/MATA α HMRα ura3Δ::KANMX   | This work.|
| YL05                     | YL09    | HMLα/MATA α HMRα ura3Δ::KANMX   | This work.|
| YL07                     | YL02    | HMLα/MATA α HMRα ura3Δ::KANMX   | This work.|
| YL09                     | YL01    | HMLα/MATA α HMRα ura3Δ::KANMX   | This work.|
| YL10                     | YL07    | HMLα/MATA α HMRα ura3Δ::KANMX   | This work.|
| SL09                     | BG87    | HMLα/MATA α HMRα ura3Δ::NeoR his3Δ | This work.|
| SL0A                     | YL01    | HMLα/MATA α HMRα ura3Δ::KANMX   | This work.|
| SL0B                     | YL04    | HMLα/MATA α HMRα ura3Δ::KANMX   | This work.|

C. glabrata strains with mutated Ho sites and/or deletion(s) of HML, MAT or HMR

| Strains                  | Parent  | Genotype                        | Reference |
|--------------------------|---------|---------------------------------|-----------|
| CGM460                   | BG14    | Δhml MATA α HMRα ura3Δ::Tn903 G418 Δ | [22]      |
| CGM390                   | BG14    | HMLα/MATA α HMRα ura3Δ::Tn903 G418 Δ | [22]      |
| SL-CG1                   | CGM390  | HMLα/MATA α Δhmr ura3Δ::Tn903 G418 Δ | This work.|
| CGM498                   | BG14    | Δhml MATA α HMRα ura3Δ::Tn903 G418 Δ | [22]      |
| SL01                     | BG87    | HMLα/MATA α Δhmr ura3Δ::NeoR his3Δ | This work.|
| SL-CG8                   | SL01    | HMLα/MATA α Δhmr ura3Δ::NeoR his3Δ | This work.|
| SL-CG9                   | CGM460  | Δhml MATA α HMRα ura3Δ::Tn903 G418 Δ | This work.|
| SL-CG10                  | SL01    | HMLα/MATA α Δhmr ura3Δ::NeoR his3Δ | This work.|
| SL-CG12                  | CGM390  | HMLα/MATA α Δhmr ura3Δ::Tn903 G418 Δ | This work.|
| SL-CG14                  | CGM460  | Δhml MATA α HMRα ura3Δ::Tn903 G418 Δ | This work.|

expression of ScHO in wild-type strains of C. glabrata, leads to the death of about 99.9% of cells and we found that both MAT and HML are efficiently cut [26]. We further analyzed surviving colonies of HM100 (HMLα/MATA α HMRα, Table 1) by determining the mating-type at each MTL locus by PCR and we found that nearly all present switching at HMR, indicative of cutting (Table 2).

In order to formally confirm that mating-type switching at each MTL locus depends on HR, we inactivated RAD51 (CAGL0I05544g) in the wild-type strain HM100 (Table 1). Inducing the Ho DSB in this strain leads to an even higher lethality than in the wild-type strain (Fig 2), and no mating-type switching is detected at any MTL locus (Table 2), confirming that switching relies on HR in C. glabrata.

As we hypothesized in our previous work [26], Ho-induced lethality in C. glabrata could be due to concomitant induction of multiple DSBs, in contrast to the situation in S. cerevisiae where HML and HMR are protected from the cut. These unrepairable cuts would lead to death by cell cycle arrest, or because cut and possibly degraded chromosomes segregating into daughter cells lack essential genes. Alternatively, we had mentioned the possibility that
switching leads to the repair of the Ho-cut locus by an intact Ho-site containing locus would cause never-ending cycles of cutting and repair that could also lead to cell death in our conditions of continuous induction on plates. We had dismissed this eventuality as unlikely, since the percentage of lethality and switched survivors is highly reproducible in our experiments. Even though the situation is the same in *S. cerevisiae* when overexpressing Ho with a galactose-inducible promoter, and no switching back and forth between the two mating-types has been reported in *S. cerevisiae* [14,19], we had no experimental proof that this did not happen in *C. glabrata*, in our experimental conditions. We thus decided to design experiments which would address both the question of the number of cut loci, and the question of the number of cuts per locus. For this, as explained below, we have used strains containing inconvertible Ho sites (*Inc*) and/or deletions of *MTL*s and we have performed a time-course experiment of induction (see Material and methods).

### Cleavage at *HML* and *HMR* is not an important contributor to lethality

As soon as we started performing experiments using strains with inconvertible *MTL*s, we noticed difference of behavior between the *MAT* locus and *HML* and *HMR*. As soon as we started performing experiments using strains with inconvertible *MTL*s, we noticed difference of behavior between the *MAT* locus and *HML* and *HMR*. We first used strains YL05 (*HMLalpha MATa-inc HMRa-inc*) and YL07 (*HMlalpha-inc MATalpha-inc HMRa*), Table 1). In these strains, we mutated the Ho sites in the region known to be essential for Ho cutting in *S. cerevisiae* [30], yielding loci *MAT-inc*, *HML-inc*, *HMR-inc* (S1 Fig), in configurations where only either the *HML* or *HMR* locus can be cut and repaired by the two non-cleavable donors *MAT-inc*, and *HMR-inc* or *HML-inc*.

Upon Ho induction, cell viability drastically increases to 35 to 55%, depending on the strain (Fig 2). Survival does not reach 100% but is more than 2.700 times higher than in the wild-type isogenic strain, HM100 (HM100 vs YL05 *P*-value < 10⁻⁴ and HM100 vs YL07 *P*-value < 10⁻⁹, Wilcoxon tests) (Fig 2). We then expressed *HO* in a strain in which only *MAT* is protected

### Table 2. Molecular structure of *MTL*s in surviving colonies after ScHo induction.

| Strain | Locus screened | PCR results | Percentage of switch |
|-------|----------------|-------------|----------------------|
| **C. glabrata strains with wild-type Ho sites** | | | |
| HM100 wt (*HMLalpha MATalpha HMRa*) | HML | 25/34 mixed HMLalpha/a 9/34 pure HMLalpha | 74 ± 6% |
| | MAT | 26/34 mixed MATalpha/a 8/34 pure MATalpha | 76 ± 6% |
| | HMR | 30/34 mixed HMRalpha/alpha 2/34 pure HMRalpha 2/34 pure HMRa | 94 ± 6% |
| HM100 Δrad51 (*HMLalpha MATalpha HMRa*) | HML | 39/39 pure HMLalpha | 0% |
| | MAT | 39/39 pure MATalpha | 0% |
| | HMR | 39/39 pure HMRa | 0% |
| **C. glabrata strains in which MAT is protected from the Ho cut** | | | |
| YL05 (*HMLalpha MATa-inc HMRa-inc*) | HML | 60/60 pure HMLa-inc | 100 ± 8% |
| YL07 (*HMLalpha-inc MATalpha-inc HMRa*) | HMR | 34/36 mixed HMRalpha-inc 2/36 pure HMRalpha-inc | 100 ± 6% |
| **C. glabrata strains in which MAT can undergo the Ho cut** | | | |
| SL-CG8 (*HMLalpha-inc MATa Δhmr*) | MAT | 36/36 pure MATalpha-inc | 100 ± 6% |
| SL-CG9 (*Δhml MATa HMRalpha-inc*) | MAT | 36/36 pure MATalpha-inc | 100 ± 6% |

Colonies surviving ScHo induction are screened by PCR at each locus that can be cut by ScHo. Percentage of switch is calculated as the ratio of the total number of pure and mixed colonies exhibiting mating-type switching divided by the total number of surviving colonies tested, expressed as percentage. The square root of the number of surviving colonies screened is used as standard error in last column.

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from the cut, while both \textit{HML} and \textit{HMR} can be cleaved by Ho (Strain YL04 \textit{HML}\textit{alpha}-\textit{inc} \textit{HMR}\textit{a}, Table 1). In this strain, cell viability reaches \textasciitilde 20\% which is 2.000 times higher than in the wild-type isogenic strain, HM100 (P-value \textless 0.001, Wilcoxon test) (Fig 2).

We analyzed the molecular structure of the \textit{HML} and \textit{HMR} loci in surviving colonies of strains YL05 and YL07 respectively, by PCR using primers specific of the mating-type carried by the \textit{MTLs} ("alpha" or "a", wt or inc, S1 Table and S2 Fig). This allows the distinction of the original \textit{HML} or \textit{HMR} locus from the repaired locus that has become resistant to cutting. We found that 100\% of surviving colonies showed mating-type switching of \textit{HML} or \textit{HMR} (Table 2). As mating-type switching reflects the efficiency of the Ho-cut, this suggests that both \textit{HML} and \textit{HMR} are efficiently cut by \textit{ScHo} and that, even though we found some mixed colonies for \textit{HMR} in strain YL07, \textit{MTLs} they are repaired by HR.

In order to confirm this, we induced expression of \textit{ScHo} in strains in which both \textit{HML} and \textit{HMR} can be cut by Ho in absence of any repair template, the two other loci being completely deleted (strain SL-CG1, \textit{HML}\textit{alpha} \textit{Delta} \textit{mat} \textit{Delta} \textit{hmr} and strain CGM498, \textit{Ahml} \textit{Delta} \textit{mat} \textit{HMRa}, Table 1) (Fig 2). Upon Ho induction, we found that survival rate does not exceed 0.2\% in both strains, suggesting that the Ho-cut is efficient at both \textit{HML} and \textit{HMR} and that the Ho-DSB at \textit{HML} and \textit{HMR} causes lethality when it cannot be repaired by HR. To explore this further, we induced \textit{ScHo} in a strain in which both \textit{HML} and \textit{HMR} can be cut and \textit{MAT} is deleted (strain CGM390 \textit{HML}\textit{alpha} \textit{Delta} \textit{mat} \textit{HMRa}, Table 1) (Fig 2). Once again, survival does not exceed 0.2\%, suggesting that \textit{HML} and \textit{HMR} are cut concomitantly and thus cannot serve as templates for

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**Fig 2. Survival to ScHo induction of strains bearing combinations of wild-type and/or mutated Ho sites.** On the left, diagram of \textit{MTL} configuration of strains is shown with the corresponding survival histogram to ScHo induction on the right. The blue box represents the Ya, the red box Yalpha, the yellow bar wild-type Ho site and the crossed circle mutated Ho site (\textit{Inc} loci) (not to scale). On the histogram, black bars are for strains from the BG87 background, grey bars are for strains from the HM100 background. Results for strains HM100 and BG87 are from (26). Values from, at least, four experiments were averaged, the SEM used as estimate of the error and the P-value was calculated using the Wilcoxon test. \(* * * : \text{P-value} < 0.001. \text{ns: non-significant.}

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one another. This reinforces the hypothesis that simultaneous DSBs, happening in wild-type strains, participate in the high lethality observed.

Altogether, these results suggest that the efficient Ho-cut at HML and HMR is not an important contributor to lethality in all configurations where they can be repaired by HR. In the absence of HR, no other mechanism such as NHEJ is able to take over the repair of the Ho-cut, and thus cell survival remains low. Results also show that protecting the MAT locus from the Ho-cut significantly increases survival. This is also confirmed by the fact that strains containing no Ho-site at any MTL locus survive the Ho induction at around 50% (strains YL10, YL09 and SL09, Table 1) (Fig 2).

A single ScHo-DSB at MAT is sufficient to induce cell death in C. glabrata

In order to measure the impact of the Ho cleavage at MAT on cell survival, we mimicked the situation in S. cerevisiae, where a single recipient of the Ho-induced DSB, the MAT locus, can be repaired by the two non-cleavable donors HML and HMR, i.e. strains YL03-MATalpha (HMLalpha-inc MATalpha HMRa-inc) and YL03-MATa (HMLalpha-inc MATa HMRa-inc) (Table 1). Expression of ScHO in those strains leads to a lethality similar to the one obtained in wild-type strains HM100 and BG87 (Fig 3) and all surviving colonies have switched, whatever the mating-type at MAT (Table 2).

Thus, a single Ho-induced DSB at MAT, whatever its mating-type, is sufficient to induce massive cell death in C. glabrata. Furthermore, this experiment allows us to reconsider the question of lethality due to never-ending cycles of cutting and repair. Indeed, if this was the reason for mortality of cells when cutting at MAT, then, since repairing with an Inc locus leads to an unswitchable locus, the mortality should be decreased in these strains upon induction.

Lethality is not due to toxic recombinational repair intermediates

Since we know that the mating-type switching system in C. glabrata is not very efficient, it is legitimate to wonder whether the degeneration of the mating-type switching mechanism

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Fig 3. Survival to ScHo induction of strains in which only the MAT locus can be cut. On the left, diagram of MTL configuration of strains is shown with the corresponding survival histogram to ScHo induction on the right. The blue box represents the Yα, the red box Yαlpha, the yellow bar wild-type Ho site and the crossed circle mutated Ho site (Inc loci) (not to scale). On the histogram, black bars are for strains from the BG87 background, grey bars are for strains from the HM100 background. Values from, at least, four experiments were averaged, the SEM used as estimate of the error and the P-value was calculated using the Wilcoxon test. ns: non-significant.

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could lead to abnormal HR intermediates. We asked whether such repair intermediates could be toxic and cause death. For example, the two ends of the broken MAT locus could invade both HML and HMR, leading to non-resolvable structures. The fact that HMR is not on the same chromosome as HML and MAT could be an additional problem, if, for example, repair of MAT occurs principally with HMR and this leads to lethal rearrangements.

In order to test this, we constructed two strains in which MAT can be cut by Ho and can only be repaired either by HML or by HMR (SL-CG8, HMLalpha-inc MATa Δhmr, and SL-CG9, Δhml MATa HMRalpha-inc, respectively, Table 1). Expression of ScHO in both strains leads to a high lethality (Fig 3), similar to the ones of the wild-type or YL03 strains (HMLalpha-inc MATalpha or MATa HMRα-inc, Table 1). We analyzed the molecular structure in surviving colonies (Table 2). All have switched, whatever the location of the repair template (HML in strain SL-CG8 and HMR in strain SL-CG9).

Thus, forcing MAT to repair solely on HML or HMR results in the same lethality as in wild-type strains. From this, we conclude that the cut MAT locus DNA ends probably do not interact with both HML and HMR in such a way that it is toxic to cells, and that there is no specific problem due to the fact that HMR is on another chromosome than MAT. It, therefore, seems unlikely that lethality could be due to non-resolvable HR intermediates.

**Time course experiments reveal that growth arrest is quick and irreversible leading to cell death**

In order to shed light on whether the toxic effect leading to cell death could be reversible and if not, whether the effect operates rapidly or not, we performed a time course experiment in which Ho is induced in liquid medium and its expression is repressed, at different time points, by plating cells on repressive medium. The survival can thus be calculated by the ratio of colonies obtained on repressive medium to the theoretical number of cells plated.

In order to easily follow events, we used again strain SL-CG9 (Δhml MATa HMRα-inc, Table 1), where only the MAT locus can be cut and a single inconvertible template for HR is present. This allows to follow a single Ho-cut and a single repair event at the MAT locus. We scored the survival along the time-course experiment as well as the percentage of survivors that have undergone switching. As shown on Fig 4, two hours upon induction of a single Ho-
DSB at MAT, survival drastically drops to less than 2%. From T = 4 hrs to T = 15 hrs, survival remains very low. Molecular analysis shows that mating-type switching in survivors reaches its maximum very rapidly after four hours of induction, at around 90%. All screened colonies display a pure genotype in PCR just as on solid medium (see Table 2). The last two points of our experiment, T = 19 hrs and T = 38 hrs show that survivors have invaded the liquid culture, thus giving rise to many colonies on repressive medium plates. These survivors thus consist of 90% of switched inconvertible clones, and around 10% of cells that have escaped Ho-induction probably by rearranging or mutating the plasmid. These results show that the toxic effect of inducing an Ho-cut at the MAT locus is irreversible even after only two hours in induction medium.

We next wanted to check whether mortality was as quick and as irreversible in wild-type strains. Induction in HM100 leads to the same pattern as SL-CG9 (Fig 4). This confirms that lethality in HM100 is not due to never-ending cycles of cutting and repair since it cannot happen in SL-CG9 and the lethality occurs at the same rate.

The lethality induced by the DSB at MAT is specific to ScHo

We wanted to investigate whether the lethality is caused by the DSB at MAT per se or by the specific combination of MAT with ScHo. Since we have previously shown that inducing other Ho endonucleases from the Nakaseomyces, including C. glabrata’s own gene, does not result in high lethality nor efficient mating-type switching [26], we decided to use the CRISPR-Cas9 system from [31]. This system relies on a unique URA3 plasmid, pCGLM1, in which the CAS9 gene is under the control of the inducible MET3 promoter, as is the case for the HO gene in the p7.1 plasmid used in the experiments above. This allows us to induce a DSB at MAT with Cas9, in the same conditions as with ScHo.

In order to allow a full comparison between ScHo and Cas9 induction, we wanted to generate a single DSB at the same locus, here MAT, whatever the endonuclease used. Indeed, the Ho site between MTLa and MTLalpha is very different while the Cas9-cut is directed with a specific gRNA, we used SL-CG8 and SL-CG9 (HMLalpha-inc MATa Δhmr, and Δhml MATa HMRalpha-inc, respectively, Table 1) with a gRNA that targets only the MAT locus, since it is directed to the Ya sequence (Fig 5A). This gRNA allows to target the Cas9-cut from 14 pb from the Ho cut (Fig 5A).

Fig 5B shows that induction of the Cas9-cut in both SL-CG8 and SL-CG9 does not lead to any lethality. We wondered whether Cas9 had indeed cut the MAT locus by screening mating-type switching of surviving colonies by PCR (Table 3).

Results show that the Cas9-induced DSB leads to mating-type switching as efficiently as the Ho protein (Table 2 and Table 3). Indeed, 72 to 85% of the colonies tested presented “alpha-inc” information at MAT, confirming the cut of this locus by Cas9 and induction of mating-type switching (Table 3). This gRNA is adjacent to an optimal NGG PAM sequence and targets a site very close to the Ho site, 14 pb away from the Ho cut.

We then asked whether we could detect a transient lethality, reflecting the Cas9-cut. Indeed, previous experiments, using the same system for inducing a Cas9-cut, but at the unrelated ADE2 locus, have shown that a transient lethality occurred in liquid culture in a time-course experiment, whereas no apparent lethality was detected on induction plates [31]. Thus, we performed a time-course experiment with Cas9 in strain SL-CG9. Fig 5D shows that Cas9 induction never leads to a sharp increase in lethality at any time point and survival remains between 65 and 92% for the whole experiment. Surprisingly, contrary to what we observe in induction on plates, screening of mating-type switching at MAT reveals that only ~20 to 36% of surviving colonies have switched mating-type (see Discussion).

These results show for the first time that mating-type switching can be induced without any lethality in C. glabrata using the CRISPR-Cas9 system. In conclusion, the fact that the MAT
Fig 5. Survival upon Cas9 induction and gRNAs used. (A) gRNA Ya2 targeting the MATa locus of *C. glabrata*. Sequence shown is a segment of the MATa locus of BG87, including the gRNA in bold and the PAM sequence in red. Plain double arrow indicates the Ho cleavage site and dashed double arrow the Cas9 cleavage site. (B) Survival of strains SL-CG8 and SL-CG9 upon Cas9-induced DSB at MAT. Induction is performed on solid medium. Results for strains SL-CG8 and SL-CG9 upon ScHo induction are from Fig 3. Values from four experiments were averaged, the SEM used as estimate of the error and the P-value was calculated using the Wilcoxon test. **: P-value < 0.001. (C) Induction was performed in liquid during a time-course experiment for strain SL-CG9 expressing Cas9 (harboring pCGLM1-Ya2). The Y-axis represents both the survival (curve) expressed as a percentage, and the percentage of switched colonies (histogram). Survival is calculated by comparing the number of colony-forming units on SC-Rep with the number of cells plated, as estimated by counting; and is normalized by dividing it by the survival rate of the control strain, i.e., strain SL-CG9 transformed by pCGLM1 for Cas9 induction, grown in the same conditions. For survival rate, values from four experiments were averaged and the SEM is used as estimate of the error. For the percentage of switched colonies, the square root of the number of surviving colonies screened is used, i.e., sqrt of 36. For time-course experiments, at points T = 17 and T = 21 hrs, no PCR of surviving colonies was performed.

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locus can be cut and repaired by HR without any accompanying high lethality demonstrates that it is ScHo cutting specifically at the MAT locus that is highly lethal in C. glabrata.

**Choice of repair template reveals a complex interplay between the MTL loci**

As the three MTLs are efficiently cut by ScHo and as the cut at MAT is the only one to lead to a high lethality, we decided to study these differences in C. glabrata by asking how each locus interacts with the two other templates.

In wild-type strains of S. cerevisiae, only the MAT locus is cut, and the mechanism of mating-type switching is productive thanks to control by the sexual identity of the cell. Indeed, the use of the donor locus of the opposite mating-type to repair the DSB at MAT is promoted (“donor preference”) [18]. Since it has been shown that sexual identity of cells is maintained in C. glabrata, at least in MATa cells (i.e., MATa cells express MATa identity, but MATα cells have no mating-type specific expression of key genes [21,22]), we asked whether this “donor preference” from S. cerevisiae is conserved in C. glabrata at the MAT locus, and also whether HML and HMR use a preferential template for repair. For this, we constructed strains that carry different and inconvertible mating-types in various combinations. First, for the MAT locus, we used strains YL03-MATα and YL03-MATa (HMLα-inc MATα Δhmr and HMLα-inc MATa HMRα-inc, respectively, Table 1). Analysis of surviving colonies from strain YL03-MATa shows that 78% display only the alpha-inc information at MAT, the remainder exhibiting various genotypes, pure a-inc for 3%, and mixed alpha-inc and a-inc for 19% (Table 4). The overwhelming percentage of pure colonies displaying only the alpha-inc information at MAT demonstrates that HML is the preferred template for repair of the MATa locus.

In strain YL03-MATα: 84% of tested surviving colonies display only the MATα-inc genotype, the others displaying various mixed or pure genotypes at MAT (Table 4). Thus, in contrast to S. cerevisiae, HML is preferentially used as template for repair in C. glabrata, whatever the mating-type at MAT.

In order to know by which template HML is preferentially repaired, we performed a molecular analysis of the HML locus, upon ScHo induction, in a strain that carries different and inconvertible mating-types at MAT and HMR (strain SL0A, HMLα-inc MATα-inc HMRα-inc, Table 1). Analysis of surviving colonies shows that 47% display pure alpha-inc information, 40% are alpha-inc and a-inc in mixed colonies and 13% are pure a-inc (Table 4). This indicates that HML preferentially repairs the DSB using MAT over HMR.

In the same way, molecular analysis of the HMR locus was performed in strain SL0B (HMLα-inc MATα-inc HMRα, Table 1). Table 4 shows that, 9% of surviving colonies are pure a-inc and 84% display both a and a-inc information We observe no repair event where
**Table 4. Analysis of template choice for repair of Ho cut at MTLs.**

| Strain                      | Locus screened | PCR results                        | Use of each locus |
|-----------------------------|----------------|------------------------------------|-------------------|
| YL03-MATα (HMLα-inc MATα HMRα-inc) | MAT            | 25/32 pure MATα-inc               | HML: 96%          |
|                            |                | 6/32 mixed MATα-inc/a-inc         | HMR: 4%           |
|                            |                | 1/32 pure MATα-inc                |                   |
| YL03-MATα (HMLα-inc MATα HMRα-inc) | MAT            | 42/50 pure MATα-inc               | HML: 95%          |
|                            |                | 5/50 mixed MATα-inc/a-inc         | HMR: 5%           |
|                            |                | 2/50 pure MATα-inc                |                   |
|                            |                | 1/50 pure MATα                     |                   |
| SL0A (HMLα-inc MATα HMRα-inc) | HML            | 33/69 pure HMLα-inc               | MAT: 79%          |
|                            |                | 20/69 mixed HMLα-inc/a-inc        | HMR: 21%          |
|                            |                | 9/69 pure HMLα-inc/pur            |                   |
|                            |                | 7/69 mixed HMLα/α-inc/a-inc       |                   |
| SL0B (HMLα-inc MATα HMRα)   | HMR            | 38/45 mixed HMRα/a-inc            | HML: ~100%        |
|                            |                | 4/45 pure HMRα-inc                | MAT: ~0%          |
|                            |                | 3/45 pure HMRα                    |                   |

In this experiment, strains are chosen so that template choice for Sc Ho-cut can be analyzed. As before, colonies are screened by PCR at the single locus that can be cut by Sc Ho. In the first three strains, since the percentage of pure colonies alone is sufficient to know which template is preferentially used for repair, the percentage of the use of each locus is calculated by only taking into account the number of pure switched surviving colonies (i.e., we did not sub-clone mixed colonies and we also omitted the pure unswitched colony obtained in strain YL03-MATα). The use of each locus is calculated as the ratio of the number of colonies showing the use of that locus on the total number of pure switched surviving colonies screened, expressed as percentage. In the case of strain SL0B, we fail to detect by PCR the use of MATα-inc to repair HMR, thus we estimate that in switched colonies, the use of HML is of the order of 100%.

**MATα-inc** was used as template. HML is thus the preferred template for the repair of HMR.

Overall, these results show that **MAT** and **HML** preferentially repair on each other and that **HMR** is preferentially repaired by **HML**.

**Exploring the residual lethality in the absence of MTL-cuts**

When cuts are non-repairable by HR; i.e., the three strains containing wild-type Ho sites: HM100 Δrad51 (HMLα MATα HMRα), SL-CG1 (HMLα Δmat HMRα) and CGM498 (Δhml Δmat HMRα) and inexplicably, the YL03 strains (HMLα-inc MATα or α HMRα-inc) (Table 1), 99.9% of the cells die. As shown above, in other strains where the Ho-cut is repaired by HR (at HML and HMR) and in strains where there is no Ho cut (Y09, YL10 and SL09, Table 1) survival varies between 20% and 61%, never reaching 100%. This residual lethality can be explained by a general toxic effect of the expression of a heterologous protein in *C. glabrata* or by of the existence of (a) cryptic unrepairable Ho site(s) elsewhere in the genome. We thus decided to test this residual lethality in other strains where no cutting occurs. For this, we constructed strains where one MTL is deleted and two are inconvertible, SL-CG10 (HMLα-inc MATα Δhmr), SL-CG12 (HMLα-inc Δmat HMRα-inc) and SL-CG14 (Δhml MATα-inc HMRα-inc) (Table 1). Upon Ho induction, survival in these three strains is around ~80%, whatever the MTL deleted (Fig 6). Comparing the survival of SL-CG10, SL-CG12 and SL-CG14 to SL09 (HMLα-inc MATα-inc HMRα-inc) (same genetic background and highest survival in our previous experiment, i.e., 60%) show that the increase in survival is significant (P-value<0.05 in each pairwise combination of SL09 with SL-CG10, SL-CG12 and SL-CG14, Wilcoxon tests). If the residual lethality observed was due to a general toxic effect or to (an) extra Ho site(s), the lethality would be the same in those strains as in strains that are inconvertible for the three MTLs (YL10, YL09 and SL09) (Fig 3) since the constructions do not modify the context outside of the MTL loci.
Since the only difference between those strains is the number of inconvertible sites and thus potential binding sites for ScHo, we believe this may explain residual lethality by binding of ScHo (see Discussion).

### Discussion

Mating-type switching is a highly regulated mechanism that relies on a chromosomal DSB. DSBs are a major threat for genome integrity [32]. Repair of such damage is thus essential and can be achieved through Rad51-dependent HR which involves many steps in order to succeed: search for homology involving Rad51 and Rad52 in *S. cerevisiae*, copy on the donor locus and displacement and resolution of the double Holliday junction [33]. In *S. cerevisiae*, the DSB at the MAT locus is repaired by HR using HMR or HML as template, depending on the original mating-type of the cell. *C. glabrata* does not switch mating types spontaneously at high frequency [24]. We have previously shown that overexpression of HO genes from *C. glabrata* and related species fails to induce efficient mating-type switching and that switching can be efficiently induced by expressing ScHO, but that it is lethal to most cells [26]. Our previous work also showed that the HML locus is cut in *C. glabrata*; something that never happens in wild-type strains of *S. cerevisiae* [26]. In this work, we aimed at understanding the link between mating-type switching and cell death in *C. glabrata*. First, we show that HMR is also cut in wild-type strains of *C. glabrata*, overexpressing ScHO. Next, we constructed strains with mutated uncuttable Ho sites (inconvertible, Inc) and with deleted MTL loci, in order to examine survival to individual DSBs at the different MTL loci as well as knowing which MTL has been used as template for repair.

In *S. cerevisiae*, the donor preference mechanism ensures an efficient mating-type switching at MAT by promoting the use of the template from the opposite mating-type, in repair [18]. We found, in *C. glabrata*, that whatever the mating-type at MAT, HML is preferentially used as template for repair.
template for repair. Thus, donor preference from *S. cerevisiae* seems not to be conserved in *C. glabrata*. In its absence, it also shows that the length of the sequence homology does not influence the use of the donor for repair. Indeed, the Ya and Yalpha segments, determining mating-type, share no homology and we observe that MATa, containing Ya, repairs preferentially using HMLalpha, containing Yalpha. We also demonstrate that HML is preferentially repaired using MAT and that HMR is preferentially repaired using HML, revealing a complex interplay between those different chromosomal segments. Loss of donor preference at MAT, along with the fact that the *C. glabrata* endogenous Ho protein fails to induce efficient mating-type switching [26], point to the possible degeneration of the mating-type switching system in *C. glabrata*. Thus, rules of DSB repair by HR observed in our inductions may reflect true preferences, independently of the MAT/Ho switching system. When the DSB can be repaired by HR using a template on the same chromosome or another chromosome, the intra-chromosomal template will be mostly chosen over the other (HML repairs preferentially with MAT and vice versa). When the DSB can only be repaired by an extra-chromosomal template, the sub-telomeric HMR was repaired preferentially using the sub-telomeric template HML. We can speculate that HR using a template on a same chromosome is preferred for DSB repair and in the absence of such a template, the sub-telomeric loci are repaired using other sub-telomeric loci.

In our previous work, we hypothesized that multiple DSBs at the MTL loci would be unrepairable and that this was the cause for lethality when mating-type switching is induced. As cited above, to mimic *S. cerevisiae*’s situation, in which MAT is the only recipient of the Ho cut, we mutated the Ho site at both HML and HMR. We are now able to demonstrate that one ScHo-DSB at the MAT locus is sufficient to induce cell death at a similar level to wild-type cells, thus invalidating our previous hypothesis. This means that, even in the presence of two intact homologous sequences, the MAT locus is not able to repair the break. More surprisingly, the DSB at MAT is only lethal when it is performed by the ScHo protein. We show, for the first time, that mating-type switching can be triggered efficiently by CRISPR-Cas9, thus independently of the Ho protein, in *C. glabrata*. This has been shown only recently in the model species *S. cerevisiae* [34]. No lethality is observed after a Cas9-DSB at MAT on plates. In liquid induction experiments, we observe a transient lethality of ~35% and a lower efficiency of switch (~30%). In both plate and liquid inductions, the Cas9 expression plasmid is constantly under selection pressure. We believe that observed discrepancies can be explained if induction is less efficient on individual cells in liquid medium than on plates, implying that cells in liquid medium can mutate the plasmid before switching (in the CAS9 gene or its promoter in such way that CAS9 is not expressed anymore, or in the gRNA sequence). These cells will never switch and can invade the culture. In contrast, some cells undergoing switching may not recover (~35% of lethality) but the ones that have survived the switch become “immune” to the Cas9-DSB, thus alleviating the need for mutating the Cas9 plasmid. Nonetheless, these cells may grow more slowly because of the maintenance of CAS9’s expression and are thus less likely to be in excess in the culture. Thus, these MAT-switched cells will be diluted and less represented on repressive plates.

To go back to the lethality induced by ScHo, unless the difference in the lethality with Cas9 is due to the 14 bp shift in cutting, which seems highly unlikely, these results suggest that the ScHo protein prevents DSB repair specifically at the MAT locus of *C. glabrata*, in such a way that 99.9% of the cells die. We have recently published that, in heterothallic strains of *N. delphensis*, overexpression of ScHo with the same plasmid as in this work leads to efficient switching, without lethality [35]. It is surprising that ScHo could have a deleterious effect in a locus- and species-specific manner. As in all three-loci-based mating-type switching systems, the three MTL loci of *C. glabrata* share identical sequences and only differ by the mating-type carried and/or their location in the genome [36]. We know that mating-type does not influence
lethality in any of our experiments. Thus, only the location of the MAT locus could explain the
specificity of lethality induced by ScHo. The MAT locus is located in a central region on chromo-
some B whereas HML and HMR are positioned in sub-telomeric regions on chromosome B and E, respectively [36]. Thus, the ScHo specificity for MAT could only be achieved either
through the structure of the chromatin or through the flanking sequences of the MAT locus. If
the ScHo protein causes lethality by preventing repair at MAT, it is perhaps because it gets
stuck at MAT, after performing the DSB, preventing recruitment of recombination proteins
and thus repair of the locus. In S. cerevisiae, it is possible to follow the fate of repairable and
non-repairable ScHo-cuts at MAT by Southern-blot analysis from a time-course experiment of
the wild-type strains, overexpressing ScHo [37,38]. S3 Fig shows that, upon Ho induction, we
are not able to visualize cut chromosomal arms at MAT. This could be explained if ScHo stays
bound on the DNA end(s) and prevents HR at this locus: continuous resection will degrae
dNA ends so that the probe cannot hybridize anymore. Indeed, in S. cerevisiae, unrepairable
ScHo-cuts are more extensively degraded than repairable ones [37]. Nonetheless, we can note
that we clearly observe the parental uncut band before induction and that this band disappears
completely as soon as we start the experiment, thus confirming that the MAT locus is effi-
ciently cut in our system.

Outside of its role in inducing a lethal DSB at MAT, ScHo displays further toxicity in C.
glabrata. Strains that are inconvertible for the three MTLs exhibit a survival of 61% at the
most, and lethality is strongly reduced by the deletion of one MTL, whatever its position,
HML, MAT or HMR. The difference between those two types of strains is the number of
invertible (Inc) Ho sites present at the MTL loci. One explanation for this would be that the
ScHo protein binds the Inc Ho sites and gets stuck there, in a way that is toxic for C. glabrata
cells. ScHo probably has a high affinity for the C. glabrata Ho sites since we know that they are
cut very efficiently and rapidly. In the case of an Inc site, the protein may be stuck there
because the substrate is not transformed into a product. Indeed, we hypothesize that the cut
releases the endonuclease and as we speculated above, this is prevented at the MAT locus, even
after cutting, possibly by the chromatin structure. On the Inc sites, the binding of ScHo does
not induce massive cell death but could, for example, physically hinder replication forks and
thus disrupt DNA replication and cell division. Performing a ChIP-PCR on the Ho protein to
examine its binding on the three MTL loci would allow us to better explore this aspect.

To put our results back into an evolutionary perspective and explore the link between
switching and sexuality, we can turn to the other Nakaseomyces species. This group comprises
C. glabrata, two other pathogens, Candida nivariensis and Candida bracarensis and three envi-
ronmental species, N. delphensis, Candida castelli, Nakaseomyces bacillisporus. This clade con-
tains species both varied in their lifestyle and in their lifecycle; all Candida species are described
as asexual and haploid and in the two sexual species, N. delphensis is an obligate haploid while
N. bacillisporus is an diploid [27]. N. delphensis undergoes natural Ho-induced mating-type
switching, just like S. cerevisiae [35]. It is striking that none of the asexual species exhibit switching,
thereby reinforcing the notion that switching is a mechanism that favours sexual reproduc-
tion. It is also remarkable, in such a case, that all asexual species have conserved the three MTL
and a highly similar HO gene [25,36,39] while switching is supposed to be a mechanism that
favours sexual reproduction. It is understandable that such a dangerous mechanism, involving a
chromosomal DSB, would be lost if it is not essential. It has been hypothesized that C. glabrata
sometimes undergoes switching [40,41]. These events could indeed be the result of very rare
Ho-induced cuts or could also be fortuitous gene conversion events independent of the Ho pro-
tein (replication accident, repair of accidental DSBs etc). If we accept that this system is largely
non-functional in C. glabrata and possibly other asexual species, the question of why they have
kept both HO and the MTL remains open. In addition, it has been reported that the HO gene is
under purifying selection in the population of *C. glabrata* strains analysed [25]. This may suggest that both Ho and the MTLs have acquired another function in the asexual *Nakaseomyces*. In *S. cerevisiae*, the MTL loci are one of the very few structured regions of the genome [42] and there remains an intriguing possibility that the conserved function of these MTLs and the Ho protein would be in structuring the chromosomes bearing them. Further studies of the 3D structure of the *C. glabrata* nucleus would shed light on this point.

**Materials and methods**

**Strains, cultures, and transformation**

*C. glabrata* strains used in this study are listed in Table 1. Strains are grown in broth or on plates at 28°C in YDP (non-selective, 1% Yeast Extract, 1% Peptone, 2% glucose), in Synthetic Complete medium lacking uracil (SC-Ura, 0.34% Yeast Nitrogen Base without amino acids, 0.7% ammonium sulfate, 2% glucose, supplemented with adenine and all amino acids except uracil) or in Synthetic Complete selective medium lacking uracil, methionine, and cysteine (induction conditions for the MET3 promoter, SC-Ind, 0.34% Yeast Nitrogen Base without amino acids, 0.7% ammonium sulfate, 2% glucose, supplemented with adenine and all amino acids except methionine and cysteine). For selection of transformants of the Ho plasmid or Cas9 plasmid and maintenance in repressive conditions for the MET3 promoter, strains are grown in SC-Ind supplemented with 2 mM each of methionine and cysteine (SC-rep) and in YPD supplemented with 2 mM each of methionine and cysteine (YPD-Rep) when repression but no selection is needed. For SC-Rep, medium is buffered by 10 mL of Na$_2$HPO$_4$ 0.05 M and NaH$_2$PO$_4$ 0.95 M per liter. For URA3 marker counter-selection, yeast strains are grown on 5-FOA medium (SC-Ura supplemented with 1 g/L of 5-fluoro-orotic acid (5-FOA) and 50 mg/L of uracil).

Transformation is done according to the “one-step” lithium acetate transformation protocol from [43].

**Induction of mating-type switching by ScHo**

The *HO* gene from *S. cerevisiae* is cloned into the pCU-MET3 plasmid under the MET3 promoter (p7.1, S2 Table) [44] and protocol for solid induction is detailed in [26]. For time-course of induction in liquid medium, transformants are grown overnight in liquid SC-Rep medium, counted, washed and resuspended in sterile water at 4.10$^7$ cells/mL. 100 μL is used to inoculate 40 mL of liquid SC-Ind medium and the culture is placed at 28°C with agitation. For each time point, a sample of the culture is counted under the microscope, diluted and plated on SC-Rep plates. Each strain that was analysed in a time-course, were also transformed with the plasmid that does not contain ScHO, pYR32 plasmid, cells were diluted, counted and plated, allowing the normalization of the survival in Ho induction.

**Induction of mating-type switching by CRISPR-Cas9**

We used the inducible CRISPR-Cas9 system for *C. glabrata* from [31] using plasmid pCGLM1. We cloned into pCGLM1 a sequence corresponding to a guide RNA (gRNA) targeting the Ya sequence (S1 Table), giving rise to plasmids pCGLM1-Ya2.

Induction of Cas9-DSB was then performed as inductions of the ScHO gene done with p7.1 (see above).

**Cell viability estimation**

Different dilutions of cultures, containing between 200 to 10$^6$ cells, are spread on both inductive and repressive media. When the survival rate is over 20%, cell viability is determined
directly as the ratio of the number of colonies counted on inductive medium to the number of colonies counted on repressive medium, for the same dilution. When the survival rate is under 1%, colonies are confluent on repressive medium at the same dilution where several colonies can be observed on induction medium. Thus, survival rate is measured by first comparing the number of colony-forming units (CFU) on inductive medium with the theoretical number of cells plated, as estimated by counting on a Thoma counting chamber. This is then corrected by the ratio of CFU to the number of cells counted, estimated by plating 200 cells on repressive medium. All the values were obtained from at least four independent transformants. Colonies number from a minimum of 2 to a maximum 746 was counted on plates. Numerical data used for drawing graphs is shown in S3 Table.

**Determining the genotype at MTL loci**

The genotype at each MTL locus is determined by PCR, when needed, directly on colonies [26] using specific primers: the forward primer is located upstream of the locus (ensuring specificity of the locus screened; HML, MAT or HMR) and a reverse primer located precisely on the Ho site (ensuring specificity of the information carried by the locus; alpha or a and wt or inc) (S1 Table, S2 Fig). In most induction experiments, we did not check switching at Inc loci since preliminary experiments showed that there was no switching, indicating that the alpha-inc and a-inc Ho sites are not cut after Ho induction. This genotyping is performed on surviving colonies directly on induction plates (for solid induction) and on repression plates (for liquid induction). As previously shown, PCRs often reveal that most surviving colonies are mixed for genotypes at MTLs [26]. In the case of Ho inductions, we have already shown that sub-cloning of such mixed colonies yields more than 80% of switched pure clones [26]. Therefore, sub-cloning has not been done on any Ho induction in this work. In the case of Cas9 induction, since we had never used this system for switching MTLs, and we have decided to subclone mixed colonies in order to assess the true efficiency of mating-type switching.

**Construction of strains**

We mutated the Ho sites in the region known to be essential for Ho cutting in S. cerevisiae [30], as shown on S1 Fig, yielding loci HML-inc, MAT-inc and HMR-inc. Modification of HML, MAT, or HMR loci was realized either by marker selection (pop-in/pop-out) [45] or by mating-type switching upon HO gene expression or by use of CRISPR-Cas9. The three methods are detailed below. Primers and plasmids are listed in S1 and S2 Tables, respectively. Method used to construct each strain is listed in S4 Table.

**Construction of PCR fragments and plasmids for pop-in.** In order to integrate the URA3 marker at the targeted locus (pop-in), we amplified the URA3 gene from S. cerevisiae under its own promoter by PCR using primers Sc-URA3-F and Sc-URA3-R and, YEp352 as template. The PCR fragment was cloned into the EcoRV-digested pBlueScript, giving rise to pURA (S2 Table).

To direct integration of the URA3 marker at the targeted locus, here the MTL loci HML, MAT or HMR, the 5' and 3' flanking regions was added to the URA3 marker in multiple steps. First, the Z sequence, shared by the three MTL loci, was amplified by PCR using primers 68/70 and HM100 strain DNA as template (S1 Table). Primers 68 and 70 contain BamHI and EcoRI restriction sites, respectively, to allow cloning of the Z PCR fragment upstream of the URA3 marker into pURA, giving rise to pZU (S2 Table).

Second, Ya and Yalpha sequences were amplified on strain HM100 by PCR, using primers 68/70 and HM100 strain DNA as template (S1 Table). Primers 68 and 70 contain BamHI and EcoRI restriction sites, respectively, to allow cloning of the Z PCR fragment upstream of the URA3 marker into pURA, giving rise to pZU (S2 Table).
into pZU, giving rise to pZUA (S2 Table). The SalI restriction site was added to primer 69 and no restriction site was added to primer 74 as the Yalpha PCR fragment already contains the HindIII restriction site 38 bp from the 5’ of the fragment. Thus, the Yalpha PCR fragment, digested by both SalI and HindIII, was cloned downstream of the URA3 marker into pZU to give rise to pZUAlpha (S2 Table).

Amplification by PCR, using universal primers M13F/M13R, on both pZUA and pZUAlpha plasmids, led to ZUA and ZUAlpha fragments, respectively. These fragments have been used for targeting HML, MAT or HMR loci (S2 Table) and Ura+ transformants were selected on SC-Ura. Correct integration of the fragment was checked by PCR.

**Construction of plasmids and PCR fragments for pop-out.** The URA3 marker is removed (pop-out) from the target locus by homologous recombination with a DNA fragment derived from the upstream and downstream sequences of that locus (S4 Table).

In order to replace the wild-type Ho site in the different MTL loci, by the inconvertible-mutated Ho site, we constructed two plasmids; pZA-inc and pZAlpha-inc (S2 Table). The pZA-inc plasmid (without URA3 gene) results from double digestion of pZUA by EcoRI and HindIII and ligation after Klenow fill-in. The pZAlpha-inc plasmid (without the URA3 gene) was constructed by cloning the BamHI/EcoRI-digested Z fragment and the EcoRI/Sall-digested Yalpha fragment into the pBlueScript double digested by BamHI and SalI. Amplification by PCR using primers M13F/M13R, from both pZA-inc and pZAlpha-inc plasmids, lead to the ZA-inc and ZAlpha-inc fragments that have been used for pop-out. The comparison of wild-type and inconvertible Ho sites is presented in S1 Fig.

In addition to the construction of Inc sites, we have also used strains with deletion of MTLs (Table 1). Strains with deletions of MAT and/or HML were directly obtained from [22] (Table 1) and Inc sites were introduced in those strains when needed (S4 Table). In addition, we constructed deletion of HMR in strains BG87 and CGM390. After pop-in of URA3 at HMR, amplification of upstream and downstream sequences (500 bp each) of HMR was performed on strain BG87, using primers Up-HMR-F/Up-HMR-R and Down-HMR-F/Down-HMR-R, respectively (S1 Table). Primer Up-HMR-R contains 40 bp of homology to the 5’ end of the downstream PCR fragment. These two fragments were then combined by fusion PCR using primers Up-HMR-F and Down-HMR-R, giving rise to the Δhmr fragment (S4 Table).

As shown in S4 Table, other fragments for pop-out experiments were obtained by direct PCR on genomic DNA.

About 1 μg of each pop-out fragment was used to transform Ura+ strains, which were then plated onto YPD, grown for 24 hrs and replica-plated onto 5-FOA plates. Resulting 5-FOA R colonies were checked by PCR for correct removal of the URA3 marker, and the locus sequenced.

**Strains obtained by mating-type switching.** When possible, we took advantage of the efficient mating-type switching induced by SchO to transpose the inc-Ho site mutation from one MTL to another, instead of doing pop-in/pop-out transformations as above. For example, an HMLalpha-inc locus can easily be used as template, during gene conversion, to repair either MAT wt or HMR wt. In addition, extra-chromosomal copies of either MATα-inc or MATApha-inc were also used as templates for mating-type switching of MTL loci, in order to insert inc-Ho sites. These copies were introduced in the p7.1 plasmid, as follows. Plasmid p7.1 [26] was digested by KpnI, and MATα-inc and MATApha-inc sequences were amplified by PCR using primers Up-Rec-MAT-F/Down-Rec-MAT-R on strains YL09 and YL07, respectively (Table 1 and S1 Table). Both primers share, respectively, 40 bp of homology to the ends of the KpnI-digested plasmid. This allows PCR fragment cloning in p7.1, at the KpnI restriction site, by homologous recombination in E. coli [46]. Correct assembly was confirmed by both analytic colony PCR and restriction digests.
Expression of Ho is induced in strains that are targeted for modification, either from the p7.1 plasmid, when a genomic MTL locus is used as template, or from p7.1-derived plasmids that contain a copy of MATa-inc or MATalpha-inc. Final loci are checked by PCR and sequencing.

**Southern-blot analysis**

Genomic DNAs were prepared using the Qiagen genomic DNA kit, according to manufacturer’s instructions. 2 μg DNA was subjected to enzymatic digestions and protocol for Southern-blot is detailed in [26]. Primers used for probe PCR amplifications are given in S1 Table.

**Construction of the Δrad51 mutant using CRISPR-Cas9**

The Δrad51 mutant of strain HM100 was constructed with the CRISPR-Cas9 system on plasmid pJH-2972 (kind donation from J. Haber, https://protocolexchange.researchsquare.com/article/nprot-5791/v1). We cloned a sequence corresponding to a gRNA targeting the RAD51 gene into plasmid pJH-2972 (S1 Table), giving rise to plasmid pJH-RAD51.

We amplified upstream and downstream sequences (500 bp each) of the RAD51 CDS (CAGL0I05544g) on strain HM100 by PCR using primers Up-Rad51-F/Up-Rad51-R and Down-Rad51-F/Down-Rad51-R, respectively (S1 Table). Primer Up-Rad51-R contains 40 bp of homology to the 5’ end of the downstream PCR fragment. These two fragments are then combined by fusion PCR using primers Up-Rad51-F and Down-Rad51-R, giving rise to the Δrad51 fragment.

The strain was then co-transformed with both 1 μg of pJH-RAD51 and 1 μg of Δrad51 fragment. Ura+ transformants were then selected on SC-Ura and checked for deletion at the RAD51 locus by PCR. Deletion was confirmed by Southern blot analysis (S4 Fig) and by sequencing.

**Supporting information**

**S1 Fig.** Comparison of wild-type and mutated Ho sites of loci carrying Yalpha (A) or Ya information (B). The wild-type Ho site is shown on top in blue letters, the mutated Ho site is shown below with mutated bp in red and deleted bp as dashes. Arrows indicate the Ho cleavage site.

(TIF)

**S2 Fig.** Mating-type screened by PCR at MAT in different strains. All strains are analyzed with primer pairs that are specific to MATa, MATalpha, MATa-inc and MATalpha-inc, respectively GS01/123, GS01/121, GS01/122 and GS01/120. Top left panel: amplification obtained on BG87 (MATa); bottom left panel: amplification obtained on YL05 (MATa-inc); top right panel: amplification obtained on HM100 (MATalpha); bottom right panel: amplification obtained on YL07 (MATalpha-inc). MM: Molecular Marker, GeneRuler 1 kb (Thermo Fisher Scientific Inc).

(TIF)

**S3 Fig.** Southern-blot analysis of the DSB at the MAT locus in the wild-type HM100 strain. Left panel: chemiluminescence image of blot of Pstl/Xhol digestion hybridized with a probe corresponding to 484 bp located 262 bp away from the first nucleotide of the Ho site. Diagram of probe in top middle panel. The regions are represented with restriction sites and size of expected fragment. Right panel: chemiluminescence image of blot of BglII/EcoRI digestion hybridized with a probe corresponding to 1,013 bp located 2,119 bp away from the first
nucleotide of the Ho site. Diagram of probe is in lower middle panel.

(TIF)

**S4 Fig. Molecular characterization of Δrad51 mutations in HM100 by Southern blot hybridization.** Left panel: chemiluminescence image of blot of HindIII digestion. Right panel: chemiluminescence image of blot of NdeI/EcoRI digestion. The probe used is 1 kb long and is composed to the 500 bp upstream of the RAD51 ORF fused to the 500 bp downstream of the RAD51 ORF.

(TIF)

**S1 Table. Primers used in this work.** Fw: Forward; Rv: Reverse. The lowercase letters represent sequences with no homology to template DNA and reverse complete regions are indicated in uppercase.

(DOCX)

**S2 Table. Plasmids used in this work.**

(DOCX)

**S3 Table. Numerical data for figures.** Tables show percentage of survival calculated for each individual transformant in experiments represented as graphs. ND: Not done. SEM: Standard error of the mean. For Figs 4 and 5C number of colonies counted during time-course experiments is also shown.

(XLSX)

**S4 Table. Methods used for strain construction.**

(DOCX)

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