Flip/flop mating-type switching in the methylotrophic yeast *Ogataea polymorpha* is regulated by an Efg1-Rme1-Ste12 pathway

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Abstract

In haploid cells of *Ogataea (Hansenula) polymorpha* an environmental signal, nitrogen starvation, induces a reversible change in the structure of a chromosome. This process, mating-type switching, inverts a 19-kb DNA region to place either MATα or MATα genes under centromeric repression of transcription, depending on the orientation of the region. Here, we investigated the genetic pathway that controls switching. We characterized the transcriptomes of haploid and diploid *O. polymorpha* by RNA-seq in rich and nitrogen-deficient media, and found that there are no constitutively α-specific or α-specific genes other than the MAT genes themselves. We mapped a switching defect in a sibling species (*O. parapolymorpha* strain DL-1) by interspecies bulk segregant analysis to a frameshift in the transcription factor *EFG1*, which in *Candida albicans* regulates filamentous growth and white-opaque switching. Gene knockout, overexpression and ChIP-seq experiments show that *EFG1* regulates *RME1*, which in turn regulates *STE12*, to achieve mating-type switching. All three genes are necessary both for switching and for mating. Overexpression of *RME1* or *STE12* is sufficient to induce switching without a nitrogen depletion signal. The homologous recombination genes *RAD51* and *RAD17* are also necessary for switching. The pathway controlling switching in *O. polymorpha* shares no components with the regulation of *HO* in *S. cerevisiae*, which does not involve any environmental signal, but it shares some components with mating-type switching in *Kluyveromyces lactis* and with white-opaque phenotypic switching in *C. albicans*.

Author summary

The molecular mechanisms of self-fertility (homothallism) vary enormously among fungal species. We previously found that in the yeast *Ogataea polymorpha*, homothallism is achieved by a novel mating-type switching mechanism that exchanges the locations of MATα and MATα genes between expression and repression contexts. Switching in this species is induced by nitrogen depletion, unlike the analogous process in *Saccharomyces cerevisiae*. Here, we show that the upstream parts of the genetic pathway controlling the
Introduction

In yeast species ( unicellular fungi ) that can reproduce sexually, the ability of a cell to mate with other cells is governed by which mating-type genes it expresses [1, 2]. In ascomycete yeasts, these genes are located at a single genomic site called the mating-type (MAT) locus. Mating generally occurs between two haploid cells with opposite genotypes (MATa and MATα) at this locus, to form a diploid zygote (MATa/α). In some ascomycete yeasts such as Saccharomyces cerevisiae, haploid cells are able to change their MAT genotypes by a process called mating-type switching [3, 4]. During this process, DNA at the MAT locus is physically replaced, exchanging a MATa allele for a MATα allele or vice versa. Mating-type switching is a form of secondary homothallism [5] because it enables a yeast strain to mate with any other strain of the same species, regardless of their initial mating types, by means of fusion between a-cells and α-cells [6, 7].

The molecular mechanism and regulation of mating-type switching in S. cerevisiae has been elucidated by extensive studies over the past several decades and is well understood [3, 8]. It involves an endonuclease (HO) that cuts the outgoing MAT locus, and two ‘silent cassettes’ (HMR and HML) that contain unexpressed copies of the MATa and MATα DNA sequences. One of the cassettes is chosen to be used as the template for synthesis of new DNA to repair the MAT locus, replacing MAT with a sequence of the opposite genotype. In contrast, until recently little was known about how other ascomycete yeasts switch mating types, other than in Schizosaccharomyces pombe [9] which is a member of a different subphylum. In 2014, Mae-kawa and Kaneko [10], and our group [11], discovered that haploid cells of Ogataea polymorpha switch mating types by a novel ‘flip/flop’ mechanism that is quite different from the mechanism used by S. cerevisiae. O. polymorpha (formerly called Hansenula polymorpha) is a methylotrophic yeast in the same subphylum as S. cerevisiae (Saccharomycotina, the budding yeasts) but quite distantly related to it (Fig 1A).

O. polymorpha chromosome 3 contains both a MATa locus and a MATα locus, approximately 19 kb apart (Fig 1B). The two MAT loci are beside two copies of an identical 2-kb DNA sequence that form an inverted repeat (IR) on the chromosome. During mating type switching, the two copies of the IR recombine, inverting the orientation of the 19-kb region relative to the rest of the chromosome. The centromere of chromosome 3 is located just to the left of the left copy of the IR (Fig 1B). The MAT locus proximal to the centromere is not transcribed, probably due to silencing by centromeric heterochromatin, whereas the distal MAT locus is transcribed. By inverting the 19-kb region, mating type switching swaps the locations of the MATa and MATα genes, repressing the MAT genes that were previously expressed, and expressing the ones that were previously repressed. Similar flip/flop mating type switching mechanisms are now known in three other Saccharomycotina species (Komagataella phaffii, Pachysolen tannophilus, and Ascoidea rubescens) [4, 11, 12].

Mating type switching in O. polymorpha is induced by an environmental signal, nitrogen depletion [10, 11]. In a culture transferred into media that contains no nitrogen, up to approximately 25% the cells in the culture switch their mating type (Fig 1C). This situation, in which an environmental signal reproducibly induces a DNA rearrangement at a specific chromosomal locus, is unusual in biology and we were motivated to investigate its mechanism. Our aim in the current study was to identify the pathway in O. polymorpha that detects the environmental induction of switching in O. polymorpha are the same as the environmental pathway that induces competence for mating in this species.
Regulation of mating-type switching in *Ogataea polymorpha*

**Fig 1.** Mating-type switching in *O. polymorpha* occurs through an inducible flip/flop chromosomal inversion mechanism [10, 11].

(A) Phylogenetic relationship among the yeast species compared in this study. The tree is re-drawn from the phylogenomic study by Shen et al. [67] and is a cladogram, i.e. branch lengths are not proportional to divergence times. All the species are in subphylum Saccharomycotina except for *Schizosaccharomyces pombe*, which is in Taphrinomycotina.

(B) Structure of the MAT region on chromosome 3 of *O. polymorpha*NCYC495 [12] in its two possible orientations. In each orientation, only the MAT genes far from the centromere are expressed. The upper part shows the orientation in which MATa genes are transcribed (green arrows), and the lower part shows the orientation in which MATα genes are transcribed (magenta arrows). Wavy lines indicate the approximate extent of transcriptional silencing around the centromere. Cyan rectangles and the arrows labeled IR represent the two identical copies of a 2-kb sequence that form the Inverted Repeat. Small rectangles represent genes: intact MATa genes (green), intact MATα genes (magenta), other genes (white), or LTRs of the Ty5-like retrotransposon Tpa5 (gray). The dark blue regions labeled SLA2’ and a1’ are non-functional truncated copies of parts of the SLA2 and a1 genes, located in the IR. Genes drawn above or below the horizontal lines are transcribed rightwards or leftwards, respectively. Arrowheads A-D indicate the locations of PCR primers used to determine mating-type.

(C) Induction of mating-type switching by nitrogen depletion. MATα strains (upper) and MATa strains (lower) were grown in YPD ‘pre-induction’ cultures, and then transferred into NaKG media, which contains no nitrogen source and strongly reduces growth rate. DNA samples were taken from the pre-induction cultures and 24 h after transfer into NaKG. PCR amplifications were performed with the primer combinations indicated. The PCR product sizes are 2.6 kb (AB), 2.7 kb (AC), 2.3 kb (BD) and 2.3 kb (CD).

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environmental signal and executes rearrangement of chromosome 3 in response. A priori, we know that the pathway in \textit{O. polymorpha} must be quite different from the pathway that regulates mating-type switching in \textit{S. cerevisiae} \cite{3, 13}, because switching in \textit{S. cerevisiae} is not regulated by the environment and occurs even in rich media, and because \textit{O. polymorpha} has no ortholog of the \textit{S. cerevisiae} HO endonuclease gene. Therefore, both the upstream (nitrogen-sensing) and downstream (DNA inversion) parts of the pathway in \textit{O. polymorpha} must be different from \textit{S. cerevisiae}. Furthermore, since the DNA rearrangements that occur during switching in \textit{S. cerevisiae}, \textit{O. polymorpha} and \textit{Kluyveromyces lactis} are all substantially different but are descendants of a common ancestral switching mechanism \cite{4, 14, 15}, we were interested to determine how the pathways that regulate these rearrangements have evolved.

To identify components of the switching pathway in \textit{O. polymorpha}, we used several strategies including transcriptomic analysis, candidate gene approaches, and mapping the defective gene in a naturally-occurring mutant that is unable to switch mating types. We identified five genes that are required for switching. Although we were unable to deduce all the steps that lead from nitrogen depletion to mating type switching, we infer that \textit{O. polymorpha} senses nitrogen depletion using the Protein Kinase A (PKA) pathway, which then transmits a signal via Ste12 to induce mating and/or mating type switching, and that recombination between the IRs is mediated by the homologous recombination pathway for DNA repair. We compare the roles of genes in the \textit{O. polymorpha} pathway to the roles of their orthologs in other species.

\section*{Results}

\subsection*{Transcriptomic response of \textit{O. polymorpha} to nitrogen depletion}

Our initial approach to search for genes involved in mating-type switching in \textit{O. polymorpha} was to look for differences between the transcriptomes of cells that are switching and cells that are not switching. Switching in several methylotrophic yeast species is induced by nitrogen depletion \cite{10–12, 16}, and in \textit{O. polymorpha} we used liquid NaKG media (0.5% NaOAc, 1% KCl, 1% glucose), which completely lacks amino acids or any other source of nitrogen, to induce switching. \textit{O. polymorpha} grows poorly in NaKG, so to induce switching we first grew ‘pre-induction’ cultures in rich media (YPD) and then transferred the cells, after washing, into NaKG. In the haploid strain NCYC495, recombination between the IRs in the \textit{MAT} region was induced within 24 hours after transfer into NaKG, whereas no recombination occurred in the YPD pre-induction cultures (Fig 1C).

To examine the transcriptional response induced by nitrogen depletion, we used mRNAseq to compare the transcriptomes of \textit{O. polymorpha} cells 2 h after transfer from a YPD pre-induction culture into NaKG, to parallel cultures transferred into fresh YPD. Furthermore, because we expect that switching occurs only in haploid cells, we conducted this experiment in parallel on haploid (\textit{MAT}a and \textit{MAT}α isogenic strains) and diploid (\textit{MAT}a/α) cells.

Growth of all three cell types in NaKG resulted in a robust transcriptional response to nitrogen depletion, with a large number of genes significantly up- or down-regulated relative to YPD (S1 Fig; S1 Table). Regardless of cell type, homologs of \textit{S. cerevisiae} genes for nitrogen starvation responses were induced, such as transporters of amino acids (\textit{DIP5}, \textit{GAP1}), urea (\textit{DUR3}), and allantoate (\textit{SEO1}), and amidases for the release of amide groups from urea (\textit{DUR1,2}), pyrimidines (\textit{PYD3}), or other substrates (\textit{AMD2}). Ribosomal protein genes were strongly repressed, as expected because of the reduced growth rate in NaKG (S1 Table). However, orthologs of \textit{S. cerevisiae} genes with mating or sporulation functions were not induced by these nitrogen depletion conditions alone, even though mating (of haploids) and sporulation (of diploids) can be induced by plating cells onto similar nitrogen-depleted solid media \cite{17}. Among the genes strongly upregulated in NaKG were two transcription factors, \textit{RME1} and...
CZF1-like3 (one of three *O. polymorpha* co-orthologs of *C. albicans* CZF1, which is a singleton zinc finger gene with no *S. cerevisiae* ortholog [18]). Both of these genes were uniformly induced in all three cell types (*MATα, MATα* and *MATa/α*), with CZF1-like3 upregulated 69- to 93-fold, and *RME1* upregulated 26- to 79-fold, upon transfer into NaKG (S1 Table).

**Lack of constitutive *MATα* - and *MATa* -specific genes**

In *S. cerevisiae*, defined sets of α- and α-specific genes that allow haploid cells to identify and respond to the presence of a mating partner are well established [19]. These genes are constitutively expressed in *S. cerevisiae* cells of the appropriate mating type. Surprisingly, comparison of gene expression between haploid *O. polymorpha* α-cells and α-cells in either NaKG or YPD media revealed that there are essentially no constitutive α- or α-specific genes in this species, apart from the *MAT* genes themselves (S1 Fig; S2 Fig). All haploid cells of *O. polymorpha* contain four *MAT* genes (*MAT α1, α2, a1*, and *a2*), and the orientation of the 19-kb region specifies whether the *α1* and *α2* genes, or the *a1* and *a2* genes, are placed at the expression site (Fig 1B). In NaKG, transcription of *α1* and *α2* was respectively 53-fold and 39-fold higher in α-cells than in a-cells; *a2* was 31-fold lower, and *a1* was just 2-fold lower. In YPD, *α1* and *α2* were 4-fold and 9-fold higher, *a1* was 6-fold lower, and *a1* showed no difference. *KAR4*, which in *S. cerevisiae* is a general pheromone-induced gene [20] required for fusion of the haploid nuclei after mating, showed moderately higher expression in α-cells than in α-cells (2 to 3-fold; S2 Fig). No other genes showed more than a 2-fold difference in transcription between α- and α-cells, in either of the two media (S2 Fig; S2 Table). This result contrasts sharply with *S. cerevisiae*, where for example several α-specific genes such as *MFA2, STE2* and *BAR1* have more than 10-fold higher expression in *MATα* than *MATα* cells in YPD [19]. It is also consistent with previous observations that expression of the pheromone receptors *STE2* and *STE3* in haploid *O. polymorpha* is independent of cell type [10].

These experiments also enabled us to identify gene expression differences between haploid and diploid cells. *O. polymorpha* is haplontic, and its diploid state is normally transient because meiosis is induced by the same conditions (nitrogen depletion) that induce mating. However, diploids can be maintained stably on YPD. We calculated the haploid-to-diploid expression ratio for each gene as the ratio between its transcription in α-cells and a/α-cells. The values of this ratio in different genes were quite consistent between YPD and NaKG media (Pearson’s *R* = 0.68; S3 Fig). Among the genes showing the strongest bias in YPD towards haploid-specific expression were several transcription factors including *CZF1-like1, CZF1-like2, CRZ1, GAT1,* and *MGA1* (S3 Table). Of these, only *CZF1-like2* was also haploid-specific in NaKG. Transcription factor *DAL81* appeared diploid-specific in both media (S3 Table).

Because mating-type switching occurs in haploid cells grown in NaKG, but not in haploids grown in YPD, and presumably not in diploids, we anticipated that genes with roles in switching might be identifiable as transcripts that are both haploid-specific and NaKG-specific. However, analysis of the genes fitting this transcription profile did not reveal any strong candidates for the downstream steps in the switching process, such as DNA recombination or endonuclease genes. Instead, most of the genes with this profile had metabolic functions (S4 Fig). The most haploid-specific and diploid-specific genes in the two media are listed in S3 Table.

**A switching defect in *O. parapolymorpha* strain DL-1 maps to *EFG1***

Next, in an alternative approach to find a component of the switching pathway, we made use of a naturally occurring mutant. When assaying the *MAT* genotypes of *Ogataea* strains, we discovered that strain DL-1 is unable to switch mating-types, even after 45 h growth in NaKG, in contrast to strainsNCYC495 and CBS4732 (Fig 2A). Strain DL-1 has previously been
described as ‘semi-sterile,’ meaning that it is very inefficient at forming diploids under nutrient-limited conditions [21]. The semi-sterility phenotype of DL-1 is therefore likely due to a loss of the signal that is induced by nitrogen depletion, upstream of the steps that normally lead to either mating or switching in response to the signal.

Strains DL-1, NCYC495 and CBS4732 were all historically classified as *Hansenula polymorpha* but it has recently been recognized, based on sequence divergence, that DL-1 is a different species from the other two. DL-1 is now classified as *Ogataea parapolymorpha*, whereas NCYC495 and CBS4732 are *O. polymorpha* [22, 23]. However, the genome sequence of *O. parapolymorpha* DL-1 [24] is completely collinear with the genome sequence of *O. polymorpha* NCYC495 [12]. Both species have 7 chromosomes, and there are no translocations or other chromosomal rearrangements between them, even though the genomes are approximately 10% different in nucleotide...
The fact that the genomes are collinear suggested to us that the mating-type switching defect in DL-1 could be mapped by using an interspecies genetic cross between it and the *O. polymorpha* laboratory strain NCYC495.

We used bulk segregant analysis [25] to map the locus causing the switching defect. We first isolated a rare diploid from a cross between DL-1 (*leu2 ura3* genotype) and an NCYC495 derivative (*ade11 met6* genotype), selecting for prototrophy. We then sporulated the diploid and isolated haploid segregants grown from random spores (Fig 2B). Segregants were screened individually for their ability to switch mating-types after 24 h in NaKG by the same PCR assay used above. We made four pools of segregants: MATa switchers, MATa non-switchers, MATα switchers, and MATα non-switchers, each pool containing between 30 and 52 haploid clones (S5A Fig), and sequenced each pool. Cultures of each clone in a pool were grown individually and then combined into pools in equal cell numbers for DNA extraction and genome sequencing. The sequence reads from the switching and non-switching pools were then mapped to the parental DL-1 and NCYC495 genome sequences (S5B Fig); only reads that were unambiguously derived from one identifiable parent were mapped. We developed an asymmetry metric (see Methods) to detect regions of the genome where biased inheritance of parental alleles correlated with the switching/non-switching phenotype in the expected direction (Fig 2C).

Two peaks of asymmetrical inheritance were detected (Fig 2C). The strongest signal was located on chromosome 6 and was centered near the gene *OPOL_95241*, which we refer to as *O. polymorpha EFG1*. It is orthologous to the *C. albicans* transcription factor *EFG1* [26] and to the *S. cerevisiae* gene pair *PHD1* and *SOK2* derived from the Whole-Genome Duplication [27–29]. Comparison of the *EFG1* sequences from the parental NCYC495 and DL-1 genomes revealed a single-base insertion at nucleotide 512 in the DL-1 gene that causes a frameshift (Fig 2D). The predicted DL-1 Efg1 protein product is truncated to 203 residues, compared to 437 residues in NCYC495. The DL-1 Efg1 protein lacks a DNA-binding domain (APSES domain [26, 30, 31]) that is conserved among Efg1 orthologs in multiple species including *C. albicans* and *S. cerevisiae* (S6A Fig). A second region of asymmetrical inheritance occurred on chromosome 7 near coordinate 330 kb (Fig 2C). Comparison of the NCYC495 and DL-1 genomes in this region did not reveal any candidate disabling mutations in genes, or differences in gene content. Considering that this analysis used a cross between two different species, it is possible that the chromosome 7 region contains a gene that interacts with a gene near *EFG1*, for which an interspecies combination of alleles is inviable, but we did not investigate this region further.

To confirm that *EFG1* plays a role in mating-type switching in *O. polymorpha*, we deleted it from both MATa and MATα strains. Gene deletions were made in *ku80Δ* derivatives from the NCYC495 genetic background [32]. PCR assays showed that, after 24 h in NaKG, almost no switched *MAT* locus products were formed in the *efg1Δ* strains, whereas extensive switching occurred in the wildtype control strains (Fig 3A). Furthermore, the *efg1Δ* strains were defective in mating, similar to the semi-sterility phenotype of DL-1. Crosses of *efg1Δ* x *efg1Δ* strains yielded no progeny, and crosses of *efg1Δ* x *EFG1* strains yielded only a small number of progeny compared to wildtype crosses (Fig 3B). This result indicates that both parents in a cross require *EFG1* activity in order to mate.

**An EFG1-dependent mechanism represses RME1 in nitrogen-rich conditions**

Because *EFG1* is required for both mating and mating-type switching in *O. polymorpha* (Fig 3A and 3B), we reasoned that it must act in an upstream part of the nutrient-sensing pathway that is shared by these two processes. Such a function is consistent with the known role of
**Fig 3.** *EFG1* is required for *O. polymorpha* mating-type switching and mating. (A) PCR determination of MAT genotypes before and after 24 h growth in NaKG of *efg1Δ* and wild-type NCYC495 strains. *efg1Δ* strains were constructed in both MATα and MATα backgrounds. PCR primers A-D are as in Fig 1. (B) Photographs of diploid growth on SD plates following crosses of haploids on MEMA for 24 h. (C) Regulation of transcription factor expression by *EFG1* and by cell type. The heatmaps show log₂(Fold Change) in expression of the genes named on the left, measured by mRNAseq, for the pairs of conditions named on the top. Columns 1–3 compare gene expression in nitrogen-poor (NaKG) to nitrogen-rich (YPD) media, in haploid MATα, haploid MATα, and diploid MATα/α strains. Column 4–7 compare *efg1Δ* to wildtype strains, in two nitrogen-rich (NaKG+N, SD) and two nitrogen-poor (NaKG, SD-N) media. Column 8 compares a pAOX-*EFG1* overexpression strain to a strain containing an empty pAOX vector, after methanol induction of pAOX. (D) *EFG1* represses *RME1* in rich media. The genomic region containing *RME1* on chromosome 3 is shown. Numbers of mapped RNAseq reads are plotted (log scale) for two strains (wildtype and *efg1Δ*) in two conditions (nitrogen-poor NaKG, and nitrogen-rich NaKG+N). Blue and red indicate transcription in the forward and reverse directions, respectively. Yellow boxes show the coding regions of genes. *RME1* has a long 3' untranslated region of approximately 850 bp.

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EFG1 in *C. albicans* as the major transcription factor of the PKA pathway [33–36], even though its *S. cerevisiae* orthologs PHD1 and SOK2 have no role in mating or switching. We therefore searched for *O. polymorpha* genes whose expression depends on Efg1.

To find genes downstream of EFG1, we compared the transcriptional profiles of wildtype and efg1Δ haploid strains, by mRNAseq in nitrogen-poor and nitrogen-rich conditions. We used two different types of paired media for this experiment. One was a comparison of transcriptomes in NaKG versus NaKG plus 40 mM ammonium sulfate, which we have previously shown abolishes switching [11]. The other, chosen to try to reduce the strong general nutrient depletion signal we observed with NaKG (S1 Fig), was a comparison of transcriptomes in synthetic defined media (SD, which includes 40 mM ammonium sulfate) versus SD lacking this nitrogen source. These mRNAseq experiments identified many nutrient transporters and enzymes that have EFG1-dependent expression in nitrogen-poor media (S4 Table), consistent with EFG1’s expected role in the PKA pathway, but no obvious candidates for direct actors in the flip/flop inversion mechanism, such as DNA recombinases or endonucleases. However, these experiments also showed that one of the genes with the largest EFG1-dependent differences in expression between nitrogen-rich and nitrogen-poor conditions was another transcription factor, RME1.

*O. polymorpha* RME1 is a gene that is substantially more highly transcribed in nitrogen-poor than in nitrogen-rich conditions, being among the top 2% of genes upregulated in NaKG (Fig 3C; S1 Table). In nitrogen-rich conditions, one of the strongest effects of deleting EFG1 was to increase the expression of RME1 from its low baseline, by factors of 4.4-fold in NaKG + ammonium sulfate, and 6.6-fold in SD, relative to wildtype cells (Fig 3C and 3D). In contrast, in nitrogen-poor conditions RME1 expression was high and unchanged between efg1Δ and wildtype cells (Fig 3D; S4 Table). In the efg1Δ strain, RME1 was expressed in both nitrogen-rich and poor media. Thus, transcription of RME1 in nitrogen-rich conditions is normally repressed by an EFG1-dependent mechanism, which could either be direct or involve intermediate proteins. EFG1 itself showed no difference in expression between NaKG and YPD (Fig 3C). In Kluyveromyces lactis, RME1 (also called MTS1) is required for both mating-type switching and mating [14, 37], so regulation of RME1 by EFG1 in *O. polymorpha* therefore suggests a mechanism connecting the nitrogen limitation response to switching and mating. In contrast, the main function of RME1 in *S. cerevisiae* is as a repressor of meiosis via repression of IME1, which has no ortholog in *O. polymorpha* [38, 39].

The set of genes showing expression changes in the efg1Δ strain also included some ‘white-opaque circuit’ genes. These genes are *O. polymorpha* homologs of genes that form a feed-forward circuit in *C. albicans* governing the phenotypic switch between mating-competent (opaque) and mating-incompetent (white) cell states. The *C. albicans* circuit includes EFG1, WOR1, WOR2 and CZF1 [40]. *O. polymorpha* has no ortholog of *C. albicans* WOR1, but has a paralogous gene (OPOL_7784) that we refer to as MIT1 because of its similarity to *S. cerevisiae* MIT1 [41]. Among the strongest effects of deleting *O. polymorpha* EFG1 were decreases of expression of CZF1-like2 and MIT1, which occurred in both nitrogen-poor and nitrogen-rich conditions (Fig 3C; S4 Table; all decreases were by less than 4-fold).

**EFG1 overexpression represses an EFG1 paralog**

In parallel to the experiments with the efg1Δ deletion strain, we also used an overexpression strain to search for genes regulated by EFG1. We placed EFG1 under the control of the *O. polymorpha* alcohol oxidase promoter (pAOX). Expression from pAOX is robustly induced when cells are switched from growth in glucose to growth in media containing methanol as the carbon source. We used mRNAseq to compare the transcriptome of the *pAOX-EFG1* strain to a control strain containing an empty pAOX construct, after overnight growth in methanol...
media. Methanol induced 7-fold higher transcription of EFG1 in the pAOX-EFG1 strain than in the control (Fig 3C). Overexpression of EFG1 led to changes in transcription of large numbers of genes (221 genes downregulated, and 26 genes upregulated, by factors of at least 8-fold; S5A Table). Notably, RME1 transcription remained unchanged in the EFG1 overexpression strain (Fig 3C).

One of the strongest effects of overexpressing EFG1 (OPOL_95241) was 53-fold repression of a related gene, OPOL_93012 (Fig 3C). Phylogenetic analysis showed that these two APSES domain proteins are the products of a gene duplication that occurred within the genus Ogataea (S6B Fig). This gene duplication is separate from an older duplication that formed the EFG1 homolog EFH1 in the Candida clade [42]. The O. polymorpha Efg1 and OPOL_93012 proteins have 49% amino acid sequence identity. The sister species O. parapolymera has orthologs of both EFG1 (with a frameshift) and OPOL_93012, but other budding yeasts including methylotrophs outside the genus Ogataea have only a single gene. EFG1 and OPOL_93012 are both transcribed in both nitrogen-poor and nitrogen-rich conditions. EFG1 has higher expression in haploids than in diploids (2.8- to 3.7-fold), whereas OPOL_93012 shows little difference between cell types (S3 Table). Expression of OPOL_93012 was unaffected in the efg1Δ strain.

Overexpression of O. polymorpha EFG1 also caused changes of expression of some white-opaque genes. CZF1-like2 was down-regulated 20-fold, and CZF1-like3 was up-regulated 7-fold (Fig 3C; S5A Table). WOR3 and WOR4, which are more recently identified components of the white-opaque circuit in C. albicans [43, 44], were down-regulated (10- and 12-fold respectively). The regulatory relationship between EFG1 and CZF1-like2 appears to be complex, because CZF1-like2 was down-regulated by both deletion and overexpression of EFG1.

RME1 and STE12, as well as EFG1, are necessary for mating-type switching and mating

Based on the results of the EFG1 deletion and overexpression mRNaseq analyses, we tested whether RME1 and the EFG1 paralog OPOL_93012 are required for mating-type switching and/or mating. We also tested STE12, which plays a central role in the mating response in other yeast species, and which shows induction by nitrogen depletion (Fig 3C; S1 Table). MATα and MATα deletion strains for each gene were constructed and tested for their ability to switch mating types, and to mate.

Deletion of RME1 severely reduced mating-type switching, as measured by PCR assay, in both MATα and MATα cells (Fig 4A), similar to the result from EFG1 deletion (Fig 3A). Deletion of STE12 completely abolished switching. Furthermore, deletion of RME1 or STE12 abolished mating, in crosses where both parents were rme1Δ or ste12Δ (Fig 4B). Crossing rme1Δ x RME1 resulted in a low number of diploid colonies, similar to efg1Δ x EFG1 crosses, whereas ste12Δ x STE12 crosses did not produce any colonies (Fig 4B). In contrast to these three genes, deleting the EFG1 paralog OPOL_93012 had no effect on switching or mating (Fig 4A and 4B).

Because EFG1 is a component of the white-opaque circuit in C. albicans, and because several O. polymorpha homologs of white-opaque genes were found to be differentially regulated in our transcriptome analyses as mentioned above (Fig 3C), we also made deletion strains of four O. polymorpha ‘white-opaque’ genes: CZF1-like2, CZF1-like3, MIT1 (WOR1), and WOR2 (S7 Fig). However, none of these deletions had any effect on either switching or mating (S7 Fig).

Overexpression of RME1 or STE12, but not EFG1, is sufficient to induce switching without an environmental signal

Since EFG1, RME1 and STE12 are all necessary for switching, we investigated whether high expression of any of them is also sufficient to induce switching, even in the absence of a
nitrogen depletion signal. We constructed methanol-inducible pAOX-RME1 and pAOX-STE12 strains similar to the pAOX-EFG1 strain described above. Switching was induced when strains containing pAOX-RME1 or pAOX-STE12 were transferred from glucose to methanol (Fig 5A), demonstrating that overexpressed RME1 and STE12 are each sufficient to induce switching. The pAOX-EFG1 strain, and a control strain containing the pAOX vector alone, did not switch under the same conditions (Fig 5A). The latter result is consistent with the observation that EFG1 is transcribed in nitrogen-rich as well as nitrogen-poor conditions, and indicates that EFG1 requires additional factors in order to induce switching.

RME1 acts upstream of STE12

The results we have presented so far show that EFG1, RME1 and STE12 are each necessary for switching, and that EFG1 acts upstream of RME1. To determine where STE12 fits into the pathway, we constructed strains that combined overexpression of one gene with deletion of another. We introduced the pAOX-RME1, pAOX-STE12, and pAOX-EFG1 constructs individually into the deletion strains rme1Δ, ste12Δ, and efg1Δ in all possible combinations (Fig 5B). Methanol induction of EFG1 was again unable to induce switching in any background, whereas STE12 overexpression induced switching in all backgrounds. RME1 overexpression, although sufficient for switching in the rme1Δ and efg1Δ backgrounds, did not induce
switching in the ste12Δ strain. This result indicates that RME1 acts upstream of STE12 in the switching pathway.

To test whether RME1 binds to the promoter of STE12, we performed ChIPseq using 3xHA-tagged Rme1, expressed from its native chromosomal locus. In addition to binding to its own promoter and 3' UTR, Rme1 bound to the promoter of STE12 (Fig 5C). Furthermore, mRNAseq analysis of the pAOX-RME1 strain shows that overexpression of RME1 results in an increase in expression of STE12 (S8 Fig; S5C Table). Together, these data suggest that RME1 directly activates transcription of STE12 by binding to its promoter, which leads to switching.
**RME1 and STE12 overexpression induces the mating pathway including pheromone genes**

Since overexpression of either RME1 or STE12 induces switching, we tried to identify components further downstream in the switching pathway by transcriptome analysis of the pAOX-RME1 and pAOX-STE12 overexpression strains, after overnight growth in methanol media. We found that the major consequence of overexpressing these transcription factors was strong induction of genes in the mating response pathway (S8 Fig; S5 Table), consistent with the essential roles of RME1 and STE12 in *O. polymorpha* mating (Fig 4). The genes induced included orthologs of the *S. cerevisiae* haploid-specific genes (STE4, GPA1, STE18, STE5, FAR1, FUS3) required for transmission of the pheromone signal. Our pAOX overexpression strains were constructed in a haploid MATa background, and we detected methanol-induced transcription of a-specific genes (BAR1, AXL1, ASG7, RAM1, RAM2, STE6) that are required for production of a-factor and modulation of the α-factor signal. The mating pathway induction by STE12 overexpression was so strong that it enabled us to annotate the α-factor gene (MFa) of *O. polymorpha* for the first time (S9 Fig). We also observed induction of the α-specific genes MATα1, MATα2 and the α-factor gene MFa, which is likely due to expression in cells that had successfully switched mating-type from MATa to MATα in the cultures (Fig 5A). In addition to the mating pathway genes, overexpression of RME1 (but not STE12) also induced transcription of genes with roles in sporulation such as RIM4, IME2, MUM2, and MEI2 (S5C Table). In contrast to RME1 and STE12, EFG1 overexpression did not significantly induce expression of mating pathway genes (S8 Fig).

Disappointingly, the RME1 and STE12 overexpression mRNAseq analyses did not reveal any clear candidates for genes that act downstream in the switching pathway. They did however show that RME1 and STE12 form a positive feedback loop. Overexpression of RME1 induced STE12 by 14-fold, and overexpression of STE12 induced RME1 by 9-fold (S5B Table, S5C Table). They also showed that overexpression of RME1 induced expression of CZF1-like3 and repressed expression of CZF1-like2 (S5A Table), similar to overexpression of EFG1, so the effect of EFG1 on these white-opaque genes is probably mediated through RME1.

**The homologous recombination pathway is required for mating-type switching**

Although the transcriptomic and ChIPseq experiments did not identify obvious candidates for downstream roles in the switching pathway, such as homologs of known DNA recombinases or endonucleases, they did uncover several *O. polymorpha* genes of unknown function whose patterns of transcription were consistent with the profile we expected switching pathway genes to have. We chose 21 candidate *O. polymorpha* genes for deletion and testing of switching phenotypes, including (i) genes of unknown function with appropriate transcription profiles, (ii) orthologs of *S. cerevisiae* genes that interact with STE12, such as TEC1, FUS3 and KSS1, and (iii) orthologs of *S. cerevisiae* genes with roles in mating-type switching, homologous recombination or DNA repair, such as ASH1, RAD51 and PMS1. Deletion strains of each of the 21 genes in an NCYC495 *ku80Δ MATα* background were tested for their ability to switch mating-types, of which 19 had no phenotype (S10 Fig).

We found that mating-type switching was almost completely abolished in strains with deletions of the orthologs of two *S. cerevisiae* genes in the homologous recombination pathway, RAD51 and RAD17 (Fig 6). In *S. cerevisiae*, Rad51 is a single-stranded DNA binding protein that mediates strand exchange during homologous recombination [45], and is necessary for mating-type switching [46]. The *O. polymorpha* RAD51 gene has previously been reported to partially complement an *S. cerevisiae* rad51 mutant, and the protein catalyzes DNA strand
exchange in vitro [47]. Rad17 is a component of the checkpoint signaling clamp called 9-1-1 in humans or Ddc1-Mec3-Rad17 in *S. cerevisiae* [45]. The Mec3 component of the clamp is not necessary for mating-type switching in *S. cerevisiae* [48], but whether Rad17 is necessary has not been investigated. The requirement for *RAD51* and *RAD17* in *O. polymorpha* switching shows that the homologous recombination pathway for repair of DNA breaks is involved in the interaction between the IRs.

**Discussion**

Our experimental results suggest a model for how mating-type switching and the mating response to pheromone are both controlled in *O. polymorpha* (Fig 7). In the presence of a nitrogen source, an *EFG1*-dependent mechanism represses transcription of *RME1* and the
whole pathway is inactive. In the absence of a nitrogen source, RME1 is active and a positive feedback loop between RME1 and STE12 expression develops. If pheromone is detected, STE12 activates the mating response pathway and mating ensues. We postulate that if no pheromone is detected, STE12 instead activates mating-type switching, which could then lead to mating with a cell of the original mating-type. The final steps in switching utilize the homologous recombination pathway, but the intermediate steps connecting STE12 to the RAD genes remain unknown.

As shown in Fig 7, RME1 also has a role in regulating ‘white-opaque’ transcription factors. We do not know if a regulatory loop similar to the C. albicans white-opaque circuit exists in O. polymorpha, but in any case our gene deletion experiments (S7 Fig) show that white-opaque genes other than EFG1 have no role in switching or mating. In C. albicans, EFG1 is the main activator of the mating-incompetent (white) state [40], whereas in O. polymorpha EFG1 is required for mating competence. Our model may be oversimplified because the connection between EFG1 and RME1 seems to be complex and may involve intermediate steps. By analysis of the efg1Δ strain we found that in nitrogen-rich conditions EFG1 causes repression of RME1, whereas in nitrogen-poor conditions EFG1 was essential for switching and mating, suggesting conversely that it causes activation of RME1. Since overexpression of EFG1 had no effect on RME1 transcription, the activity of EFG1 may depend on other factors such as the presence of partner proteins, or post-translational modification of Efg1. The Efg1 proteins of some yeast species are known to be phosphorylated [49–51]. C. albicans Efg1 can act as both a repressor and an activator [26, 52], so it is possible that O. polymorpha Efg1 can both positively and negatively affect RME1 transcription in different conditions.

A fundamental difference between O. polymorpha and S. cerevisiae is that in S. cerevisiae, detection of pheromone is the only signal necessary to trigger a mating response, whereas in O. polymorpha a nitrogen depletion signal is needed as well. Ste12 is the probable point of integration of these two signals in O. polymorpha (Fig 7), with the nitrogen-depletion signal (communicated through Efg1 and Rme1) increasing the level of STE12 transcription, and the pheromone-induced MAP kinase cascade activating Ste12 by releasing the ortholog of the inhibitor proteins Dig1/Dig2 [53, 54]. We suggest that O. polymorpha cells initiate switching if Ste12 protein becomes abundant but no pheromone has been detected.

Comparing the networks that contain EFG1, RME1 and STE12 in different ascomycete species shows that there has been extensive reorganization during evolution (Fig 8). These networks are complex because they integrate information about the cell’s nutrient status (from the PKA pathway), the presence of pheromone (from the MAPK pathway), and the cell’s ploidy (from the a1/a2 repressor), to decide whether the cell responds by mating, switching, sporulating, or filamentous growth [55]. In S. cerevisiae, the nutrient status of the cell is primarily signaled by modulating PKA activity, which occurs via cyclic AMP for glucose sensing, and independently of cAMP for sensing other nutrients such as nitrogen [36, 56]. Much of the transcriptional response to changes in PKA activity in S. cerevisiae is mediated by the stress-response transcription factors Msn2 and Msn4 [56, 57]. However, there is no ortholog of Msn2/4 in O. polymorpha, and in C. albicans the major PKA-regulated transcription factor is Efg1, not Msn2/4 [33–36, 58]. It is likely that in O. polymorpha PKA regulates EFG1 to signal nitrogen depletion, because PKA is known to regulate Efg1 orthologs in Eremothecium (Ashbya) gossypii [59] and S. cerevisiae [28, 57], as well as C. albicans [51, 60]. One of the functions of S. cerevisiae Sok2 is to repress the master inducer of meiosis IME1 in rich conditions [49], but IME1 also has no ortholog in O. polymorpha. Thus the role of O. polymorpha EFG1 may be quite unlike the roles of S. cerevisiae SOK2 and PHD1. The MAPK and PKA pathways may be more interconnected in other ascomycetes than in S. cerevisiae, because C. albicans Efg1 also plays a role in mating and interacts with the Dig1/2 ortholog [54].
Regulation of mating-type switching in *Ogataea polymorpha*

**Saccharomyces cerevisiae**
- Bem4
- a1/c2
- Ste12
- Tec1
- Filamentous growth
- Mating response
- Rme1
- Efg1
- Ime1
- Ime2
- Meiosis, Sporulation
- Ash1, Swi5
- Switching (HO)
- (Phd1/Sok2)
- Msn2/4

**Ogataea polymorpha**
- Pheromone Signal
- MAPK
- a1/c2
- Ste12
- Mating response
- Switching (inversion)
- Efg1
- Rme1
- Meiosis not studied
- Ime1
- Msn2/4

**Kluyveromyces lactis**
- Pheromone Signal
- MAPK
- a1/c2
- Ste12
- Mating response
- Msn2/4
- Rme1
- Switching (α3, Kat1)
- Meiosis
- Ime1

**Eremothecium gossypii**
- Pheromone Signal
- MAPK
- Ime1
- a1/c2
- Ste12
- Mating response
- Efg1
- (Sok2)
- Sporulation (in haploids, mitotic?)
- Meiosis not studied
- Switching not studied
- Rpe1

**Candida albicans**
- Pheromone Signal
- MAPK
- a1/c2
- Ime1
- W/O circuit
- Opaque state
- Wor2, Wor1
- White state
- Czf1
- Ste12
- (Cph1)
- Mating response
- Filamentous growth / Biofilm
- No meiosis
- No mating-type switching
- Rme1

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The mating response pathway of *O. polymorpha* is similar to that in *K. lactis* in the sense that Rme1 conveys the nutrient depletion signal that is needed to activate STE12 for mating [20, 37], but their pathways for induction of switching are different (Fig 8). In *K. lactis*, Ste12 has no known role in switching, and upon nutrient depletion Rme1 induces switching either by activating transcription of *KAT1* or by binding to the α3 locus, depending on the direction of switching [14, 15]. Furthermore, the connection between nutrient signaling and switching in *K. lactis* has been proposed to occur via Msn2 rather than Efg1 [61]. Nevertheless, the pathway that regulates switching in *O. polymorpha* has more similarity to that in *K. lactis* than to that in *S. cerevisiae*. Switching in *S. cerevisiae* via HO endonuclease is highly regulated in terms of cell cycle and cell lineage [3, 4, 13], but has no connection to PKA signaling or STE12 (Fig 8).

Is there an endonuclease or site-specific recombinase for mating-type switching in *O. polymorpha*? At the outset of this project we assumed that the flip/flop mechanism would employ a specific enzyme to initiate recombination between the two IRs, but we have been unable to find such an enzyme. In retrospect, we realize that a site-specific recombinase is unlikely because recombinases generally recognize sites that are much shorter than the 2-kb IRs [62]. It now seems probable that during switching a site-specific DNA break is induced in one copy of the IR, followed by repair by recombination with the other copy, which can be resolved as either a crossover (inversion of the 19-kb region) or a non-crossover (no switching). Site-specific breaks are made in *S. cerevisiae* by HO, and in *K. lactis* by Kat1 and α3, but the *O. polymorpha* genome contains no homologs of any of these proteins. One possible hypothesis for *O. polymorpha* is that a site-specific break might be formed during attempted replication of a fragile DNA site, similar to switching in *Schizosaccharomyces pombe* [4, 9], but if this is correct, the site must be fragile only in nitrogen-poor conditions. Alternatively, *O. polymorpha* might use a recombinase or endonuclease that is activated post-transcriptionally. Further characterization of the switching mechanism in *O. polymorpha* may require biochemical approaches or genetic screens to identify mutants that switch constitutively.

**Materials and methods**

**Strain and plasmids**

Strains and plasmids used in this study are listed in S6 Table. Constructs for gene deletions contained 700–1000 base pairs of sequence flanking the target locus and an antibiotic resistance marker. Flanking and marker sequences were amplified using a high-fidelity DNA polymerase (Phusion or Q5, New England Biolabs), purified (PCR Purification Kit, Qiagen), and assembled by fusion PCR. PCR products were introduced into cells by electroporation, as described previously [63]. Gene deletions were made in *ku80Δ* backgrounds to increase efficiency of homologous recombination [32]. Successful integration was tested by antibiotic selection on YPD plates containing 200 μg/mL G418, 200 μg/mL hygromycin B, 100 μg/mL nourseothricin, or 100 μg/mL zeocin, as appropriate. Colony PCR was performed on resistant colonies to test for integration at the correct locus (GoTaq G2 polymerase, Promega). Plasmids for overexpression were constructed using pHIPH4 [32]. *EFG1, STE12*, and *RME1* coding sequences were amplified using a high fidelity polymerase and primers containing restriction
enzyme sites (SbfI, XmaI, HindIII, or XbaI). The purified PCR products and plasmid were digested, ligated, and transformed into E. coli. Clones were purified and digested with StuI enzyme overnight at 37°C for electrotransformation into O. polymorpha.

**Mating-type switching PCR assay**

To induce mating-type switching, overnight ‘pre-induction’ cultures grown at 37°C in YPD were centrifuged at 3400 x g for 2 min, washed once in NaKG (0.5% sodium acetate, 1% potassium chloride, 1% glucose), and resuspended in 10 mL NaKG at A600 0.5. NaKG cultures were then incubated on a shaker at 28°C for 24 h. DNA was isolated from the pre-incubation and NaKG-grown cultures by phenol:chloroform extraction. The MAT locus orientation was determined by PCR amplification using GoTaq G2 polymerase (Promega) for 30 cycles with 55°C annealing temperature and 3 min elongation. PCR products were visualized on 1% agarose gel with ethidium bromide staining.

**Mating assay**

Cells were streaked in parallel lines on YPD agar and crossed on MEMA (2.5% maltose, 0.5% malt extract, 2% agar) by replica plating. MEMA plates were incubated at 28°C for 24 h before replica plating to SD agar. SD plates were incubated at 37°C for 48 h to observe growth of diploids.

**Methanol induction**

Induction of expression from the AOX promoter was achieved by growing overnight cultures at 37°C in mineral media [64] containing 0.5% glucose (MMG). Overnight cultures were diluted in fresh MMG to A600 0.2 and grown to A600 >1.5. Cultures were diluted again in fresh MMG to A600 0.2 and grown to A600 >2.0. Cultures were diluted in mineral media + 0.4% methanol (MMM) to A600 0.2 and grown on shaker overnight at 37°C. RNA samples were isolated from these cultures with two biological replicates by hot acid phenol extraction and DNase I (Invitrogen) treatment.

**Bulk segregant analysis**

A diploid prototrophic colony obtained from the O. polymorpha NCYC495 x O. parapolyomorpha DL-1 interspecies cross was sporulated by streaking on ME agar (2% malt extract, 2% agar) and incubating at 25°C. Random spores were isolated by ether treatment: sporulating culture was suspended in sterile water before addition of an equal volume of diethyl ether and incubation at 30°C for 45 min. Ether-treated cells were diluted, plated on YPD agar, and incubated at 37°C for 48 h. Haploid clones grown from spores were tested for the ability to switch mating types using the PCR assay described above, with the following modification: DNA extractions were performed by treatment of cells with 700 units lyticase, incubation at 37°C for 30 min, followed by extraction using a Promega Maxwell 16 according to manufacturer’s instructions. Clones were identified as MATα or MATα, and as switchers or non-switchers, by PCR assay. Clones with clear phenotypes were assigned to four pools for sequencing: MATα switchers (30 clones), MATα non-switchers (35 clones), MATα switchers (35 clones) and MATα non-switchers (52 clones) (S5A Fig). Clones for each pool were grown individually and the pools were then made by combining equal A600 units for phenol:chloroform DNA extraction. DNA was also extracted from the parental strains NCYC495 and DL-1. All DNA samples were purified using a Genomic DNA Clean and Concentrator kit (Zymo). Genomic DNA
library preparation and Illumina HiSeq 2500 sequencing were performed at the University of Missouri DNA Core Facility.

We first created new reference genome sequences for our \textit{O. polymorpha} NCYC495 (\textit{ade}11 \textit{met}6) and \textit{O. parapolymorpha} DL-1 (\textit{leu}2 \textit{ura}3) parental strains, by mapping the reads from these strains onto the published genome sequences \cite{12, 24} using BWA \cite{65}. We did this because we discovered that our \textit{ade}11 \textit{met}6 derivative of NCYC495 (obtained from Dr. Kantcho Lahtchev, Bulgarian Academy of Sciences) contains regions with significant numbers of differences relative to the reference sequence of strain NCYC495 \textit{leu}1.1 (obtained from Prof. Andriy Sibirny, National Academy of Sciences of Ukraine) that was sequenced by Riley et al. \cite{12}. Our ‘NCYC495’ \textit{ade}11 \textit{met}6 stock appears to be the product of a cross between a genuine NCYC495 background and an \textit{O. polymorpha} strain with a slightly divergent genome, possibly strain CBS4732.

We then mapped the Illumina reads from each of the four pools to these \textit{O. polymorpha} and \textit{O. parapolymorpha} reference genome sequences. Only reads that had a single perfect match to one species, but no perfect match to the other, were retained for analysis. We divided the \textit{Ogataea} genome into 7824 segments, where each segment is either a pair of orthologous genes in NCYC495 and DL-1, or a pair of ‘intergenic’ regions in the interval between two consecutive pairs of orthologs. These ‘intergenic’ regions can include genes that are present in one species but absent in the other. For each segment in each species, we calculated the numbers of reads from each pool, and from the parental strains, that mapped to it. Preliminary analysis showed no significant differences between the two mating types, so we merged the data from \textit{MATa} and \textit{MATa} clones. We then calculated four ratios for each genomic segment:

\begin{itemize}
  \item $SW_{\text{NCYC495}}$ is the number of reads from switchers that mapped exclusively to the NCYC495 reference genome in this segment, divided by the number of reads from the NCYC495 parental strain that mapped exclusively to the NCYC495 reference genome in this segment, normalized by the total numbers of mapped reads in each library.
  \item $NS_{\text{NCYC495}}$ is the number of reads from non-switchers that mapped exclusively to the NCYC495 reference genome in this segment, divided by the number of reads from the NCYC495 parental strain that mapped exclusively to the NCYC495 reference genome in this segment, normalized by the total numbers of mapped reads in each library.
  \item $SW_{\text{DL-1}}$ is the number of reads from switchers that mapped exclusively to the DL-1 reference genome in this segment, divided by the number of reads from the DL-1 parental strain that mapped exclusively to the DL-1 reference genome in this segment, normalized by the total numbers of mapped reads in each library.
  \item $NS_{\text{DL-1}}$ is the number of reads from non-switchers that mapped exclusively to the DL-1 reference genome in this segment, divided by the number of reads from the DL-1 parental strain that mapped exclusively to the DL-1 reference genome in this segment, normalized by the total numbers of mapped reads in each library.
\end{itemize}

These four ratios are plotted in S5B Fig. We defined the Asymmetry metric (Fig 2C) of a genomic segment as

\[
\text{Asymmetry} = \max(SW_{\text{NCYC495}} - 1, 0) \cdot \max(1 - NS_{\text{NCYC495}}, 0) \cdot \max(NS_{\text{DL-1}} - 1, 0) \cdot \max(1 - SW_{\text{DL-1}}, 0)
\]

This metric has a value of zero, except in genomic segments where four criteria are met simultaneously: the proportion of NCYC495-derived DNA is higher than expected by chance in the switcher pool but lower than expected in the non-switcher pool, and the proportion of
DL-1-derived DNA is higher than expected by chance in the non-switcher pool but lower than expected in the switcher pool.

**RNAseq and ChIPseq**

Strains for nitrogen depletion samples and efg1 deletion samples were grown in YPD at 37°C overnight, diluted to an OD600 0.1 and grown to log phase (OD600 1.0). Cultures were pelleted by centrifugation, washed once in YPD, NaKG, NaKG + 40mM ammonium sulfate, SD, or SD minus ammonium sulfate, before resuspending in the same media and culturing for 2 h at 28–30°C. RNA samples from these cultures were prepared with the MasterPure Yeast RNA Purification Kit (Epicentre, Illumina) or by hot acid phenol extraction and DNase I (Invitrogen) treatment. Nitrogen depletion samples were performed in triplicate, overexpression and efg1 deletion samples were performed in duplicate.

Chromatin immunoprecipitation (ChIP) was performed by formaldehyde crosslinking of log phase cells for 20 min with glycerol addition used to stop the reaction. Cells were lysed using glass beads and chromatin was fragmented by sonicating with a Bioruptor Standard (Diagenode). EZview Red Anti-HA Affinity Gel (Sigma-Aldrich) was used to immunoprecipitate chromatin fragments, and bound DNA was eluted using HA peptide (Sigma-Aldrich). Crosslink reversal was followed by phenol:chloroform extraction. ChIP samples were performed with three biological replicates that were pooled prior to sequencing.

Stranded mRNAseq and ChIPseq library preparation and sequencing services were performed at the University of Missouri DNA Core Facility. 50–51 bp unpaired Illumina reads (RNAseq and ChIPseq) were mapped to the *Ogataea polymorpha* (NCYC495 leu1-1 [12]) genome using Bowtie v1.1.2 using the following options: -v = 3, to report end-to-end hits with < = 3 mismatches; -k = 10, to report up to 10 good alignments per read;—best, so hits guaranteed best stratum with ties broken by quality; -M = 1, to report just 1 random hit out of the good alignments for a read; -S, to write hits in SAM format; -p = 10, to use 10 processors. Aligned hits were split into reads that mapped to the forward and reverse strand (SAM FLAG = 0 and 16) before proceeding. Samtools v0.1.12a (r862) was used to create sorted and indexed BAM files of the results. Bedtools v2.19.0 was used to create genome coverage Bedgraph files, which were converted to BigWig files using bedGraphToBigWig v4 for visualization as tracks in Jbrowse v1.11.2.

For RNAseq data htseq-count v0.6.0 was used to calculate for each feature the number of reads mapping to it. We mapped to a feature file based on the original JGI NCYC495 annotation with extensive manual modification. We counted against both the forward and reverse strand mapping SAM files, creating sense and antisense counts for each feature, but only retained sense counts for further analysis. We then calculated Transcripts Per Million (TPMs) [66] for each feature and used DESeq2 in R v3.2.1 to calculate differential expression between conditions.

**Supporting information**

**S1 Fig.** Cell-type and media dependent gene expression patterns in *O. polymorpha*. Heatmap shows the log2 (fold change) in expression of all genes in the genome from mRNAseq of *O. polymorpha* haploid MATa, haploid MATα, and diploid MATa/α strains grown in rich media (YPD), or 2 h after transfer from YPD into nitrogen depletion media (NaKG). (EPS)

**S2 Fig.** Gene expression differences between a-cells and α-cells. Each gene’s expression ratio between a-cells and α-cells is plotted, for NaKG media (X-axis) and YPD media (Y-axis). Only
the 475 genes for which the expression ratio was significantly different from 1 in at least one of the conditions are plotted (unadjusted \( P < 1\text{e}-3 \)).

\( \text{(EPS)} \)

\textbf{S3 Fig. Gene expression differences between haploid a and diploid a/\( \alpha \) \textit{O. polymorpha} cells.}\n
For each gene in the genome, its expression ratio between a-cells and a/\( \alpha \)-cells is plotted, in NaKG media (X-axis) and YPD media (Y-axis). The \( \text{MAT}a1 \) and \( \text{MAT}a2 \) genes appear diploid-enriched in this experiment because they have higher expression in diploid a/\( \alpha \)-cells than in haploid a-cells. Only genes for which the expression difference was significant at \( P < 1\text{e}-3 \) (adjusted for multiple testing with the Benjamini-Hochberg correction) for at least one of the media are plotted.

\( \text{(EPS)} \)

\textbf{S4 Fig. Search for haploid-specific, NaKG-induced genes.}\n
Environmental control of gene expression in haploid cells (X-axis) is compared to cell type control of gene expression in nitrogen limitation media (Y-axis). Genes with a role in mating-type switching are expected to lie in the bottom-left quadrant.

\( \text{(EPS)} \)

\textbf{S5 Fig. (A) Phenotypes of haploids used in bulk segregant analysis.}\n
Random spores isolated from a DL-1 x NCYC495 diploid were grown overnight in YPD before transfer to NaKG for 24 h. Gels show PCR analysis of the \text{MAT} locus to determine the original mating type and switching phenotype of the haploid isolates. Haploids were classified into four groups as shown. (B) Inheritance of genomic regions derived from NCYC495 and DL-1 in the sequenced pools of switching and non-switching progeny. The upper panel shows reads that mapped exclusively to the NCYC495 reference genome, and the lower panel shows reads that mapped exclusively to the DL-1 reference genome, from switcher (blue) and non-switcher (red) pools. The Y-axis is the ratio between the normalized number of mapped reads from a pool, relative to the number from the parental strain sample, in each of the 7824 genomic segments. Chromosome numbering and orientation follows the convention for \textit{O. polymorpha} [12].

\( \text{(TIF)} \)

\textbf{S6 Fig. (A) Multiple sequence alignment of Efg1 and related proteins.}\n
The APSES domain is highlighted in blue. The orange triangle indicates the site of the frameshift mutation in \textit{O. parapolymerpha} strain DL-1. (B) Phylogenetic tree of the Efg1 protein family. \textit{O. polymorpha} has two \textit{EFG1}-like genes, which we refer to as \textit{EFG1} (\textit{OPOL}_95241) and \textit{OPOL}_93012. This gene duplication is specific to the genus \textit{Ogataea}, shared by \textit{O. polymorpha} and \textit{O. parapolymerpha}. The tree was constructed by Maximum Likelihood using an LG model with an alignment of 1377 amino acids in 55 taxa from Saccharomycotina and Pezizomycotina. Branch support was determined by 100 bootstrap replicates.

\( \text{(EPS)} \)

\textbf{S7 Fig. White-opaque circuit genes other than \textit{EFG1} are not required for mating-type switching or mating in \textit{O. polymorpha}. (A)}\n
PCR determination of \text{MAT} genotypes before and after 24 h growth in NaKG for wild-type, \textit{czf1-like2}\( \Delta \), \textit{czf1-like3}\( \Delta \), \textit{czf1-like2aczf1-like3}\( \Delta \), \textit{mit1}\( \Delta \) and \textit{wor2}\( \Delta \) \textit{O. polymorpha} strains in \text{MAT}a (left) and \text{MAT}a (right) backgrounds. PCR primers A-D are as in Fig 1. (B) Photographs of diploid growth on SD plates following crosses on MEMA plates for 24 h.

\( \text{(EPS)} \)
S8 Fig. *RME1* and *STE12* overexpression induces the mating pathway in *O. polymorpha*. **(Left)** Heatmap showing the log$_2$(fold change) of expression of all genes in mRNAseq of strains overexpressing *RME1*, *STE12*, or *EFG1* from the *pAOX* methanol-inducible promoter, relative to a control strain containing empty *pAOX* vector. **(Right)** Genes with the highest expression increases in *STE12* and *RME1* overexpression strains include those with α-specific (gene names in green), α-specific (pink), haploid-specific (purple), and other mating functions (orange). Genes with Rme1 signals in ChIPseq are indicated by orange boxes.

EPS

S9 Fig. Alignment of a-factor (MFa) sequences from *O. polymorpha*, *S. cerevisiae* and *C. albicans*. The *O. polymorpha* gene is located immediately upstream of *RAD17* on chromosome 5, at position complement(253651..253761) of NCBI accession number AECK0100005.1 [12].

EPS

S10 Fig. Nineteen candidate genes not required for mating-type switching. Genes were selected based on their expression patterns in *O. polymorpha* or their known roles in other species. Deletion strains in a *MATα ku80Δ* background were generated by electrotransformation. The *MAT* locus was PCR amplified from each strain before, and 24 h after, transfer from a YPD pre-induction culture into NaKG. The wildtype (WT) panels in this figure are reproduced from Fig 1C and Fig 6 because these assays were all done together.

EPS

S1 Table. *O. polymorpha* genes induced and repressed on nitrogen-depleted (NaKG) vs. rich (YPD) media.

(XLSX)

S2 Table. Gene expression differences between *O. polymorpha* MATα and MATα haploid cells.

(XLSX)

S3 Table. Genes with highest expression differences between haploid (*MATα*) and diploid (*MATα/α*) *O. polymorpha* cells, in YPD media and NaKG media.

(XLSX)

S4 Table. *O. polymorpha* genes with largest expression differences in *efg1Δ* vs. WT cells in different media: (A) NaKG media plus ammonium sulfate (nitrogen-replete); (B) NaKG media (nitrogen-poor); (C) SD media (nitrogen-replete); (D) SD media without nitrogen (nitrogen-poor).

(XLSX)

S5 Table. *O. polymorpha* genes with largest expression differences in (A) *pAOX-EFG1*, (B) *pAOX-STE12*, and (C) *pAOX-RME1*, vs. *pAOX* control upon induction of expression in methanol media.

(XLSX)

S6 Table. Strains and plasmids used in this study.

(XLSX)

S7 Table. Primers used in this study.

(XLSX)

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