Published in final edited form as:
Fungal Genet Biol. 2011 September ; 48(9): 858–865. doi:10.1016/j.fgb.2011.04.003.

Milestones in *Candida albicans* Gene Manipulation

Dhanushki P. Samaranayake[a] and **Steven D. Hanes**[b]

[a]Department of Biomedical Sciences, School of Public Health, State University of New York, Albany, NY 12208, USA

[b]Department of Biochemistry and Molecular Biology, Upstate Medical University, Syracuse, NY 13210, USA

Abstract

In the United States, candidemia is one of the most common hospital-acquired infections and is estimated to cause 10,000 deaths per year. The species *Candida albicans* is responsible for the majority of these cases. As *C. albicans* is capable of developing resistance against the currently available drugs, understanding the molecular basis of drug resistance, finding new cellular targets, and further understanding the overall mechanism of *C. albicans* pathogenesis are important goals. To study this pathogen it is advantageous to manipulate its genome. Numerous strategies of *C. albicans* gene manipulation have been introduced. This review evaluates a majority of these strategies and should be a helpful guide for researchers to identify gene targeting strategies to suit their requirements.

Keywords

Pathogenic fungi; *Candida albicans*; gene manipulation; selectable marker; homologous recombination

1. Introduction

Candida species are serious human fungal pathogens among immune compromised individuals (Odds, 1994). Susceptible patients include those undergoing cancer chemotherapy or organ transplantation, patients infected with HIV, and pre-mature infants (Schelenz et al., 2011; Epstein et al., 2003; Muller et al., 1999). Candida blood stream infections (candidemia) are life threatening among hospitalized immune-compromised patients, including neonates (Fridkin et al., 2006). The incidence rate of hospital-acquired candidemia is as high as 8 in every 100, 000 people (Kao et al., 1999). Today, candidemia is one of the leading bloodstream infections in the United States with an annual cost of treatment approaching $1 billion (Miller et al., 2001). Although infections with non-albicans *Candida* species have emerged in recent years (Miceli et al., 2011), the species, *C. albicans* is still responsible for the majority of cases (Horn et al., 2009; Ruhnke, 2006).

Corresponding author: Steven D, Hanes, Ph.D., Department of Biochemistry and Molecular Biology, SUNY-Upstate Medical University, 750 E. Adams Street, Syracuse, NY 13210, haness@upstate.edu, (315) 464-9887.
co-corresponding authors: S.D. Hanes: haness@upstate.edu; D.P. Samaranayake: dhashchin@yahoo.com

Researchers have studied this pathogen for decades. As a result, there are several clinically available drugs to treat *C. albicans* infections (De Rosa et al., 2009; Shao et al., 2007; Lai et al., 2008). However, *C. albicans* has the ability to acquire resistance against many of these drugs (Cannon et al., 2007; Casalinuovo et al., 2004). Therefore, even today there is a need for additional drugs to inhibit this pathogen.

Molecular analysis of *C. albicans* has been particularly challenging for many reasons. *C. albicans* has no clearly defined sexual cycle (Noble and Johnson, 2007). Moreover genetic manipulation of *C. albicans* is not simple. First, the organism is diploid (Barnett, 2008; Jones et al., 2004) and in most cases both alleles of a gene must be manipulated. Second, compared to the highly studied yeast, *Saccharomyces cerevisiae, C. albicans* lacks natural plasmids, such as the 2-micron plasmid, for use in transformation and shows lower transformation and recombination frequencies making the manipulation of both alleles of a gene even more challenging (De Backer et al., 2000; Magee et al., 2003). Third, *C. albicans* shows non-conventional codon usage where the CUG codon is decoded as serine, not leucine (Santos and Tuite, 1995). Therefore, when using marker gene sequences from other organisms for gene manipulation in *C. albicans*, the codons need to be optimized. Fourth, the use of selectable markers has been particularly challenging because some markers have effects on virulence of the pathogen. In addition, *C. albicans* shows natural resistance to drugs such as G418, hygromycin B and cycloheximide, which are used for selection in *S. cerevisiae* (De Backer et al., 2000). This review highlights the milestones achieved for effective methods to manipulate the *C. albicans* genome.

2. Nutritional Markers for Selection

Early researchers introduced mutations in the *C. albicans* genome by exposure to UV or chemical mutagens. These methods left behind undesirable mutations that could not be traced or repaired (Fonzi and Irwin, 1993).

Fonzi and Irwin (1993) used a clinical isolate of *C. albicans* (SC5314) to make a strain (CAI4) that was auxotrophic for growth on uracil-deficient media. To do this, they disrupted both copies of the *URA3* gene in *C. albicans* using the *imm434* region of the λgt10 bacterial phage. This was the first time in the field of *C. albicans* research that a reliable parent strain was available to introduce mutations into the genome of *C. albicans*.

In *C. albicans*, the *URA3* gene encodes the enzyme, orotidine 5'-monophosphate decarboxylase that catalyzes the conversion of orotidine 5'-monophosphate to uridine 5'-monophosphate in the *de-novo* pyrimidine biosynthesis pathway (Lay et al., 1998). The resulting CAI4 strain is unable to grow in the absence of uracil, hence *URA3* gene could be used as a dominant nutritional selectable marker in *C. albicans*.

Because of the use of *URA3* as a selectable marker to disrupt genes, the method is popularly known as the “URA Blaster method”. Here, the gene of interest is disrupted using a cassette that carries the *URA3* gene flanked by two *hisG* sequences from *Salmonella typhimurium* (Fig. 1A-1). After the initial transformation, the transformants are selected on uracil-deficient media. Because the placement of two direct repeats of the *hisG* sequence at close proximity in the genome is unstable, spontaneous recombination events can occur between these *hisG* sequences. Such recombination events will loop out the *URA3* gene and leave behind one copy of the *hisG* sequence in the genome. The loopout event allows the re-cycling of the *URA3* marker for the disruption of the second allele. The use of *URA3* is specifically advantageous because it can also be counter selected. Therefore, in the next step the cells that excise the *URA3* gene by a recombination event between the *hisG* direct repeats can be selected on 5-FOA (5-Fluoroorotic acid). An additional second

transformation step potentially generates cells that are null for the gene of interest, if recombination occurs at the wild-type allele.

The “URA Blaster” method was the method of choice for not only genetic manipulation of *C. alibicans*, but also for constructing other parent strains that lacked additional nutritional markers (Negredo et al., 1997; Wilson et al., 1999). However, during the construction of the parent strain CAI4, the deletion construct also removed a portion of the 3’ end of the adjacent gene, *IRO1* (García et al., 2001). *IRO1* is a gene associated with iron utilization (García et al., 2001). Although there is no direct evidence that the loss of the 3’ portion of the *IRO1* gene affects the virulence of the pathogen, iron uptake is known to be important for growth of *C. albicans* (Ramanan and Wang, 2000). Further, the CAI4 strain is known to show defective expression of 14 different proteins when compared to its parent, SC5314 (Brand et al., 2004).

When using the “URA Blaster” method for gene manipulation, the recombination between *hisG* repeats leaves a scar (one copy of the *hisG* sequence) in the genome. In instances where a promoter region of an adjacent gene is within or just following the termination region of the gene of interest, leaving behind a scar could be detrimental as it might affect the expression of the adjacent gene. Foreign DNA sequences can also cause rearrangements in the genome or make further manipulations or evaluations more complex (Fehér et al., 2008). For these reasons, gene manipulation without leaving a scar has become a goal in other organisms as well (Storici et al., 2001; Cox et al., 2007; Liang and Liu, 2010).

Another drawback of using the “URA Blaster” method is that exposure to 5-FOA in the second step is potentially mutagenic and can introduce chromosomal rearrangements (Wellington et al., 2006). In addition, this method leaves a single copy of *URA3* at the locus of the gene of interest. Unfortunately almost half a decade later, researchers discovered that the expression of *URA3* at ectopic loci in the genome can affect the virulence of *C. albicans* (Lay et al., 1998; Brand et al., 2004). *URA3* gene expression in some strains disrupted by the “URA Blaster” method, hence carrying *URA3* in an ectopic location of the genome, showed a 2 to 18 fold reduction in orotidine 5'-monophosphate decarboxylase enzyme activity (Lay et al., 1998). Overall the change in the chromosomal location of the *URA3* gene affected the *C. albicans* Ura3 activity, hyphal morphogenesis, adherence and lethality in mice (Cheng et al., 2003). Phenotypic changes in about 30% of the published papers at that time were, upon reevaluation, due to the expression of *URA3* at an ectopic location in the genome and not due to the deletion of the gene of interest *per se* (Brand et al., 2004).

To overcome this problem, the single remaining copy of *URA3* was removed from the locus of the gene of interest and placed at a highly expressed locus such as, *RPS1*, *ENO1*, *ARG4* or at the native locus of *URA3* itself (Murad et al., 2000; Brand et al., 2004; Sundstrom et al., 2002; Davis et al., 2000; Ramon and Fonzi, 2003). It was shown that 40% expression from *URA3* was adequate for full virulence affects of *C. albicans* on mice (Lay et al., 1998; Brand et al., 2004). Therefore, replacing one copy of *URA3* was considered adequate for a reliable experiment. It was around this time that “reconstitution” of a mutant strain by introduction of a wild-type copy of the gene to rescue the mutant phenotype was considered an essential part of a *C. albicans* experiment (Magee et al., 2003; Brand et al., 2004).

As a result of the problems associated with the “URA Blaster” method, many researchers developed alternative versions of the “URA Blaster” cassette or entirely new deletion cassettes. Morschhauser et al. (1999) introduced the use of *FLP* recombinase system to make a recyclable *URA3* cassette to disrupt both copies of the gene of interest in the parent strain CAI4 (URA Flipper). In this method, the *URA3* marker is flanked by two direct repeats of the Flp recombinase recognition site, FRT (Fig. 1A-2). The expression of the *FLP*

recombinase gene is controlled by the inducible *SAP2* promoter (secreted aspartyl proteinase family 2). After transformation, the *SAP2* promoter is activated and *FLP* is expressed. To activate the *SAP2* promoter the cells should be grown in YCB-BSA (**Y**east **C**arbon **B**ase and **B**ovine **S**erum **A**lbumin, pH4.0) media. The expressed Flp recombinase then recognizes the FRT sites and loops out the *URA3* gene by homologous recombination, making the auxotrophic marker available for the second transformation (Fig. 1B). This method avoids the use of 5-FOA and its associated mutagenic potential to counter select for *URA3* gene. However, similar to the "URA Blaster" cassette, the "URA Flipper" cassette also leaves behind a scar (a single FRT sequence) in the genome, at the locus of the gene of interest. Nevertheless the scar left behind is very small - only 34 base pairs. In addition, the method requires that the cells are grown in YCB-BSA media to express Flp recombinase, which could be undesirable for specific gene manipulations, such as genes associated with certain metabolic processes and should be carefully considered.

Both "URA Blaster" and "URA Flipper" cassettes require several cloning steps to be customized for the use of one's own gene of interest. Wilson et al. (1999) tested the use of relatively rapid PCR-amplified deletion cassettes to disrupt genes of interest in parent strains that were derivatives of CAI4. They were, RM1000, auxotrophic for *URA3* and *HIS1* (Negredo et al., 1997) and BWP17, auxotrophic for *URA3*, *HIS1* and *ARG4* (Wilson et al., 1999). A 50–60 base pair overhang of homologous sequences of the gene of interest on either side of the selectable marker was found to be sufficient for disruption. Because this gene cassette was not recyclable, they used two nutritional markers from the available *URA3, HIS1* or *ARG4* to disrupt the two alleles.

Wilson et al. (2000) introduced a recyclable *URA3* cassette that could be efficiently PCR amplified. Since the cassette was recyclable a single nutritional marker could be used for gene manipulation in *C. albicans*. In addition, this cassette saved an enormous amount of time as it could be rapidly PCR amplified. The cassette is similar to the "Ura Blaster" cassette with the exception that it does not carry the bulky *hisG* sequences, but instead carries a 200 base pair region of the 3' end of the *URA3* gene, at its 5' end (Fig. 1A-3). However, the method does leave behind a scar (200bp) after the recombination between the repeating 3' end sequences of the *URA3* gene and also requires the use of 5-FOA.

Enloe et al. (2000) constructed a UAU1 cassette as a further development of this cassette and implemented it on the parent strain, BWP17 (Fig. 1A-4). The importance of the UAU1 cassette is that it allows the disruption of both copies of the gene of interest with a single transformation step. UAU1 cassette has the *URA3* gene disrupted by the *ARG4* gene, but the 5' end sequence of the *URA3* gene also carries a 530 base pair sequence homology to its 3' end sequence. Therefore, when transformed into the genome, recombination loops out the *ARG4* gene and makes a functional copy of *URA3*. This unique feature of the UAU1 cassette allows selection of rare instances where both alleles of the gene are replaced by mitotic recombination or gene conversion, in a single transformation step. These rare double-delete cells are detected due to the presence of both, *ARG4* and *URA3* markers. However, these potential homozygous deletion mutants need to be confirmed to rule out possible allelic triplications, in which a wild-type allele is retained in spite of two alleles been replaced by the cassette. This condition may arise due to known triplicated alleles in the genome, increase in ploidy, tandem duplications or translocations (Enloe et al., 2000).

It is known that *C. albicans* has remarkable tolerance for aneuploidy (Rustchenko, 2007). Aneuploidy refers to the gain or loss of full or parts of chromosomes that leads to an alteration in the normal complement of chromosomes. Aneuploidy can affect the virulence of *C. albicans* (Chen et al., 2004). The chromosome alterations were initially tested through contour-clamped homogenous electric field gels (CHEF gels) and quantitative Southern blot

hybridization, two fairly laborious methods (Thrash-Bingham and Gorman, 1992; Navarro-García et al., 1995; Chen et al., 2004). Selmecki et al. (2005) tested aneuploidy in *C. albicans* laboratory strains using comparative genome hybridization (CGH). CGH allows comprehensive analysis of genomic alterations across over 6000 ORFs of *C. albicans* in a microarray format. Aneuploidy of the laboratory strains (CAI4 and BWP17) were tested compared to the clinical isolate SC5314. The results suggested that the laboratory strain, CAI4 had an unstable trisomy at chromosome 2 as it was not seen in the successive strains derived from it. In addition, the laboratory strain BWP17 and its parent (RM1000#6) showed a heterozygous deletion of the distal portion of right arm of chromosome 5. Thereafter, more attention was paid to constructing parents strain that had no or minimal karyotypic changes.

Noble and Johnson (2005) constructed parent strains that were auxotrophic for *LEU2*, *HIS1* and *ARG4* nutritional markers. The main strains constructed were, SN87 (auxotrophic for *LEU2* and *HIS1*), SN95 (auxotrophic for *HIS1* and *ARG4*) and SN152 (auxotrophic for *LEU2*, *HIS1* and *ARG4*). Unlike many of the previously constructed parent strains, these showed no karyotypic changes. All these strains have one copy of *URA3* expressed at the native locus. Using these strains, *C. albicans* genes are disrupted without the use of the *URA3* marker. These markers are not recyclable and are left at the locus of the gene of interest (Fig. 1A-5). However, extensive tests indicate that expression of any of the above nutritional markers in ectopic genomic locations did not affect the virulence of *C. albicans*. They also initiated the use of heterologous marker genes from *C. maltosa* or *C. dubliniensis* strains when disrupting genes in *C. albicans* to decrease recombination events at the endogenous locus of the marker gene.

Dennison et al. (2005) adapted the popular Cre-*loxP* system for *C. albicans* gene disruption in the BWP17 parent strain. In two separate transformation steps, the two copies of the gene of interest are disrupted by nutritional markers such as *HIS1* and *ARG4*, which are flanked by *loxP* sequences. In a third step, a fragment flanked by *arg4* sequences that carry both the Cre recombinase under the control of the *MET3* promoter and the nutritional marker *URA3* is transformed into *C. albicans* (Fig. 1A-6). This fragment integrates into the *ARG4* gene that is disrupting one copy of the gene of interest. Next, activation of *MET3* promoter produces Cre recombinase, which catalyses the recombination between *loxP* sites, looping out both the *HIS1* and the Cre recombinase-*URA3* fragment. The method requires three transformation steps and leaves the null strain auxotrophic for all three nutritional markers. The method also leaves behind only a 34 base pair scar (a single *loxP* sequence) at the locus of the gene of interest.

Many deletion cassettes that were developed since the “URA blaster” method, allow one to remove *URA3* from the locus of the gene of interest and replace it at its native locus. It should be noted that in addition to the multiple steps required to create the null mutant (gene deletion and the subsequent step to loop out the *URA3* gene), the *URA3* gene replacement step requires an additional transformation step. Unlike these methods, the UAU1 cassette leaves the *URA3* gene at the locus of the gene of interest (Enloe et al., 2000). This method can still be useful so long as a reconstituted strain is created and shown to restore the wild-type phenotype. Further, this method is faster (creates *C. albicans* null mutants with a single transformation step) and will not leave behind any chromosomal rearrangements that may arise due to the use of, 5-FOA since it avoids the use of this drug. Therefore, the UAU1 cassette seems to be prominent among the gene disruption methods that use *URA3*. However, if one prefers to avoid the *URA3* marker for selection altogether, the use of parent strains that are auxotrophic for *LEU2*, *HIS1* and *ARG4* nutritional markers may be useful (Noble and Johnson, 2005). This method uses two transformation steps, but does not require additional steps to loop out the nutritional markers. To date, no drawbacks of retaining the

LEU2, *HIS1* or *ARG4* markers at ectopic loci have been identified. Nevertheless the resulting null mutant phenotypes should be validated by comparing phenotypes with an isogenic strain that is reconstituted for the gene of interest.

In 2004, *C. albcians* was selected as the first eukaryotic pathogen for genome sequencing. Jones et al. (2004) used the heterozygous diploid genome of the widely-used clinical isolate, SC5314 to sequence the genome of *C. albcians* by using a whole-genome shotgun approach. Thereafter, sequences of a majority of the *C. albcians* genes were available making gene targeting in this organism much easier, faster, more efficient and, precise. Soon after, d'Enfert et al. (2005) established the Candida Genome Database (CGD) using the genome sequence of *C. albicans* and also enriched it with published literature on *C. albicans*. The CGD (www.candidagenome.org) is a freely available valuable resource for researches in the field because it is a reliable source of organized data, tools for data analysis and information about the current research community.

3. Drug Markers for Selection

Some researchers have chosen to avoid entirely the use of nutritional markers to manipulate the *C. albicans* genome. This is necessary if the genes of interest are in pathways affected by nutritional status. Instead, drug resistance markers can be used. The use of drug makers also opens way for testing clinical isolates as is, without creating any auxotrophic mutations. This advantage heavily benefits the researchers that test *C. albicans* clinical isolates. For example, clinical isolates can be tested to identify mechanisms of drug resistance, which will be another phase of addressing the current problem of drug resistance seen in this pathogen (Heilmann et al., 2010). The milestones in the development of these drug markers are discussed below.

Goshorn and Scherer (1989) first identified dominant mycophenolic acid (MPA) resistant mutants in *C. albicans*. MPA inhibits the inosine monophosphate dehydrogenase (Imh3) enzyme that directs *de novo* synthesis of GMP. These mutants were used to study *C. albicans* natural variants. Kohler et al. (1997) developed a strategy using *IMH3* as a selectable marker for *C. albicans* genetic manipulations. They overexpressed *IMH3* from a plasmid in the strain CAI4 and found that the successful transformants were far more resistant to MPA compared to the wild-type strains. However, the method was not developed to the level of chromosomal integration. Wirsching et al. (2000) used MPA resistance as a dominant selectable marker for chromosomal gene disruptions. Here, they used a form of the *IMH3* gene that was mutated so as to avoid re-integration of the fragment into the normal chromosomal copy of *IMH3* in *C. albicans*. They adapted the "URA flipper" strategy to this system whereby FRT recombination sites were positioned to flank the *FLP* recombinase gene (driven by the *SAP2* promoter) and the *IMH3* gene (replacing *URA3* selectable marker) to generate a "MPA^R flipper" cassette (Fig. 1A-7). Using the MPA^R flipper method, genes of *C. albicans* can be disrupted even in a clinical isolate without the use of any other auxotrophic markers. However, the method leaves behind a small 34 base pair FRT sequence at the locus of the gene of interest. In addition, the transformants are slow to appear and recombination is favored at the chromosomal *IMH3* locus making the process of screening for successful transformants time consuming and tedious. Deleting the native *IMH3* gene would be useful to reduce integration at this locus.

Reuss et al. (2004) used the drug nourseothricin as a dominant selectable marker for gene disruption. They introduced into *C. albicans* cells the streptothricin acetyltransferase (*SAT1*) gene (from bacterial transposon Tn1825) that confers resistance to the drug nourseothricin. A FLP-mediated recyclable marker system was used with the *FLP* recombinase gene regulated by the *MAL2* (maltase) promoter and the *SAT1* gene regulated by the *ACT1*

promoter (SAT1 flipper) (Fig. 1A-8). However, the *MAL2* promoter that is usually activated when cells are grown in maltose instead of glucose was later found to be a leaky promoter. Therefore *FLP* recombinase was expressed even when cells were grown in media with glucose.

Shen et al. (2005) used the *Streptomyces noursei nat1* (nourseothricin acetyltransferase) gene adapted to suit *C. albicans* in a CaNAT1-FLP cassette. The Ca*NAT1* gene (regulated by the *Ashbya gossypii TEF1* promoter) and the *FLP* recombinase gene (regulated by the *SAP2* promoter) are flanked by FRT recombination sites, as in the "URA flipper" cassette (Fig. 1A-9). Nourseothricin was found to have minimal affects on filamentation or cell growth. However, the CaNAT1-FLP cassette also leaves a genomic scar in the form of a single FRT sequence.

4. Other Markers for Selection

Limited uses of several other markers were also recorded in *C. albicans* gene disruption experiments. Morschhauser et al. (1998) used green fluorescence protein (GFP) in a reporter system with promoter and termination sequences from *ACT1* gene to disrupt *C. albicans* genes. The first transformation is selected by the expression of GFP. However the selection of the second transformation depends on the increase in the intensity of GFP signal or the use of a GFP variant with different spectral properties in the second round of transformation. In addition, limited use of codon optimized firefly luciferase and renilla luciferase have been recorded as methods of *C. albicans* gene disruption (Doyle et al., 2006; Srikantha et al., 1996).

5. Disruption of Essential *C. albicans* Genes

In most cases, the disruption of non-essential genes is relatively straight forward, compared to disruption of essential genes. Initially, the inability to obtain homozygous deletions was used as evidence that a gene was essential. This was inherently tentative because it is a negative result. Instead, conditional mutants were used to test the essentiality of genes. Here one copy of the gene is deleted and the expression of the second copy is made conditional. The essentiality of the gene is determined by measuring survival of *C. albicans* after a shift to non-permissive conditions.

For example, Devasahayam et al. (2002) engineered a conditional allele of a gene of interest (*ESS1*) based on prior work in *S. cerevisiae*. The mutated *C. albicans* gene, was first demonstrated to be temperature sensitive (*ts*) in *S. cerevisiae* prior to its analysis in *C. albicans*. The investigators then generated a *ts* strain of *C. albicans* by deleting one copy of *ESS1* and replacing the other with the engineered allele. The strain grew normally at permissive temperature, but did not grow at restrictive temperature, demonstrating the essentiality of *ESS1* in *C. albicans*.

Roemer et al. (2003) developed the "GRACE method" (**G**ene **R**eplacement **A**nd **C**onditional **E**xpression) for conditional expression of essential genes. In this method, the first copy of the gene is disrupted by a cassette containing the *HIS3* selectable marker (Roemer et al., 2003). This cassette also has two distinct bar codes placed on either side allowing the rapid and simple identification of the strain by PCR amplification. The second copy is placed under the control of a tetracycline (Tet) regulatable promoter system using a codon optimized *SAT1* selectable maker (from *E. coli*) driven by the *ACT1* promoter (Fig. 1A-10). Under normal conditions the binding of the transactivator protein to the Tet responsive promoter allows constitutive expression of the essential gene of interest. However, if the cells are provided with tetracycline, the association between the transactivatior protein and

the Tet responsive promoter is disrupted leading to repression of the gene. The method is useful to identify a gradient of phenotypes from cidal, to static, to no growth phenotype.

Park and Morschhäuser (2005) also used a tetracycline-regulatable system to make conditional null mutants of essential genes. Instead of the "Tet-Off" system (the gene regulated by tetracycline is turned off in the presence of the drug) explained above, Park and Morschhäuser adapted a "Tet-On" system (the gene regulated by tetracycline is turned on in the presence of the drug). The "Tet-On" system was made possible when Gossen et al. (1995) recognized the reverse Tet repressor. In the "Tet-On" system, the reverse Tet repressor (from *E. coli*) is fused to an activation domain (*GAL4* form *S. cerevisiae*) to create a reverse tetracycline-controlled transactivator (rtTA), which binds to the Tet responsive promoter in the presence of the drug. This is in contrast to the usual behavior of the transactivator that binds to the Tet responsive promoter in the absence of the drug.

To use this system, the essential gene of interest and the MPA resistance marker flanked by FRT sites is transformed, disrupting one copy of the *ACT1* gene. This copy covers the requirement of the essential gene, allowing the native copies to be deleted. Next, a construct that carries the *URA3* selectable marker, the rtTA (codon optimized to be used in *C. albicans*) and the *FLP* recombinase gene placed under a Tet-responsive promoter is transformed, disrupting one copy of the *ADH1* gene (Fig. 1A-11). Park and Morschhäuser used the tetracycline derivative, doxycycline instead of tetracycline in all of their experiments, as the rtTA shows higher sensitivity to this derivative.

Under normal conditions, in the absence of doxycycline the *FLP* recombinase gene is not expressed. However, if the cells are provided with doxycycline, the rtTA binds the Tet-responsive promoter and transcribes the *FLP* recombinase gene. Flp recombinase recognizes the FRT sites and loops out the essential gene along with the MPA resistance marker and the loss of the essential gene can be identified by the gain of MPA susceptibility. Deletion of an essential gene gives rise to a nonviable strain, as assessed by the number of colony forming units formed in the presence or absence of doxycycline.

One advantage of the method is that the expression of the *FLP* recombinase gene is not depended on nutrient changes in the media as in the "URA flipper" (Morschhauser et al. 1999), "Cre-*loxP* system" (Dennison et al. 2005), "MPA^R flipper" (Wirsching et al. 2000) or "CaNAT1-FLP cassette" (Shen et al. 2005) methods, where the recombinase gene is under the regulation of a promoter that shows media-dependent activation. Therefore, in instances where nutritional changes are not desirable, this "Tet-On" system could be an attractive method for gene manipulation in *C. albicans*. However, the method requires the deletion of both copies of the essential gene before or after the expression of the extra copy at the *ACT1* locus, demanding the need for more transformations steps than required for in the "GRACE method".

6. Gene Manipulation in Other Fungal Species

Similar to *C. albicans*, gene manipulation in many other clinically important *Candida*, *Cryptococcus* and *Aspergillus* species continues to evolve (Magee et al., 2003; Hull and Heitman, 2002; Brookman and Denning, 2000; Brakhage and Langfelder, 2002). For example, the *URA5* gene is a popular marker for gene disruption in *Cryptococcus* species. In the past, *URA5* function was reintroduced at random locations in the genome (Hull and Heitman, 2002), however, today, it can be restored to its native locus. For example, Narasipura et al. (2006) constructed a *Cryptococcus gattii* parent strain that has a non-functional *URA5* gene due to a point mutation, which can subsequently be restored to functionality through rare spontaneous reversion events. Uracil auxotrophs are also used for gene manipulation in *Aspergillus* species. For this purpose, mutants of *pyrG* gene that

encodes orotidine-5′-monophosphate decarboxylase that is essential for uracil synthesis are used (Brakhage and Langfelder, 2002). Comparable to *C. albicans*, the use of drug selectable markers has also become popular in both, *Cryptococcus* and *Aspergillus* species (Hua et al., 2000; McDade and Cox, 2001; Brakhage and Langfelder, 2002).

RNA interference techniques are also proving to be successful in *Cryptococcus neoformans*, *Aspergillus fumigatus* and *Aspergillus nidulans* giving a new dimension to gene manipulation in these species (Liu et al., 2002; Mouyna et al., 2004; Barton and Prade, 2008). RNA interference techniques are yet to be proven successful in *C. albicans* (Staab et al., 2011). There is evidence for the presence of RNAi in *C. albicans* (Drinnenberg et al., 2009) and the presence of a majority of the proteins involved in the RNAi pathway (Drinnenberg et al., 2009; Staab et al., 2011). However, the mere presence of these proteins does not seem sufficient to trigger the RNAi pathway by dsRNA that are exogenously introduced (Staab et al., 2011). It is suggested that the RNAi pathway in *C. albicans* may have evolved in a species-specific way that it is no longer triggered by exogenous dsRNA (Staab et al., 2011).

7. Concluding Remarks

Over the last decade or so, a variety of methods have been developed to manipulate the *C. albicans* genome in useful ways. However, special attention should be paid to the method of choice, the selectable markers used, and their collective consequences on virulence or other phenotypes to be studied. Constructing an isogenic strain reconstituted for the gene of interest remains the strongest means of validating the method of choice and mutant phenotypes.

While great progress has been made, it is still discomforting that a scar-less method for disrupting *C. albicans* genes has not yet been established. A “delitto perfecto” method has been developed to disrupt genes in *S. cerevisiae*, the highly studied cousin of *C. albicans* (Storici et al., 2001). Here, a counter selectable reporter (CORE) cassette flanked by homologous sequences flanking the gene of interest is first integrated in to the genome, to replace the gene. In a second step, two 80 bp integrative recombinant oligonucleotides (with 70bp homology to sequence extending from either end of the CORE cassette in the genome) that are designed to have at least a 20 bp overlap between their 3’ end sequences are annealed together, extended and transformed. These molecules will occasionally recombine at the locus of interest replacing the CORE cassette, leaving no scar behind. The cells that are perfectly null at the gene of interest, with no scar left behind are typically counter-selected for using two markers, 5-FOA and a drug selectable marker. The drug selectable markers available in a CORE cassette thus far are, G418 and hygromycin B (Storici and Resnick, 2006).

This method is yet to be adapted for *C. albicans* gene disruption. The main drawback of implementing the method is that *C. albicans* is inherently resistant to the drug selection makers (G418 and hygromycin B) that are currently used. However, *C. albicans* does show susceptibility to the drug, nourseothricin suggesting that it may be possible to implement a different version of this method for *C. albicans* gene disruption. One can expect that a “delitto perfecto” method for *C. albicans* gene disruptions will be available in the not too distant future.

The *C. albicans* research community is benefiting from large-scale and genome-wide approaches developed in *S. cerevisiae*. Two examples are given. First, Homann et al. (2009) created a homozygous deletion library of 143 non-essential transcriptional regulators using the parent strain SN152 constructed by Noble and Johnson (2005). This deletion library

carries two independent isolates for each gene knockout, and is a valuable resource available to study the role of these transcription regulators in drug sensitivity, morphogenetic switching, to identify binding partners to these regulators through chromatin immunoprecipitation and more. By 2010 this homozygous deletion library expanded to include the deletion of 647 genes (Noble et al., 2010), signifying rapid growth in this research field.

Second, Oh et al. (2010) constructed a tagged heterozygous transposon deletion library that included 3633 strains. They modified the Tn5 transposon to include a Gateway conversion cassette, the UAU1 cassette (Enloe et al., 2000) and a kanamycin resistance gene. The modified transposon was introduced to a *C. albicans* genomic DNA library and the individual mutagenized clones were tested for the disruption of a gene of interest. Each gene insertion was maximized to a have a unique tag to make the process of strain tracking and quantitation easier. Finally, they transformed the transposon flanked by sequences from the gene of interest in to the parent strain BWP17 constructed by Wilson et al. (1999). The correct heterozygous transformants were selected by arginine prototrophy. This publicly available resource is useful to detect haploinsufficient phenotypes, gene function, drug sensitivity and more. Moreover, because the strains are tagged, the experiments can be multiplexed and conducted in a high-throughput manner. Collectively, these resources open the way for comprehensive genome-wide analysis as well as systematic analysis of *C. albicans* (Noble et al., 2010; Oh et al., 2010).

It is noteworthy that the *C. albicans* research field is evolving with great caution. Bouchonville et al. (2009) discovered that simple and popular DNA transformation procedures such as, the lithium acetate protocols or electroporation can introduce aneuploidy to the *C. albicans* strains. It is therefore advisable that all *C. albicans* mutant strains be tested for aneuploidy before conducting extensive studies (Arbour et al., 2009). Arbour et al. (2009) introduced a simple and straight forward multiplex quantitative PCR assay to test the ploidy of any new *C. albicans* mutant strain.

In summary, the *C. albicans* research field is evolving rapidly, and making use of ever more sophisticated and precise genetic tools. One can expect increased use of genome-wide and systematic analysis of *C. albicans*. Advances in high-throughput data generation and analysis will be a useful step in the development of therapies to manage *C. albicans* as a human pathogen.

Acknowledgments

Dhanushki Samaranayake is a Ph.D. student in the laboratory of SDH. We thank the reviewers for constructive advice. This work was supported by a grant from the National Institutes of Health (R01-GM055108) to SDH.

References

Arbour M, Epp E, Hogues H, Sellam A, Lacroix C, Rauceo J, Mitchell A, Whiteway M, Nantel A. Widespread occurrence of chromosomal aneuploidy following the routine production of *Candida albicans* mutants. FEMS Yeast Res. 2009; 9:1070–1077. [PubMed: 19732157]

Barnett JA. A history of research on yeasts 12: medical yeasts part 1, *Candida albicans*. Yeast. 2008; 25:385–417. [PubMed: 18509848]

Barton LM, Prade RA. Inducible RNA Interference of brlAβ in *Aspergillus nidulans*. Euk. Cell. 2008; 7:2004–2007.

Bouchonville K, Forche A, Tang KE, Selmecki A, Berman J. Aneuploid chromosomes are highly unstable during DNA transformation of *Candida albicans*. Euk. Cell. 2009; 8:1554–1566.

Brakhage AA, Langfelder K. Menacing mold: the molecular biology of *Aspergillus fumigatus*. Annu. Rev. Microbiol. 2002; 56:433–455. [PubMed: 12142473]

Brand A, MacCallum DM, Brown AJP, Gow NAR, Odds FC. Ectopic expression of *URA3* can influence the virulence phenotypes and proteome of *Candida albicans* but can be overcome by targeted reintegration of *URA3* at the *RSP10* locus. Euk. Cell. 2004; 3:900–909.

Brookman JL, Denning DW. Molecular genetics in *Aspergillus fumigatus*. Curr. Opin. Microbiol. 2000; 3:468–474. [PubMed: 11050444]

Cannon RD, Lamping E, Holmes AR, Niimi K, Tanabe K, Niimi M, Monk BC. *Candida albicans* drug resistance – another way to cope with stress. Microbiology. 2007; 153:3211–3217. [PubMed: 17906120]

Casalinuovo IA, Di Francesco P, Garaci E. Fluconazole resistance in *Candida albicans*: a review of mechanism. Eur. Rev. Med. Pharmacol. Sci. 2004; 8:69–77. [PubMed: 15267120]

Chen X, Magee BB, Dawson D, Magee PT, Kumamoto CA. Chromosome 1 trisomy compromises the virulence of *Candida albicans*. Mol Microbiol. 2004; 51:551–565. [PubMed: 14756793]

Cheng S, Nguyen MH, Zhang Z, Jia H, Handfield M, Clancy CJ. Evaluation of the roles of four *Candida albicans* genes in virulence by using gene disruption strains that express URA3 from the native locus. Infect Immun. 2003; 71:6101–6103. [PubMed: 14500538]

Cox MM, Layton SL, Jiang T, Cole K, Hargis BM, Berghman LR, Bottje WG, Kwon YM. Scarless and site-directed mutagenesis in *Salmonella enteritidis* chromosome. BMC Biotechnol. 2007; 7:59. [PubMed: 17875218]

Davis D, Edwards JE Jr, Mitchell AP, Ibrahim AS. *Candida albicans* RIM101 pH response pathway is required for host-pathogen interactions. Infect. Immun. 2000; 68:5953–5959. [PubMed: 10992507]

De Backer MD, Magee PT, Pla J. Recent developments in molecular genetics of *Candida albicans*. Annu. Rev. Microbiol. 2000; 54:463–498. [PubMed: 11018135]

d'Enfert C, Goyard S, Rodriguez-Arnaveilhe S, Frangeul L, Jones L, Tekaia F, Bader O, Albrecht A, Castillo L, Dominguez A, Ernst JF, Fradin C, Gaillardin C, Garcia-Sanchez S, de Groot P, Hube B, Klis FM, Krishnamurthy S, Kunze D, Lopez MC, Mavor A, Martin N, Moszer I, Onésime D, Perez Martin J, Sentandreu R, Valentin E, Brown AJ. CandidaDB: a genome database for *Candida albicans* pathogenomics. Nucleic Acids Res. 2005; 33(Database issue):D353–D357. [PubMed: 15608215]

Dennison PM, Ramsdale M, Manson CL, Brown AJ. Gene disruption in *Candida albicans* using a synthetic, codon-optimised Cre-loxP system. Fungal Genet. Biol. 2005; 42:737–748. [PubMed: 16043373]

De Rosa FG, Garazzino S, Pasero D, Di Perri G, Ranieri VM. Invasive candidiasis and candidemia: new guidelines. Minerva Anestesiol. 2009; 75:453–458. [PubMed: 19078900]

Devasahayam G, Chaturvedi V, Hanes SD. The Ess1 prolyl isomerase is required for growth and morphogenetic switching in *Candida albicans*. Genetics. 2002; 160:37–48. [PubMed: 11805043]

Doyle TC, Nawotka KA, Purchio AF, Akin AR, Francis KP, Contag PR. Expression of firefly luciferase in *Candida albicans* and its use in the selection of stable transformants. Microb. Pathog. 2006; 40:69–81. [PubMed: 16427765]

Drinnenberg IA, Weinberg DE, Xie KT, Mower JP, Wolfe KH, Mower JP, Wolfe KH, Fink GR, Barte lDP. RNAi in budding yeast. Science. 2009; 326:544–550. [PubMed: 19745116]

Enloe B, Diamond A, Mitchell AP. A single-transformation gene function test in diploid *Candida albicans*. J. Bacteriol. 2000; 182:5730–5736. [PubMed: 11004171]

Epstein JB, Hancock PJ, Nante lS. Oral candidiasis in hematopoietic cell transplantation patients: an outcome-based analysis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2003; 96:154–163. [PubMed: 12931087]

Fehér T, Karcagi I, Gyorfy Z, Umenhoffer K, Csörgo B, Pósfai G. Scarless engineering of the *Escherichia coli* genome. Methods Mol Biol. 2008; 416:251–259. [PubMed: 18392972]

Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics. 1993; 134:717–728. [PubMed: 8349105]

Fridkin SK, Kaufman D, Edwards JR, Shetty S, Horan T. Changing incidence of Candida bloodstream infections among NICU patients in the United States: 1995–2004. Pediatrics. 2006; 117:1680–1687. [PubMed: 16651324]

Goshorn AK, Scherer S. Genetic analysis of prototrophic natural variants of *Candida albicans*. Genetics. 1989; 123:667–673. [PubMed: 2575557]

Gossen M, Freundlieb S, Bender G, Müller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science. 1995; 268:1766–1769. [PubMed: 7792603]

García MG, O'Connor JE, García LL, Martínez SI, Herrero E, del Castillo Agudo L. Isolation of a *Candida albicans* gene, tightly linked to URA3, coding for a putative transcription factor that suppresses a *Saccharomyces cerevisiae* aft1 mutation. Yeast. 2001; 18:301–311. [PubMed: 11223939]

Heilmann CJ, Schneider S, Barker KS, Rogers PD, Morschhäuser J. An A643T mutation in the transcription factor Upc2p causes constitutive ERG11 upregulation and increased fluconazole resistance in *Candida albicans*. Antimicrob Agents Chemother. 2010; 54:353–359. [PubMed: 19884367]

Homann OR, Dea J, Noble SM, Johnson AD. A phenotypic profile of the *Candida albicans* regulatory network. PLoS Genet. 2009; 5:e1000783. [PubMed: 20041210]

Horn DL, Neofytos D, Anaissie EJ, Fishman JA, Steinbach WJ, Olyaei AJ, Marr KA, Pfaller MA, Chang CH, Webster KM. Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. Clin Infect Dis. 2009; 48:1695–1703. [PubMed: 19441981]

Hua J, Meyer JD, Lodge JK. Development of positive selectable markers for the fungal pathogen *Cryptococcus neoformans*. Clin. Diagn. Lab. Immunol. 2000; 7:125–128. [PubMed: 10618292]

Hull CM, Heitman J. Genetics of *Cryptococcus neoformans*. Annu. Rev. Genet. 2002; 36:557–615. [PubMed: 12429703]

Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT, Davis RW, Scherer S. The diploid genome sequence of *Candida albicans*. Proc. Natl. Acad. Sci. USA. 2004; 101:7329–7334. [PubMed: 15123810]

Kao AS, Brandt ME, Pruitt WR, Conn LA, Perkins BA, Stephens DS, Baughman WS, Reingold AL, Rothrock GA, Pfaller MA, Pinner RW, Hajjeh RA. The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. Clin Infect Dis. 1999; 29:1164–1170. [PubMed: 10524958]

Köhler GA, White TC, Agabian N. Overexpression of a cloned IMP dehydrogenase gene of *Candida albicans* confers resistance to the specific inhibitor mycophenolic acid. J. Bacteriol. 1997; 179:2331–2338. [PubMed: 9079920]

Lai C, Tan C, Huang Y, Shao P, Hsueh P. Current challenges in the management of invasive fungal infections. J. Infect. Chemother. 2008; 14:77–85. [PubMed: 18622668]

Lay J, Henry LK, Clifford J, Koltin Y, Bulawa CE, Becker JM. Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. Infect. Immun. 1998; 66:5301–5306. [PubMed: 9784536]

Liang R, Liu J. Scarless and sequential gene modification in *Pseudomonas* using PCR product flanked by short homology regions. BMC Microbiol. 2010; 10:209. [PubMed: 20682065]

Liu H, Cottrell TR, Pierini LM, Goldman WE, Doering TL. RNA interference in the pathogenic fungus *Cryptococcus neoformans*. Genetics. 2002; 160:463–470. [PubMed: 11861553]

Magee PT, Gale C, Berman J, Davis D. Molecular genetic and genomic approaches to the study of medically important fungi. Infect. Immun. 2003; 71:2299–2309. [PubMed: 12704098]

McDade HC, Cox GM. A new dominant selectable marker for use in *Cryptococcus neoformans*. Med. Mycol. 2001; 39:151–154. [PubMed: 11270405]

Miceli MH, Díaz JA, Lee SA. Emerging opportunistic yeast infections. Lancet Infect Dis. 2011; 11:142–151. [PubMed: 21272794]

Miller LG, Hajjeh RA, Edwards JE Jr. Estimating the cost of nosocomial candidemia in the united states. Clin Infect Dis. 2001; 32:1110. [PubMed: 11264044]

Morschhäuser J, Michel S, Hacker J. Expression of a chromosomally integrated, single-copy GFP gene in *Candida albicans*, and its use as a reporter of gene regulation. Mol. Gen. Genet. 1998; 257:412–420. [PubMed: 9529522]

Morschhäuser J, Michel S, Staib P. Sequential gene disruption in *Candida albicans* by FLP-mediated site-specific recombination. Mol. Microbiol. 1999; 32:547–556. [PubMed: 10320577]

Mouyna I, Henry C, Doering TL, Latgé JP. Gene silencing with RNA interference in the human pathogenic fungus *Aspergillus fumigatus*. FEMS Microbiol. Lett. 2004; 237:317–324. [PubMed: 15321679]

Müller FM, Groll AH, Walsh TJ. Current approaches to diagnosis and treatment of fungal infections in children infected with human immuno deficiency virus. Eur J Pediatr. 1999; 158:187–199. [PubMed: 10094436]

Murad AM, Lee PR, Broadbent ID, Barelle CJ, Brown AJ. CIp10, an efficient and convenient integrating vector for *Candida albicans*. Yeast. 2000; 16:325–327. [PubMed: 10669870]

Narasipura SD, Ren P, Dyavaiah M, Auger I, Chaturvedi V, Chaturvedi. S. An efficient method for homologous gene reconstitution in *Cryptococcus gattii* using *URA5* auxotrophic marker. Mycopathologia. 2006; 162:401–409. [PubMed: 17146584]

Navarro-García F, Pérez-Diaz RM, Magee BB, Pla J, Nombela C, Magee P. Chromosome reorganization in *Candida albicans* 1001 strain. J Med Vet Mycol. 1995; 33:361–366. [PubMed: 8683403]

Negredo A, Monteoliva L, Gil C, Pla J, Nombela C. Cloning, analysis and one-step disruption of the ARG5,6 gene of *Candida albicans*. Microbiology. 1997; 143:297–302. [PubMed: 9043106]

Noble SM, Johnson AD. Strains and strategies for large-scale gene deletion Studies of the diploid human fungal pathogen *Candida albicans*. Euk. Cell. 2005; 4:298–309.

Noble SM, Johnson AD. Genetics of *Candida albicans*, a diploid human fungal pathogen. Annu. Rev. Genet. 2007; 41:193–211. [PubMed: 17614788]

Noble SM, French S, Kohn LA, Chen V, Johnson AD. Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. Nat Genet. 2010; 42:590–598. [PubMed: 20543849]

Odds FC. Candida species and virulence. ASM News. 1994; 60:313–318.

Oh J, Fung E, Schlecht U, Davis RW, Giaever G, St Onge RP, Deutschbauer A, Nislow C. Gene annotation and drug target discovery in *Candida albicans* with a tagged transposon mutant collection. PLoS Pathog. 2010; 6 pii: e1001140.

Park YN, Morschhäuser J. Tetracycline-inducible gene expression and gene deletion in *Candida albicans*. Euk. Cell. 2005; 4:1328–1342.

Ramanan N, Wang Y. A high-affinity iron permease essential for *Candida albicans* virulence. Science. 2000; 288:1062–1064. [PubMed: 10807578]

Ramón AM, Fonzi WA. Diverged binding specificity of Rim101p, the *Candida albicans* ortholog of PacC. Euk. Cell. 2003; 2:718–728.

Reuss O, Vik A, Kolter R, Morschhäuser J. The SAT1 flipper, an optimized tool for gene disruption in *Candida albicans*. Gene. 2004; 341:119–127. [PubMed: 15474295]

Roemer T, Jiang B, Davison J, Ketela T, Veillette K, Breton A, Tandia F, Linteau A, Sillaots S, Marta C, Martel N, Veronneau S, Lemieux S, Kauffman S, Becker J, Storms R, Boone C, Bussey H. Large-scale essential gene identification in *Candida albicans* and applications to antifungal drug discovery. Mol. Microbiol. 2003; 50:167–181. [PubMed: 14507372]

Ruhnke M. Epidemiology of *Candida albicans* infections and role of non-*Candida-albicans* yeasts. Curr Drug Targets. 2006; 7:495–504. [PubMed: 16611037]

Rustchenko E. Chromosome instability in *Candida albicans*. FEMS Yeast Res. 2007; 7:2–11. [PubMed: 17311580]

Santos MA, Tuite MF. The CUG codon is decoded in vivo as serine and not leucine in *Candida albicans*. Nucleic Acids Res. 1995; 23:1481–1486. [PubMed: 7784200]

Schelenz S, Abdallah S, Gray G, Stubbings H, Gow I, Baker P, Hunter PR. Epidemiology of oral yeast colonization and infection in patients with hematological malignancies, head neck and solid tumors. J Oral Pathol Med. 2011; 40:83–89. [PubMed: 20923440]

Selmecki A, Bergmann S, Berman J. Comparative genome hybridization reveals widespread aneuploidy in *Candida albicans* laboratory strains. Mol Microbiol. 2005; 55:1553–1565. [PubMed: 15720560]

Shao PL, Huang LM, Hsueh PR. Recent advances and challenges in the treatment of invasive fungal infections. Int. J. Antimicrob. Agents. 2007; 30:487–495. [PubMed: 17961990]

Shen J, Guo W, Köhler JR. CaNAT1, a heterologous dominant selectable marker for transformation of *Candida albicans* and other pathogenic *Candida* species. Infect. Immun. 2005; 73:1239–1242. [PubMed: 15664973]

Srikantha T, Klapach A, Lorenz WW, Tsai LK, Laughlin LA, Gorman JA, Soll DR. The sea pansy Renilla reniformis luciferase serves as a sensitive bioluminescent reporter for differential gene expression in *Candida albicans*. J. Bacteriol. 1996; 178:121–129. [PubMed: 8550405]

Staab JF, White TC, Marr KA. Hairpin dsRNA does not trigger RNA interference in *Candida albicans* cells. Yeast. 2011; 28:1–8. [PubMed: 20737430]

Storici F, Lewis LK, Resnick MA. *In-vivo* site-directed mutagenesis using oligonucleotides. Nat. Biotechnol. 2001; 19:773–776. [PubMed: 11479573]

Storici F, Resnick MA. The delitto perfetto approach to in vivo site-directed mutagenesis and chromosome rearrangements with synthetic oligonucleotides in yeast. Methods Enzymol. 2006; 409:329–345. [PubMed: 16793410]

Sundstrom P, Cutler JE, Staab JF. Reevaluation of the role of *HWP1* in systemic candidiasis by use of *Candida albicans* strains with selectable marker *URA3* targeted to the *ENO1* locus. Infect. Immun. 2002; 70:3281–3283. [PubMed: 12011025]

Thrash-Bingham C, Gorman JA. DNA translocations contribute to chromosome length polymorphisms in *Candida albicans*. Curr Genet. 1992; 22:93–100. [PubMed: 1423721]

Wellington M, Kabir MA, Rustchenko E. 5-fluoro-orotic acid induces chromosome alterations in genetically manipulated strains of *Candida albicans*. Mycologia. 2006; 98:393–398. [PubMed: 17040068]

Wilson RB, Davis D, Mitchell AP. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. J. Bacteriol. 1999; 181:1868–1874. [PubMed: 10074081]

Wilson RB, Davis D, Enloe BM, Mitchell AP. A recyclable *Candida albicans URA3* cassette for PCR product-directed gene disruptions. Yeast. 2000; 16:65–70. [PubMed: 10620776]

Wirsching S, Michel S, Morschhäuser. J. Targeted gene disruption in *Candida albicans* wild-type strains: the role of the MDR1 gene in fluconazole resistance of clinical *Candida albicans* isolates. Mol. Microbiol. 2000; 36:856–865. [PubMed: 10844673]

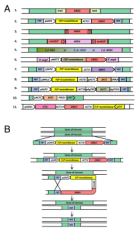

Figure 1. (A) Concepts underlying gene disruption constructs of *C. albicans*

The figure shows the various constructs used for gene manipulation in *C. albicans*. The gene of interest and/or flanking sequences are shown in green. All sequences coming from different species have been codon optimized to be used in *C. albicans*. **Line 1.** URA blaster (Fonzi and Irwin,1993) **2.** URA flipper cassette. p; promoter region of given gene, t; termination sequence of given gene. (Morschhauser et al.,1999) **3.** PCR amplifiable *URA3* disruption cassette (Wilson et al., 2000) **4.** UAU1 cassette (Enloe et al., 2000). **5.** PCR amplifiable marker cassettes from non-*albicans* species. *C. d.*; *Candida dubliniensis*, *C. m.*; *Candida maltosa*. (Noble and Johnson, 2005) **6.** Cre-*loxP* system (Dennison et al., 2005), note that when this construct integrates, the *arg4* sequences will be flanked by *loxP* sites. **7.** "MPA^R flipper" cassette (Wirsching et al., 2000) **8.** SAT1 flipper cassette (Reuss et al., 2004) **9.** CaNAT1-FLP cassette. *Ag; Ashbya gossypii*. (Shen et al., 2005) **10.** "Tet-Off" system (Roemer et al., 2003) **11.** "Tet-On" system (Park and Morschhäuser, 2005). **(B) Schematic representation of the *URA*-flipper method,** adapted from Morschhauser et al. (1999). ***Step 1***: Exogenously added insert DNA replaces one copy of the gene of interest by homologous recombination. ***Step 2***: Expression of *FLP* recombinase promotes loss of *URA3*, leaving behind one copy of *FRT*. ***Steps 3–4***: Repeating steps 1 and 2 enables the replacement of the remaining allele and results in a knockout of the gene of interest.