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Shuttle vectors for facile gap repair cloning and integration into a neutral locus in *Candida albicans*

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Candida albicans is the most prevalent fungal pathogen of humans. The current techniques used to construct *C. albicans* strains require integration of exogenous DNA at ectopic locations, which can exert position effects on gene expression that can confound the interpretation of data from critical experiments such as virulence assays. We have identified a large intergenic region, *NEUT5L*, which facilitates the integration and expression of ectopic genes. To construct and integrate inserts into this novel locus, we re-engineered yeast/bacterial shuttle vectors by incorporating 550 bp of homology to *NEUT5L*. These vectors allow rapid, facile cloning through *in vivo* recombination (gap repair) in *Saccharomyces cerevisiae* and efficient integration of the construct into the *NEUT5L* locus. Other useful features of these vectors include a choice of three selectable markers (*URA3*, the recyclable *URA3-dpl200* or *NAT1*), and rare restriction enzyme recognition sites for releasing the insert from the vector prior to transformation into *C. albicans*, thereby reducing the insert size and preventing integration of non-*C. albicans* DNA. Importantly, unlike the commonly used *RPS1/RP10* locus, integration at *NEUT5L* has no negative effect on growth rates and allows native-locus expression levels, making it an ideal genomic locus for the integration of exogenous DNA in *C. albicans*.

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INTRODUCTION

Candida albicans, a commensal of most individuals, is also one of the most prevalent fungal pathogens of humans. Most individuals are colonized with *C. albicans* by approximately 6 weeks of age. As a commensal, *C. albicans* inhabits a range of niches within the human body, including the skin, the oral-gastrointestinal tract and the uro-genital tracts. Normally, *C. albicans* causes non-lethal, superficial mucosal infections. However, conditions of immunosuppression, prolonged antibiotic treatment, or the use of invasive catheters, among other risk factors, can allow *C. albicans* to reach the bloodstream, leading to systemic infections that are associated with high mortality rates (values range from ~10–15 % to ~40 % mortality due to candidaemia in the USA) (Falagas *et al.*, 2006; Gudlaugsson *et al.*, 2003; Zaoutis *et al.*, 2005). Thus, current antifungal therapies are not sufficiently effective and there is a great need to better understand the biology and pathogenicity of *C. albicans*.

C. albicans research routinely requires *in vivo* studies, and it is important to ensure that mutant strains are neither auxotrophic nor haploinsufficient for any genes required for virulence. For example, strains that express only an ectopic copy of *URA3*, a commonly used auxotrophic marker, can exhibit reduced virulence, a fact that has confounded the interpretation of experiments using this marker (Brand *et al.*, 2004; Cheng *et al.*, 2003; Lay *et al.*, 1998; Staab & Sundstrom, 2003). Because *C. albicans* lacks a conventional meiotic sexual cycle and has a diploid genome, constructing strains with disrupted or replaced genes relies upon transformation with exogenous DNA. Furthermore, since neither an RNA interference (RNAi) system, nor stable extrachromosomal plasmids with useful markers are efficient for use in *C. albicans* (Huber & Rustchenko, 2001; Kurtz *et al.*, 1987), genetic alterations must be done by integration of DNA into the fungal genome. Thus, major approaches to strain construction utilize DNA transformation by homologous recombination of PCR-amplified inserts or of plasmid DNA.

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Abbreviation: RES, restriction enzyme site.

Four supplementary figures, a set of supplementary methods, with supplementary references, and the complete nucleotide sequences of the shuttle vectors targeting the *NEUT5L* region are available with the online version of this paper.

The number and variety of molecular tools available for genetic manipulation of *C. albicans* have increased greatly in the past decade with the development of auxotrophic and drug-resistance markers, epitope tags, fluorescent tags and constitutive or regulatable promoters (Backen *et al.*, 2000; Barelle *et al.*, 2004; Basso *et al.*, 2010; Beckerman *et al.*, 2001; Care *et al.*, 1999; Doyle *et al.*, 2006; Enloe *et al.*, 2000; Finkel *et al.*, 2011; Gerami-Nejad *et al.*, 2001, 2009, 2004, 2012; Gola *et al.*, 2003; Keppler-Ross *et al.*, 2008; Lai *et al.*, 2011; Lebel *et al.*, 2006; Murad *et al.*, 2000; Nobile & Mitchell, 2009; Oh *et al.*, 2010; Park & Choi, 2002; Reijnst *et al.*, 2011; Reuß *et al.*, 2004; Sánchez-Martínez & Pérez-Martín, 2002; Shen *et al.*, 2005; Srikantha *et al.*, 1996; Staab *et al.*, 2003; Stynen *et al.*, 2010; Umeyama *et al.*, 2002; Vieira *et al.*, 2010; Wilson *et al.*, 2000; Xu *et al.*, 2011). Available vectors for the integration of genes into ectopic locations in the *C. albicans* genome target the *HIS1* promoter (orf19.4026), the *ENO1* promoter (orf19.935) or the widely used *RPS1* ORF (orf19.3002, also referred to as *RPS10* or RP10) (Care *et al.*, 1999; Davis *et al.*, 2002; Dennison *et al.*, 2005; Murad *et al.*, 2000; Spreghini *et al.*, 2003; Staab *et al.*, 2003; Vieira *et al.*, 2010). However, there are several important limitations to these approaches. First, many of these vectors rely on markers useful only in auxotrophic strains (e.g. *his1*Δ/Δ or *ura3*Δ/Δ strains) and not in prototrophic clinical isolates. Second, gene expression at some genomic loci is subject to position effects that alter the level of expression relative to what is seen at the native locus (Pirrotta & Gross, 2005; Tartof, 1994). Third, insertion at *RPS1* disrupts the ORF encoding the Rps1 ribosomal protein resulting in hemizygosity of *RPS1*, which has been reported to cause growth defects (Oh *et al.*, 2010) (and see below). Thus, it would be ideal to have a 'neutral' locus with no coding or other known function and no effect on growth rates, for use as a conventional site for insertion of exogenous DNA in *C. albicans*.

Here, we describe the identification of a neutral locus, *NEUT5L*, which facilitates strain construction by allowing for high-frequency integration of exogenous DNA and efficient expression of the integrated constructs. To facilitate the cloning and integration at this locus, we constructed a series of integrative shuttle vectors that exploit homologous recombination *in vivo* (gap repair) in *Saccharomyces cerevisiae* and carry *NAT1*, the dominant drug resistance marker that can be used in clinical isolates, a *URA3* counterselectable auxotrophic marker, or a recyclable *URA3* auxotrophic marker (*URA3-dpl200*) (Wilson *et al.*, 2000). Cloning into these vectors can also be achieved by classical restriction enzyme digestion and ligation. Furthermore, convenient restriction enzyme sites have been engineered such that most of the non-*C. albicans* vector sequence is excluded from the integrative fragment prior to transformation (this includes the *Escherichia coli* and *S. cerevisiae* selectable markers and the *S. cerevisiae* centromeric and autonomous replication sequences). Similar to shuttle vectors that target the *RPS1* locus (Vieira *et al.*, 2010), fragments cloned into these *NEUT5L* shuttle vectors can be PCR-amplified directly from *S. cerevisiae* DNA extracts, thereby obviating the amplification step in *E. coli*, which may be difficult for large plasmids and/or toxic inserts. Importantly, expression levels of genes inserted at *NEUT5L* are similar to levels of expression from the native locus, and do not appear to be subject to any position effects. Furthermore, no fitness cost is incurred when exogenous DNA is integrated at this locus. Therefore, we have identified *NEUT5L*, a neutral locus in the *C. albicans* genome, for the integration and expression of ectopic DNA, and we have generated shuttle vectors containing convenient selectable markers for facile and rapid cloning and integration into *NEUT5L*.

METHODS

Strains, media and growth conditions. *C. albicans* was routinely grown at 30 °C in rich YPAD medium (1 % yeast extract, 2 % peptone, 2 % glucose). Transformants of *S. cerevisiae* were selected in synthetic complete medium (SC) [0.17 % yeast nitrogen base without ammonium sulfate (Q-BioGene), 0.5 % ammonium sulfate, 2 % glucose, and supplemented with a dropout mix containing amino and nucleic acids except tryptophan or uracil, depending on the auxotrophic requirement for the selection (Adams & Stearns, 1997)]. All media were supplemented with uridine (80 μg ml^{-1}) except when used for selection of *ura*$^-$ strains. For solid media, 2 % Bactoagar was used.

All *C. albicans* strains derive from strain SC5314 (YJB11562), and are listed in Table 1. All *C. albicans* transformations were performed following the LiAc protocol (Wilson *et al.*, 1999). *nat*+ transformants were selected by plating the transformation mix on YPAD and replica plating the following day onto YPAD plates supplemented with 400 μg nourseothricin ml^{-1} (Werner Bioagents).

Strains YJB11372, YJB12780 and YJB12257 containing an *ENO1–GFP–NAT1* cassette at *ENO1* (orf19.395), at *RPS1* (orf19.3002) or at *NEUT5L*, respectively, were constructed by transforming *C. albicans* strain SC5314 as follows. Strain YJB11372 was constructed by C-terminally tagging Eno1 with a *GFP–NAT1* cassette PCR amplified from plasmid pMG2120 with primers 714 and 3185 (Tables 2 and 3), which contain 70 bp of homology to the *ENO1* 3′ region. The correct integration of the *GFP–NAT1* cassette was verified by PCR of genomic DNA using the primers 716 and 658 (Table 2). Strains YJB12257 and YJB12780 were constructed by transforming the *ENO1–GFP–NAT1* cassette, including 1000 bp of *ENO1* promoter, PCR-amplified from strain YJB11372 using primers 5359 and 5360, which have 70 bp of homology to *RPS1*, or primers 4730 and 4731, which have 70 bp of homology to *NEUT5L* (Table 2). The correct integration of the *ENO1–GFP–NAT1* cassette was verified by PCR of genomic DNA using the primer pairs 5361 and 968, and 2040 and 5401, for integration into *RPS1* and 4577 and 968, and 2040 and 4580, for integration into *NEUT5L* (Table 2).

Strain YJB12563 (Table 1) was constructed by transforming *Sfi*I-digested pDUP3-*RBP1* into the *rbp1*Δ/Δ strain YJB12543 (Zacchi *et al.*, 2010). Similarly, strains YJB12565 and YJB12566 (Table 1) were constructed by transforming *Sfi*I-digested pDIS3-*RBP1* or pDIS3-*RBP1*^{G62D}, respectively, into the *rbp1*Δ/Δ strain YJB12543. The correct integration of the inserts was verified by performing PCR from genomic DNA. Because the insert from pDIS vectors integrates at *NEUT5L* in the opposite orientation to the insert from pDUP (Fig. 2c, d), the PCR analysis used *NAT1* primer 3118 (Table 2) and two primers annealing outside *NEUT5L*: primer 4969 (Table 2) for strain

Table 1. *C. albicans* and *S. cerevisiae* strains used in this study

Strain (YJB)	Parent	Genotype	Reference
11562 (SC5314)		Wild-type (clinical isolate)	Kurtz *et al.* (1986)
3018 (CAI4)	SC5314	*ura3::λimm434/ura3::λimm434*	Fonzi & Irwin (1993)
1873 (CAF2-1)	SC5314	*ura3::λimm434/ura3::λimm434*	Fonzi & Irwin (1993)
12780	SC5314	*RPS1/rps1::ENO1–GFP–NAT1*	This study
11372	SC5314	*ENO1/ENO1–GFP–NAT1*	This study
12257	SC5314	*NEUT5L/neut5l::ENO1–GFP–NAT1*	This study
12982 (YAG134)	CAI4	*ura3::λimm434/ura3::λimm434 rbp1::MX3/RBP1*	Cruz *et al.* (2001)
12543 (DAY1230)	CAI4	*ura3::λimm434/ura3::λimm434 rbp1::MX3/rbp1::MX3*	Zacchi *et al.* (2010)
12563	12543	*ura3::λimm434/ura3::λimm434 rbp1::MX3/rbp1::MX3 NEUT5L/NEUT5L–NAT1–RBP1–NEUT5L*	This study
12565	12543	*ura3::λimm434/ura3::λimm434 rbp1::MX3/rbp1::MX3 NEUT5L/neut5l::RBP1-NAT1*	This study
12566	12543	*ura3::λimm434/ura3::λimm434 rbp1::MX3/rbp1::MX3 NEUT5L/neut5l::RBP1^{G62D}-NAT1*	This study
12984	CAI4	*ura3::λimm434/ura3::λimm434 NEUT5L/NEUT5L–URA3–NEUT5L*	This study
12985	CAI4	*ura3::λimm434/ura3::λimm434 NEUT5L/neut5l::URA3*	This study
12046		*MATα his3Δ200 trp1-901 leu2-3,-112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-lacZ GAL4*	Vojtek *et al.* (1993)

YJB12563 (for pDUP insert, 1.1 kb PCR product), and primer 4926 (Table 2) for strains YJB12565-6 (for pDIS inserts, 1.6 kb PCR product) (Fig. 3a).

Strains YJB12984 and YJB12985 (Table 1) containing a *URA3* cassette inserted at *NEUT5L* were constructed by transforming *C. albicans* strain CAI4 as follows. To construct strains YJB12984 and YJB12985, vectors pDUP4 and pDIS4 (Table 3) were digested with *Nae*I and transformed into CAI4 strain. The correct integration of the *URA3* cassette was verified by PCR of genomic DNA using the primers 324 and 4969 (Table 2) for integration at *NEUT5L* using the pDUP vector, and 324 and 4926 (Table 2) for integration at *NEUT5L* using the pDIS vector.

Tests for rapamycin resistance were performed with single colonies picked from YPAD plates and resuspended in 300 µl sterile water, 200 µl of this dilution was spread on YPAD and plates were left to dry at room temperature for 1 h. Sterile Whatman paper discs spotted with 10 µl 5 % DMSO or 1 µg rapamycin (LC Laboratories) in 5 % DMSO were placed on the inoculated agar surface. Plates were incubated at 30 °C for 24 h.

Vector construction. The shuttle vectors pDUP3–pDUP5 and pDIS3–pDIS5 for cloning through gap repair and integration into *NEUT5L* (Figs 1 and 2 and S1 and S2, available with the online version of this paper) were constructed as follows. The 550 bp region located in the middle of the intergenic region between orf19.1961 and orf19.1963 was chosen as the locus for ectopic DNA integration (Fig. 1a, white box). This 550 bp fragment (designated *NEUT5L*) was PCR amplified in two consecutive fragments of 300 bp (NEUT5L 3′) and 250 bp (NEUT5L 5′) each from *C. albicans* SC5314 DNA (strain YJB11562). Each PCR fragment was sequentially cloned into the *S. cerevisiae* shuttle vector pRS314 (GenBank: U03440.1) (Sikorski & Hieter, 1989) by *in vivo* recombination, such that the NEUT5L 5′ and 3′ fragments flanked the vector's polylinker region (for more details on the *in vivo* recombination technique see the Supplementary Methods) (Figs 2 and S1). The PCR fragments were integrated into the vector in opposite orientations, to generate vectors that will integrate into the genome by duplicating *NEUT5L* (pDUP vectors) or by disrupting *NEUT5L* (pDIS vectors) (see Results and Discussion for more details). The 300 bp NEUT5L 3′ fragment was PCR amplified using the primers 4574 and 4575 (to construct pDUP1) or 4580 and 4581 (to construct pDIS1), and each fragment was *in vivo* recombined into *Ngo*MIV-digested pRS314 (p2308, Table 3) in the *trp*$^-$ *S. cerevisiae* strain L40 (YJB12046) (Fig. S1) (Sikorski & Hieter, 1989; Vojtek *et al.*, 1993). The 250 bp NEUT5L 5′ fragment was PCR amplified using the primers 4576 and 4577 (for pDUP2) or 4582 and 4583 (for pDIS2), and each fragment was *in vivo* recombined into *Kpn*I-digested pDUP1 or pDIS1, respectively (Fig. S1). The pDUP2/pDIS2 vectors containing the two fragments comprising the 550 bp *NEUT5L* region cloned in different orientations were verified by sequencing. To incorporate each of the auxotrophic markers *URA3* and *URA3dpl200* and the drug resistance marker *NAT1* into pDUP2 and pDIS2, each marker was PCR amplified using primers 4658 and 4659 (Table 2) using as templates the plasmids p1374 (*URA3*), p1653/pDDB57 (*URA3-dpl200*) and p2105/pJK795 (*NAT1*) (Table 3) and was *in vivo* recombined into *Sac*I/*Sac*II digested pDUP2 and pDIS2 in *S. cerevisiae* strain L40 to generate pDUP3–pDUP5 and pDIS3–pDIS5, respectively (Table 3, Figs 2a, b, S2). All *in vivo* recombination 'reactions' were plated on SC-TRP medium, except for the construction of plasmids carrying the *URA3-dpl200* cassettes (pDUP5 and pDIS5) which were plated on SC-URA to ensure the integrity of the recyclable cassette and to prevent the selection of plasmids carrying only the *dpl200* portion of the marker. All PCRs were performed using high-fidelity polymerases (Pfx Platinum, Invitrogen; *Pfu* Turbo, Agilent). All vectors containing the selectable markers were sequenced using primers 5429 and 5430 (Table 2) that anneal to the pRS314 sequence flanking the *NEUT5L* fragments. The complete sequence and maps of pDUP3–pDUP5/pDIS3–pDIS5, as well as detailed protocols for transformation of *S. cerevisiae* and general procedures and important notes for performing a successful *in vivo* recombination are available in the Supplementary Material.

Vectors pDUP3/pDIS3-*RBP1* and pDIS3-*RBP1*^{G62D} were constructed as follows. The *RBP1* (orf19.6452) ORF, including 914 bp of promoter and 463 bp of terminator region, was PCR amplified from *C. albicans* SC5314 DNA using primer pairs 5227 and 5228 (for *RBP1*), or 5227 and 5230, and 5228 and 5229 (for *RBP1*^{G62D}) (Fig. S4), and the PCR fragments were *in vivo* recombined into *Sma*I-digested and phosphatase-treated pDUP3 and pDIS3 in *S. cerevisiae* strain L40, to generate pDUP3/pDIS3-*RBP1* and pDIS3-*RBP1*^{G62D},

Table 2. Primers used in this study

Lower case, primer tail homologous to pRS314 for *in vivo* recombination; underlined, *Sfi*I–*NgoM*IV restriction sites; bold, point mutation (G62D). All primers were designed for this study.

Primer	Sequence 5′–3′
324	CTTCAACTGTAAAAGGGGCG
658	TTTGTACAATTCATCCATACCATG
714	GAGAATCGAAGAAGAATTAGGTTCTGAAGCTATCTACGCTGGTAAAGATTTCCAAAAGGCTTCTCAATTGGGT-GGTGGTTCTAAAGGTGAAGAATT
716	GCTTTGTTGTTGAAGGTTAACC
986	AATAAAGGGAGTAAGGTGGT
2040	GCATCACCTGGAACAGAAGTTCTGTATC
3118	CCCAGATGCGAAGTTAAGTGCGCAG
3185	TTTAATTAGTTCATATATTCAAGATGTTCCTATAAAAGAAAAAAAAAAGCACCAGCTTTTTTTTATTTAATCGTAAAA-CGACGGCCAGTGAATTC
3951	AGATCCAGATATTGAAGGTAAAAGGTA
3952	GAAAATCTCCCCCTTCACATT
4423	AATACCACGTTACCGTCATTGAT
4424	CCAGTGATCATATTCTTGATGAAATC
4574	cctaaagggagcccccgatttagagcttgacggggaaagcGGCCGGCTAGGCCTTAAACAAGTGGTATTCAAGCAC
4575	ctttcgctttcttcccttccttctcgccacgttcgccggTAACCCACTGAATTCTACATCG
4576	accccaggctttacactttatgcttccggctcctatgttgGGCCGGCTAGGCCTGGAAGGACGATGAAGGAGAG
4577	atatcaagcttatcgataccgtcgacctcgaggggggggccGTAATTGTAGTAAGAATGAC
4580	cctaaagggagcccccgatttagagcttgacggggaaagcGGCCGGCTAGGCCTTAACCCACTGAATTCTACATCG
4581	ctttcgctttcttcccttccttctcgccacgttcgccggTAAACAAGTGGTATTCAAGCAC
4582	accccaggctttacactttatgcttccggctcctatgttgGGCCGGCTAGGCCTGTAATTGTAGTAAGAATGAC
4583	atatcaagcttatcgataccgtcgacctcgaggggggggccGGAAGGACGATGAAGGAGAG
4658	Ttaagttgggtaacgccagggttttcccagtcacgacgtt
4659	ctgcagcccgggggatccactagttctagagcggccgccaTGTGGAATTGTGAGCGGATA
4730	GCTCGGAGGAGGCTCCCCAAAGGTTTTATCACCAATGGTGGTACCACTAACCCAAGAACAGAAAAAGCATTGTAG-ACATGATTGTGCAATACCGTG
4731	CAGAGTATGTGAAGCAATTGCAATTGCAATTATTAGAGATCCAGAAAACTGAATTGTGCTTGAATACCACGATAT-CAAGCTTGCCTCGTCCCCG
4926	AAAAGGCCTGATAAGGAGAGATCCATTAAGAGCA
4969	GGATTTAGTTCCATTATGG
5227	ggtcatagctgtttcctgtgtgaaattgttatccgctcacCATCAGTTAGTGTCATGGAG
5228	ggcccccctcgaggtcgacggtatcgataagcttgatatAGAAAATCCAACTCCGAGAG
5229	TGTTGGTCAAGTTATTAAAG**A**TTGGGATATTTCTTTAACT
5230	AGTTAAAGAAATATCCCAA**T**CTTTAATAACTTGACCAACA
5359	GGTAAAACTTTGATCAACAGATCTACCGGTTTAAAGAATGCCGCTGATGGCTTGAAAGGTAGAGTTTTCGGTAGAC-ATGATTGTGCAATACCGTG
5360	ACCTTGGACTTCATCAACTCTCAATTTGATTTTTCTGTAAGAGTGGTCTTCGGAACCTTGTAAGTCGGCCGATATCA-AGCTTGCCTCGTCCCCG
5361	GTGTGTGTGTTCCAAGTCCCAGCTCTCAC
5401	CTCAATTTAGAGGATTGAGCGTAAGTAG
5429	GTGCCGTAAAGCACTAAATCGG
5430	GTTACCTCACTCATTAGGCACCCC
5930	GTCTGAAGAACTTCCACAAATTG
5931	TGGTTACTGTATCACCTGGC

respectively. Primers 5230 and 5229 carry the point mutation that leads to *RBP1*^{G62D} (bold type in Table 2).

Growth rate experiments. Three independent colonies from each strain were inoculated into three culture tubes containing 2 ml YPAD and grown overnight at 30 °C. Fifty microlitres of each culture was washed with 1 ml water and resuspended in 1 ml water, and 3 µl were inoculated into 150 µl media using 96-well round-bottom sterile polystyrene plates (Corning). Growth conditions tested were: YPAD, YPAD buffered at pH 3 (100 mM HEPES), YPAD buffered at pH 6 (100 mM HEPES), YPAD buffered at pH 9 (100 mM HEPES), YPAD + 1 M NaCl and YPAD + 4 mM H_2O_2. The plate was incubated at 30 °C in a Tecan Sunrise microplate incubator/spectrophotometer with high-speed shaking. Readings of OD_{595} were collected every 15 min (for at least 24 h). Data were processed with an in-house script written in Matlab software (Mathworks), in which the raw OD data were pre-processed by subtraction of a blank measurement and shortening of curves that end with long saturation

Table 3. Plasmids used in this study

Plasmid	Alias	Characteristics	Reference
p2308	pRS314	Amp, *CEN6/ARS4*, *TRP1*	Sikorski & Hieter (1989)
p1374		pGEM-*URA3*	Wilson *et al.* (1999)
p1653	pDDB57	*URA3-dpl200*	Wilson *et al.* (2000)
p2105	pJK795	*NAT1*	Shen *et al.* (2005)
pMG2120	pMG2120	*GFP-NAT1*	Gerami-Nejad *et al.* (2012)
pDUP2	p2365	pRS314-*NEUT5L*^{DUP} (for insertion/duplication)	This study
pDUP3	p2366	pRS314-*NEUT5L*^{DUP}-*NAT1*	This study
pDUP4	p2367	pRS314-*NEUT5L*^{DUP}-*URA3*	This study
pDUP5	p2368	pRS314-*NEUT5L*^{DUP}-*URA3dpl200*	This study
pDUP3-*RBP1*	p2369	pRS314-*NEUT5L*^{DUP}-*NAT1-RBP1*	This study
pDIS2	p2372	pRS314-*NEUT5L*^{DIS} (for insertion/disruption)	This study
pDIS3	p2373	pRS314-*NEUT5L*^{DIS}-*NAT1*	This study
pDIS4	p2374	pRS314-*NEUT5L*^{DIS}-*URA3*	This study
pDIS5	p2375	pRS314-*NEUT5L*^{DIS}-*URA3dpl200*	This study
pDIS3-*RBP1*	p2376	pRS314-*NEUT5L*^{DIS}-*NAT1-RBP1*	This study
pDIS3-*RBP1*^{G62D}	p2377	pRS314-*NEUT5L*^{DIS}-*NAT1-RBP1*^{G62D}	This study

periods; the time-dependent growth of the log-transformed OD was fitted to a piecewise linear function with three pieces: (1) constant OD over time, representing the lag phase; (2) a linear growth of the log-transformed OD; and (3) constant OD over time, representing saturation of growth. The free parameters of the function are: the starting OD during lag phase, the doubling time, the time at which half-maximum saturation is reached and the maximal OD at saturation. The fitting procedure minimized the sum of absolute differences between the experimental data and the estimated curve. The highest growth rate (lowest doubling time) of the culture corresponds to the slope of the inflection point of the resulting fitted sigmoid curve. Graphs were made using Microsoft Office Excel 2007. Statistical analyses were performed on two independent experiments with three replicates (total $n=6$), using standard statistical operations manually implemented in Microsoft Office Excel. A *t*-test was used to determine if there were differences between experiments (no difference found, *P*-value >0.32). An ANOVA test was used to determine the likelihood that strains had identical doubling times (not identical, *P*-value <0.0061). A pair-wise *t*-test using Bonferroni correction was employed to identify differences between the doubling times of the strains within each experimental condition (six comparisons, significant *P*-value $<0.05/6=0.0083$).

Protein extraction and Western blot analysis. Strains were grown in YPAD at 30 °C to exponential phase, and equal amounts of the cultures, as determined spectrophotometrically (OD_{600}), were resuspended in 400 µl Thorner Buffer (40 mM Tris pH 8, 5 % SDS, 8 M Urea, 100 µM EDTA) supplemented with 2 mM DTT. Three hundred microlitres of 500 µM acid-washed glass beads were added to the samples, and samples were heated at 95 °C for 8 min and vortexed for 30 min to lyse cells. 5× non-reducing sample buffer (0.5 M Tris pH 8, 50 % glycerol v/v, 0.0125 % Bromophenol blue, 5.4 % SDS) was added to the samples to a 1× final concentration with 5 % β-mercaptoethanol. Samples were heated at 95 °C for 8 min, centrifuged to pellet insoluble cell debris, and equal amounts of protein (as determined spectrophotometrically) were separated in a 12 % acrylamide gel and transferred to PVDF membranes for Western blotting. Mouse anti-GFP (Roche) and rabbit anti-*S. cerevisiae* histone H4 (which cross-reacts with *C. albicans* histone H4) (Glowczewski *et al.*, 2004) were used to probe the membranes. Images of the membranes were obtained using a digital camera documentation system (Chemiimager 5500, ProteinSimple). GFP signal intensity was quantified using Image J 1.44d (Wayne Rasband, National Institutes For Health); final values displayed in Fig. 1(b) were normalized first to the loading control signal and later to the GFP signal intensity of Eno1-GFP integrated at *ENO1* (value set to 1).

RNA extraction and real-time quantitative PCR (qPCR). RNA was prepared from strains grown to exponential phase in YPAD at 30 °C by using the MasterPure RNA Purification kit (Epicentre). The samples were treated with DNase (Epicentre) to remove contaminating genomic DNA, and cDNA was prepared using the ProtoScript M-MuLV First Strand cDNA Synthesis kit (New England BioLabs), using oligo d(T) primers. cDNA was measured by qPCR using SYBR Green I (Roche Applied Sciences). Gene expression was calculated as the amount of *RBP1* (primers 5930 and 5931, Table 2) or *URA3* (primers 3951 and 3952, Table 2) cDNA relative to the amount of *TEF1* (primers 4423 and 4424, Table 2) cDNA in the same sample using the LightCycler 480 software system (Roche Applied Science). Each *RBP1* or *URA3* experiment was performed at least twice. The data from each experiment were normalized to the *RBP1* or *URA3* expression of the wild-type strain, and the mean ± SD of the normalized data of multiple experiments was plotted using Microsoft Excel.

RESULTS AND DISCUSSION

Identification of a neutral locus for DNA integration into *C. albicans* genome

Integration of DNA into *C. albicans* at an exogenous locus has been classically done by targeting the *HIS1* promoter (Davis *et al.*, 2002), the *ENO1* promoter (Staab *et al.*, 2003) (less commonly) and the *RPS1* ORF (Dennison *et al.*, 2005; Murad *et al.*, 2000; Vieira *et al.*, 2010). However, integration at the *HIS1* or *ENO1* promoters using the available vectors requires specific auxotrophies (*his-* or *ura-*, respectively) (Davis *et al.*, 2002; Staab *et al.*, 2003). Integration at *RPS1* disrupts one *RPS1* allele, causing hemizygosity which is associated with a haploinsufficient growth defect (Oh *et al.*, 2010). Thus, we sought an alternative locus in the *C. albicans*

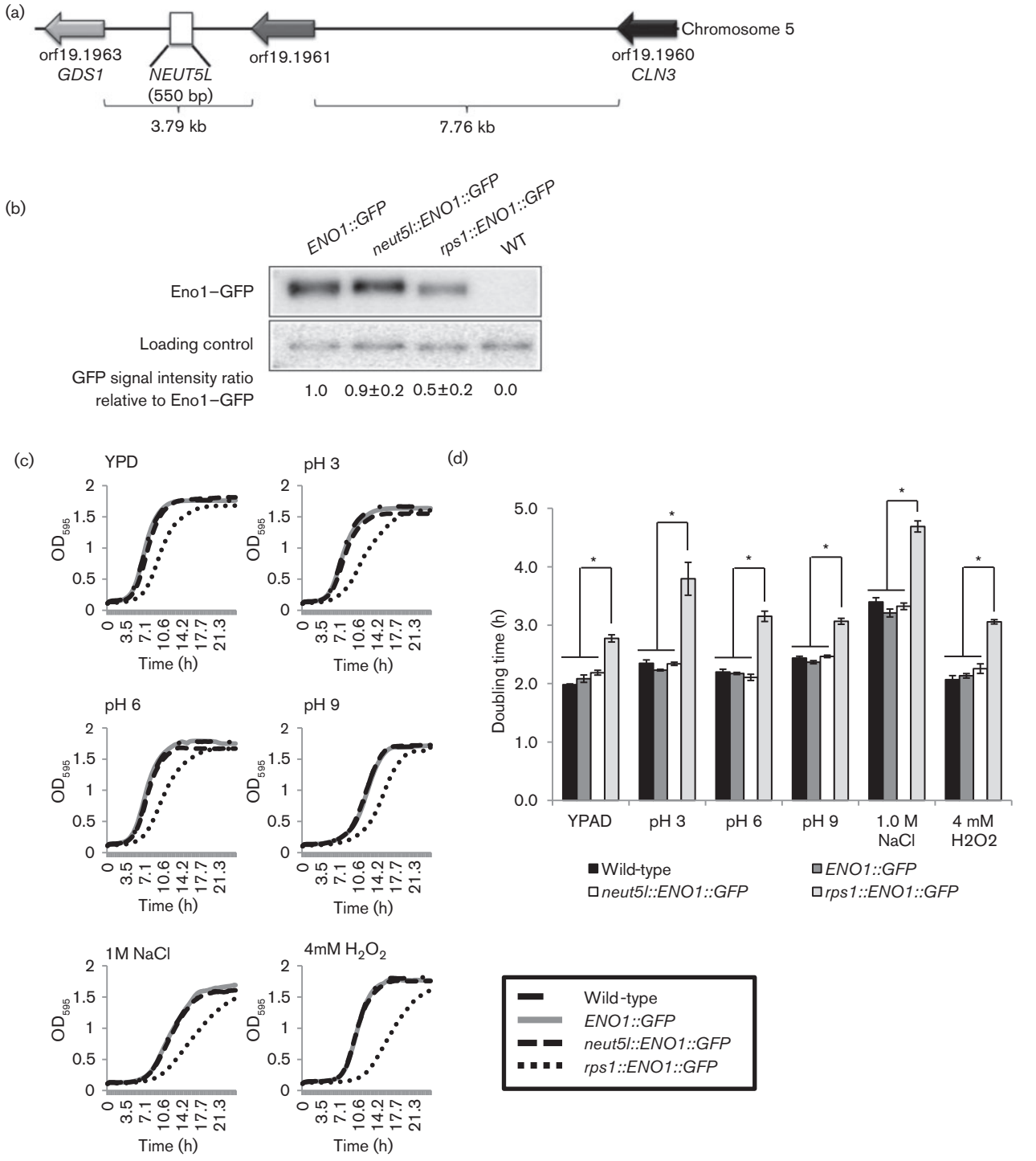

Fig. 1. The intergenic region between orf19.1961 and orf19.1963 is a neutral locus for integration and expression of exogenous DNA. (a) Region of chromosome 5 showing *CLN3*/orf19.1960, orf19.1961, *GDS1*/orf19.1963, and the 550 bp *NEUT5L* region (white box, which corresponds to Chromosome 5L coordinates 232782–233331); (b) Western blot showing expression of Eno1–GFP integrated at *ENO1* (strain YJB11372), *NEUT5L* region (YJB12257), or *RPS1* (YJB12780) detected with anti-GFP antibody. The wild-type strain SC5314 was used as a negative control. Anti-H4 antibody was used as a loading control. Indicated below is the mean ± SD of the GFP signal intensity normalized to the loading control and to the *ENO1* native locus of two independent experiments. (c) Overnight YPAD cultures of strains SC5314 (WT, dashed and dotted black line), YJB11372 (*ENO1*::*GFP*, continuous grey line), YJB12257 (*neut5l*::*ENO1*::*GFP*, dashed black line), and YJB12780 (*rps1*::*ENO1*::*GFP*, dotted black line) were diluted 1 : 1000 in YPAD, YPAD buffered at pH 3, 6 or 9, or YPAD containing

1 M NaCl or 4 mM H_2O_2, and incubated for 24 h 25 min at 30 °C with high-speed shaking. OD_{595} was measured every 15 min using a Tecan Sunrise microplate. The growth curves show the combined results of two independent experiments in which each strain was tested in triplicate. (d) Doubling times calculated from the growth curves of two independent experiments in which each strain was tested in triplicate (shown is the mean ± SE, total $n=6$). * P-value <0.0083.

genome that permits the efficient integration and expression of exogenous DNA without a detectable effect of the integration on the strain fitness.

To this end, we identified a large region (approximately 13 kb) on Chromosome 5L between ORFs *CLN3*/orf19.1960 and *GDS1*/orf19.1963 that contains only one other ORF, the uncharacterized orf19.1961 (approximately 1.6 kb long) (Fig. 1a). The intergenic region between orf19.1961 and *CLN3* is approximately 7.8 kb, and the intergenic region between orf19.1961 and *GDS1* is approximately 3.8 kb, both much longer than the average size of *C. albicans* intergenic regions (865 bp) (A. Koren and J. Berman, unpublished results). The region between *CLN3* and orf19.1961 appeared attractive as a locus for exogenous DNA integration due to its length and to the absence of other coding elements, but detection of spontaneous neocentromere formation at this location (L. S. Burrack and J. Berman, unpublished observation) suggested that the region may have uncharacterized features that could interfere with gene expression. Thus, we used a region located approximately midway between orf19.1961 and *GDS1* (located 1.71 kb from the *GDS1* start codon and 1.53 kb from the orf19.1961 stop codon), which has diverged extensively in *Candida dubliniensis*, suggesting that it is under minimal purifying selective pressure (unpublished data). We refer to this 550 bp long region as the *NEUT5L* region (Fig. 1a, white box), based on its position on Chr5L and its neutral character with respect to gene expression and strain fitness, as characterized below.

To determine the efficiency of expression of exogenous DNA integrated at *NEUT5L*, we compared the expression of an *ENO1–GFP* cassette integrated at three different loci: the *ENO1* (native) locus (YJB11372), *NEUT5L* locus (YJB12257) and *RPS1* locus (YJB12780). Integration of *ENO1–GFP* disrupted *NEUT5L* and *RPS1* loci, but not *ENO1*, where *GFP* is integrated in-frame at the 3′ end of the *ENO1* ORF (see Methods). Western blot analysis of Eno1–GFP expression revealed that normalized expression levels of Eno1–GFP at the *ENO1* locus and at the *NEUT5L* locus were similar (signal ratio of $1:0.9\pm0.2$ for *ENO1* versus *NEUT5L*) (Fig. 1b). In contrast, expression levels of Eno1–GFP inserted at *RPS1* were lower than at the *ENO1* locus (signal ratio of $1:0.5\pm0.2$ for *ENO1* versus *RPS1*) (Fig. 1b). Similar results were obtained with an independently constructed set of strains with the same genotype (data not shown). This suggests that integration of *ENO1–GFP* at *RPS1* had a position effect, reducing the level of *ENO1–GFP* expression relative to its native locus (Henikoff, 1992; Tartof, 1994), while integration at *NEUT5L* enabled expression levels similar to those observed at the native locus. In support of this conclusion, we observed that expression of *RBP1* and of *URA3* from the *NEUT5L* locus was also similar to native locus levels (Fig. 3c, d).

To determine if integration at *NEUT5L* caused any obvious growth defects in *C. albicans*, we compared growth rates of a wild-type strain (SC5314, no insertions) with the growth rates of the three strains described above expressing *ENO1–GFP* at the *ENO1*, *NEUT5L*, or *RPS1* loci. Growth rates of the different strains were measured in rich medium (YPAD) and under stress conditions, including YPAD buffered at different pH (pH 3, pH 6 and pH 9), high salinity (1 M NaCl) and oxidative stress (4 mM H_2O_2) (Fig. 1c, d). These assays were performed at 30 °C. Strains with ENO1–GFP integrated at the *ENO1* native locus (YJB11372) or at *NEUT5L* (YJB12257) exhibited similar growth curves (Fig. 1c) and similar doubling times (Fig. 1d) to the wild-type strain (YJB11562) under all the conditions tested. In contrast, the strain containing an *ENO1–GFP* cassette integrated at *RPS1* (YJB12780) exhibited a more pronounced lag phase (average approximately 7 h, Fig. 1c) and a significantly longer doubling time (P-value <0.0083, Fig. 1d) than the other three strains under all the conditions tested (average approximately 5.0 h lag phase, Fig. 1c). Essentially similar results were obtained when using an independently constructed set of strains with the same genotype and when using a different cassette inserted at *RPS1* (*ENO1–GFP–2W1S–OVA*$_{323–339}$*–OVA*$_{257–264}$*–I-E*$\alpha_{50–66}$*–NAT1*) [from pMG2271 (Igyártó *et al.*, 2011); data not shown]. The reduced growth observed in the YJB12780 strain (*RPS1*/*rps1*::*ENO1–GFP*) is consistent with previous results showing that *RPS1* is haploinsufficient (Oh *et al.*, 2010). Therefore, the phenotypes of strains constructed by integration at the *RPS1* locus should be compared with those of strains that carry control inserts in the *RPS1* locus as well. Importantly, the ability of strains containing an integration of *ENO1*::*GFP* at *NEUT5L* to filament in solid or liquid Spider medium and in medium containing serum was similar to that of the wild-type strain (data not shown). Taken together, our results indicate that insertion at *NEUT5L* permits expression of inserts at near-native locus levels and that it incurs no fitness cost during growth *in vitro*.

Construction of shuttle vectors for gap-repair-mediated cloning and integration at the *NEUT5L* locus

To efficiently integrate exogenous DNA into the *NEUT5L* locus, we constructed two sets of integrative shuttle vectors: vectors pDUP (Fig. 2a) and pDIS (Fig. 2b), which only differ in how the construct integrates into *NEUT5L* [i.e. integration

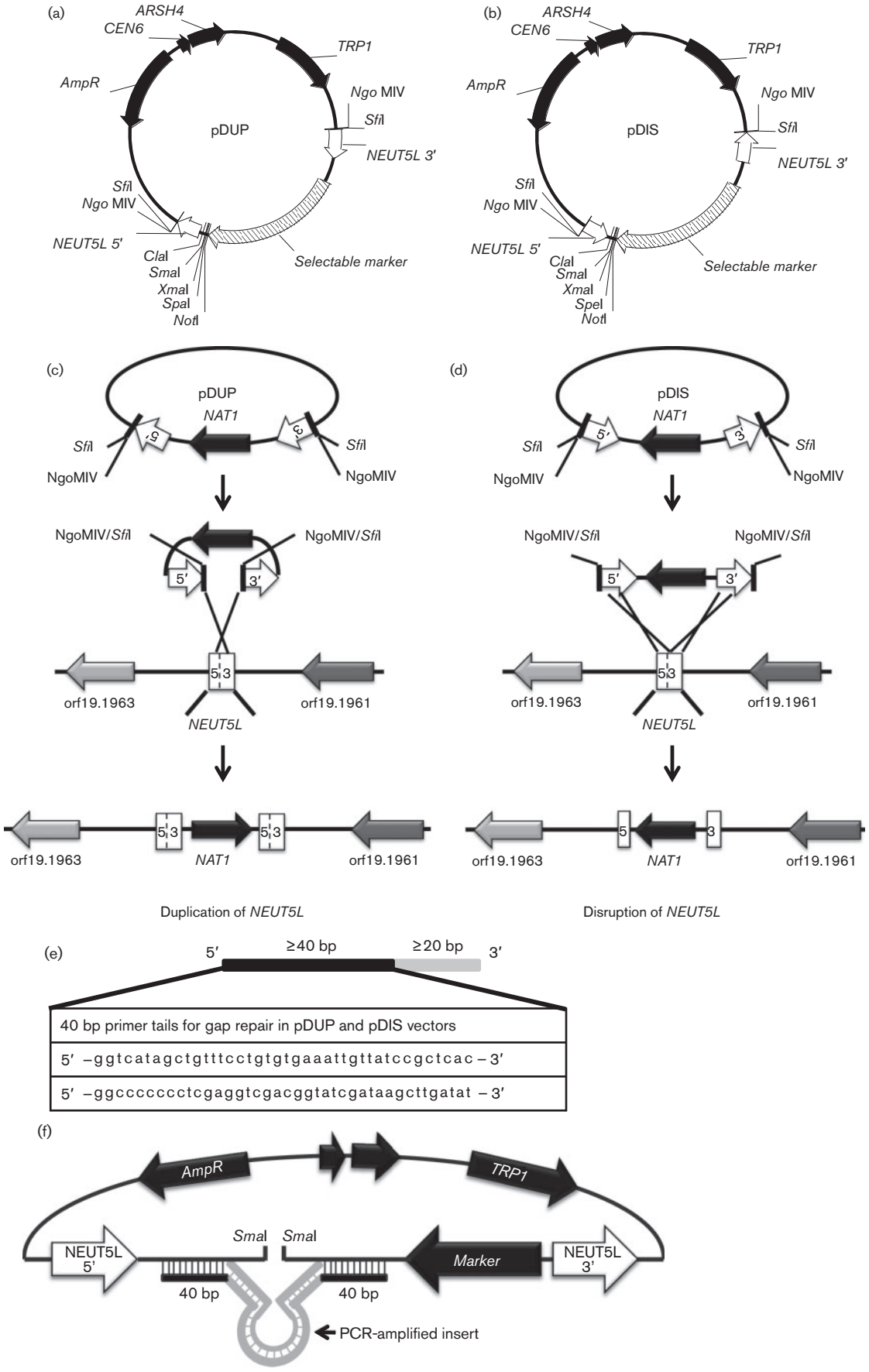
(a)
ARSH4
CEN6
TRP1
AmpR
Ngo MIV
SfiI
pDUP
NEUT5L 3′
SfiI
Ngo MIV
NEUT5L 5′
ClaI
SmaI
XmaI
SpaI
NotI
Selectable marker
(b)
ARSH4
CEN6
TRP1
AmpR
Ngo MIV
SfiI
pDIS
NEUT5L 3′
SfiI
Ngo MIV
NEUT5L 5′
ClaI
SmaI
XmaI
SpeI
NotI
Selectable marker
(c)
pDUP
NAT1
5′
3′
SfiI
NgoMIV
SfiI
NgoMIV
NgoMIV/SfiI
NgoMIV/SfiI
5′
3′
5
3
orf19.1963
orf19.1961
NEUT5L
5
3
5
3
orf19.1963
NAT1
orf19.1961
Duplication of NEUT5L
(d)
pDIS
NAT1
5′
3′
SfiI
NgoMIV
SfiI
NgoMIV
NgoMIV/SfiI
NgoMIV/SfiI
5′
3′
5
3
orf19.1963
orf19.1961
NEUT5L
5
3
orf19.1963
NAT1
orf19.1961
Disruption of NEUT5L
(e)
5′
≥40 bp
≥20 bp
3′
40 bp primer tails for gap repair in pDUP and pDIS vectors
5′ –ggtcatagctgtttcctgtgtgaaattgttatccgctcac– 3′
5′ –ggccccccctcgaggtcgacggtatcgataagcttgatat – 3′
(f)
AmpR
TRP1
SmaI
SmaI
NEUT5L 5′
Marker
NEUT5L 3′
40 bp
40 bp
PCR-amplified insert

Fig. 2. Maps of the *S. cerevisiae–E. coli* shuttle vectors for integration into the *C. albicans NEUT5L* locus. (a) pDUP shuttle vector; and (b) pDIS shuttle vector. Common features of the vectors include the Amp^R Ampicilin resistance gene for selection in *E. coli*, the *TRP1* marker for selection on *S. cerevisiae trp-* strains, and a centromeric (*CEN6*) and autonomous replicating sequence (*ARSH4*) to ensure replicative stability in *S. cerevisiae*. The selectable markers for the resulting vectors are: pDUP3 and pDIS3, *NAT1*; pDUP4 and pDIS4, *URA3*; pDUP5 and pDIS5, *URA3-dpl200*. (c, d) Representation of *Sfi*I/*NgoM*IV restriction enzyme digestion of inserts from pDUP and pDIS vectors and integration into *NEUT5L*. (e) Schematic representation of the primer design for gap repair. These primers work in both pDUP and pDIS vectors. Primers are $\geqslant$60 bp long, and consist of $\geqslant$20 bp with homology to the insert required for PCR amplification of the insert (grey line), followed by $\geqslant$40 bp with homology to pRS314 added as the primer tails (black line). The box contains the 40 bp tails from primers 5227 (top) and 5228 (bottom) that should be added to the primers to clone inserts into pDUP3-5/pDIS3-5 (Table 2). (f) Representation of the *in vivo* recombination between a *Sma*I-linearized pDIS vector and a PCR amplified insert containing 40 bp of homology to pDIS.

by duplication of *NEUT5L* (Fig. 2c) versus disruption of *NEUT5L* (Fig. 2d)]. Integration by duplication of the region targeted for recombination (Nunes *et al.*, 1999) is a common approach used in diverse organisms, including the *C. albicans* vectors Cip10, Cip10-2μ, pHWP1-GFP3 and pDDB78 (Dennison *et al.*, 2005; Spreghini *et al.*, 2003; Staab *et al.*, 2003; Vieira *et al.*, 2010). Integration by disruption of the region targeted for recombination is also an approach commonly used in *C. albicans* (Sasse & Morschhäuser, 2012; Wilson *et al.*, 1999). Here, vectors were designed so that either one of these approaches can be used to target integration into the *NEUT5L* locus.

The backbone of the pDUP and pDIS vectors (Fig. 2a, b) is the *S. cerevisiae–E. coli* shuttle vector pRS314 (Sikorski & Hieter, 1989) which has already been used for integration into the *C. albicans* genome (at the *HIS1* locus) (Davis *et al.*, 2002; Ganguly & Mitchell, 2012; Spreghini *et al.*, 2003; Wolf & Davis, 2010; Zacchi *et al.*, 2010). Important features of pRS314 include: an ampicillin resistance (*AmpR*) gene for selection in bacterial strains, the pBLUESCRIPT (Stratagene) polylinker region for convenient cloning and three elements from *S. cerevisiae* that permit replication, maintenance and selection in this budding yeast [an autonomous replicating sequence (*ARSH4*) centromeric DNA (*CEN6*) and an auxotrophic marker (*TRP1*) (Fig. 2)].

To construct the vectors, we utilized an *in vivo* recombination or gap repair approach (Muhlrad *et al.*, 1992). Gap repair seals double-strand breaks in plasmid DNA by recombining DNA fragments that share homology at the junction regions. In *S. cerevisiae*, gap repair facilitates highly efficient cloning of DNA fragments into plasmid backbones, and has been utilized to generate vectors for use in diverse organisms, including *C. albicans* (Davis *et al.*, 2002; Ma *et al.*, 1987; Nunes *et al.*, 1999; Spreghini *et al.*, 2003; Vieira *et al.*, 2010). There are two important requirements for cloning by *in vivo* recombination into any vector using *S. cerevisiae*. First, the DNA fragments to be recombined must share at least 40 bp of homology at the junctions. This region of homology can be added to the 5′ end of primers used to PCR amplify the insert of choice (Fig. 2e). For clarity, the sequences of all primers used for *in vivo* recombination in this work display the sequences with homology to pRS314 in lowercase type (Table 2). Second, the restriction enzyme site (RES) used to linearize the vector must be located between the regions homologous to the desired insertion site (e.g. within the DNA region flanked by the two 40 bp primer tails) (Figs 2f and S1). Using this methodology, six different vectors were constructed for integration into the *NEUT5L* locus in *C. albicans*, as described below (Figs 2 and S2).

To construct the pDUP/pDIS vectors, we sequentially integrated the *NEUT5L* region and one of three different selectable markers (*NAT1*, *URA3* and *URA3-dpl200*) into pRS314 by *in vivo* recombination (Fig. S1). The 550 bp *NEUT5L* region was cloned in two fragments (NEUT5L 3′ and NEUT5L 5′) each flanking the pRS314 polylinker region (Figs 2 and S1). *Sfi*I and *NgoM*IV RES (underlined in primers in Table 2) were incorporated during the PCR so that they flank the NEUT5L 5′ and NEUT5L 3′ fragments (Figs 2 and S1). Finally, three different markers were incorporated into the pDUP and pDIS backbones: *NAT1* (pDUP3, pDIS3), *URA3* (pDUP4, pDIS4) and the *URA3-dpl200* recyclable marker (pDUP5, pDIS5) (Figs 2 and S2) (Shen *et al.*, 2005; Wilson *et al.*, 2000). These six shuttle vectors are designed for cloning any DNA of interest within the remaining RES of the polylinker located between NEUT5L 5′ and the selectable marker (Fig. 2). Cloning into these vectors can be achieved with both classical restriction enzyme digestion/ligation (at the *Not*I–*Cla*I RES) and by *in vivo* recombination (at the *Sma*I RES) (Fig. 2). To integrate a construct from pDUP/pDIS vectors into the *C. albicans* genome, the entire region to be integrated is isolated from the pDUP/pDIS backbone by digestion at the *Sfi*I/*NgoM*IV RES that flank the two *NEUT5L* fragments (Fig. 2c, d). This last step has two advantages: first, it reduces the size of the integrating DNA fragment and, second, it reduces the likelihood of integration of non-*C. albicans* DNA (*AmpR*, *CEN6*, *ARSH4* and *TRP1*) into the *C. albicans* genome (data not shown). Integration of inserts from pDUP and pDIS vectors not only results in the duplication or disruption of *NEUT5L*, respectively, but also leads to integration of the insert in opposite orientations (Fig. 3a). In sum, we

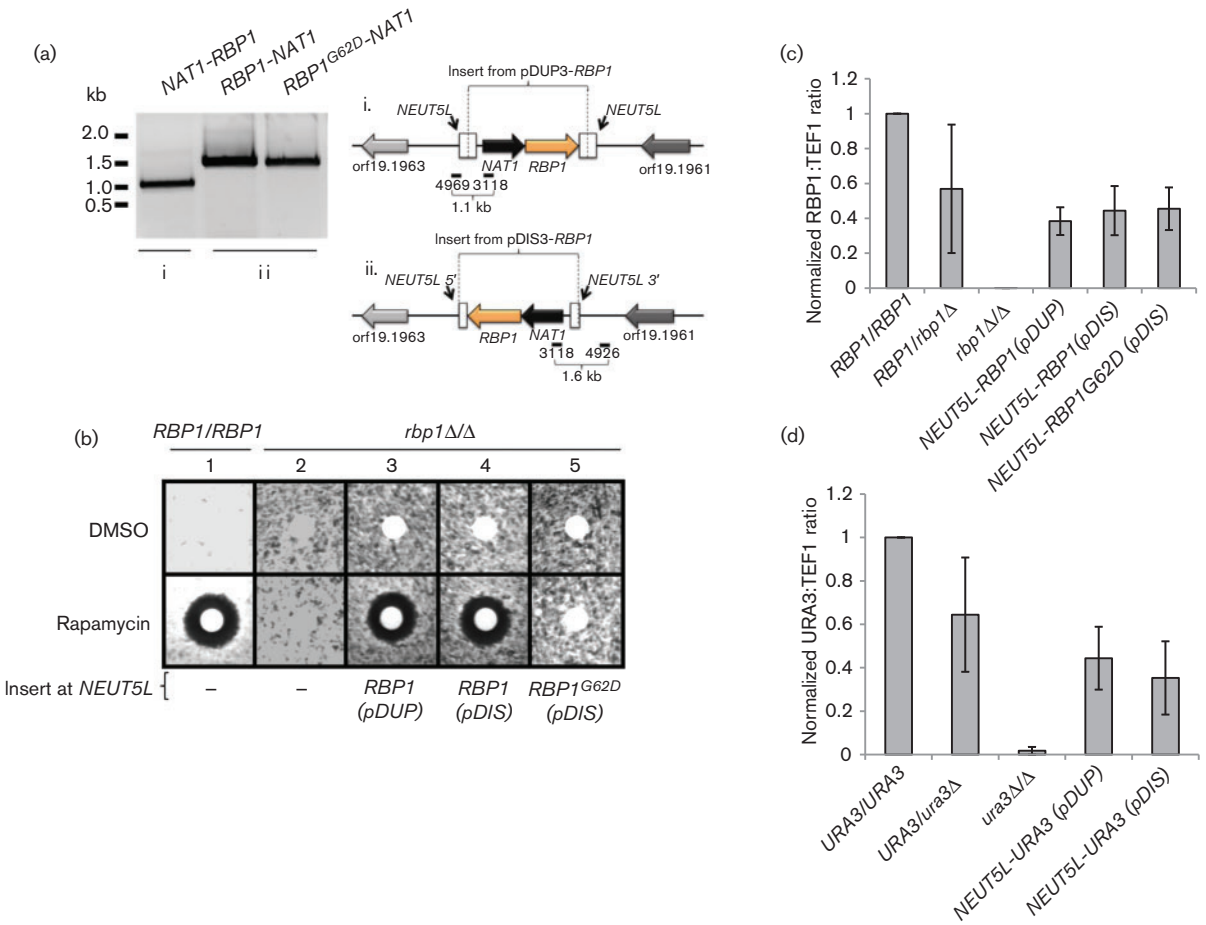

Fig. 3. Inserts from pDUP/pDIS vectors are efficiently integrated and expressed from *NEUT5L*. (a) PCR from genomic DNA of *NAT*$^{+}$ transformants showing proper integration into *NEUT5L* of the *RBP1–NAT1* cassette from pDUP3/pDIS3-*RBP1* and pDIS3-*RBP1*^{G62D}. (i) Primer set 3118+4969 was used to PCR amplify the genomic DNA of strain YJB12563 [*rbp1*Δ/Δ *NEUT5L-RBP1* (pDUP)], while (ii) primer set 3118+4926 was used to PCR amplify the genomic DNA of strains YJB12565 [*rbp1*Δ/Δ *NEUT5L-RBP1*(pDIS)] and YJB12566 [*rbp1*Δ/Δ *NEUT5L-RBP1*^{G62D} (pDIS)]. (b) Phenotypic test showing that the *RBP1* and *RBP1*^{G62D} constructs integrated via duplication (pDUP vector, column 3) or disruption (pDIS vectors, columns 4 and 5) of the *NEUT5L* region are expressed. Strains SC5314 (*RBP1*/*RBP1*), YJB12543 (*rbp1*Δ/Δ), and YJB12563-6 were plated onto YPAD medium and grown in the presence of discs embedded with 5 % DMSO (upper panels) or with 1 μg rapamycin ml^{-1} in 5 % DMSO (bottom panels). Pictures showing the zone of growth inhibition were taken after 24 h of incubation at 30 °C. (c) and (d) Quantitative RT-PCR showing that *RBP1* and *RBP1*^{G62D} (c) and *URA3* (d) are expressed to native-locus levels when integrated at the *NEUT5L* region. *RBP1* expression levels at its native locus in the wild-type SC5314 (*RBP1*/*RBP1*), the *RBP1*/*rbp1*Δ (YJB12982), and *rbp1*Δ/Δ (YJB12543) strains were compared with *RBP1* expression at *NEUT5L* from strains YJB12563 [*rbp1*Δ/Δ *NEUT5L-RBP1*(pDUP)], YJB12565 [*rbp1*Δ/Δ *NEUT5L-RBP1*(pDIS)], and YJB12566 [*rbp1*Δ/Δ *NEUT5L-RBP1*^{G62D} (pDIS)]. *URA3* expression levels at its native locus in the wild-type (CAF2-1) (*URA3*/*URA3*), the *URA3*/*ura*Δ (CAI4), and *ura3*Δ/Δ SC5314 strains were compared with *URA3* expression at *NEUT5L* from strains YJB12984 [*ura3*Δ/Δ *NEUT5L-URA3* (pDUP)] and YJB12985 [*ura3*Δ/Δ *NEUT5L-URA3* (pDIS)]. qRT-PCR was performed on RNA samples obtained from the different strains grown to exponential phase in YPD at 30 °C. Shown is the mean ± SD of the target gene (*RBP1* or *URA3*)/reference gene (*TEF1*) ratio for each strain normalized to *RBP1* or *URA3* levels of expression of the wild-type strain of at least two independent experiments.

have engineered six *S. cerevisiae/E. coli* shuttle vectors based on the pRS314 backbone that allow for rapid and facile cloning through *in vivo* recombination and target the insert for integration into the *NEUT5L C. albicans* locus.

Proof of principle: expression of *RBP1* and *RBP1*^{G62D} from *NEUT5L*

To test the utility of the plasmids, we cloned and inserted into *NEUT5L* the gene *RBP1* (orf19.6452), which encodes

the rapamycin binding protein (known as FKBP12 in mammals and Fpr1 in *S. cerevisiae*). *RBP1* confers a readily detectable phenotype to the transformed cells: $RBP1^{+}$ cells are sensitive to rapamycin, while rbp^{-} cells (*rbp1*Δ/Δ) are resistant to rapamycin (Cruz *et al.*, 2001). Rapamycin is a bacterial macrolide that, when in complex with Rbp1, blocks Tor kinase function, triggering a major cellular response through the inhibition of the TOR signalling pathway that leads to cell growth arrest (Cruz *et al.*, 2001; Wullschleger *et al.*, 2006). Mutations in *RBP1*, such as a spontaneous recessive point mutation in a highly conserved glycine residue ($fpr1^{G65D}$ in *S. cerevisiae*) also confer rapamycin resistance (Cruz *et al.*, 2001; Ferrara *et al.*, 1992; Heitman *et al.*, 1991). In *C. albicans*, the equivalent residue to *S. cerevisiae FPR1*-G65 is *RBP1*-G62 (Fig. S3, black box). Thus, if *C. albicans* Rbp1 has functions like *S. cerevisiae* Fpr1, reintroduction of *RBP1*, but not of $RBP1^{G62D}$, into *rbp1*Δ/Δ *C. albicans* cells is expected to restore the wild-type, rapamycin-sensitive phenotype.

The wild-type *RBP1* gene, including the *RBP1* promoter and terminator regions, was cloned into both pDUP3 and pDIS3 by *in vivo* recombination to produce pDUP3-*RBP1* and pDIS3-*RBP1*. The NEUT5L 5′–*RBP1*–*NAT1*–NEUT5L 3′ insert was released from pDUP3-*RBP1* and pDIS3-*RBP1* by *Sfi*I digestion and transformed into the *C. albicans rbp1*Δ/Δ strain (YJB12543), yielding approximately 50 nourseothricin-resistant colonies (NAT^{+}) μg^{-1} digested plasmid DNA. PCR screening of the transformants detected correct integration in 100 % (four out of four) of the independent transformants tested for each vector (Fig. 3a). Similar levels of efficiency of integration were obtained with other DNA inserts as well (data not shown), indicating that both vectors direct highly efficient integration at the *NEUT5L* locus.

Next, we tested the effect of integration at *NEUT5L* on *RBP1* expression. Growth of the wild-type *RBP1*/*RBP1* SC5314 strain is inhibited by rapamycin and not by solvent alone (DMSO) (Fig. 3b, first column, halo of growth inhibition), while *rbp1*Δ/Δ cells are rapamycin-resistant (Fig. 3b, second column; (Cruz *et al.*, 2001)). If *RBP1* is indeed expressed at the *NEUT5L* locus, the *rbp1*Δ/Δ strains transformed with pDUP3-*RBP1* (Fig. 3b, third column) and pDIS3-*RBP1* (Fig. 3b, fourth column) should become rapamycin-sensitive. As expected, these strains showed a halo of growth inhibition indicating that constructs from both pDUP and pDIS shuttle vectors are efficiently expressed from the *NEUT5L* locus. In order to directly verify that the level of *RBP1* gene expression in these strains was similar when integrated at *NEUT5L* compared with its native locus, we extracted RNA from cells grown in YPD at 30 °C and performed quantitative RT-PCR to measure *RBP1* mRNA levels (Fig. 3c). To aid in the comparison, we normalized all the data to the expression level of the *RBP1*/*RBP1* wild-type strain. *RBP1* expression level in the *RBP1*/*rpb1Δ* strain was approximately one half of the expression level in the wild-type strain (0.57 ± 0.37) (Fig. 3c). When integrated at *NEUT5L*, *RBP1* expression was similar to that of the *RBP1*/*rpb1*Δ strain (0.38 ± 0.08 for the pDUP construct and 0.44 ± 0.14, for the pDIS construct) (Fig. 3c). No *RBP1* expression was detected in the *rpb1*Δ/Δ strain, as expected. These results indicate that *RBP1* integrated at *NEUT5L* using the pDUP and pDIS vectors is indeed expressed at native-locus levels, supporting the conclusion that *NEUT5L* is a neutral locus for expression of exogenous DNA (see above, Fig. 1b). Furthermore, these results also suggest that gene expression at *NEUT5L* is similar independently of the direction of integration and of the duplication of the *NEUT5L* region caused by using either the pDUP or pDIS systems.

Because the auxotrophic marker *URA3* is susceptible to position effects (Brand *et al.*, 2004; Cheng *et al.*, 2003; Lay *et al.*, 1998; Staab & Sundstrom, 2003), we verified that integration at *NEUT5L* allowed for *URA3* native-locus level of expression. To do this we integrated *URA3* cassettes from pDUP4 and pDIS4 vectors (Fig. 2a, b) into *NEUT5L* and compared *URA3* expression in these strains with the wild-type strain and an *URA3*/*ura3*Δ heterozygote strain grown in YPD at 30 °C (Fig. 3d). To aid in the comparison, we normalized all the data to the expression level of the *URA3*/*URA3* wild-type strain. *URA3* expression level in the *URA3*/*ura3*Δ strain was approximately one half of the expression level in the wild-type strain (0.64 ± 0.26) (Fig. 3d). When integrated at NEUT5L, *URA3* expression was similar to the expression levels of the *URA3*/*ura3*Δ heterozygote strain (0.44 ± 0.14 for the pDUP4 construct and 0.35 ± 0.17 for the pDIS4 construct) (Fig. 3d). Almost no detectable *URA3* expression was observed in the *ura3*Δ/Δ strain, as expected. These results indicate that *URA3* is efficiently expressed from *NEUT5L*, and that this auxotrophic marker can be safely used when integrating constructs at *NEUT5L* to perform virulence studies.

Cloning by *in vivo* recombination is particularly useful because desired mutations, including point mutations as well as fusion with epitope tags or fluorescent proteins, can be introduced into the insert in a single step, obviating the need to design specific RES and perform sequential cloning steps [e.g. (Wolf & Davis, 2010; Zacchi *et al.*, 2010)]. For example, we utilized this approach to introduce the *Sfi*I and *NgoM*IV RES bordering the *NEUT5L* fragments of the pDUP/pDIS vectors (Figs 2 and S1). To provide another example of the advantages of the gap repair technique, we constructed pDIS3-$RBP1^{G62D}$ vectors in a single step. *RBP1* was amplified as two fragments, using the same external primers employed to clone wild-type *RBP1* into pDIS3 and new, internal, perfectly overlapping 40 bp primers carrying the GGT (glycine) to GAT (aspartic acid) mutation present in the $RBP1^{G62D}$ allele (Fig. S4). The two PCR fragments were then *in vivo* recombined into *Sma*I-digested pDIS3 by co-transformation into *S. cerevisiae*. The *NEUT5L*-$RBP1^{G62D}$-*NAT1* constructs were released from the pDIS vector by *Sfi*I digestion and transformed into the *rbp1*Δ/Δ strain. PCR screening of transformants showed 100 % efficiency

Table 4. Advantages and applications of pDUP/pDIS vectors

Advantages of the pDUP/pDIS vectors
1) Intergenic region between orf19.1961 and orf19.1963: the NEUT5L locus a. Efficient insertion and expression of exogenous DNA. b. Insertion at this locus does not detectably affect growth or filamentation. **2) pDUP/pDIS vectors** a. Markers: counterselectable *URA3*; recyclable *URA3-dpl200*; dominant drug resistance marker *NAT1*. b. Convenient RES for preventing introduction of foreign DNA into *C. albicans*. c. High efficiency of integration into *NEUT5L* (550 bp total homology to integration locus). d. Two modes of integration: duplication and disruption. **3) Gap repair in *S. cerevisiae*** a. Facilitates cloning of multiple overlapping fragments (Eckert-Boulet *et al.*, 2012; Iizasa & Nagano, 2006; Marykwas & Passmore, 1995). b. One-step cloning simultaneously incorporates desired mutations or epitope tags. c. Does not require shared specific RES in the insert and vector. d. Obviates *E. coli* cloning step thereby facilitating cloning of inserts that are toxic to bacteria. e. Lower cost relative to site-directed mutagenesis.
Applications of the pDUP/pDIS vectors
1) Rapid and facile construction of mutant/chimeric inserts. Recombining inserts into pDUP/pDIS vectors while simultaneously incorporating: a. Mutations: point mutations, insertions, deletions, etc. b. Epitope and fluorescent tags. c. Regulatable promoters, or other modifications. **2) Reversible integration**. a. Pop-in/pop-out inserts integrated at NEUT5L using pDUP4 vectors. b. Test for lethality of a homozygous null deletion by reversible insertion of a third copy at NEUT5L prior to deleting both copies at the native locus.

of integration at *NEUT5L* (four out of four nourseothricin-resistant strains contained *NAT1* at the *NEUT5L* locus) (Fig. 3a). We had hypothesized that the $RBP1^{G62D}$ mutation would confer rapamycin resistance, similar to the phenotype of the equivalent $FPR1^{G65D}$ mutation in *S. cerevisiae* [(Heitman *et al.*, 1991) and Fig. S3, black box]. Consistent with this, integration of $RBP1^{G62D}$ into the *C. albicans rbp1*Δ/Δ strain at the *NEUT5L* locus did not confer a rapamycin-sensitivity phenotype (Fig. 3b, fifth column), while integration of wild-type *RBP1* did (Fig. 3b, fourth column). Importantly, $RBP1^{G62D}$ expression level at *NEUT5L* was similar to *RBP1* native-locus levels (0.45 ± 0.12 versus 0.57 ± 0.37), indicating that the rapamycin resistance phenotype was caused by the G62D mutation and not by the lack of expression of the construct (Fig. 3c). Thus, *in vivo* recombination and integration into *NEUT5L* enables efficient hypothesis-testing through site-directed mutagenesis and cloning coupled in a single step.

In summary, the work presented here describes two new important features for efficient genetic manipulation of the *C. albicans* genome (Table 4). First, we identified a novel neutral region that permits the efficient integration of exogenous DNA and the expression of inserts to levels similar to their expression at their native locus. Importantly, no growth or filamentation defects have been detected following disruption of one copy of the *NEUT5L* locus under a range of growth conditions. Second, we constructed a series of six shuttle vectors for efficient one-step cloning and integration of constructs into *C. albicans*. The vectors are based on the pRS314 plasmid backbone, which is commonly used for expression and complementation studies in *S. cerevisiae*, and has been used for cloning and integration of constructs into the *C. albicans HIS1* locus. These re-engineered vectors provide a number of advantages over other available plasmids (Table 4). First, the 550 bp region of homology to *NEUT5L* contained in the vectors yields highly efficient targeting to this specific locus. Second, the three markers in these vectors expand the range of selection schemes available by incorporating the counterselectable auxotrophic *URA3* or the recyclable *URA3-dpl200* markers, and the dominant *NAT1* drug resistance marker that is ideal for use in the transformation of clinical isolates and other prototrophic strains. Third, the two alternative RES (*Sfi*I and *NgoM*IV) bordering the insert release the construct from the vector prior to transformation, thereby preventing integration of non-*C. albicans* DNA into the *C. albicans* genome and reducing the size of the insert. Fourth, the vectors allow for integration of the insert such that the region of insertions is either duplicated or disrupted. Fifth, when using the pDUP vectors, duplication of the region of insertion can be used to drive the reversible integration of the construct. Sixth, inserts can be cloned into the vectors

by *in vivo* recombination or by using classical RES digestion/ligation approaches. Seventh, fragments cloned into the pDUP/pDIS vectors can be PCR-amplified directly from *S. cerevisiae* DNA extracts, thereby skipping the requirement for subcloning in *E. coli,* which may be impractical for large plasmids and/or toxic inserts. Finally, the steps described here to generate the pDUP/pDIS vectors can be used to design new vectors that direct integration into any chosen region of the genome.

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REFERENCES

Adams, A., Gotschling, D., Kaiser, C. & Stearns, T. (1997). *Methods in Yeast Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Backen, A. C., Broadbent, I. D., Fetherston, R. W., Rosamond, J. D., Schnell, N. F. & Stark, M. J. (2000). Evaluation of the *CaMAL2* promoter for regulated expression of genes in *Candida albicans. Yeast* **16**, 1121–1129.

Barelle, C. J., Manson, C. L., MacCallum, D. M., Odds, F. C., Gow, N. A. & Brown, A. J. (2004). GFP as a quantitative reporter of gene regulation in *Candida albicans. Yeast* **21**, 333–340.

Basso, L. R., Jr, Bartiss, A., Mao, Y., Gast, C. E., Coelho, P. S., Snyder, M. & Wong, B. (2010). Transformation of *Candida albicans* with a synthetic hygromycin B resistance gene. *Yeast* **27**, 1039–1048.

Beckerman, J., Chibana, H., Turner, J. & Magee, P. T. (2001). Single-copy *IMH3* allele is sufficient to confer resistance to mycophenolic acid in *Candida albicans* and to mediate transformation of clinical *Candida* species. *Infect Immun* **69**, 108–114.

Brand, A., MacCallum, D. M., Brown, A. J., Gow, N. A. & Odds, F. C. (2004). 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 *RPS10* locus. *Eukaryot Cell* **3**, 900–909.

Care, R. S., Trevethick, J., Binley, K. M. & Sudbery, P. E. (1999). The *MET3* promoter: a new tool for *Candida albicans* molecular genetics. *Mol Microbiol* **34**, 792–798.

Cheng, S., Nguyen, M. H., Zhang, Z., Jia, H., Handfield, M. & Clancy, C. J. (2003). 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* **71**, 6101–6103.

Cruz, M. C., Goldstein, A. L., Blankenship, J., Del Poeta, M., Perfect, J. R., McCusker, J. H., Bennani, Y. L., Cardenas, M. E. & Heitman, J. (2001). Rapamycin and less immunosuppressive analogs are toxic to *Candida albicans* and *Cryptococcus neoformans* via FKBP12-dependent inhibition of TOR. *Antimicrob Agents Chemother* **45**, 3162–3170.

Davis, D. A., Bruno, V. M., Loza, L., Filler, S. G. & Mitchell, A. P. (2002). *Candida albicans* Mds3p, a conserved regulator of pH responses and virulence identified through insertional mutagenesis. *Genetics* **162**, 1573–1581.

Dennison, P. M., Ramsdale, M., Manson, C. L. & Brown, A. J. (2005). Gene disruption in *Candida albicans* using a synthetic, codon-optimised Cre–*loxP* system. *Fungal Genet Biol* **42**, 737–748.

Doyle, T. C., Nawotka, K. A., Purchio, A. F., Akin, A. R., Francis, K. P. & Contag, P. R. (2006). Expression of firefly luciferase in *Candida albicans* and its use in the selection of stable transformants. *Microb Pathog* **40**, 69–81.

Eckert-Boulet, N., Pedersen, M. L., Krogh, B. O. & Lisby, M. (2012). Optimization of ordered plasmid assembly by gap repair in *Saccharomyces cerevisiae. Yeast* **29**, 323–334.

Enloe, B., Diamond, A. & Mitchell, A. P. (2000). A single-transformation gene function test in diploid *Candida albicans. J Bacteriol* **182**, 5730–5736.

Falagas, M. E., Apostolou, K. E. & Pappas, V. D. (2006). Attributable mortality of candidemia: a systematic review of matched cohort and case-control studies. *Eur J Clin Microbiol Infect Dis* **25**, 419–425.

Ferrara, A., Cafferkey, R. & Livi, G. P. (1992). Cloning and sequence analysis of a rapamycin-binding protein-encoding gene (RBP1) from *Candida albicans. Gene* **113**, 125–127.

Finkel, J. S., Yudanin, N., Nett, J. E., Andes, D. R. & Mitchell, A. P. (2011). Application of the systematic ''DAmP'' approach to create a partially defective *C. albicans* mutant. *Fungal Genet Biol* **48**, 1056–1061.

Fonzi, W. A. & Irwin, M. Y. (1993). Isogenic strain construction and gene mapping in *Candida albicans. Genetics* **134**, 717–728.

Ganguly, S. & Mitchell, A. P. (2012). Mini-blaster-mediated targeted gene disruption and marker complementation in *Candida albicans. Methods Mol Biol* **845**, 19–39.

Gerami-Nejad, M., Berman, J. & Gale, C. A. (2001). Cassettes for PCR-mediated construction of green, yellow, and cyan fluorescent protein fusions in *Candida albicans. Yeast* **18**, 859–864.

Gerami-Nejad, M., Hausauer, D., McClellan, M., Berman, J. & Gale, C. (2004). Cassettes for the PCR-mediated construction of regulatable alleles in *Candida albicans. Yeast* **21**, 429–436.

Gerami-Nejad, M., Dulmage, K. & Berman, J. (2009). Additional cassettes for epitope and fluorescent fusion proteins in *Candida albicans. Yeast* **26**, 399–406.

Gerami-Nejad, M., Forche, A., McClellan, M. & Berman, J. (2012). Analysis of protein function in clinical *C. albicans* isolates. *Yeast* **29**, 303–309.

Glowczewski, L., Waterborg, J. H. & Berman, J. G. (2004). Yeast chromatin assembly complex 1 protein excludes nonacetylatable forms of histone H4 from chromatin and the nucleus. *Mol Cell Biol* **24**, 10180–10192.

Gola, S., Martin, R., Walther, A., Dünkler, A. & Wendland, J. (2003). New modules for PCR-based gene targeting in *Candida albicans*: rapid and efficient gene targeting using 100 bp of flanking homology region. *Yeast* **20**, 1339–1347.

Gudlaugsson, O., Gillespie, S., Lee, K., Vande Berg, J., Hu, J., Messer, S., Herwaldt, L., Pfaller, M. & Diekema, D. (2003). Attributable mortality of nosocomial candidemia, revisited. *Clin Infect Dis* **37**, 1172–1177.

Heitman, J., Movva, N. R. & Hall, M. N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253**, 905–909.

Henikoff, S. (1992). Position effect and related phenomena. *Curr Opin Genet Dev* **2**, 907–912.

Huber, D. & Rustchenko, E. (2001). Large circular and linear rDNA plasmids in *Candida albicans*. *Yeast* **18**, 261–272.

Igyártó, B. Z., Haley, K., Ortner, D., Bobr, A., Gerami-Nejad, M., Edelson, B. T., Zurawski, S. M., Malissen, B., Zurawski, G. & other authors (2011). Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. *Immunity* **35**, 260–272.

Iizasa, E. & Nagano, Y. (2006). Highly efficient yeast-based in vivo DNA cloning of multiple DNA fragments and the simultaneous construction of yeast/*Escherichia coli* shuttle vectors. *Biotechniques* **40**, 79–83.

Keppler-Ross, S., Noffz, C. & Dean, N. (2008). A new purple fluorescent color marker for genetic studies in *Saccharomyces cerevisiae* and *Candida albicans*. *Genetics* **179**, 705–710.

Kurtz, M. B., Cortelyou, M. W. & Kirsch, D. R. (1986). Integrative transformation of *Candida albicans*, using a cloned *Candida ADE2* gene. *Mol Cell Biol* **6**, 142–149.

Kurtz, M. B., Cortelyou, M. W., Miller, S. M., Lai, M. & Kirsch, D. R. (1987). Development of autonomously replicating plasmids for *Candida albicans*. *Mol Cell Biol* **7**, 209–217.

Lai, W. C., Tseng, T. L., Jian, T., Lee, T. L., Cheng, C. W. & Shieh, J. C. (2011). Construction of *Candida albicans* Tet-on tagging vectors with a Ura-blaster cassette. *Yeast* **28**, 253–263.

Lay, J., Henry, L. K., Clifford, J., Koltin, Y., Bulawa, C. E. & Becker, J. M. (1998). Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. *Infect Immun* **66**, 5301–5306.

Lebel, K., MacPherson, S. & Turcotte, B. (2006). New tools for phenotypic analysis in *Candida albicans*: the *WAR1* gene confers resistance to sorbate. *Yeast* **23**, 249–259.

Ma, H., Kunes, S., Schatz, P. J. & Botstein, D. (1987). Plasmid construction by homologous recombination in yeast. *Gene* **58**, 201–216.

Marykwas, D. L. & Passmore, S. E. (1995). Mapping by multi-fragment cloning *in vivo*. *Proc Natl Acad Sci U S A* **92**, 11701–11705.

Muhlrad, D., Hunter, R. & Parker, R. (1992). A rapid method for localized mutagenesis of yeast genes. *Yeast* **8**, 79–82.

Murad, A. M., Lee, P. R., Broadbent, I. D., Barelle, C. J. & Brown, A. J. (2000). CIp10, an efficient and convenient integrating vector for *Candida albicans*. *Yeast* **16**, 325–327.

Nobile, C. J. & Mitchell, A. P. (2009). Large-scale gene disruption using the *UAU1* cassette. *Methods Mol Biol* **499**, 175–194.

Nunes, A., Thathy, V., Bruderer, T., Sultan, A. A., Nussenzweig, R. S. & Ménard, R. (1999). Subtle mutagenesis by ends-in recombination in malaria parasites. *Mol Cell Biol* **19**, 2895–2902.

Oh, J., Fung, E., Schlecht, U., Davis, R. W., Giaever, G., St Onge, R. P., Deutschbauer, A. & Nislow, C. (2010). Gene annotation and drug target discovery in *Candida albicans* with a tagged transposon mutant collection. *PLoS Pathog* **6**, e1001140.

Park, N. H. & Choi, W. (2002). Vectors designed for efficient molecular manipulation in *Candida albicans*. *Yeast* **19**, 1057–1066.

Pirrotta, V. & Gross, D. S. (2005). Epigenetic silencing mechanisms in budding yeast and fruit fly: different paths, same destinations. *Mol Cell* **18**, 395–398.

Reijnst, P., Walther, A. & Wendland, J. (2011). Dual-colour fluorescence microscopy using yEmCherry-/GFP-tagging of eisosome components Pil1 and Lsp1 in *Candida albicans*. *Yeast* **28**, 331–338.

Reuß, O., Vik, A., Kolter, R. & Morschhäuser, J. (2004). The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene* **341**, 119–127.

Sánchez-Martínez, C. & Pérez-Martín, J. (2002). Site-specific targeting of exogenous DNA into the genome of *Candida albicans* using the FLP recombinase. *Mol Genet Genomics* **268**, 418–424.

Sasse, C. & Morschhäuser, J. (2012). Gene deletion in *Candida albicans* wild-type strains using the *SAT1*-flipping strategy. *Methods Mol Biol* **845**, 3–17.

Shen, J., Guo, W. & Köhler, J. R. (2005). *CaNAT1*, a heterologous dominant selectable marker for transformation of *Candida albicans* and other pathogenic *Candida* species. *Infect Immun* **73**, 1239–1242.

Sikorski, R. S. & Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27.

Spreghini, E., Davis, D. A., Subaran, R., Kim, M. & Mitchell, A. P. (2003). Roles of *Candida albicans* Dfg5p and Dcw1p cell surface proteins in growth and hypha formation. *Eukaryot Cell* **2**, 746–755.

Srikantha, T., Klapach, A., Lorenz, W. W., Tsai, L. K., Laughlin, L. A., Gorman, J. A. & Soll, D. R. (1996). The sea pansy *Renilla reniformis* luciferase serves as a sensitive bioluminescent reporter for differential gene expression in *Candida albicans*. *J Bacteriol* **178**, 121–129.

Staab, J. F. & Sundstrom, P. (2003). *URA3* as a selectable marker for disruption and virulence assessment of *Candida albicans* genes. *Trends Microbiol* **11**, 69–73.

Staab, J. F., Bahn, Y. S. & Sundstrom, P. (2003). Integrative, multifunctional plasmids for hypha-specific or constitutive expression of green fluorescent protein in *Candida albicans*. *Microbiology* **149**, 2977–2986.

Stynen, B., Van Dijck, P. & Tournu, H. (2010). A CUG codon adapted two-hybrid system for the pathogenic fungus *Candida albicans*. *Nucleic Acids Res* **38**, e184.

Tartof, K. D. (1994). Position effect variegation in yeast. *Bioessays* **16**, 713–714.

Umeyama, T., Nagai, Y., Niimi, M. & Uehara, Y. (2002). Construction of FLAG tagging vectors for *Candida albicans*. *Yeast* **19**, 611–618.

Vieira, N., Pereira, F., Casal, M., Brown, A. J., Paiva, S. & Johansson, B. (2010). Plasmids for *in vivo* construction of integrative *Candida albicans* vectors in *Saccharomyces cerevisiae*. *Yeast* **27**, 933–939.

Vojtek, A. B., Hollenberg, S. M. & Cooper, J. A. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**, 205–214.

Wilson, R. B., Davis, D. & Mitchell, A. P. (1999). Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J Bacteriol* **181**, 1868–1874.

Wilson, R. B., Davis, D., Enloe, B. M. & Mitchell, A. P. (2000). A recyclable *Candida albicans URA3* cassette for PCR product-directed gene disruptions. *Yeast* **16**, 65–70.

Wolf, J. M. & Davis, D. A. (2010). Mutational analysis of *Candida albicans SNF7* reveals genetically separable Rim101 and ESCRT functions and demonstrates divergence in bro1-domain protein interactions. *Genetics* **184**, 673–694.

Wullschleger, S., Loewith, R. & Hall, M. N. (2006). TOR signaling in growth and metabolism. *Cell* **124**, 471–484.

Xu, T., Bharucha, N. & Kumar, A. (2011). Genome-wide transposon mutagenesis in *Saccharomyces cerevisiae* and *Candida albicans*. *Methods Mol Biol* **765**, 207–224.

Zacchi, L. F., Gomez-Raja, J. & Davis, D. A. (2010). Mds3 regulates morphogenesis in *Candida albicans* through the TOR pathway. *Mol Cell Biol* **30**, 3695–3710.

Zaoutis, T. E., Argon, J., Chu, J., Berlin, J. A., Walsh, T. J. & Feudtner, C. (2005). The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis. *Clin Infect Dis* **41**, 1232–1239.

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