Original Article

Deletions of the *Idh1*, *Eco1*, *Rom2*, and *Taf10* Genes Differently Control the Hyphal Growth, Drug Tolerance, and Virulence of *Candida albicans*

(CRISPR-Cas9 / Idh1 / Eco1 / Rom2 / Taf10 / gene deletion / virulence / pathogenicity)

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Abstract. The most recent genome-editing system called CRISPR-Cas9 (clustered regularly interspaced short palindromic repeat system with associated protein 9-nuclease) was employed to delete four non-essential genes (i.e., Caeco1, Caidh1, Carom2, and Cataf10) individually to establish their gene functionality annotations in pathogen *Candida albicans*. The biological roles of these genes were investigated with respect to the cell wall integrity and biogenesis, calcium/calcineurin pathways, susceptibility of mutants towards temperature, drugs and salts. All the mutants showed increased vulnerability compared to the wild-type background strain towards the cell wall-perturbing agents, (antifungal) drugs and salts. All the mutants also exhibited repressed and defective hyphal growth and smaller colony size than control CA14. The cell cycle of all the mutants decreased enormously except for those with *Carom2* deletion. The budding index and budding size also increased for all mutants with altered bud shape. The disposition of the mutants towards cell wall-perturbing enzymes disclosed lower survival and more rapid cell wall lysis events than in wild types. The pathogenicity and virulence of the mutants was checked by adhesion assay, and strains lacking *rom2* and *eco1* were found to possess the least adhesion capacity, which is synonymous to their decreased pathogenicity and virulence.

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Abbreviations: CIP – calf intestinal phosphatase, CWI – cell wall integrity, DUB – deubiquitination, HAT – histone acetylation, Nat – nourseothrycin, OD – optical density, SD – synthetic defined, YPD – yeast peptone dextrose.

Introduction

Calcium ions are important regulators in eukaryotic cells as these ions are involved in determination of programmed cell death, growth, and muscle contraction in the heart and mouth. Eukaryotic cells possess highly conserved calcium/calcineurin signalling and transportation pathways that are responsible for important biofunctions both in microbial and mammalian cells (Clapham, 2007; Liu et al., 2015). In both kinds of eukaryotic cells, functional counterparts (i.e., calcium channels, pumps, transporters, sequestrators, and exchangers) not only exist, but also function in a similar manner. These functional counterparts, under non-stressed growth conditions, regulate Ca^{2+} homeostasis in mammals, plants, yeasts, etc. In non-stressed physiological conditions, Ca^{2+} enters the cytosol of *Saccharomyces cerevisiae* via two unknown transporters in the plasma membrane, which results in a transient rise in cytosolic Ca^{2+}, leading to activation of the calcineurin/calcium ion signalling pathway (Cui et al., 2009). Continual and prolonged cytosolic Ca^{2+} accumulation is considered fatal to cells and its level is countered by the calcium sequestration system (i.e., calcium pump Pmc1; vacuolar Ca exchanger Vcx; calcium pump Pmr1; and

Ca^{2+}/H^{+} exchanger Gdt1 of the ER/Golgi secretory pathway). The calcineurin/Ca^{2+} signalling pathway is highly conserved and controls the morphogenesis and cell wall biogenesis in various infectious yeast/fungal species including *Candida albicans*.

Candida albicans is a common human commensal in immuno-compromised patients with the capability of growing on/colonizing skin and mucosal surfaces (Southern et al., 2008). A striking feature of *Candida albicans* biology is its ability to grow as filamentous/hyphal, pseudohyphal and yeast forms, in which the hyphal/filamentous form is considered to be vital for its pathogenicity as the filamentous form can grow and invade epithelial cells (Fu et al., 1998; Sandovsky-Losica et al., 2006; Malic et al., 2007). The complete sequencing of *Candida albicans* (diploid) genome project has provided a foundation for studies related to its biology, pathogenesis, and therapeutic interventions (Odds et al., 2004). For this purpose, functionally annotating the *Candida albicans* genes directly or indirectly related to Calcineurin/Ca^{2+} pathways, filamentation and pathogenesis is critical. However, it is very difficult to functionally annotate such genes using classical genetics or other large-scale genetic analyses, owing to the natural diploid state and lack of plasmid system and manipulatable sexual cycle. Being a diploid microorganism, sequential modification of single loci was required to create homozygous deletion mutants, which is quite a laborious, time-consuming and less efficient way to annotate the physiology and biochemistry of genes. However, recently, some authors accelerated the genetic analysis of *Candida albicans* by employing CRISPR and the CRISPR-associated gene 9 (CRISPR-Cas9) systems to rapidly create *C. albicans* homozygous mutants (Vyas et al., 2015; Min et al., 2016).

We have employed the same CRISPR system in *C. albicans* to successfully develop homozygous mutants for the genes *ECO1* (orf19.5053), *TAF10* (orf19.3242), *IDH1* (orf19.4826), and *ROM2* (orf19.906). Eco1 is an acetyltransferase protein in *Saccharomyces cerevisiae* with a vital role in filamentous growth, chromatin binding, (regulation of) DNA replication, cohesion of mitotic sister chromatids, etc. (Toth et al., 1999; Kenna and Skibbens, 2003; Brands and Skibbens, 2005). Deletion of *ScECO1* has been found to cause growth defects in *S. cerevisiae* (Bell and Labib, 2016). Similarly, NAD-dependent isocitrate dehydrogenase (Idh1) plays a significant role in ethanol fermentation in budding yeast and Idh1 was previously designated as the rate-limiting enzyme in flux through the TCA cycle. Idh1 catalyses the isocitrate to α-ketoglutarate transformation in the TCA cycle. Besides that, Idh1 was also identified as the second most significant enzyme, controlling cell growth under furfural stress (Unrean, 2017). Therefore, deletion of *IDH1* was shown to decrease the degree of growth and reduce ethanol fermentation. Furthermore, Rom2 (RHO1 multicopy suppressor) usually located in the bud neck in polarity sites for bud formation during cytokinesis, and deletion of *ROM2* was found to leave lethal effects on morphogenesis of *S. cerevisiae*. Rom2 is activated by the cell wall integrity (CWI) pathway, which in turn activates the local glucan synthesis in the bud region (Lai et al., 2018). Moreover, Rho1 activity in the CWI pathway is precisely controlled by modulation of the levels of mRNA for Rho1-GEF (guanine nucleotide exchange factors), Rom2 and Rho1-GAP (GTPase-activating proteins), and Lrg1 (Li et al., 2016). Taf10 is actually a member of the TAFIID (TATA-binding protein-associated factor/transcription factor) complex, which is a subunit of the SAGA (a conserved transcription coactivator) complex involved in TBP binding, activator binding, deubiquitination (DUB), and histone acetylation (HAT). Deletion of *TAF10* led to unviability with arrested cell cycle progression in budding yeast (Han et al., 2014).

To date, *S. cerevisiae* has been the major subject of all gene functionality annotations and genome analysis studies in the literature, and no gene functionality annotation studies have been performed by creating homozygous mutants of *Candida albicans* via CRISPR-Cas9 with respect to *CaECO1*, *CaIDH1*, *CaROM2*, and *CaTAF10*. Furthermore, substantial variations in gene essentiality and genomic organization of *S. cerevisiae* and *C. albicans* were revealed by genomic studies (Xu et al., 2007). In part, these variations reflected the distinct lifestyles and evolutionary divergence of *S. cerevisiae* and *C. albicans* as saprophytic yeast and opportunistic fungal pathogen, respectively (Xu et al., 2007). So, ultimately the objective of this work was to create homozygous mutants of *C. albicans* by using state of the art CRISPR-Cas9 technology to establish gene functionality annotation for the deleted genes (i.e., *ECO1*, *IDH1*, *ROM2*, *TAF10*) and their influence on filamentation, virulence and drug tolerance.

Material and Methods

Strains, media, plasmids and primers

Tables 1 and 2 list all the existing and newly constructed strains of *C. albicans* and plasmids used. The list of primers designed and used in this entire work is mentioned in Table 3. Yeast peptone dextrose (YPD) medium (2% peptone, 2% glucose, 1% yeast extract) or synthetic defined (SD) medium (auxotrophic amino acids as needed, 0.67% yeast nitrogen base without amino acids, and 2% glucose) were used to maintain all the strains of *C. albicans,* which were grown at 30 °C (Lee et al., 2004). *E. coli* competent cells (DH5α) were used for replication purposes in LB media (yeast extract, tryptone, sodium chloride) and were purchased from New England Biolabs (Ipswich, MA). Glycerol, salmon sperm DNA, *Bsm*BI, *Eco*RI, *Xho*II, calf intestinal phosphatase (CIP), T4 polynucleotide kinase/T4-ligase/Exnase enzyme, (hi-fi) Taq polymerase were supplied by New England Biolabs. NAT, ampicillin, polyethylene glycol, uridine, lithium acetate, SDS, agarose, dextrose, uracil, bacto-agar, all amino acids (purines and

Table 1. Strains used and constructed in this study

Strain	Genotype	Source
CAI4	ura3::λimm434/ura3::λimm434	Fonzi and Erwin, 1993
AHCA29	CAI4 idh1/idh1::natR	This study
AHCA47	CAI4 rom2/rom2::natR	This study
AHCA113	CAI4 eco1/eco1:: natR	This study
AHCA67	CAI4 taf10/taf10::natR	This study
XDCA06	CAI4-CIp10	Xu et al., 2007
AHCA11	CAI4 idh1/idh1::frt/CIp10-IDH1	This study
AHCA79	CAI4 rom2/rom2::frt/CIp10-ROM2	This study
AHCA54	CAI4 eco1/eco1::frt/CIp10-ECO1	This study
AHCA41	CAI4 taf10/taf10::frt/CIp10-TAF10	This study

Table 2. Plasmids used and constructed in this study

Plasmid	Description	Source
pV1093	Vector with CaCAS9 expression cassette with natR marker	Viyas et al., 2015
CIp10	*C. albicans* integration vector with URA3 marker	Brand et al., 2004
pV1095	pV1093 vector with repairing template of Idh1	This study
pV1096	pV1093 vector with repairing template of Rom2	This study
pV1097	pV1093 vector with repairing template of Eco1	This study
pV1098	pV1093 vector with repairing template of Taf10	This study
CIp10-IDH1	Full-length *Idh1* gene in CIp10	This study
CIp10-ECO1	Full-length *Eco1* gene in CIp10	This study
CIp10-ROM2	Full-length *Rom2* gene in CIp10	This study
CIp10-TAF10	Full-length *Taf10* gene in CIp10	This study

pyrimidines) were purchased from Thermo Fisher Scientific (Waltham, MA). The drugs used for the drug tolerance work were purchased from Sigma-Aldrich, Shanghai, P. R. China.

DNA/plasmid manipulation

Plasmids for CaCas9 solo systems are listed in Table 2. We designed and ordered the 20-bp guide RNAs (gRNAs) of the respective genes (*ECO1*, *ROM2*, *IDH1*, *TAF10*) to be deleted according to Vyas et al. (2015). The gRNAs were cloned with the aid of Exnase HSE (New England Biolabs) into the purified pV1093 plasmid after its linearization with *Bm*HBI enzyme to yield pEco1-gRNA, pRom2-gRNA, pIdh1-gRNA, pTaf10-gRNA after replication in DH5α. After confirmation and verification of gRNA cloning into pV1093 via restriction digestion (*Eco*RI/*Pst*I) and sequencing, the pEco1-gRNA, pRom2-gRNA, pIdh1-gRNA, pTaf10-gRNA were digested with *Sac*I and *Kpn*I and co-transformed with their respective repair-DNAs into *E. coli* (DH5α). The positive colonies of *E. coli* having successful homologous recombination and containing repair DNA (with destruction of the PAM site and introduction of the restriction site, stop codons, and least possible modifications in the original gene sequence) were further verified with (colony) PCR, restriction analysis and sequencing. Purified plasmids containing the repair templates (5 μg) were transformed into *C. albicans* using the lithium acetate method. Repair templates were generated with 60-bp oligonucleotide primers containing a 20-bp overlap at their 3′ ends centred on the desired mutation point. Primers were extended by thermocycling with ExTaq.

Most guides were either immediately adjacent to or within 15 bp of the desired mutagenesis point. Following transformation, *C. albicans* was grown in YPD medium supplemented with 0.27 mM uridine and selected using nourseothrycin (Nat) at a concentration of 200 μg/ml. The potential *C. albicans* colonies with homozygous mutations at the desired locus were verified by restriction analysis, sequencing, complementation, and phenotypic assays. For complementation purposes, the respective genes with promoters (i.e., *ECO1*, *ROM2*, *IDH1*, *TAF10*) were cloned into the linearized (*Hind*III and

Table 3. List of primers used in this study

Gene name/orf.xx.xxxx	Primer type	Primers
ECO1/Candidate gene 01 (orf19.5053)	gRNA primers	1sgR: GCTATTTCTAGCTCTAAAACGGGTATTCCATTGATGAAATC
		1sgR: GCTATTTCTAGCTCTAAAA**C**GGGTATTCCATTGATGAAAT**C**
	Repair/edited DNA primers	1Rf:ATATCTCAAGATAAATTACTACATAACAAATACCACACAAATTTC ATC TAA TGG TAA ACC CTG CAG
		1RR:AATTCTCAATTATTAAAACATCATTATCAGTTTTATAATTCTGCA-GGGTTTACCATTAGATGAAAT
	Screening primers	ScFR1: GGAACCAAATTTGTGTGGAG
		ScRev1: AGTGGTGGATTGACAGCCAC
	Complementary primers	Forward Primer1: GTGAATTCGATATCAAGCTTTTGAGTGGCAACCCCTGATG
		Reverse Primer 1: GGGAACAAAAGCTGGGTACCAACCTGCTTTATCGCGACTC
TAFF10, 25/Candidate gene 07 (orf19.3242)	gRNA primers	7sgF: CGTAAACTATTTTTAATTTGGCGGAGACAAAGATCGACAC
		7sgR: GCTATTTCTAGCTCTAAAACGTGTCGATCTTTGTCTCCGC
	Repair/edited DNA primers	7RF:ACAGGACGACCAACAACCCCAACAATCCCAGAGCCAAACAG-CGTGATGACAAAGATCGACACTCGAG
		7RR:AGTTGATGAAGTAGCATCATTTTGAGGTTTTGCGCTATCACTC-GAGTGTCGATCTTTGTCATCACGC
	Screening primers	ScFR7: CCTAGCGTGGGTTAGTCAGG
		ScRev7: CAACAGTTTGGTGGTATGGG
	Complementary primers	Forward Primer 7: GTGAATTCGATATCAAGCTTCCTGACTTGCACCAG-CTCTC
		Reverse Primer 7: GGGAACAAAAGCTGGGTACCAATGTCACAAGTGG-GAGACG
IDH1/Candidate gene 08 (orf19.4826)	gRNA primers	8sgF: CGTAAACTATTTTTAATTTGGTTACTTTGATTCCAGGTGA
		8sgR: GCTATTTCTAGCTCTAAAACTCACCTGGAATCAAAGTAAC
	Repair/edited DNA primers	8RF: AGAAGCCGTTTTACCAAAGAAATATGGTGGCAGATACACT GT TAA TAA GAT CTC GAG GTG ATC G
		8RR:GAAAATTTGTTTGACGGAATCAGTGATTTCTTGACCAGCACG-ATCACCTCGAGATCTTATTAAC
	Screening primers	ScFR8: CCCTGGATACCAAGAGCTTA
		ScRev8: GCAGTACCACCAATATCAGC
	Complementary primers	Forward Primer 8: GTGAATTCGATATCAAGCTTCCATAAGCAGTGTATCTAGTCAGGG
		Reverse Primer 8: GGGAACAAAAGCTGGGTACCGGTTTGGTTGGTTGGTGGTG
ROM2/Candidate gene 11 (orf19.906)	gRNA primers	11sgF: CGTAAACTATTTTTAATTTGCTGAGGAACAATCCTAATAA
		11sgR: GCTATTTCTAGCTCTAAAACTTATTAGGATTGTTCCTCAGCA
	Repair/edited DNA primers	11RF: GTCGAGTAATAGTTCTTGGTCTAACAACGATTCTTACCAA CTG AGG AAC AAT CCT TAA TAA CTG CAG
		11RR:TGAGTGTTGTTGTGACATTAAATGTGGGTTATGGTTATTAC TT- GGTCTAACAACGATTCTTACCA
	Screening primers	ScFR11: GGAACCGTAAGTCCCTCTTT
		ScRev11: TTGAATTGGGGGCAAGTTGC
	Complementary primers	Forward Primer 11: GTGAATTCGATATCAAGCTTGTCACCCTTTGAATT-GGGCA
		Reverse Primer 11: GGGAACAAAAGCTGGGTACCGTTGCAATTATTGT-CGCGCG

*Kpn*I) integrating plasmid CIp10, yielding CIp10-Eco1, CIp10-Rom2, CIp10-Idh1, and CIp10-Taf10, which were used to complement the homozygous mutants of *C. albicans* for the respective genes. Following complementary transformation, *C. albicans* was grown on SDS-URA media. The complemented strains of *C. albicans* were verified by restriction analysis, PCR, sequencing, and dilution assays.

Phenotype tests

The homozygous mutant strains and the background strain (CAI4) were grown in liquid medium at 30 °C overnight, and serially diluted and spotted sequentially by 10-fold dilution onto plates as mentioned by Liu et al. (2010). Following incubation for 2–3 days, the plates were pictured. Filamentation induction media were used to assess the filamentation by inoculating the cells at 37 °C with shaking. For morphological studies of colonies, 20 cells from each strain were plated on YPD-10% serum, Lee and Spider medium (Lee et al., 1975) for 5–7 days at 37 °C.

Growth curve assay

Cells of homozygous mutants (eco1/eco1, rom2/rom2, idh1/idh1, taf10/taf10), revertant strains (eco1/eco1+CIp10-CaEco1, rom2/rom2+CIp10-CaRom2, idh1/idh1+CIp10-CaIdh1, taf10/taf10+CIp10-CaTaf10), wild-type background (CAI4), and CAI4-CIp10 were cultured on SD-URA medium overnight at 30 °C, and 10-fold diluted serially in freshly prepared YPD for further 10 h growth. Optical density (OD) of the cultured samples was measured after every 2 h at 600 nm. Data were the average of three independent experiments and analysed by GraphPad Prism software. P values of < 0.05 were considered to be significant.

Drug susceptibility and tolerance assays

We conducted drug tolerance and susceptibility assays using the solid agar-based spot method as described by Yang et al. (2017). The drugs used for this purpose and the concentrations used are mentioned in Table 4. The cells of the strains were taken out of a –80 °C freezer and

Table 4. Summary of the drug sensitivity assays conducted for the C. albicans homozygous mutants

Drugs	CA14	Ca idh1	Ca eco1	TAF10	ROM2
CFW (150 µg/ml)	–	+ +	+ +	+ –	+ –
CFW (300 µg/ml)	–	+ +	+ +	+ +	+ +
0.5 M KCl	– –	+ +	+ +	–	–
1 M KCl	–	+ +	+ +	+ +	+ +
2% DMSO	+	+ +	+ +	+ –	+ –
10% DMSO	+ +	+ +	+ +	+ +	+ +
Rapamycin 2 ng/ml	+ +	+ +	+ +	+ –	+ –
Rapamycin 5 ng/ml	+ +	+ +	+ +	+ +	+ +
CR 150 µg/ml	–	–	–	+ +	+ +
CR 300 µg/ml	–	–	–	+ +	+ +
0.05% SDS	+ +	+ +	+ +	+ +	+ +
0.1% SDS	+ +	+ +	+ +	+ +	+ +
0.2 M LiCl	– +	+ +	+ +	+ +	– +
0.4 M LiCl	+ +	+ +	+ +	+ +	+ +
0.5 M NaCl	– –	– –	– –	– –	– –
1 M NaCl	– +	+ +	+ +	+ +	– +
1 mM H_2O_2	– –	+ +	+ +	– –	– –
0.5 mM H_2O_2	– –	+ +	+ +	– –	– –
9% ethanol	+ +	+ +	+ +	+ +	+ +
Caspo 10 µg/ml	– –	– +	– –	– –	– –
1 M KCl	– –	+ +	+ +	+ +	+ +
0.5 M $MnCl_2$	– –	+ +	+ +	+ +	+ +
10% DMSO	+ +	+ +	+ +	+ +	+ +
30 Mm DTT	– –	– –	– –	– –	– –
10 mM Cap	– –	– –	– –	– –	– –

– –: no effect on growth; + +: pronounced effect on growth; + –: mildly influenced

incubated overnight at 30 °C in liquid YPD medium. Following overnight incubation, the cells of each strain were serially diluted and spotted sequentially by 10-fold dilution onto YPD agar plates containing the specified quantity of each drug. After spotting, the plates were incubated for 2–3 days at 30 °C, after which the plates were taken out of the incubator, observed and photographed.

Zymolyase and chitinase sensitivity assays

These assays were performed according to the method of Ovalle et al. (1998). The cells of all mutants and wild-type strain were grown to exponential phase followed by washing three times in 1X TE buffer. After washing, the cells were suspended in TE buffer and their OD_{600} was set at 0.9 followed by the triplicate pipetting into a 96-well plate with 0% volume 1× TE (no-enzyme control), or with 10% volume 1× TE containing 100T zymolyase (Zymo Research, Irvine, CA) to a final concentration of 50 µg/ml. We calculated the OD_{600} every 4 min for a total of 160 min at room temperature. The chitinase enzyme activity assay was performed using the chitinase enzyme from *Streptomyces griseus* (Sigma-Aldrich, St. Louis, MO), which was dissolved in 200 mM potassium phosphate buffer (pH 6) with $CaCl_2$ (2 mM) at a final concentration of 1 unit/ml of chitinase. After 3 h of incubation, OD_{600} of the grown cells were adjusted to 0.5 in phosphate buffer containing chitinase and incubated for 2 h. The OD was then taken after each 15 min independently in triplicate.

XTT Assay to analyse cell adhesion

An inoculum of each overnight incubated strain was prepared in YPD, and further incubated for 2 h at 30 °C with rotation at 250 rpm. After 2 h, the cells were resuspended in RPMI 1640 medium with uracil/L-glutamine and MOPS (morpholinepropanesulphonic acid) (165 mM) (pH 7.0) at a concentration of 1×10^7 cells/ml. Aliquots of each strain were then further incubated individually in wells of a 96-well microtitre plate (Corning, Sigma-Aldrich, St. Louis, MO) at 37 °C for 2 h without shaking. Following the designated time, non-adherent cells were flushed out by aspirating the planktonic cells with a pipette from one half of the wells, whereas adherent cells were washed with 3× PBS. The other half of unwashed wells were taken as the unwashed controls. XTT reduction assay was used to measure the metabolic activities of the washed cells and total cells, as described previously by Sardi et al. (2019). The adherence (%) was then measured by dividing the metabolic activity of the adhered cells by the metabolic activity of the total cells.

Results and Discussion

Deletion of ECO1, ROM2, IDH1, TAF10 by the CRISPR-Cas9 transient system

We employed the CRISPR-Cas9 solo system to substitute or knockout the gene-sized genomic regions in *C. albicans*. Four genes (i.e., *ECO1*, *ROM2*, *IDH1*, *TAF10*) were targeted and successfully deleted individually to obtain homozygous mutants of each gene. The CRISPR system was used with the aid of plasmid pV1093 containing functional CaCas9 as a baseline to construct vectors with sgRNAs (i.e., pEco1-gRNA, pRom2-gRNA, pIdh1-gRNA, pTaf10-gRNA), which were later used to be directed against each gene specifically (Fig. 1). The plasmids also have a 2-kb arm for homologous integration at the *ENO1* locus in addition to the *NAT* selection marker (Fig. 1). We used the sgRNA vectors of respective genes, i.e., pEco1-gRNA, pRom2-gRNA, pIdh1-gRNA, pTaf10-gRNA, to finally construct the targeting vectors (pV1097, pV1095, pV1096, and pV1098, respectively) for the deletion of each gene (i.e. *ECO1*, *ROM2*, *IDH1*, *TAF10,* respectively) following introduction of the respective repair DNAs (see Fig. 1 and Table 3) as described by Vyas et al. (2015).

The targeting vectors directed the CaCas9 activity towards 169 bp, 123 bp, 60 bp, and 162 bp downstream from the start codons of *ECO1*, *ROM2*, *IDH1*, *TAF10*, respectively. The *eco12Δ*::*NAT*, *rom22Δ*::*NAT, idh12Δ*::*NAT,* and *taf102Δ*::*NAT* constructs served as templates to create deletions of *ECO1*, *ROM2*, *IDH1*, *TAF10* genes, respectively. The *eco12Δ*::*NAT*, *idh12Δ*::*NAT, rom22Δ*::*NAT,* and *TAF102Δ*::*NAT* constructs had 80-bp arms, which were homologous to upstream or otherwise downstream sequences from the *ECO1*, *IDH1*, *ROM2*, *TAF10* coding region, respectively, and the targeted sites were directed by pEco1-gRNA, pIdh1-gRNA, pRom2-gRNA, pTaf10-gRNA guide RNAs, which were adjacent to the deletion end point of *eco1*2Δ::*NAT*, *idh12Δ*::*NAT, rom22Δ*::*NAT,* and *taf102Δ*::*NAT,* respectively. The *eco12Δ*::*NAT*, *idh12Δ*::*NAT, rom22Δ*::*NAT,* and *taf102Δ*::NAT were transformed along linearized and purified pECO1-gRNA, pIDH1-gRNA, pROM2-gRNA, pTAF10-gRNA into *C. albicans* strain CAI4 using the lithium acetate transformation method after growth on YPD + NAT plates for two days at 30 °C. The NAT-resistant transformants were selected and verified genotypically and phenotypically (Fig. 2). The genotypes of potential transformants were confirmed by restriction analysis, sequencing, and gene complementation methods. We used PCR to determine whether *CaCAS9* or the sgRNA expression cassette was detectable in genomic DNA of the transformants. A high frequency (almost 10-fold) of NAT-resistant transformant colonies was found, which were subsequently checked by PCR and restriction analysis to confirm the presence of *eco12Δ*::*NAT*, *idh12Δ*::*NAT, rom22Δ*::*NAT,* and *taf102Δ*::*NAT* constructs in the genome of the transformants.

Deletion of ECO1, ROM2, IDH1, TAF10 genes and sensitivity to salts and drugs

To understand the biological role of the *ECO1*, *ROM2*, *IDH1*, and *TAF10* genes in morphogenesis, cell wall biogenesis, pathogenesis, and calcineurin/Ca^{2+} signalling pathway, we disrupted the two alleles of each of

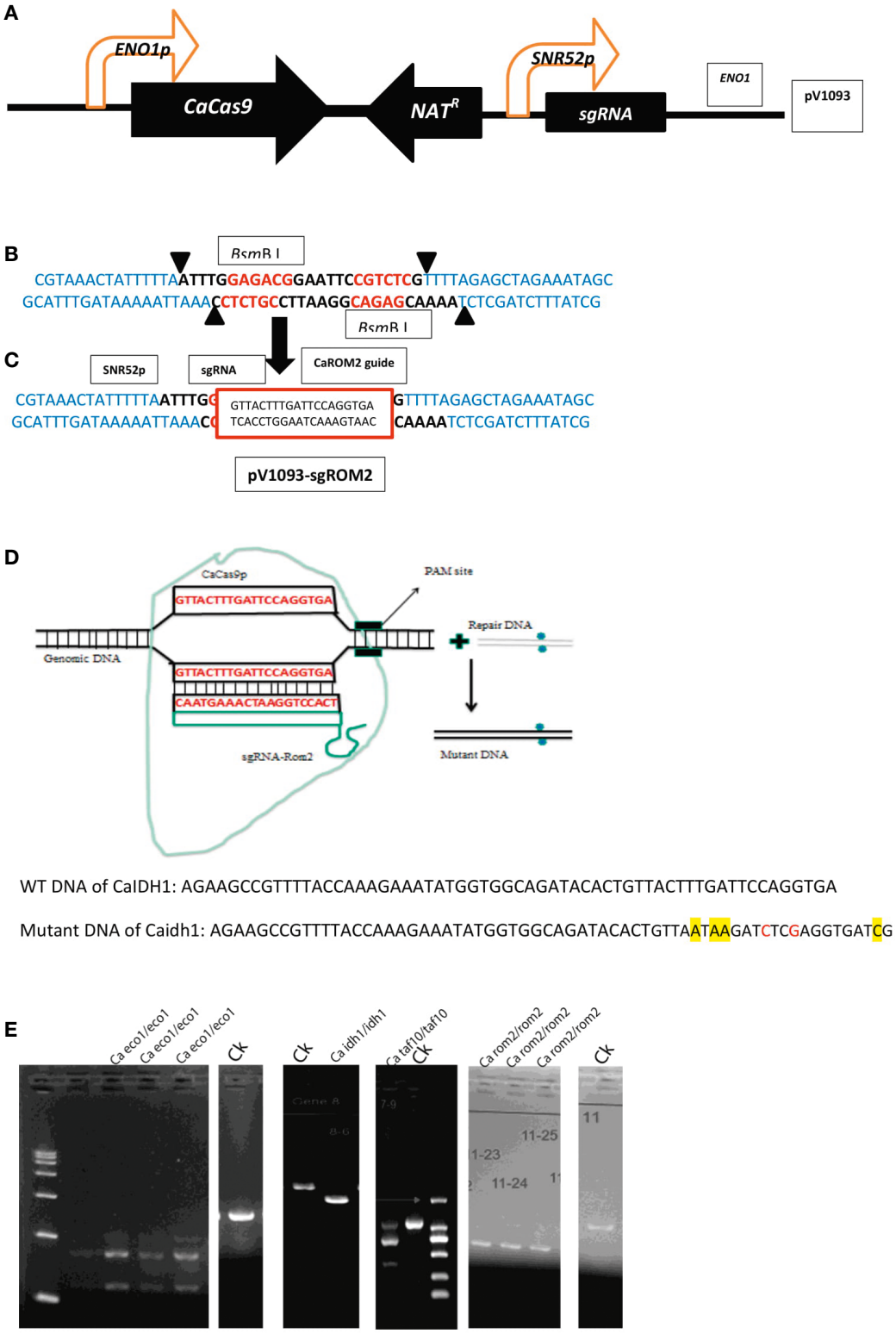

Fig. 1. **A)** The solo system consists of plasmid pV1093, which targets ENO1. The *Cas9* gene is fused to sequences encoding the 3X SV40 nuclear localization signal and 3X FLAG tag for in-frame fusion to the 3' end of the gene. The Cas9p from this construct is expressed from the constitutive ENO1 promoter at the plasmid integration site. The RNA polymerase III (Pol III) promoter SNR52p was used to express sgRNAs**. B)** The guide expression system permits rapid cloning by digestion with *Bsm*BI followed by ligation of annealed oligos (shaded sequences) with the desired guide sequence (IDH1 guide sequence in red box). **C)** Schematic of the *Cas9* mutagenesis method. The system can create homozygous mutations in the gene (*, PAM site) and simultaneously mutate sequences to prevent repeated cleavage subsequent to integration. **D)** DNA sequences of *IDH1* locus in wild-type and mutant isolates. Two consecutive stop codons are in frame within the *IDH11* ORF. A *Xho*I restriction enzyme site is introduced at the PAM region. **E)** *Pst*I/*Xho*I restriction digestion confirmation of PCR products of respective genes amplified from mutants before sequencing.

these four genes. Compared to wild-type, the homozygous mutants of *ECO1*, *ROM2*, *IDH1*, *TAF10* became more sensitive to calcium (0.2–0.6 M), lithium and cadmium (100 μM), magnesium (10 mM), sodium (1.5 M), manganese (1 mM), and potassium (1.5 M) ions (Figs. 2 and 3). All the homozygous mutants also showed reduced or no tolerability towards SDS (0.05–0.1%), ethanol (9%), DMSO (10%), DTT (30 mM), and caspofungin (10 mM). The exhibition of repressed growth by all mutants (*Sceco1, Scidh1, Scrom2,* and *Sctaf10*) in the presence of calcium ions clearly implies their involvement in the CWI calcineurin/Ca^{2+}signalling pathways.

The repressed growth in calcium salt was reversed by addition of cyclosporine (CsA) (Fig. 2). Among all the homozygous mutants, the mutants of acetyl transferase (*Sceco1*) were found to be most sensitive to all the above-mentioned ions and drugs (Table 4 and Figs. 1 and 2). This could possibly be due to the cited essential involvement of Eco1 in eleven important (sub)cellular cell cycle processes of *S. cerevisiae*, ranging from (regulation of) DNA repair/replication/organization to telomere organization, with two important functions of acetyl transferase activity and chromatin binding (Unal et al., 2007; Lyons and Morgan, 2011). Eco1 helps to establish cohesion among sister chromatids by acetylating the Smc3 subunit of cohesin during S phase, after which the levels of Eco1 were found to drop. The degradation of Eco1 is essential in the activation of Cdc4 (cell division control protein 4) and Cdk1 (cyclin-dependent kinase 1), which happens at the phosphorylation sites in Eco1. The consequent phosphorylation of Eco1 generates the phosphodegrons that network with Cdc4. Cdc4 and Cdk1 subsequently control cohesion by promoting chromosome segregation.

Nevertheless, in case of DNA damage, Eco1 can initiate cohesion by acetylation of cohesion subunit Scc1 (Unal et al., 2007; Lyons and Morgan, 2011). However, such reactivation of cohesion strongly relies on the stability of Eco1. In the case that Eco1 loses stability, the whole replication process may collapse in the damaged cells. Instead of having multiple phosphorylation sites, only two phosphorylation sites of Eco1 (i.e., Ser98 and Ser99) promote the binding of Cdc4 and Cdk1, respectively (Lyons et al., 2013). The phosphorylation of Eco1 is necessary for correct polarization of the actin cytoskeleton and bud emergence. The lack of Cdc4 or non-phosphorylation of Cdc4 with Eco1 can result in defective polarization and arrested bud formation (Schmidt et al., 1997). Lyons et al. (2013) also found that deletion of Eco1 or one of its crucial phosphorylation sites did not disturb the whole and related cell cycle processes, but could also cause increased/decreased sensitivity/tolerability towards different harsh conditions.

Among all the homozygous mutants, only *rom2* showed the least sensitivity towards the different ions (including calcium) and drugs (Fig. 3 and Table 4). Our finding is in conflict with previous findings of *ROM2* null mutants of *S. cerevisiae* (Schmidt et al., 1997). Schmidt and his colleagues reported that the *rom2* mutant of *S. cerevisiae* showed severe growth defects. The *Carom2* mutants also showed growth defects compared to the wild type (*CAI4*): having smaller colonies than the wild-type background. However, these growth defects were of a lesser degree than the growth defects of *Scrom2* mutants. Moreover, disruption of *ROM2* was found to enhance the temperature sensitivity phenotypes of *Scrom2* mutants, whereas disruption of both *ROM1* and *ROM2* resulted in lethality in *S. cerevisiae* (Ozaki et al., 1996). *Scrom2* was also found not to grow at temperatures above 40 °C and below 10 °C (data not shown). Rosenwald et al. (2016) also checked the caspofungin phenotype of *Candida galbarata* and found that *Carom2* was mildly sensitive to the drug caspofungin compared with wild type. We found that *Carom2* was also mildly sensitive to caspofungin (10 mM). Conditional *rom2* mutants of *Aspergillus fumigatus* were also noted for impaired growth and sporulation. The exposure of conditional *rom2* mutants of *Aspergillus fumigatus* towards cell wall-perturbing agents (Congo red and calcofluor white) was observed along with great sensitivity, which was reversed by addition of sorbitol (1.2 M) (Samantaray et al., 2013).

To our surprise, the *Carom2* mutant did not show any sensitivity towards Congo red (150-300μg/ml), but it was sensitive to calcofluor (300 μg/ml). We also checked the vulnerability of the mutants (*Caeco1, Caidh1, Carom2,* and *Cataf10*) towards other cell wall-perturbing agents such as SDS (0.05–0.1%), DMSO (2–10%), ethanol (2–9%), caffeine (20 μl/ml), caspofungin (20 μg/ml), fluconazole (128 μg/ml), and rapamycin (2–5 ng/ml), which were found to be repressors of *C. albicans* mutants (Figs. 1 and 2, Table 4). Phenotypes and growth patterns of *Caidh1* and *Cataf10* towards cell wall-perturbing agents were identical to each other. Previously, Lin et al. (2007) also asserted the growth phenotypes of *S. cerevisiae* lacking *IDH1*. The *Scidh1* mutants were found to be defective, with slow growth, inability to assimilate non-fermentable sugars and respiration-deficient diminutive isolated colonies. Regarding TAFs, all the TAFs are considered essential for growth in yeast barring *TAF14*, and all the yeast genes depend upon one or more *TAFs* for their growth in nutrient-rich medium. Deletion of any *TAF* including *TAF10* can repress normal growth of yeasts and make them more susceptible to salts and drugs (Sinha et al., 2017). Furthermore, revertant strains of *C. albicans* were also produced by re-introduction of *CaECO1*, Ca*IDH1*, Ca*ROM2*, and Ca*TAF10.* All the revertants moderately reinstated the growth phenotype of homozygous mutants. The colonies of mutants and revertants were somewhat smaller than those of the wild-type background on agar YPD plates.

Influence of deletion of ECO1, ROM2, IDH1, TAF10 genes on cell cycle and budding

Filamentous growth of *C. albicans* is considered to be closely related to its virulence. Therefore, to examine the influence of deletion of the *ECO1*, *ROM2*, *IDH1*,

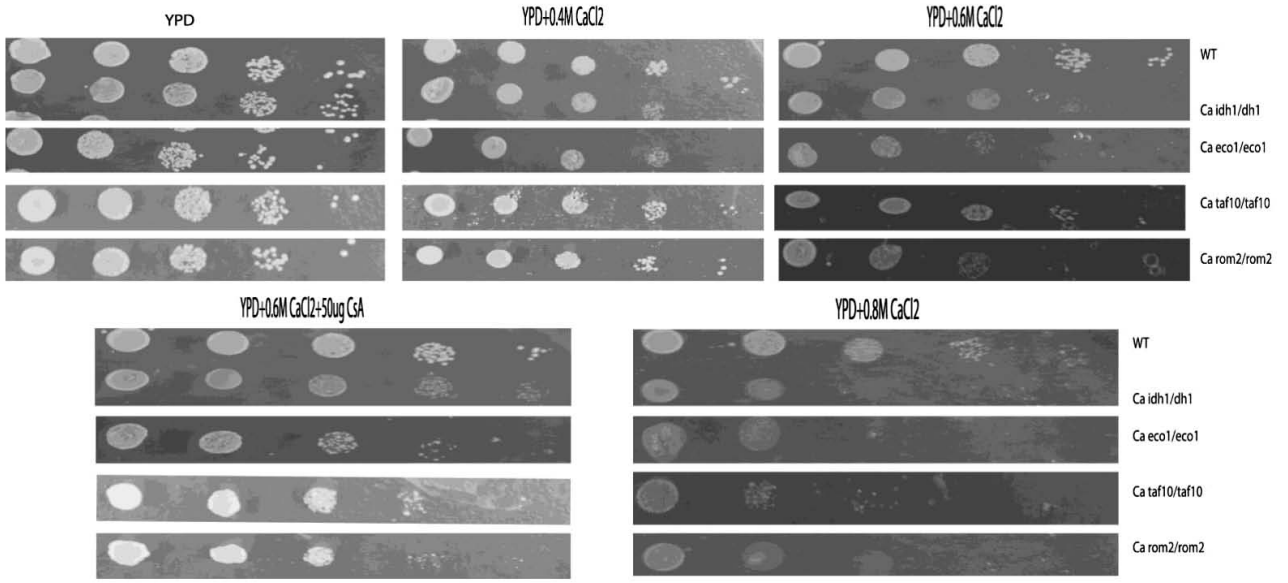

Fig. 2. Representation of the calcium-sensitive phenotypes of the wild-type CAI4 and its isogenic mutant of the respective genes in the absence or presence of 50 µg/ml CsA, respectively. Test strains were inoculated in liquid YPD and cultured overnight at 30 °C. Overnight cultures were serially diluted 10 times, and cells of 2 µl from each dilution were spotted onto plates. Plates were incubated at 30 °C for 2–3 days and photographed.

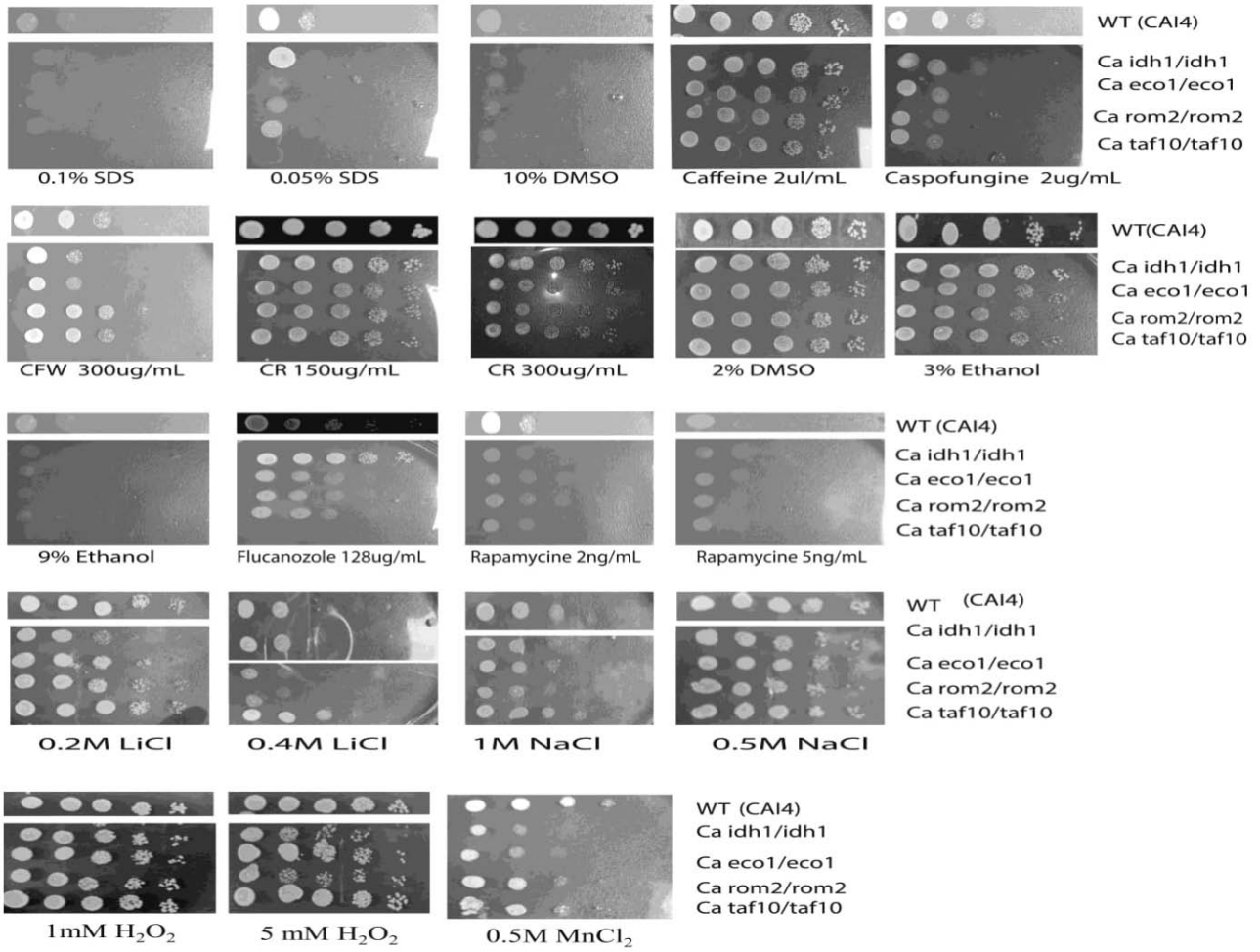

Fig. 3. Representation of the sensitivity of wild-type *C. albicans* and its isogenic mutants lacking the genes *Idh1*, *Eco1*, *Rom2*, and *Taf10*, respectively, against different concentrations of varying drugs, salts and cell wall-perturbing agents.

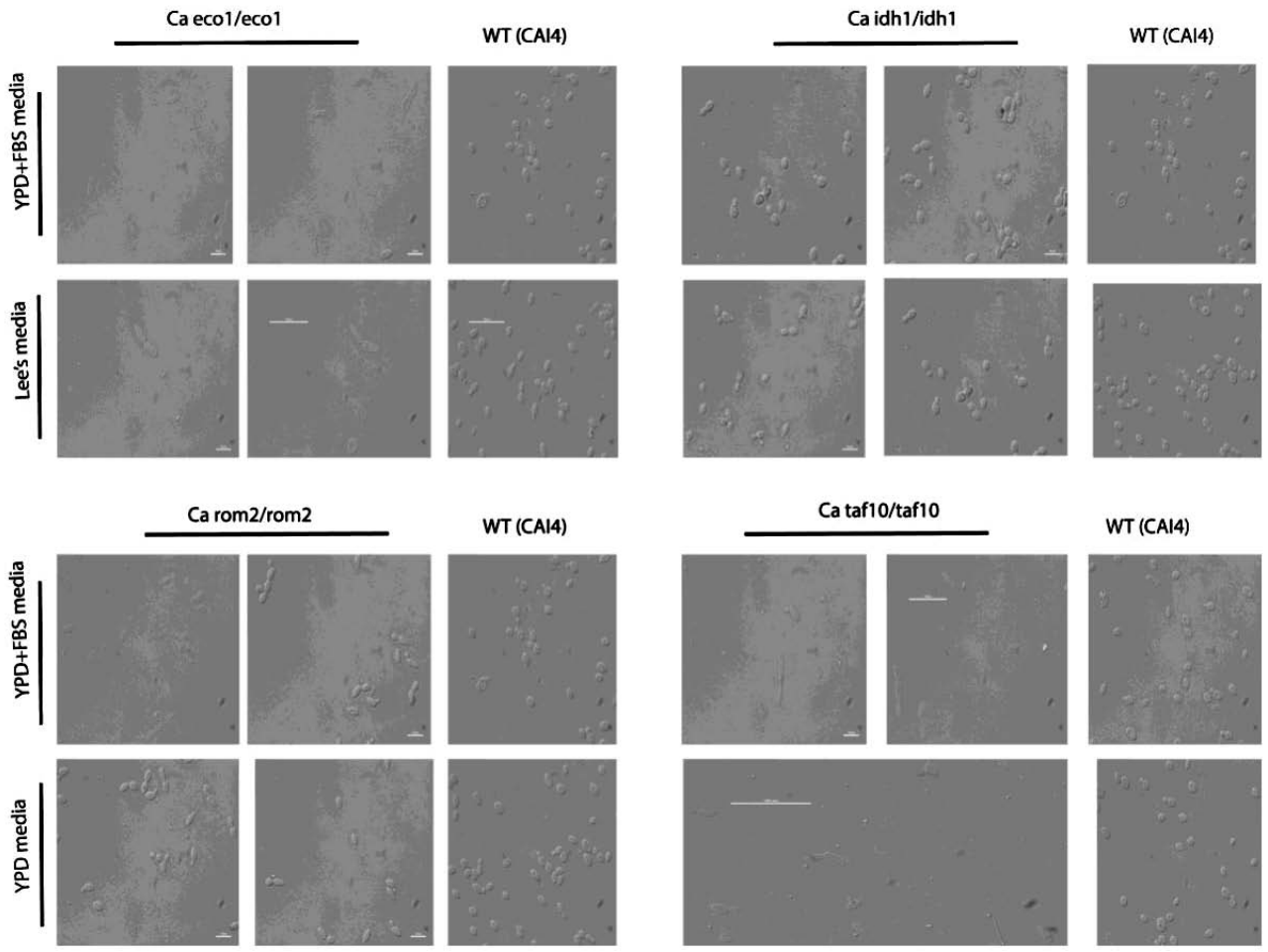

Fig. 4. Effect on the filamentation, budding index and cell cycle of *C. albicans* lacking *Idh1*, *Eco1*, *Rom2*, and *Taf10* genes. Cells were grown overnight in SD-URA medium, and inoculated to liquid YPD medium containing 10% foetal bovine serum (FBS) (**A**) and Lee's medium (**B**) for the filamentation assay. Cells were photographed after being incubated for 1.5 h and 3 h, respectively, at 37 °C.

TAF10 genes on filamentation and growth rate, we first checked the cell cycle of all mutant strains spectrophotometrically in addition to the spot assays (Fig. 4). The cell cycles of all mutant strains were found to be decreased compared to the wild-type strain except for *Carom2*. To our amazement, the cell cycle of *Carom2* mutants exceeded the control strain. Increased budding index and budding size were also seen, which we can postulate as a possible reason for the increased cell cycle of *Carom2* mutants. It is quite possible that the effect of *Carom2* deletion was suppressed by higher expression of Tor2/cAMP phosphodiesterase Pde2p or otherwise depleted protein kinase A (PKA) activity (Park et al., 2005; Yan and Kang, 2014). Additionally, the *Scrom2* mutant was also reported to have an increased budding percentage compared with that of the wild type, which suggested a putative inhibitory role of rom2 in START conversion. The budding shape of *Carom2* mutants was longitudinal or otherwise long cylindrical. The cell cycle and budding index of *Caidh1* was decreased (Fig. 4). Previously, Lu et al. (2017) reported that the reduced growth of Sc*idh1* was also associated with increased sensitivity towards DNA damaging drugs. The *eco1* deletion was found to result in chromosome compaction, reduced Smc3 acetylation and reciprocal crossing over, immeasurable sister-chromatid cohesion defects, reduced and defective growth, increased sensitivity to drugs and genotoxic stress, and inability of DNA repair.

Smc3 with reduced acetylation signalled towards less cohesive complexes, which affected DNA repair and replication. In addition, in the *Sceco1* mutant, origin firing happened not only at the origin of replication sites (ARS), but also at non-ARS sites (Lu et al., 2010). The budding shape of *Caidh1* and *Caeco1* was oval, with elongated and speared shape (Fig. 4). The growth of all the mutants displayed defective filamentation in YPD + 10% FBS media with reduced colony size on YPD agar media. Likewise, deletion of *ScIDH*1, a rate-limiting protein of the TCA cycle, also showed defective filamentation with reduced growth (Tao et al., 2017). The main reason was that TCA cycle/*idh1* mutants displayed reduced intracellular ATP- and GTP-bound Ras1 pro-

tein. The Ras1 protein is correlated with the ATP level and needs ATP for its activation. The activation of Ras1 protein activates the Ras1-cAMP pathway, which is associated with hyphal growth and CO_2 sensing. The reduced level of ATP in the TCA cycle/*idh1* mutants downregulates the Ras1-cAMP pathway, resulting in reduced and defective hyphal growth (Tao et al., 2017).

Sensitivity to zymolyase, chitinase treatment and cell adhesion

The cell wall integrity was found to be influenced by zymolyase and chitinase enzymes, which have β-1,3-glucanase or otherwise residual protease and chitinase activities, respectively. We employed different concentrations of zymolyase to explore whether the β-1,3-glucanase variably affected cell wall damage in the homozygous mutants and wild-type strains. Figure 5A clearly demonstrates that zymolyase decreased the cell survival of all mutants. Cells lacking Rom2 were found to be substantially more vulnerable to zymolyase enzymatic activity than other mutants and the wild-type background. Among other mutants, *Cataf10* showed the least vulnerability towards the cell wall disruption by zymolyase. *Caidh1* and *Caeco1* were also sensitive to zymolyase, but their sensitivity towards the zymolyase enzyme was found to be intermediate between those of *Carom2* and the wild type. These results suggested that deletion of *ROM2* increased the fragility of *rom2* mutants, depicting the vital role of Rom2 in the CWI pathways. For the chitinase activity assay, the mutant and wild-type strains were incubated for 120 min in YPD medium + designated amount of chitinase enzyme (Fig. 5B). Optical density (OD) of the treated cells of all strains was measured spectrophotometrically at 600 nm during different time intervals from 0 to 120 min, and the drop in OD was synonymous to degradation of the cell wall. OD measurements of all mutants were remarkably lower than the background strain (control) grown in YPD + chitinase medium after 0.5 h. The mutant strains were more prone to cell lysis, due to which the OD of mutant cells declined even after 0.25 h of treatment. Among the mutants, *Carom2* displayed the most significant cell wall lysis and degradation pattern of all the mutants. The OD of *taf10* and *idh1* mutants were less than that of the control, but differed non-significantly from each other. The degradation process of cell wall chitin of *eco1* mutants was somewhat similar to that of cells lacking *TAF0* or *IDH1* until 1 h, after which a sudden drop in the OD of *eco1* mutants was observed.

Cell adhesion

Cell adhesion is an important phenotype of *C. albicans*, which is related to its pathogenesis and virulence,

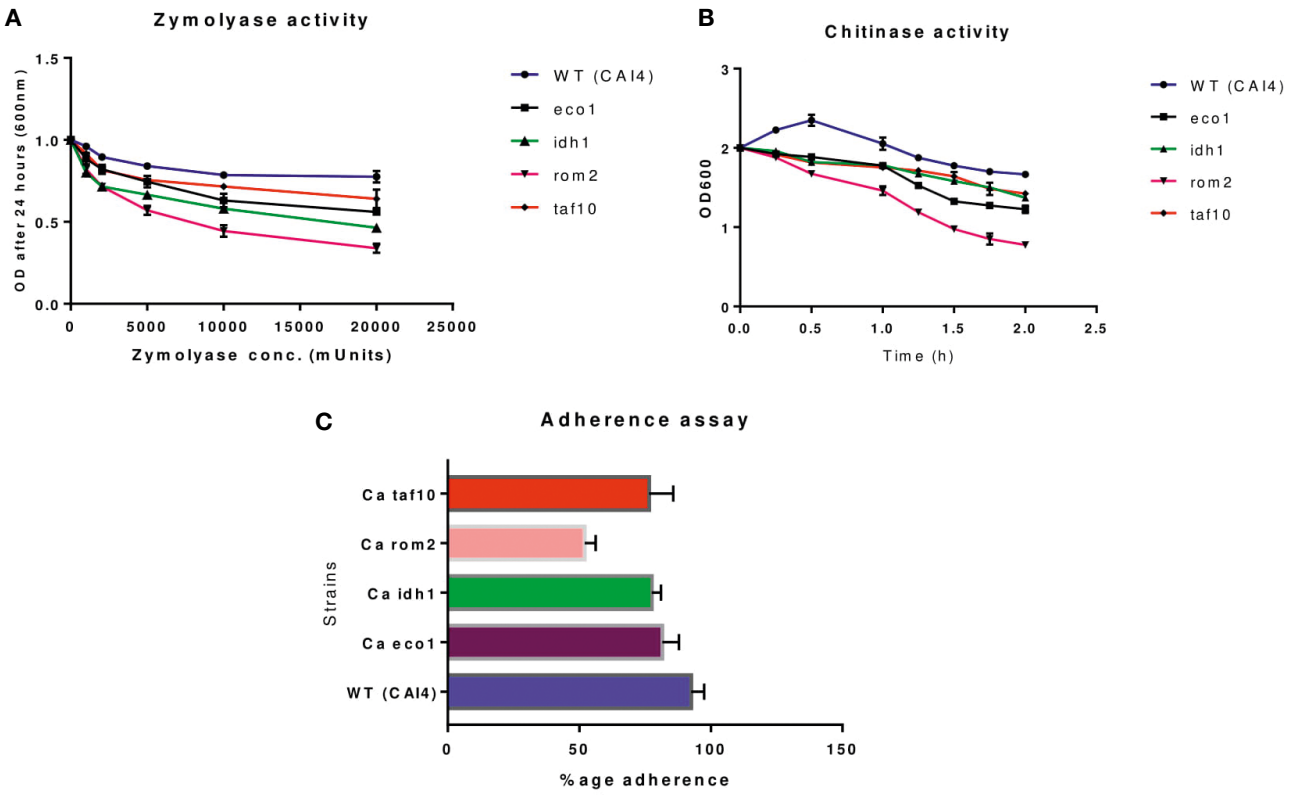

Fig. 5. **A)** Representation of the cell wall vulnerability of all mutants and wall types against the zymolyase enzymatic activity. **B)** Representation of the cell wall vulnerability of all mutants and wall types against the chitinase enzymatic activity. **C)** Representation of the influence of the gene deletion on the adherence ability of all mutants.

and we therefore also evaluated the cell adhesion of all strains in RPMI 1640 media at pH 7.0. The adhesion assay was carried out in stationary phase, as the adhesion ability is found to vary according to the growth phase, with highest adhesion during the stationary phase. The deletion of genes also variably influenced the adhering ability of all mutants (Fig. 5C). All the mutants showed reduced adherence to polystyrene surfaces in comparison to the wild-type strain. The *rom2* mutant of *C. albicans* showed the least adherence value out of other mutants, whereas the adhesiveness of *Cataf10* and *Caidh1* was non-significantly different from each other. It was clear that *Cataf10* and *Caidh1* mutants also showed non-significantly differing behaviour in the chitinase activity assay. The cells with deleted *ECO1* were similar to the wild-type strain in maintaining the adherence ability. In summary, *rom2* mutants were the most affected strains with respect to cell wall degradation, higher susceptibility, and least virulence potency followed by *Caeco1*.

Conclusion

CRISPR-Cas9 successfully created homozygous mutants for genes *ECO1*, *IDH1*, *ROM2*, and *TAF10* in *Candida albicans* CAI4. The strains lacking the genes (*Caeco1*, *Caidh1*, *Carom2*, and *Cataf10*) showed higher susceptibility towards different salts (including calcium), cell wall-perturbing chemicals and drugs. Phenotypic assays showed that *Caeco1* was the most vulnerable mutant, while *Carom2* exhibited the least sensitivity towards the above-mentioned salts and drugs. Deletion of the selected genes also resulted in repressed growth, decreased cell cycle and colony size (except for *Carom2*), altered budding shape, and increased budding index (except for *Caeco1*). Zymolyase and chitinase assays revealed higher fragility of cell walls of the mutant strains, especially in the case of *Carom2*, due to which these stains underwent more extensive and more rapid cell-wall-lysis events. The observed pathogenicity, and hence virulence potential, of the newly constructed strains was much lower than that of the wild-type background strain. We can therefore deduce that these genes, especially *ROM2* and *ECO1*, regulate homeostasis of the cell wall integrity/biogenesis, calcium, drugs, salts, filamentation, and virulence of pathogenic *C. albicans.* Further work is needed to clearly identify *Caeco1*, *Caidh1*, *Carom2*, and *Cataf10* as potential antifungal drug targets.

Conflict of interest

Authors declare no competing interests.

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Author contributions

A. H.[#] and S. A. H.[#] are considered equal first authors for this manuscript. A. H. and S. A. H. performed all the experimental work. S. U. and A. B. F. helped in spot assays and plasmid preparation. M. U. I. helped in the sequencing data interpretation. A. H. wrote this manuscript. M. U. edited and reviewed the whole manuscript and provided suggestions to the main authors about overall research and research plan. All authors read and approved the final manuscript.

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