

Distinct and Redundant Roles of the Two MYST Histone Acetyltransferases Esa1 and Sas2 in Cell Growth and Morphogenesis of *Candida albicans*

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Candida albicans is associated with humans, as both a harmless commensal organism and a pathogen. Adaption to human body temperature is extremely important for its growth and morphogenesis. *Saccharomyces cerevisiae* Esa1, a member of the MYST family HATs (histone acetyltransferases) and the catalytic subunit of the NuA4 complex, and its homologues in other eukaryotes have been shown to be essential for cell growth. To investigate the functional roles of two MYST family HATs, Esa1 and Sas2 in *C. albicans*, we deleted *ESA1* and *SAS2* in the *C. albicans* genome and performed cell growth analyses. Our results demonstrated that *C. albicans* Esa1 is not essential for general growth but is essential for filamentous growth. The *esa1/esa1* mutant cells exhibited sensitivity to thermal, genotoxic, and oxidative stresses but tolerance to cold, osmotic, and cell wall stresses. In contrast, the *sas2/sas2* mutant adapted to growth at higher temperatures and promoted filament formation at lower temperatures, resembling the phenotype of a *C. albicans* strain overexpressing *ESA1*. Cells with deletions of both *ESA1* and *SAS2* were inviable, reflecting the functional redundancy in cell growth. *C. albicans* Esa1 and Sas2 have distinct and synergistic effects on histone acetylation at H4K5, H4K12, and H4K16. Esa1 contributes mainly to acetylation of H4K5 and H4K12, whereas Sas2 contributes to acetylation of H4K16. Our findings suggest that *C. albicans* Esa1 and Sas2 play opposite roles in cell growth and morphogenesis and contribute coordinately to histone acetylation and gene regulation.

The MYST proteins, which represent the largest family of HATs (histone acetyltransferases) (1) and are named for the founding members MOZ (2), Ybf2/Sas3 (3), Sas2 (4), and TIP60 (5), are conserved from yeasts to humans and are involved in diverse biological functions, including gene regulation, DNA repair, cell cycle regulation, and development (6). Esa1 (Essential SAS family acetyltransferase, also called KAT5), the human TIP60 orthologue in *Saccharomyces cerevisiae*, is essential for cell growth. Inactivation of the Esa1 HAT activity renders the yeast temperature sensitive (37°C) and leads to abnormal cell morphology (7, 8). Moreover, Esa1 is required for transcriptional silencing at the rDNA locus (9). As the catalytic subunit of the NuA4 HAT complex, Esa1 is involved in acetylation of four conserved lysines of the histone H4 N-terminal tail (lysines 5, 8, 12, and 16) (10). Furthermore, Esa1 is involved in acetylation of nonhistone substrates and contributes to the regulation of metabolism and stress response (11). Recently, Esa1 has been shown to function as a lysine acetyltransferase (KAT) required for autophagy via acetylation of the autophagy signaling component Atg3 (12).

Another MYST family member in yeast, Sas2 (Something About Silencing, also called KAT8), is the catalytic subunit of the SAS HAT complex (Sas2p-Sas4p-Sas5p) and can acetylate both free histones and nucleosomes and is involved in regulation of transcriptional silencing (13, 14). Sas2 is mainly required for acetylation of histone H4 at lysine 16 (H4K16) and plays an important role in maintaining the boundary of telomeric heterochromatin by opposing effects of Sir2 on deacetylation of H4K16Ac. Sas2 and Sir2 function in a concerted manner with transcriptional regulation in yeast, by acetylating and deacetylating H4K16 via a mechanism that may be common to all eukaryotes (15, 16). The human MYST family member MOF (Males Absent on the First), exists in at least two distinct complexes, MOF-MSL and MOF-MSL1v1,

which have indistinguishable activities on H4K16 but differ dramatically in acetylating the nonhistone substrate p53 (17).

The MYST proteins have well-established roles on histone acetylation; however, their substrate spectrum is broader, and new nonhistone protein substrates are continuously being discovered (18). Other typical substrates of the MYST enzymes are components of the multisubunit MYST complexes, including the MYST proteins themselves (6). Discovering novel substrates of MYST proteins is pivotal for understanding the diverse functions of these essential acetyltransferase in nuclear processes, signaling, stress response, and metabolism.

Candida albicans is a common fungal pathogen. It colonizes skin and mucosal surfaces of the majority of healthy individuals in the human population but can cause various forms of candidiasis, ranging from superficial mucosal infections to life-threatening systemic diseases in immunocompromised patients (19, 20). The ability of *C. albicans* to undergo morphological transition to survive in different human niches contributes to its pathogenicity and adaptability. So far, the biological functions of the MYST proteins in *C. albicans* have not been studied, although the components of the NuA4 and SAS complexes have been predicted and sequenced. We previously reported that Yng2, a component of the

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
SC5314	Wild type	24
CAI4	<i>ura3::λ imm434/ura3::λ imm434</i>	24
BWP17	<i>ura3::λ imm434/ura3::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>	23
CWX1	<i>ura3::λ imm434/ura3::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG ESA1/esa1::HIS1</i>	This study
CWX2	<i>ura3::λ imm434/ura3::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG esa1::HIS1/esa1::hisG-URA3-hisG</i>	This study
CWX3	<i>ura3::λ imm434/ura3::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG esa1::HIS1/esa1::hisG</i>	This study
CWX4	<i>ura3::λ imm434/ura3::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG SAS2/sas2::HIS1</i>	This study
CWX5	<i>ura3::λ imm434/ura3::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG sas2::HIS1/sas2::hisG-URA3-hisG</i>	This study
CWX6	<i>ura3::λ imm434/ura3::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG sas2::HIS1/sas2::hisG</i>	This study
CWX7	<i>ura3::λ imm434/ura3::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG esa1::ARG4 Ptet-ESA1::URA3</i>	This study
CWX8	<i>ura3::λ imm434/ura3::λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG sas2::HIS1/sas2::hisG-ura3-hisG esa1::ARG4 Ptet-ESA1::URA3</i>	This study
Plasmids		
pCUB6	Replacement of <i>S. cerevisiae</i> URA3 by <i>C. albicans</i> URA3 in pNKY50	24
pESA1-KO	0.53- and 0.6-kb KO fragments of <i>C. albicans</i> ESA1 and <i>hisG-URA3-hisG</i> in pBluescript SK(+)	This study
pSAS2-KO	0.55- and 0.5-kb KO fragments of <i>C. albicans</i> SAS2 and <i>hisG-URA3-hisG</i> in pBluescript SK(+)	This study
pBES116	<i>C. albicans</i> URA3 vector for integration at ADE2 locus	53
pBES116-ESA1	2.18-kb CaESA1 in pBES116 for ESA1 expression under control of ESA1 promoter	This study
pBA1	<i>C. albicans</i> ADH1 promoter in BES116	25
pBA1-WX1 (pBA1-ESA1)	1.56-kb full-length CaESA1 in pBA1 for ESA1 expression under control of ADH1 promoter	This study
pBA1-WX2	1.2 kb of CaESA1 with deletion of chromo domain in pBA1	This study
pBA1-WX3	0.9 kb of CaESA1 with deletion of HAT domain in pBA1	This study
pBA1-WX4	1.3 kb of CaESA1 with deletion of insertion sequence in pBA1	This study
pBA1-WX5	1.45-kb full-length ScESA1 in pBA1	This study
pBA1-WX6	ScESA1 G315E mutation in pBA1	This study
pBA1-WX7	ScESA1 L327S mutation in pBA1	This study
pBA1-WX8	1.45-kb full-length human TIP60 cDNA in pBA1	This study
pBA1-WX9	Hybrid of CaEsa1 chromo domain and hTIP60 MYST domain	This study
pBA1-WX10	Hybrid of hTIP60 chromo domain and CaEsa1 MYST domain	This study
p671	<i>C. albicans</i> ACT1 promoter, 13×myc-FLAG1, HIS1	25
p671-ARG4	HIS1-to-ARG4 substitution in p671	This study
pCaUME6-3	Supplies ADH1p-cartTA-GAL4AD fragment	54
pCPC39	Supplies tet-on promoter and URA3 marker	This study
pCPC42	Supplies tet-off promoter and URA3 marker	This study
pCPC97	Infusion of pUC18 and fragment containing CaESA1 (bp -521 to +1979)	This study
pCPC98 (pPtet-ESA1)	Infusion of pCPC97 and pCPC42 for doxycycline-controlled ESA1 expression	This study

Piccolo NuA4, plays an important role in morphogenesis of *C. albicans* (21). In this study, we report the characterization of two MYST proteins in *C. albicans*, namely, *C. albicans* Esa1 (CaEsa1), the catalytic subunit of the NuA4 complex, and *C. albicans* Sas2 (CaSas2), the catalytic subunit of the SAS complex. We demonstrate that CaEsa1 and CaSas2 have distinct and redundant effects on histone H4 acetylation and cell growth. In addition, we describe their functional roles in morphogenesis, stress response, and other cellular processes of *C. albicans*.

MATERIALS AND METHODS

Strains and culture conditions. The *C. albicans* strains used in this study are listed in Table 1. Yeast strains were routinely grown on YPD medium (1% yeast extract, 2% peptone, and 2% glucose). Synthetic complete media with different carbon sources were used for the growth assay. YPD plus 10% bovine serum was used for hyphal induction. YPS medium (1% yeast extract, 2% peptone, and 2% sucrose) with 1% agar was used for the colony morphology assay under embedded conditions (22).

Plasmids and strain constructions. SC5314 genomic DNA was used as the template for PCR amplification of all *C. albicans* genes. All constructs were verified by DNA sequencing. The plasmids used in this study are listed in Table 1. The primers used for PCR amplification are listed in Table S1 of the supplemental material.

For construction of the *esa1/esa1* mutant, the first copy of *ESA1* was deleted by PCR-based homologous recombination (23). *C. albicans* *HIS1* was amplified from plasmids pGEM-HIS1 and substituted for *ESA1* in *C. albicans* parent strain BWP17. The second copy of *ESA1* was disrupted by the “URA-blaster” method (24). PstI-digested pESA1-KO was transformed into the *ESA1/esa1* mutant to generate the *esa1/esa1* mutant (CWX2) (see Fig. S1A in the supplemental material). The same strategy was used for construction of the *sas2/sas2* mutant. The first copy of *SAS2* was deleted by the PCR-based method, and the second copy of *SAS2* was disrupted by the URA-blaster method. A PstI-linearized pSAS2 knockout (pSAS2-KO) was introduced into the *SAS2/sas2* mutant to produce the *sas2/sas2* mutant (CWX5) (see Fig. S1B). All constructs were verified by PCR analysis (see Fig. S1C and D). To generate the *sas2 esa1* double mutant, the first copy of *ESA1* in the *sas2/sas2* mutant was deleted by replac-

ing *ESA1* with *ARG4* by using a PCR-based knockout strategy, and the second copy of *ESA1* was replaced by a PCR fragment amplified from pCPC98 (pTet-*ESA1*), in which *ESA1* expression is under doxycycline control (CWX8).

pESA1-KO for knockout of *ESA1*, pSAS2-KO for knockout of *SAS2*, and pCPC98 (pTet-*ESA1*) for doxycycline-controlled *C. albicans* *ESA1* expression are described in the supplemental material. pBA1-WX1 (pBA1-*ESA1*) to pBA1-WX10 were constructed for the ectopic expression of genes under the control of the *ADH1* promoter (see the supplemental material).

Western blot analysis. Protein extraction and immunoblotting were performed as described previously (25). *C. albicans* cells were grown in YPD and harvested and resuspended in 0.5 ml of lysis buffer (10 mM Tris-HCl [pH 8.0], 250 mM NaCl, 0.1% NP-40, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 0.5 g/ml leupeptin, 1.4 g/ml pepstatin, 2.4 μ g/ml chymostatin, and 17 μ g/ml aprotinin). Proteins were separated by 7.5% SDS-PAGE, transferred to a polyvinylidenedifluoride membrane and then subjected to Western blotting. Histone H4 acetylation levels were analyzed by using peroxidase-conjugated antibodies that specifically recognized unacetylated H4 (Sigma), acetylated H4 (H4Ac), acetylated H4 at lysine 5 (H4K5Ac), lysine 12 (H4K12Ac), and lysine 16 (H4K16Ac) (Abcam).

Quantitative reverse transcription-PCR (RT-PCR) analysis. RNA extraction was performed as described by Lane et al. (26). Total RNA was reverse transcribed to cDNA with the ReverTra Ace qPCR RT kit from Toyobo. Quantitative PCR was performed on a StepOne qPCR instrument (Applied Biosystems) with a SYBR green master mix containing Fast Start *Taq* DNA polymerase (Roche) and SYBR green nucleic acid stain (Roche). Transcription levels of genes in different samples were normalized against the levels of *ACT1*. The data were measured in three independent experiments.

Stress response assay. The susceptibilities of the mutants to hydroxyurea (HU), methyl methanesulfonate (MMS), camptothecin (CPT), and H₂O₂ were tested by using cells grown in YPD medium at room temperature (22°C) to exponential phase. Cells were serially diluted 10-fold, and 5 μ l was spotted onto a YPD plate with or without 10 mM hydroxyurea, 0.01% methyl methanesulfonate, 15 μ g/ml camptothecin, or 2 mM hydrogen peroxide and then incubated at room temperature for 3 days. For analysis of resistance of the mutants to NaCl (5 M), Congo red (100 μ g/ml), caspofungin (0.32 μ g/ml), nonfermentable carbon sources (2% glucose, 2% maltose, 2% ethanol, or 2% glycerol), and thermal stress (42°C or 45°C), cells grown in YPD medium at room temperature to exponential phase (optical density at 600 nm [OD₆₀₀], 0.6 to 0.8) were serially diluted in YPD, YP, or synthetic complete medium (SC) plates containing the appropriate supplements and examined after incubation for 56 h at room temperature or the temperatures specified.

Growth rate analysis. For analysis of the cell growth rate above 10°C, the doubling time was determined by measuring OD₆₀₀ values of growing cells. Overnight cultures were reinoculated into fresh liquid YPD and grown at the specified temperature, and OD₆₀₀ values were measured during the log phase at various time points and used for calculation of generation times. The data were measured in three independent experiments. The growth curves, with doubling times in minutes, were drawn using the Origin 6.1 software (MicroCal) or sigmaplot10 for data statistical analysis. For analysis of the cell growth rate at 4°C, the doubling time was determined by a method used for a yeast replicative life span assay (27). Strains were patched onto fresh solid medium and grown for 2 days at 25°C. Cells were arrayed onto YPD plates by using a micromanipulator, allowed to grow for 3 h at 25°C, and then incubated at 4°C. Virgin daughter cells were isolated as buds from mother cells and subjected to life span analysis. The doubling time was calculated between generations. Each experiment consisted of more than 10 mother cells and was independently repeated at least twice.

RESULTS

***Candida albicans* Esa1 plays a role in cell growth in sensing temperature.** To identify the MYST family members of *C. albicans*, we searched the *C. albicans* genome database (<http://www.candidagenome.org/>) and found a protein designated Esa1 (orf19.5416), which shares the highest similarity with *S. cerevisiae* Esa1 (ScEsa1), and a protein designated Sas2 (orf19.2087), which shares the highest similarity with *S. cerevisiae* Sas2 (ScSas2). Compared with ScEsa1, *C. albicans* Esa1 (CaEsa1) contains a MYST domain at the C terminus with 84% identity and a chromo domain (CHD) at the N terminus with 61% identity. However, there is an insertion sequence between residues 402 to 458 in the MYST domain of CaEsa1 (Fig. 1A).

Esa1 and its homologues have been shown to be essential in all eukaryotes examined, including *S. cerevisiae* (7, 8), *Aspergillus nidulans* (28), *Schizosaccharomyces pombe* (29), *Drosophila melanogaster* (30), and *Mus musculus* (31). To examine whether *C. albicans* *ESA1* is also essential for cell growth, we knocked out the *ESA1* gene in *C. albicans* by using a combined PCR-based and “URA-blaster” method. Surprisingly, the mutant cells with deletion of both copies of *ESA1* were still viable (see Fig. S1A and C in the supplemental material), indicating that CaEsa1 is not essential for general growth. Furthermore, we found that the *esa1/esa1* mutant strains could grow on YPD plates in the cold room (4°C) with a doubling time of 486 min, in contrast to the wild-type cells, which entered into the quiescent stage (phase) and stopped growing in the cold room (4°C). On the other hand, the *esa1/esa1* mutant cells stopped growing at the high temperature (37°C) (Fig. 1B). These unusual phenomena could be attributed to the *ESA1* deletion as reintroducing *ESA1* back into its own locus under the control of the *ESA1* endogenous promoter and rescuing the wild-type phenotype. The *ESA1/esa1* heterozygote showed a similar phenotype to the revertant (Fig. 1B). These results indicated that deletion of *C. albicans* *ESA1* caused reduced cell growth at high temperatures (>25°C) but increased growth at low temperatures (<25°C), suggesting that CaEsa1 is required for cell survival at high temperatures (37°C) and for cell quiescence at cold temperatures (4°C).

S. cerevisiae Esa1 is a HAT required for H4 and H2A acetylation *in vivo* (7, 10, 32, 33). To test if CaEsa1 exhibited HAT activity, we performed Western blot analysis that detected acetylation levels of histone H4 in cells lacking *ESA1*. At all temperatures examined, the *esa1/esa1* mutant cells exhibited a significantly decreased overall level of H4 acetylation. In particular, the acetylation levels of H4K5 and H4K12 were dramatically reduced, but that of H4K16 was not (Fig. 1C), suggesting that CaEsa1 is required for H4 acetylation at H4K5 and H4K12 independent of the growth temperature.

***C. albicans* Esa1 is a functional homologue of ScEsa1 and hTIP60.** The MYST domain of CaEsa1 shares a high sequence identity with that of ScEsa1 (84% identity) and human TIP60 (hTIP60; 79% identity), and its N-terminal chromo domain shares relatively low sequence identity with that of ScEsa1 (61% identity) and hTIP60 (52% identity). In addition, CaEsa1 contains an insertion sequence (residues 402 to 458) in the MYST domain. To determine whether CaEsa1 is a functional homologue (orthologue) of ScEsa1 or hTIP60, we examined the abilities of ScEsa1 and hTIP60 to complement the growth defect in the *Caesa1* mutant cells. We constructed a series of ectopic expression vectors for

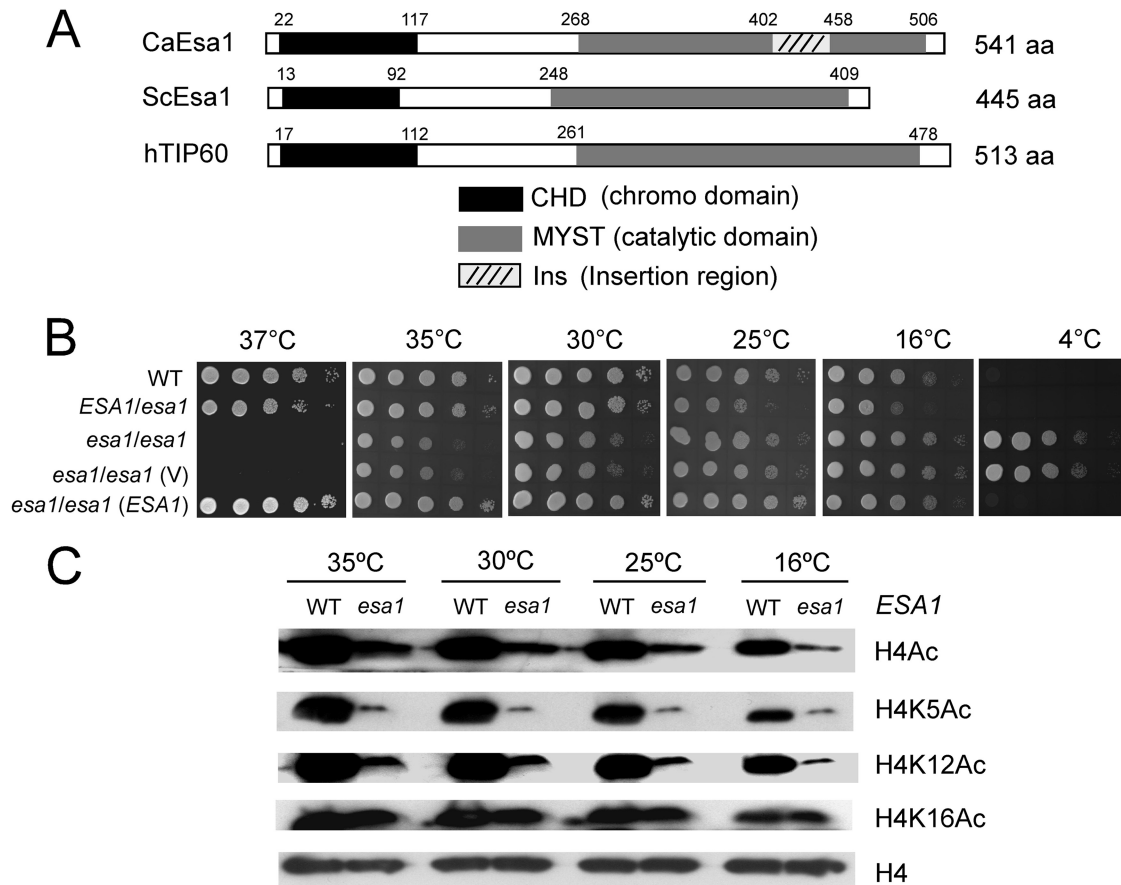


FIG 1 *Candida albicans* open reading frame 19.5416 encodes a conserved MYST protein, Esa1, that plays a role in cell growth. (A) Schematic depiction of the MYST domains and chromo domains in *C. albicans* Esa1, *S. cerevisiae* Esa1, and human TIP60. (B) Growth of *esa1/esa1* mutant strains on YPD plates at different temperatures. Strains are wild type (WT; BWP17/pBES116), *ESA1/esa1* (CWX1/pBES116), *esa1/esa1* (CWX2), *esa1/esa1* (V) (CWX3/pBES116), and *esa1/esa1* (*ESA1*) (CWX3/pBES116-*ESA1*). The cells were spotted onto YPD plates and incubated at 37°C and 35°C for 2 days, at 30°C and 25°C for 3 days, or at 16°C for 5 days and at 4°C for 2 weeks. (C) Western analyses of histone H4 acetylation levels in the wild type and the *esa1/esa1* mutant. Cells were grown in liquid YPD at 35°C, 30°C, 25°C, or 16°C and harvested for Western analysis with antibodies that specifically recognized acetylated H4 (H4Ac), H4 acetylated at lysine residues 5 (H4K5Ac), 12 (H4K12Ac), 16 (H4K16Ac) (all from Abcam), and histone H4 (Sigma).

functional complementary assays (Fig. 2A; Table 1). ScESA1 and hTIP60 were introduced into *C. albicans* and expressed under the control of the *ADH1* promoter. At 37°C, the ectopically expressed ScESA1 and hTIP60 suppressed the lethal phenotype of the *Caesa1* mutant (Fig. 2B, left panel); at 4°C, they suppressed the cold-resistant phenotype of the *Caesa1* mutant (Fig. 2B, right panel). These results together showed that both ScESA1 and hTIP60 can rescue the growth defect of the *Caesa1* mutant cells at high temperature and suppress the abnormal growth at low temperature (Fig. 2B), indicating that CaEsa1 is a functional homologue of ScEsa1 and hTIP60.

We further analyzed the contributions of the chromo domain, the MYST domain, and the insertion sequence of CaEsa1 to cell growth. As shown in Fig. 2B, deletion of the CHD or MYST domain failed to rescue the phenotype of the *Caesa1* mutant, but deletion of the insertion sequence had no effect on cell growth, suggesting that both the CHD and MYST domain are required for CaEsa1 function. It has been shown that two *S. cerevisiae* variants containing mutant ScEsa1 (G315E and L327S) lacking HAT activity are temperature sensitive (37°C) (7, 8). As expected, our complementation assay results showed that neither the *Scesa1* G315E

mutant nor the *Scesa1* L327S mutant could rescue the growth defect of the *Caesa1* mutant (Fig. 2B, lower panels).

Unlike the MYST domain, the CHDs of ScEsa1, CaEsa1, and TIP60 are less conserved. The CHDs have been considered to mediate chromatin interactions in a variety of different protein contexts. The CHDs can also mediate interactions with histones, DNA, and RNA (34). Interaction of the TIP60 CHD with the trimethylated H3K9 (H3K9me3) links DNA damage detection to activation of Tip60 (35). Considering the low sequence similarity between the CHDs, we constructed two hybrid proteins to exchange the CHD and MYST domain of CaEsa1 with those of TIP60, and we tested their effects on cell growth. Both hybrid proteins suppressed the growth deficiency of the *Caesa1* mutant (Fig. 2B, lower panels), albeit not to the same extent. The TIP60-MYST domain fully complemented the CaEsa1 MYST domain, whereas TIP60-CHD partially complemented the CaEsa1 CHD at 37°C, reflecting a functional diversity of the chromo domains which may add an additional layer to their functional regulation.

***C. albicans* Esa1 is essential for filamentous growth.** Esa1 is the catalytic subunit of the NuA4 HAT complex, which acetylates histones H4 and H2A. In *S. cerevisiae*, among the three subunits of

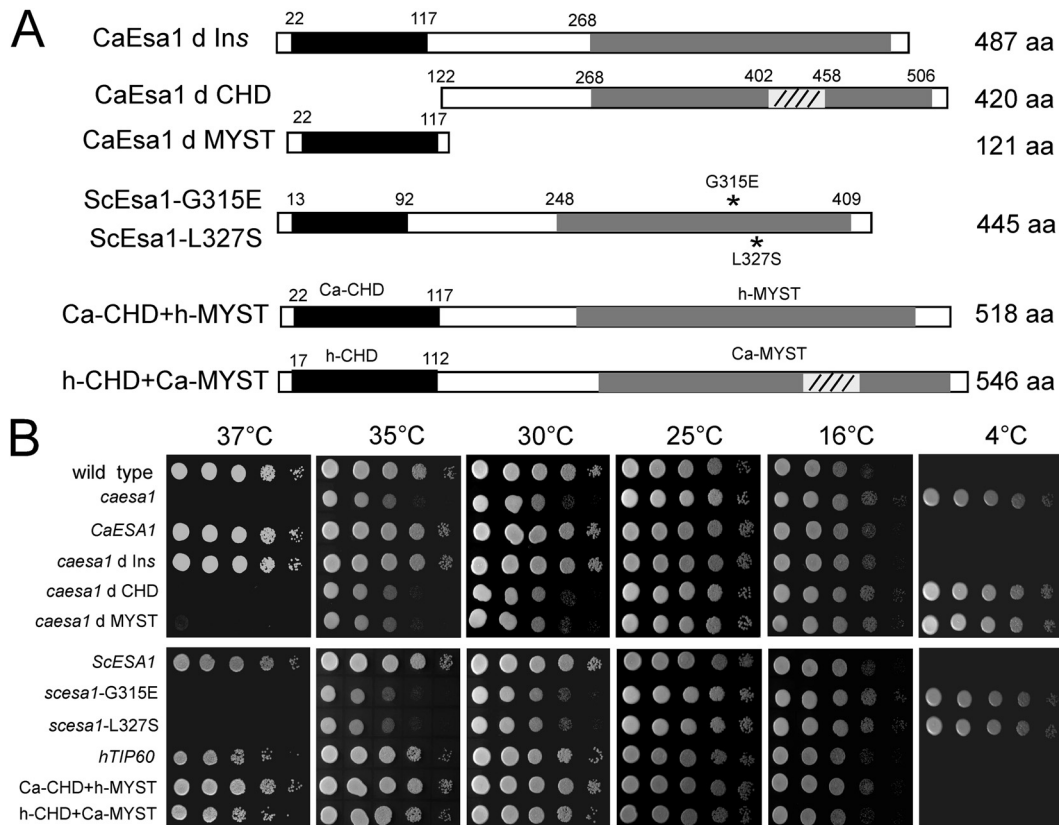


FIG 2 Expression of both *S. cerevisiae* *ESA1* and human *TIP60* can complement the growth defect of the *esa1/esa1* mutant in *C. albicans*. (A) Strategy for ectopic expression. CaEsa1, wild-type *C. albicans* Esa1; CaEsa1 d Ins, deletion of the insertion region in CaEsa1; CaEsa1 d CHD, deletion of the chromo domain in CaEsa1; CaEsa1 d MYST, deletion of the MYST domain in CaEsa1; ScESA1, wild-type *S. cerevisiae* Esa1; ScEsa1-G315E, G315E substitution in *S. cerevisiae* Esa1; ScEsa1-L327S, L327S substitution in *S. cerevisiae* Esa1; hTIP60, wild-type human TIP60; Ca-CHD+h-MYST, hybrid of *C. albicans* Esa1 chromo domain and human TIP60 MYST domain; h-CHD+Ca-MYST, hybrid of human TIP60 chromo domain and *C. albicans* Esa1 MYST domain. (B) Growth of strains on YPD plates at different temperatures. The exponentially growing cells were spotted onto YPD plates and incubated at the temperatures indicated. Ectopic expression vectors pBA-WX1 to pBA-WX10 were introduced into *C. albicans* *esa1/esa1* mutant (CWX3) cells for the complementation assay.

the NuA4 core module (Piccolo NuA4), Yng2 is the only one not essential for cell growth. Our previous study showed that, like the yeast homologue, *C. albicans* Yng2 is not essential for cell growth but is involved in hyphal regulation via NuA4 HAT activity (21). Thus, we predicted that the catalytic subunit Esa1 plays a critical role in hyphal development. As expected, deletion of *C. albicans* *ESA1* blocked filament formation under all hyphal induction conditions examined (Fig. 3). Since the *esa1* mutant could not grow at 37°C, we lowered the temperature to 35°C. At 35°C, wild-type cells formed pseudohyphae in liquid YPD medium without serum and formed true hyphae in the presence of serum, whereas the *esa1/esa1* mutant cells displayed the yeast form (Fig. 3A). In solid serum-containing medium, the wild-type strain produced florid filamentous colonies, while the *esa1/esa1* mutant formed small smooth yeast colonies (Fig. 3B). On solid surfaces of YPD plates, the wild-type strain invaded into the agar and formed filaments below the surface, whereas the *esa1/esa1* mutant failed in invasion and was defective in invasive growth (Fig. 3C). To analyze the role of Esa1 in filamentous growth under embedded conditions, we examined the phenotype of the *esa1/esa1* mutant at high and low temperatures under microaerophilic conditions. After growth in YPS agar at 35°C for 1 day, the wild-type colonies generated long heterogeneous filaments, but the *esa1/esa1* mutant produced

smooth colonies (Fig. 3D). At 25°C, the wild-type colonies produced limited amounts of filaments after 3 days incubation, while the *esa1/esa1* mutant formed smooth colonies (Fig. 3D). These observations indicate that Esa1 is essential for hyphal development and filamentous growth of *C. albicans*.

***C. albicans* Esa1 is involved in regulation of gene expression.** *C. albicans* Esa1, as a HAT, was predicted to have effects on gene regulation. *HWP1* and *ECE1* are the first few hypha-specific genes identified (36, 37). Consistent with the defect in hyphal morphogenesis, the *esa1/esa1* mutant was also defective in the expression of hypha-specific genes. *HWP1* and *ECE1* were highly induced in the wild-type cells under hypha-inducing conditions (YPD plus 10% serum; 35°C), but they were undetectable under yeast growth conditions (YPD; 25°C) (Fig. 4A). The expression levels of *HWP1* and *ECE1* in the *esa1/esa1* mutant were undetectable under hypha-inducing conditions (Fig. 4A). These results indicated that Esa1 is essential for hyphal development and the induction of hypha-specific genes.

To characterize the molecular basis of the Esa1 effects on *C. albicans* growth at different temperatures, we examined the expression levels of several heat shock response genes, including *HSP90*, *HSP70* and *HSP104* in the *esa1/esa1* mutant cells. Heat shock proteins play key roles in protein biogenesis and have dif-

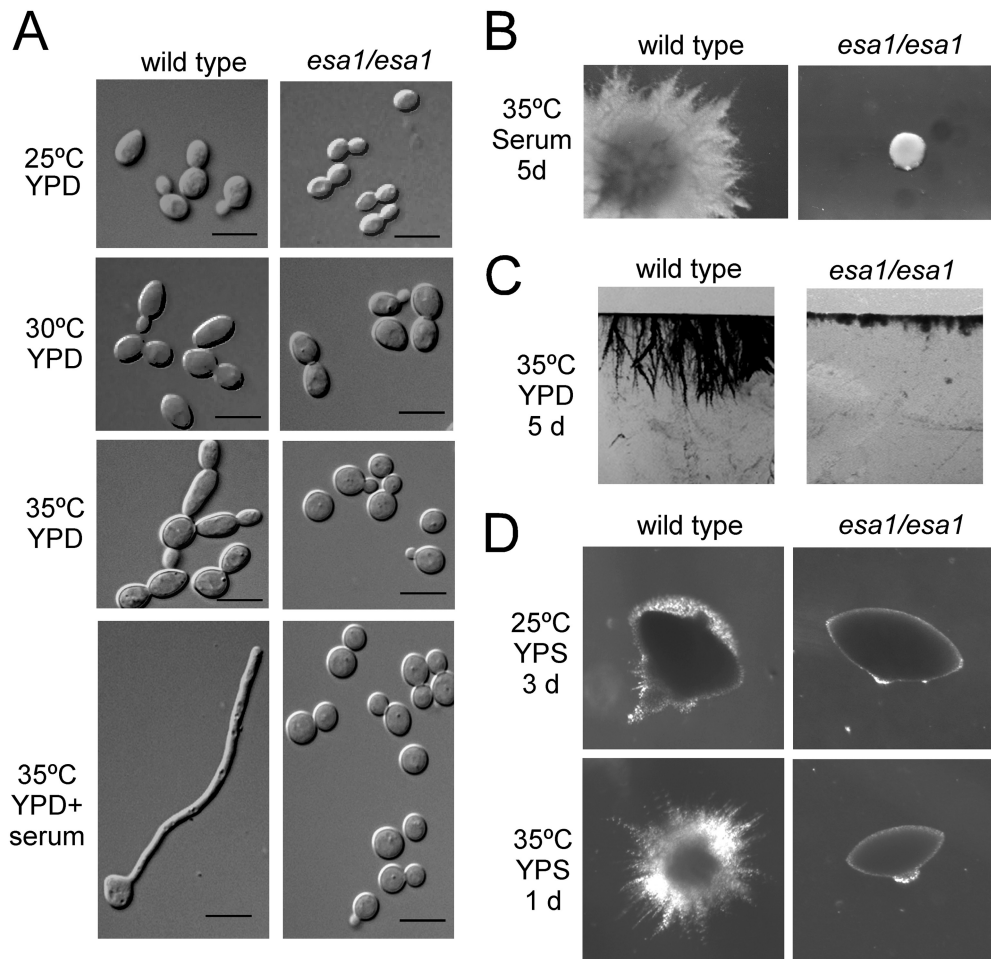


FIG 3 *C. albicans* Esa1 is essential for hyphal development. (A) Cell morphology of wild-type (BWP17/pBES116) and *esa1/esa1* mutant (CWX3/pBES116) cells under liquid conditions. The cells were cultured in liquid YPD medium at 25°C, 30°C, or 35°C for 6 h for yeast growth and in YPD plus 10% serum at 35°C for 3.5 h for hyphal growth. Bars, 5 μ m. (B) Colony morphologies of the strains. The cells were plated on solid serum-containing medium and incubated at 35°C for 5 days. (C) Invasive growth on agar. The strains were patched on YPD plates and grown at 35°C for 5 days. After washing with water, the agar containing cells was cut into slices for photographing. (D) Filamentous growth under embedded conditions. The cells were plated with molten YPS agar and grown at 25°C for 3 days or 35°C for 1 day.

ferent functions in cell growth, morphogenesis, and pathogenesis of *C. albicans* (38–40). All three heat shock genes, *HSP90*, *HSP70*, and *HSP104*, were induced in response to heat shock stress (25 to 35°C) in the wild-type *C. albicans* cells but failed to be induced in the *esa1/esa1* mutant cells (Fig. 4B). Requirement of Esa1 for induction of the heat shock genes may correlate its essential role in cell growth at a high temperature (37°C).

We further examined the expression levels of several stress response genes, including *HSP12*, *TPS1*, and *TPS2* in the *esa1/esa1* mutant cells. Hsp12, a small heat shock protein conserved among fungal species (41, 42), is induced during stationary-phase growth and under stress conditions, including heat shock, osmotic, and oxidative stress in *C. albicans* (42). In the wild-type cells, *HSP12* was greatly induced in response to both heat shock stress (25°C to 35°C) and cold shock stress (25°C to 4°C) (Fig. 4C). Interestingly, the expression of *HSP12* was 10-fold derepressed at 25°C in log-phase *esa1/esa1* mutant cells. The derepression was enhanced by cold shock, but not by heat shock (Fig. 4C). Trehalose, an important stress protectant, is synthesized in a two-step process in fungi by trehalose-6-phosphate (Tre6P) synthase and Tre6P phosphatase,

which are encoded by *TPS1* and *TPS2*, respectively (43). *C. albicans* Tps1 and Tps2 are proteins that are enriched during and play roles in osmotic and thermal stresses (44–46). Under exponential growth conditions at 25°C, the expression levels of both *TPS1* and *TPS2* were derepressed in the *esa1/esa1* mutant cells compared to the wild-type cells (Fig. 4C). Like *HSP12*, the *TPS* derepression was also enhanced by cold shock, but not by heat shock (Fig. 4C). Together, our data indicate that Esa1 has negative effects on transcription of *HSP12*, *TPS1*, and *TPS2*. Derepression of these genes under cold stress may largely account for the inhibitory role of Esa1 on cell growth at cold temperatures.

***C. albicans* Esa1 is required for stress responses.** Adaption to the host environment is important for survival of *C. albicans* (47, 48). The *esa1/esa1* mutant cells displayed sensitivity to DNA damage induced by HU, MMS, and CPT (Fig. 5A). This sensitivity was not due to the growth defect of the mutant, as we used the growth conditions were YPD medium and room temperature (22°C), under which both wild-type and *esa1/esa1* mutant cells have similar growth rates. The viability of the *esa1/esa1* mutant cells was at least 5-fold lower than that of the wild-type cells when spotted on the

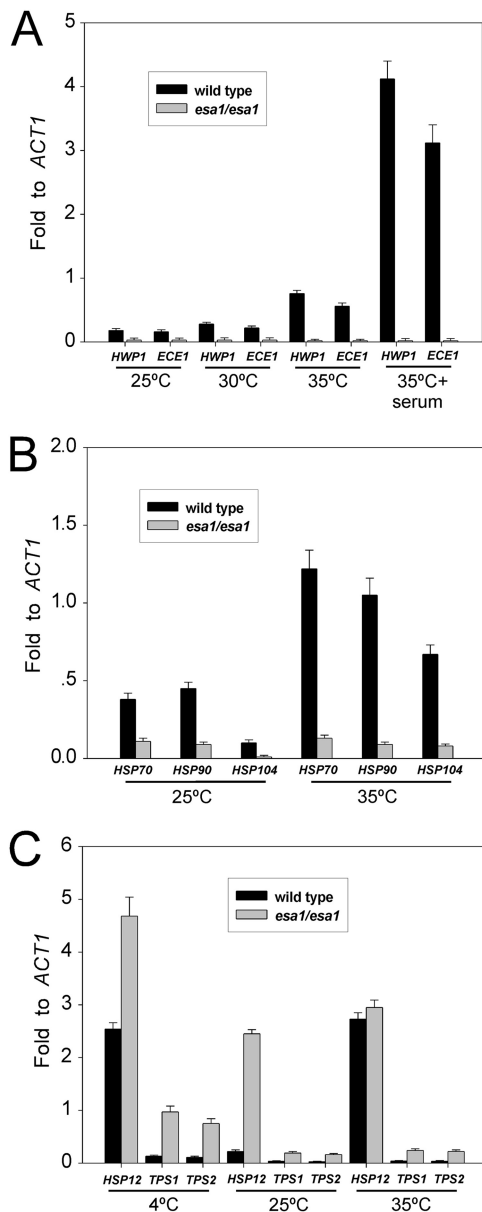


FIG 4 Effects of *C. albicans* Esa1 on gene regulation. (A) Quantitative RT-PCR analyses showed defective induction of hypha-specific genes *HWP1* and *ECE1* in *esa1/esa1* mutants. Cells were grown in YPD plus 10% serum at 35°C for 3.5 h or grown in YPD at 25°C, 30°C, or 35°C for 6 h. (B) Expression levels of heat shock genes *HSP90*, *HSP70*, and *HSP104* were inhibited in *esa1/esa1* mutants. Cells were grown in YPD at 25°C and then heat shocked at 35°C for 0.5 hour. (C) Expression levels of stress response genes *HSP12*, *TPS1*, and *TPS2* were derepressed in *esa1/esa1* mutants. Cells were cultured in YPD at 25°C and then upshifted to 35°C or downshifted to 4°C for 0.5 hour, and total RNAs were extracted for qRT-PCR analyses.

plates containing HU, MMS, or CPT (data not shown), suggesting that CaEsa1 is required for DNA damage repair in *C. albicans*. The *esa1/esa1* mutant cells were also sensitive to peroxide (Fig. 5A), suggesting that Esa1 plays a critical role in protecting *C. albicans* from H₂O₂-induced cell death.

In addition to temperature, genotoxic, and oxidative stresses, we extended our study to the impact of Esa1 on other stress responses. On solid medium containing 5 M NaCl (YPD plus NaCl

or YP plus NaCl) or high concentration of sucrose (YP plus 10% sucrose), the *esa1/esa1* mutant cells grew much faster than the wild-type cells at room temperature (22°C) (Fig. 5B and C), suggesting that inactivation of Esa1 increases the ability of *C. albicans* to resist osmotic stress. The *esa1/esa1* mutant cells also exhibited cell wall stress-resistant phenotypes and grew faster in the presence of Congo red and the antifungal drug caspofungin, both of which interfere with glucan biosynthesis (Fig. 5B). Consistent with their resistance to cold stress, the *esa1/esa1* mutant cells displayed osmotic and cell wall stress resistance.

We further sought to study the function of Esa1 in metabolic regulation. Compared to the wild-type cells, the *esa1/esa1* mutant cells grew slowly in media containing a fermentable sugar, including glucose and maltose, but grew faster in media containing a nonfermentable carbon source, such as ethanol and glycerol (Fig. 5D). A similar phenomenon was observed in synthetic growth medium (SC plates with fermentable and nonfermentable carbon sources), excluding the effect of nutrients (Fig. 5E). The faster growth phenotype of the *esa1/esa1* mutant in nonfermentative media was observed not only at room temperature (22°C) but also at 30°C and 16°C, but it was hardly observed at 35°C, due to a reduced growth rate of the *esa1/esa1* mutant. Lack of Esa1 enhanced the ability of *C. albicans* to utilize nonfermentable carbon sources. In some synthetic growth media, including SCLD (SC plus 0.1% glucose) and SLAD (SD plus low ammonia), the *esa1/esa1* mutant cells grew poorly, suggesting a defect in nutrient uptake (Fig. 5C). Taken together, these data indicate that Esa1 mediates multiple cellular processes and plays important roles in temperature adaptation, DNA damage repair, the oxidative stress response, osmotic stress tolerance, cell wall assembly, and metabolism regulation.

***C. albicans* Sas2 plays a role opposite to Esa1 in cell growth and morphogenesis.** We suspected that the nonlethal phenotype of the *esa1/esa1* mutant might be due to the existence of a functionally redundant MYST HAT in *C. albicans*. Thus, we searched the *C. albicans* genome database and found another putative MYST protein (orf19.2087) that was homologous to *S. cerevisiae* Sas2. Unlike most of the other MYST family proteins, such as human MOF, ScSas2 and CaSas2 contain only a MYST domain at the C terminus, with 83% sequence identity, but lack a chromo domain at the N terminus (Fig. 6A). To test the role of *C. albicans* SAS2 in cell growth, we deleted both copies of *SAS2* in *C. albicans*. Deletion of *C. albicans* *SAS2* did not cause a lethal phenotype, but the cells exhibited resistance to high temperature. The *sas2/sas2* mutant cells grew much faster than the wild-type cells at high temperature, including 37°C, and especially at 42°C and 45°C (Fig. 6B). The *ESA1*-overexpressing strain displayed a phenotype similar to the *sas2/sas2* mutant and grew faster at high temperature (Fig. 6B), in contrast to the sensitive phenotype of the *esa1/esa1* mutant. These results indicate that CaSas2 and CaEsa1 play opposite roles in cell growth at high temperature.

To confirm the data, we analyzed the doubling time of the strains (see Fig. S2 in the supplemental material). Above room temperature (22°C), the *esa1/esa1* mutant cells grew much slower than the wild-type cells; below room temperature, the *esa1/esa1* mutant cells grew faster than the wild-type cells (see Fig. S2A). The *sas2/sas2* mutant cells showed a slightly higher growth rate above 30°C but a slightly lower rate below 16°C. As expected, overexpression of *ESA1* reversed the phenotype of the *esa1/esa1* mutant

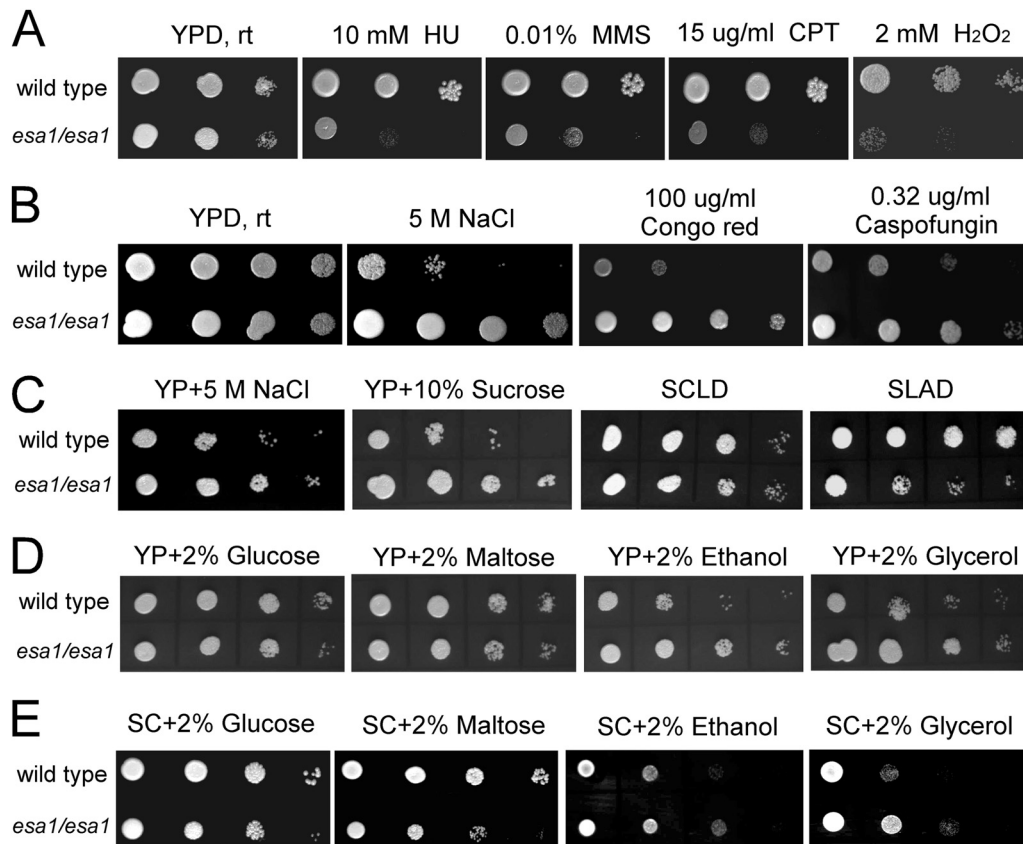


FIG 5 *C. albicans* Esa1 is involved in multiple stress responses. (A) *esa1/esa1* mutants are sensitive to genotoxic and oxidative stresses. Wild-type (BWP17/pBES116) and *esa1/esa1* mutant (CWX3/pBES116) cells were serially diluted 10-fold, spotted onto YPD plates containing DNA-damaging agents (10 mM HU, 0.01% MMS, and 15 μ g/ml CPT) or 2 mM H₂O₂, and incubated at room temperature (rt; 22°C) for 3 days. (B and C) *esa1/esa1* mutants were resistant to osmotic and cell wall stresses. YPD plates containing 5 M NaCl, 100 μ g/ml Congo red, or 0.32 μ g/ml caspofungin (B), or YP with 5 M NaCl or 10% sucrose (C) were used for growth assays. SC plates containing 0.1% glucose (SCLD) and low ammonia (SLAD) were used for a nutrient-sensing assay. (D and E) YP plates (D) or SC plates (E) containing 2% glucose, maltose, ethanol, or glycerol were used for a carbon source-sensing assay.

and the phenotype resembled that of the *sas2/sas2* mutant (see Fig. S2B).

CaSas2 has an inhibitory effect on hyphal growth. At high temperature (37°C), the wild-type cells could not sustain hyphal growth in YPD media without serum, whereas the *sas2/sas2* mutant cells formed true hyphae (Fig. 6C). At an intermediate temperature (30°C), the wild-type cells could not produce hyphae in YPD media with or without serum, but the *sas2/sas2* cells promoted filament formation even in the absence of serum (Fig. 6C). At a lower temperature (25°C), the *sas2/sas2* cells exhibited a phenotype similar to wild-type cells and grew in yeast form (Fig. 6C). Overexpression of *ESA1* resulted in a similar phenotype as the *sas2/sas2* mutant in hyphal production, opposite to the phenotype of the *esa1/esa1* mutant (Fig. 6C). Consistent with their opposite effects on cell growth, CaEsa1 and CaSas2 also have opposite effects on hyphal development.

Esa1 and Sas2 link histone H4 acetylation to cell growth. Deletion of *C. albicans* *ESA1* caused dramatic reductions in acetylation at H4K5 and H4K12, but not at H4K16 (Fig. 1C and 7A), probably due to the existence of a redundant HAT for H4K16, similar to *S. cerevisiae* (15, 16). In *S. cerevisiae*, Sas2 is mainly required for H4K16 acetylation, and Sas2 links H4K16 acetylation to inhibition of the spread of subtelomeric heterochromatin

through opposing Sir proteins binding to euchromatin (15, 16). In mammals, MOF also plays important roles in transcription activation by acetylating H4K16 (17). Thus, we predicted that CaSas2 also contributes to H4K16 acetylation. Indeed, deletion of *C. albicans* *SAS2* reduced the acetylation level of H4K16 but had subtle effects on acetylation of H4K5 and H4K12 (Fig. 7A). Interestingly, at a higher temperature (35°C), CaSas2 seemed to have inhibitory effects on acetylation of H4K5 and H4K12. Deletion of *SAS2* slightly increased the acetylation levels of H4K5 and H4K12 at higher temperature (35°C); however, the increase was not obvious at lower temperature (16°C) (Fig. 7A).

To determine whether the two MYST proteins have synergistic effects on H4 acetylation, we tried to delete both the *ESA1* and *SAS2* genes in *C. albicans*, but we failed to obtain the *esa1/esa1 sas2/sas2* double mutant. Hence, we constructed a conditional knockdown *esa1 sas2* double mutant by downregulating the expression of *ESA1* in a tet-off system. At 25°C, the *ESA1* mRNA levels in both *esa1/Ptet-ESA1* and *sas2/sas2 esa1/Ptet-ESA1* mutant cells decreased greatly after addition of doxycycline (see Fig. S3A in the supplemental material), indicating that the *ESA1* expression can be specifically repressed by doxycycline. After treatment with doxycycline at 25°C, the acetylation level of H4 in the *sas2/sas2 esa1/Ptet-ESA1* mutant was decreased. At 6 h, the acetylation levels

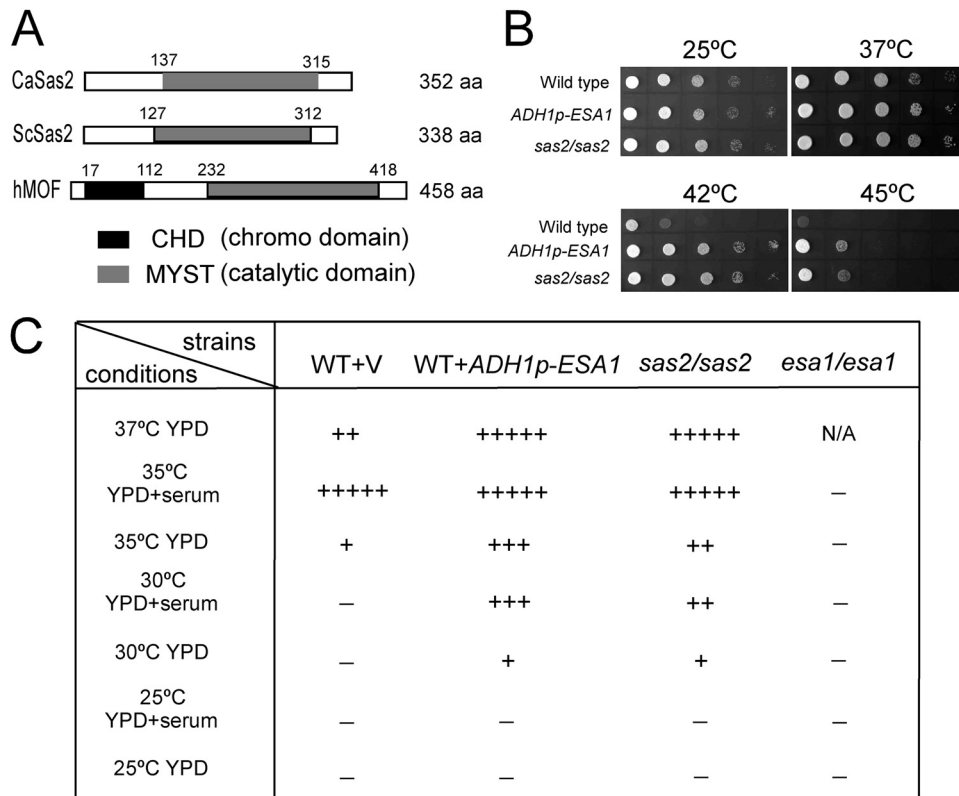


FIG 6 *Candida albicans* open reading frame 19.2087 encodes MYST protein Sas2, which plays a role opposite to that of Esa1 in cell growth and morphogenesis. (A) Schematic depiction of the MYST domain and chromo domain in *C. albicans* Sas2, *S. cerevisiae* Sas2, and human MOF. (B) *sas2/sas2* mutant cells were tolerant to high temperature. The cells were spotted onto YPD plates and incubated at 25°C, 37°C, 42°C, or 45°C for 2 days. (C) Sas2 and Esa1 have opposite effects on filament formation. Wild-type (BWP17/pBA1), wild-type with *ADH1p-ESA1* (BWP17/pBA1-*ESA1*), *esa1/esa1* mutant (CWX3/pBA1), and *sas2/sas2* mutant (CWX6/pBA1) cells were grown in liquid YPD medium with or without 10% serum at different temperatures for 3 h. —, yeast growth; +, filamentous growth. The level of cell elongation is indicated by the number of + symbols. N/A, not available (cells failed to grow).

of H4K5, H4K12, and H4K16 were all significantly decreased (see Fig. S3B). When we extending the treatment time to 24 h, *ESA1* expression under *tet* promoter control was almost completely repressed, and the mutant cells stopped budding and displayed growth arrest. At this time point, H4K16 acetylation was completely abolished, and H4K5 and H4K12 acetylation was also decreased to very low levels at the temperatures examined (Fig. 7A). All of these results together indicate that the two MYST HATs Esa1 and Sas2 have redundant and synergistic effects on H4 acetylation.

To link H4 acetylation to cell growth, we tested the growth of the wild-type, *esa1/Ptet-ESA1*, and *sas2/sas2 esa1/Ptet-ESA1* cells on solid medium containing doxycycline. The *ESA1*-downregulated cells were sensitive to high temperature (37°C) and resistant to cold temperature (4°C) but could grow at intermediate temperatures (Fig. 7B), i.e., showed a phenotype resembling the phenotype of the *esa1/esa1* mutant cells. However, downregulating *ESA1* in the *sas2/sas2 esa1/Ptet-ESA1* mutant cells blocked cell growth at all temperatures (Fig. 7B). Inactivating both Esa1 and Sas2 caused a synthetic lethal phenotype, suggesting that Esa1 and Sas2 have synergistic effects on cell growth. Based on these results, we conclude that the two MYST HATs Esa1 and Sas2 play distinct and redundant roles in cell growth and morphogenesis of *C. albicans*.

DISCUSSION

Esa1 and Sas2 have distinct and redundant effects on H4 acetylation and cell growth. In this study, we identified two MYST proteins, Esa1 and Sas2, in the human pathogen *C. albicans*. *C. albicans* Esa1 is a functional homologue (orthologue) of *S. cerevisiae* Esa1 and human TIP60 and is the catalytic subunit of the NuA4 HAT complex (49). *C. albicans* Sas2 is the orthologue of *S. cerevisiae* Sas2 and is the catalytic subunit of the SAS HAT complex (15, 16). Esa1 and Sas2 have opposite effects on cell growth. Loss of Esa1 changes gene expression in a way that supports growth at low temperature. In contrast, loss of Sas2 supports cell growth at high temperature. Deletion of *ESA1* is not lethal, but deletion of both *ESA1* and *SAS2* causes lethality, indicating that Esa1 and Sas2 function redundantly in cell growth. Esa1 and Sas2 also play opposite roles in *C. albicans* morphogenesis, similar to the opposite roles of two histone deacetylases (HDACs), HOS2 and HDA1, in the regulation of hyphal formation (50). The synergistic effects of Esa1 and Sas2 on H4 acetylation at H4K5, H4K12, and H4K16 are correlated with their essential roles in cell growth. *S. cerevisiae* Esa1 has been proven a major HAT for acetylation of H4K5 and H4K12 and a secondary HAT for acetylation of H4K16 (10). The capability of *S. cerevisiae* Esa1 to acetylate H4K5, H4K12, and H4K16 and the synergy of *C. albicans* Esa1 and Sas2 on H4

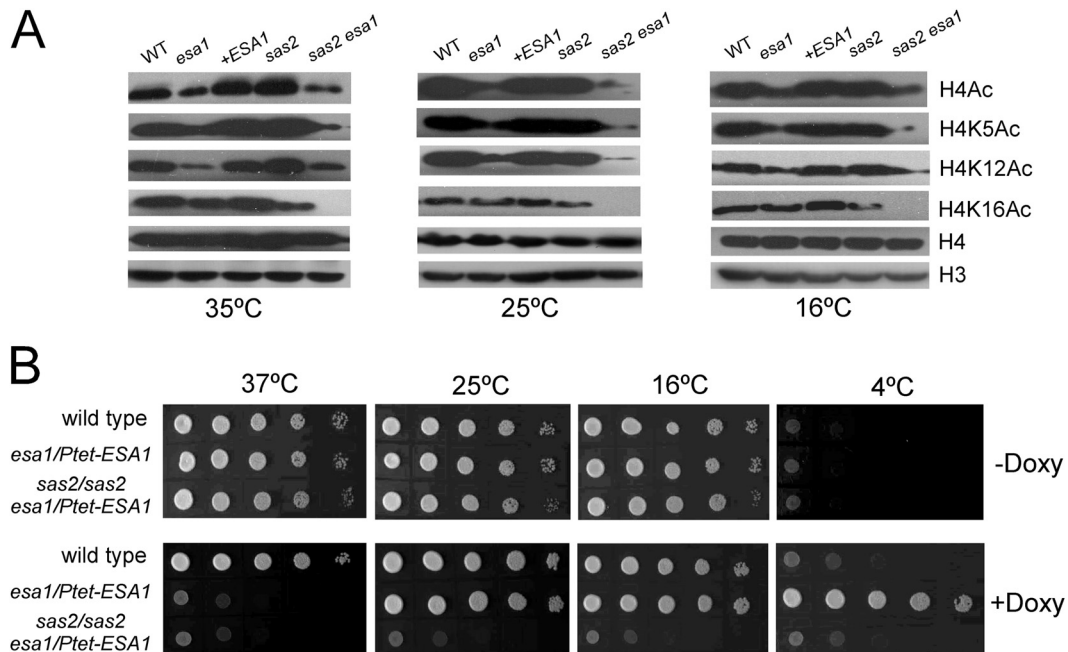


FIG 7 *C. albicans* Esa1 and Sas2 have synergistic effects on histone acetylation and cell growth. (A) Western analyses of histone H4 acetylation levels in the wild type and mutants. (B) Double deletion of *ESA1* and *SAS2* showed synthetic lethality. The cells were precultured in YPD medium containing 50 $\mu\text{g/ml}$ doxycycline for 24 h and then spotted onto YPD plates in the absence or presence of doxycycline and incubated at the indicated temperatures. Strains are wild type (BWP17/pBA1), wild type with *ADH1p-ESA1* (BWP17/pBA1-*ESA1*), *esa1/esa1* (CWX3/pBA1), *sas2/sas2* (CWX6/pBA1), *esa1/Ptet-ESA1* (CWX7), and *sas2/sas2 esa1/Ptet-ESA1* (CWX8). All *Ptet-ESA1* cells were treated with doxycycline to knock down the *ESA1*.

acetylation link the acetylation of H4 at the K5, K12, and K16 sites to essential roles in cell growth.

Esa1 and Sas2 are involved in regulation of multiple cellular processes of *C. albicans*. The MYST family of HATs is associated with diverse functions. *C. albicans* Esa1 and Sas2 target histone tails and are involved in transcriptional regulation of genes responsible for hyphal induction, heat shock, and stress resistance. The requirement of *C. albicans* Esa1 for the expression of hypha-specific genes is consistent with its essential role in *C. albicans* hyphal development. The activation of *C. albicans* Esa1 during cell growth at high temperatures correlates with the expression of heat shock genes. The repression of *C. albicans* Esa1 in response to stress is linked to the expression of stress response genes. Therefore, *C. albicans* Esa1 could function as a transcription coactivator as well as a transcription corepressor via modulation of histone acetylation.

C. albicans displays robust stress responses to promote survival in the host. Trehalose and glycerol are stress protectants, and accumulation of trehalose and glycerol is induced under stress conditions, including osmotic stress (43), high temperature (51), and cold stress (52). Loss of Esa1 caused cells to superaccumulate trehalose in stationary phase at all temperatures examined (see Fig. S4A in the supplemental material), which correlated well with its stress resistance and nonlethal phenotypes. Loss of Sas2 leads to accumulation of more trehalose at high temperature (42°C), which is associated with its heat-resistant phenotype (see Fig. S4C). Interestingly, the *esa1/esa1* mutant cells did not accumulate more glycerol than the wild-type cells (see Fig. S4B), suggesting that the glycerol level may not correlate with survival of the *esa1/esa1* mutant.

In the *esa1/esa1* mutant, trehalose accumulation does not seem

to be correlated with *TPS* gene expression in sensing temperature. *TPS1* and *TPS2* are expressed at the highest level at 4°C, yet the trehalose concentration is the highest at 35°C and the lowest at 4°C. The reason is that different states of the *esa1/esa1* mutant cells were collected for measurement of *TPS* gene expression and the trehalose concentration. *TPS* gene expression was measured under cold shock or heat shock conditions, but the trehalose concentration was measured in cells from stationary phase. In stationary-phase cells, *TPS* gene expression in the *esa1/esa1* mutant is hard to detect due to low level of *TPS* mRNA. On the other hand, superaccumulation of trehalose in the *esa1/esa1* mutant is not increased by cold shock or heat shock (data not shown). Higher *TPS* gene expression and a higher trehalose concentration in the *esa1/esa1* mutant may account for the inhibitory role of Esa1 on cell growth at cold temperatures but could not account for the decreased fitness of the mutant at high temperatures. A reasonable explanation might be that the downregulation of heat shock proteins plays a predominant role in the heat sensitivity of the *esa1/esa1* mutant, which is supported by the essential role of Hsp90 for *C. albicans* viability. Indeed, downregulated *HSP90* expression causes a lethal phenotype (40).

C. albicans Esa1 and Sas2 may target nonhistone substrates and function as KATs in the regulation of multiple cellular processes. *S. cerevisiae* Esa1 has been proven to be able to acetylate a variety of substrates, including Pck1, Yng2, and Atg3 (1, 11, 12), which are involved in multiple cellular processes. More substrates of the MYST proteins have been identified, including factors involved in transcription, heterochromatin formation, the cell cycle, DNA repair, and gluconeogenesis, and other subunits of MYST protein complexes (6). The mediation of several cellular processes, including temperature adaptation, stress response, cell cycle progress,

metabolism, and morphogenesis, suggests that *C. albicans* Esa1 and Sas2 are involved in multiple regulatory processes via histone and/or nonhistone modification cross talk.

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