Research Article

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SDH2 is involved in proper hypha formation and virulence in *Candida albicans*

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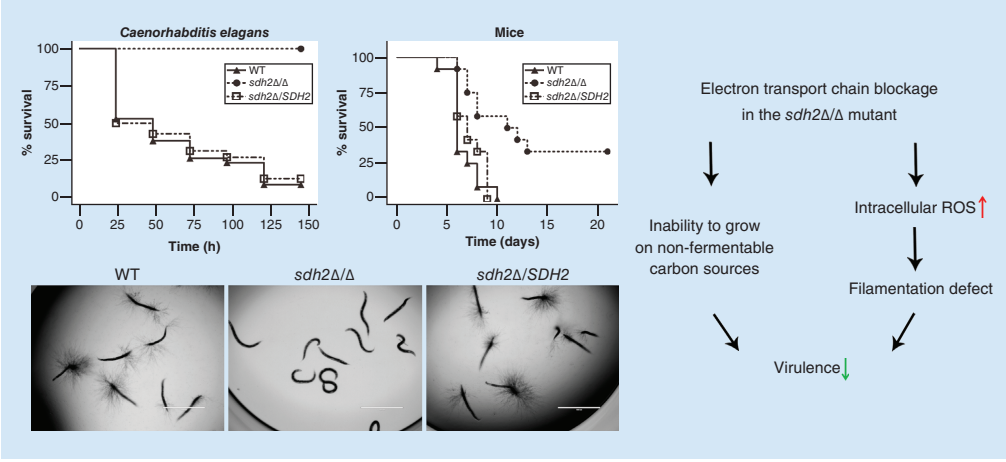
Aim: To investigate the role of *SDH2* in *Candida albicans* filamentation and virulence. **Materials & methods:** *Caenorhabditis elegans* and mouse candidiasis models were used to assess the virulence of a *sdh2*Δ/Δ mutant. Various hypha-inducing media were used to evaluate the hyphal development of *C. albicans*. DCFH-DA was used to measure intracellular Reactive Oxygen Species (ROS) levels. **Results:** The *sdh2*Δ/Δ mutant was avirulent in the *C. elegans* model, hypovirulent in a murine candidiasis model, and defective to form filaments both *in vitro* and *in vivo*. Intracellular ROS level increased in the *sdh2*Δ/Δ mutant, and the filamentation defects of *sdh2*Δ/Δ were rescued by decreasing intracellular ROS. **Conclusion:** *SDH2* plays an important role in *C. albicans* filamentation and virulence probably through affecting intracellular ROS.



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Candida albicans is a major fungal pathogen, causing diseases varying from superficial mucosal disorders to life-threatening bloodstream infections with a more than 40% mortality rate [1,2]. In contrast to the model yeast *Saccharomyces cerevisiae* which preferentially ferments, *C. albicans* is a Crabtree-negative species exclusively using respiration when oxygen is available [3]. Respiration is comprised of a set of metabolic reactions and processes including glycolysis, tricarboxylic acid (TCA) cycle, electron transport chain (ETC), and ATP synthesis [3] and appears to

play a significant role in *C. albicans* biology [4–7]. Several respiration-related genes contribute to the morphological transition and pathogenicity of *C. albicans* [4–7]. For example, deletion of *CaNDH51* [6], a gene functioning in utilizing NADH for ATP production, results in filamentation defects. Also, deletion of the mitochondrion-related genes *GOA1* [4] and *MCU1* [5] causes respiration defects and strikingly attenuated virulence in a murine candidiasis model. It is still unclear whether the contributions of *CaNDH51*, *GOA1* and *MCU1* to filamentation and virulence are based on their roles in ATP production.

We have developed a *Caenorhabditis elegans*-based infection model that offers a different approach for high-throughput screening work and yeast-to-hypha transition studies [8–10]. In this study, we utilized this model system in order to investigate the role of *SDH2* that encodes a putative iron-sulfur subunit of succinate dehydrogenase (SDH, also named respiratory complex II), which is involved in both the TCA cycle and the ETC [11,12].

Materials & methods

Strains & growth conditions

Caenorhabditis elegans glp-4; sek-1 strain was propagated on nematode growth medium on lawns of *Escherichia coli* OP50 by using standard methods [8]. The *C. albicans* strains used in this study and the generation of the *sdh2*Δ/Δ mutant, the *SDH2* reintegrated strain, the *fum*12Δ/Δ and *adh1*Δ/Δ mutant are described in Supplementary Table 1 and Text 1. *Candida albicans* strains were routinely propagated in YPD (1% yeast extract, 2% peptone and 2% dextrose) liquid medium at 30°C in a shaking incubator. To investigate the role of *SDH2* in yeast-to-hypha morphological transition, *C. albicans* strains were grown in media known to induce the morphological transition, including YPD + serum, Lee, Spider, Spider + glucose and YPS. YPD + serum medium contains YPD and 10% (vol/vol) fetal calf serum. Lee medium is rich in amino acids and glucose. Spider medium contains 1% (wt/vol) nutrient broth, 1% (wt/vol) mannitol and 0.2% (wt/vol) K_2HPO_4. Spider + glucose medium is Spider medium with 100 mM glucose supplemented. YPS medium contains 1% yeast extract, 2% peptone, 2% sucrose and 1% agar [13].

Virulence assays using a *Caenorhabditis elegans* candidiasis model

As described previously [8], approximately 400 synchronized adult *C. elegans glp-4; sek-1* nematodes were added to the center of the *C. albicans* lawns on brain heart infusion (BHI) media and incubated at 25°C for 4 h [14]. After a careful wash, worms were pipetted into wells of six-well tissue culture plates containing 2 ml of liquid medium (80% M9, 20% BHI) and kanamycin (45 μg/ml). Dead worms were scored and removed daily.

Virulence assay using a murine candidiasis model

Mice were infected with *C. albicans* according to an established protocol [15]. CD-1 female mice (6-week old, 18–22 g) were infected with 1.5×10^6 colony-forming units (CFUs) of *C. albicans* suspended in Phosphate-Buffered Saline (PBS) via a tail vein injection in a 100 μl volume. The murine protocol was approved by the Massachusetts General Hospital Committee on Research, Subcommittee on Research Animal Care (SRAC; OLAW Assurance # A3596-01). The protocol (2011N000175) conformed to the USDA Animal Welfare Act, Policy on Humane Care and Use of Laboratory Animals (PHS Policy), the *ILAR Guide for the Care and Use of Laboratory Animals* and other applicable laws and regulations. Special attention was given to minimize the suffering of the mice.

Kidney CFU assay

The kidney CFU assay was performed to assess the fungal burden in the kidneys. The kidney is the most frequent target of *C. albicans*, and the kidney CFU assay is the most commonly used method to investigate the infection with *C. albicans* [16,17]. Briefly, fungal cells were prepared and injected as described above for the disseminated murine candidiasis model. 2 days after inoculation, kidneys were removed aseptically, weighed and homogenized in 1 ml sterile PBS. Serial dilutions were plated on YPD agar with antibiotics to determine the CFU/g kidney.

Fungal cells staining with periodic acid Schiff

Histopathological analysis was performed to assess kidney damage. Kidney tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. Thin sections were stained using periodic acid Schiff (PAS) to reveal the hyphal structure of the fungal pathogens [17].

Measurement of intracellular ethanol content

Candida albicans cells were grown on Spider + glucose (100 mM) at 30°C for 5 day or YPD + serum medium at 37°C for 3 day. *Candida albicans* cells (0.1–0.3 g) were picked up, accurately weighted, and suspended with 1 ml sterilized water. Glass beads (0.5 mm, 700 µl) were added to each suspension, and the *C. albicans* cells were lysed by vigorous vortexing, interrupted by cooling on ice. Glass beads were removed by centrifugation and the ethanol concentrations in the supernatants were determined by Enzytec™ fluid ethanol kit (R-Biopharm AG, Darmstadt, Germany).

Measurement of intracellular ATP levels

The experiment was carried out according to the protocol described previously [18]. *Candida albicans* cells were adjusted to 1×10^6 cells/ml with culture medium and incubated at 30°C with shaking for 30 min. Intracellular ATP was detected using BacTiter-Glo reagent (Promega, WI, USA). Luminescent signals were determined on a Tecan Infinite® 200 Pro Multilabel Counter. A standard curve for ATP increments (from 100 nM to 10 pM) was constructed, and the ATP contents were calculated from the standard curve.

Measurement of intracellular ROS levels

Intracellular ROS levels were assessed as reported before [18–20]. In each group, *C. albicans* cells (1×10^7 cells/ml) were incubated with 20 µg/ml of 2,7-dichlorofluorescin diacetate (DCFH-DA; , Molecular Probes, Eugene, OR, USA) at 30°C for 3 h. After being washed and resuspended in PBS buffer, 100 µl *C. albicans* sample was used for intracellular ROS measurement. Fluorescence intensity (FI) values were detected on the Infinite 200 Pro (Tecan, Männedorf, Switzerland) with excitation wavelength at 480 nm and emission wavelength at 530 nm. The detected FI value can reflect the intracellular ROS level. ROS levels were assessed by subtracting the FI value of cells without DCFH-DA from that of cells with DCFH-DA.

Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) and *post hoc* (Bonferroni and Student–Newman–Keuls') tests. Animal survival was examined using the Kaplan–Meier method and differences were determined using the log-rank test (STATA 6; STATA, TX, USA). A p-value <0.05 was considered statistically significant.

Results

SDH2 is required for *C. albicans* virulence *in vivo*

We used the *C. elegans* candidiasis model to screen libraries of homozygous mutants from the Fungal Genetics Stock Center (http://www.fgsc.net/candida/FGSCcandidaresources.htm). Of particular interest was the identification of a mutant lacking *SDH2* that was avirulent in this model; the mutant lacked hypha piercing the worm cuticle (data not shown). To confirm the role of *SDH2* in the virulence of *C. albicans*, we constructed a *sdh2*Δ/Δ mutant and the reintegrated strain *sdh2*Δ/*SDH2* using the *SAT1* flipping method [21] (Supplementary Table 1, Text 1). The growth of the wild-type, *sdh2*Δ/Δ and *sdh2*Δ/*SDH2* strains in routine YPD culture medium and RPMI 1640 medium was monitored and there was no growth defect (Supplementary Table 2). In the *C. elegans* candidiasis model, more than 60% of the worms died within the first 48 h after infection with the wild-type or the reintegrated strains (Figure 1A), while at 120 h, more than 85% of the worms were dead, and every dead worm had visible hyphae piercing the cuticle (Figure 1B). In contrast, no dead worms and no hyphae were observed in the *sdh2*Δ/Δ group (Figure 1A & B).

In a murine candidiasis model, the survival data indicated that the *sdh2*Δ/Δ mutant was significantly less virulent than the wild-type strain ($p < 0.01$; Figure 1C). The CFU data indicated that the fungal burden in the mouse kidneys was less in the *sdh2*Δ/Δ mutant infected group compared with the control group ($p < 0.05$; Figure 1D). PAS staining of the mouse kidneys indicated clusters of hyphae in the wild-type and reintegrated strain infection groups. In contrast, no obvious hyphal cluster was observed in the *sdh2*Δ/Δ group (Figure 1E). Collectively, the *sdh2*Δ/Δ mutant was avirulent in the *C. elegans* candidiasis model and hypovirulent in the murine candidiasis model.

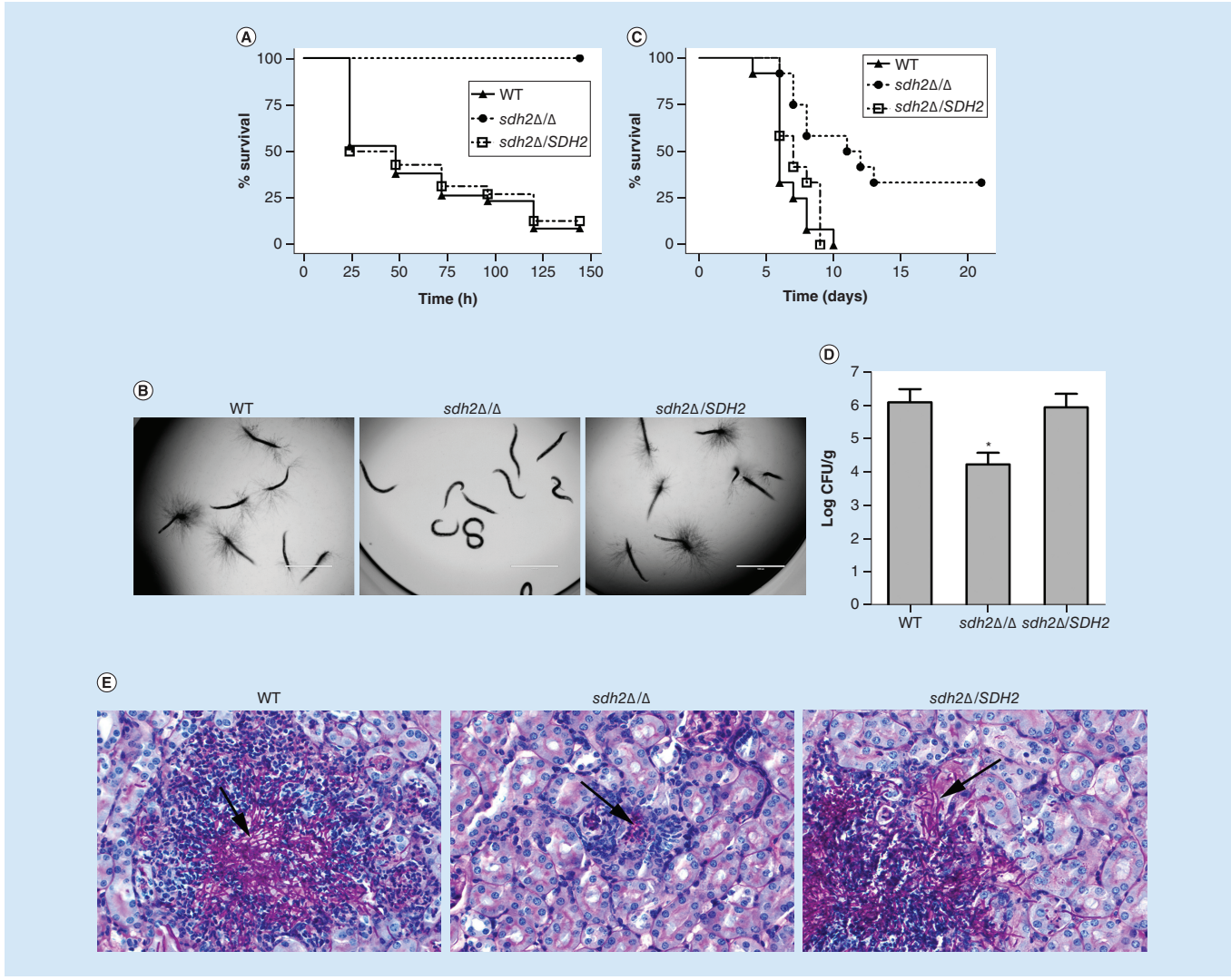

Figure 1. The *sdh2*Δ/Δ mutant is avirulent in the *Caenorhabditis elegans* candidiasis model and hypovirulent in the murine candidiasis model. (A) *Caenorhabditis elegans* survival was followed for 144 h after *Candida albicans* infection (n = 60–70 worms per strain of *C. albicans*). Compared with the *C. albicans* wild-type strain SC5314, the *sdh2*Δ/Δ mutant is avirulent to *C. elegans* ($p < 0.001$). The reintegration of *SDH2* restored the virulence of *C. albicans* to wild-type levels. **(B)** The *C. elegans glp-4*; *sek-1* nematodes were infected by *C. albicans* wild-type, *sdh2*Δ/Δ or *sdh2*Δ/*SDH2* strain, respectively. On day 3, the worms were photographed. **(C)** Mice survival was followed over 21 days (n = 12 mice per strain of *C. albicans*). Mice were inoculated via the tail vein with 1.5×10^6 CFUs of the indicated strains, and observed twice daily. Compared with the wild-type strain, the *sdh2*Δ/Δ mutant is hypovirulent ($p < 0.01$). **(D)** Fungal burden of the kidneys of mice infected with the wild-type, *sdh2*Δ/Δ and *sdh2*Δ/*SDH2* reintegrated strains 2 days after inoculation (n = 4 mice per strain of *C. albicans*). Statistical analyses were performed using ANOVA and *post hoc* (Bonferroni and Student–Newman–Keuls') tests. $p < 0.05$ was considered significant. *$p < 0.05$ compared with mice infected with the wild-type strain. **(E)** PAS stained thin sections of kidneys 2 days after inoculation with the indicated strains of *C. albicans* demonstrate differences in filamentation within the organ. Tissues were examined microscopically. Arrows indicate *C. albicans* filaments in the tissues.
CFU: Colony-forming unit; PAS: Periodic acid Schiff.

The *sdh2*Δ/Δ mutant exhibits filamentation defects

As described above, the *sdh2*Δ/Δ mutant did not result in the characteristic puncture of filaments through the *C. elegans* cuticle or in obvious hyphal clusters within the kidneys of mice. To study the invasive hyphal development, we used an agar system that mimics some of the features of tissue invasion [22,23]. The *sdh2*Δ/Δ mutant displayed severe filamentation defects on/within all the hypha-inducing agar media tested, including YPD + serum, Lee, Spider (with mannitol as carbon source), Spider + glucose and YPS. More specifically, the *sdh2*Δ/Δ mutant formed smooth-edged colonies, in contrast to wild-type and the reintegrated strains that developed wrinkled colonies and

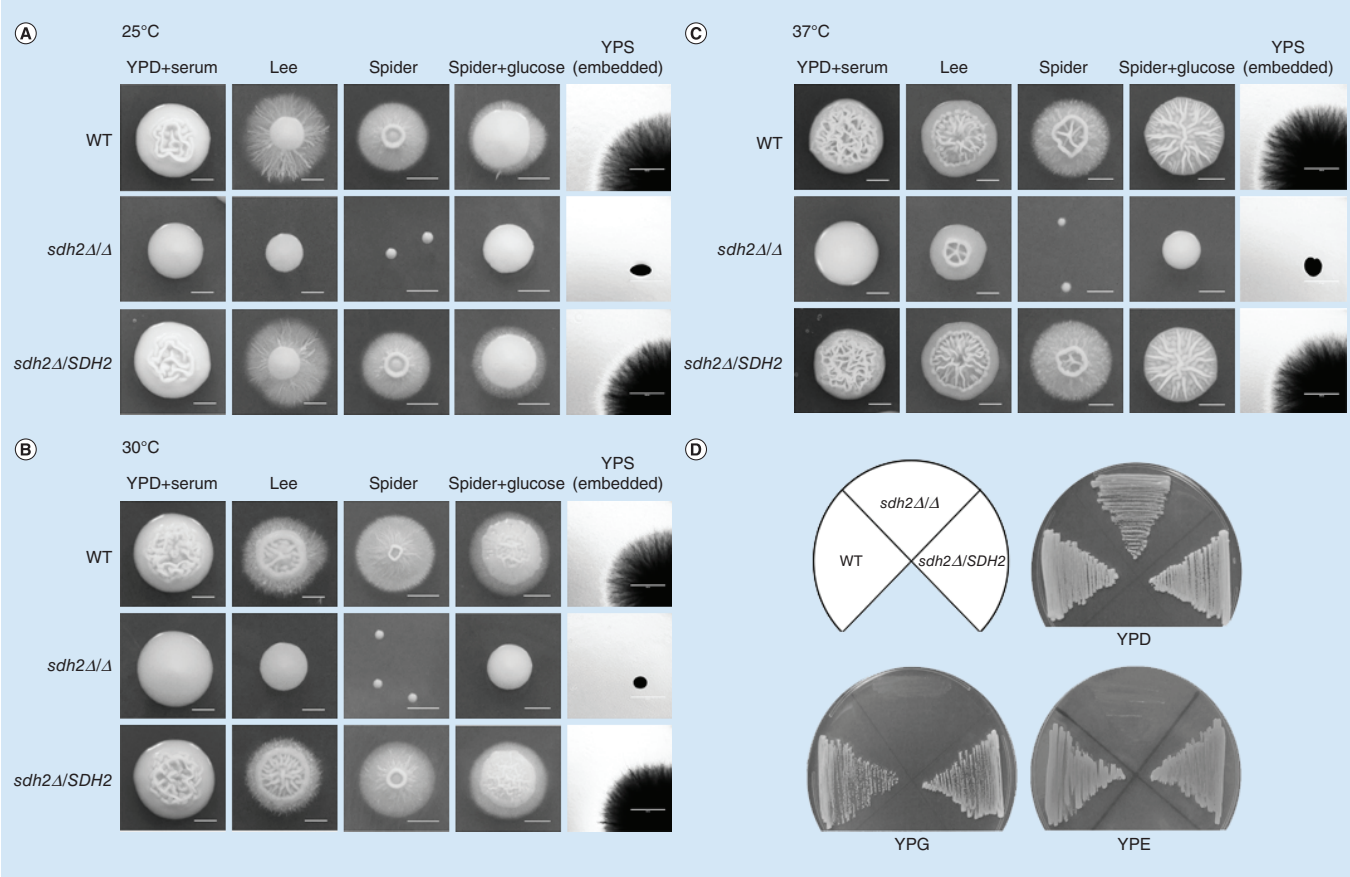

Figure 2. The *sdh2*Δ/Δ mutant exhibits filamentation defects. (A) Filamentous growth of *Candida albicans* at 25°C. Times of incubation were as follows: YPD + serum, 9 days; Lee, 11 days; Spider, 11 days; Spider + glucose, 8 days; YPS, 3 days. **(B)** Filamentous growth of *C. albicans* at 30°C. Times of incubation were as follows: YPD + serum, 6 days; Lee, 9 days; Spider, 9 days; Spider + glucose, 5 days; YPS, 3 days. **(C)** Filamentous growth of *C. albicans* at 37°C. Times of incubation were as follows: YPD + serum, 3 days; Lee, 6 days; Spider, 6 days; Spider + glucose, 4 days; YPS, 3 days. **(D)** The *sdh2*Δ/Δ mutant cannot grow on nonfermentable carbon sources.
YPD: Contains fermentable glucose as carbon source; YPE: Contains nonfermentable ethanol as carbon source; YPG: Contains nonfermentable glycerol as carbon source.

radial hyphae at all tested temperatures (25, 30 and 37°C) (Figure 2A–C), although the *sdh2*Δ/Δ colonies were still wrinkled at 37°C (Figure 2C).

In addition, the *sdh2*Δ/Δ colonies appeared to be small on Spider agar (with nonfermentable mannitol as carbon source), suggesting a specific growth defect on this medium. Moreover, after incubation at 30°C for 24 h, no obvious growth was observed of the *sdh2*Δ/Δ mutant on YPG (with nonfermentable glycerol as carbon source) and YPE (with nonfermentable ethanol as carbon source) agar (Figure 2D). In contrast, the *sdh2*Δ/Δ mutant grew on YPD (with fermentable glucose as carbon source), YPM (with fermentable maltose as carbon source) and YPS (with fermentable sucrose as carbon source) agar (Supplementary Figure 1), suggesting that the *sdh2*Δ/Δ mutant prefers fermentable carbon sources.

Ethanol accumulates in *sdh2*Δ/Δ cells grown on a fermentable carbon source, but this is not responsible for their defect in filamentation

Based on the findings above, our initial hypothesis was that the *sdh2*Δ/Δ mutant depends on fermentation for growth. Since ethanol is the main product of fermentation in yeast [24], we measured the ethanol content within different *C. albicans* colonies. As expected, ethanol content significantly increased in the *sdh2*Δ/Δ mutant, compared with the wild-type strain grown on hypha-inducing media (Spider + glucose, $p < 0.05$; YPD + serum, $p < 0.05$; Figure 3A).

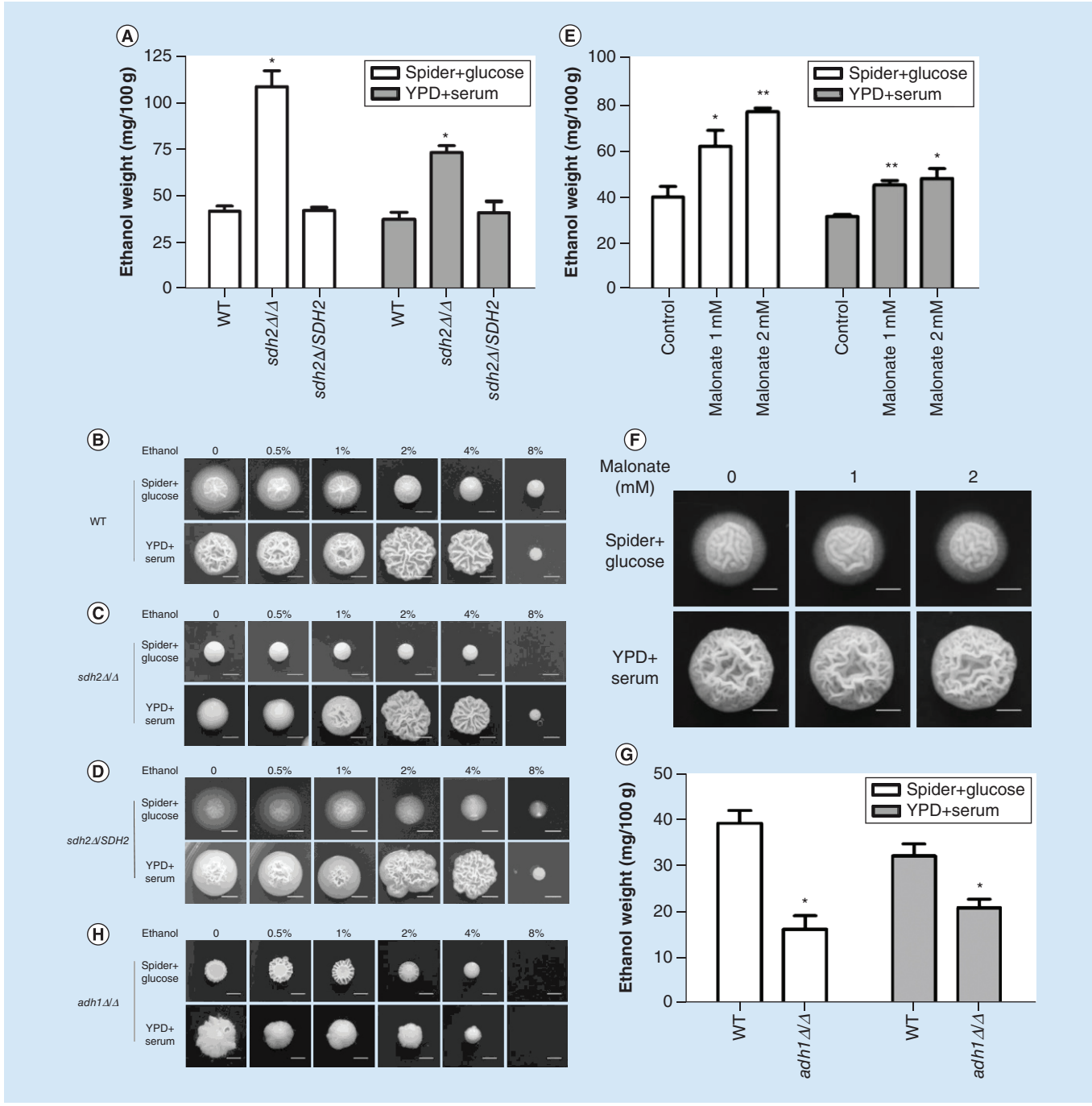

Figure 3. Ethanol accumulates in the *sdh2*Δ/Δ mutant with fermentable carbon source, but this is not related to the filamentation defects of the *sdh2*Δ/Δ mutant. (A) The deletion of *SDH2* results in ethanol accumulation in *Candida albicans*. The ethanol content per 100 g *C. albicans* is shown. *$p < 0.05$ compared with the wild-type control group. **(B–D)** Ethanol did not inhibit the filamentation of *C. albicans* in a dose-dependent manner. Ethanol at a range of concentrations was added to the media. WT, *sdh2*Δ/Δ, *sdh2*Δ/*SDH2* cells plated on Spider + glucose medium were incubated at 30°C for 3 days; *C. albicans* on YPD: + serum medium was incubated at 37°C for 3 days. **$p < 0.01$ compared with the control group without treatment. **(E & F)** Malonate treatment affects ethanol accumulation in *C. albicans*, but the treatment does not result in filamentation defect. **(G & H)** The *adh1*Δ/Δ mutant has less intracellular ethanol content, while this strain does not exhibit stronger filamentation phenotype.

Previous studies reported that ethanol could inhibit germ tube formation and the elongation of germ tubes [25]. To investigate whether the increased ethanol content is associated with the filamentation defects of *C. albicans*, we supplemented ethanol into hypha-inducing media. Unexpectedly, ethanol did not inhibit the filamentation of *C. albicans* in a dose-dependent manner, and 2 and 4% ethanol even enhanced the filamentous growth of *C. albicans* on YPD + serum medium (Figure 3B–D). To confirm that ethanol could not effectively suppress filamentation, we used malonate to disrupt the TCA cycle and thereby increase the intracellular ethanol content [26]. Although ethanol indeed accumulated in malonate-treated cells (Figure 3E), this was not accompanied by a defect in filamentation (Figure 3F). Moreover, we examined the *adh1*Δ/Δ mutant, which despite having a lower ethanol content (Figure 3G), actually displays a defect in filamentation rather than an enhancement (Figure 3H). Taken together, these findings suggested that ethanol accumulation is not associated with the filamentation defects of *C. albicans*. Thus, another mechanism must account for the filamentation defects of the *sdh2*Δ/Δ mutant.

A succinate dehydrogenase inhibitor specifically disrupting the electron transport chain mimics the impact of *SDH2* deletion

As a succinate: ubiquinone oxidoreductase, SDH has two distinct inhibitors: malonate and carboxin [27,28]. Malonate is an analog of succinate that binds to the site where succinate normally binds, inhibiting the TCA cycle [12,26–27], while carboxin selectively binds to the site where quinone normally binds, disrupting respiration by interfering with the ETC [12].

Since *SDH2* is predicted to encode an iron–sulfur subunit of SDH that is involved in both the TCA cycle and the ETC [11,12], we used carboxin, an SDH inhibitor targeting the ETC [12], to investigate if the virulence and filamentation defects of the *sdh2*Δ/Δ mutant is caused by the blockage of the ETC. Indeed, carboxin dose-dependently decreased virulence (Figure 4A) and abolished the filamentation of wild-type *C. albicans* in the *C. elegans* model (Figure 4B), as well as *in vitro* (Figure 4C). Moreover, carboxin (at 1.5 mM) treatment also inhibited the growth of wild-type *C. albicans* on media containing nonfermentable glycerol (YPG medium) or ethanol (YPE medium) as carbon sources (Figure 4D). These findings are in accordance with the phenotype observed in the *sdh2*Δ/Δ mutant, suggesting that the attenuated virulence, filamentation defects, and inability to grow on nonfermentable carbon sources of the *sdh2*Δ/Δ mutant may be caused by the blockage of the ETC.

To investigate if the phenotype of *sdh2*Δ/Δ is also associated with the blockage of the TCA cycle, we further used malonate to inhibit the TCA cycle [12,27]. In contrast to carboxin, malonate neither improved the survival of *C. elegans* with candidiasis nor abolished the filamentous growth of *C. albicans* (Figure 5A–C). Moreover, malonate did not affect the growth of the wild-type *C. albicans* on nonfermentable carbon sources (Figure 5D). In order to confirm that the virulence and filamentation defects are not associated with the blockage of the TCA cycle, we also investigated the role of a TCA cycle specific gene *FUM12* which encodes the fumarate hydratase (http://www.candidagenome.org/). In accordance with the findings on malonate, the deletion of *FUM12* did not affect the virulence of *C. albicans* in the *C. elegans* model (Figure 5E), and the *fum12*Δ/Δ mutant exhibited similar filamentous growth with the wild-type strain (Figure 5F & G).

ATP content is normal, while ROS accumulates intracellularly in the *sdh2*Δ/Δ mutant, leading to the filamentation defects of the mutant

As detailed so far, we found that carboxin decreased the virulence of *C. albicans* in the *C. elegans* candidiasis model and inhibited the filamentation of *C. albicans*, which is in accordance with the phenotype observed in the *sdh2*Δ/Δ mutant, whereas malonate treatment neither attenuated virulence nor inhibited filamentation. Given that ETC is a critical process in respiration [29], a major energy provider to the cell [29], we next investigated the influence of *SDH2* on the abundance of intracellular ATP. Interestingly, the ATP content in the *sdh2*Δ/Δ mutant was similar to that in the wild-type strain in the culture media tested in this study (Figure 6A & B).

Previous studies suggest that SDH plays an important role in delivering electrons in the ETC, and suppressing the function of Sdh2p (SdhB) may increase ROS production in mammalian cells [30]. We thereby investigated the intracellular ROS abundance of *C. albicans*. Notably, the *sdh2*Δ/Δ mutant exhibited significantly increased intracellular ROS level compared with the wild-type or the reintegrated strains (Figure 7A). We further clarified the relationship between intracellular ROS abundance and filamentation using pharmacological tools and various culture conditions. To investigate whether the increased intracellular ROS is associated with the filamentation defects of *C. albicans*, we supplemented ROS-inducing agents, including H_2O_2, and menadione into the *C. albicans* culture media. As expected, the ROS inducing agents dose-dependently led to ROS accumulation in

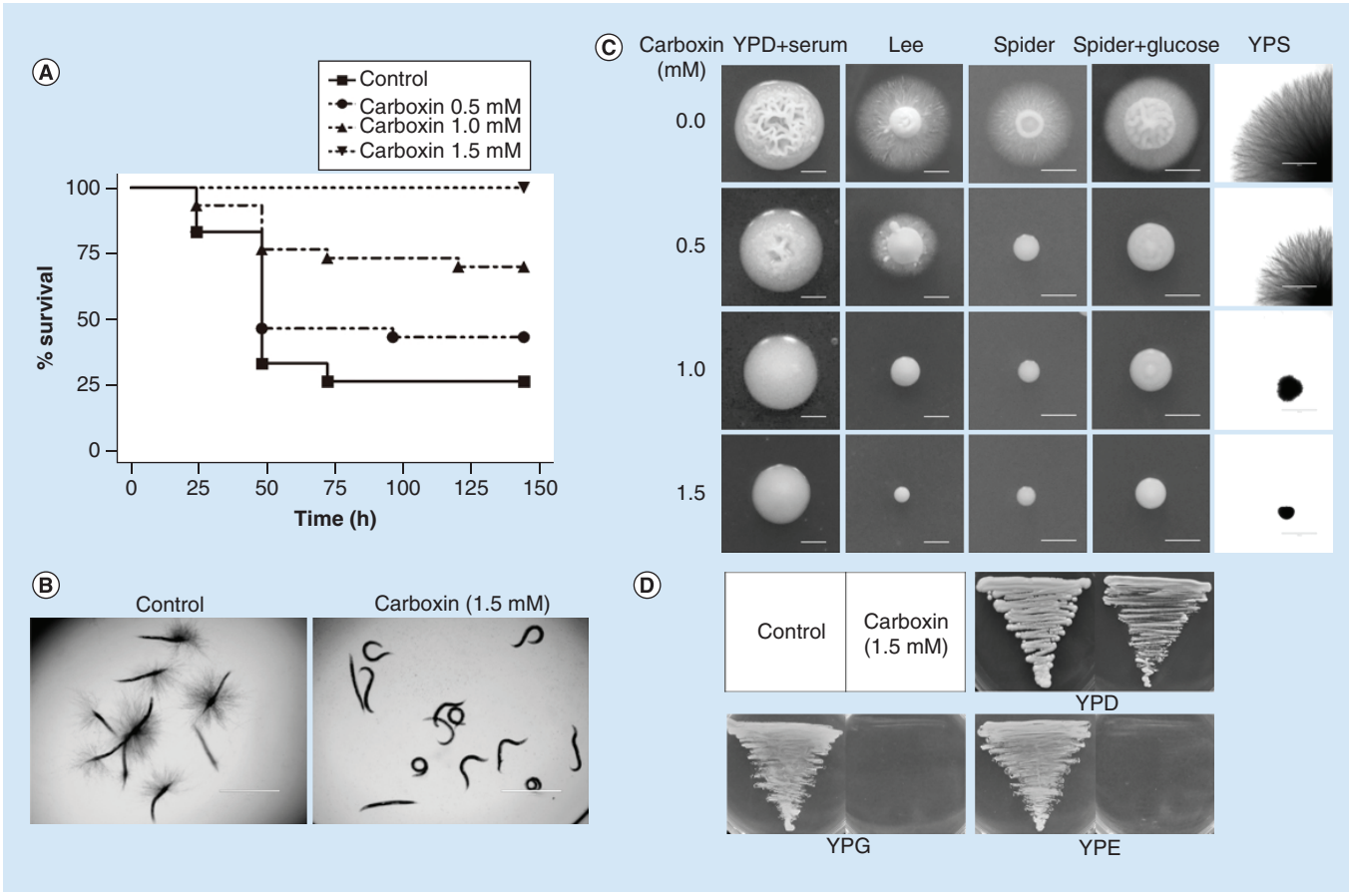

Figure 4. Carboxin attenuates the virulence and filamentous growth of the wild-type *Candida albicans*. (A) Carboxin decreases the virulence of *C. albicans* and dose-dependently improves the survival of worms in the *Caenorhabditis elegans* candidiasis model. 1.5 mM carboxin protects the *C. elegans* completely. **(B)** 1.5 mM carboxin completely inhibits the filamentation of *C. albicans in vivo* in the *C. elegans* candidiasis model. **(C)** Carboxin dose-dependently inhibits the filamentous growth of *C. albicans in vitro*. **(D)** Carboxin at 1.5 mM completely abolishes the growth of wild-type *C. albicans* on nonfermentable carbon sources.
YPD: Contains fermentable glucose as carbon source; YPE: Contains nonfermentable ethanol as carbon source; YPS: With fermentable sucrose as carbon source.

C. albicans (Figure 7B). Accordingly, the ROS-inducing agents inhibited the filamentous growth of *C. albicans* dose-dependently (Figure 7C).

Moreover, we evaluated if decreasing intracellular ROS could rescue the filamentation of the *sdh2*Δ/Δ mutant. First, we used antioxidative vitamin E and proanthocyanidins. As expected, the antioxidants decreased intracellular ROS of *C. albicans* (Figure 7D). Meanwhile, the filamentation of the *sdh2*Δ/Δ mutant was actually rescued by these antioxidants, and radial hyphae were seen on the *sdh2*Δ/Δ colonies (Figure 7E). Given that respiration is the main resource of intracellular ROS [31,32], we further used low oxygen or anoxia culture condition to suppress respiration and thereby inhibit intracellular ROS production. Under both the low oxygen and the anoxia conditions, the *sdh2*Δ/Δ mutant exhibited similar filamentous growth to the wild-type strain (Figure 7E), as expected. In contrast, the filamentation defect of a *efg1*Δ/Δ *cph1*Δ/Δ strain could not be rectified by low oxygen or anoxia (Figure 7F), which served as a control in this experiment. Taken together, these series of experiments indicate that the *SDH2* deletion results in ETC disruption and thereby ROS accumulation, and the increased intracellular ROS leads to filamentation defects of *C. albicans*.

Discussion

In this study, we found that the deletion of *SDH2* significantly attenuates the virulence of *C. albicans*, as evidenced by the increased survival and decreased fungal burden of the *sdh2*Δ/Δ mutant infected hosts. A prominent reason for the attenuated virulence is the filamentation defects of the *sdh2*Δ/Δ mutant. The ability of *C. albicans* to form

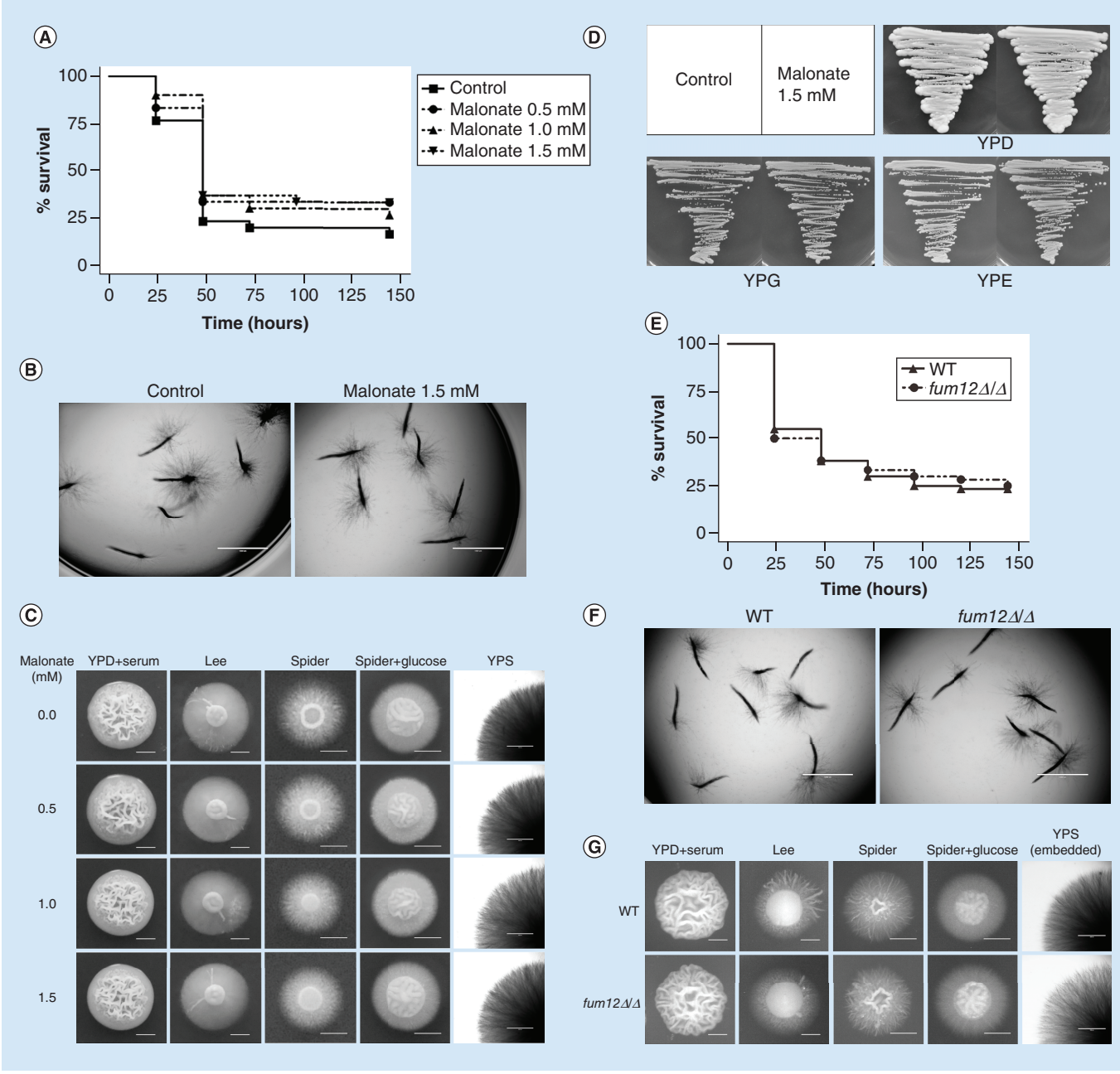

Figure 5. Malonate and *FUM12* do not affect the virulence and filamentous growth of *Candida albicans*. (A & B) Malonate does not attenuate the virulence and filamentation of wild-type *C. albicans* in the *Caenorhabditis elegans* candidiasis model. **(C)** Malonate does not affect the filamentous growth of wild-type *C. albicans in vitro*. **(D)** Malonate does not affect the growth of wild-type *C. albicans* on YPD, YPG and YPE agar plates. **(E & F)** The deletion of *FUM12* does not attenuate the virulence and filamentation of *C. albicans* in the *C. elegans* candidiasis model. **(G)** The deletion of *FUM12* does not affect the filamentous growth of *C. albicans in vitro*.
YPD: Contains fermentable glucose as carbon source; YPE: Contains nonfermentable ethanol as carbon source; YPG: Contains nonfermentable glycerol as carbon source; YPS: With fermentable sucrose as carbon source.

filaments is an important virulence attribute [33]. Mutants unable to form hyphae are reported to be avirulent [34]. In this study, the *sdh2*Δ/Δ mutant exhibited obvious filamentation defects: the *sdh2*Δ/Δ mutant could not develop filaments to puncture the cuticle of the *C. elegans* or form hyphal clusters in the kidneys of mice, and it exhibited obvious filamentation defects on all tested solid hypha-inducing media. The filamentation defects of the *sdh2*Δ/Δ mutant may well contribute to its attenuated virulence. Another contributing factor to the attenuated virulence may be the inability of the *sdh2*Δ/Δ mutant to grow on nonfermentable carbon sources. Recent studies have suggested

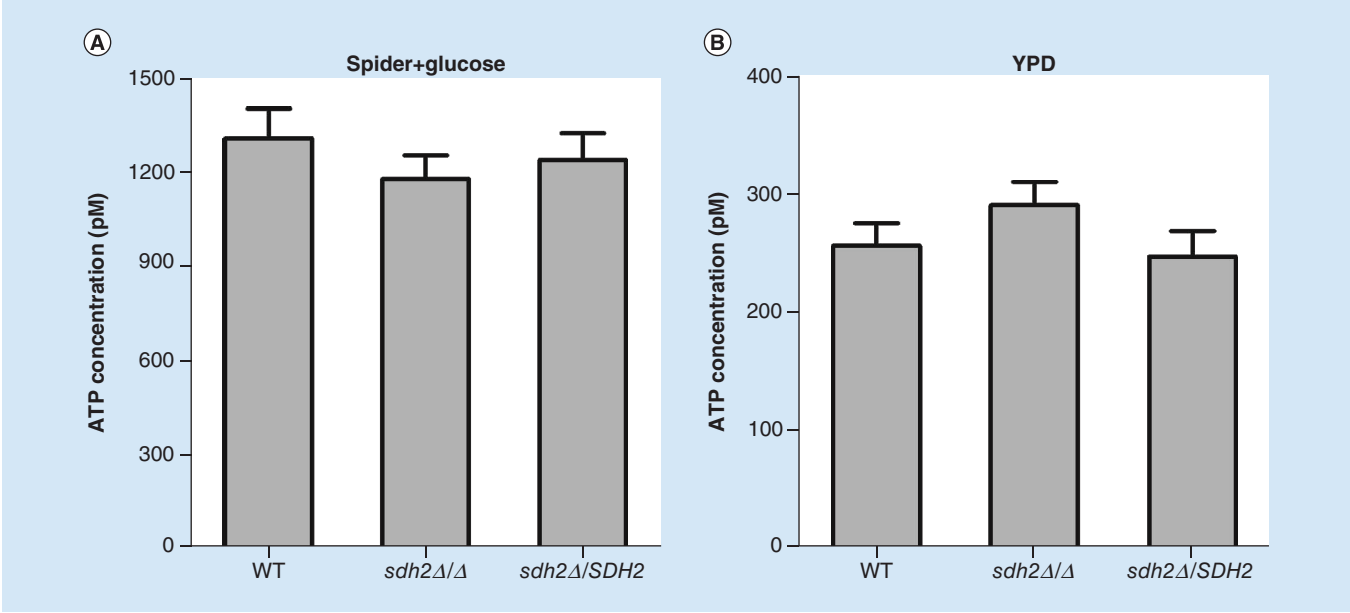

Figure 6. Intracellular ATP content in *Candida albicans* wild-type, *sdh2*Δ/Δ and *sdh2*Δ/*SDH2* strains. (A) ATP content of the strains in Spider + glucose medium. **(B)** ATP content of the strains in YPD medium. The ATP content in the *sdh2*Δ/Δ mutant was not less than that in the wild-type strain.
YPD: Contains fermentable glucose as carbon source.

that during systemic infections of *C. albicans*, few niches are rich in glucose and pathogenic microbes must assimilate a range of carbon sources to grow and colonize their hosts [35,36]. In particular, upon phagocytosis by macrophages *C. albicans* has to induce alternative carbon metabolism and form hyphae to escape from macrophages [37]. Thereby, the ability of *C. albicans* to assimilate a range of carbon sources is also important in virulence. The *sdh2*Δ/Δ mutant could not grow on nonfermentable carbon sources (Supplementary Figure 1) and this could have contributed in the virulence phenotype.

SDH2 encodes a subunit of SDH that is involved in both the TCA cycle and the ETC [11,12]. SDH catalyzes the oxidation of succinate to fumarate in the TCA cycle [11,12], which is coupled to the reduction of ubiquinone to ubiquinol as a part of the ETC [11,12]. Thereby, SDH is also known as mitochondrial complex II [12]. In the present study, we found that the role of *SDH2* in virulence is specifically related to its function in ETC, but not TCA. Disruption of the SDH function in TCA using malonate had no obvious influence on virulence, filamentation, and growth on nonfermentable sources. In contrast, disruption of the SDH function in ETC in wild-type *C. albicans* using carboxin mimics the phenotype from deletion of *SDH2*, including the virulence in *C. elegans* candidiasis model, filamentation, and growth observation using nonfermentable carbon sources.

Since suppressing the function of Sdh2p (SdhB) disrupts its role in delivering electrons in the ETC and thereby leads to ROS accumulation [30], we analyzed the association between intracellular ROS content and filamentation. We used ROS-inducing agents including H_2O_2 and menadione to increase intracellular ROS abundance, and antioxidants, such as vitamin E and proanthocyanidins, to decrease intracellular ROS; furthermore, we used low oxygen or anoxia culture condition to suppress respiration and thereby inhibit the intracellular ROS production. With the increase of intracellular ROS, filamentation is inhibited, while with the decrease of intracellular ROS, filamentation is enhanced. Importantly, the filamentation defects of the *sdh2*Δ/Δ mutant were rescued by decreasing intracellular ROS under various conditions, including supplementation with antioxidants, and low oxygen or anoxia culture conditions. Thus, it can be inferred that intracellular ROS accumulation results in the filamentation defects of the *sdh2*Δ/Δ mutant.

Interestingly, although *SDH2* deletion disrupts ETC in aerobic respiration which plays a leading role in supplying ATP [38], the deletion of *SDH2* did not result in reduced ATP levels in this study. A possible explanation is that ATP can be produced through fermentation [39]. In our experiment setting that enough glucose (at least 100 mM) was supplied, sufficient ATP could be generated through fermentation.

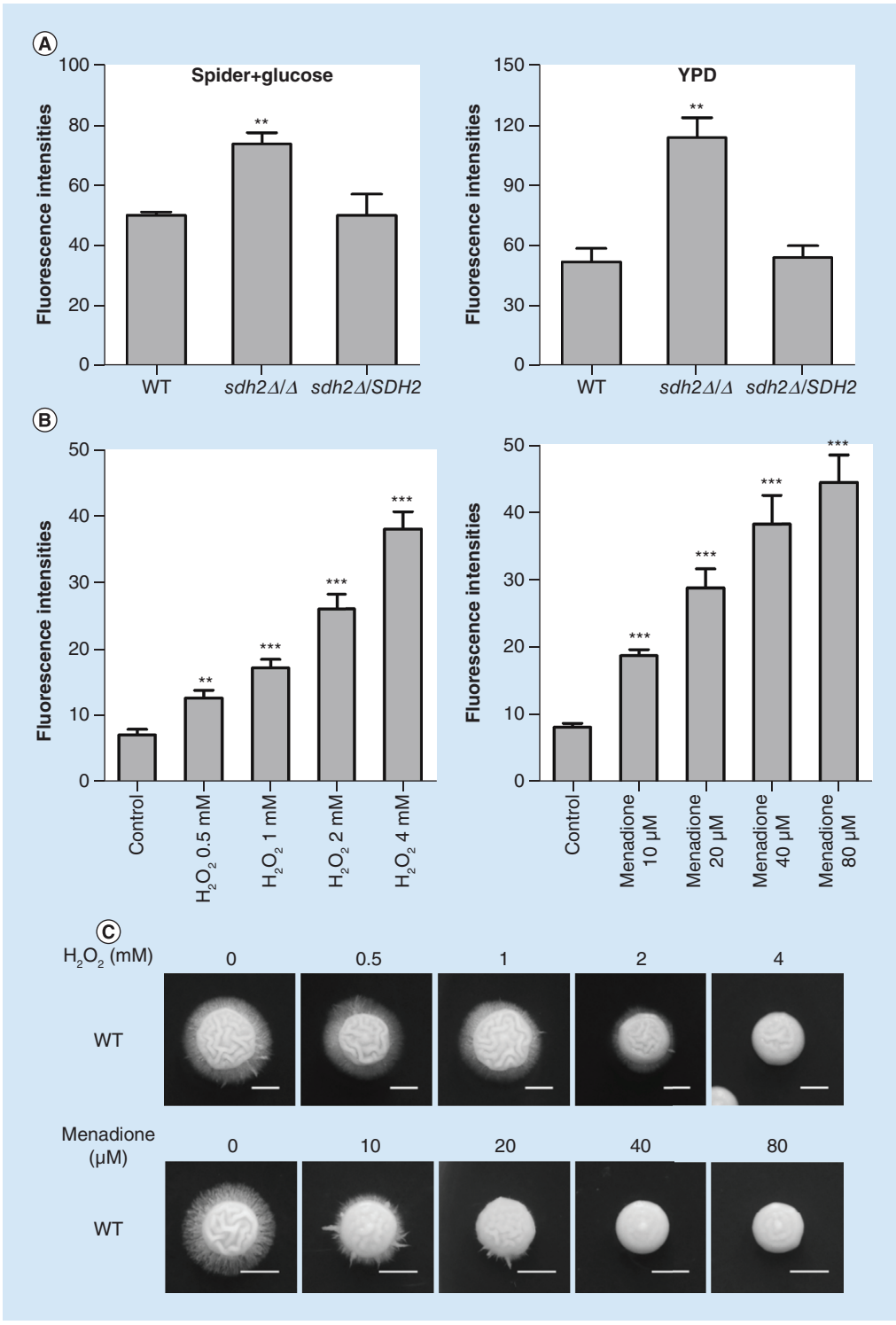

Figure 7. Increased intracellular eactive oxygen species leads to filamentation defects of *Candida albicans*, and decreasing intracellular reactive oxygen species can rescue the filamentation of *sdh2*Δ/Δ. (A) The *sdh2*Δ/Δ mutant exhibits significantly increased intracellular reactive oxygen species (ROS). **p < 0.01 compared to the wild-type control group. **(B)** ROS-inducing agents dose-dependently lead to ROS accumulation in wild-type *C. albicans*. ROS-inducing agents include H_2O_2 and menadione. **p < 0.01; ***p < 0.001 compared with the wild-type *C. albicans* cells without treatment. **(C)** The ROS-inducing agents inhibit the filamentous growth of *C. albicans* dose-dependently. **(D)** Antioxidative vitamin E and proanthocyanidins decreased intracellular ROS of *C. albicans*. *p < 0.05; **p < 0.01 compared with the group without treatment. #p < 0.05 compared with wild-type *C. albicans*. **(E)** The filamentation of *sdh2*Δ/Δ is rescued by the antioxidants (vitamin E and proanthocyanidins). **(F)** The filamentation of *sdh2*Δ/Δ is rescued under both the low oxygen and the anoxia conditions, while *efg1*Δ/Δ *cph1*Δ/Δ remains filamentation defective.

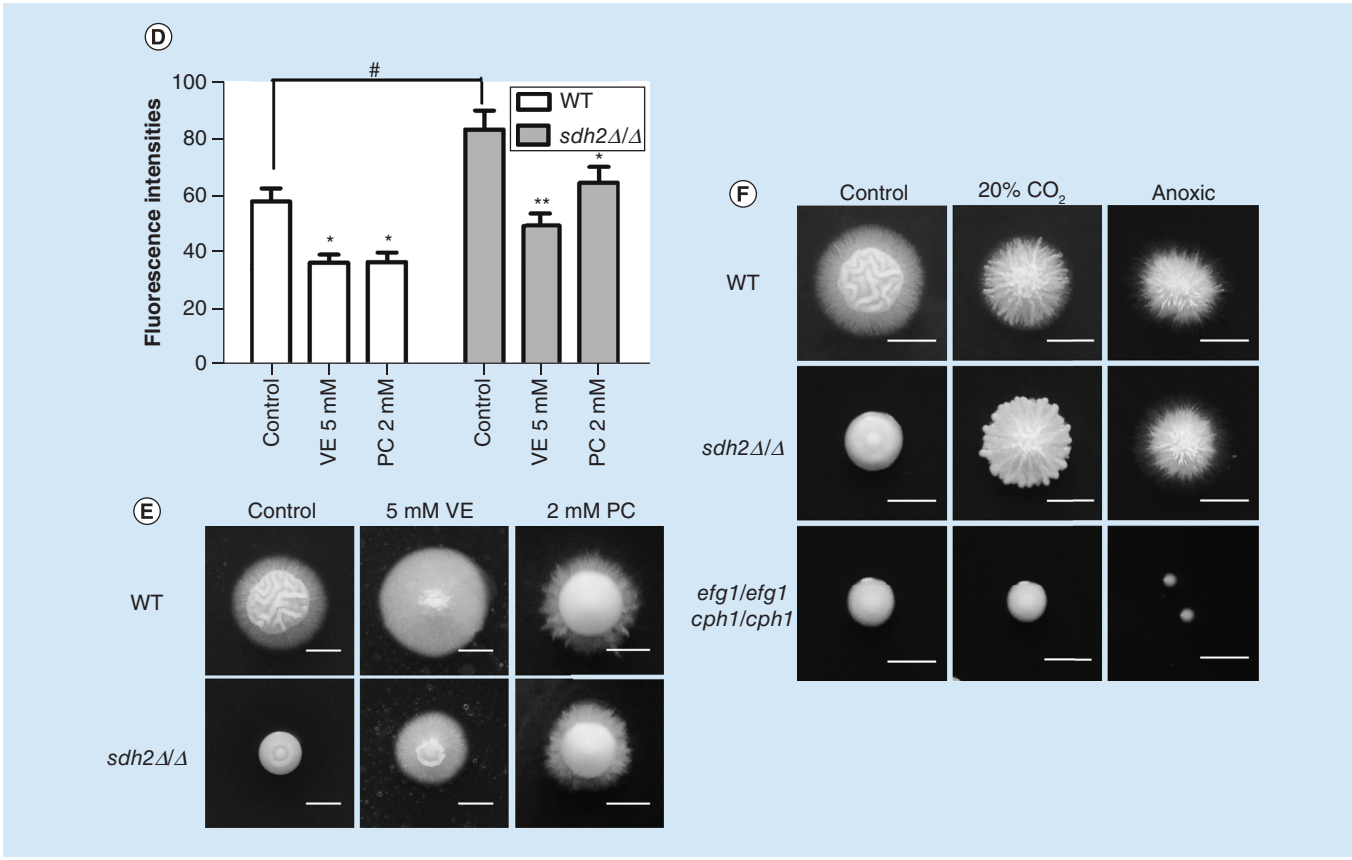

Figure 7. Increased intracellular eactive oxygen species leads to filamentation defects of *Candida albicans*, and decreasing intracellular reactive oxygen species can rescue the filamentation of *sdh2*Δ/Δ (cont.). (A) The *sdh2*Δ/Δ mutant exhibits significantly increased intracellular reactive oxygen species (ROS). **$p < 0.01$ compared to the wild-type control group. **(B)** ROS-inducing agents dose-dependently lead to ROS accumulation in wild-type *C. albicans*. ROS-inducing agents include H_2O_2 and menadione. **$p < 0.01$; ***$p < 0.001$ compared with the wild-type *C. albicans* cells without treatment. **(C)** The ROS-inducing agents inhibit the filamentous growth of *C. albicans* dose-dependently. **(D)** Antioxidative vitamin E and proanthocyanidins decreased intracellular ROS of *C. albicans*. *$p < 0.05$; **$p < 0.01$ compared with the group without treatment. #$p < 0.05$ compared with wild-type *C. albicans*. **(E)** The filamentation of *sdh2*Δ/Δ is rescued by the antioxidants (vitamin E and proanthocyanidins). **(F)** The filamentation of *sdh2*Δ/Δ is rescued under both the low oxygen and the anoxia conditions, while *efg1*Δ/Δ *cph1*Δ/Δ remains filamentation defective.

Another notable finding is that the ATP content of the cells in Spider + glucose medium is approximately four-times higher compared with that in YPD medium. Looking at this difference, the nutrients in the two media are different. YPD is nutrient-rich, while Spider is a hypha-inducing medium. In this series of experiments, the intracellular ATP content of cells in Spider + glucose medium was higher. This finding is consistent with previous reports indicating that high intracellular ATP content is associated with hypha-inducing signals [40]. Ras1, Cyr1 and Ira2 may be the major regulators of this reported association [40]. More specifically, Ras1, Cyr1 and Ira2 are upstream of a critical hypha-inducing signaling pathway, the cAMP-PKA pathway [23]. In hypha-inducing medium, Ras1, Cyr1 and Ira2 may form a master-regulatory complex, integrating different environmental and intracellular signals, including metabolic status [40]. Therefore, hypha-inducing signals are associated with high intracellular ATP levels, such as that provided by Spider + glucose medium in this study.

Notably, we observed that on YPD + 10% serum agar 1–4% ethanol could rescue the filamentation of the *sdh2*Δ/Δ mutant. Some previous studies have revealed that fusel alcohols and ethanol could induce a switch from vegetative to pseudohyphal growth [41]. More specifically, in nutrition-rich YPD medium, 2–6% ethanol could inhibit protein synthesis and promote the filamentation of *C. albicans*, even in the *efg1*Δ/Δ mutant and *cph1*Δ/Δ mutant, indicating a nonclassical filament-regulating pathway [41]. Our finding that ethanol could induce filamentous growth on YPD + 10% serum agar, even in the *sdh2*Δ/Δ mutant, may also be due to the translation-inhibitory effect of ethanol.

Taken in its totality, this study sheds light on the role of SDH in the pathogenicity of *C. albicans* and suggests that disrupting the ETC through blocking the quinone binding site could serve as a novel target for antifungal drug discovery.

Conclusion & future perspective

SDH plays an important role in *C. albicans* filamentation and virulence, mediated through its role of SDH in the ETC, probably through affecting intracellular ROS. The electron transport site of SDH may thus provide a target for antifungal drug discovery. Future studies on the association between the *C. albicans* redox system and filamentation may help identify additional *C. albicans* virulence factors that could serve as potential antifungal targets.

Summary points

SDH2* is required for *Candida albicans* virulence *in vivo

- Based on *C. albicans* mutant libraries screening, the *SDH2 null* mutants was avirulent in a *Caenorhabditis elegans* candidiasis model.
- The *sdh2*Δ/Δ mutant lacked hypha piercing the nematode worm cuticle in the *C. elegans* candidiasis model.
- The *sdh2*Δ/Δ mutant was hypovirulent in mouse candidiasis model.
- The *sdh2*Δ/Δ mutant exhibits filamentation defects.
- The *sdh2*Δ/Δ mutant exhibits growth defects on nonfermentable carbon sources.
- Ethanol accumulation in *sdh2*Δ/Δ cells grown on a fermentable carbon sources is not responsible for filamentation defects.
- A succinate dehydrogenase inhibitor specifically disrupting the electron transport chain (ETC) mimics the impact of the *SDH2* deletion.
- Carboxin, an SDH inhibitor targeting the ETC, mimics the impact of the *SDH2* deletion.
- Malonate, an SDH inhibitor targeting the tricarboxylic acid cycle does not affect the virulence and filamentation of *C. albicans*.
- The contribution of *SDH2* to filamentation and virulence is specifically associated with the ETC, but not the tricarboxylic acid cycle.
- The filamentation defects of the *sdh2*Δ/Δ mutant are not due to ATP insufficiency in cells grown on a fermentable carbon source.
- ROS accumulated in the *sdh2*Δ/Δ mutant, leading to filamentation defects.
- The *sdh2*Δ/Δ mutant exhibited significantly increased intracellular ROS.
- ROS-inducing agents inhibited the filamentous growth of *C. albicans* dose-dependently.
- The filamentation defects of the *sdh2*Δ/Δ mutant were rescued by antioxidative agents decreasing intracellular ROS.
- The filamentation defects of the *sdh2*Δ/Δ mutant were rescued by decreasing intracellular ROS under low oxygen or anoxia culture conditions.

Conclusion

- *SDH2* is involved in proper hypha formation and virulence in *C. albicans*.
- The impact of *SDH2* on filamentation and virulence is mediated by its role in the ETC.
- The role of *SDH2* in filamentation is through affecting intracellular ROS.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fmb-2018-0033

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all animal experimental investigations. No human subjects were involved in this study.

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