AMPKα Subunit Ssp2 and Glycogen Synthase Kinases Gsk3/Gsk31 are involved in regulation of sterol regulatory element-binding protein (SREBP) activity in fission yeast

Abstract

Sterol regulatory element-binding protein (SREBP), a highly conserved family of membrane-bound transcription factors, is an essential regulator for cellular cholesterol and lipid homeostasis in mammalian cells. Sre1, the homolog of SREBP in the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), regulates genes involved in the transcriptional responses to low sterol as well as low oxygen. Previous study reported that casein kinase 1 family member Hhp2 phosphorylated the Sre1 N-terminal transcriptional factor domain (Sre1N) and accelerated Sre1N degradation, and other kinases might exist for regulating the Sre1 function. To gain insight into the mechanisms underlying the Sre1 activity and to identify additional kinases involved in regulation of Sre1 function, we developed a luciferase reporter system to monitor the Sre1 activity through its binding site called SRE2 in living yeast cells. Here we showed that both ergosterol biosynthesis inhibitors and hypoxia-mimic CoCl2 caused a dose-dependent increase in the Sre1 transcription activity, concurrently, these induced transcription activities were almost abolished in Δsre1 cells. Surprisingly, either AMPKα Subunit Ssp2 deletion or Glycogen Synthase Kinases Gsk3/Gsk31 double deletion significantly suppressed ergosterol biosynthesis inhibitors- or CoCl2-induced Sre1 activity. Notably, the Δssp2Δgsk3Δgsk31 mutant showed further decreased Sre1 activity when compared with their single or double deletion. Consistently, the Δssp2Δgsk3Δgsk31 mutant showed more marked temperature sensitivity than any of their single or double deletion. Moreover, the fluorescence of GFP-Sre1N localized at the nucleus in wild-type cells, but significantly weaker nuclear fluorescence of GFP-Sre1N was observed in Δssp2, Δgsk3Δgsk31, Δssp2Δgsk3, Δssp2Δgsk31 or Δssp2Δgsk3Δgsk31 cells. On the other hand, the immunoblot showed a dramatic decrease in GST-Sre1N levels in the Δgsk3Δgsk31 or the Δssp2Δgsk3Δgsk31 cells but not in the Δssp2 cells. Altogether, our findings suggest that Gsk3/Gsk31 may regulate Sre1N degradation, while Ssp2 may regulate not only the degradation of Sre1N but also its translocation to the nucleus.
**Introduction**

Sterol homeostasis is essential for eukaryotic cells to maintain the normal structure and fluidity of cell membrane as well as to regulate the function of membrane proteins and sterol synthesis. Sterol regulatory element binding protein (SREBP), a subfamily of basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factors that are widely conserved from fungi to human, is an important factor that regulates sterol levels in cells [1, 2]. SREBP, as a non-activated precursor protein synthesized in the endoplasmic reticulum (ER), consists of an N-terminal transcription factor domain and a C-terminal domain that forms a complex with a sterol sensing protein, SREBP cleavage activating protein (Scap) [3]. Under sterol replete conditions, Scap binds cholesterol, and the SREBP-Scap complex is retained in the ER [4]. Upon sterol depletion, Scap undergoes a conformational change and SREBP-Scap enters COPII vesicles for transport to the Golgi [5, 6], and then SREBP is cleaved sequentially in the Golgi, resulting in the release of the SREBP transcription factor domain, which then translocate into the nucleus and bind to the specific DNA sequence (sterol regulatory element, SRE) of the target genes involved in sterol synthesis [7].

In fission yeast, Sre1, the homologue of mammalian SREBP, is not only a factor for controlling sterol homeostasis but also a principal regulator of low oxygen gene expression [2, 8]. It has been reported that, upon conditions of low oxygen, ergosterol biosynthesis decreases, and Scp1, the homologue of Scap, transports Sre1 from the ER to the Golgi where Sre1 is proteolytically cleaved, releasing the active Sre1 N-terminal transcription factor fragment (Sre1N). Under low oxygen or low sterols conditions, the Sre1-Scp1 transports and cleavages at the Golgi increase dramatically. After released, Sre1N enters the nucleus and promotes transcription of the target genes involved in sterol synthesis as well as oxygen responsive genes. Upon reintroduction of oxygen or sterol, Sre1N degradation is accelerated through a proteasome-dependent pathway, allowing rapid down-regulation of Sre1N [9, 10]. Factors controlling Sre1 cleavage and activation have been largely studied [11, 12], but the mechanism underlying Sre1 degradation remains not fully understand.

The initial characterization of the fission yeast SREBP pathway revealed that the activated transcription factor Sre1N could be hyper-phosphorylated, indicating potential regulation of sterol homeostasis by kinases [8]. Recent studies have shown that a highly conserved casein kinase 1 family member Hhp2 phosphorylates Sre1N and accelerates Sre1N degradation, but it seems like that Hhp2 is not the sole Sre1N kinase. Sre1N contains at least 22 phosphorylated serine and threonine residues [13], suggesting that additional kinases might be involved in Sre1 activity regulation.

Here, our studies focus on whether additional protein kinases are involved in regulation of Sre1 activity. We monitored the transcriptional activity of Sre1 in living cells by using a luciferase reporter system with three tandem repeats of SRE2 fused to firefly luciferase gene. We found that ergosterol biosynthesis inhibitors- or hypoxia-mimic CoCl$_2$-induced Sre1 transcriptional activity was significantly suppressed in AMPK$_\alpha$ Subunit Ssp2 deletion or Glycogen Synthase Kinases Gsk3/Gsk31 double deletion as well as double deletion of Ssp2 and Gsk3 or Gsk31, respectively. In particular, the nuclear fluorescence of GFP-Sre1N were dramatically reduced in these deletion cells. In addition, the immunoblot showed a dramatic decrease in Sre1N levels in the Δgsk3/Δgsk31 or Δssp2Δgsk3/Δgsk31 cells but not in the Δssp2, Δssp2Δgsk3 or Δssp2Δgsk31 cells. Our findings reveal the involvement of AMPK$_\alpha$ Subunit Ssp2 and Glycogen Synthase Kinases Gsk3/31 in regulation of SREBP activity in fission yeast, which may pave a way for further studying similar mechanisms in higher eukaryotes.
Materials and methods

Strains, media, and genetic and molecular biology methods

*S. pombe* strains used in this study are listed in Table 1. The normal minimal medium EMM (Edinburgh minimal medium), the complete medium yeast extract-peptone-dextrose (YPD) and the rich yeast extract with supplements (YES) have been described previously [14]. Standard genetic and recombinant-DNA methods [15] were used except where noted. Gene disruptions are denoted by lower-case letters representing the disrupted gene followed by two colons and the wild type gene marker used for disruption (for example, *sre1*:::ura4+). Gene disruptions are abbreviated by the gene preceded by Δ (for example, Δ*sre1*). Proteins are denoted by Roman letters and only the first letter is capitalized (for example, Sre1) [16].

Plasmids constructions

A multicopy plasmid (pKB7665) containing the *nmt1* promoter without its *cis* element, three tandem repeats of SRE2-like sequence (ATCACCCCAT) which is the binding core of the Sre1 transcriptional activator identified in the *sre1*+ promoter, and the destabilized luciferase from pGL3 (R2.2) version containing PEST, CL1, and AU-rich repeats was constructed as described previously [17], except that the CDRE oligonucleotides were replaced by the SRE2-like oligonucleotides (sense, 5’-GGC TTA TCA CCC CAT ATA CAA TCA CCC CAT ATA CAC AAT CAC CCC ATA TGC AC-3’; antisense, 5’-TCG AGT GCA TAT CAC CCC ATT GTG TAT ATC ACC CCA TTG TAT ATC ACC CCA TAA GCC TGC A-3’, SRE2-like sequence underlined). Then, an integration 3×SRE2::luc (R2.2) plasmid was constructed by inserting the SRE2-like oligonucleotides and *arg1*+ into pBC SK(+) (Stratagene) to give pSY291 [18]. The multicopy plasmid and integration plasmid of 3×SRE2::luc (R2.2) were all used for real-time monitoring assay of Sre1-mediated transcriptional activity.

The truncated fragment of *sre1*+ gene, encoding the active N-terminal transcription factor domain of Sre1 (Sre1N) was amplified by PCR with the genomic DNA of *S. pombe* as a template. The sense primer used for PCR was 5’-CGC GGA TCC ATG CAA AGC TCA ATT

| Strain | Genotype | Reference |
|--------|----------|-----------|
| HM123  | h- leu1-32 | Our stock |
| KP1737 | h- leu1-32 ura4-D18 gk3::ura4’ gsk31::KanMX6 | [27] |
| KP1813 | h- leu1-32 ura4-D18 gk3::ura4’ | [27] |
| KP2101 | h- leu1-32 arg1 | [18] |
| KP2310 | h- leu1-32 ura4-D18 gk31::KanMX6 | [27] |
| KP3089 | h- leu1-32 ura4-D18 sp2::ura4’ | [27] |
| KP4275 | h- leu1-32 ura4-D18 sp2::ura4’ gsk31::KanMX6 | [22] |
| KP4283 | h- leu1-32 ura4-D18 sp2::ura4’ gk3::ura4’ | [27] |
| KP4304 | h- leu1-32 ura4-D18 ass1::loxP sp2::ass11’ | This study |
| KP5683 | h+ his2 leu1-32 ura4-D18 asn1::loxP gk3::ura4’ gsk31::KanMX6 | This study |
| KP6544 | h- leu1-32 ura4-D18 sre1::ura4’ | This study |
| CM109  | h- leu1-32 scp1::KanMX4 | This study |
| CM125  | h- leu1-32 hhp2::KanMX4 | This study |
| CM134  | h- leu1-32 ura4-D18 asn1::loxP sp2::asn1 gk3::ura4’ gsk31::KanMX6 | This study |
| CM150  | h- leu1-32 arg1::arg1-3×SRE2-Luciferase(type(2.2)) | This study |
| CM156  | h- leu1-32 ura4-D18 arg1 sre1::ura4’ | This study |
| CM172  | h- leu1-32 ura4-D18 sre1::ura4 arg1::arg1-3×SRE2-Luciferase(type(2.2)) | This study |

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CCG -3’ (BamHI site are underlined), and the antisense primer was 5’ -ACG CGT CGA CTT ATG GAG ACA TAA GAA AAG-3’ (SalI site are underlined). The amplified product was digested with BamHI/SalI, and the resulting fragment was subcloned into Bluescript SK (+) (Stratagene, USA). To assess the subcellular localization and the total protein levels of Sre1N, the truncated fragment of sre1+gene which encoding N-terminal transcripational factor domain was ligated to the C terminus of the GFP or GST expressing GFP-Sre1N or GST-Sre1N and subcloned into the pREP1 expression vector containing a thiamine-repressible nmt1 promoter [19]. Expression was repressed by the addition of 4 μM thiamine to EMM.

Real-time monitoring assay of Sre1-mediated transcriptional activity
The multicopy 3×SRE2::luc (R2.2) reporter plasmid pKB7665 was transformed into fission yeast wide-type cells and mutations to perform the luciferase reporter assays as described previously [20]. In addition, to obtain the chromosome-borne 3×SRE2::luc (R2.2), the integration reporter plasmid linearized with StuI was integrated into the chromosome at the arg1+ locus of both KP2101 (h− leu1-32 arg1) and CM156 (h− leu1-32 ura4-D18 arg1 sre1::ura4+) as described previously [18]. The cells transformed with the multicopy reporter plasmid or the chromosome-borne cells were selected and cultured in EMM or EMM with leucine to midlog phase at 30˚C and recovered by centrifugation respectively. Then the cells were resuspended in refresh EMM or EMM with leucine containing different concentrations of drugs. Luciferin was used as a substrate for Firefly luciferase, and yielding luminescence was detected using a luminometer (AB-2350; ATTO Co., Tokyo, Japan) at 1-min intervals and reported as relative light units (RLU).

Gene deletion
To delete the sre1+ gene, a one-step gene disruption by homologous recombination [21] was performed. The sre1::ura4+ disruption was constructed as follows. The cloned open reading frame of the sre1+ gene in pBluescript SK (Stratagene) was digested with HindIII, and the resulting fragment containing the sre1+ gene was subcloned into the HindIII site of the pBluescript vector. Then a BamHI fragment containing the ura4+ gene was inserted into the BamHI site of the previous construct, causing the interruption of the open reading frame. The fragment containing the disrupted sre1+ gene was transformed into haploid cells. Stable integrants were selected on medium lacking uracil, and disruption of the gene was checked by Southern blotting.

The deletion of either scp1+ or hhp2+ gene with a genetic background of h+ leu1-32 ura4-D18 ade6-M210 or M216 and the KanMX cassette was purchased from BioNEER (South Korea) [22]. We constructed scp1 or hhp2 deletion cells that were not auxotrophic for uracil and adenine by the genetic cross between wild-type cells HM123 and the above strains to make CM109 or CM125, respectively (Table 1). We constructed Δssp2Δgsk3Δgsk31 triple deletion by the genetic cross between KP4304 (h+ leu1-32 ura4-D48 asn1::loxp ssp2::asn1+) and KP5683 (h+ his2 leu1-32 ura4-D48 asn1::loxp gsk3::ura4+ gsk31::KanMX6) to make CM134 (h+ leu1-32 ura4-D48 asn1::loxp ssp2::asn1+gsk3::ura4+ gsk31::KanMX6) (Table 1).

Fluorescence microscopy
Cells transformed with pREP1-GFP-Sre1N were grown in EMM medium with 4 μM thiamine to attenuate the expression for 16 h at 30˚C, GFP-Sre1N was detected by its own fluorescence expressed in living cells by fluorescence microscope using a Nikon Eclipse Ni-U microscope equipped with a DS-Q2 camera (Nikon Instruments Inc., Japan). For measurement of fluorescence intensities, images of the cells expressing GFP-Sre1N were taken using an oil-immersion
objective lens (UAp0 100×, NA 1.3, Nikon) at NA = 0.65; The best-focused image of the eight optical sections was selected for quantification. Quantification of fluorescence intensities was determined as follows. Specify a region including the nucleus, and calculate the mean fluorescence intensity (Mean\(N\)) and area values (Area\(N\)) of the nuclear region. Calculate the mean fluorescence intensity in a cytoplasmic region of the same cell as the background mean fluorescence intensity (Mean\(B\)). Calculate GFP-Nucleus fluorescence intensity on NE (\(F_{NE}\)) according to the following formula: 
\[
F_{N} = (\text{Mean}_N - \text{Mean}_B) \times \text{Area}_N.
\]
Measure the fluorescence intensities of about 50 cells in each strain, and calculate average values and standard deviations. Wide-type cells fluorescence intensity was set to one in calculating relative fluorescence intensities [23, 24].

**Cell extract preparation and immunoblot analysis**

For the analysis of the total Sre1N protein in various mutants, whole cell extracts were prepared from cultures of wild-type cells or mutants harboring pREP1-GST-Sre1N plasmid grown at 30˚C to mid-log phase. Total cell lysates were prepared as follows. Approximately 2 × 10\(^7\) cells were resuspended in 500 μl of homogenizing buffer (92.5% 2N NaOH, 7.5% β-mercaptoethanol). After cooling on ice for 10 min, the proteins were precipitated by the addition of 500 μl of 50% trichloroacetic acid. Then, cellular debris was removed by centrifugation at 14000 rpm for 5 min. The resulting protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [25]. We used separating acrylamide gels (10.0%) with a mono/bis ratio of 29:1 to detect Sre1N. Purified polyclonal anti-GST was used as the primary antibodies, and goat anti-rabbit immunoglobulin G (IgG) Fc fragments was used as the secondary antibodies. Enhanced chemiluminescence was used for immunodetection on the membrane.

**Statistical analyses**

Quantitative data were expressed as means ± S.D. Multiple comparisons were statistically analyzed by one-way ANOVA followed by Tukey’s test. The difference was considered to be significant, if P value is less than 0.05. All statistical analyses were performed using the SPSS 16.0 software package (SPSS, Inc., Chicago, IL, USA).

**Results**

**Real-time monitoring of Sre1 activity in living cells**

Mammalian SREBPs bind a 10-bp DNA sequence in the promoters of target genes, called the sterol regulatory element (SRE) [7]. In fission yeast, two DNA elements (SRE2 and SRE3) in the promoter of \(sre1^+\) gene were identified to be necessary and sufficient for oxygen-dependent, Sre1-dependent transcription, but the binding of Sre1 to SRE2 was stronger than to SRE3 [26]. In order to monitor the real-time Sre1 activity for further studying the regulation mechanisms underlying the Sre1 activity, we constructed multicopy reporter plasmid containing three tandem repeats of SRE2 fused to firefly luciferase gene. As shown in Fig 1A–1C, ergosterol biosynthesis inhibitors including clotrimazole (CLZ), terbinafine (TER), and fenpropimorph (FEN), increased the luciferase activity with a peak rise at about 12–13 hours through the SRE2 motif in a dose-dependent manner (Fig 1A–1C). Since it is known that Sre1 can bind to the SRE2 motif for its transcriptional control, we examined the involvement of Sre1 in ergosterol biosynthesis inhibitors-induced activation of SRE2 reporter. The results showed that these ergosterol biosynthesis inhibitors-induced increase in SRE2 reporter activity was completely abolished in \(Δsre1\) cells (Fig 1D–1F), suggesting that the multicopy reporter
assay reflected Sre1 activity upon low ergosterol conditions. Given that Scp1 is required for Sre1 cleavage in fission yeast [8], we also examined the effect of Scp1 deletion on ergosterol
biosynthesis inhibitors-induced SRE2 reporter activity. As shown in Fig 1D–1F, ergosterol biosynthesis inhibitors-induced increase in SRE2 reporter activity was also abolished in Δscp1 cells (Fig 1D–1F), further strengthening the reliability of our reporter system.

CoCl2 activates Sre1-dependent SRE2 reporter activity

It has been known that Sre1 functions as an important oxygen sensor in fission yeast and Sre1 can be proteolytically cleaved and activated under low oxygen conditions [8]. We then tested whether hypoxia mimic Cobalt chloride (CoCl2) could activate SRE2 reporter. As shown in Fig 2A, CoCl2 also caused a marked dose-dependent increase in the SRE2 reporter response with a peak at about 13 hours in wild-type cells (Fig 2A). Consistently, in Δsre1 cells, the multicopy reporter response was reduced by two orders of magnitude compared to wild-type cells, indicating that CoCl2 could activate Sre1-dependent SRE2 reporter activity (Fig 2B). Likewise, the Δscp1 mutant showed significantly lower SRE2 reporter activity (Fig 2B).

Considering that the copy number of the multicopy plasmid stably maintained cells might be affected by various factors, we constructed wild-type and Δsre1 chromosome-borne 3×SRE2::luc (R2.2) cells, respectively (CM150 and CM172 listed in Table 1). Similar to the results of cells harboring the multicopy 3×SRE2::luc (R2.2) reporter, the wild-type chromosome-borne 3×SRE2::luc (R2.2) cells could be activated by various concentrations of CLZ, TER or CoCl2 (Fig 3A–3C) in a dose-dependent manner with a similar peak rise time. Likewise, an extremely low response upon stimulation was observed in Δsre1 chromosome-borne 3×SRE2::luc (R2.2) cells (Fig 3D–3F). These results indicate that both the episomal multicopy and the chromosome integration 3×SRE2::luc (R2.2) reporter could reflect Sre1 activity in living cells.

Deletion of Ssp2 or/and Gsk3/Gsk31 markedly suppressed CLZ, TER or CoCl2-induced Sre1 activity

Next, our studies focused on whether additional protein kinases were involved in regulation of Sre1 activity. Consistent with the notion that casein kinase 1 family member Hhp2 accelerates

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Fig 2. CoCl2 activates Sre1-dependent SRE2 reporter activity. CoCl2 (0.08 mM to 0.32 mM) induced a marked increase in Sre1 transcriptional activity. Wild-type cells harboring the multicopy reporter plasmid were cultured and assayed as described in Fig 1A–1C. (B) Two orders of magnitude reduced from wild type cells were observed in Δsre1 and Δscp1 cells. The Δsre1 and Δscp1 cells harboring the multicopy reporter plasmid were cultured and assayed as described in Fig 1A–1C. Y-axis values are the relative light units (RLU) of peak height normalized to cell density (OD660) of each sample at the peak time. The data were averaged from three independent experiments, and each sample was done in triplicate. Error bars indicate means (n = 3) ± S.D. **P<0.01 compared with the vehicle condition for the respective genotype.

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Sre1N degradation [13], we found that deletion of hhp2 significantly increased SRE2 reporter activity in the presence/absence of CLZ, TER or CoCl2 (Fig 4A–4C), thus further validating our reporter system. Surprisingly, in deletion of ssp2 gene, encoding the AMP-activated
Role of Ssp2 and Gsk3/31 in Sre1 activity

(A) CLZ (μg/ml)

(B) TER (μg/ml)

(C) CoCl2 (mM)
Given that Sre1N exists as a hyperphosphorylated protein that contains at least 22 phosphorylated serine and threonine residues [8], and protein kinase Hhp2 regulates Sre1N degradation, we then wanted to know whether deletion of Ssp2, Gsk3 or Gsk31 affect Sre1N degradation. GFP-Sre1N clearly localized at the nucleus in the wild-type cells (Fig 6B). In Δgsk3 or Δgsk31 cells, GFP-Sre1N also localized at the nucleus, similar to that of wild-type cells. However, in Δssp2 cells, GFP-Sre1N also localized at the nucleus, similar to that of wild-type cells. Therefore, GFP-Sre1N also localized at the nucleus, similar to that of wild-type cells (Fig 6A). It is known that Sre1N is released at Golgi and enters the nucleus for further promoting transcription of the target genes [28]. As expected, GFP-Sre1N clearly localized at the nucleus in the wild-type cells (Fig 6B). In Δgsk3 or Δgsk31 cells, GFP-Sre1N also localized at the nucleus, similar to that of wild-type cells. However, in Δssp2, ΔΔgsk3Δgsk31, ΔΔgsk2Δgsk3, ΔΔgsk3Δgsk31 or ΔΔgsk2Δgsk3Δgsk31 deletion cells, significantly weaker fluorescence intensity of GFP-Sre1N was observed at the nucleus compared with that of the wild-type cells.
Fig 5. Deletion of Ssp2 or and Gsk3/Gsk31 markedly suppressed CLZ, TER or CoCl2-induced Sre1 activity. The Δssp2, Δgsk3Δgsk31, Δssp2Δgsk3, Δssp2Δgsk31 or Δssp2Δgsk3Δgsk31 cells showed a significant decrease in Sre1 activity and delay in the peak rising of the SRE2 reporter stimulated with 8 μg/ml CLZ (A), 8 μg/ml TER (B) or 0.32 mM CoCl2 (C). Cells harboring the multi copy reporter plasmid were cultured and assayed as described in Fig 1A–1C. Y-axis values are the relative light units (RLU) of peak height normalized to the cell density (OD660) of each sample at the peak time. The data were averaged from three independent experiments, and each sample was done in triplicate. Error bars indicate means (n = 3) ± S.D. *P<0.05 and **P<0.01 compared with wild-type cells. #P<0.05 and ##P<0.01 compared between
of wild-type cells (Fig 6B and 6C). As these data seem consistent with changes in Sre1N activity as well as Sre1N protein levels, we further performed the immunoblot analysis to detect the total protein levels of Sre1N in wild-type cells and the deletion mutants. GST-Sre1N is also functional as its expression complemented the CoCl$_2$-sensitive growth defect of the Δsre1 cells.
(Fig 6A). Consistent with their weak fluorescence intensity, the whole amount of GST-Sre1N was reduced in the mutants except that in Δgsk3 or Δgsk31 cells (Fig 6D). However, the total amount of GST-Sre1N was markedly different between Δssp2 and Δgsk3Δgsk31 deletion cells. The immunoblot showed a dramatic decrease in Sre1N levels in the Δgsk3Δgsk31 double mutant cells, while deletion of Ssp2 has just a minor effect on Sre1N levels (Fig 6D). This was different from the nuclear localization, where Δssp2 mutant had a major effect that was similar to the Δgsk3Δgsk31 double mutant (Fig 6B and 6C). In comparing the Δgsk3Δgsk31 double mutant versus the Δssp2Δgsk3Δgsk31 triple mutant, there is no additive defect in Sre1N levels and the two strains look identical (Fig 6D). These results suggest that Gsk3/Gsk31 might play the primary role in regulating Sre1N degradation, whereas Ssp2 might regulate not only Sre1N degradation but also nuclear localization of Sre1N.

Discussion

In fission yeast, Sre1, the homologue of mammalian SREBP, regulates sterol homeostasis and hypoxia adaptation [29]. It was known that, as a negative regulator of Sre1, casein kinase 1 family member Hhp2 accelerates Sre1N degradation. However, studies on additional kinases involved in Sre1 activity regulation are still limited. Here, we identified AMPK family member Hhp2 accelerates Sre1N degradation. However, studies on additional kinases and the two strains look identical (Fig 6D). These results suggest that Gsk3/Gsk31 might play the primary role in regulating Sre1N degradation, whereas Ssp2 might regulate not only Sre1N degradation but also nuclear localization of Sre1N.

Three evidences support that our luciferase reporter system could reflect Sre1 activity. First, ergosterol biosynthesis inhibitors, namely CLZ, TER and FEN could induce a marked increase in transcriptional activity of Sre1 in a dose-dependent manner. Second, ergosterol biosynthesis inhibitors-induced Sre1 activity was abolished in Δsre1 or Δscp1 cells. Third, loss of Hhp2, a negative regulator of Sre1, significantly increased transcriptional activity of Sre1 in the presence/absence of ergosterol biosynthesis inhibitors, such as CLZ or TER.

Our previous studies found that Gsk3 and Gsk31 function redundantly in cell growth at restrictive temperatures and sexual differentiation [27]. In present study, several lines of evidence support the hypothesis that Gsk3 and Gsk31 function redundantly in regulation of Sre1 activity, as well as Ssp2 and Gsk3/31 act on parallel in regulation of Sre1 activity. First, CLZ, TER or CoCl2-induced Sre1 activity in Δgsk3Δgsk31 cells was significantly reduced compared to wild-type cells, but slightly reduced in Δgsk3 or Δgsk31 cells. Second, CLZ, TER or CoCl2-induced Sre1 activity in Δssp2Δgsk3 or Δssp2Δgsk31 was only slightly lower than or almost equal to Δssp2 cells. Third, the deletion of ssp2+ gsk3+ gsk31+, ssp2+ and gsk3+, or ssp2+ and gsk31+ significantly delayed the peak rising of the SRE2 reporter, but the deletion of gsk3+ or gsk31+ did not. Forth, the Δssp2Δgsk3Δgsk31 cells showed the lowest Sre1 activity compared to any of their single or double deletions. These results suggested that there is a genetic interaction between Ssp2 and Gsk3/Gsk31, and Ssp2 and Gsk3/Gsk31 may act on parallel pathway in regulation of Sre1 activity.

Furthermore, we found that the fluorescence of GFP-Sre1N at the nucleus observed in Δssp2, Δgsk3Δgsk31, Δssp2Δgsk3, Δssp2Δgsk31 or Δssp2Δgsk3Δgsk31 cells was significantly weakened compared with that in wild-type cells. To our surprises, while nuclear accumulation of GFP-Sre1N appeared to be diminished to a similar extent in the Δssp2 mutant and the Δgsk3Δgsk31 double mutant, the total amount of GST-Sre1N was markedly different in these two mutants. Thus, it seems possible that Ssp2 regulates not only the degradation of Sre1N but also its translocation to the nucleus, whereas Gsk3/Gsk31 regulate mainly its degradation. Since casein kinase 1 family member Hhp2 accelerates Sre1N degradation, our results
suggested that Ssp2/Gsk3/Gsk31 might act as inhibitors of Hhp2, or alternatively act on Sre1N activity independently of Hhp2. Previous studies suggested that Sre1 cleavage, Sre1N stability and Sre1N DNA binding are involved in the regulation of Sre1 activity [30–32]. Based on our present results, we propose that Ssp2 and Gsk3/Gsk31 might affect Sre1N stability to regulate Sre1 activity. However, it is currently undetermined whether Ssp2 and Gsk3/31 are involved in Sre1 cleavage or Sre1N DNA binding for regulation of Sre1 activity.

In conclusion, our findings establish new functional link between Sre1 and three protein kinases, namely Ssp2, Gsk3 and Gsk31. The present data strongly suggest that Ssp2 and Gsk3/Gsk31 play cooperative but distinct roles in the regulation of Sre1 activity in fission yeast. Understanding whether Ssp2 or Gsk3/Gsk31 directly phosphorylates Sre1N and inhibits its degradation is important questions to be addressed in the future.

## Supporting information

S1 Appendix. The original uncropped and unadjusted western blotting images and all individual data points within curve and column graphs.

(ZIP)

## Author Contributions

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