THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 5, pp. 2725–2735, January 31, 2014

Casein Kinase 1 Regulates Sterol Regulatory Element-binding Protein (SREBP) to Control Sterol Homeostasis*

Received for publication, August 19, 2013, and in revised form, November 20, 2013 Published, JBC Papers in Press, December 10, 2013, DOI 10.1074/jbc.M113.511899

Rita T. Brookheart, Chih-Yung S. Lee, and Peter J. Espenshade[1]

From the Department of Cell Biology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Background: SREBP transcription factor controls sterol homeostasis.
Results: Fission yeast casein kinase 1 (CK1) binds and accelerates degradation of active SREBP, inhibiting expression of target genes and decreasing ergosterol.
Conclusion: CK1 controls nuclear SREBP stability to regulate sterol homeostasis in fission yeast.
Significance: CK1 is the first kinase shown to regulate fungal SREBP and functions in the control of SREBP and sterol homeostasis.

Sterol homeostasis is tightly controlled by the sterol regulatory element-binding protein (SREBP) transcription factor that is highly conserved from fungi to mammals. In fission yeast, SREBP functions in an oxygen-sensing pathway to promote adaptation to decreased oxygen supply that limits oxygen-dependent sterol synthesis. Low oxygen stimulates proteolytic cleavage of the SREBP homolog Sre1, generating the active transcription factor Sre1N that drives expression of sterol biosynthetic enzymes. In addition, low oxygen increases the stability and DNA binding activity of Sre1N. To identify additional signals controlling Sre1 activity, we conducted a genetic overexpression screen. Here, we describe our isolation and characterization of the casein kinase 1 family member Hhp2 as a novel regulator of Sre1N. Deletion of Hhp2 increases Sre1N protein stability and ergosterol levels in the presence of oxygen. Hhp2-dependent Sre1N degradation by the proteasome requires Hhp2 kinase activity, and Hhp2 binds and phosphorylates Sre1N at specific residues. Our results describe a role for casein kinase 1 as a direct regulator of sterol homeostasis. Given the role of mammalian Hhp2 homologs, casein kinase 1δ and 1ϵ, in regulation of the circadian clock, these findings may provide a mechanism for coordinating circadian rhythm and lipid metabolism.

Maintenance of cholesterol homeostasis in mammals is imperative for proper cell membrane fluidity and structure, regulation of membrane protein function, and the biosynthesis of bile acids and steroids (1). Altered levels of cholesterol result in hypercholesterolemia and contribute to the manifestation of several human diseases (2, 3). Thus, maintaining tight control of cholesterol levels is important for health and cell survival. A master regulator of cellular cholesterol levels is the family of proteins known as sterol regulatory element-binding proteins (SREBPs)[2]; the specific isoforms SREBP-2 and -1a are important for cholesterol metabolism (4, 5). SREBPs are highly conserved from fungi to mammals, and under normal sterol conditions they exist as inactive endoplasmic reticulum transmembrane proteins. Depletion of cellular sterol results in translocation of SREBP to the Golgi where it undergoes proteolytic cleavage, liberating the N terminus which then localizes to the nucleus to function as a transcription factor (4, 6). Once in the nucleus, active SREBP promotes expression of genes required for sterol homeostasis by binding to specific sterol regulatory elements (SREs) in the promoters of target genes.

SREBPs modulate cellular sterol levels in response to extracellular cues that may alter or compromise sterol metabolism, including changes in oxygen and iron availability, exposure to insulin, and light-dark cycles (7–12). Oxygen is an essential substrate for sterol synthesis and many other metabolic pathways (13). In *Schizosaccharomyces pombe*, reduction in environmental oxygen supply activates the SREBP homolog Sre1 to restore sterol synthesis and cell growth under low oxygen (8, 14). We previously demonstrated that low oxygen stimulates cleavage of Sre1, increasing production of the active N-terminal Sre1N transcription factor and expression of its target genes (15, 16). In addition, low oxygen stimulates Sre1N DNA binding activity and prevents Sre1N degradation by the proteasome (17–19). Finally, the *sre1*$^+$ promoter contains SREs, allowing Sre1 to induce its own gene expression via a positive feedback loop (14). Through these regulatory mechanisms, yeast SREBP controls sterol homeostasis and cell growth in response to low environmental oxygen.

Proper transcriptional regulation of sterol homeostasis necessitates the integration of information from multiple pathways, such as energy supply, nutrient availability, and cell growth signals. To date, only the candidate prolyl hydroxylase Ofd1 and its regulator Nro1 have been identified as modulators of Sre1N activity (17, 18). Ofd1 alters Sre1N function through two oxygen-dependent mechanisms, promotion of Sre1N protein degradation and inhibition of Sre1N DNA binding (17–20). Other factors controlling Sre1N function are not known. To elucidate the mechanisms by which Sre1N is regulated, we performed a high copy plasmid screen in the presence of oxygen using an *S. pombe* cDNA library and isolated clones that decreased Sre1N activity. From our screen, we identified the casein kinase 1 Hhp2 as a negative regulator of Sre1N function.

* This work was supported, in whole or in part, by National Institutes of Health Grants HL077588 (to P. J. E.) and HL106971 (to R. T. B.).
[1] To whom correspondence should be addressed: Dept. of Cell Biology, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Physiology 107B, Baltimore, MD 21205. Tel.: 443-287-5026; Fax: 410-502-7826; E-mail: peter.espenshade@jhmi.edu.
[2] The abbreviations used are: SREBP, sterol regulatory element-binding protein; CK1, casein kinase 1; SRE, sterol regulatory element; 5-FOA, 5-fluoroorotic acid; BZ, bortezomib; EMM, Edinburgh 20 minimal medium; DSP, dithiobis(succinimidyl propionate).

Hhp2 is a member of the casein kinase 1 (CK1) family, a highly conserved group of serine/threonine kinases present in eukaryotes spanning yeast to humans (21). Hhp2 is the *S. pombe* homolog of mammalian CK1 isoforms δ and ϵ. Hhp2 has been implicated in the response to DNA damage and is required for chromosomal segregation during meiosis (22, 23). In mammals, CK1 isoforms δ and ϵ have well described roles in the control of circadian rhythm and Wnt signaling (21, 24). Recently, CK1s have been shown to influence cell metabolism through control of the transcriptional co-activator peroxisome proliferator-activated receptor γ co-activator-1α and hypoxia-inducible factor (25, 26). Here, we demonstrate a novel requirement for CK1 in maintaining sterol homeostasis and control of SREBP degradation.

EXPERIMENTAL PROCEDURES

Reagents—Edinburgh 20 minimal medium (EMM) was obtained from MP Biologicals; yeast extract was from BD Biosciences; amino acids and protease inhibitors (leupeptin, PMSF, and pepstatin A) were from Sigma; horseradish peroxidase-conjugated affinity-purified donkey anti-rabbit and anti-mouse IgG were from Jackson ImmunoResearch, oligonucleotides were from Integrated DNA Technologies; dithiobis(succinimidyl propionate) (DSP) cross-linker was from Thermo Scientific; mouse monoclonal Myc antibody (9E10) and β-actin antibody (C4) were from Santa Cruz Biotechnology; and rabbit polyclonal Myc antibody (06-549) was from Upstate. Sre1 antibody has been previously described (8); cholesterol was from Steraloids; [γ-^{32}P]ATP was from Perkin-Elmer Life Sciences, and bortezomib was from LC Laboratories.

Yeast Strains—Wild-type haploid *S. pombe* strain KGY425 (*h-*, *his3-D1*, *leu1–32*, *ura4-D18,* and *ade6-M210*) was obtained from American Type Culture Collection (27). *S. pombe* strains *sre1*Δ, *sre1N*, *sre1N-MP*, and *sre1N ubr1*Δ have been described previously (19). *S. pombe* strains *sre1N hhp2*Δ, *sre1 hhp2*Δ, *sre1N ubr1*Δ *hhp2-myc,* and *sre1N*Δ *hhp2-myc ubr1*Δ were generated by homologous recombination from haploid KGY425 yeast using established techniques (28). Yeast strains expressing plasmids were generated by transformation.

Strains were grown to exponential phase at 30 °C in rich medium (YES) or EMM plus appropriate supplements for selection (225 μg/ml each of histidine, uracil, leucine, adenine, and lysine) using established techniques (8). An In Vivo$_2$ 400 workstation (Biotrace Inc.) was used to maintain anaerobic conditions as described previously (8, 14). EMM containing 0.1% 5-fluoroorotic acid (5-FOA) (Toronto Research Chemicals) was made as described (29).

Plasmids—hhp2, ofd1, and *tom70* constructs driven by the *adh* promoter were isolated from our screen and were from an *S. pombe* cDNA library (30). Plasmids expressing wild-type or kinase-dead (K41N) Hhp2 from the thiamine-repressible *nmt* promoter were generated by subcloning wild-type or mutant *hhp2*$^+$ cDNA fragments into pSLF172 and pSLF272 (31). Bacterial constructs expressing His$_6$-tagged wild-type or kinase-dead Hhp2 and Sre1N (amino acids 1–440) were produced by inserting appropriate *S. pombe* cDNAs into pET15b (Novagen). The His$_6$-tagged Cnp3-N (amino acids 1–277) bacterial construct was generated by subcloning *cnp3-N*$^+$ *S. pombe* cDNA into pProExHTb (Invitrogen).

Plasmid cDNA Library Screen—sre1N 4×SRE-ura4$^+$ cells were transformed with the SPLE-1 *S. pombe* cDNA library (30, 32). Expression of the cDNA library was driven by the *adh* promoter in the pLEV3 vector that contains the *S. cerevisiae LEU2* gene for plasmid selection. Transformed cells were plated onto EMM plates lacking leucine and containing 0.1% 5-FOA. A total of 2.4 × 10^5 transformants were screened for growth on selection plates for 6 days and isolated by single colony purification. Plasmid DNA was extracted from positive clones, amplified in *Escherichia coli*, and sequenced as described previously (18).

Northern and Western Blotting—Total RNA was isolated, and Northern blotting was performed as described previously (8). Design and synthesis of radiolabeled RNA probes for *sre1N*$^+$, *hem13*$^+$, and *tub1*$^+$ were performed as described previously (14, 19). Whole-cell yeast protein extraction and Western blotting were performed as described previously using primary antibodies and appropriate horseradish peroxidase-conjugated affinity-purified immunoglobulin (8). Where mentioned, lysates were treated with 1 unit/μl alkaline phosphatase (Roche Applied Science) for 1 h at 37 °C prior to Western blot analysis.

Measurement of Protein Half-life—Experiments were performed as described previously (18). Briefly, yeast cells were grown in the absence of oxygen for 4 h to exponential phase prior to being treated with cycloheximide (200 μg/ml) and shifted to normoxic conditions. Samples were harvested at 0, 5, 10, 15, and 20 min post-cycloheximide addition. Whole-cell lysis was performed followed by Western blotting. Blots were imaged with the VersaDoc Imaging System (Bio-Rad), and band intensities were quantified using Quantity One software (Bio-Rad). Sre1N protein half-life was calculated using the equation $t_{½} = (\ln 2)/k$, where k is the slope of the line calculated using an exponential trendline (33).

Co-immunoprecipitation—Immunoprecipitation of endogenous yeast protein was performed as described previously (8). Briefly, cells were grown for 4 h in the presence of oxygen and then treated with 2 mM DSP cross-linker in PBS for 30 min. Cross-linking was stopped by treatment with 20 mM Tris-HCl, pH 7.5, for 15 min. Exponentially growing cells (5 × 10^7 cells) were lysed with glass beads (0.5 mm) in 100 μl of Nonidet P-40 lysis buffer (50 mM HEPES-HCl, pH 7.4, 100 mM NaCl, 1.5 mM $MgCl_2$, 1% Nonidet P-40) plus 2× protease inhibitors (PMSF, leupeptin, and pepstatin A) for 14 min. Insoluble material was removed by centrifugation at 14,000 rpm for 2 min. The supernatant was saved and incubated with 5 μg of monoclonal Myc antibody (9E10) in 1 ml of Nonidet P-40 lysis buffer for 15 min prior to the addition of 40 μl of protein A-Sepharose beads (Repligen). Samples were rotated for 2.5 h at 4 °C followed by three washes with Nonidet P-40 lysis buffer and resuspended in 1× SDS-PAGE loading buffer prior to Western blot analysis. Immunoprecipitation of exogenously expressed proteins was performed as above except cells were grown for 4 h in the absence of oxygen prior to treatment with DSP under normoxic conditions.

In Vitro Kinase Assay—Plasmids expressing recombinant His$_6$-tagged Hhp2, Hhp2-K41N, and Sre1N were transformed into *E. coli* BL21-CodonPlus (DE3) cells (Agilent). The His$_6$-

tagged Cnp3-N plasmid was transformed into *E. coli* BL21-(DE3) *ompT*. Protein expression in *E. coli* was induced by treatment of cells with 0.6 mM isopropyl β-D-thiogalactopyranoside at an A_{600} of 0.5 and shifted to 30 °C for 1–3 h. Cells were lysed by sonication in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole at pH 8.0) with 1× Protease Complete tablets (Roche Applied Science). Tagged proteins were purified by nickel affinity chromatography using nickel-nitrilotriacetic acid-agarose beads per the manufacturer's instructions (Qiagen). Purified recombinant proteins (50 ng of kinase and 0.5 μg of substrate) were incubated in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1% Triton X-100, 100 μM ATP, 0.1 μg/μl BSA) at 30 °C for 1 h with 1 μCi of [γ-^{32}]ATP. Reactions were stopped by addition of cold SDS-loading dye followed by boiling for 5 min. Proteins were resolved by SDS-PAGE, and incorporation of radioactive phosphate was detected by autoradiography.

Mass Spectrometry—In vitro kinase assay was carried out as described above but using unlabeled ATP. Proteins were visualized by staining gels with GelCode Blue (Thermo Scientific), and bands corresponding to substrates and kinases were excised and analyzed by MS/MS at the Johns Hopkins University Mass Spectrometry and Proteomics Facility and the Taplin Mass Spectrometry Facility. A radiolabeled kinase assay was performed in parallel to confirm the reaction efficiency.

Sterol Analysis—Sterols were extracted from cells as described previously with the following modifications (34). Cell pellets (1×10^8 cells) were resuspended in 1 ml of methanol and transferred to disposable glass tubes to which 8 ml of methanol and 4.5 ml of 60% (w/v) KOH were added along with 5 μg of cholesterol as a recovery standard. Samples were vortexed and sterol esters were saponified at 75 °C for 2 h with gentle agitation. Samples were cooled to room temperature, and nonsaponifiable sterols were extracted with 4 ml of petroleum ether (Sigma) and dried under nitrogen gas. Samples were resuspended in 300 μl of heptane, and 2 μl was injected into an automatic liquid sampler on an Agilent 6850 gas chromatograph equipped with an HP-1 column and flame ionization detector. Sterol species were identified, and peak areas were normalized to the cholesterol standard.

RESULTS

Identification of Sre1N Negative Regulators—Decreased oxygen or ergosterol synthesis stimulates proteolytic cleavage of Sre1 to produce the active Sre1N transcription factor (Fig. 1*A*) (8, 35). We previously demonstrated that Sre1N activity is negatively regulated in the presence of oxygen by the candidate prolyl hydroxylase Ofd1 (17–19). To identify additional genes involved in Sre1N regulation, we conducted a genetic overexpression screen in *S. pombe* using an integrated reporter gene regulated by Sre1N activity (Fig. 1*B*). The reporter gene consisted of four tandem Sre1N DNA-binding sites driving expression of *ura4*$^+$ that codes for orotidine 5-monophosphate decarboxylase and is required for growth in the absence of uracil (18, 36). Orotidine 5-monophosphate decarboxylase also enables the cells to metabolize the compound 5-FOA to the toxin 5-fluorouracil; thus, cells that highly express *ura4*$^+$ fail to grow in the presence of 5-FOA (29). Previously, we isolated

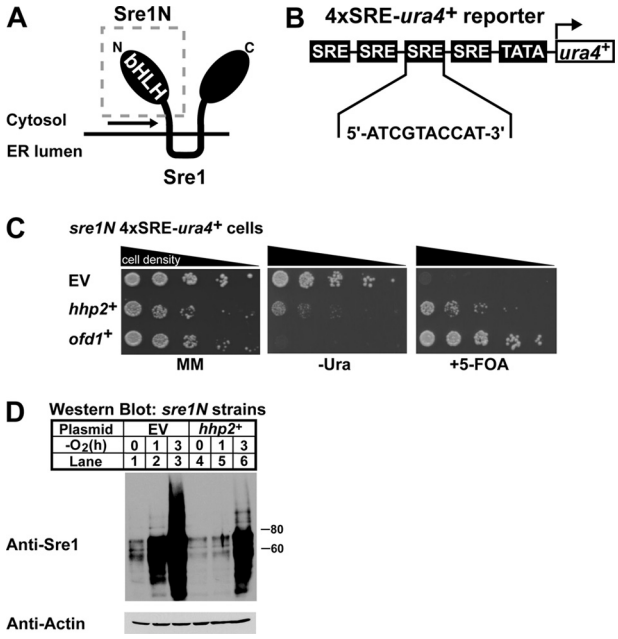

FIGURE 1. **Genetic screen identifies negative regulators of Sre1N.** *A,* schematic of Sre1 with soluble Sre1N (*gray box*) and region of cleavage (*black arrow*) highlighted. *ER*, endoplasmic reticulum. *B,* diagram of the integrated *4×SRE-ura4*$^+$ reporter gene containing four consecutive SRE sequences upstream of a minimal promoter and the *ura4*$^+$ gene. *C,* growth assay of *sre1N 4×SRE-ura4*$^+$ reporter strains containing empty vector (*EV*), *hhp2*$^+$, or *ofd1*$^+$ constructs plated onto minimal medium (*MM*), minimal medium lacking uracil (−*Ura*), and minimal medium containing 5-FOA. Cells were plated in 5-fold serial dilutions. *D,* Western blot analysis of Sre1N expression in the *sre1N 4×SRE-ura4*$^+$ reporter strain expressing *adh*-driven empty vector or *hhp2*$^+$ constructs. Cells were grown in the absence of oxygen for the indicated times. Actin served as a loading control.

positive regulators of Sre1N using a related reporter gene carrying two SRE sites and selecting for growth on medium lacking uracil (18).

To identify negative regulators of Sre1N, we generated a strain that expressed the *4×SRE-ura4*$^+$ reporter construct and Sre1N (amino acids 1–440) from the endogenous *sre1*$^+$ locus (18). Expression of Sre1N was achieved by insertion of a stop codon upstream of the first transmembrane domain segment of Sre1 (Fig. 1*A*). This approach enabled the expression of Sre1N independently of proteolytic cleavage. The *sre1N 4×SRE-ura4*$^+$ reporter strain was able to grow in complete medium and in medium lacking uracil; however, the strain failed to grow in the presence of 5-FOA (Fig. 1*C*) (18). In contrast, we previously reported that *sre1N 3×SRE-ura4*$^+$ reporter cells grew on 5-FOA medium, suggesting that modest inhibition of Sre1N activity might rescue growth of *sre1N 4×SRE-ura4*$^+$ cells on 5-FOA medium (18).

To screen for negative regulators of Sre1N, we transformed *sre1N 4×SRE-ura4*$^+$ cells with the SPLE-1 *S. pombe* plasmid cDNA library (32). Each cDNA plasmid was driven by the alcohol dehydrogenase promoter (*adh*) and contained the *S. cerevisiae LEU2* gene, which allowed for selection of transformants on medium lacking leucine (32). A total of 2.4×10^5 transformants were screened for growth on leucine-free medium containing 5-FOA. Cells expressing cDNAs that decreased Sre1N transcriptional activity would grow in the presence of 5-FOA, whereas those containing cDNAs that either enhanced or had

TABLE 1
cDNAs isolated from genetic screen

Name	Description	No. of clones isolated
hhp2$^+$	Casein kinase 1 family protein	4
ofd1$^+$	2-Oxoglutarate and Fe(II) dioxygenase domain containing protein 1	8
tom70$^+$	Mitochondrial TOM complex subunit Tom70	1

no effect on Sre1N activity would fail to grow. Clones growing in the presence of 5-FOA were isolated, and plasmids were extracted and sequenced to identify the expressed cDNAs. Several clones were isolated that contained plasmids coding for the same gene. From our screen we isolated several genes that promoted growth in the presence of 5-FOA. Notably *ofd1*$^+$ and *hhp2*$^+$ were isolated multiple times, and *tom70*$^+$ was isolated once (Table 1). *ofd1*$^+$ is a transcriptional target and negative regulator of Sre1N, thus validating our screen, and *tom70*$^+$ is a predicted subunit of the mitochondrial TOM complex (14, 17). *hhp2*$^+$, a homolog of the mammalian casein kinase 1 (CK1) family, was isolated four times (22, 37). Hhp2, like Sre1N, localizes to the nucleus and has been shown to function in response to DNA damage and to be required for chromosomal segregation during meiosis (22, 23). A role for Hhp2 in sterol homeostasis has not been described.

Overexpression of hhp2$^+$ *Reduces Sre1N Protein*—We verified that the 5-FOA-resistant phenotype of the *hhp2*$^+$-expressing clones was plasmid-dependent by retransforming the *hhp2*$^+$ library plasmid into the *sre1N 4×SRE-ura4*$^+$ strain and plating cells onto minimal medium, minimal medium lacking uracil, and minimal medium with 5-FOA (Fig. 1*C*). Strains containing appropriate empty or *ofd1*$^+$ plasmids served as controls. Cells with empty vector grew on both minimal medium and minimal medium lacking uracil plates, but cells failed to grow on plates that contained 5-FOA. Cells expressing *hhp2*$^+$ showed reduced growth in the absence of uracil compared with cells containing empty vector but more growth relative to cells expressing *ofd1*$^+$. Additionally, like Ofd1, expression of *hhp2*$^+$ promoted growth on medium containing 5-FOA relative to empty vector cells. These data suggest that *hhp2*$^+$ expression negatively affects Sre1N activity, resulting in decreased Ura4 production compared with empty vector cells, allowing for growth in the presence of 5-FOA. Compared with *ofd1*$^+$-expressing cells, *hhp2*$^+$-expressing cells grew better on uracil-free medium but more slowly on 5-FOA medium.

To test if *hhp2*$^+$ altered Sre1N levels, we measured Sre1N by Western blot in *sre1N 4×SRE-ura4*$^+$ cells expressing either empty vector or *hhp2*$^+$ grown under normoxia or low oxygen (Fig. 1*D*). Empty vector cells expressed Sre1N in the presence of oxygen, and upon oxygen depletion, the Sre1N increased. However, *hhp2*$^+$-expressing cells contained less Sre1N than empty vector cells both in the presence of oxygen and upon being shifted to low oxygen. These data, along with the growth assay, indicate that *hhp2*$^+$ is a negative regulator of Sre1N.

We performed a parallel study to verify the phenotype for *tom70*$^+$. Compared with cells with empty vector, *tom70*$^+$-expressing cells were resistant to 5-FOA (Fig. 2*A*) and contained less Sre1N in the absence of oxygen relative to cells with empty

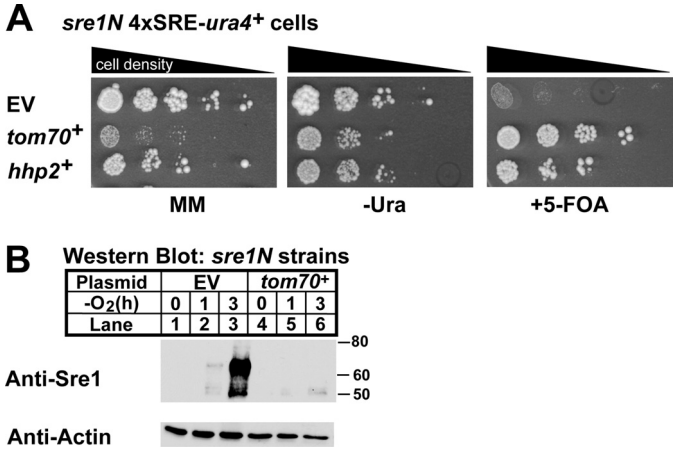

FIGURE 2. **Verification of *tom70*$^+$ as a negative regulator of Sre1N.** *A,* growth assay of *sre1N 4×SRE-ura4*$^+$ reporter strains containing empty vector (*EV*), *tom70*$^+$, or *hhp2*$^+$ constructs plated as in Fig. 1*C*. *B,* Western blot analysis of Sre1N expression in the *sre1N 4×SRE-ura4*$^+$ reporter strain expressing *adh*-driven empty vector or *tom70*$^+$ constructs. Cells were treated as in Fig. 1*D*. *MM,* minimal medium.

vector (Fig. 2*B*). These data suggest that, like *hhp2*$^+$, *tom70*$^+$ negatively regulates Sre1N. However, *tom70*$^+$-expressing cells exhibited slow growth relative to cells carrying empty vector, necessitating additional experiments to determine whether these effects are direct. These studies are ongoing.

Hhp2 Regulates Sre1N and Sterol Homeostasis—Overexpression of *hhp2*$^+$ decreased Sre1N. To confirm the role of Hhp2 in Sre1N regulation, we generated an *sre1N* strain with a deletion of *hhp2*$^+$ and assessed Sre1N levels in *sre1N* and *sre1N hhp2*Δ cells by Western blot under normoxic and low oxygen conditions (Fig. 3*A*, *top panel*). In the presence of oxygen, Sre1N levels were low in *sre1N* cells and increased upon shifting cells to low oxygen conditions. However, *sre1N hhp2*Δ cells showed elevated levels of Sre1N in the presence of oxygen and a slight increase during low oxygen.

Sre1N directly activates its own transcription through a positive feedback loop (14). To determine whether the Sre1N present in *sre1N hhp2*Δ cells was functional, we examined Sre1N activity. We assessed mRNA levels of the Sre1N target genes *hem13*$^+$ and *sre1N*$^+$ by Northern blot and observed that, relative to *sre1N* cells, *sre1N hhp2*Δ cells exhibited higher levels of target mRNAs under normoxic conditions (Fig. 3*A*, *bottom panel*). Because Sre1N induces expression of genes required for ergosterol biosynthesis, we also measured ergosterol levels of *sre1N* and *sre1N hhp2*Δ cells in the presence of oxygen (Fig. 3*B*) (14). Compared with *sre1N* cells, ergosterol was 47% higher in *sre1N hhp2*Δ cells. Together, these results confirm our overexpression studies showing that Hhp2 is a negative regulator of Sre1N, and we demonstrate that deletion of *hhp2*$^+$ increases functional Sre1N and ergosterol.

Hhp2 Controls Sre1N Stability—Oxygen regulates both the stability of Sre1N and its DNA binding activity (17, 19). Given that *sre1N* expression involves a positive feedback loop, changes at multiple regulatory steps could affect Sre1N expression. To test whether Hhp2 regulates Sre1N stability, we utilized a previously characterized *sre1N-MP* (mutant promoter) strain in which the Sre1 DNA-binding sites (SREs) in the *sre1N* promoter have been mutated, inhibiting the Sre1N-positive

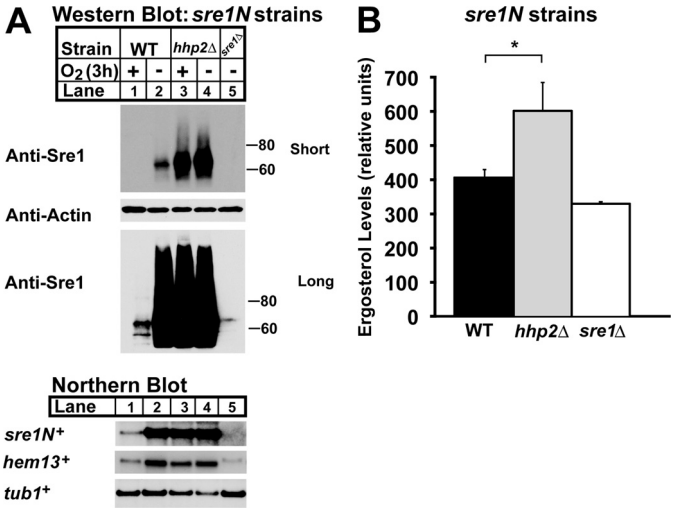

FIGURE 3. **Hhp2 regulates Sre1N and sterol homeostasis.** *A,* Western (*top panels*) and Northern blot (*bottom panel*) analyses of *sre1N*, *sre1N hhp2*Δ, and *sre1*Δ cells grown in the presence or absence of oxygen for 3 h. Whole-cell lysates were subjected to Western blotting to assess Sre1N and β-actin expression. Total RNA was analyzed by Northern blot and probed for $sre1N^+$, $hem13^+$, and $tub1^+$ expression. Blots shown are representative of three independent experiments. *B,* sterol levels of *sre1N*, *sre1N hhp2*Δ, and *sre1*Δ cells grown under normoxic conditions were quantified by gas chromatography. Recovery was normalized to an internal cholesterol standard. All data are expressed as mean ± S.E. of three independent experiments. *, $p < 0.03$.

feedback loop (17). Consequently, changes in Sre1N are due to post-transcriptional regulation in *sre1N-MP* cells. Under low oxygen, *sre1N-MP* cells showed increased Sre1N, but no change in *sre1N* mRNA, reflecting the oxygen regulation of Sre1N stability (Fig. 4*A*) (17). *sre1N-MP hhp2*Δ cells showed increased Sre1N in both the presence and absence of oxygen relative to *sre1N-MP* cells (Fig. 4*A*, *lanes 3* and *4*). $sre1N^+$ mRNA was equal under all conditions, consistent with Hhp2 post-transcriptional regulation of Sre1N. As expected, increased Sre1N resulted in elevated mRNA for the Sre1N target gene $hem13^+$, indicating that the Sre1N accumulating in *sre1N-MP hhp2*Δ cells was functional.

To test further whether Hhp2 controls Sre1N stability, we measured Sre1N half-life using a cycloheximide chase assay (Fig. 4*B*) (17). *sre1N* and *sre1N hhp2*Δ cells were grown in the absence of oxygen for 4 h to accumulate Sre1N. Cells were then treated with the translation inhibitor cycloheximide and shifted to normoxic conditions, and whole-cell lysates were harvested every 5 min. Western blot analysis indicated that the half-life of Sre1N in wild-type cells was ~8 min, similar to what has been reported previously (17, 18). In contrast, *hhp2*Δ cells had an average Sre1N half-life of ~27 min (Fig. 4*B*). These results coupled with our studies in the *sre1N-MP* strain show that Hhp2 regulates Sre1N by accelerating its degradation.

Hhp2 Kinase Activity Is Required to Accelerate Sre1N Degradation—Next, we sought to elucidate the mechanism by which Hhp2 regulates Sre1N stability. Hhp2 is the *S. pombe* homolog of mammalian casein kinase 1δ (CK1δ) and CK1ϵ that have been shown to phosphorylate and subsequently promote the degradation of target proteins; therefore, we hypothesized that Hhp2 may regulate Sre1N stability through Sre1N phosphorylation (22, 25, 37–39).

To investigate whether Hhp2 kinase activity was required for Sre1N regulation, we performed Western blot analysis of whole-cell lysates from *sre1N hhp2*Δ strains expressing empty vector, $hhp2^+$*-myc*, or kinase-dead $hhp2^+$*-K41N-myc* (generated by a lysine to asparagine mutation at residue 41) (Fig. 5*A*). *sre1N hhp2*Δ cells containing an empty vector exhibited highly elevated levels of Sre1N relative to control *sre1N* cells, and expression of $hhp2^+$ in the mutant resulted in a significant decrease of Sre1N. However, when kinase-dead Hhp2 K41N was expressed to an equivalent level in the mutant, we observed no change in Sre1N compared with empty vector *hhp2*Δ cells. The inability of Hhp2 K41N to alter levels of Sre1N indicated that Hhp2 kinase activity is required to accelerate Sre1N degradation. Additionally, the data further confirm Hhp2 as a negative regulator of Sre1N protein, because expression of $hhp2^+$ largely complemented the *hhp2*Δ strain. Incomplete complementation was most likely due to cell-to-cell variation in plasmid copy number due to the fact that *S. pombe* expression systems lack centromere-based plasmids (40).

Sre1N is degraded via the proteasome in the presence of oxygen, and thus we sought to determine whether Hhp2 accelerated Sre1N degradation through this pathway (17, 18). We assayed Sre1N levels in *hhp2*Δ strains carrying empty vector or expressing $hhp2^+$*-myc* after treatment for 3 h with the proteasome inhibitor bortezomib (BZ) or vehicle (DMSO); *sre1N* cells containing empty vector were included as a positive control (Fig. 5*B*). Two independent *hhp2-myc hhp2*Δ strains were analyzed. In *sre1N* vehicle-treated cells, Sre1N was undetectable by Western blot, and treatment with BZ resulted in accumulation of Sre1N indicating proteasome inhibition (Fig. 5*B*, *lanes 1* and *2*). *hhp2*Δ cells showed highly elevated levels of Sre1N in the absence of BZ (Fig. 5*B*, *lane 7*). Expression of $hhp2^+$ in *hhp2*Δ cells decreased Sre1N as expected due to Sre1N accelerated degradation. Importantly, the Hhp2-dependent degradation was blocked by addition of BZ, consistent with Hhp2 promoting Sre1N degradation by the proteasome (Fig. 5*B*, *lanes 3–6*). BZ treatment of *hhp2*Δ cells carrying $hhp2^+$ vector increased Sre1N, but levels did not equal that of the BZ-treated *hhp2*Δ strain carrying empty vector (Fig. 5*B*, *lanes 4, 6,* and *8*). The failure of BZ treatment to completely block the effects of Hhp2 on Sre1N suggested incomplete complementation from a lack of centromere-based plasmids in *S. pombe* or that Hhp2 may have proteasome-independent effects on Sre1N (40). Interestingly, the *hhp2*Δ strain carrying empty vector contained high levels of Sre1N, and upon treatment with BZ, the Sre1N levels increased further (Fig. 5*B*, *lanes 7* and *8*), suggesting the existence of an Hhp2-independent mechanism for Sre1N degradation.

Hhp2 Binds and Phosphorylates Sre1N—Sre1N exists as a hyperphosphorylated protein that contains at least 22 phosphorylated serine and threonine residues[3] (8). This fact, along with the requirement for Hhp2 kinase to accelerate Sre1N degradation, suggested that Sre1N may be an Hhp2 substrate. To address this, we first tested whether Hhp2 interacts with Sre1N by performing a co-immunoprecipitation assay using *sre1N* cells that expressed either untagged or Myc-tagged Hhp2 at the

[3] B. T. Hughes and P. J. Espenshade, unpublished observations.

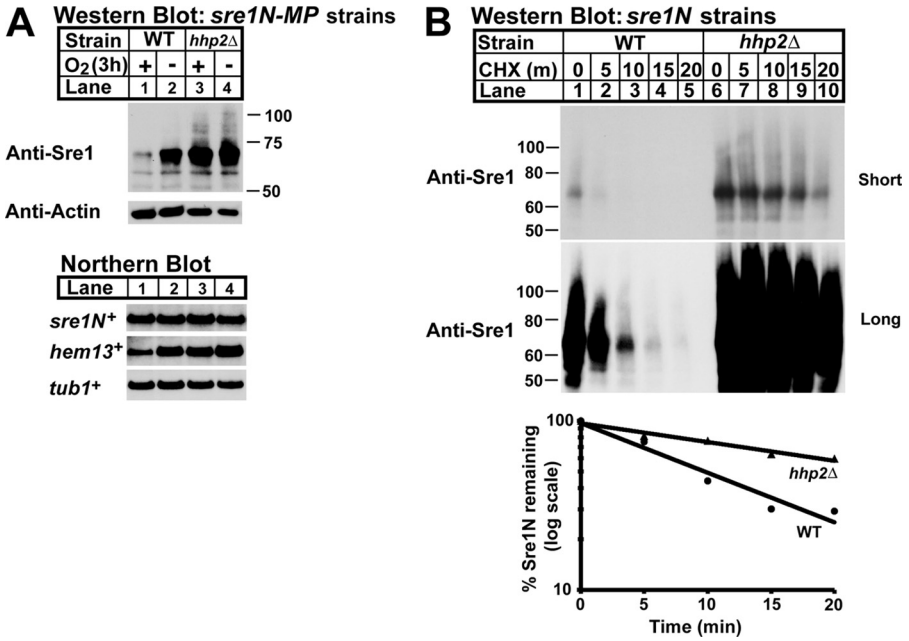

FIGURE 4. **Hhp2 accelerates Sre1N degradation.** *A,* Western (*top panel*) and Northern (*bottom panel*) blot analyses of *sre1N-MP* and *sre1N-MP hhp2*Δ strains grown in the presence or absence of oxygen for 3 h. Whole-cell lysates and total RNA were analyzed as in Fig. 3. Blots shown are representative of three independent experiments. *B,* cycloheximide chase experiment of *sre1N* and *sre1N hhp2*Δ cells grown in the absence of oxygen for 4 h, treated with cycloheximide (*CHX*) (200 μg/ml) at $t = 0$, and shifted to normoxic conditions for the indicated time. *Graph* shows percentage of Sre1N remaining at each time point. *Blot* and *graph* are representative of three independent experiments.

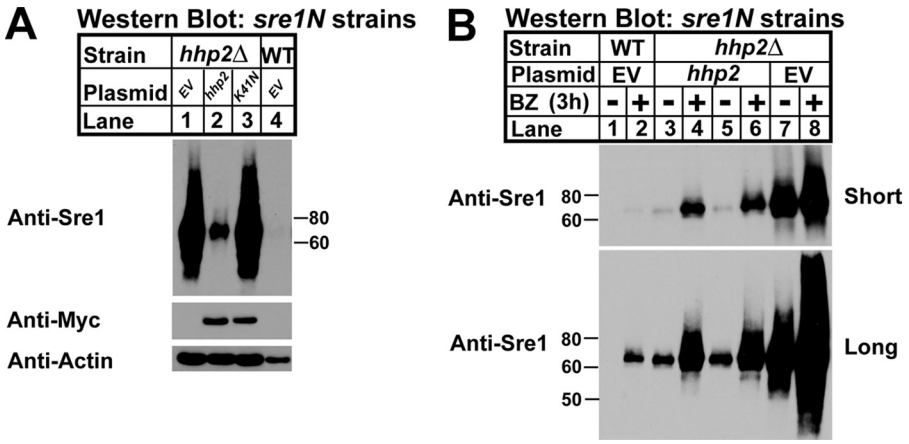

FIGURE 5. **Hhp2 kinase activity is required for Sre1N degradation.** *A,* Western blot of whole-cell lysates from *sre1N hhp2*Δ cells carrying empty vector, *hhp2*$^+$, or *hhp2-K41N* constructs and *sre1N* cells with empty vector. Blots were probed for Sre1N, Myc, and β-actin expression and are representative of three independent experiments. *B,* Western blot of whole-cell lysates from *sre1N* or *sre1N hhp2*Δ cells carrying empty vector or *hhp2*$^+$ plasmid treated with DMSO or 1 mM bortezomib (*BZ*) for 3 h in the presence of oxygen. *Lanes 3–6* show two independent plasmid transformants. Blots were probed for Sre1N expression and are representative of three independent experiments.

hhp2$^+$ locus (Fig. 6*A*). Sre1N is rapidly degraded under normoxic conditions through the action of the E3 ubiquitin ligase Ubr1 (17, 19). To improve our ability to detect an interaction between Hhp2 and Sre1N, we used a *ubr1*Δ strain that accumulates Sre1N in the presence of oxygen. To test whether Hhp2 interacted with the precursor Sre1 or the activated transcription factor Sre1N, we performed these studies in cells that expressed endogenous full-length, cleavable Sre1. Immunoprecipitation of Hhp2 from cross-linker-treated cells specifically pulled down Sre1N, but not Sre1 precursor, indicating that Hhp2 interacts with Sre1N *in vivo* in the presence of oxygen (Fig. 6*A*).

We next investigated whether Hhp2 kinase activity was required for binding to Sre1N. Co-immunoprecipitation assays were performed using *hhp2*Δ cells carrying either empty vector, *hhp2*$^+$*-myc*, or kinase-dead *hhp2*$^+$*-K41N-myc* plasmids (Fig. 6*B*). Sre1N co-immunoprecipitated with both Hhp2 and Hhp2-K41N demonstrating that kinase activity is not required for the Sre1N-Hhp2 interaction. However, relative to the input lane, kinase-dead Hhp2 bound less Sre1N compared with the wild-type enzyme (Fig. 6*B*, *lane 3*), suggesting that kinase activity or the Lys-41 side chain is required for optimal Sre1N binding.

To determine whether Sre1N is an Hhp2 substrate, we tested the effect of *hhp2*$^+$ deletion on Sre1N phosphorylation by Western blot (Fig. 6*C*). Western blot analysis of *sre1N* cell lysates showed Sre1N as a smear (Fig. 6*C*, *lane 1*). Treatment of cell lysates with alkaline phosphatase collapsed the smear to a single band indicating that Sre1N is hyperphosphorylated as

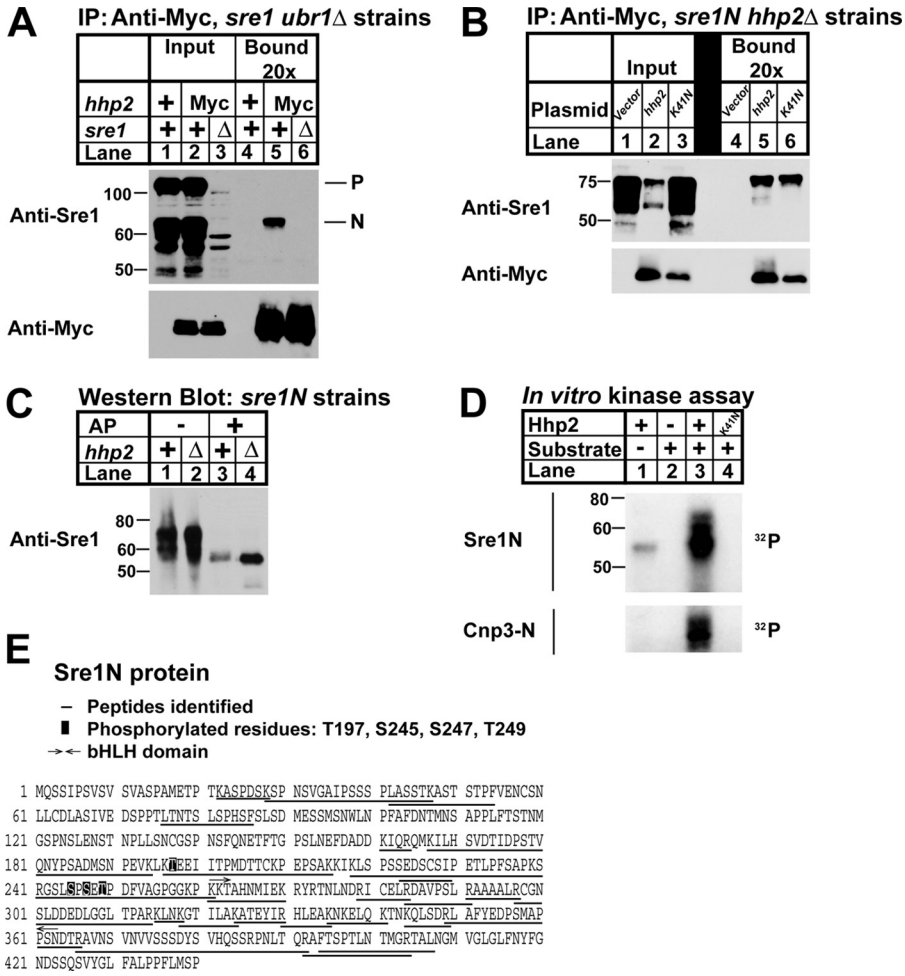

FIGURE 6. **Hhp2 binds and phosphorylates Sre1N.** *A,* co-immunoprecipitation (*IP*) of Hhp2-Myc and Sre1 from *ubr1*Δ, *ubr1*Δ *hhp2-myc*, and *ubr1*Δ *hhp2-myc sre1*Δ cells treated with 2 mM DSP cross-linker in PBS for 30 min was carried out using anti-Myc antibody and Nonidet P-40-solubilized whole-cell lysates. *Input* and *bound* (20× overloaded) fractions were analyzed by Western blot using anti-Myc and anti-Sre1. Blots are representative of three independent experiments. *P* and *N* denote the precursor and nuclear forms of Sre1, respectively. *B,* co-immunoprecipitation of Hhp2 and Sre1N in *sre1N hhp2*Δ cells carrying the indicated plasmids was carried out as in *A* except cells were grown for 4 h in the absence of oxygen, switched to normoxic conditions, and treated with 2 mM DSP cross-linker in PBS for 30 min prior to lysis. Anti-Myc bound is 10× overloaded. Blots are representative of three independent experiments. *C,* Western blot of whole-cell lysates from *sre1N* and *sre1N hhp2*Δ cells grown in the presence of oxygen and treated with 1 mM bortezomib (*BZ*) for 3 h. Lysates in *lanes 3* and *4* were treated with alkaline phosphatase (*AP*). *sre1N* samples (*lanes 1* and *3*) were 12× overloaded relative to mutant samples to enable visualization of Sre1N protein. *D, in vitro* kinase assay was performed with the indicated purified recombinant Hhp2 and either Sre1N or Cnp3-N as substrate. Kinase reaction products were resolved by SDS-PAGE, and autoradiograms are shown. *E,* sequence of Sre1N showing mass spectrometry results of Sre1N-Hhp2 *in vitro* kinase reaction. Peptides identified are *underlined*. Phosphorylated residues are *boxed*.

reported previously (Fig. 6*C*, *lane 3*) (8). Sre1N mobility was unaltered in *hhp2*Δ cells, and the hyperphosphorylated species collapsed to a single band upon alkaline phosphatase treatment (Fig. 6*C*, *lanes 2* and *4*). These data suggest that Hhp2 is not the sole Sre1N kinase and that Hhp2 likely phosphorylates Sre1N at either a single or a small number of residues whose effect on the mass of Sre1N cannot be seen due to the heavily phosphorylated state of Sre1N.

We next decided to employ a direct approach to test whether Hhp2 phosphorylated Sre1N by performing an *in vitro* kinase assay using purified, bacterially expressed Hhp2 and Sre1N (Fig. 6*D*). Recombinant Hhp2 was active insomuch as it phosphorylated the known substrate Cnp3-N that served as a positive control (Fig. 6*D*, *lane 3, bottom panel*) (23). No kinase activity was detected in the reaction containing kinase-dead Hhp2-K41N and Sre1N (Fig. 6*D*, *lane 4, bottom panel*). Hhp2 undergoes autophosphorylation, and we observed this in the Hhp2-only reaction (Fig. 6*D*, *lane 1*) (41). This signal was absent from the Cnp3-N panel due to the different masses of Hhp2 (~55 kDa) and Cnp3-N (~30 kDa). Incubation of Sre1N with Hhp2 resulted in robust phosphorylation of Sre1N that required Hhp2 kinase activity (Fig. 6*D*, *lanes 3* and *4*). Hhp2-phosphorylated Sre1N species did not display the same extent of mobility shift as the smear observed in Fig. 6*C*, suggesting that Hhp2 phosphorylates a smaller number of Sre1N residues.

To confirm the *in vitro* phosphorylation of Sre1N, we performed tandem mass spectrometry analysis of excised gel slices from Sre1N–Hhp2 and Sre1N–Hhp2-K41N *in vitro* kinase assays. Purified Hhp2 and Sre1N have similar molecular masses and thus co-migrated on SDS-PAGE. Mass spectrometry analysis confirmed that the bands visualized by autoradiography in the Sre1N–Hhp2 sample corresponded to phosphorylated Sre1N peptides. Peptide coverage of Sre1N was 65%, and we

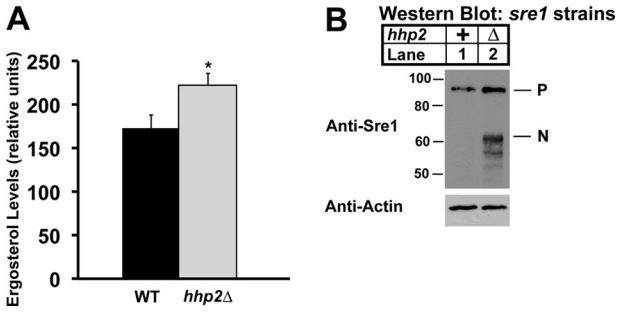

FIGURE 7. **Hhp2 regulates sterol homeostasis.** *A,* ergosterol level in wild-type and *hhp2*Δ cells was measured by gas chromatography. Recovery was normalized to an internal cholesterol standard. All data are expressed as mean ± S.E. of three independent experiments. *, $p < 0.04$. *B,* Western blot of whole-cell lysates from wild-type and *hhp2*Δ cells. Blots probed for Sre1 and β-actin expression are representative of three independent experiments. Uncleaved Sre1 precursor (*P*) and cleaved Sre1N nuclear form (*N*) are designated.

identified four phosphorylated Sre1N residues as follows: Thr-197, Ser-245, Ser-247, and Thr-249 that are all upstream of the basic helix-loop-helix DNA binding domain of Sre1N (Fig. 6*E*). Several serine and threonine residues are present in the undetected Sre1N peptides; therefore, it is possible that not all phosphorylated residues were identified. We were unable to identify autophosphorylated Hhp2 residues in the Sre1N–Hhp2 sample, possibly due to the low abundance of phosphorylated Hhp2. No phosphorylated peptides for either Sre1N or Hhp2-K41N were identified in the Sre1N–Hhp2-K41N sample. Together, these data demonstrate that Hhp2 binds and phosphorylates Sre1N.

Hhp2 Is a Regulator of Sterol Homeostasis—Thus far, our studies have been conducted in cells expressing Sre1N but not the endogenous full-length Sre1 protein. To evaluate the function of Hhp2 in sterol regulation under physiological conditions, we generated an *hhp2* deletion strain from wild-type *S. pombe* that expresses a full-length Sre1. Measurement of ergosterol levels in the *hhp2*Δ cells grown in the presence of oxygen revealed a 29% increase in ergosterol relative to wild-type cells (Fig. 7*A*). We next assessed Sre1N levels in these cells and observed an accumulation of Sre1N in the *hhp2*Δ strain relative to wild-type cells, consistent with a role for Hhp2 in Sre1N degradation (Fig. 7*B*). We also observed an increase in Sre1 precursor in *hhp2*Δ cells, likely due to increased Sre1N and positive feedback on the $sre1^+$ promoter. Together, these data demonstrate that Hhp2 is a key negative regulator of Sre1N that is required to maintain sterol homeostasis under nonstress conditions. In the presence of oxygen, Hhp2 functions in the degradation of Sre1N that results from a constitutive, low level of Sre1 cleavage.

DISCUSSION

SREBPs regulate sterol homeostasis in yeast and mammals (42). The initial characterization of the fission yeast SREBP pathway revealed that the active Sre1N transcription factor was hyperphosphorylated, suggesting potential regulation of sterol homeostasis by kinases/phosphatases (8). Using a high copy plasmid screen, we identified the mammalian casein kinase 1δ/ϵ homolog, Hhp2, as a novel regulator of Sre1N stability and sterol homeostasis in fission yeast. Hhp2 binds Sre1N and accelerates its degradation via the proteasome. Hhp2 phosphorylates Sre1N *in vitro,* and kinase activity is required for Sre1N degradation, suggesting that Hhp2 phosphorylation of Sre1N targets it for degradation. Consistent with the role of Sre1N in activating sterol synthesis, deletion of Hhp2 increased sterols in wild-type cells and cells expressing only the truncated Sre1N. Importantly, Hhp2 functions independently from changes in oxygen, providing a novel mechanism for control of SREBP activity.

The SREBP pathway is highly conserved in pathogenic fungi that cause infections in immunocompromised individuals (43). Indeed, Sre1 is required for virulence of the fungal pathogens *Cryptococcus neoformans* and *Aspergillus fumigatus* in mouse models of disease (44–46). As a regulator of Sre1, Hhp2 may also be required for virulence of these clinically important pathogens. Current antifungal drugs target the ergosterol pathway (47). Given that Hhp2 regulates ergosterol homeostasis, modulation of Hhp2 activity may improve the efficacy of existing antifungals.

Using mass spectrometry, we identified four Sre1N residues phosphorylated by Hhp2 *in vitro* (Fig. 6*E*), and these data validated that Hhp2 binds Sre1N directly. To test whether these residues were endogenous substrates required for Sre1N stability, we mutated all four amino acids to alanine. Stability of this mutant Sre1N was unchanged, indicating that the residues are *in vitro* substrates but likely not Hhp2 sites *in vivo* (data not shown). CK1s prefer pre-phosphorylated substrates that have been primed by action of another kinase and recognize the consensus sequence (pS/pT)*XX*(S/T) (21). The Sre1N substrate used in our studies was purified from *E. coli* and likely lacked any priming phosphorylation that occurs in fission yeast cells. Future studies will determine the endogenous Hhp2 phosphorylation sites to dissect the role of Hhp2-dependent degradation in the overall control of the Sre1 pathway. Importantly, the requirement for priming phosphorylation for Hhp2 substrate recognition provides an opportunity for additional kinase regulation of Sre1N turnover.

Sre1N is a short lived protein with a half-life of ~8 min in wild-type cells (17). This rapid turnover of Sre1N is mediated by the E2 ubiquitin-conjugating enzyme Rhp6 and the E3 ubiquitin ligase Ubr1 that target Sre1N for proteasomal degradation (19). Hhp2 accelerates Sre1N proteasomal degradation, and $hhp2^+$ deletion increased Sre1N half-life to ~27 min (Fig. 4*B*). Sre1N degradation in *hhp2*Δ cells is still faster than in *ubr1*Δ cells (~100 min), and addition of proteasome inhibitor to *hhp2*Δ cells further stabilized Sre1N (Fig. 5*B*). Together, these data indicate that Sre1N is degraded by both Hhp2-dependent and Hhp2-independent pathways. The candidate prolyl hydroxylase Ofd1 accelerates Sre1N in an oxygen-regulated manner (17, 20). Future studies will investigate the relationship between Hhp2- and Ofd1-dependent mechanisms for Sre1N degradation through Rhp6/Ubr1. The presence of Hhp2-dependent and -independent pathways would enable cells to fine-tune ergosterol metabolism in response to various stimuli such as changes in oxygen concentration and nutrient availability. In addition to ergosterol biosynthesis, Sre1N regulates genes required for heme and sphingolipid biosynthesis, electron transport, and amino acid metabolism, suggesting that differ-

ent Sre1N regulatory pathways may exist to modulate specific arms of metabolism (14). Here, we describe a mechanism of Hhp2-dependent degradation of Sre1N, yet it is possible that Hhp2 may also alter Sre1N activity at the level of *sre1N* translation. Because Hhp2 binds to Sre1N and based on reports demonstrating the direct role of CK1s on protein stability, it is unlikely Hhp2 impacts Sre1N solely at the level of translation (25, 38, 39).

The ability of Hhp2 to accelerate Sre1N degradation provides a mechanism for integration of non-oxygen signals into the control of sterol metabolism. But the signals that control Hhp2 activity are unknown. Autophosphorylation increases Hhp2 activity *in vitro*, whereas CK1δ/ϵ autophosphorylation is inhibitory (41, 48–50). One possibility is that Hhp2 could be controlled by a protein phosphatase through removal of regulatory phosphorylation. In addition, it was recently reported that mammalian CK1ϵ enzymatic activity is activated upon binding to the DEAD box RNA helicase DDX3 (51). Although the precise mechanism behind this regulation is not known, discovery of DDX3 as a CK1 enhancer suggests that Hhp2 activity may also be regulated in this fashion. Additional genetic screens may identify factors that regulated Hhp2 activity, providing clues to the upstream signals that control Sre1N turnover.

In addition to Hhp2, *S. pombe* codes for a second CK1δ/ϵ homolog called Hhp1 that shares 75% amino acid sequence identity with Hhp2 (22). Both kinases have been shown to serve redundant cellular functions (22, 23, 52). However, previously, we identified Hhp1 in a high copy plasmid screen to identify positive regulators of Sre1N activity (18). It will be interesting to test whether Sre1N is also a substrate for Hhp1 and whether the two kinases have opposing effects on Sre1N activity.

The CK1 Hhp2 is the first kinase reported to function in the fungal SREBP pathway, but multiple kinases have been implicated in the regulation of mammalian SREBPs, including GSK3, CDK8, and AMP-activate protein kinase (53–56). Interestingly, like Hhp2, GSK3 and CDK8 phosphorylate SREBP, promoting its degradation via the proteasome (53, 55). The high degree of SREBP pathway conservation between *S. pombe* and mammals and the existence of mammalian Hhp2 homologs CK1δ and CK1ϵ suggest that CK1s may also regulate cholesterol homeostasis by controlling SREBP stability in humans. Indeed, a recent high throughput cDNA overexpression screen for SREBP regulators identified CK1ϵ as a negative regulator of SREBP activity in HEK-293 cells (57). Should CK1 regulation of SREBP be conserved, this finding would have important implications for regulation of lipid metabolism. Multiple lines of evidence link circadian rhythm to control of cell metabolism (25, 58–61). Mammalian CK1δ/ϵ are key regulators of the mammalian molecular circadian clock; mutation of CK1ϵ is responsible for the short period mutation in the tau mutant Syrian hamster, and a missense mutation in CK1δ results in human familial advanced sleep phase syndrome (62–64). Identification of CK1s as regulators of mammalian SREBPs would provide a mechanism for the coordinate regulation of lipid and circadian-dependent metabolism. Studies are underway to test whether SREBP regulation by CK1 is conserved.

Acknowledgments—We kindly thank Charlie Hoffman (Boston College) for providing the S. pombe cDNA plasmid library and the Taplin Mass Spectrometry Facility and the Johns Hopkins University Mass Spectrometry and Proteomics Facility for mass spectrometry analyses. We also thank Diedre Ribbens for reviewing the manuscript.

REFERENCES

1. Ikonen, E. (2008) Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.* **9,** 125–138
2. Tabas, I. (2002) Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J. Clin. Invest.* **110,** 905–911
3. Maxfield, F. R., and Tabas, I. (2005) Role of cholesterol and lipid organization in disease. *Nature* **438,** 612–621
4. Goldstein, J. L., DeBose-Boyd, R. A., and Brown, M. S. (2006) Protein sensors for membrane sterols. *Cell* **124,** 35–46
5. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **109,** 1125–1131
6. Osborne, T. F., and Espenshade, P. J. (2009) Evolutionary conservation and adaptation in the mechanism that regulates SREBP action: what a long, strange tRIP it's been. *Genes Dev.* **23,** 2578–2591
7. Blatzer, M., Barker, B. M., Willger, S. D., Beckmann, N., Blosser, S. J., Cornish, E. J., Mazurie, A., Grahl, N., Haas, H., and Cramer, R. A. (2011) SREBP coordinates iron and ergosterol homeostasis to mediate triazole drug and hypoxia responses in the human fungal pathogen *Aspergillus fumigatus*. *PLoS Genet.* **7,** e1002374
8. Hughes, A. L., Todd, B. L., and Espenshade, P. J. (2005) SREBP pathway responds to sterols and functions as an oxygen sensor in fission yeast. *Cell* **120,** 831–842
9. Matsumoto, E., Ishihara, A., Tamai, S., Nemoto, A., Iwase, K., Hiwasa, T., Shibata, S., and Takiguchi, M. (2010) Time of day and nutrients in feeding govern daily expression rhythms of the gene for sterol regulatory element-binding protein (SREBP)-1 in the mouse liver. *J. Biol. Chem.* **285,** 33028–33036
10. Peterson, T. R., Sengupta, S. S., Harris, T. E., Carmack, A. E., Kang, S. A., Balderas, E., Guertin, D. A., Madden, K. L., Carpenter, A. E., Finck, B. N., and Sabatini, D. M. (2011) mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. *Cell* **146,** 408–420
11. Hatori, M., Hirota, T., Iitsuka, M., Kurabayashi, N., Haraguchi, S., and Kokame, K., Sato, R., Nakai, A., Miyata, T., Tsutsui, K., and Fukada, Y. (2011) Light-dependent and circadian clock-regulated activation of sterol regulatory element-binding protein, X-box-binding protein 1, and heat shock factor pathways. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 4864–4869
12. Kotzka, J., Lehr, S., Roth, G., Avci, H., Knebel, B., and Muller-Wieland, D. (2004) Insulin-activated Erk-mitogen-activated protein kinases phosphorylate sterol regulatory element-binding protein-2 at serine residues 432 and 455 *in vivo*. *J. Biol. Chem.* **279,** 22404–22411
13. Rosenfeld, E., and Beauvoit, B. (2003) Role of the non-respiratory pathways in the utilization of molecular oxygen by *Saccharomyces cerevisiae*. *Yeast* **20,** 1115–1144
14. Todd, B. L., Stewart, E. V., Burg, J. S., Hughes, A. L., and Espenshade, P. J. (2006) Sterol regulatory element binding protein is a principal regulator of anaerobic gene expression in fission yeast. *Mol. Cell. Biol.* **26,** 2817–2831
15. Stewart, E. V, Nwosu, C. C., Tong, Z., Roguev, A., Cummins, T. D., Kim, D. U., Hayles, J., Park, H. O., Hoe, K. L., Powell, D. W., Krogan, N. J., and Espenshade, P. J. (2011) Yeast SREBP cleavage activation requires the Golgi Dsc E3 ligase complex. *Mol. Cell* **42,** 160–171
16. Stewart, E. V., Lloyd, S. J., Burg, J. S., Nwosu, C. C., Lintner, R. E., Daza, R., Russ, C., Ponchner, K., Nusbaum, C., and Espenshade, P. J. (2012) Yeast sterol regulatory element-binding protein (SREBP) cleavage requires Cdc48 and Dsc5, a ubiquitin regulatory X domain-containing subunit of the Golgi Dsc E3 ligase. *J. Biol. Chem.* **287,** 672–681
17. Hughes, B. T., and Espenshade, P. J. (2008) Oxygen-regulated degradation

of fission yeast SREBP by Ofd1, a prolyl hydroxylase family member. *EMBO J.* **27,** 1491–1501
18. Lee, C. Y., Stewart, E. V, Hughes, B. T., and Espenshade, P. J. (2009) Oxygen-dependent binding of Nro1 to the prolyl hydroxylase Ofd1 regulates SREBP degradation in yeast. *EMBO J.* **28,** 135–143
19. Lee, C. Y., Yeh, T. L., Hughes, B. T., and Espenshade, P. J. (2011) Regulation of the Sre1 hypoxic transcription factor by oxygen-dependent control of DNA binding. *Mol. Cell* **44,** 225–234
20. Porter, J. R., Lee, C. Y., Espenshade, P. J., and Iglesias, P. A. (2012) Regulation of SREBP during hypoxia requires Ofd1-mediated control of both DNA binding and degradation. *Mol. Biol. Cell* **23,** 3764–3774
21. Cheong, J. K., and Virshup, D. M. (2011) Casein kinase 1: Complexity in the family. *Int. J. Biochem. Cell Biol.* **43,** 465–469
22. Dhillon, N., and Hoekstra, M. F. (1994) Characterization of two protein kinases from *Schizosaccharomyces pombe* involved in the regulation of DNA repair. *EMBO J.* **13,** 2777–2788
23. Ishiguro, T., Tanaka, K., Sakuno, T., and Watanabe, Y. (2010) Shugoshin-PP2A counteracts casein-kinase-1-dependent cleavage of Rec8 by separase. *Nat. Cell Biol.* **12,** 500–506
24. Price, M. A. (2006) CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. *Genes Dev.* **20,** 399–410
25. Li, S., Chen, X.-W., Yu, L., Saltiel, A. R., and Lin, J. D. (2011) Circadian metabolic regulation through crosstalk between casein kinase 1δ and transcriptional coactivator PGC-1α. *Mol. Endocrinol* **25,** 2084–2093
26. Kalousi, A., Mylonis, I., Politou, A. S., Chachami, G., Paraskeva, E., and Simos, G. (2010) Casein kinase 1 regulates human hypoxia-inducible factor HIF-1. *J. Cell Sci.* **123,** 2976–2986
27. Burke, J. D., and Gould, K. L. (1994) Molecular cloning and sequence characterization of the *Schizosaccharomyces pombe* his3 gene for use as a selectable marker. *Mol. Gen. Genet.* **242,** 169–176
28. Bähler, J., Wu, J. Q., Longtine, M. S., Shah, N. G., McKenzie, A., 3rd., Steever, A. B., Wach, A., Philippsen, P., and Pringle, J. R. (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14,** 943–951
29. Boeke, J. D., Trueheart, J., Natsoulis, G., Fink, and G. R. (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* **154,** 164–175
30. Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K., and Okayama, H. (1990) High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **18,** 6485–6489
31. Forsburg, S. L., and Sherman, D. A. (1997) General purpose tagging vectors for fission yeast. *Gene* **191,** 191–195
32. Janoo, R. T., Neely, L. A., Braun, B. R., Whitehall, S. K., and Hoffman, C. S. (2001) Transcriptional regulators of the *Schizosaccharomyces pombe fbp1* gene include activation complex. *Genetics* **157,** 1205–1215
33. Belle, A., Tanay, A., Bitincka, L., Shamir, R., and O'Shea, E. K. (2006) Quantification of protein half-lives in the budding yeast proteome. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 13004–13009
34. Hughes, A. L., Lee, C. Y., Bien, C. M., and Espenshade, P. J. (2007) 4-Methyl sterols regulate fission yeast SREBP-Scap under low oxygen and cell stress. *J. Biol. Chem.* **282,** 24388–24396
35. Porter, J. R., Burg, J. S., Espenshade, P. J., and Iglesias, P. A. (2010) Ergosterol regulates sterol regulatory element binding protein (SREBP) cleavage in fission yeast. *J. Biol. Chem.* **285,** 41051–41061
36. Sehgal, A., Lee, C. Y., and Espenshade, P. J. (2007) SREBP controls oxygen-dependent mobilization of retrotransposons in fission yeast. *PLoS Genet.* **3,** e131
37. Kearney, P. H., Ebert, M., and Kuret, J. (1994) Molecular cloning and sequence analysis of two novel fission yeast casein kinase-1 isoforms. *Biochem. Biophys. Res. Commun.* **203,** 231–236
38. Akashi, M., Tsuchiya, Y., Yoshino, T., and Nishida, E. (2002) Control of intracellular dynamics of mammalian period proteins by casein kinase Iϵ (CKIϵ) and CKIδ in cultured cells. *Mol. Cell. Biol.* **22,** 1693–1703
39. Eide, E. J., Woolf, M. F., Kang, H., Woolf, P., Hurst, W., Camacho, F., Vielhaber, E. L., Giovanni, A., and Virshup, D. M. (2005) Control of mammalian circadian rhythm by CKIϵ-regulated proteasome-mediated PER2 degradation. *Mol. Cell. Biol.* **25,** 2795–2807
40. Hayles, J., and Nurse, P. (1992) Genetics of the fission yeast. *Schizosaccharomyces* pombe. *Annu. Rev. Genet.* **26,** 373–402
41. Hoekstra, M. F., Dhillon, N., Carmel, G., DeMaggio, A. J., Lindberg, R. A., Hunter, T., and Kuret, J. (1994) Budding and fission yeast casein kinase I isoforms have dual-specificity protein kinase activity. *Mol. Biol. Cell* **5,** 877–886
42. Espenshade, P. J., and Hughes, A. L. (2007) Regulation of sterol synthesis in eukaryotes. *Annu. Rev. Genet.* **41,** 401–427
43. Bien, C. M., and Espenshade, P. J. (2010) Sterol regulatory element binding proteins in fungi: hypoxic transcription factors linked to pathogenesis. *Eukaryot. Cell* **9,** 352–359
44. Lee, H., Bien, C. M., Hughes, A. L., Espenshade, P. J., Kwon-Chung, K. J., and Chang, Y. C. (2007) Cobalt chloride, a hypoxia-mimicking agent, targets sterol synthesis in the pathogenic fungus *Cryptococcus neoformans*. *Mol. Microbiol.* **65,** 1018–1033
45. Chun, C. D., Liu, O. W., and Madhani, H. D. (2007) A link between virulence and homeostatic responses to hypoxia during infection by the human fungal pathogen *Cryptococcus neoformans*. *PLoS Pathog.* **3,** e22
46. Willger, S. D., Puttikamonkul, S., Kim, K. H., Burritt, J. B., Grahl, N., Metzler, L. J., Barbuch, R., Bard, M., Lawrence, C. B., and Cramer, R. A. (2008) A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in *Aspergillus fumigatus*. *PLoS Pathog.* **4,** e1000200
47. Kanafani, Z. A., and Perfect, J. R. (2008) Resistance to antifungal agents: mechanisms and clinical impact. *Clin. Infect. Dis.* **46,** 120–128
48. Cegielska, A., Gietzen, K. F., Rivers, A., and Virshup, D. M. (1998) Autoinhibition of casein kinase Iϵ (CKIϵ) is relieved by protein phosphatases and limited proteolysis. *J. Biol. Chem.* **273,** 1357–1364
49. Gietzen, K. F., and Virshup, D. M. (1999) Identification of inhibitory autophosphorylation sites in casein kinase Iϵ. *J. Biol. Chem.* **274,** 32063–32070
50. Swiatek, W., Tsai, I. C., Klimowski, L., Pepler, A., Barnette, J., Yost, H. J., and Virshup, D. M. (2004) Regulation of casein kinase Iϵ activity by Wnt signaling. *J. Biol. Chem.* **279,** 13011–13017
51. Cruciat, C. M., Dolde, C., de Groot, R. E., Ohkawara, B., Reinhard, C., Korswagen, H. C., and Niehrs, C. (2013) RNA helicase DDX3 is a regulatory subunit of casein kinase 1 in Wnt/β-catenin signaling. *Science* **339,** 1436–1441
52. Rumpf, C., Cipak, L., Dudas, A., Benko, Z., Pozgajova, M., Riedel, C. G., Ammerer, G., Mechtler, K., and Gregan, J. (2010) Casein kinase 1 is required for efficient removal of Rec8 during meiosis I. *Cell Cycle* **9,** 2657–2662
53. Zhao, X., Feng, D., Wang, Q., Abdulla, A., Xie, X. J., Zhou, J., Sun, Y., Yang, E. S., Liu, L. P., Vaitheesvaran, B., Bridges, L., Kurland, I. J., Strich, R., Ni, J. Q., Wang, C., Ericsson, J., Pessin, J. E., Ji, J. Y., and Yang, F. (2012) Regulation of lipogenesis by cyclin-dependent kinase 8–mediated control of SREBP-1. *J. Clin. Invest.* **122,** 2417–2427
54. Li, Y., Xu, S., Mihaylova, M. M., Zheng, B., Hou, X., Jiang, B., Park, O., Luo, Z., Lefai, E., Shyy, J. Y., Gao, B., Wierzbicki, M., Verbeuren, T. J., Shaw, R. J., Cohen, R. A., and Zang, M. (2011) AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell Metab.* **13,** 376–388
55. Sundqvist, A., Bengoechea-Alonso, M. T., Ye, X., Lukiyanchuk, V., Jin, J., Harper, J. W., and Ericsson, J. (2005) Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCF(Fbw7). *Cell Metab.* **1,** 379–391
56. Punga, T., Bengoechea-Alonso, M. T., and Ericsson, J. (2006) Phosphorylation and ubiquitination of the transcription factor sterol regulatory element-binding protein-1 in response to DNA binding. *J. Biol. Chem.* **281,** 25278–25286
57. Chatterjee, S., Szustakowski, J. D., Nanguneri, N. R., Mickanin, C., Labow, M. A., Nohturfft, A., Dev, K. K., and Sivasankaran, R. (2009) Identification of novel genes and pathways regulating SREBP transcriptional activity. *PLoS One* **4,** e5197
58. Lamia, K. A., Storch, K. F., and Weitz, C. J. (2008) Physiological significance of a peripheral tissue circadian clock. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 15172–15177
59. Rudic, R. D., McNamara, P., Curtis, A. M., Boston, R. C., Panda, S., Ho-

genesch, J. B., and Fitzgerald, G. A. (2004) BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol.* **2,** e377

60. Marcheva, B., Ramsey, K. M., Buhr, E. D., Kobayashi, Y., Su, H., Ko, C. H., Ivanova, G., Omura, C., Mo, S., Vitaterna, M. H., Lopez, J. P., Philipson, L. H., Bradfield, C. A., Crosby, S. D., JeBailey, L., Wang, X., Takahashi, J. S., and Bass, J. (2010) Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinemia and diabetes. *Nature* **466,** 627–631
61. Turek, F. W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D. R., Eckel, R. H., Takahashi, J. S., and Bass, J. (2005) Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* **308,** 1043–1045
62. Ralph, M. R., and Menaker, M. (1988) A mutation of the circadian system in golden hamsters. *Science* **241,** 1225–1227
63. Xu, Y., Padiath, Q. S., Shapiro, R. E., Jones, C. R., Wu, S. C., Saigoh, N., Saigoh, K., Ptácek, L. J., and Fu, Y. H. (2005) Functional consequences of a CKIδ mutation causing familial advanced sleep phase syndrome. *Nature* **434,** 640–644
64. Lowrey, P. L., Shimomura, K., Antoch, M. P., Yamazaki, S., Zemenides, P. D., Ralph, M. R., Menaker, M., and Takahashi, J. S. (2000) Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science* **288,** 483–492