Supplementary information

Supplementary materials and methods

Mass spectrometry

For lipid analysis by mass spectrometry, heme-deficient say1Δ (YRS1853) mutant cells were grown in cholesterol containing media. Lipids were extracted from the cell pellet, concentrated and analyzed in the positive ion mode on a Bruker Esquire HCT ion trap mass spectrometer (ESI) with a flow rate of 120 μl/h and a capillary tension of -250 V. Ion fragmentation was induced with argon as collision gas at a pressure of 8 mbar.

Lipid labeling and analysis

To examine ergosterol dependent export into the culture media, cells were grown in SC media containing Tween 80 (0.05 mg/ml) and [14C]cholesterol (0.025 μCi/ml), for 16 h at 24°C. Cells were collected by centrifugation, washed twice with SC media to remove the unincorporated label. Cells were then diluted to OD600 of ~1 in media either containing no ergosterol, 2 μg/ml, 20 μg/ml or 200 μg/ml of cold ergosterol and cells were cultivated for 24 h. Cells were collected and lipids were extracted from the cell pellet and culture supernatant. Lipids were separated by thin-layer chromatography on silica gel 60 plates (TLC; Merck, Darmstadt, Germany) with the solvent system petroleum ether/diethylether/acetic acid (70:30:2; per vol.) and radiolabeled lipids were analyzed and quantified by scanning with a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer (Berthold Technologies, Bad Wildbad, Germany).

To examine the substrate specificity of the acetylation and export pathway,
heme-deficient cells were cultivated at 24°C for 16 h in YPD media containing
Tween 80 (0.05 mg/ml) and either [3H]7-ketocholesterol, [3H]lanosterol at 1
(American Radiolabeled Chemicals Inc) at 0.025 μCi/ml. Cells were collected by
centrifugation and lipids were extracted from the cell pellet and the culture
supernatant. Samples were dried and analyzed by TLC.

**Oxygen-dependence of Say1 and Atf2 levels**

To examine the expression levels of Say1 and Atf2 under aerobic and
heme-deficient conditions, hem-deficient cells expressing C-terminally myc- or
GFP-tagged versions of Say1 and Atf2, respectively, were cultivated in YPD
media containing either Tween 80 (5 mg/ml) and cholesterol (20 μg/ml) or 10
μg/ml aminolevulinic acid (ALA) for 16 h. Cells were diluted to OD₆₀₀ of ~0.5 in
fresh media and samples were removed after 0, 2, 8 and 16 h of growth at 24°C.
Proteins were extracted from equal OD units of cells, precipitated, separated by
electrophoresis, blotted and probed with antibodies against myc, GFP or Pgk1.

**Supplementary references**

Nakamura, K., Niimi, M., Niimi, K., Holmes, A.R., Yates, J.E., Decottignies, A.,
Candida albicans drug efflux pump Cdr1p in a *Saccharomyces cerevisiae*
strain deficient in membrane transporters. *Antimicrob Agents Chemother*, 45,
3366-3374.

Bangham, R., Benito, R., Boeke, J.D., Bussey, H., Chu, A.M., Connelly, C.,
Davis, K., Dietrich, F., Dow, S.W., El Bakkoury, M., Foury, F., Friend, S.H.,
Gentalen, E., Giaevar, G., Hegemann, J.H., Jones, T., Laub, M., Liao, H.,
Acetylation controls sterol export

### Supplementary tables

**Table SI: S. cerevisiae strains used in this study**

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<tr>
<th>Strain</th>
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Acetylation controls sterol export

YRS2529  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ SAY1- This study MYC-HIS3MX6 hem1::LEU2

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YRS2538  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ GAL1- This study GFP-ATF2-HIS3MX6 hem1::LEU2

YRS2539  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ GAL1- This study GFP-ATF2-HIS3MX6 say1::KanMX4

YRS2550  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 EUROSCARF; Say1::kanMX4

YRS2551  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 EUROSCARF; Say1::kanMX4

YRS2655  MATα ade2Δ0 his3Δ1 leu2Δ0 ura3Δ0 sec12n This study say1::HIS3MX6 hem1::LEU2 sec12n

YRS2656  MATα pdr1-3 his1 ura3 yor1::hisG snq2::hisG Nakamura et al., 2001
pdr5::hisG pdr10::hisG pdr11::hisG ycf1::hisG
pdr3::hisG pdr15::hisG

YRS2776  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 This study ygr263cT/G757::MYC-HIS3MX6

YRS2846  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 This study say1::HIS3MX6 erg4::kanMX4

YRS2851  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 This study atf2::HIS3MX6 erg4::kanMX4

YRS2857  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 This study ygr263cT/G757::MYC-HIS3MX6 hem1::LEU2

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YRS2891  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ Say1::KanMX4 hem1::LEU2 pRecAADACL1::[URA3]

YRS2985  MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ Say1::KanMX4 hem1::LEU2 pRecAADAC::[URA3]
Acetylation controls sterol export

YRS3018  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ0  This study
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YRS3019  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ0  This study
trp1::URA3

YRS3052  MATa  his3Δ11,15  leu2Δ3,112  trp1Δ-1  This study
HDEL::URA3  GFP-SAY1-HIS3MX6

YRS3100  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ  This study
are1::kanMX4  are2::kanMX4  hem1::LEU2

YRS3101  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ  This study
are1::kanMX4  are2::kanMX4  say1::HIS3MX6
hem1::LEU2

YRS3102  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ  This study
are1::kanMX4  are2::kanMX4  say1::HIS3MX6

YRS3275  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ0  This study
say1::kanMX4  trp1::URA3

YRS3276  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ0  This study
atf2::kanMX4  trp1::URA3

YRS3277  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ  GAL1-  This study
GFP-SAY1-HIS3MX6  trp1::URA3

YRS3278  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ  GAL1-  This study
GFP-ATF2-HIS3MX6  trp1::URA3

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YRS3280  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ  This study
are1::kanMX4  are2::kanMX4  atf2::HIS3MX6
hem1::LEU2

YRS3281  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ0  This study
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trp1::URA3

YRS3282  MATα  his3Δ1  leu2Δ0  ura3Δ0  lys2Δ0  ATF2-  This study
GFP-HIS3MX6  hem1::LEU2
Supplementary figure legends

Figure S1. Mass spectrometric analysis of cholesterol acetate.
A) *say1Δ* mutant cells accumulate an ion with $m/z$ ratio identical to that of cholesterol acetate. Heme-deficient *say1Δ* (YRS1853) mutant cells were grown in media containing cholesterol. Lipids were extracted from the cell pellet and analyzed by mass spectrometry. The sodium adduct of cholesterol acetate (428.69 Da) employed as chemical standard gives a major peak at $m/z=451.3$. *say1Δ* mutant cells contain a lipid with identical $m/z$ ratio.

B) The fragmentation profile of the lipid present in *say1Δ* mutant cells is identical to that of cholesterol acetate. Fragmentation of the cholesterol acetate ($m/z$ 451.3) reveals a daughter ion of $m/z=396.3$, which corresponds to a loss of sodium acetate. Fragmentation of the ion at $m/z=451.3$ in the lipid extract of *say1Δ* mutant cells reveals the same daughter ion at $m/z=396.3$. CA, cholesterol acetate.

Figure S2. Export of cholesterol acetate is stimulated by the presence of ergosterol in the media.
Heme-deficient *say1Δ* (YRS1853) mutant cells were labeled with $[^{14}C]$cholesterol for 16 h, diluted into fresh media containing the indicated concentration of cold ergosterol and cultivated for 24 h. Lipids were extracted from the cell pellet and the culture media, analyzed by TLC and quantified. The proportion of cholesterol acetate (CA), free cholesterol (FC), and steryl esters (STE) that is exported into the culture media is plotted as a function of ergosterol concentration in the media.
Figure S3. Formation of cholesterol acetate is independent of Are1 and Are2, but efficient export of cholesterol acetate requires long-chain steryl esters.

A) Formation of cholesterol acetate is independent of Are1 and Are2. Heme-deficient wild-type (YRS1849), say1Δ (YRS1853), atf2Δ (YRS2135), are1Δ are2Δ (YRS3100), are1Δ are2Δ say1Δ (YRS3101), and are1Δ are2Δ atf2Δ (YRS3280) mutant cells were labeled with [14C]cholesterol for 16 h. Lipids were extracted, separated by TLC and visualized using a phosphorimager. FC, free cholesterol; CA, cholesteryl acetate; STE, steryl ester.

B) Export of sterol acetate is strongly reduced in cells lacking Are1 and Are2. Heme-deficient say1Δ (YRS1853), are1Δ are2Δ (YRS3100), and are1Δ are2Δ say1Δ (YRS3101) mutant cells were labeled with [14C]cholesterol for 16 h, diluted into fresh media containing cold ergosterol and cultivated for 6 h. Lipids were extracted from the cell pellet (intracellular) and the culture media (extracellular) and analyzed by TLC. FC, free cholesterol; CA, cholesteryl acetate; STE, steryl ester. The open star indicates an unidentified secreted sterol derivative.

Figure S4. Say1 or Atf2 are not essential in cells that form no long-chain steryl esters.

Deletion of either Say1 or Atf2 in an are1Δ are2Δ mutant background does not impair growth. Heme-competent and heme-deficient wild-type (YRS1533, YRS1849), say1Δ (YRS2550, YRS1853), atf2Δ (YRS2551, YRS2135), are1Δ are2Δ (YRS2486, YRS3100), are1Δ are2Δ say1Δ (YRS3102, YRS3101), and are1Δ are2Δ atf2Δ (YRS3279, YRS3280) mutant cells were serially diluted 10-fold and spotted on YPD plates or media supplemented with cholesterol and Tween (Chol/Tween) or with ALA. Plates were incubated at 30°C for 4 d.
Figure S5. Substrate specificity of the sterol acetylation and export pathway.

Heme-deficient wild-type (YRS1849), saylΔ (YRS1853), atf2Δ (YRS2135), and are1Δ are2Δ (YRS3100) mutant cells were labeled with either [14C]β-sitosterol, [14C]progesterone, [14C]25-hydroxycholesterol, [3H]7-ketocholesterol, or [3H]lanosterol for 16 h. Lipids were extracted from the cell pellet (intracellular) and the culture media (extracellular), separated by TLC and visualized using a phosphorimager. FSito, free sitosterol; STE, steryl ester; FProg, free progesterone; OH-CA, hydroxycholesteryl acetate; FOH-C, free hydroxycholesterol; FKC, free 7-ketocholesterol; FL, free lanosterol.

Figure S6. Expression of Say1 and Atf2 is independent of oxygen availability.

Heme-deficient cells expressing chromosomally tagged versions of Say1-myc (YRS2529) or Atf2-GFP (YRS3282) were cultivated in YPD media containing either cholesterol/Tween or ALA. Cells were diluted to an OD of 0.5 and samples were removed after growth for the time indicated, proteins were extracted, separated by electrophoresis, and probed with antibodies against myc or GFP. Phosphoglycerate kinase (Pgk1) was used as a loading control.
Figure S1
Figure S2
Figure S3
Figure S4
25-hydroxycholesterol

sitosterol

progesterone

25-hydroxycholesterol

7-ketocholesterol

lanosterol

Figure S5
Figure S6