Supplementary information

Supplementary materials and methods

Mass spectrometry

For lipid analysis by mass spectrometry, heme-deficient *say1*Δ (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 [^{14}C]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 OD_{600} 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 [^{3}H]7-ketocholesterol, [^{3}H]lanosterol at 1 μCi/ml or [^{14}C]25-hydroxycholesterol, [^{14}C]ß-sitosterol, or [^{14}C]progesterone (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 OD_{600} 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., Monk, B.C., Goffeau, A. and Cannon, R.D. (2001) Functional expression of Candida albicans drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters. *Antimicrob Agents Chemother*, **45**, 3366-3374.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., Andre, B., 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., Giaever, G., Hegemann, J.H., Jones, T., Laub, M., Liao, H.,

Davis, R.W. and et al. (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science*, **285**, 901-906.

Supplementary tables

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

Strain	Relevant genotype	Source or reference
YRS1533	*BY4742; MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	EUROSCARF; Winzeler et al., 1999
YRS1845	*MATa ade2Δ0 his3Δ1 leu2Δ0 ura3Δ0 sec12^{ts}*	This study
YRS1849	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 hem1::LEU2*	This study
YRS1853	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 say1::kanMX4 hem1::LEU2*	This study
YRS2133	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 atf2::kanMX4 say1::HIS3MX6*	This study
YRS2135	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ atf2::kanMX4 hem1::LEU2*	This study
YRS2136	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 atf2::kanMX4 say1::HIS3MX6 hem1::LEU 2*	This study
YRS2209	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 atf1::kanMX4 say1::HIS3MX6 hem1::LEU2*	This study
YRS2211	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ GAL1-GFP-SAY1-HIS3MX6*	This study
YRS2212	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ GAL1-GFP-SAY1-HIS3MX6 hem1::LEU2*	This study
YRS2483	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 ATF2-GFP-HIS3MX6*	This study
YRS2486	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 are1::kanMX4 are2::kanMX4*	This study
YRS2495	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 say1::HIS3MX6 hem1::LEU2 pdr5::kanMX4 snq2::kanMX4*	This study
YRS2528	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ SAY1-MYC-HIS3MX6*	This study
YRS2529	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ SAY1-*	This study

YRS2529	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ SAY1-MYC-HIS3MX6 hem1::LEU2*	This study
YRS2537	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ GAL1-GFP-ATF2-HIS3MX6*	This study
YRS2538	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 GAL1-GFP-ATF2-HIS3MX6 hem1::LEU2*	This study
YRS2539	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ GAL1-GFP-ATF2-HIS3MX6 say1::KanMX4 hem1::LEU 2*	This study
YRS2550	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 say1::kanMX4*	EUROSCARF; Winzeler et al., 1999
YRS2551	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 atf2::kanMX4*	EUROSCARF; Winzeler et al., 1999
YRS2655	*MATa ade2Δ0 his3Δ1 leu2Δ0 ura3Δ0 sec12^{ts} say1::HIS3MX6 hem1::LEU2 sec12^{ts}*	This study
YRS2656	*MATα pdr1-3 his1 ura3 yor1::hisG snq2::hisG pdr5::hisG pdr10::hisG pdr11::hisG ycf1::hisG pdr3::hisG pdr15::hisG*	Nakamura et al., 2001
YRS2776	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 ygr263cT/G757::MYC-HIS3MX6*	This study
YRS2846	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 say1::HIS3MX6 erg4::kanMX4*	This study
YRS2851	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 atf2::HIS3MX6 erg4::kanMX4*	This study
YRS2857	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 ygr263cT/G757::MYC-HIS3MX6 hem1::LEU2*	This study
YRS2862	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 erg4::kanMX4*	EUROSCARF; Winzeler et al., 1999
YRS2890	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ say1::KanMX4 hem1::LEU2 pYES [URA3]*	This study
YRS2891	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ say1::KanMX4 hem1::LEU2 pRecAADACL1::[URA3]*	This study
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YRS3019	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1::URA3*	This study
YRS3052	*MATa his3Δ11,15 leu2Δ3,112 trp1Δ-1 HDEL::URA3 GFP-SAY1-HIS3MX6*	This study
YRS3100	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ are1::kanMX4 are2::kanMX4 hem1::LEU2*	This study
YRS3101	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ are1::kanMX4 are2::kanMX4 say1::HIS3MX6 hem1::LEU2*	This study
YRS3102	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ are1::kanMX4 are2::kanMX4 say1::HIS3MX6*	This study
YRS3275	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 say1::kanMX4 trp1::URA3*	This study
YRS3276	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 atf2::kanMX4 trp1::URA3*	This study
YRS3277	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ GAL1-GFP-SAY1-HIS3MX6 trp1::URA3*	This study
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YRS3279	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ are1::kanMX4 are2::kanMX4 atf2::HIS3MX6*	This study
YRS3280	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ are1::kanMX4 are2::kanMX4 atf2::HIS3MX6 hem1::LEU2*	This study
YRS3281	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 are1::kanMX4 are2::kanMX4 atf2::HIS3MX6 trp1::URA3*	This study
YRS3282	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 ATF2-GFP-HIS3MX6 hem1::LEU2*	This study

Supplementary figure legends

Figure S1. Mass spectrometric analysis of cholesterol acetate.

A) *say1*Δ mutant cells accumulate an ion with *m/z* ratio identical to that of cholesterol acetate. Heme-deficient *say1*Δ (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. *say1*Δ mutant cells contain a lipid with identical *m/z* ratio.

B) The fragmentation profile of the lipid present in *say1*Δ 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 *say1*Δ 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 *say1*Δ (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), *say1*Δ (YRS1853), *atf2*Δ (YRS2135), *are1*Δ *are2*Δ (YRS3100), *are1*Δ *are2*Δ *say1*Δ (YRS3101), and *are1*Δ *are2*Δ *atf2*Δ (YRS3280) mutant cells were labeled with [^{14}C]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 *say1*Δ (YRS1853), *are1*Δ *are2*Δ (YRS3100), and *are1*Δ *are2*Δ *say1*Δ (YRS3101) mutant cells were labeled with [^{14}C]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 *are1*Δ *are2*Δ mutant background does not impair growth. Heme-competent and heme-deficient wild-type (YRS1533, YRS1849), *say1*Δ (YRS2550, YRS1853), *atf2*Δ (YRS2551, YRS2135), *are1*Δ *are2*Δ (YRS2486, YRS3100), *are1*Δ *are2*Δ *say1*Δ (YRS3102, YRS3101), and *are1*Δ *are2*Δ *atf2*Δ (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.

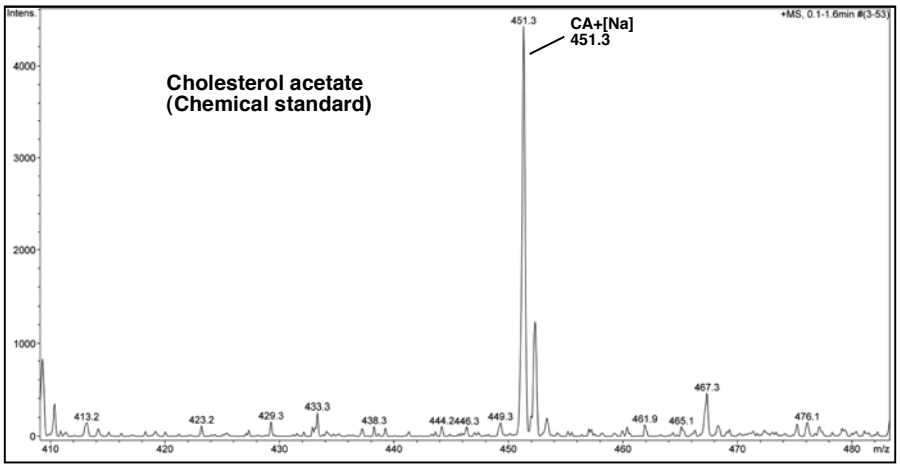
Figure S5. Substrate specificity of the sterol acetylation and export pathway.

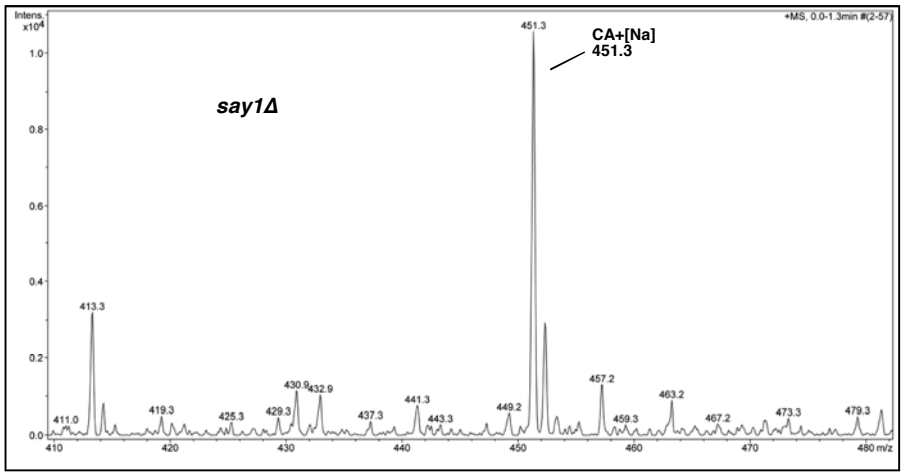
Heme-deficient wild-type (YRS1849), *say1*Δ (YRS1853), *atf2*Δ (YRS2135), and *are1*Δ *are2*Δ (YRS3100) mutant cells were labeled with either [^{14}C]ß-sitosterol, [^{14}C]progesterone, [^{14}C]25-hydroxycholesterol, [^{3}H]7-ketocholesterol, or [^{3}H]lanosterol for 16 h. Lipids were extracted from 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.

A





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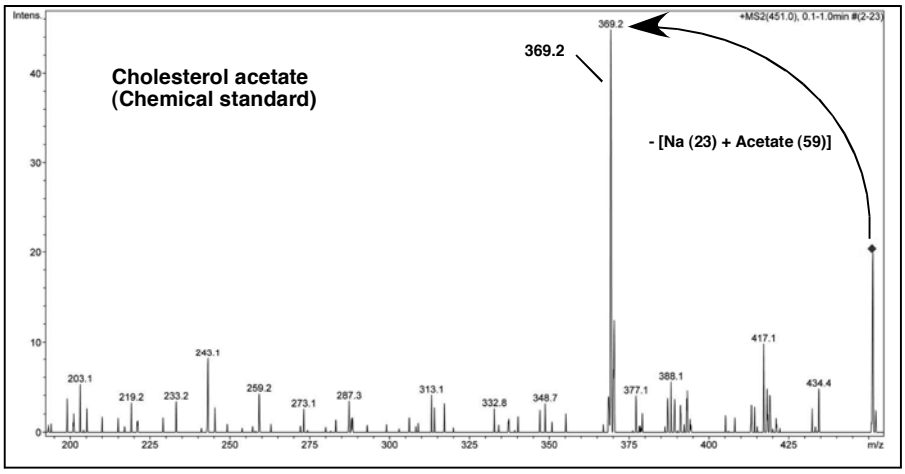
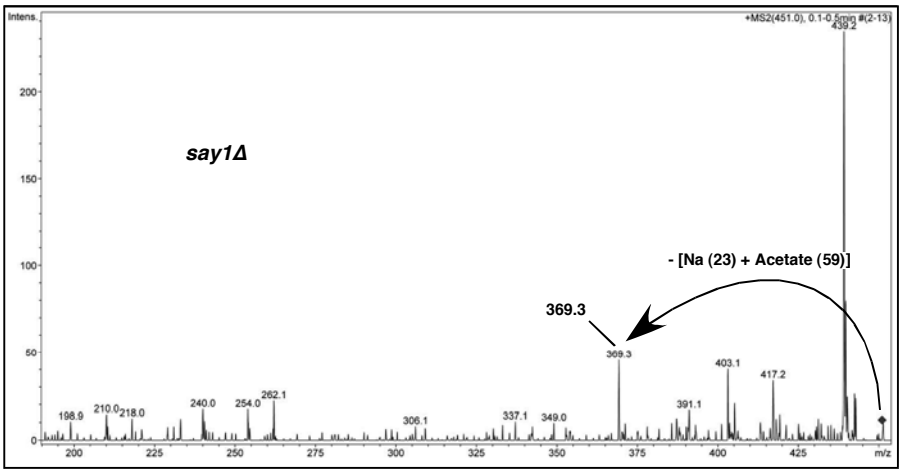
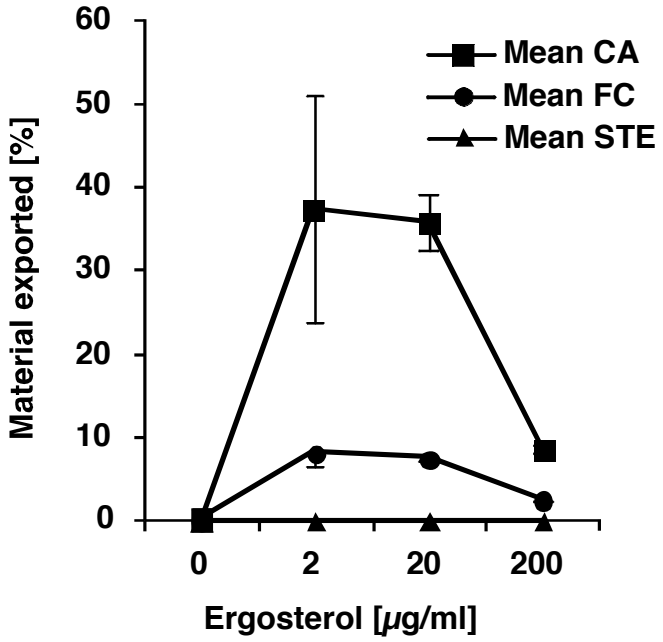


Daughter ions of CA (451.3)



Daughter ions of CA (451.3)

Figure S1


60
50
40
30
20
10
0
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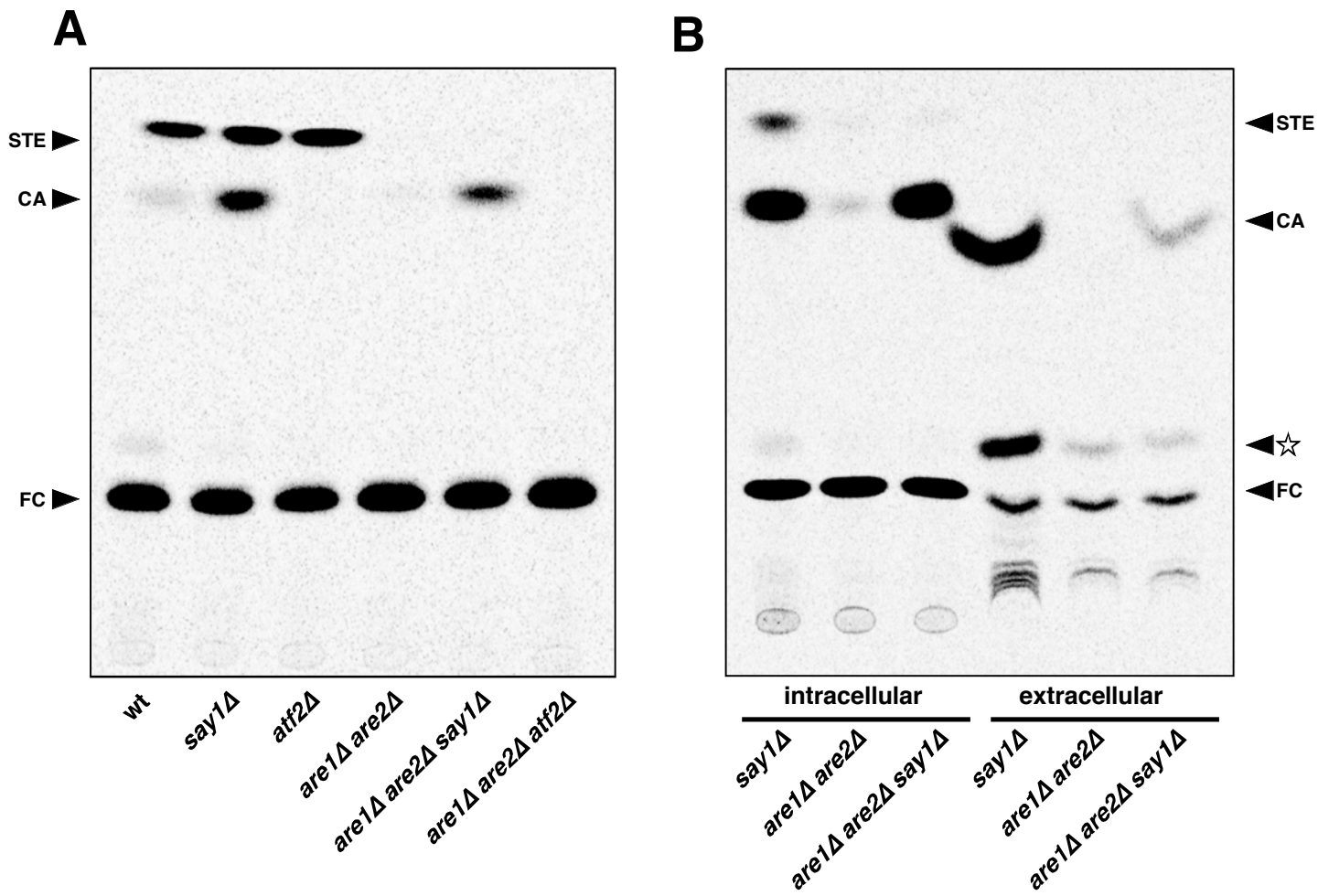
Mean CA
Mean FC
Mean STE
0
2
20
200
Ergosterol [µg/ml]

Figure S2


A
STE
CA
FC
wt
say1Δ
atf2Δ
are1Δ are2Δ
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STE
CA
☆
FC
intracellular
extracellular
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are1Δ are2Δ say1Δ
say1Δ
are1Δ are2Δ
are1Δ are2Δ say1Δ

Figure S3

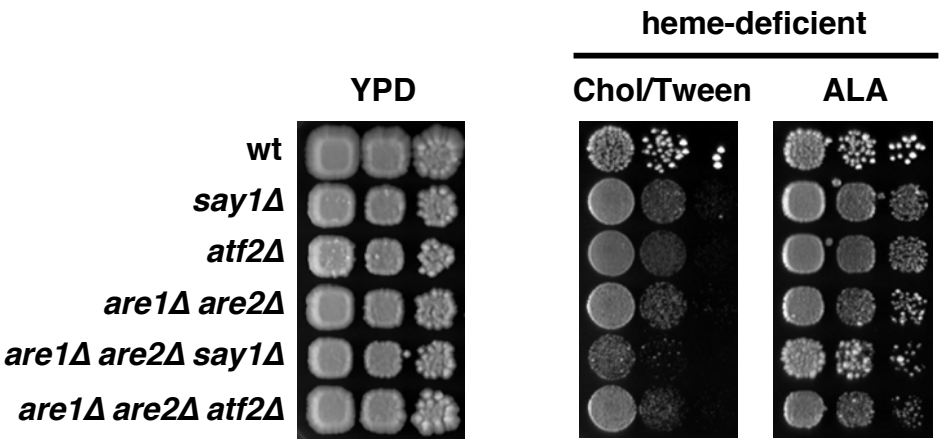
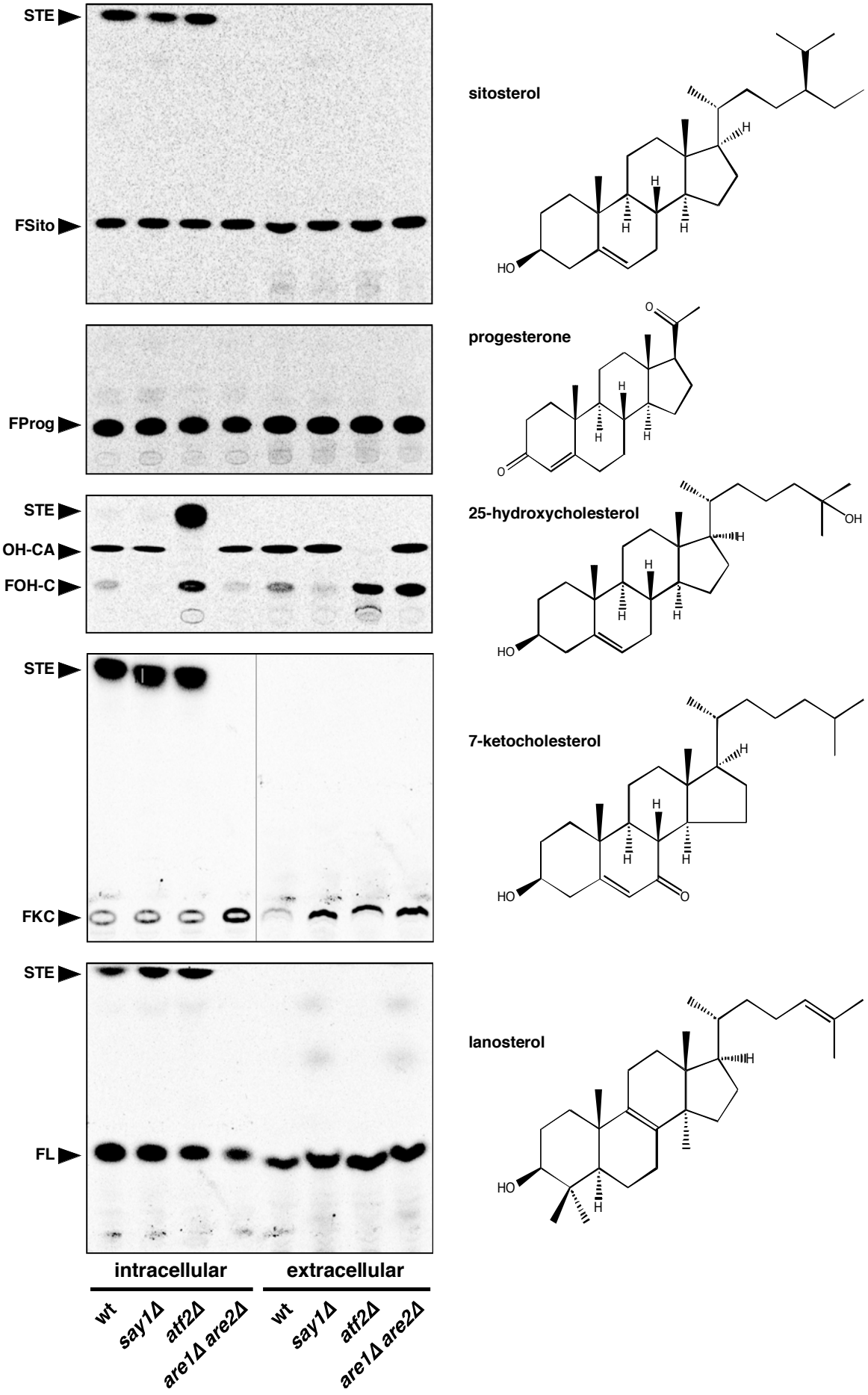

heme-deficient
YPD
Chol/Tween
ALA
wt
say1Δ
atf2Δ
are1Δ are2Δ
are1Δ are2Δ say1Δ
are1Δ are2Δ atf2Δ

Figure S4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atf2Δ
are1Δ are2Δ
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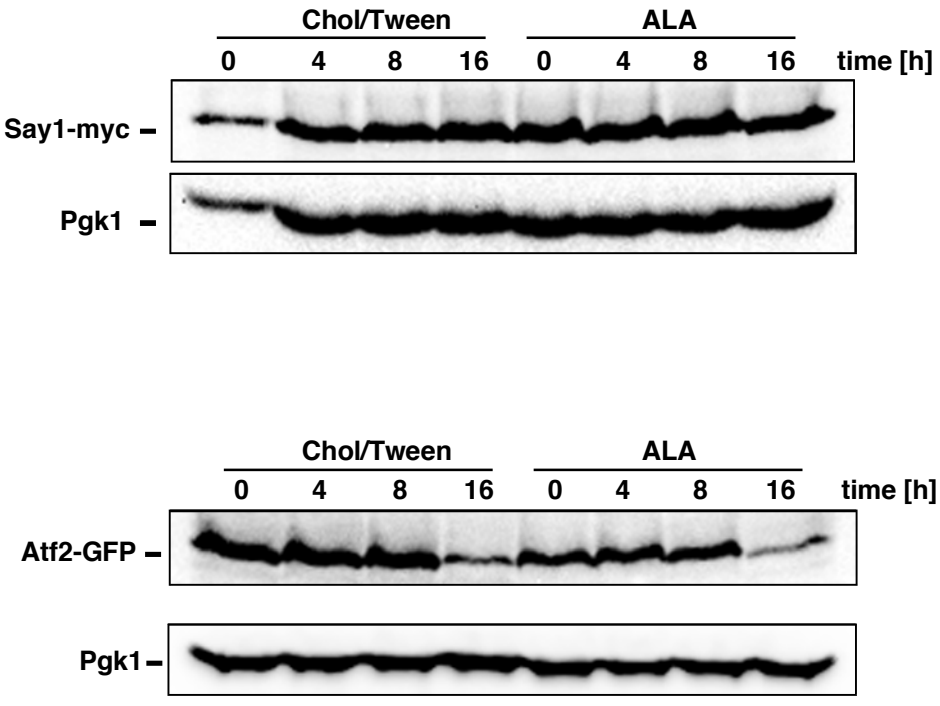
atf2Δ
are1Δ are2Δ
sitosterol
progesterone
25-hydroxycholesterol
7-ketocholesterol
lanosterol

Figure S5


Chol/Tween
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8
16
0
4
8
16
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Say1-myc
Pgk1
Chol/Tween
ALA
0
4
8
16
0
4
8
16
time [h]
Atf2-GFP
Pgk1

Figure S6