

Yeast lacking the sterol C-5 desaturase Erg3 are tolerant to the anti-inflammatory triterpenoid saponin escin

Authors

Emily J. Johnston^{1*}, Jess Tallis¹, Edward Cunningham-Oakes², Tessa Moses³, Simon J. Moore⁴, Sarah Hosking⁵, Susan J. Rosser^{1*}

Author affiliations

¹Centre for Engineering Biology, University of Edinburgh, Edinburgh, EH9 3BD, UK

²Department of Infection Biology and Microbiomes, Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, L69 7ZB, UK

³EdinOmics, RRID:SCR_021838, University of Edinburgh, Max Born Crescent, Edinburgh EH9 3BF, UK

⁴Genetic Science Division, Thermo Fisher Scientific, 7 Kingsland Grange, Warrington, Cheshire, WA1 4SR, UK

⁵Unilever R&D Port Sunlight, Quarry Road East, Bebington, Wirral, CH63 3JW, UK

***Contact information**

Emily J. Johnston: emily.johnston@ed.ac.uk.

Susan J. Rosser: susan.rosser@ed.ac.uk

Supplementary Information

Methods

Gas Chromatography Time-Of-Flight Mass Spectrometry (GC/QTOF-MS)

GC/QTOF-MS was conducted using an Agilent 7200B GC/Q-TOF mass spectrometer with Gerstel Multi-Purpose Sampler (MPS) robotics (Anatune). Samples were derivatised directly prior to GC/QTOF-MS using the MPS; to each vial, 50 µL of pyridine:N-methylsilyltrifluoroacetamine (1:4) was added, vortexed (30 seconds), incubated (30 min, 37 °C, 750 rpm), and then centrifuged (2 min, 4,500 rpm). Trimethylsilylated samples of 1 µL were injected with a 1:10 split ratio into a DB-5ms 30 m × 250 µm × 0.25 µm GC column (Agilent Technologies). Helium was used as the carrier gas at a flow rate of 1 mL/min. The inlet was set to 250 °C, and the GC oven was programmed to hold at 100 °C for 2 min, then increase to 300 °C (at 25 °C/min), where it was held for 25 min. The ion source was set to 230 °C, with 35 µA filament current and 70 eV electron energy. A mass range of 35-600 *m/z* was scanned at an acquisition rate of 5 spectra/s with a solvent delay of 13 min.

RNA extraction and sequencing

Cell pellets of approximately 2×10^7 cells were lysed using 100 U of lyticase (Sigma L2524), and RNA was extracted using RNeasy Mini Kit (QIAGEN 74104) following the manufacturer's instructions, with on-column DNase treatment (RNase-free DNase set; QIAGEN 79254). The RNA was eluted into 50 µL DEPC-treated water (Ambion AM9915G).

cDNA synthesis

500 ng of RNA was reverse transcribed to cDNA using SuperScript IV (Invitrogen 18091200) and Oligo d(T)₂₀ primers according to manufacturer's instructions, with RiboLock RNase Inhibitor (ThermoScientific EO0381) and Ribonuclease H (Invitrogen 18021071).

RNA sequencing

At the University of Liverpool Centre for Genomic Research, total RNA samples were treated concomitantly with DNase (Turbo DNA-freeTM kit; AM1907; Ambion by Life Technologies) and SUPERase•In RNase Inhibitor (AM2694; Invitrogen). After treatment, the samples were purified by use of Agencourt RNAClean XP beads (A66882; Beckman-Coulter). rRNA depletion was carried out by use of the NEBNext® rRNA Depletion Kit (E7850L; New England BioLabs), but substituting the bacterial baits with yeast baits. NEBNext Ultra II Directional RNA libraries were prepared from rRNA depleted RNA (E7760L; New England BioLabs). Paired-end RNA-Seq reads were generated using an Illumina NovaSeq6000.

Analysis of RNA sequencing data

First, raw FASTQ files were trimmed for the presence of Illumina adapter sequences (wherever the 3' end matched the adapter sequence for ≥3 bp) using Cutadapt (v1.2.1). Reads were further trimmed using Sickle (v1.200), with a minimum window quality score of 20, and reads <10 bp after trimming were removed. Additional read quality checks were performed using FastQC (v0.11.9) and MultiQC (v1.10). Reads were then mapped to a representative genome annotation for BY4741 (<http://sgd->

archive.yeastgenome.org/sequence/strains/BY4741/BY4741_Toronto_2012/) from the Saccharomyces Genome Database, using the universal RNA-sequence aligner, STAR (v2.7.7a). Resultant BAM files were quality-filtered, using a mapping quality (MAPQ) cut-off of 244. Quality filtered files were then assessed for quality using the depth utility of Samtools (v1.9). Quality-assessed BAM files were then used with the genome annotation for BY4741, to generate a table of aligned read counts, using featureCounts (v2.0.1) from the subread package, with the minimum number of overlapping bases in a read required for read assignment set to 150 bp. Analysis of Differentially Expressed Genes was conducted using iDEP (v9.2)¹. Gene Set Enrichment analysis was carried out using ShinyGO (v0.61)². Heatmaps for selected gene sets were generated using Morpheus (<https://software.broadinstitute.org/morpheus>).

Tables

Figures

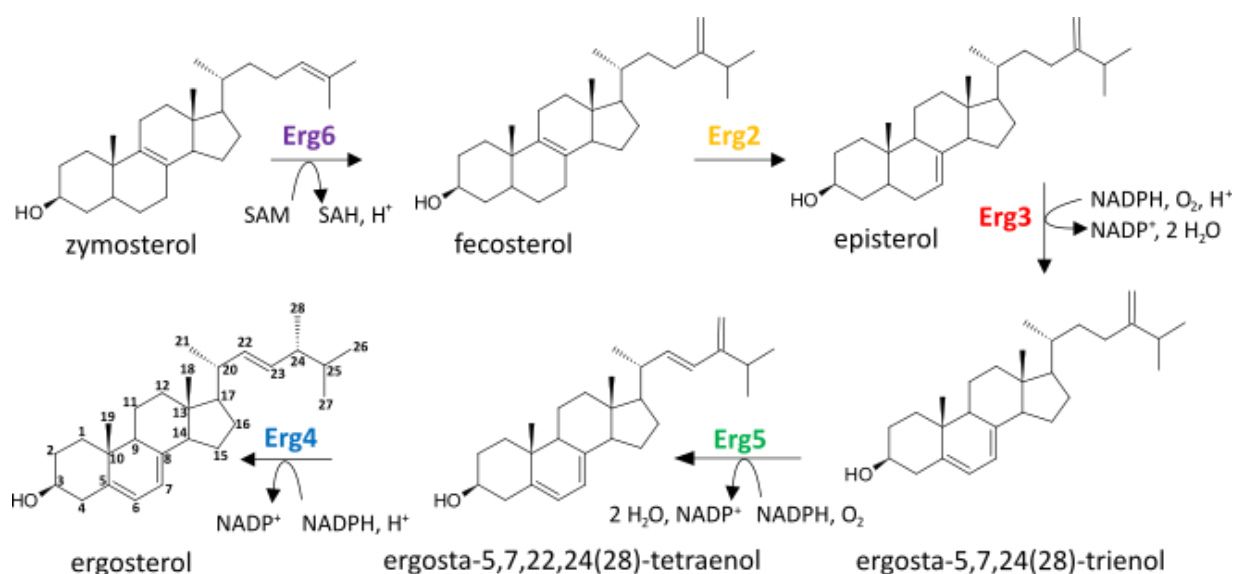


Figure S1: Reactions catalysed in the late steps of ergosterol biosynthesis

The five reactions in the conversion of zymosterol to ergosterol are often depicted sequentially as shown here, however the enzymes involved exhibit some promiscuity in sterol substrate. Erg6 is a C-24 sterol methyltransferase. Erg2 is a C-8 sterol isomerase. Erg3 is a sterol C-5 desaturase. Erg5 is a C-22 sterol desaturase. Erg4 is a C-24 sterol reductase. SAM, S-adenosyl-L-methionine. SAH, S-adenosyl-L-homocysteine.

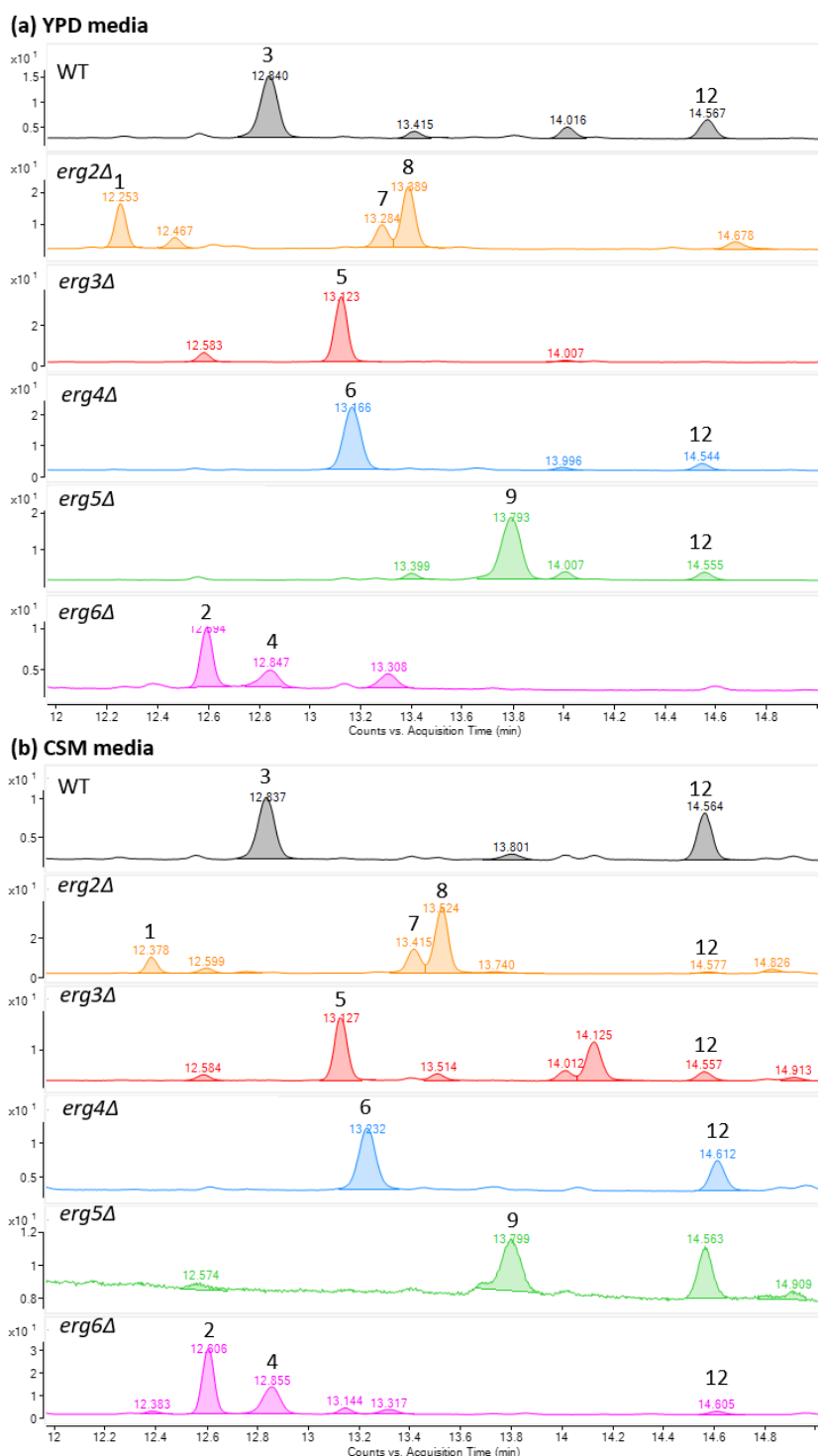
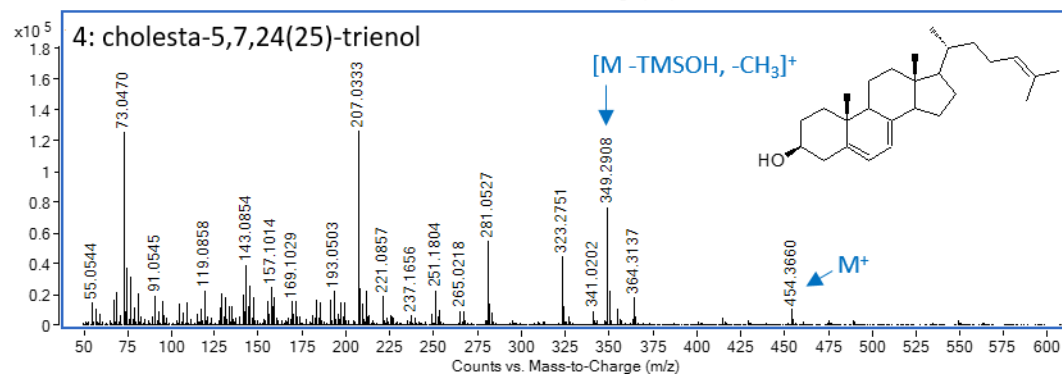
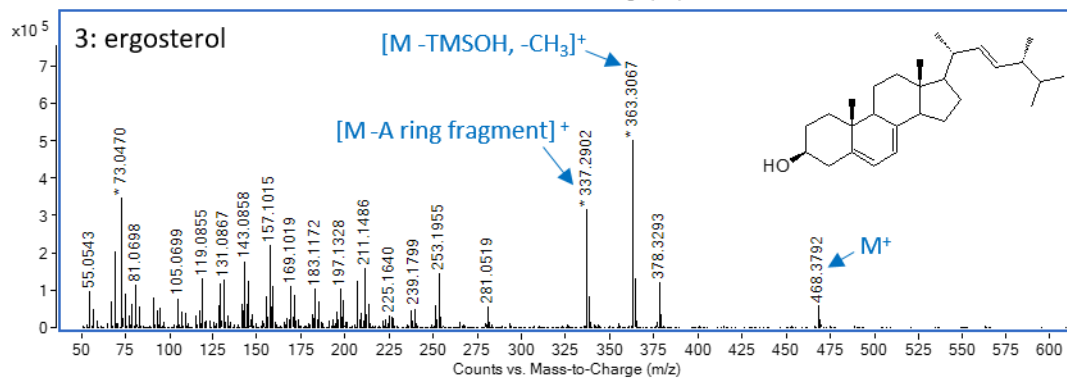
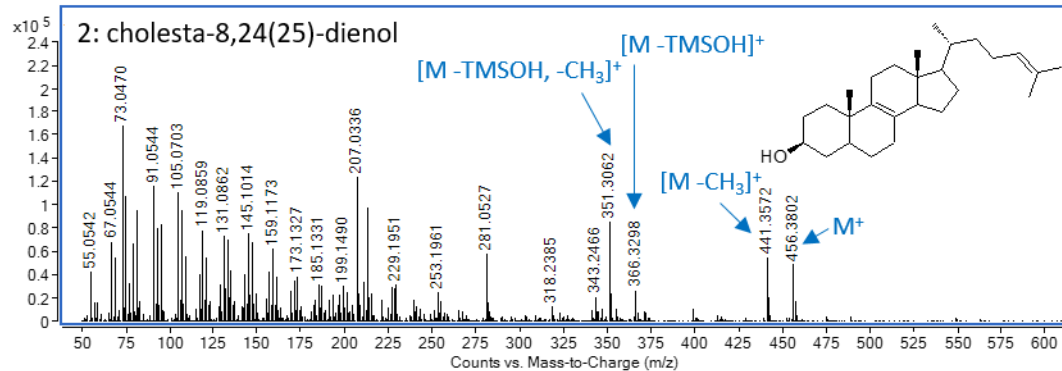
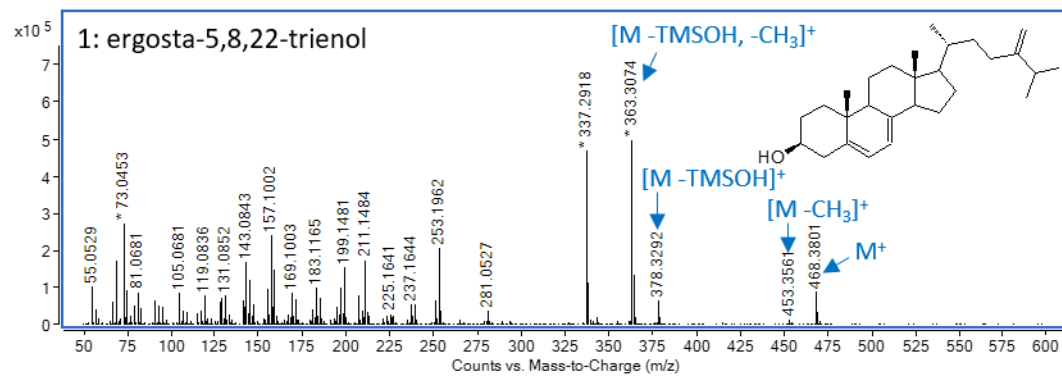
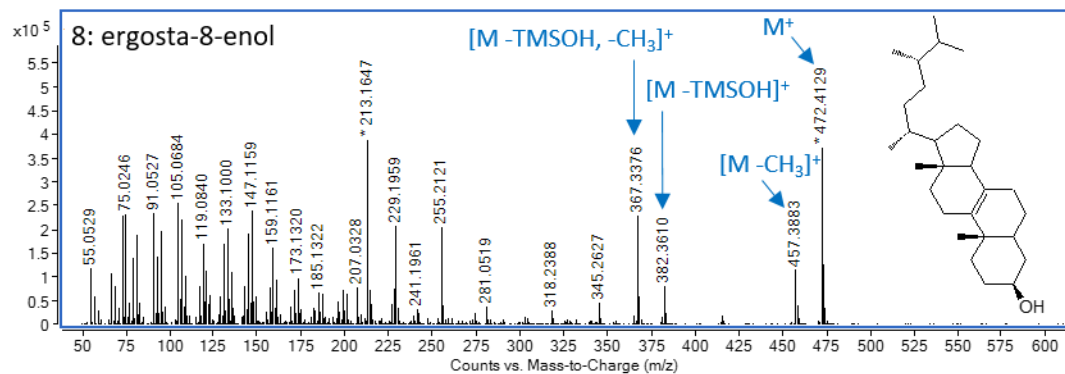
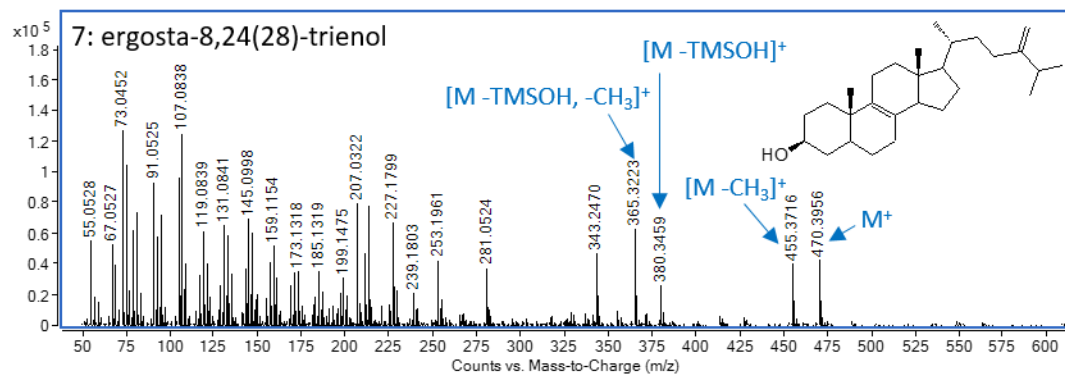
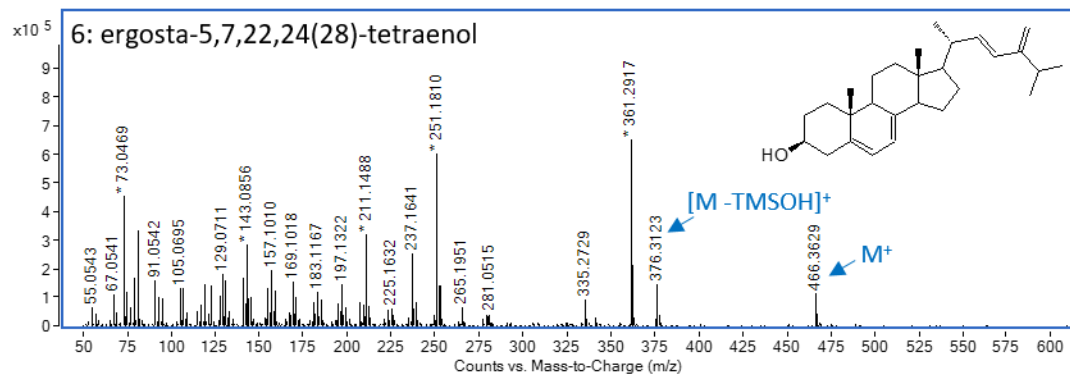
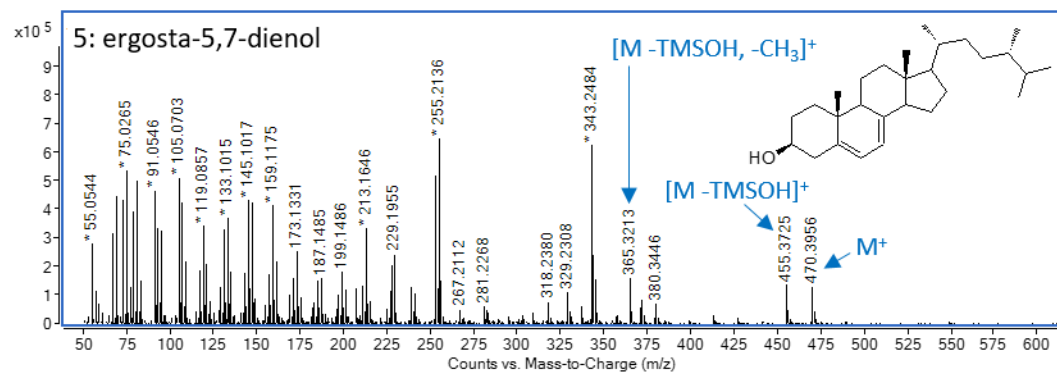


Figure S2: Total Ion Chromatograms for sterol extracts

Total Ion Chromatograms (TICs) showing peaks corresponding to sterols extracted from yeast strains cultured in either (a) YPD or (b) CSM media for 24 hours. Peak annotation based on ion m/z , relative retention times and relative abundances which have been previously reported³⁻⁶: (1) ergosta-5,8,22-trienol, (2) cholesta-8,24(25)-dienol (zymosterol), (3) ergosterol, (4) cholesta-5,7,24(25)-trienol, (5) ergosta-7,22-dienol, (6) ergosta-5,7,22,24(28)-tetraenol, (7) ergosta-8,24(28)-trienol (fecosterol), (8) ergosta-8-enol, (9) ergosta-5,7-dienol, (10) ergosta-7,24(28)-dienol (episterol), (11) ergosta-7-enol and (12) lanosterol. Ergosterol and lanosterol were verified using reference standards.





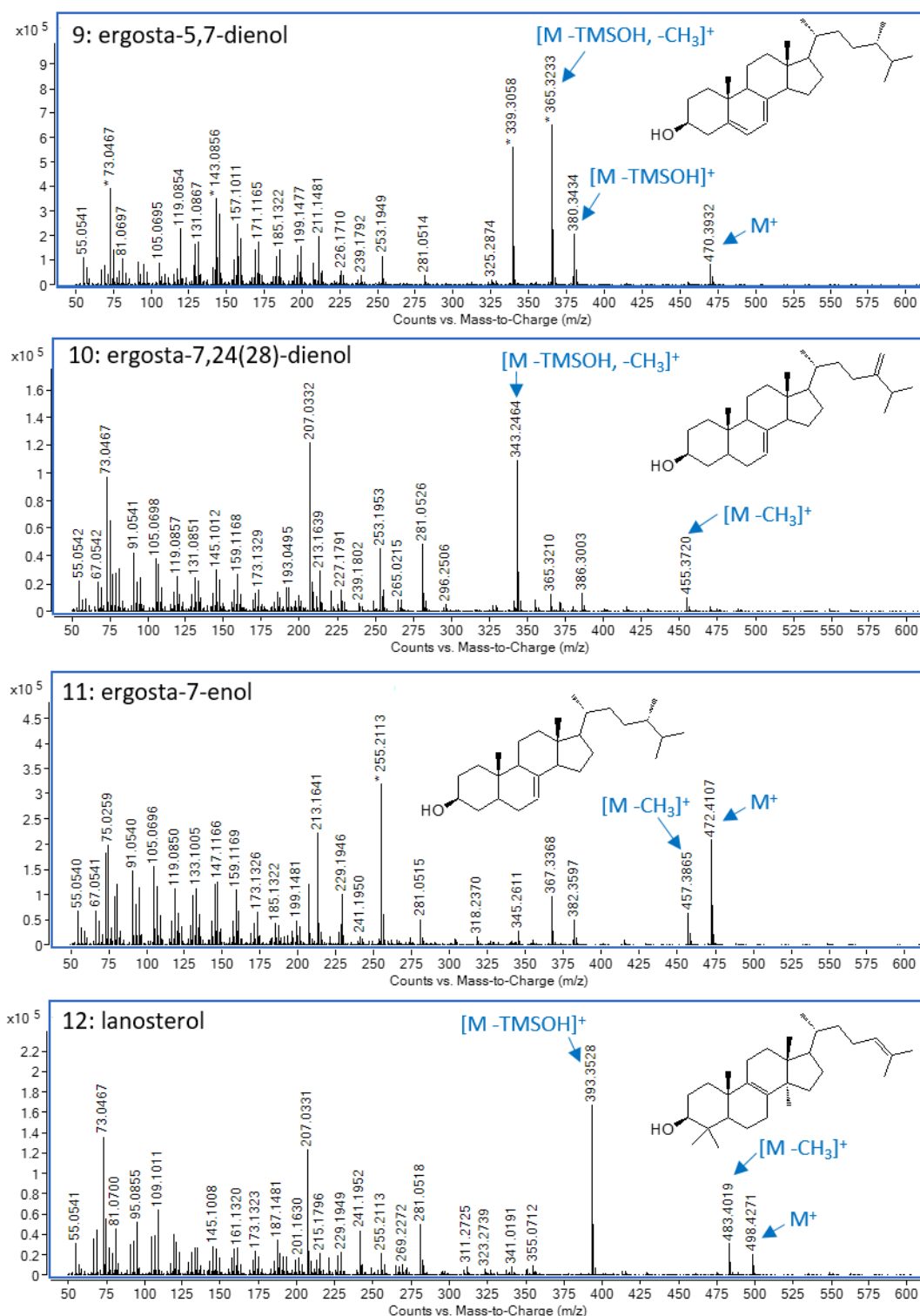


Figure S3: Sterol fragmentation patterns

Ions at the TIC peaks annotated 1 to 12 in Figure S2. M, parent ion with trimethylsilylation of the hydroxyl groups. TMSOH, trimethylsilanol. The characteristic ergosterol fragment ion at m/z 337 results from fragmentation of the A ring with loss of carbon atoms 1 to 3 and their substituents, and transfer of hydrogen from C-9⁷. Except for ergosterol and lanosterol which were verified using reference standards, sterol annotation is speculative based on ion m/z, relative retention times and abundances which have been previously reported³⁻⁶.

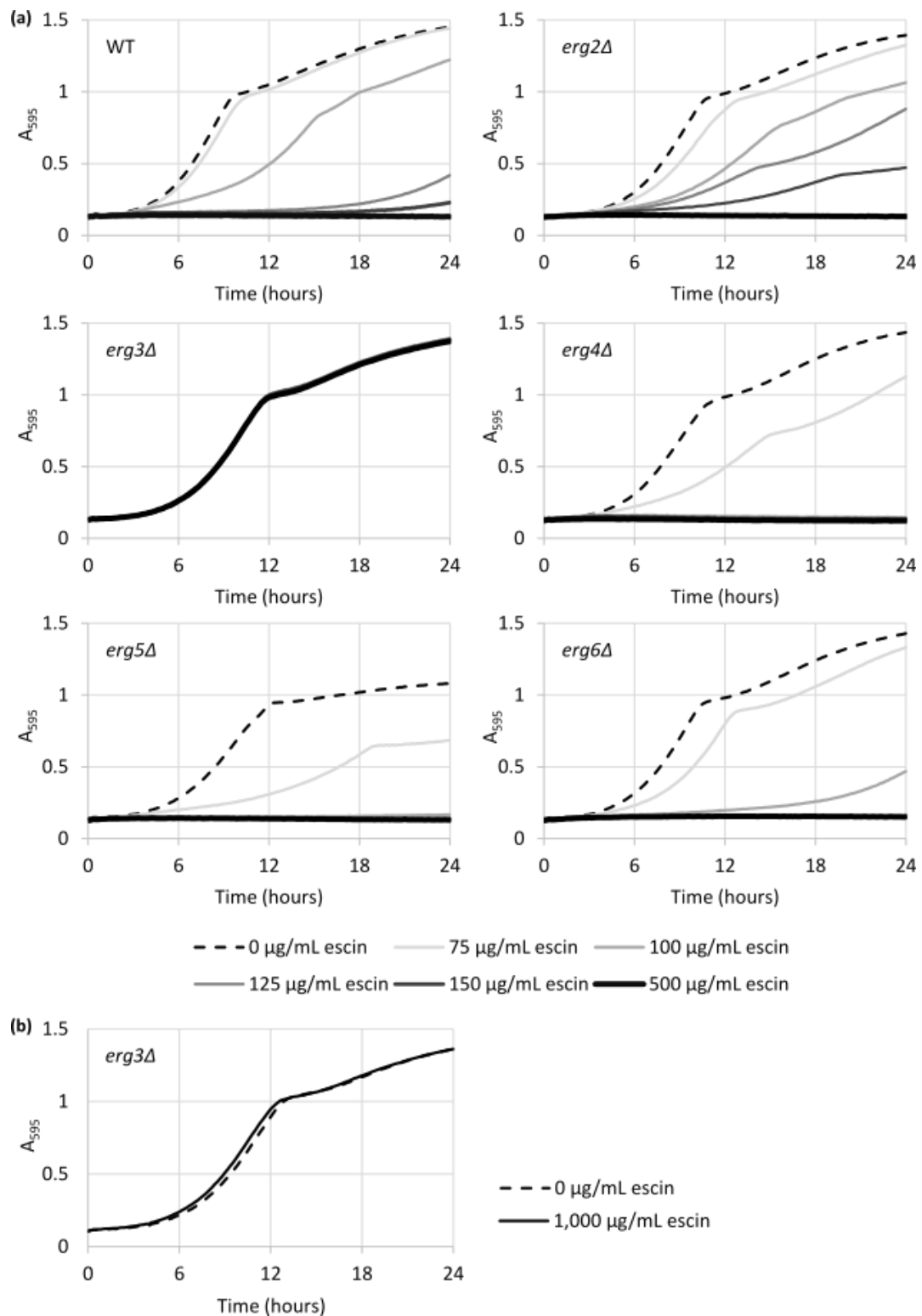


Figure S4: Growth of ergosterol biosynthesis mutants in the presence of escin

a, Growth of microplate cultures indicated by A_{595} , in the presence of different concentrations of escin. YPD media 1.25 % methanol. Average of three biological replicates shown. **b**, Growth of the *erg3Δ* strain in the presence of 0 and 1,000 $\mu\text{g/mL}$ escin, YPD media 2.5 % methanol. Average of three biological replicates.

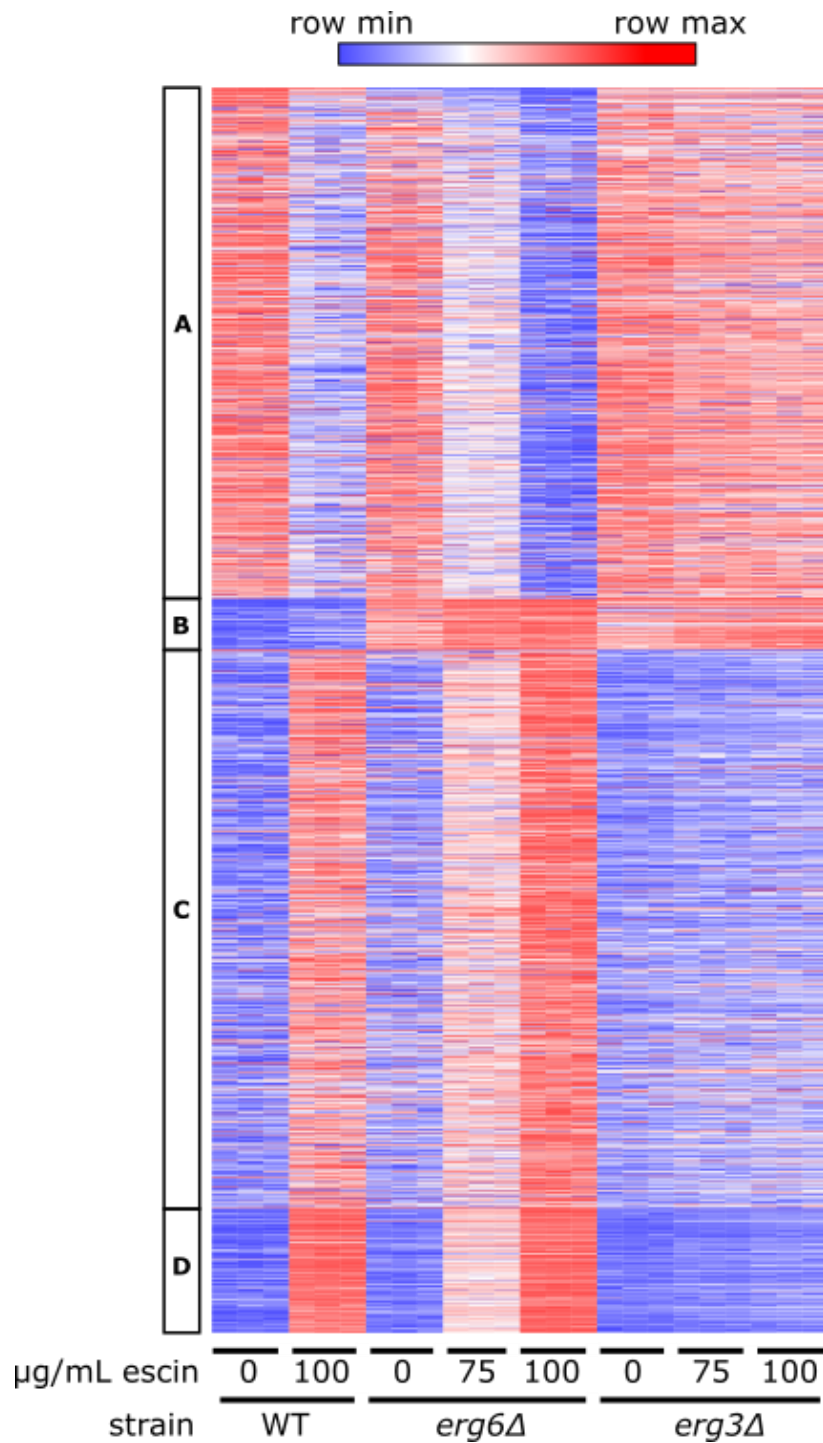


Figure S5: K means clustering of the 2,000 most variable genes

Expression as $\log_2(\text{CPM}+4)$. Cluster A ($n=821$) is enriched in genes relating to ribosome biogenesis and RNA processing. Cluster B ($n=82$) is enriched in genes relating to sterol biosynthesis, sterol transport, siderophore transport, and regulation of transcription by glucose. Cluster C ($n=897$) is enriched in genes relating to late nucleophagy, lipid catabolism, sulphur assimilation, the tricarboxylic acid cycle and gluconeogenesis. Cluster D ($n=200$) is enriched in genes relating to trehalose, mannose, fructose and glutamate metabolism, glycolysis and cell wall organisation. Cluster D is also enriched in genes associated with responses to osmotic, oxidative, temperature and starvation stress.

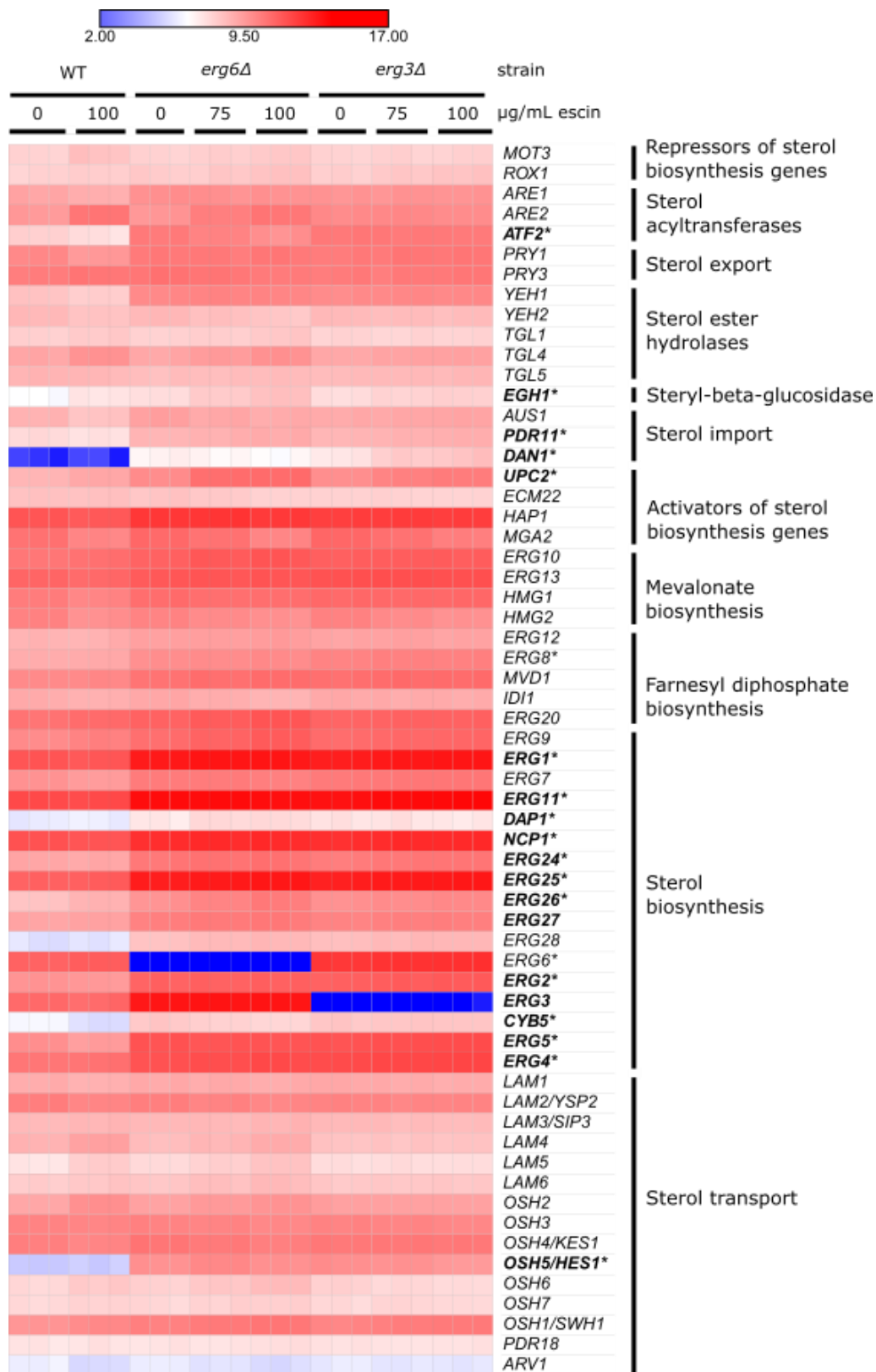


Figure S6: Expression of genes relating to sterol biosynthesis and transport

Expression scale $\log_2(\text{CPM}+4)$. Bold; upregulated ≥ 2 -fold in the comparison *erg6Δ* vs. WT (0 μg/mL escin). Asterisk; upregulated ≥ 2 -fold in the comparisons *erg3Δ* vs. WT (0 μg/mL escin). Comparison FDR ≤ 0.1 .

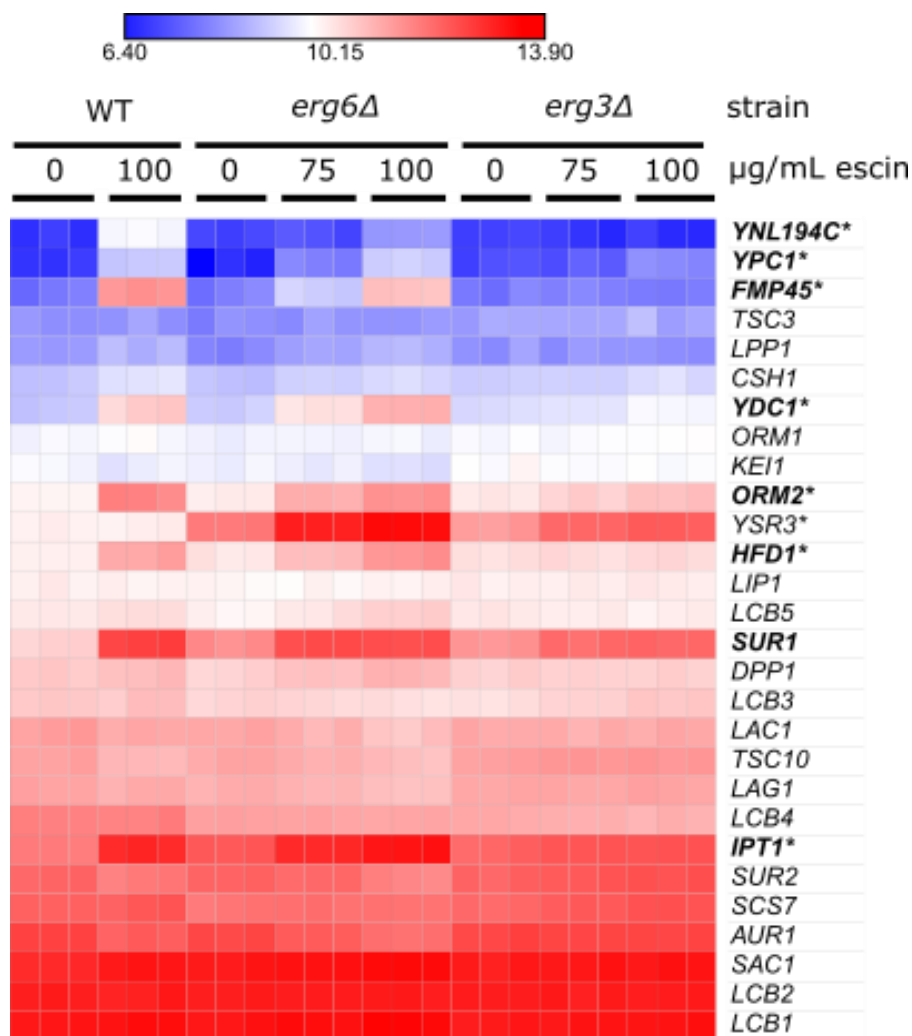


Figure S7: Expression of genes relating to sphingolipid supply

Expression scale log2(CPM+4). Bold; upregulated ≥ 2 -fold in the comparison WT 100 vs. 0 $\mu\text{g/mL}$ escin. Asterisk; upregulated ≥ 2 -fold in the comparisons *erg6Δ* 100 vs. 0 $\mu\text{g/mL}$ escin. Comparison FDR ≤ 0.1 .

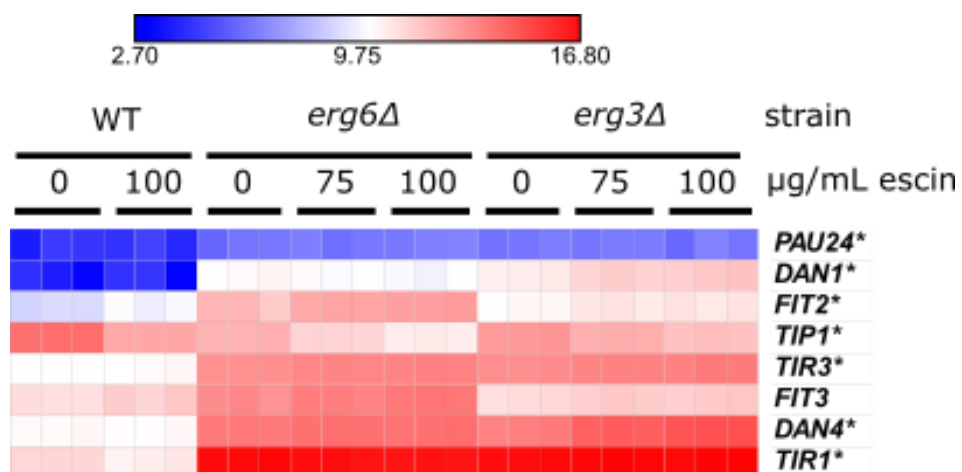


Figure S8: Expression of differentially expressed cell wall mannoprotein genes

Expression of mannoprotein genes which are upregulated ≥ 2 -fold in the comparison *erg6Δ* vs. WT (0 μg/mL escin). Asterisk; upregulated ≥ 2 -fold in the comparisons *erg3Δ* vs. WT (0 μg/mL escin). Scale $\log_2(\text{CPM}+4)$. Comparison FDR ≤ 0.1 .

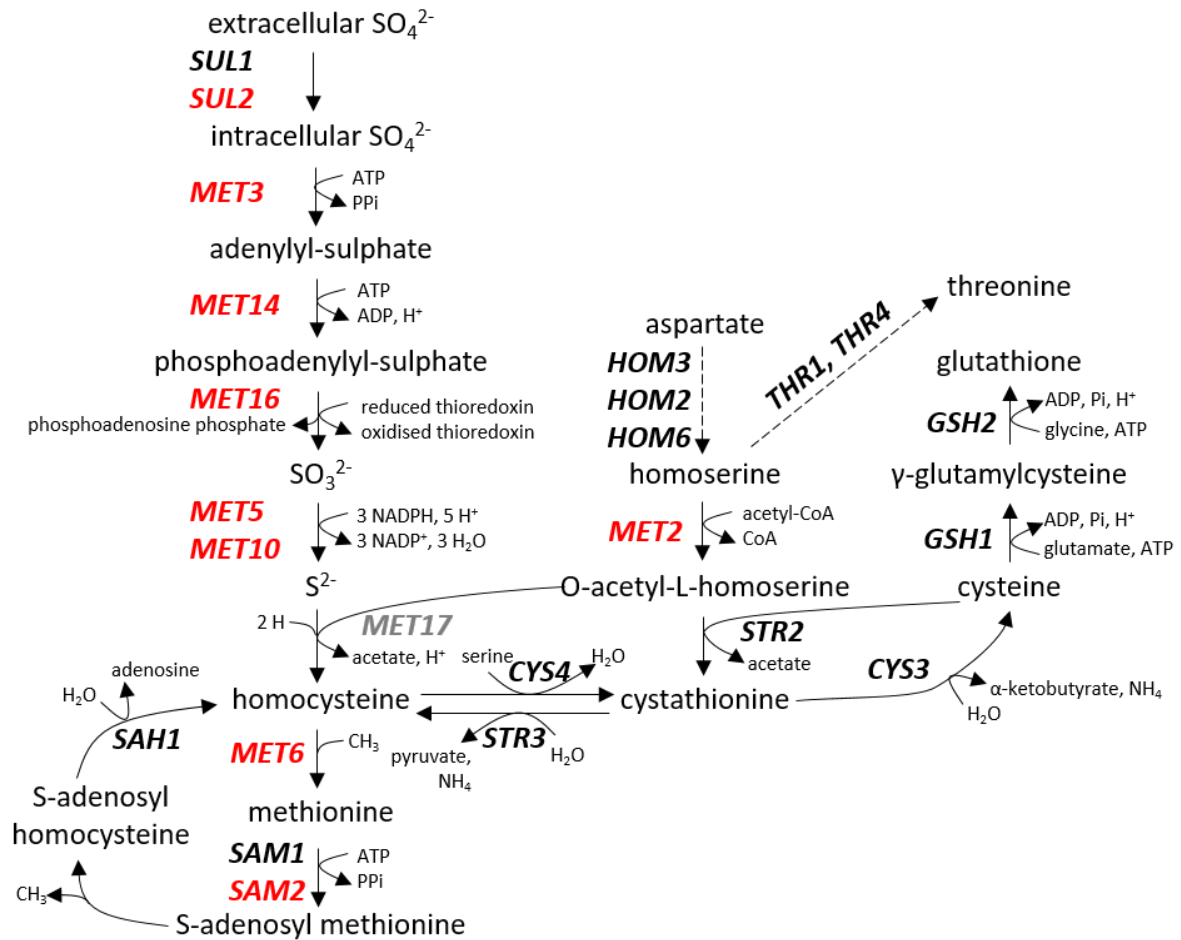


Figure S9: Overview of the methionine biosynthesis pathway. Red; gene upregulated ≥ 2 -fold in *erg6Δ* 0 $\mu\text{g/mL}$ escin versus WT 0 $\mu\text{g/mL}$ escin (FDR ≤ 0.1). Dashed arrow; summary of multiple biosynthesis steps. The *MET17* gene (alias *MET15*) is disrupted in BY4741.

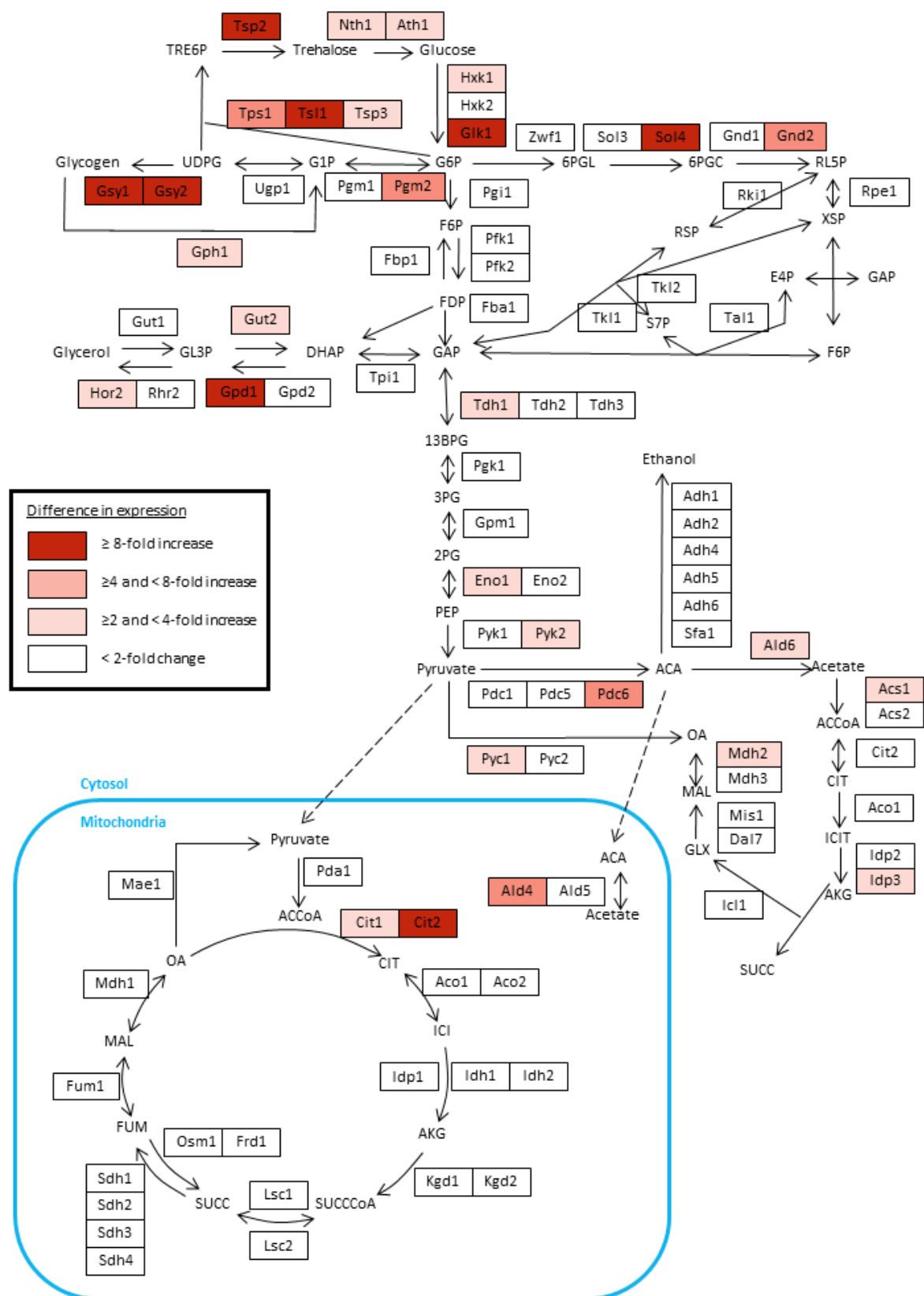


Figure S10: Impact of escin treatment on yeast central carbon metabolism

Overview of yeast central carbon metabolism, adapted from previously published diagrams^{8,9}. Colour indicates difference in expression between WT 100 and 0 µg/mL escin treatments. None of the depicted genes are downregulated ≥2-fold in response to escin treatment.

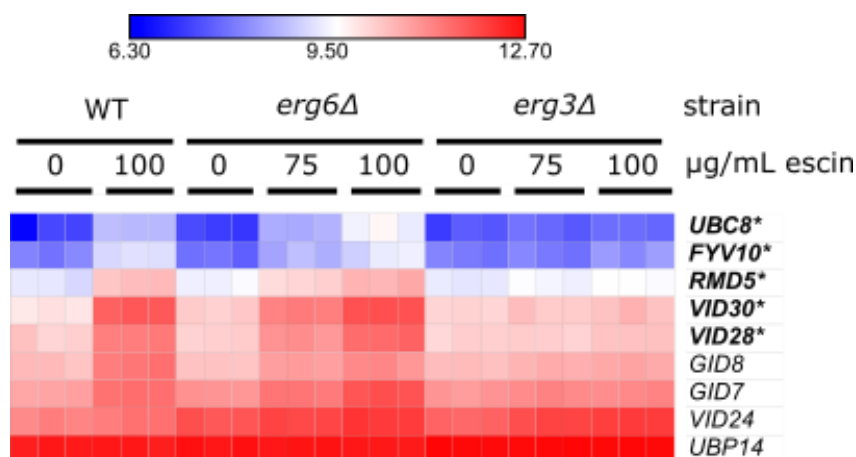


Figure S11: Expression of GID complex genes

Expression of genes encoding components of the GID complex¹⁰, scale $\log_2(\text{CPM}+4)$. Bold; upregulated ≥ 2 -fold in the comparison WT 100 vs. 0 μg/mL escin. Asterisk; upregulated ≥ 2 -fold in the comparisons *erg6Δ* 100 vs. 0 μg/mL escin. Comparison FDR ≤ 0.1 .

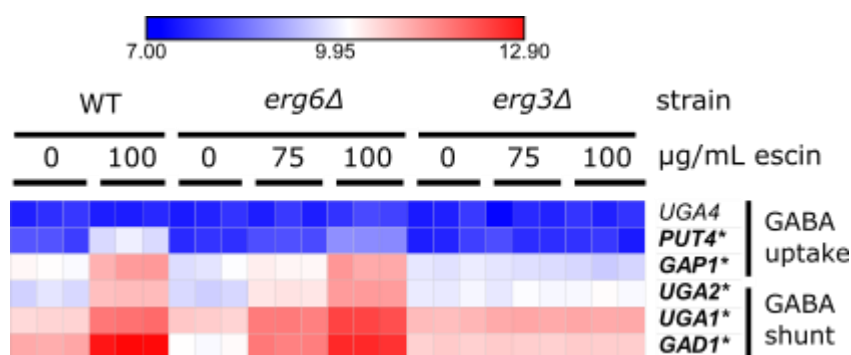


Figure S12: Expression of genes of the GABA shunt pathway

Expression of genes involved in γ -aminobutyric acid (GABA) uptake and the GABA shunt, scale $\log_2(\text{CPM}+4)$. Bold; upregulated ≥ 2 -fold in the comparison WT 100 vs. 0 μg/mL escin. Asterisk; upregulated ≥ 2 -fold in the comparisons *erg6Δ* 100 vs. 0 μg/mL escin. Comparison FDR ≤ 0.1 . *GAD1* encodes a glutamate decarboxylase, *UGA1* encodes a GABA transaminase and *UGA2* encodes a succinate semialdehyde dehydrogenase.

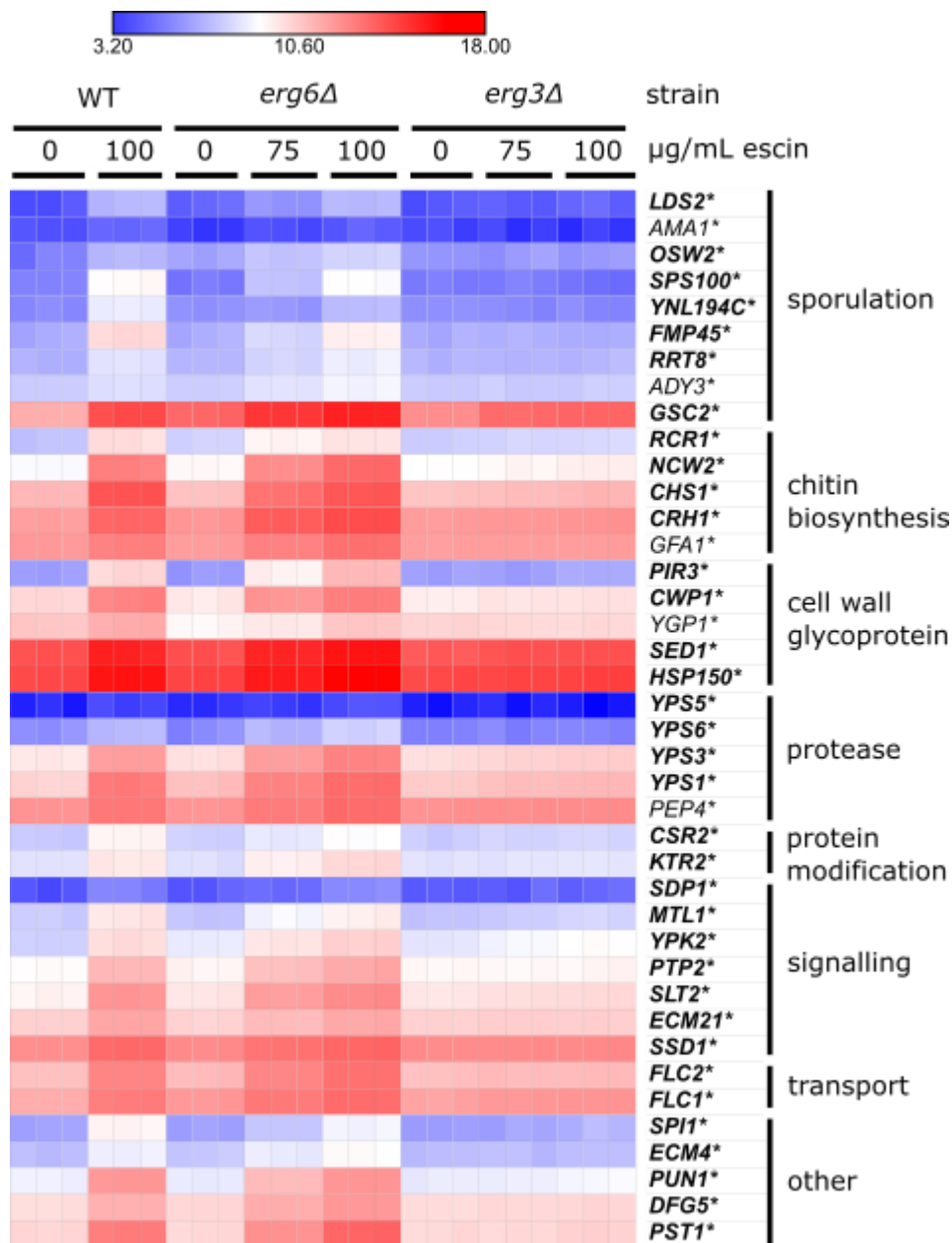


Figure S13: Escin response genes relating to cell wall organisation

Expression of genes relating to cell wall organisation which are upregulated ≥ 2 -fold (FDR ≤ 0.1) in response to 100 $\mu\text{g/mL}$ escin treatment in either BY4741 (bold) and/or *erg6Δ* (asterisk). Scale $\log_2(\text{CPM}+4)$

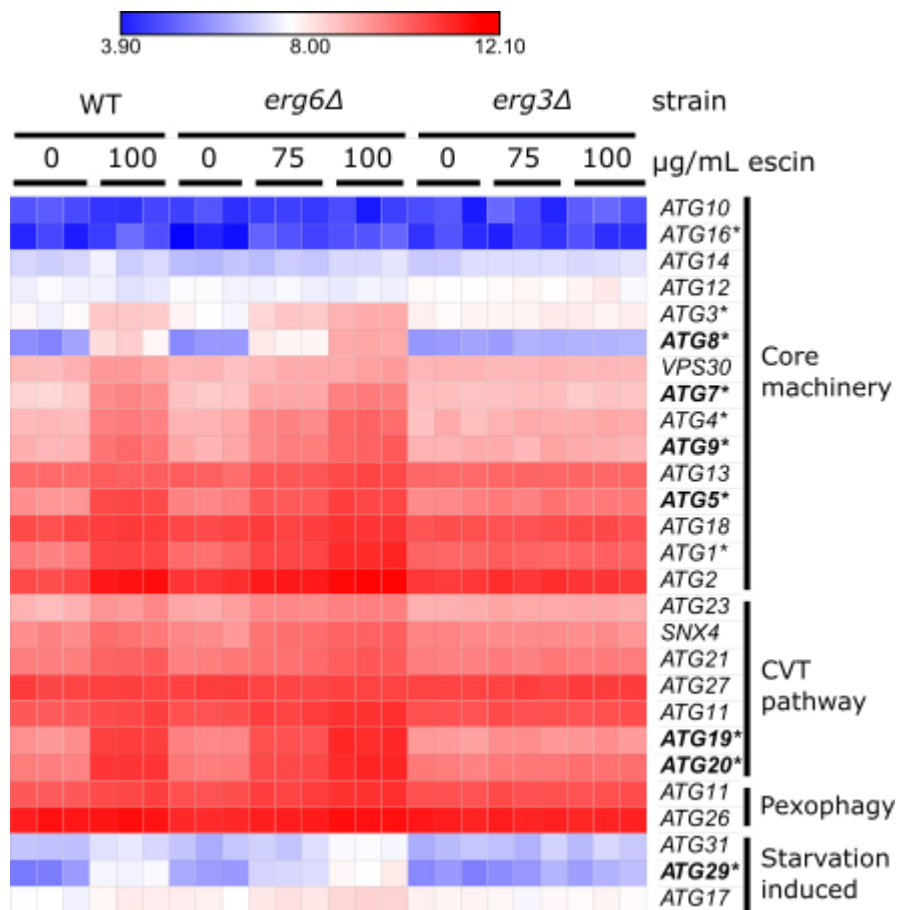


Figure S14: Expression of genes related to autophagy

Genes encoding proteins of the core machinery for membrane formation, the cytoplasm-to-vacuole (CVT) pathway, pexophagy and starvation induced autophagy¹¹. The Atg11 protein is involved in both the CVT pathway and pexophagy. Expression scale $\log_2(\text{CPM}+4)$. Bold; upregulated ≥ 2 -fold in the comparison WT 100 vs. 0 $\mu\text{g/mL}$ escin. Asterisk; upregulated ≥ 2 -fold in the comparisons *erg6Δ* 100 vs. 0 $\mu\text{g/mL}$ escin. Comparison FDR ≤ 0.1 .

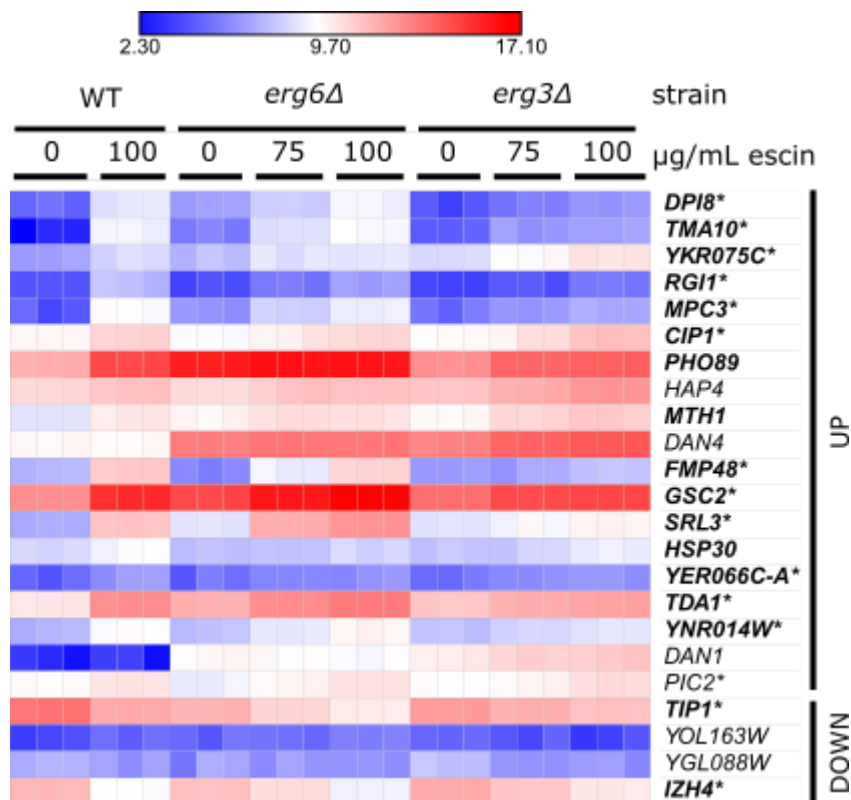


Figure S15: Expression of genes which are differentially regulated in the *erg3Δ* strain in response to escin treatment

Expression of genes which are up or downregulated ≥ 2 -fold (FDR ≤ 0.1) in *erg3Δ* in the comparison 100 vs. 0 $\mu\text{g/mL}$ escin, ordered from the largest to smallest $\log_2$ fold change. Expression scale $\log_2(\text{CPM}+4)$. Bold; also differentially expressed ≥ 2 -fold in the comparison WT 100 vs. 0 $\mu\text{g/mL}$ escin. Asterisk; also differentially expressed ≥ 2 -fold in the comparison *erg6Δ* 100 vs. 0 $\mu\text{g/mL}$ escin.

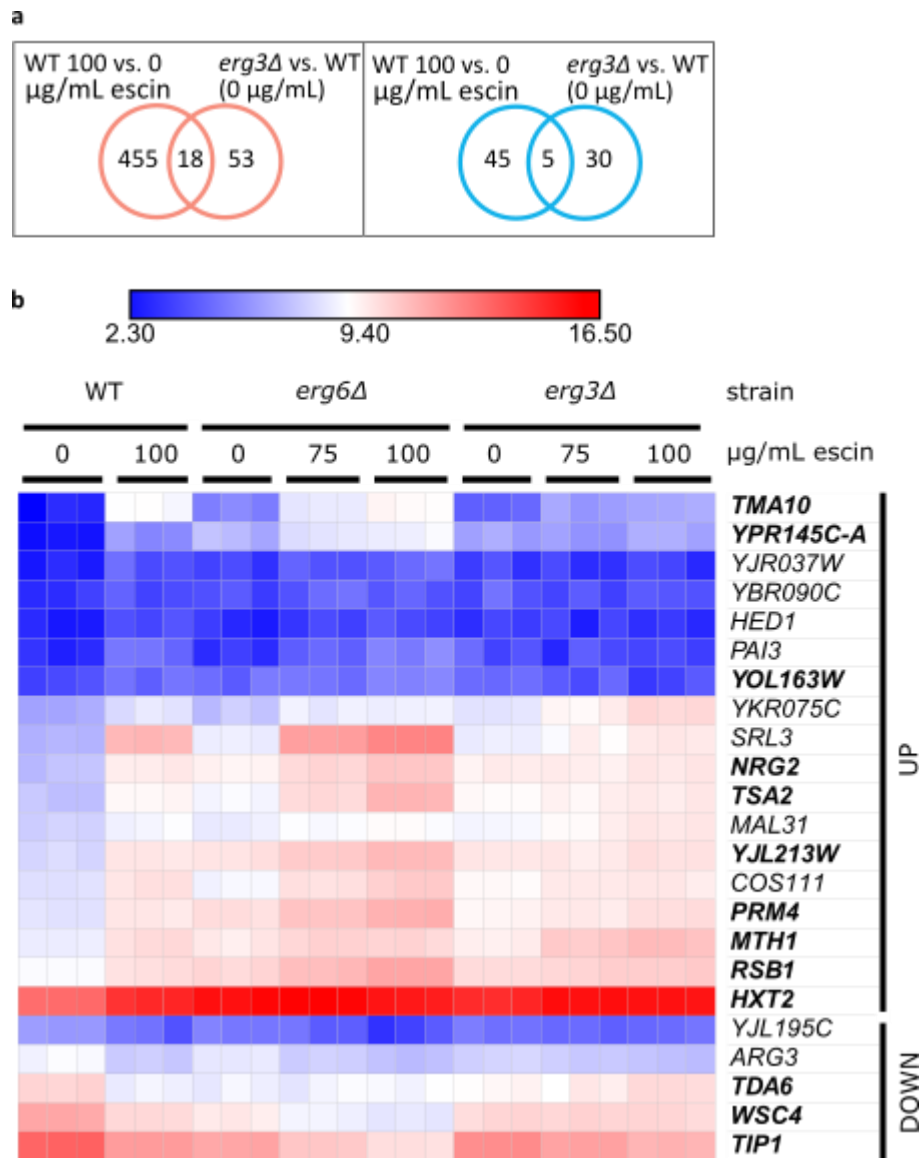


Figure S16: Overlap between escin response DEGs in the WT, and genes which are differentially expressed in *erg3Δ* under control conditions

a, Venn diagram for genes which are differentially expressed ≥ 2 -fold in the comparison WT 100 vs. WT 0 $\mu\text{g/mL}$ escin treatment, and the comparison *erg3Δ* 0 $\mu\text{g/mL}$ vs. WT 0 $\mu\text{g/mL}$ escin treatment ($\text{FDR} \leq 0.1$). This comparison explores whether the *erg3Δ* strain could be ‘primed’ for the stress of escin treatment. **b**, Expression of the overlapping genes in (a), scale $\log_2(\text{CPM}+4)$. Genes in bold are also differentially regulated ≥ 2 -fold in the comparison *erg6Δ* 0 $\mu\text{g/mL}$ vs. WT 0 $\mu\text{g/mL}$ escin treatment.

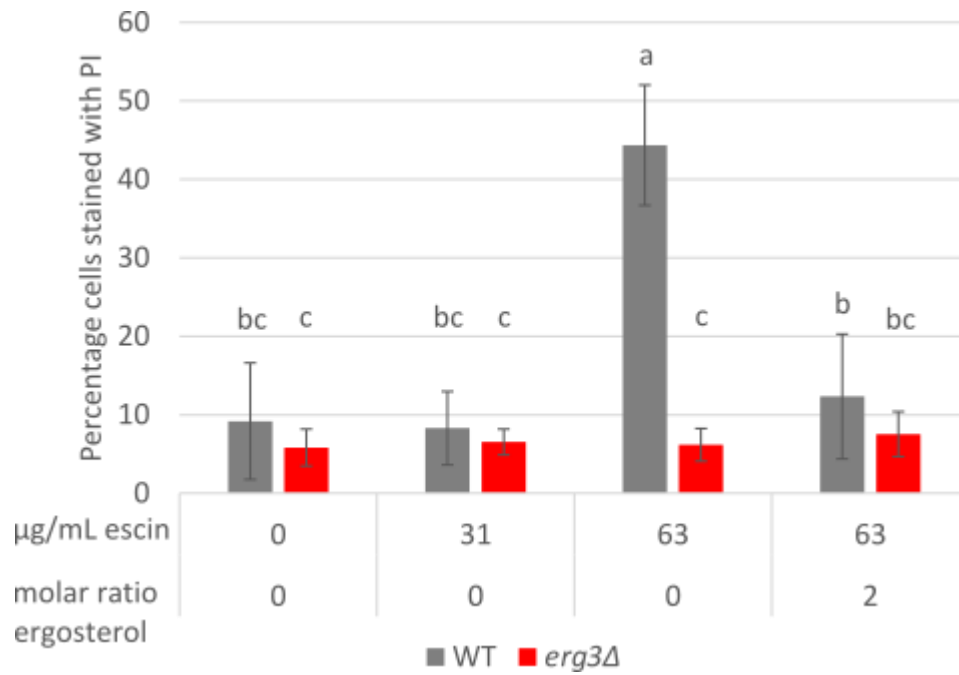


Figure S17: Staining of cells with propidium iodide

Mid-log cells were immobilised in chambered slides, treated with escin, escin:ergosterol mix, or control (1.25 % methanol) in PBS for 1 hour, washed, treated with propidium iodide for 20 minutes, washed, and then imaged. The average percentage of cells stained with PI is shown, $\pm$ SD for the 16 images analysed (4 biological replicates and 4 technical replicates). Statistics: one-way ANOVA with post-hoc Tukey HSD test, conditions not connected by the same letter are significantly different ($p \leq 0.05$).

References

1. Ge, S. X., Son, E. W. & Yao, R. iDEP: an integrated web application for differential expression and pathway analysis of RNA-Seq data. *BMC Bioinformatics* **19**, 534 (2018).
2. Ge, S. X., Jung, D. & Yao, R. ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics* **36**, 2628–2629 (2020).
3. Heese-Peck, A. *et al.* Multiple Functions of Sterols in Yeast Endocytosis. *Mol. Biol. Cell* **13**, 2664–2680 (2002).
4. Guan, X. L. *et al.* Functional interactions between sphingolipids and sterols in biological membranes regulating cell physiology. *Mol. Biol. Cell* **20**, 2083–2095 (2009).
5. Bean, T. P. *et al.* Sterol content analysis suggests altered eburicol 14 α -demethylase (CYP51) activity in isolates of *Mycosphaerella graminicola* adapted to azole fungicides. *FEMS Microbiol. Lett.* **296**, 266–273 (2009).
6. Martel, C. M. *et al.* Identification and Characterization of Four Azole-Resistant erg3 Mutants of *Candida albicans*. *Antimicrob. Agents Chemother.* **54**, 4527–4533 (2010).
7. Kenny, P. T. M. & Wetzel, J. M. Letter: Fragmentation Studies of Ergosterol. The Formation of the Fragment Ion at m/z 337. *Eur. Mass Spectrom.* **1**, 411–413 (1995).
8. Oliveira, A. P. *et al.* Regulation of yeast central metabolism by enzyme phosphorylation. *Mol. Syst. Biol.* **8**, 623 (2012).
9. Williams, T. C., Peng, B., Vickers, C. E. & Nielsen, L. K. The *Saccharomyces cerevisiae* pheromone-response is a metabolically active stationary phase for bio-production. *Metab. Eng. Commun.* **3**, 142–152 (2016).
10. Santt, O. *et al.* The Yeast GID Complex, a Novel Ubiquitin Ligase (E3) Involved in the Regulation of Carbohydrate Metabolism. *Mol. Biol. Cell* **19**, 3323–3333 (2008).
11. Nakatogawa, H., Suzuki, K., Kamada, Y. & Ohsumi, Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat. Rev. Mol. Cell Biol.* **10**, 458–467 (2009).