Upregulating the mevalonate pathway and repressing sterol synthesis in *Saccharomyces cerevisiae* enhances the production of triterpenes

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Abstract

Pentacyclic triterpenes are diverse plant secondary metabolites derived from the mevalonate (MVA) pathway. Many of these molecules are potentially valuable, particularly as pharmaceuticals, and research has focused on their production in simpler and more amenable heterologous systems such as the yeast *Saccharomyces cerevisiae*. We have developed a new heterologous platform for the production of pentacyclic triterpenes in *S. cerevisiae* based on a combinatorial engineering strategy involving the overexpression of MVA pathway genes, the knockout of negative regulators, and the suppression of a competing pathway. Accordingly, we overexpressed *S. cerevisiae ERG13*, encoding 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, and a truncated and deregulated variant of the rate-limiting enzyme HMG-CoA reductase 1 (tHMGR). In the same engineering step, we deleted the *ROX1* gene, encoding a negative regulator of the MVA pathway and sterol biosynthesis, resulting in a push-and-pull strategy to enhance metabolic flux through the system. In a second step, we redirected this enhanced metabolic flux from late sterol biosynthesis to the production of 2,3-oxidosqualene, the direct precursor of pentacyclic triterpenes. In yeast cells transformed with a newly isolated sequence encoding lupeol synthase from the Russian dandelion (*Taraxacum koksaghyz*), we increased the yield of pentacyclic triterpenes by 127-fold and detected not only high levels of lupeol but also a second valuable pentacyclic triterpene product, β-amyrin.

Keywords Metabolic engineering · MVA pathway · Sterol biosynthesis · tHMGR · Pentacyclic triterpenes · *Saccharomyces cerevisiae*

Introduction

Isoprenoids are a diverse group of natural compounds found in all living organisms, with at least 50,000 different structures already reported (Hemmerlin et al. 2012; Liao et al. 2016). In plants, these products are derived from the plastidial 2C-methyl-d-erythritol 4-phosphate (MEP) and the cytosolic mevalonate (MVA) pathway. In the latter, acetyl-CoA is converted to the isoprenoid precursor isopentenyl diphosphate (IPP) via six enzymatic steps. Two important MVA pathway enzymes are the sequentially acting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase (HMGS) and HMG-CoA reductase (HMGR), the latter representing the rate-limiting step (Demierre et al. 2005). IPP is isomerized to form dimethylallyl pyrophosphate (DMAPP), and together, IPP and DMAPP can act as substrates for various isoprenoid-derived pathways. For example, two molecules of IPP and one of DMAPP can be converted into farnesyl pyrophosphate (FPP) which in turn can be converted into squalene by squalene synthase (SQS). The oxidized form of squalene (2,3-oxidosqualene) is a precursor for the synthesis of sterols (leading to the production of lanosterol in fungi and animals, or cycloartenol in plants) and also pentacyclic triterpenes (Fig. 1a), the latter involving various oxidosqualene cyclases (OSCs) such as lupeol synthase in the dandelion *Taraxacum officinale* and β-amyrin synthase in the wormwood plant *Artemisia annua* (Shibuya et al. 1999; Kirby et al. 2008). The products of these enzymes can be further metabolized by acylation or oxidation. The efficient triterpene oxidation of, e.g., lupeol to betulin and betulinic acid by P450 enzymes could be demonstrated in yeast (Zhou...
et al. 2016). FPP is also a precursor for the synthesis of sesquiterpenes, e.g., farnesene or amorpha-4,11-diene, a precursor of the anti-malarial drug artemisinin (Martin et al. 2003). The value of isoprenoids, particularly as pharmaceuticals, has prompted the development of heterologous production systems including the yeast *Saccharomyces cerevisiae* (reviewed by Liao et al. 2016 and Vickers et al. 2017). The potential of the yeast MV A pathway for the production of isoprenoids was first demonstrated by overexpressing the catalytic domain of HMGR (tHMGR), which increased the yield of squalene (Donald et al. 1997). The consequences of overexpressing other MV A pathway genes were determined by combinatorial library screening for the overexpression of ERG10 (acetoacetyl CoA thiolase; AACT), ERG13 (HMGS), and ERG12 (mevalonate kinase) which enhanced the production of amorpha-4,11-diene (Yuan and Ching 2014). The MV A pathway has also been targeted using the CRISPR/Cas9 system, revealing loci that trigger the accumulation of mevalonate and triterpenes when knocked out (Jakočiūnas et al. 2015; Arendt et al. 2017). The targets included ROXI, encoding a transcriptional regulator that inhibits genes involved in the MVA pathway and sterol biosynthesis (Henry et al. 2002; Montañés et al. 2011; Özaydin et al. 2013; Jakočiūnas et al. 2015). The insertion of regulated promoters into the yeast genome can also suppress genes involved in endogenous but competitive isoprenoid pathways to redirect the metabolic flux in a more precise and desirable manner. Accordingly, the methionine sensitive MET3 promoter was used to downregulate the expression of the lanosterol synthase gene (*ERG7*), which represents the first committed step in the late sterol biosynthesis pathway, enhancing the production of β-amyrin (Kirby et al. 2008). A similar strategy was used to suppress the squalene synthase gene (*ERG9*), allowing the enhanced production of artemisinin (Ro et al. 2006; Westfall et al. 2012) until the authors changed their strategy and used the copper transporter 3 (CTR3) promoter, which allows transcriptional repression to be induced by adding CuSO4 to the medium, thus reducing the costs of industrial processes (Paddon et al. 2013).

Here, we developed a new yeast-based platform for the synthesis of triterpenes, combining the overexpression of the MVA pathway genes ERG13 (HMGS) and a truncated version of HMG1 (tHMGR), the disruption of the ROXI gene, and the copper-regulated repression of ERG7 using the CTR3

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**Fig. 1** Triterpene accumulation in the yeast *S. cerevisiae* expressing TkLUP. a Schematic representation of the MVA pathway leading to the synthesis of sterols and pentacyclic triterpenes via oxidosqualene cyclases (OSCs). Dashed arrows represent multiple enzymatic reactions. AACT = acetyl-CoA C-acetyltransferase; DMAPP = dimethylallyl pyrophosphate; IPP = isopentenyl diphosphate; HMGS = 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase; HMGR = HMG-CoA reductase; SQS = squalene synthase; SQE = squalene epoxidase. b Schematic representation of the TkLUP coding sequence under the control of the GAL1 promoter (P<sub>GAL1</sub>) and CYC1 terminator (T<sub>CYC1</sub>). c Yeast cells carrying the TkLUP coding sequence showed two additional peaks in the GC-MS spectrum (m/z = 218, arrows), probably representing β-amyrin (retention time = 17.95 min) and lupeol (retention time = 18.25 min) because they match the corresponding standards. d Yeasts carrying the TkLUP coding sequence accumulated 0.16 mg/g CDW of the putative lupeol but the quantification of the β-amyrin peak was not possible. Wild-type (WT) and pAG424<sub>P<sub>GAL1</sub>,<sub>cclb</sub> vector control CEN.PK2-1C cells served as controls. The standard deviation was calculated from *n* = 3 individual transformants; CDW = cell dry weight.
promoter. By implementing this platform, we were able to enhance the productivity of the MVA pathway and redirect metabolic flux from late sterol biosynthesis, resulting in a 127-fold increase in the yield of lupeol using lupeol synthase from the Russian dandelion *Taraxacum kokssaghzy* (TkLUP) as a model enzyme.

**Materials and methods**

**Cloning of constructs**

The construct pAG424_P*GAL1*-TkLUP was prepared by amplifying the *TLK* coding sequence (GenBank MG646375) from *T. kokssaghzy* cDNA using forward primer 5′-AAA GTC GAC TAA AAA AAT GTG GAA GCT GTA AAT AAC GTA AGA TTA CA-3′ and reverse primer 5′-AAA CTC GAG ATA TAT TTT GAA CAA TAC GA-3′ (restriction sites are underlined). The PCR product was purified, digested, and inserted into pENTR3c (Invitrogen, Carlsbad, USA). The *TKL* coding sequence was then introduced into pAG424_P*GAL1*-ccdB (Alberti et al. 2007; Addgene, Cambridge, USA) by LR recombination.

The construct pESC-rox1-KIURA3_tHMGR/ERG13 was generated by amplifying the *ROX1* coding sequence from *S. cerevisiae* genomic DNA using forward primer 5′-AAA GGA GCC GCA TGA ATC CTA AAT CCT CTAC-3′ and reverse primer 5′-AAA GCG GCC GCT CAT TGC GGA AAT ACT AGG-3′ (restriction sites are underlined). Furthermore, pESC-URA (Agilent Technologies, Santa Clara, USA) was used as a template to amplify a pESC-URA vector backbone containing *NorI* restriction sites using forward primer 5′-AAA GCG GCC GCC CAG CTG CAT TAA TGA ATC CCT CTAC-3′ and reverse primer 5′-AAA GCG GCC GCC CAG CTG CAT TAA TGA ATC CCT CTAC-3′ (restriction sites are underlined). After digestion with *NorI*, the restriction fragments were ligated to obtain pESC-rox1. This vector was digested with *BglII* and a synthetic DNA fragment (Invitrogen), consisting of a KIURA3 marker cassette (Gueldener et al. 2002), and an *AsI/Nb.BsmI* uracil-specific excision reaction (USER) cassette (Hansen et al. 2011) was inserted to obtain pESC-rox1-KIURA3. In a parallel approach, the coding sequences of the truncated *HMGl* gene (*tHMGR*) and *ERG13* were amplified from the *S. cerevisiae* genomic DNA using forward primer 5′-AAA GGA TCC AAA AAA ATG GTT TTA ACC AAT AAA AC-3′ and reverse primer 5′-AAA GTC GAC TAA TGA TTT AAT GCA GGT GAC-3′ for *tHMGR* and forward primer 5′-AAA GGA TTC AAA AAA ATG AAA CTC TCA ACT AAA CTT TG-3′ and reverse primer 5′-AAA GCG GCC GCT TAT TTT TTA ACA TCG TAA GAT C-3′ for *ERG13* (restriction sites are underlined). The PCR products were digested with BamHI/SalI and EcoRI/NorI, and ligated into pESC-URA to generate pESC-URA-tHMGR/ERG13. The *NorI* restriction site was removed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer’s protocol.

In the final step, the expression cassette, containing the *tHMGR* and *ERG13* coding sequences, as well as the bidirectional GAL1/GAL10 promoter and the ADH1/CYC1 terminators, was amplified using forward primer 5′-CGT GGC AUT CAG GAC GCT ATG CTA TAC-3′ and reverse primer 5′-CAC GGC AUC TTC GAG CTG CCC AAA ACC-3′ (uracil base for USER cloning shown in bold) and was introduced into pESC-rox1-KIURA3 (digested with *AsI*) according to the USER protocol.

The construct pESC_P*ERG7*-KILEU2_PCTR3_erg7 was generated in a three-step process. In the first step, an *ERG7* fragment was amplified from *S. cerevisiae* genomic DNA using forward primer 5′-CAC ATT TAA GGG CTA TAC AAA GAT GAC AGA ATT TTA TTT TGA CA-3′ and reverse primer 5′-AAA GGC GCC GCC CCA ATA AAC GTA AGA TTA CA-3′, and a *CTR3* promoter fragment was amplified using forward primer 5′-AAA GCC GCC GCC AGC TGA AAT CTG CTG CTA TTC GAG ATG CAC GGC ACC CCA ATA AAC GTA AGA TTA CA-3′, and reverse primer 5′-TGT CAG AAT AAA ATT CTG TCA TCT TGT TAT AGC CCT TAA ATG T-3′ (italic letters indicate the overlapping region; restriction sites are underlined). The products were fused by overlapping PCR using the *CTR3* promoter forward primer and the *ERG7* reverse primer. The spliced product was digested with *SfiI* and transferred to the pESC-URA vector linearized with the same enzyme to obtain pESC_PCTR3_erg7. In the second step, the upstream *ERG7* promoter fragment was amplified from the *S. cerevisiae* genomic DNA using forward primer 5′-AAA CAG CTG AAT CTG CTG CTA TCT CTG GTG-3′ and reverse primer 5′-AAA GGA TCC CCT GCA GGT CCG CAG ATA TCA AAT CTA G-3′, and was transferred to the *PvuII/BamHI* sites of pESC_PCTR3_erg7 to obtain pESC_P*ERG7*-PCTR3_erg7. In the final step, a synthetic DNA fragment (Invitrogen) containing a KILEU2 auxotrophy cassette (Gueldener et al. 2002) was ligated at the *SbfI/BamHI* restriction sites to obtain pESC_P*ERG7*-KILEU2_PCTR3_erg7.

The integrity of all constructs was verified by sequencing (Sanger et al. 1977) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). Yeast strain CEN.PK2-1C was obtained from EUROSCARF (Oberursel, Germany). Restriction enzymes were obtained from New England Biolabs GmbH (Frankfurt a.M., Germany).

**Strain construction and culture conditions**

The *S. cerevisiae* strain CEN-PK2-1C was transformed using the lithium acetate method (Gietz and Schiestl 2007) with *URA3* (pESC-rox1-KIURA3_tHMGR/ERG13), *LEU2* (pESC_P*ERG7*-KILEU2_PCTR3_erg7), and *TRP1*.
lated to a final cell density of 10^5 cells/ml and incubated at the culture reached a cell density of 0.4 × 10^6 cells/ml, the 30 °C shaking at 140 rpm in a 250-ml Erlenmeyer flask. When CuSO4 when repressing the expression of cultivated overnight at 30 °C on a rolling platform. From this colony was picked, inoculated into 5 ml SD medium, and both ends of the integrated construct if needed. checked for integrity by colony PCR using primers spanning Mountain View, USA) and incubated at 30 °C. Clones were not expressed galactose-inducible genes, a single colony was picked, inoculated into 5 ml SD medium, and cultivated overnight at 30 °C on a rolling platform. From this culture, 50 ml of fresh SD medium (containing 150 μM CuSO4 when repressing the expression of ERG7) was inoculated to a final cell density of 10^5 cells/ml and incubated at 30 °C shaking at 140 rpm in a 250-ml Erlenmeyer flask. When the culture reached a cell density of 0.4 × 10^6 cells/ml, the medium was changed to SD medium containing galactose instead of glucose to induce gene expression. The cells were grown to a density of 4 × 10^6 cells/ml and harvested by centrifugation (10 min, 1000×g).

Squalene and triterpene extraction and quantitation

Yeast metabolites were extracted as described by Rodriguez et al. (2014). Briefly, freeze-dried yeast cells were incubated at 80 °C in a water bath for 5 min after adding 1 ml 6% [w/v] KOH in methanol (Carl Roth, Karlsruhe, Germany) and 100 μg cholesterol as an internal standard (Sigma, St. Louis, USA) to each sample. To extract the metabolites from the methanol mixture, 1 ml of n-hexane (Carl Roth) was added. After vortexing, the upper phase was transferred to a new vial and the extraction was repeated two times using 500 μl n-hexane. The n-hexane of the pooled extracts was removed by evaporation. The samples were re-solubilized in 1 ml acetone (Carl Roth) and analyzed by gas chromatography mass spectrometry (GC-MS) using a GC-MS-QP 2010 Ultra (Shimadzu, Duisburg, Germany) equipped with a 30-m Rtx-5MS column. After a 1-min hold at 120 °C, the temperature was increased to 330 °C at 21 °C per min (pressure = 58.8 kPa) followed by a hold of 330 °C for 10 min. Different compounds were identified according to their ion mass/charge ratios (43, 55, 69, 95, 109, 189, 204, 207, 218, 271, 285, and 411 m/z) by peak integration using LabSolution software (Shimadzu) and matching to the National Institute of Standards and Technology library. The total ion current of the detected substances was normalized against the cholesterol internal standard and the dry weight of the sample. The statistical significance of the results was confirmed using a two-sample t test at p < 0.05.

Results

Identification of TkLUP as a lupeol synthase from T. koksaghyz

We chose a lupeol synthase from the rubber-producing dandelion T. koksaghyz as a model enzyme for the new yeast platform. Using primers designed for the amplification of TRX, a lupeol synthase gene from T. officinale (Shibuya et al. 1999), we were able to amplify a 2277-bp open reading frame from T. koksaghyz cDNA encoding a polypeptide of 759 amino acids, showing 99.3% sequence identity to the T. officinale lupeol synthase. For expression in the wild-type (WT) S. cerevisiae strain CEN.PK2-1C, we inserted the sequence into pAG424_PGAL1-ccdB, which allows expression under the control of the GAL1 promoter (Fig. 1 b). The empty vector pAG424_PGAL1-ccdB served as the vector control in the expression experiments. After cultivation and triterpene extraction, GC-MS analysis revealed two additional peaks (m/z = 218) in the extracts from three independent transformants expressing the lupeol synthase sequence, but these peaks were not observed in the WT or vector control samples (Fig. 1c). The retention times matched those of the β-amyrin and lupeol standards, indicating that the peaks reflected the accumulation of trace amounts of β-amyrin and ~0.16 mg/g cell dry weight (CDW) of lupeol (Fig. 1d). These data strongly supported the annotation of the new T. koksaghyz sequence as lupeol synthase (TkLUP).

The deletion of ROX1 and the overexpression of HMG1 and ERG13 enhance the accumulation of lupeol by 16.5-fold

To enhance the production of lupeol, we overexpressed two yeast MVA pathway genes that have previously been shown to improve the yield of isoprenoids in heterologous yeast systems (Kirby et al. 2008; Asadollahi et al. 2010; Paddon et al. 2013). HMG1 (HMG1) catalyzes the rate-limiting step of the pathway and is subject to strict feedback control, so we overexpressed a truncated form of the enzyme (tHMG1) which no longer responds to feedback inhibition by enzyme degradation due to a missing ubiquitination signal (DeBose-Boyd 2008). We also overexpressed HMGS (ERG13) which promotes isoprenoid biosynthesis by supplying the substrate for HMG1 (Yuan and Ching 2014). Finally, we knocked out the ROX1 gene, which encodes a negative regulator of the MVA pathway and late sterol biosynthesis (Henry et al. 2002; Montañés et al. 2011; Özyaydin et al. 2013; Jakočiūnas et al. 2015). We expressed the truncated HMG1 gene and ERG13 vector control samples (Fig. 1c). The retention times matched those of the β-amyrin and lupeol standards, indicating that the peaks reflected the accumulation of trace amounts of β-amyrin and ~0.16 mg/g cell dry weight (CDW) of lupeol (Fig. 1d). These data strongly supported the annotation of the new T. koksaghyz sequence as lupeol synthase (TkLUP).
under the control of the bidirectional GAL1/GAL10 promoter and integrated the entire overexpression cassette into the ROX1 locus to concurrently knock out this gene (Fig. 2a). As expected, the overexpression of tHMGR and HMGS and the knockout of ROX1 in yeast cells already expressing TkL UP resulted in a significant 8.2-fold increase in the squalene content as well as a 16.5-fold higher yield of lupeol compared to cells carrying the TkLUP sequence alone (Fig. 2b). There was also a 3.6-fold increase in the abundance of lanosterol (Table 1).

The transformed yeast cells grew at a slower rate, as the time to reach the cell density for harvesting extended from 17 to 18.5 h, perhaps reflecting the toxicity of the higher squalene content (Donald et al. 1997; Asadollahi et al. 2010). However, this engineering step allowed us to compare the putative lupeol peak (Fig. 2c) with that of the lupeol standard (Fig. 2d). The comparable masses of the peaks supported

Fig. 2 Accumulation of squalene and lupeol after the deletion of ROX1 and the overexpression of MVA pathway genes. a Schematic representation of the construct for the deletion of ROX1 and the overexpression of tHMGR and ERG13. The tHMGR and ERG13 coding sequences were placed under the control of a bidirectional GAL1/GAL10 promoter (PGAL1;PGAL10). KlURA3 was used to complement the uracil auxotrophy during the integration of the construct into the yeast genome. The locus for integration was defined by sequences flanking the construct (target-up; target-down) that were homologous to the ROX1 target site. Transformation of the NotI-linearized construct led to the knockout of ROX1 by homologous recombination. b Yeast strains carrying the integrated construct in addition to the TkLUP coding sequence (rox1::PGAL1-tHMGR PGAL10-ERG13 TkLUP) accumulated higher levels of the lupeol precursor squalene in contrast to cells carrying only the TkLUP sequence (p = 0.0137). Cells containing the empty vector pAG424 PGAL1_ccdB served as controls (rox1::PGAL1-tHMGR PGAL10-ERG13 vector control). The deletion of ROX1 and the overexpression of tHMGR and ERG13 resulted in a 16.5-fold increase in the lupeol content (p = 0.00893). c Mass spectrum of the designated lupeol peak. d Mass spectrum of the measured external lupeol standard. Standard deviation was calculated from n = 3 individual transformants. CDW = cell dry weight; one asterisk = p ≤ 0.05; two asterisks = p ≤ 0.01
amyrin peak was still too weak for detailed mass analysis.

Alternatively, competition from the endogenous late sterol biosynthesis pathway is primarily responsible for the low pentacyclic triterpene content in our cells. We therefore set out to suppress the expression of ERG7 (lanosterol synthase) representing the first committed step in the late sterol biosynthesis pathway.

ERG7 repression using the CTR3 promoter causes 2,3-oxidosqualene to accumulate and achieves a further 7.6-fold increase in the lupeol content

The engineering steps outlined above resulted in the accumulation of squalene, but not of 2,3-oxidosqualene (the substrate of the TkLUP). This indicates that squalene synthase (ERG9) may have a higher metabolic capacity than squalene epoxidase (ERG1) as previously suggested by Asadollahi et al. (2010), perhaps exacerbated by the rapid conversion of the limited 2,3-oxidosqualene pool by TkLUP itself. Alternatively, competition from the endogenous late sterol biosynthesis pathway could draw flux away from the pentacyclic triterpenes. Yeast cells overexpressing ERG1 accumulate 2,3-oxidosqualene but not downstream pentacyclic triterpenes (Veen et al. 2003), suggesting that competition from the endogenous late sterol biosynthesis pathway is primarily responsible for the low pentacyclic triterpene content in our cells. We therefore set out to suppress the expression of ERG7 (lanosterol synthase) representing the first committed step in the late sterol biosynthesis pathway.

![Image](https://via.placeholder.com/150?text=Image+Link)
The effect was even stronger in the yeast growing in the presence of 150 μM CuSO₄. The amount of squalene declined significantly \( (p = 0.00613) \) as the accumulation of 2,3-oxidosqualene increased by 4.7-fold \( (p = 0.00507) \), which may reflect the absence of \( \text{ERG9} \) and \( \text{ERG1} \) repression by ergosterol, as well as the enhanced induction of \( \text{ERG1} \) due to the lower levels of lanosterol (Table 1) \( \text{M'Baya et al. 1989} \). A further significant decrease in squalene levels \( (p = 0.00061) \) was observed when the yeast were cultivated in the presence of 375 μM CuSO₄. However, there was no significant change in 2,3-oxidosqualene levels but the time to reach the cell density for harvesting extended from 20 to 35 h. This indicates that high levels of CuSO₄ are toxic. We concluded that sufficient repression of \( \text{ERG7} \) was achieved in the presence of 150 μM CuSO₄, allowing us to overcome the bottleneck in 2,3-oxidosqualene synthesis. We therefore chose 150 μM CuSO₄ for the enhanced production of lupeol in a strain combining \( \text{rox1::PGAL1-tHMGR PGAL10-ERG13 PERG7Δ::PCTR3} \) and the coding sequence of \( \text{TkLUP} \), or the pAG424_PGAL1_ccdB empty plasmid serving as a control (Fig. 4).

As expected, the yeast strain carrying all three constructs, when cultivated in the presence of CuSO₄, showed a shift in squalene and 2,3-oxidosqualene levels compared to its parental strain lacking the \( \text{CTR3} \) promoter fragment. The squalene content of these cells was 2.6-fold lower \( (p = 0.04422) \), but 2,3-oxidosqualene accumulated instead. Furthermore, the sterol content declined, as shown by the 6.5-fold lower levels of lanosterol \( (p = 0.02384) \) and 3.9-fold lower levels of ergosterol \( (p = 0.00941) \), confirming that copper-based repression was sufficient for the regulation of \( \text{ERG9} \) and \( \text{ERG1} \) in the strain expressing \( \text{TkLUP} \). Moreover, introducing the copper-repressible promoter enhanced the lupeol content by a further 7.6-fold \( (p = 0.00637) \). No additional peaks representing products of \( \text{TkLUP} \), but the already described β-amyrin peak, could be observed in the comparison of the total ion count.

**Fig. 3** Repression of \( \text{ERG7} \) leads to the accumulation of 2,3-oxidosqualene. **a** Schematic representation of the construct used for the integration of the copper-sensitive \( \text{CTR3} \) promoter (\( \text{P}_{\text{CTR3}} \)). To introduce the promoter into the yeast genome, leucine auxotrophy was complemented by the \( \text{KILEU2} \) gene. The construct was flanked by sequences homologous to the \( \text{ERG7} \) promoter (target-up; \( \text{P}_{\text{ERG7}} \)) and coding sequence (target-down; \( \text{ERG7} \)) to place the \( \text{CTR3} \) promoter upstream of the endogenous \( \text{ERG7} \) coding sequence. **b** The yeast strain carrying the \( \text{CTR3} \) promoter construct showed a reduction in squalene levels and the accumulation of 2,3-oxidosqualene even without exposure to CuSO₄ \( (\text{rox1::PGAL1-tHMGR PGAL10-ERG13 PERG7Δ::PCTR3 0 \ μ\text{M CuSO}_4}) \). This effect was significantly enhanced in the presence of 150 μM CuSO₄ \( (\text{rox1::PGAL1-tHMGR PGAL10-ERG13 PERG7Δ::PCTR3 150 \ μ\text{M CuSO}_4}) \). Higher concentrations of copper (375 μM CuSO₄) reduced squalene levels and increased the abundance of 2,3-oxidosqualene \( (\text{rox1::PGAL1-tHMGR PGAL10-ERG13 PERG7Δ::PCTR3 375 \ μ\text{M CuSO}_4}) \). Standard deviation was calculated from \( n = 3 \) individual transformants. CDW = cell dry weight; one asterisk \( = p \leq 0.05 \); two asterisks \( = p \leq 0.01 \); three asterisks \( = p \leq 0.001 \).
squalene (peak 1) 2,3-oxidosqualene (peak 2)
g/g CDW

lanosterol (peak 5) ergosterol (peak 4)
g/g CDW

lupeol

rox1::PGAL1-tHMGR PGAL10-ERG13
PERG7Δ::PCTR3
TkLup vector control

m/z

ion count [m/z 218]

ret. time [min.]

β-amyrin (peak 6) lupeol

m/z

ion count [m/z 218]
To accomplish our push-and-pull strategy, we overexpressed the MVA pathway genes ERG13 (HMGS) and a truncated form of HMG1 (HMGR) because others have shown that HMGR is the key rate-limiting step in the pathway and its deregulated form tHMGR can enhance squalene accumulation and isoprenoid levels in yeast platforms (Kirby et al. 2008; Asadollahi et al. 2010; Westfall et al. 2012; Scalciati et al. 2012; Paddon et al. 2013; Lv et al. 2014; Yuan and Ching 2014). Furthermore, the overexpression of ERG13 increases flux through the pathway, given that a 13-fold increase in amorph-4,11-diene production was observed when ERG13 was overexpressed in combination with tHMGR, ERG10, and ERG12 (Yuan and Ching 2014).

We chose the ROX1 locus as an integration site for our overexpression cassette, thus knocking out this negative regulator of the MVA pathway and late sterol biosynthesis (Henry et al. 2002; Montañés et al. 2011; Özaydın et al. 2013; Jakočiūnas et al. 2015). The knockout should up-regulate the MVA pathway in general, although the higher levels of squalene resulting from the increased flux were in part consumed by the simultaneously upregulated late sterol biosynthesis pathway. Using this strategy, we were able to increase the accumulation of squalene, lanosterol, ergosterol, and lupeol (Fig. 2b and Table 1). This came at the cost of a slight reduction in growth of our squalene-accumulating yeast strain, consistent with some previous studies (Donald et al. 1997; Asadollahi et al. 2010) but not others (Veen et al. 2003). We did not observe the accumulation of 2,3-oxidosqualene, the direct precursor of pentacyclic triterpene synthesis, so we redirected the flux from late sterol biosynthesis towards the production of pentacyclic triterpenes by replacing the endogenous ERG7 promoter with the copper-repressible CTR3 promoter (Labbé et al. 1997). The absence of 2,3-oxidosqualene is therefore likely to reflect the lower capacity of ERG1 compared to ERG9 (Asadollahi et al. 2010) and the rapid conversion of 2,3-oxidosqualene into lanosterol by ERG7 (Veen et al. 2003) or into pentacyclic triterpenes by TlLUP (this study).

As expected, we observed lower squalene levels and the accumulation of 2,3-oxidosqualene following the repression of endogenous ERG7 in our squalene and pentacyclic triterpene accumulating yeast strain. This may reflect the lower expression of ERG7 itself, but the regulatory mechanism of ERG1 and ERG9 may also contribute to our observations. Because we detected lower levels of ergosterol and lanosterol, the deregulation of a described negative feedback loop may also contribute to the enhanced expression of ERG9 and ERG1. Therefore, the lower sterol content may prevent the suppression of ERG9 and ERG1 due to limited ergosterol levels, and may enhance the expression of ERG1 due to the lack of...
suppression by lanosterol (M‘Baye et al. 1989). By providing the bulk of the direct substrate 2,3-oxidosqualene by the described metabolic engineering steps for the TkLUP enzyme, we were able to enhance the accumulation of lupeol even further and also annotate a thus far uncharacterized peak in the GC-MS spectrum as β-amyrin. In the literature, the product specificity of lupeol synthases ranges from very specific with minor byproducts depending on cultivation conditions (e.g., OEW lupeol synthase from *Olea europaea*, TRW lupeol synthase from *Taraxacum officinale*; Shibuya et al. 1999) to moderate specificity with approximately 20% β-amyrin byproduct (lupeol synthase from *Arabidopsis thaliana*; Herrera et al. 1998). We calculate the β-amyrin byproduct of TkLUP to be approximately 0.6% of total product, classifying it to the higher specificity enzymes.

In conclusion, we were able to enhance the synthesis of pentacyclic triterpenes 127-fold in the case of lupeol within two engineering steps. In addition, we were able to characterize a second pentacyclic triterpene (β-amyrin) synthesized by our model enzyme TkLUP. The unused bulk of 2,3-oxidosqualene could be used as a substrate by other oxidosqualene cyclases to produce even higher amounts of oxidosqualene derivatives. Optimal cultivation techniques could also be used to increase the yields of these molecules (reviewed by Liao et al. 2016).

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**Compliance with ethical standards**

**Conflict of interest** Jan Niklas Bröker, Nicole van Deenen, Boje Müller, and Christian Schulze Gronover are named as inventors on a submitted patent application (EP18166374.1) which relates to this study.
Prüfer has no conflict of interest, as he is not an inventor of the mentioned patent application.

**Ethical statement** This article does not contain any studies involving human participants or animals.

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