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Using the yeast three-hybrid system for the identification of small molecule-protein interactions with the example of ethinylestradiol

https://doi.org/10.1515/hsz-2021-0355
Received August 27, 2021; accepted February 10, 2022;
published online February 28, 2022

Abstract: Since the first report on a yeast three-hybrid system, several approaches have successfully utilized different setups for discovering targets of small molecule drugs. Compared to broadly applied MS based target identification approaches, the yeast three-hybrid system represents a complementary method that allows for the straightforward identification of direct protein binders of selected small molecules. One major drawback of this system, however, is that the drug has to be taken up by the yeast cells in sufficient concentrations. Here, we report the establishment of a yeast three-hybrid screen in the deletion strain ABC9Δ, which is characterized by being highly permeable to small molecules. We used this system to screen for protein binding partners of ethinylestradiol, a widely used drug mainly for contraception and hormone replacement therapy. We identified procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (PLOD2 or lysyl hydroxylase, LH2) as a novel direct target and were able to confirm the interaction identified with the yeast three-hybrid system by a complementary method, affinity chromatography, to prove the validity of the hit. Furthermore, we provide evidence for an interaction between the drug and PLOD2 *in vitro* and *in cellulo*.

Keywords: ethinylestradiol; lysyl hydroxylase 2; target identification; yeast three-hybrid screen.

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Introduction

Many fundamental processes in biology rely on small ligand-protein interactions which form the basis for pharmacological intervention of human diseases. Once the bioactivity of a compound has been critically evaluated, the challenge remains to identify its molecular target. In contrast to target-based identification of bioactive small molecules, in which large libraries of small molecules are typically screened against a selected (disease related) target, cell-based screenings of new biologically active small molecules are increasingly employed. Anti-tumoral compounds are very often discovered in phenotypic screens, looking at cytotoxicity or inhibition of proliferation. This 'bottom-up' strategy struggles with the bottleneck of target identification, which is often laborious and time-consuming (Schenone et al. 2013). Nevertheless, an increased understanding of tumor biology has enabled the development of molecularly targeted cancer drugs, many of which are based on a natural product scaffold. An example for a success story of a natural product in clinical use to treat cancer is paclitaxel (Taxol®), which is an inhibitor of microtubule disassembly (Band 1992). At the same time, albeit potent anti-tumor activities are observed, the molecular targets of numerous other small molecules often remain elusive, as do the molecular mechanisms that lead to the observed phenotype in cells. For developing small molecules as potential lead structures for novel therapeutics, knowledge of the identity of the target protein is crucial (Bunnage et al. 2015). Indeed, affected networks and downstream or upstream signaling pathways are often the missing link to completely understand the mechanisms leading to the activity of a small molecule and to explain the cellular effects observed upon small molecule treatment.

Although various approaches for target identification have proven successful, the combination of complementary approaches is required for success in many cases. Current approaches for target identification rely on genetic mutant screens (Han and Nijhawan 2019) or chemical proteomics based on mass spectrometry. Chemical

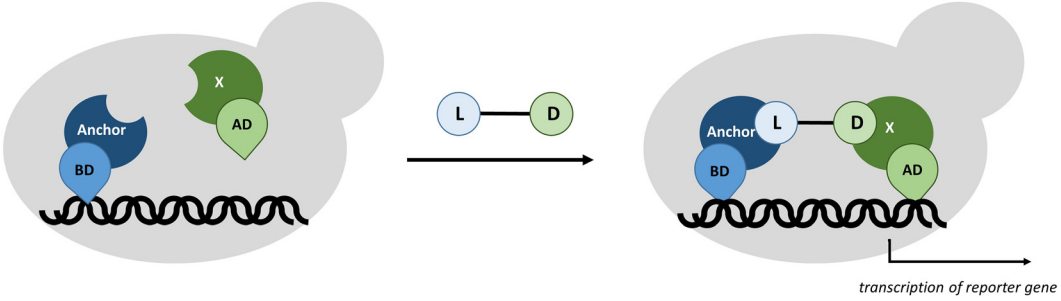
proteomic approaches have proven very powerful methods by employing small molecule probes to fish for protein targets. Among these approaches, affinity chromatography is probably the most widely used; other recently described chemical proteomics methods include drug affinity responsive target stability (DARTS) (Lomenick et al. 2011) or activity based protein profiling (ABPP) (Chen et al. 2017).

Affinity chromatography represents an un-biased method that allows for identification of a direct binding partner of small molecules (Rix and Superti-Furga 2008). To select for binding partners of a small molecule with this approach, the compound needs to be immobilized onto a solid support. Whole cell lysates are then applied to the surface exposing the small molecule. Non-binding proteins are removed by a washing step and proteins with an affinity to the small molecule are retained. After elution, the potential binding partners are resolved by SDS-PAGE and analysed using state-of-the-art mass spectrometry and statistical analysis. In contrast to high-throughput screening efforts, which often only focus on selected subproteomes such as kinases, the compound of interest is exposed to the entire variety of cellular proteins including all naturally occurring posttranslational modifications and activation states. The shortcomings of this approach are mainly that transient interactions are missed out, and that the method selects for high abundance proteins, whereas proteins that are only expressed at low levels are easily missed.

Conceptually, the yeast three-hybrid system (Y3H) is similar to affinity based target identification. Both approaches share the disadvantage that they require the derivatization of the active molecule. Compared to affinity based target identification, however, the Y3H system offers several advantages, such as its application in living cells; furthermore, the Y3H system is based on cDNA libraries, hence allows for the identification of proteins underrepresented in cell lysates used for affinity chromatography due to low expression levels; furthermore, no high end mass spectrometer or other costly device is necessary; the identity of the protein of interest can be obtained by standard DNA sequencing, for which ample service providers are available at very low costs.

The Y3H system builds on the yeast-2-hybrid (Y2H) system, which is used to screen for protein-protein interactions of a selected organism in baker's yeast (Fields and Song 1989). In this system, a protein of interest, the 'bait' is expressed as fusion protein to the DNA binding domain of a split transcription factor, whereas a pool of potential binding partners or cDNA libraries ('preys') are expressed as fusion proteins of the activation domain. Only if an interaction takes place, is the transcription of a reporter gene activated, leading to survival of the yeast cells on selective plates (Brückner et al. 2009). In the Y3H system, a small molecule-ligand hybrid is added to the Y2H setup (Scheme 1) (Licitra and Liu 1996). The small molecule of interest is chemically coupled to an anchor molecule, which can be a small molecule substrate with high affinity to the enzyme it inhibits, such as the methotrexate-dihydrofolate reductase interaction pair (Becker et al. 2004; Henthorn et al. 2002), which has been used to screen for kinase inhibitors, or the substrate of a protein tag such as SNAP tag (Chidley et al. 2011). The SNAP tag Y3H system has successfully been applied to clinically approved drugs against human cDNA libraries, yielding not only the confirmation of 16 previously identified tyrosine kinases but also a previously unknown non-kinase hit in the case of the tyrosine kinase inhibitor dasatinib. Similarly, screening of atorvastatin against cDNA libraries has revealed a number of novel targets. Moreover, the Y3H system has proven useful in the screening of protein targets of anti-*Mycobacterium tuberculosis* drugs (Moser and Johnsson 2013).

The advantage of the Y3H screening over conventional target fishing methods (like affinity chromatography or whole cell screenings) is that the identity of the target



Scheme 1: Setup of a yeast three-hybrid system.
A small molecule (D) is coupled to a linker (L) that allows for binding to an anchor protein coupled with the DNA binding domain of a split transcription factor (BD). In this setup, the small molecule is screened against a library of potential binding partners (X) fused to the activation domain of the same transcription factor (AD). Transcription of a reporter gene and thus survival of the yeast cell on selective plates only occurs upon interaction of the small molecule (D) with a protein (X).

protein is readily revealed by isolating the plasmid DNA from the hit yeast colony followed by sequencing. This fact also facilitates the confirmation of the hits, since the gene encoding for the potential protein binding partner can be directly cloned from the Y3H vector into a vector for protein expression. One disadvantage of using this system is a high false positive rate; one way to identify false positives is to verify that growth of the yeast depends on the presence of the small molecule probe. Depending on the nature of the identified interaction, complementary assays can be performed to confirm the interaction and analyze downstream effects.

In this study, we set up and tested a Y3H platform for its suitability to identify protein binders of small molecule drugs. For a proof-of-concept screening, we exemplarily chose ethinylestradiol (EE) as small molecule candidate, with the rationale that it features an ethynyl moiety that allows for a simple modification of the molecule.

Estrogens are widely used in combined oral contraceptives (COCs). As a member of the estrogens family, EE was first applied in COCs because it was confirmed to have better oral bioavailability (38–48%) than other estrogens (Kuhl 2005) while exerting the same functions: it regulates vaginal bleeding, replaces lost estradiol after suppression of ovarian function, and also reduces the amount of progestin which is necessary to inhibit ovulation effectively (Kluft et al. 2017). Activities of estrogens are mainly mediated via estrogen receptor (ER) α, a nuclear hormone receptor that is responsible for endogenous estrogen associated regulation and signaling (Brzozowski et al. 1997). EE binds with high affinity to ERα, and modifying the molecule via 'click' chemistry at its ethynyl functional group does not hinder binding to the receptor (Tang et al. 2018).

Results

In order to be able to perform un-biased target identification screens we established a Y3H system; as an anchor, we used the trimethoprim-DHFR interaction, which has already been validated before (Gallagher et al. 2007). The interaction of purvalanol B with the kinase CDK5 was selected as positive control, since this interaction pair has already been validated in a Y3H setup (Wegner et al. 2015). We therefore synthesized a purvalanol B derivative linked to trimethoprim via a PEG (polyethyleneglycol) linker using newly designed (see the Supplementary Material) trimethoprim-PEG-amine (Figure 1).

Dhfr (*E. coli*) and *cdk5* (human) genes were cloned into the Y2H bait and prey vectors, respectively, from the Matchmaker Gold Y2H system (Takara Bio) and transformed into the modified yeast reporter strain ABC9Δ, which was obtained from the A*STAR institute in Singapore. This strain has nine efflux pump deletions, and has been proven to possess superior properties for yeast based screenings, since it has a higher permeability for small molecules, which is a major challenge of the yeast based system (Wong et al. 2017). The yeast was then spotted on unselective plates (-LW), and on selective plates (-LWH) with or without the purvalanol B probe. The spot

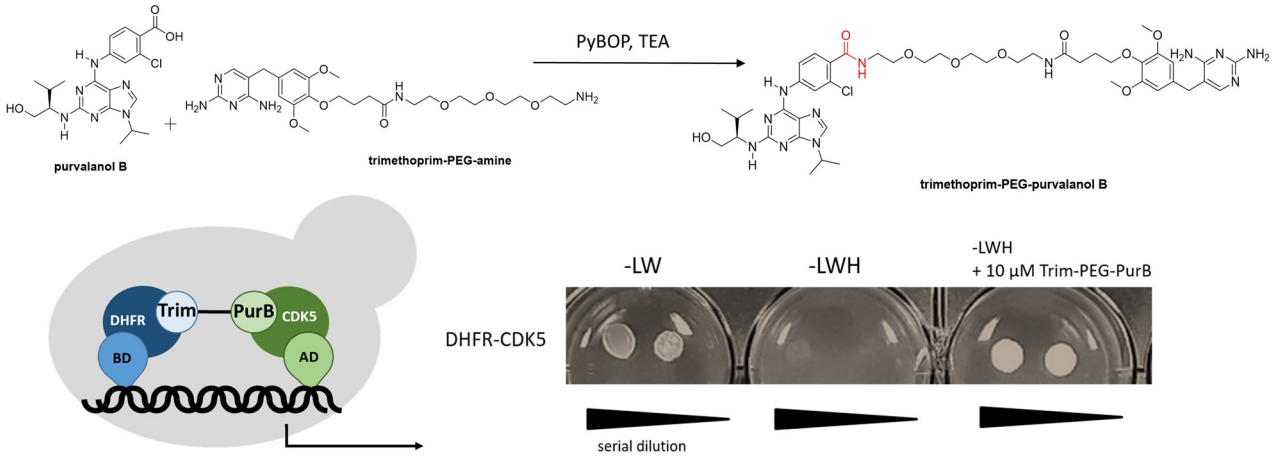


Figure 1: Establishing the Y3H system based on a dihydrofolate synthase (DHFR) anchor that binds tightly to the drug trimethoprim (Trim). For proof of principle, the known interaction of cyclin dependent kinase 5 (CDK5) with the CDK5 inhibitor purvalanol B (PurB) was tested in the system. For synthesis of the Y3H probe, PurB was coupled to amino-functionalized trimethoprim via a PEG linker (see Supplementary Material). The yeast reporter strain expressing CDK5 fused to the activation domain, and DHFR fused to the DNA binding domain was grown on medium selecting for the two plasmids (-LW) and plates selecting for the interaction (-LWH) in the presence and absence of the trimethoprim-PEG-purvalanol B probe.

test showed that the yeast grew well on selective plates only in the presence of the probe (Figure 1).

Having confirmed that our Y3H setup can indeed detect small molecule-protein interactions, we next wanted to employ this system for the identification of protein binding partners of small molecules out of a pool of proteins. Therefore, we used conventional cDNA libraries for Y2H screens, which are commercially available. We used an electroporation protocol (Benatuil et al. 2010) to transform the ABC9Δ strain containing the DHFR bait plasmid with a mammary gland and pancreas cDNA library, respectively. The viability of the transformed yeast strains was determined by cfu plating and was found to be larger than 10^9 cfu/ml for both libraries (see Supplementary Material).

A clickable trimethoprim probe with a PEG linker and an azide moiety was synthesized as described in the Supplementary Material. In brief, the 4″-hydroxy analogue (TMP-OH) of trimethoprim was connected with a butyrate spacer (Li et al. 2018), which in turn was amidated with 11-azido-3,6,9-trioxaundecan-1-amine. The trimethoprim-PEG-azide was coupled to EE via a copper-catalyzed alkyne-azide 'click' reaction generating a triazole unit at C-17 of EE (see Figure 2) and the Y3H screen was then performed similar to previously described procedures (Chidley et al. 2011; Moser and Johnsson 2013). In brief, yeast library stocks were spread onto selective plates (-LWH containing 2.5 mM 3-aminotriazole [3-AT]) containing 10 μM trimethoprim-PEG-EE. 3-AT is an inhibitor of the *his3* reporter gene product, and addition of 3-AT was found to lead to a lower false positive rate. More than 5×10^7 yeast cells were used per plate to ensure a larger than 10 fold of library coverage (complexity of the library indicated as 3×10^6 by the manufacturer). One 15 cm diameter plate was used per screen and library. The plates were incubated for 10 days at 30 °C; after this time, colonies were picked and subjected to growth dependence tests by spotting them on selective plates, and plates with and without the EE probe, which allows for the elimination of further false positives. Only colonies that grew on plates containing the drug conjugate, but showed no growth on plates without the drug conjugate, were classified as hits and analyzed further (Figure 2). From the mammary gland library, 18 hits were obtained, while the pancreas library yielded 12 colonies classified as hits.

Colony PCR of all hits was performed (see Supplementary Material). Sequencing analyses of all cDNA inserts led to the exclusion of further false positives, due to the obtained sequence being either out of frame with the GAL4 AD, or due to the fragment not being part of a coding sequence. Eventually, the screening of the pancreas library yielded no real hit, while one confirmed hit was obtained from the mammary gland library screen. This hit was identified twice and encoded for the last 81 amino acids of procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (PLOD2, alternative name lysyl hydroxylase 2 [LH2]). A high false-positive rate is inherent to yeast based screenings, since many proteins can activate the reporter without an interacting partner (Shivhare et al. 2021). In contrast to the Y2H screen, in the Y3H system false positives can be eliminated in one simple spotting step as performed in this study, which only selects yeast colonies that grow depending on the presence of the probe, thereby eliminating auto-activators.

In order to validate PLOD2 as hit and interaction partner of EE, we performed affinity chromatography using a biotin-

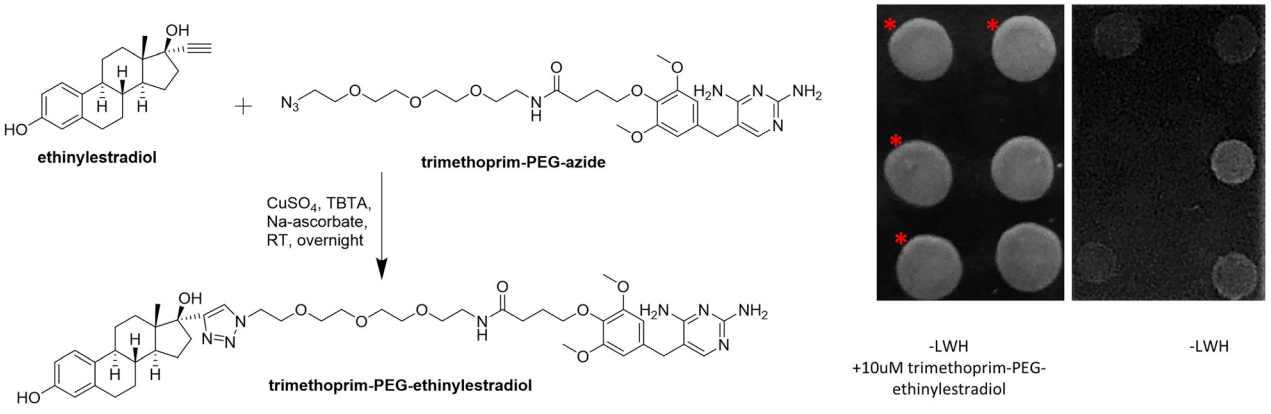


Figure 2: Using the Y3H system to screen for binding partners of EE.
First, a trimethoprim-PEG-EE probe was synthesized to allow for DHFR anchoring in the Y3H setup, and then screened against two different human cDNA libraries in the Y3H setup. Colonies were picked und subjected to a growth dependence test in the presence and absence of the probe to eliminate false positives (right panel). Only colonies that showed growth on the plates containing the probe but no growth on the plate without the probe, were classified as 'hits' (marked with a red asterisk*).

PEG-EE probe (see Supplementary Material). The probe allowed for the immobilization of EE on streptavidin beads. The beads were then incubated with human recombinant PLOD2, or whole cell lysates of yeast overexpressing the identified PLOD2 fragment as GAL4 AD fusion (see the Materials and methods section and Supplementary Material). As a negative control, a modified biotin carrying the same linker as the EE probe was immobilized on the streptavidin beads. After washing, bound proteins were eluted with SDS and heat, and analyzed by SDS-PAGE and Western Blotting using a PLOD2 antibody for staining, or a GAL4 antibody for the detection of the GAL4 AD fusion protein from the Y3H vector. Indeed, the Western Blot revealed that the PLOD2 fragment identified in our Y3H screen can be enriched on EE modified beads, although only a faint band was observed, while no band was detected in the eluate from the beads modified with pegylated biotin as negative control (see the Supplementary Material). Having confirmed the interaction between EE and the 81 aa fragment of PLOD2 identified in the Y3H screen, we performed the same experiment using human recombinant PLOD2, showing that also the whole protein can be enriched with the EE probe in a pull-down setup (Figure 3A), yielding a more intense band compared to the fragment.

PLOD2 is involved in collagen cross-linking by hydroxylating lysyl residues in telopeptides of fibrillary collagens. In the course of this reaction, succinate is created that in turn can be detected in a luminescence based *in vitro* assay and allows for monitoring of PLOD2 activity. Using the assay developed by Devkota et al. (2019), the influence of EE on PLOD2 activity was determined. Indeed, using human recombinant PLOD2, a decrease in the formation of succinate was observed upon treatment with EE (Figure 3B).

PLOD2 catalyzes the hydroxylation of lysine residues in the telopeptides of fibrillar collagens, which leads to the formation of stable collagen cross-links. A recent study also showed that PLOD2 can be secreted by tumor cells and modify collagen in the extracellular space (Chen et al. 2016). To further investigate the effect of EE on PLOD2, we conducted a collagen I staining to see whether EE treatment can alter extracellular collagen fibers. As a known inhibitor of PLOD2 expression, minoxidil (Xu et al. 2017) was also included as a positive control. From the confocal microscopy images (Figure 4A), we can see that without treatment, cells actively remodel the collagen fibers creating a cage-like structure around themselves. The addition of EE and the control substance minoxidil, however, significantly impaired matrix remodeling. At a higher concentration of EE or minoxidil, the cells appear to interact less with the underlying collagen, so that a cage around the cells can no longer be observed.

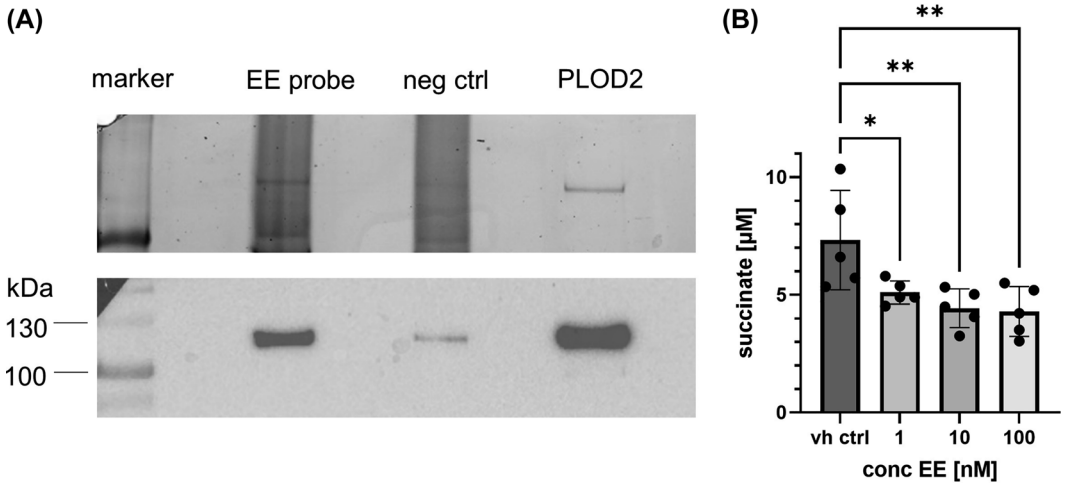


Figure 3: Validation of the EE-PLOD2 interaction pair using commercially available human recombinant PLOD2 (whole protein).
(A) Affinity chromatography. A biotin-PEG-EE probe was immobilized on streptavidin beads. Next, 200 ng of human recombinant PLOD2 were added. After incubation and washing, bound proteins were eluted and analyzed by SDS-PAGE. A Western Blot using a PLOD2 antibody revealed an enrichment for the eluate from the EE modified solid phase compared to beads modified with pegylated biotin as negative control. Top panel: SDS-PAGE loading control. Lower panel: Exemplary photo of a Western Blot (Western Blots of all three replicates can be found in the Supplementary Material). (B) PLOD2 activity assay. An activity assay was performed that measures the succinate formed by PLOD2 in a bioluminescence based assay (Devkota et al. 2019). An impairment of PLOD2 activity is observed upon addition of EE in comparison to a vehicle control (vh ctrl). $n = 5$, error bars represent standard deviations. $*p < 0.05$, $**p < 0.01$.

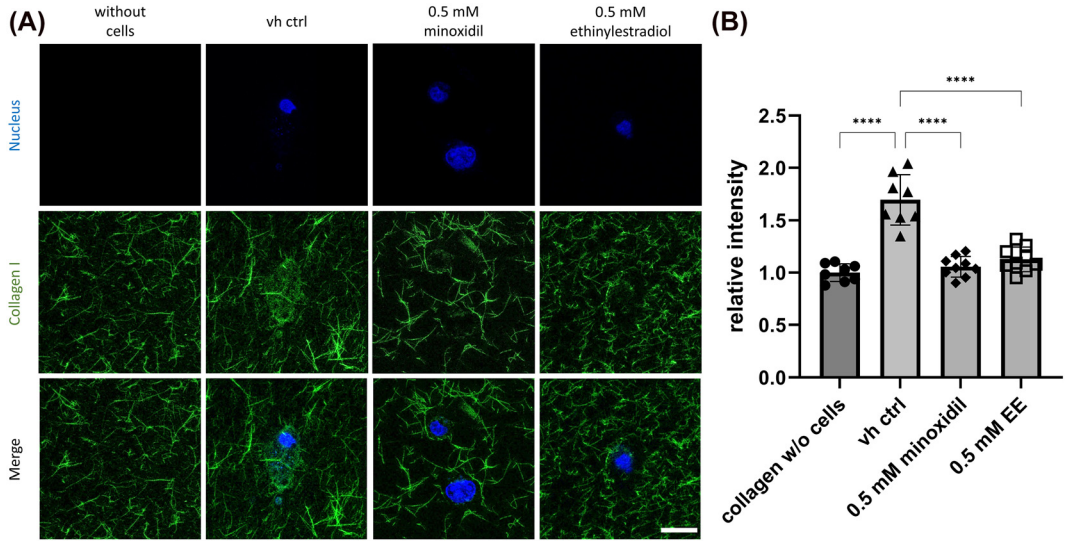


Figure 4: EE inhibits collagen remodeling.
HeLa cells were seeded onto FITC labeled collagen I (2 mg/ml) and treated with either EE, minoxidil (an inhibitor of PLOD2 expression; Xu et al. 2017) as positive control, or a DMSO vehicle control (vh ctrl). (A) Images taken 24 h after cell seeding are shown with the nuclei in blue (live cell Hoechst staining) and the collagen fibers in green (scale bar 25 µm). (B) Evaluation of collagen fluorescence intensity shows that treatment with EE or the positive control minoxidil leads to reduced density of collagen fibers in close proximity of the cells, indicating a potential effect of EE on PLOD2 in comparison to a vehicle control. Error bars represent standard deviations (vh ctrl, ****$p < 0.0001$).

Discussion

The identification of protein targets of a small molecule drug is crucial for its further development. Not only downstream mode of action studies, also medicinal chemistry approaches are facilitated in case an interaction partner is known. Among the many approaches to fish for targets of small molecules, the Y3H system has not found a broad application, despite its technical simplicity. Here, we report the setup of a Y3H platform for target identification using an already validated drug-protein interaction pair as anchor. The interaction between trimethoprim and DHFR is known to be strong; *E. coli* DHFR has a K_i of 0.17 nM for inhibition by trimethoprim (Cammarata et al. 2017), rendering this pair ideal for a Y3H setup. In order to facilitate drug uptake, we used the recently published *Saccharomyces cerevisiae* ABC9Δ strain featuring 9 efflux pump deletions (Wong et al. 2017) as a reporter strain. Indeed, using a trimethoprim-purvalanol B chemical inducer of dimerization and CDK5 as prey completing the setup of two known interaction pairs, the yeast grew only on selective plates containing the purvalanol B probe, validating our approach in living yeast cells.

We then introduced two commercially available cDNA libraries intended for Y2H studies, a mammary gland and pancreas cDNA library, respectively, into the reporter strain containing the DHFR bait plasmid. Performing a Y3H screen with EE (linked to trimethoprim) as a candidate drug yielded several colonies that passed the growth dependence test; after sequencing, however, none of the inserts from the pancreas library screening turned out to be real positive hits. Nevertheless, from the mammary gland library screening, two colonies among those classified as hits both carried a library plasmid with an insert encoding for an 81 amino acid sequence in the coding sequence of PLOD2. PLOD2 is a 737 amino acid protein, whereby the first 25 amino acids are part of a signal peptide (https://www.uniprot.org/uniprot/O00469). The 81 amino acid sequence identified in our screen covers a region towards the C-terminus of the protein, and contains the metal binding domain as well as the active site of PLOD2. Whether the identified fragment is correctly folded is not clear. Since this fragment was identified in the screen looking for binding partners of EE, we assume that it is at least folded correctly to a degree which allows for the binding to take place. This is underlined by the pull-down experiments, in which a stronger band is obtained when using whole recombinant PLOD2 compared to the fragment identified in the screen (Figure 3A and Supplementary Material). PLOD2 has received increased attention recently, since it has been found to aid tumor metastasis by increasing the stiffness of the collagen matrix (Chen et al. 2015, 2016). For a first confirmation of PLOD2 as a binding partner of EE, a complementary affinity chromatography

approach was employed. Using immobilized EE, the PLOD2 fragment identified in the Y3H screen could be pulled down from yeast cell lysates overexpressing the fragment (see Supplementary Material, Figure 2). Since the cDNA insert from the Y3H screen did not cover the entire coding sequence, we wanted to validate the interaction between EE and PLOD2 using human recombinant PLOD2. Therefore, we repeated the pull-down experiment using the commercially available whole PLOD2 and a PLOD2 antibody for Western Blot analysis. Compared to the negative control (pegylated biotin), a clear enrichment was observed when PLOD2 was washed over beads with immobilized EE (Figure 3), which confirmed the validity of the interaction identified in the Y3H screen. Since the fragment identified in the Y3H screen contains the catalytic site of PLOD2, we assessed the effect of EE on PLOD2 activity. PLOD2 catalyzes the formation of hydroxyl-lysine residues in collagens, a reaction in which succinate is formed. By performing a bioluminescence based PLOD2 activity assay that is based on Promega's Succinate-Glo™ Assay (Devkota et al. 2019), the formation of succinate can be monitored. Using this assay and commercially available whole PLOD2, addition of EE led to a decreased formation of succinate, suggesting an impairment of PLOD2 activity by EE (Figure 3). To further underline our results, we have assessed PLOD2 expression levels using a PLOD2 antibody and have found that both, minoxidil and EE, led to a decrease of PLOD2 expression levels (see Supplementary Material).

PLOD2 is secreted by tumor cells and modifies collagen in the extracellular space (Chen et al. 2016). The decrease of collagen intensity around the cells indicates that the extracellular collagen crosslinking is impaired upon treatment with EE (Figure 4).

Albeit yielding a previously unknown binding partner of EE, interestingly, ERα, the classical target of EE, has not been identified in the Y3H screen. This fact could be due to the size of the receptor which probably has excluded it in the process of library construction, which is limited to cDNA of a smaller size range. Another possible explanation could be that – in case the receptor or parts of it are included in the library – increasing the number of plates used for screening might lead to the identification of ERα in the screen. Nevertheless, using the Y3H screen allowed for the identification of a hitherto unknown protein binding partner of the widely used contraceptive EE. The implications of the interaction between PLOD2 by EE as identified in a biological context, especially in cancer biology, is the subject of future studies.

Despite its simplicity, the Y3H has the drawback that a small molecule of interest has to be derivatized by chemical synthesis, and that this probe has then to be taken up by the yeast cells in sufficient concentration. Here, we report the establishment of a yeast-3-hybrid screen in the efflux pump deletion strain ABC9Δ, which has been proven previously to enable a more efficient drug uptake into the yeast cell (Wong et al. 2017). In this study, we confirmed the validity of our Y3H setup by testing the known interaction pair purvalanol B-CDK5, and by performing a proof-of-principle screen, we revealed a previously un-identified binding partner of EE, which we confirmed by conventional affinity chromatography. Hence, our Y3H is now ready to be applied for target ID screens and represents a complementary, simple, and straightforward approach for the identification of direct targets of small molecule drugs.

Materials and methods

Chemicals and materials

HPLC grade methanol (MeOH), HPLC grade acetonitrile (ACN), dichloromethane (DCM), dimethylsulfoxide (DMSO), hydrochloric acid (HCl), acetic acid (AcOH), sodium hydroxide (NaOH), and silica gel 60 were obtained from VWR (Ismaning, Germany) and ultra-pure water (18 $M\Omega \cdot cm^{-1}$) from a Millipore S.A.S. Milli-Q Academic system (Molsheim, France). Ammonium acetate (NH_4AcO), bovine serum albumin (BSA), calcium chloride ($CaCl_2$), sorbitol, d_6-DMSO, and ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) were purchased from Merck KGaA (Ismaning, Germany). SepPak Plus C18 cartridges were obtained from Waters Associates (Milford, USA). Magnesium chloride ($MgCl_2$) and glycerol were purchased from AppliChem (Darmstadt, Germany). TBTA, Agar–Agar, ampicillin, α-ketoglutaric acid, potassium chloride (KCl), Roti®-Quant (5× concentrate) Bradford solution and sodium dodecyl sulfate (SDS) were purchased from Carl Roth (Karlsruhe, Germany). Purvalanol B from Cayman chemicals was purchased from biomol (Hamburg, Germany). Streptavidin beads, $[IKG]_3$ peptide, Collagen Type I, Page Ruler™ Plus Prestained Protein Ladder and Abnova™ PLOD2 (Human) Recombinant Protein were obtained from Thermo Fisher Scientific (Schwerte, Germany). Anti-GAL4 AD antibody and Anti-PLOD2/LH2 antibody were purchased from Abcam (Berlin, Germany). Lithium acetate was purchased from BioScience GmbH (Dümmer, Germany). 50% PEG was purchased from Takara Bio. Dithiothreithol (DTT) was purchased from SERVA Electrophoresis (Heidelberg, Germany). The Succinate-Glo™ JmjC Demethylase/Hydroxylase Assay Kit was purchased from Promega GmbH (Walldorf, Germany). Ethinylestradiol, minoxidil, Collagen Type I-FITC Conjugate, biotin-PEG_3-N_3, copper(II) sulfate pentahydrate ($CuSO_4 \times 5H_2O$), yeast nitrogen base, yeast synthetic drop-out medium supplements, adenine, tryptophan, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), triethylamine, glass beads, imidazole, β-mercaptoethanol, potassium phosphate dibasic (K_2HPO_4), potassium phosphate monobasic (KH_2PO_4), magnesium sulfate ($MgSO_4$), phenylmethylsulfonyl fluoride (PMSF), Tris-HCl, pyridine, HEPES, EDTA-2Na, sodium chloride (NaCl), ferrous chloride ($FeCl_2$), Nonidet P-40, sodium

deoxycholate, sodium ascorbate, ascorbic acid and Triton-X 100 were purchased from Sigma-Aldrich (Taufkirchen, Germany). Goat anti-Rabbit IgG secondary antibody was purchased from Dianova (Hamburg, Germany). Anti-Mouse IgGk light chain BP secondary antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Chromatography

(i) Analytical HPLC: Agilent 1260 Infinity II LC system with a 1260 Infinity Degasser, a 1260 Series quaternary pump and 1260 Series diode array detector; Merck LiChrospher® 100 RP-18 (5 µm) LiChroCART® 125-4, protected by a Merck LiChrospher® 100 RP-18 (5 µm) LiChroCART® 4-4 i.d. pre-column; injection volume: 100 µl (unless stated otherwise). Solvent system: mobile phase A = NH_4AcO buffer 10 mM pH 7, B = ACN, flow 0.5 ml/min; Solvent composition: 0–2 min 5% B, 2–17 min 5–100% B, 17–20 min 100% B, 20–22 min 100–5% B. Data were processed with OpenLab CDS Data Analysis 2.3.

(ii) Semi-preparative HPLC: Büchi Pure C-830 with prep HPLC pump 300 bar, fraction collector, and prep sample injection valve. Gynkotek LC-System with manual sampler, M480 pump, Phenomenex DG-301 online degasser, Gynkotek UVD 640 diode array detector and a Rheodyne injection valve with 5 ml loop. Column: Supelco Ascentis® C18, 5 µm, 15 cm × 10 mm, with a Phenomenex pre-column ODS 9 × 16 mm; mobile phase A = NH_4AcO buffer 10 mM pH 7, B = ACN, flow 2.5 ml/min; solvent composition: 0–2 min 12% B, 2–12 min 12–20% B, 12–30 min 20–80% B, 30–40 min 80–100% B. Data were processed with Gynkosoft 5.50 or Büchi Pure software 1.5.

Spectroscopy

HR-MS were measured at the MS facility of the Department of Chemistry, University of Munich. Data were processed using Xcalibur. NMR spectra were recorded on an Avance III HD 500 MHz NMR spectrometer from Bruker BioSpin equipped with a CryoProbe™ Prodigy broadband probe holder. NMR data were analyzed with Mestre Nova.

Spot test with trimethoprim-PEG-purvalanol B

The *S. cerevisiae* reporter strain ABC9Δ was transformed with bait and prey plasmid in two steps. In the first step, the bait plasmid was introduced into the yeast using the quick and easy protocol from Gietz and Schiestl (2007b). The transformation mix was spread on 10 mm plates containing complete minimal medium minus tryptophan (-W). In a second step, the yeast containing the bait plasmid were transformed using the high efficiency transformation protocol from Gietz and Schiestl (2007a). Selection of transformants was carried out on plates containing complete minimal medium minus leucine tryptophan (-LW).

One colony of each transformation was restreaked on a -LW plate to ensure robust growth. The yeast was then picked from the plate, and suspended in 1× TE in three dilutions. A 2 µl portion of each dilution was spotted on -LW plates as growth control, and on plates containing complete minimal medium minus leucine tryptophan histidine (-LWH) and on -LWH plates containing 10 µM trimethoprim-PEG-purvalanol B (in DMSO). The plates were incubated at 30 °C and pictures were taken after 4 days.

Preparation of the Y3H reporter strains containing cDNA libraries

The mammary gland and pancreas cDNA library in pACT2 were purchased from Takara Bio (traditional Y2H libraries). Plasmids were isolated after cultivation in Luria-Bertani (LB) medium containing ampicillin using a Qiagen MaxiPrep kit. The ABC9Δ strain containing the bait plasmid (eDHFR in pGBKT7) was transformed by electroporation based on the protocol of Benatuil et al. (2010) with modifications: An overnight culture of yeast in -W was diluted 5× to 1 l in two 500 ml baffled flasks. After incubation on a shaker for 5–6 h, the cultures were centrifuged at 4 °C (500 g) for 2 min. The pellets were washed twice with 200 ml of ice cold MilliQ water each and once with 200 ml of ice cold 1 M sorbitol. The pellet was suspended in 80 ml of 0.1 M lithium acetate/10 mM DTT and incubated at 30 °C for 30 min with shaking. After centrifugation, the pellet was washed with 80 ml of ice cold 1 M sorbitol and centrifuged again. The competent cells were cautiously suspended in 400 µl of ice cold 1 M sorbitol (total volume about 1 ml) and used within 1 h. 1.5 µg of library plasmid were used for electroporation. The electroporation was carried out using an Eppendorf Eporator with the settings for *S. cerevisiae*. 1 ml of pre-warmed YPAD medium was added directly after the pulse, and the transformation mixture was incubated at 30 °C on a thermoshaker (400 rpm). After 1 h, the mixtures were centrifuged (1 min, 10 000 rpm). The pellets were suspended in -LW medium (total 1 l) and incubated at 30 °C overnight on a shaker. The cultures were centrifuged and washed twice with one volume of 1× TE. About two pellet volumes of freezing solution (65% glycerol, 100 mM $MgSO_4$, 25 mM Tris–HCl pH 8) were added. The viability of the libraries was determined by plating dilutions on -LW plates to be 2×10^9 cfu/ml for the mammary gland library and 4×10^9 cfu/ml for the pancreas library.

Y3H screening

The Y3H screening was performed following a previously published protocol (Moser and Johnsson 2013). In brief, both mammary gland cDNA library and pancreas cDNA library with the amount of about 25–50 µl stock (5×10^7–2×10^8 cfu/ml) were diluted in 1× TE and then homogeneously spread on large plates (150-mm diameter) containing complete minimal medium minus leucine tryptophan histidine (-LWH) + 2.5 mM 3-amino-1,2,4-triazole (3-AT) + 10 µM trimethoprim-PEG-EE (diluted from a 10 mM stock in DMSO) + 1% (w/v) agarose. After 10 days at 30 °C, all colonies larger than 1 mm in diameter were picked and resuspended in 100 µl 1× TE each in 96-well plates. Using a metal 96-prong replicator, the resuspended colonies were transferred onto -LWH plates with 2.5 mM 3-AT firstly and then onto -LW plates. After 5 days at 30 °C, all colonies which did not grow in -LWH plates but grew in -LW plates were picked and resuspended in 100 µl 1× TE in 96-well plates. The resuspended colonies were transferred onto -LWH plates only with 1 mM 3-AT firstly and then onto -LWH plates with 1 mM 3-AT and 10 µM trimethoprim-PEG-EE using a metal 96-prong replicator. After 3 days at 30 °C, all colonies which grew better in plates with trimethoprim-PEG-EE than in plates without the compound were picked and resuspended in 100 µl 1× TE in 96-well plates. Two µl of the resuspended yeast cells were spotted on a -LW plate to ensure robust

growth. After 3 days at 30 °C, the colonies were picked with tips and resuspended in 1 ml -LW medium. Yeast cultures were then incubated at 30 °C overnight. Plasmid DNAs were isolated with a commercial kit according to the manufacturer's instructions (EZ-10 Spin Column Yeast Plasmid DNA Mini-Preps Kit, Bio Basic). The cDNA inserts were amplified by colony PCR and analyzed by sequencing (Eurofins Scientific). The cDNA identity was determined by BLAST analysis.

Amplification of hit plasmids in *E. coli*

Plasmid DNAs of five potential target proteins were transferred into *E. coli* cells using electroporation. After incubation at 37 °C for 1 h, the *E. coli* cells were homogeneously spread on plates (100 mm diameter) containing LB medium + 50 µg/ml ampicillin. After overnight incubation at 37 °C, single colonies were picked from each plate and suspended in 5 ml LB medium with 50 µg/ml ampicillin. After overnight culture at 37 °C and 100 rpm in the thermoshaker, *E. coli* cell pellets were recovered by centrifugation. Plasmid DNAs were then isolated with a commercial kit according to the manufacturer's instructions [QIAprep® Spin Miniprep Kit (250), Qiagen]. The cDNA inserts were analyzed by sequencing (Eurofins Scientific) and subsequent BLAST analysis. The standard GAL4 AD primer was used for DNA sequencing.

Yeast transformation with hit plasmids

Transformations were carried out following the protocol by Gietz and Schiestl (2007b). An overnight culture of the *S. cerevisiae* Y2H Gold strain (Takara Bio) in 10 ml of YPAD medium was used.

Yeast cell lysates for affinity chromatography

For each transformed yeast strain, a 5 ml of overnight culture in YPAD medium was prepared. After incubation, the YPAD medium was discarded by centrifugation. The pellet was resuspended in -L medium and vortexed for 30 s to disperse cell clumps. Then the cell culture was diluted with -L medium to an OD600 value of about 0.2. The cells were incubated at 30 °C and 120 rpm for the protein expression until the OD600 value reached 0.4–0.6. The culture was poured into pre-chilled 50 ml falcons halfway filled with ice. The tubes were immediately placed in a pre-chilled rotor and centrifuged at 1000*g* for 5 min at 4 °C. The cell pellet was washed with 50 ml of ice-cold H_2O.

Glass beads of the same volume as the cell pellet were added to the pellet. A 150 µl portion of RIPA buffer (150 mM NaCl, 50 mM Tris–HCl pH 8, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) with 1 mM PMSF was added to resuspend the pellet. The mixture was vortexed for 1 min and incubated on ice for 1 min afterwards. The vortex/chill cycle was repeated four more times. The supernatant was collected after centrifugation at 18,000 rpm for 10 min at 4 °C. The remaining pellet was extracted with another 150 µl of RIPA buffer with 1 mM PMSF and the lysates were pooled. The protein concentration of the lysate was determined by a Bradford assay.

Biotin-tag-based EE pull-down

100 µl of streptavidin beads suspension were washed with washing buffer (10 mM HEPES-NaOH, 50 mM KCl, 1 mM EDTA-2Na, 10% glycerol, pH 7.9) and resuspended in 50 µl of washing buffer. 50 µl of 2 mM biotin-PEG-EE conjugate (see Supplementary Material) or biotin-PEG_3-N_3 (Sigma-Aldrich) as negative control were added to the beads. The mixture was incubated at room temperature for 30 min. After washing three times with 1 ml of washing buffer each, 100 µl of either yeast cell lysate (total 1 mg of protein as determined by a Bradford assay) or 200 ng of human recombinant PLOD2 (Abnova™, full-length ORF AAH37169, amino acids 27–758, with GST tag at N-terminal) were added. The mixture was incubated at 4 °C for 1 h. The beads were washed 2 times with 0.5 ml of washing buffer each. The supernatant was discarded by centrifugation at 3000 rpm for 3 min at 4 °C. 45 µl of 1× Western Blot sample buffer was added. The samples were boiled at 99 °C for 5 min. The eluates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on discontinuous polyacrylamide gels (12%). Proteins from the gels were transferred to 0.2 µm polyvinylidene difluoride (PVDF) membranes by Western Blotting. For detection, either a GAL4 AD primary antibody (Abcam) was used for the fragment (GAL4 AD-PLOD2 fusion protein), or a PLOD2 antibody (Abcam) for the detection of human recombinant PLOD2. Protein bands were detected by chemiluminescence using a ChemiDoc™ Touch Imaging System (Bio-Rad, Munich, Germany). Protein bands were analyzed by comparison with the Page Ruler™ Plus Prestained Protein Ladder and intensities of protein bands were analyzed using Image Lab™ software (Bio-Rad, Munich, Germany).

PLOD2 activity assay

The activity assay was performed as described by Devkota et al. (2019) with minor adaptations. In brief, 15 µl of 0.47 µM PLOD2 in assay buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 0.5% DMSO, 0.01% Triton-X 100) were added to a white 96-well plate. Then, 5 µl of 5× compound stock or DMSO in assay buffer were added. After incubation for 50 min at r.t., 5 µl of 5× substrate mix (1 mM $[IKG]_3$ peptide, 0.1 mM α-ketoglutarate, 1 mM ascorbic acid, 0.1 mM $FeCl_2$ (freshly prepared) in assay buffer) were added to the well. The plate was incubated for 3 h at r.t. The succinate detection reagents from Promega's Succinate-Glo™ Assay kit were added according to the manufacturer's instruction. Luminescence was measured on an Orion II Microplate Luminometer.

Collagen I staining

A collagen gel with a concentration of 2 mg/ml was prepared. For that, 58.8 µl of collagen were mixed with 30 µl of 10× PBS, 1.4 µl of 1 N NaOH and 209.8 µl of H_2O. 2.5% of FITC labelled collagen were added to the collagen solution for visualization. 30 µl of collagen were added to each well of an 8-well ibidi slide and distributed with a pipette tip to coat the whole bottom of the well. The slide was placed in a 10 cm petri dish and a wet paper towel was added to the petri dish to avoid drying out of the collagen. The collagen was incubated at 37 °C/5% CO_2 for 30 min to allow polymerization. In the meantime, HeLa cells were prepared at a concentration of 27,000 cells/ml. 230 µl of the cell suspension were seeded to each well after the collagen had polymerized. Afterwards, 20 µl of the compound samples were added to each well. The slide was incubated at 37 °C/5% CO_2. After 24 h, cellular nuclei were stained using 2.5 mg/ml Hoechst 33,342 (37 °C/5% CO_2 for 20 min). Afterwards, the cells were washed with PBS once and 250 µl of medium was added to each well again. The cells were imaged on a Leica TCS SP8 confocal microscope. A HC PL APO CS2 63×/1.4 NA oil objective was applied. The pinhole size was set to 1.0 airy units and the pixel size was set to 2048 × 2048. Following lasers were employed: 405 nm (diode) for excitation of Hoechst 33342, 488 nm (argon) for

excitation of FITC labelled collagen. Mean intensities of collagen fibers around cells were evaluated using ImageJ (Version 1.53c) and normalized to a no cell control.

PLOD2 expression levels

Details are presented in the online Supplementary Material.

Synthesis

Details are presented in the online Supplementary Material.

Acknowledgements: The authors would like to thank Prakash Arumugam (Bioinformatics Institute, A*STAR Singapore) for providing the yeast strain ABC9Δ and for helpful discussions, Lars Allmendinger for NMR measurements, and Teresa Gruber, Eva Staschik, Maria-Teresa Lluesma, Rita Socher, Jana Peliskova, and Julia Blenninger for technical support.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: Funding from the German Research Foundation (DFG, Project-ID 448289381 to S.M.) and the Mentoring program at the Faculty of Chemistry and Pharmacy at LMU is gratefully acknowledged. P. Wang is supported by a Fellowship from the Chinese Scholarship Council.

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

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Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/hsz-2021-0355).