Humanizing yeast sterol biosynthesis to understand evolution and disease

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1. Abstract

Baker's yeast (*S. cerevisiae*) and humans (*H. sapiens*) have a relatively high level of genetic conservation in essential pathways including the proteasome, cytoskeleton, and sterol synthesis due to sharing a common ancestor approximately 1.1 billion years ago. This thesis focuses on the sterol biosynthesis pathway, which produces the related membrane lipids of ergosterol in yeast and cholesterol in humans, both of which are critical in maintaining cell membrane structure and fluidity. Yeast sterol biosynthesis genes are essential for yeast viability in standard lab growth conditions, however, previous studies in our laboratory have shown that yeast strains lacking genes for ergosterol biosynthesis can live if cholesterol is supplemented in the growth medium, suggesting these lipids may be interchangeable in yeast. To understand the extent to which yeast sterol biosynthesis can be humanized we systematically aimed to engineer this human pathway into yeast in place of its own genes. We took advantage of the CRISPR-Cas9 genetic engineering technique to systematically substitute yeast genes in the sterol pathway with their human equivalents. To date, we have humanized 80% of the yeast sterol biosynthesis pathway. Besides informing us about the evolution of core metabolic pathways across vast periods of divergence, a humanized yeast strain that contains the entire cholesterol biosynthesis pathway should serve as a remarkable new reagent for downstream medical research to help us better understand human lipid metabolic disease (including heart disease) and to perform high-throughput allelic variant and drug screens in this pathway.

2. Background

All organisms on Earth are related in some manner, diverging from a single common ancestor some billions of years ago (Darwin, 1859). Humans (*H. sapiens*) and baker's yeast (*S. cerevisiae*) are distantly related on an evolutionary time scale and last shared a common ancestor approximately 1.1 billion years ago (from *timetree.org*). It has been posited by the ortholog-function conjecture that orthologous genes across species, that is, genes that are related by vertical descent—in contrast to horizontally descended genes, or paralogs—tend to perform the same or similar function across species (Gabaldón and Koonin 2013). This was corroborated in 2015 when Kachroo, *et al*. assayed functional replacement of yeast genes by their human versions, with a success rate of 47% (Kachroo, *et al.* 2015). Moreover, this study found that there was modularity to the ability of yeast genes to be humanized: the yeast genes tended to be replaceable by their human orthologs if other genes in the same biological process were also humanizable. It was found that the sterol biosynthesis pathway, composed of 19 enzymatic steps, was nearly entirely humanizable in yeast, with all but two genes (*FDPS* and *FDFT1*) individually replaceable by their human orthologs. The current study focuses on this pathway, which ultimately produces ergosterol in yeast and cholesterol in humans, both of which are vital to life for both species.

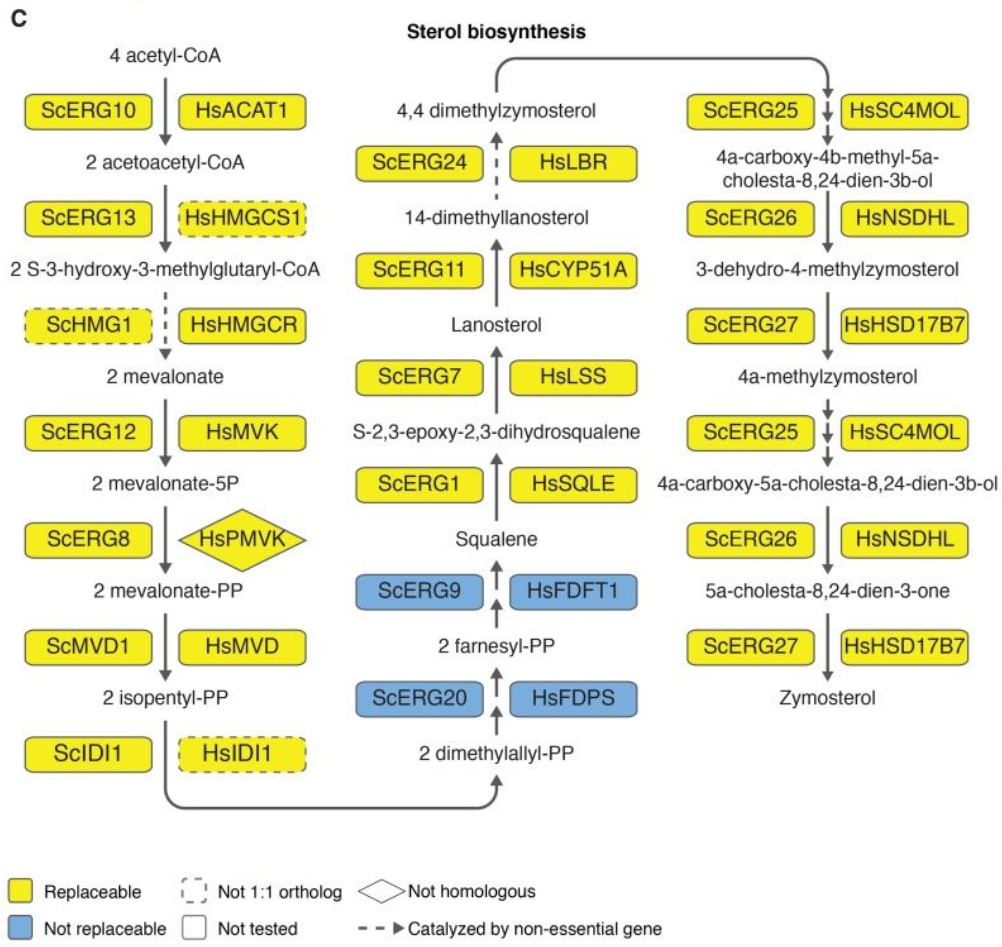

Figure 2.1: The sterol synthesis pathway which produces the common intermediate, zymosterol, in both yeast and humans. Following the production of zymosterol, four additional steps produce ergosterol from zymosterol in yeast, and cholesterol from zymosterol in humans. Assay of the sterol pathway revealed that 17 of the 19 genes were replaceable with human orthologs (from Kachroo, *et al.* 2015).

The sterol pathway can be considered an essential pathway to eukaryote existence. Ergosterol and cholesterol modulate cell membrane fluidity, with cholesterol being the principal sterol produced in higher animal membranes. Ergosterol, a similar lipid in structure (Figure 2.2) and in function, is present in the membranes of fungi as well as some protozoans and insects (Czub and Bazinski, 2006). Cholesterol is exceedingly important to animal life, being the

precursor to many other molecules—including bile salts used in digestion, Vitamin D, and the five major classes of steroid hormones: progestogens, glucocorticoids, mineralocorticoids, androgens, and estrogens (Berg, *et al.* 2002). These hormones are signal molecules which regulate a wide variety of organismal functions.

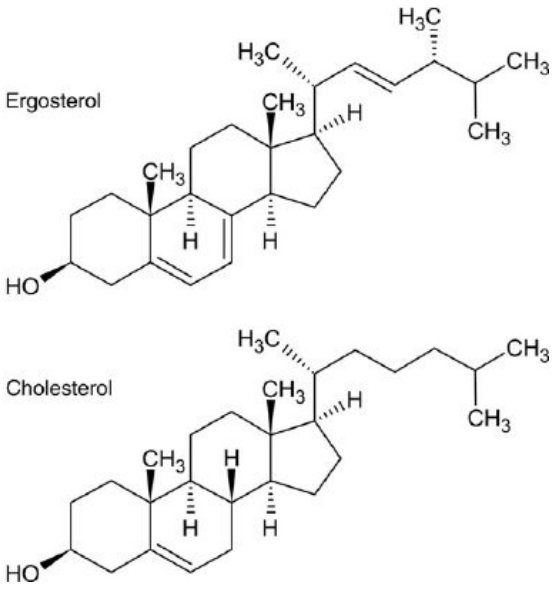

Figure 2.2: Structures of ergosterol and cholesterol, the end product of the sterol pathway in yeast and humans, respectively (from *researchgate.com*).

In humans, malfunctioning cholesterol biosynthesis or immoderate cholesterol levels are known to be causative to many different diseases resulting from hereditary effects as well as a sedentary lifestyle. These diseases include coronary heart disease, stroke, peripheral artery disease, Type 2 diabetes, and high blood pressure (from *clevelandclinic.org*). According to a new report published by the American Heart Association in 2018, high cholesterol at any age increases an individual's lifetime risk for heart disease and stroke (Grundy, *et al.* 2018), and currently, nearly one-third of all Americans have high levels of low-density lipoprotein cholesterol (LDL-C), which contributes to fatty plaque buildup in the arteries (from the

American Heart Association, *heart.org*). With heart disease being the leading cause of death in the United States (from *cdc.org*), research into this pathway should be beneficial to improve population health and individualized care. Statins, the current gold standard drug used to lower LDL-C which targets the sterol pathway gene *Hs-HMGCR*, often work well in only certain individuals, and can have deleterious side effects including muscle pain and damage, liver damage, increased blood sugar and Type 2 diabetes, as well as neurological side effects such as memory loss or confusion (from *mayoclinic.org*). The cholesterol pathway is difficult to study directly in humans, which require barriers such as IRB approval in order to undergo testing. The ability to study this pathway in a simpler, rapidly reproducing microbe would enable us to test potential diagnostics, drugs, and patient mutation effects without the extensive time and monetary costs associated with animal studies.

While ergosterol has slight differences from cholesterol in membrane effects, including altered lipid acyl chain and slightly thicker membrane bilayers (Czub and Baginski, 2006), an ongoing study in our labs have shown that ergosterol can be completely substituted by cholesterol in yeast living in laboratory growth conditions, with yeast thriving using cholesterol when yeast ergosterol genes are disabled (Kachroo, personal communication). Taking advantage of this observation in the current study, we attempted to completely humanize the sterol pathway in yeast, that is, replacing the yeast genes in this pathway with their human orthologs using the gene editing technology CRISPR-Cas9. The previous study conducted by Kachroo, *et al*. showed that most genes in the sterol pathway could be individually replaced in yeast when expressing the human gene from a plasmid and disabling the yeast gene *via* deletion or conditional alleles, but did not confirm whether this would be possible when excising the yeast protein coding sequence

and genomically integrating the protein coding sequence of the human ortholog using a genetic engineering technique such as CRISPR-Cas9 (Kachroo, *et al.* 2015). Moreover, the two genes which were previously not replaceable by plasmid, *FDPS* and *FDFT1* might be replaceable when using genomic integration. Now that it has been shown that most individual genes in sterol synthesis *can* be replaced, we wanted to take it a step further—is it possible to build the *entire* sterol pathway in yeast using human orthologs? And furthermore, if this were possible, could a yeast strain which synthesized and lived off cholesterol (rather than ergosterol) be engineered? Engineering a strain which contains part of, or the entirety of, the human cholesterol pathway would introduce a new way to conduct research to modify or regulate this human pathway in a simple yeast model by being able to more closely examine its gene, enzyme, and intermediate functionality and how they affect the ability for the organism to survive. The ability to study the human sterol pathway in yeast would also allow us to determine the how human genes work in a non-native background, and whether or not they interface with yeast sterol-relevant networks comparably to the yeast's wild-type counterparts with any phenotypic differences. Strains with different combinations of humanized loci would also enable us to study gene-gene interactions and their effects on survivability that might eventually be translated to humans. Finally, a humanized yeast model could be used to study human genetic variation in this pathway, and how they can be modified by drugs or disease.

3. Methods

3.1 *CRISPR-Cas9 Genome Editing*

3.1.1 *Preparation of Reagents*

3.1.1.1 *Preparation of Primers*

Repair template primers, CRISPR plasmids, and confirmation primers were designed according to Akhmetov, *et al*. 2018. Repair templates included the human ortholog as well as a region of yeast homology flanking the human gene in order to induce homology directed repair in the yeast. Confirmation primers for each *S. cerevisiae* ortholog included a yeast forward and yeast reverse primer; confirmation primers for each *H. sapiens* ortholog included a yeast forward and human reverse primer to ensure the human ortholog was integrated into the genome. Use of *S. cerevisiae* primers provided a negative control, and design of the *H. sapiens* primers ensured that contamination from human researcher DNA did not yield false positives.

3.1.1.3 *Preparation of Media*

Sigma Life Science YPD Broth was prepared at 50% W/W diH_2O and autoclaved for 15 min at 220°C. YPD broth was used to culture yeast cells without selection.

Synthetic Complete (SC) uracil dropout plates were prepared at W/W 0.15% YNB w/o amino acids or ammonium sulfate, 0.5% ammonium sulfate, 2% dextrose, 2% agar, 0.2% uracil dropout powder in diH_2O and autoclaved for 15 min at 220°C then poured. –ura plates were used to culture yeast colonies with selection for those containing the CRISPR plasmid with a ura marker. SC –lys–met plates were prepared using the same method, but substituting –ura dropout powder with –lys–met dropout powder.

5-Fluoroorotic Acid (5FOA) plates were prepared using W/W 0.17% YNB w/o amino acids or ammonium sulfate, 0.1% sodium glutamate, 2% glucose or dextrose, 0.8% uracil, and 2% agar in diH_2O. The solution was autoclaved for 15 min at 220°C while a solution of 100% 5-fluoroorotic acid in DMSO was prepared and syringe tip filtered. The filtered 5FOA was added to the autoclaved solution and poured. 5FOA plates were used to culture colonies with selection for those which did not contain the CRISPR plasmid in preparation for iterative CRISPR-Cas9 transformation.

3.1.1.3 *Preparation of Competent Cells*

CRISPR-Cas9 transformation reactions were performed using Zymo Research Frozen-EZ Yeast Transformation II kits. Competent cells were prepared first by culturing yeast cells overnight in YPD. 1 ml of culture was then centrifuged at 7000 rcf for 3 min, and the supernatant discarded. 1 ml of Frozen EZ Solution 1 was added to the cells and the cells resuspended, vortexing as necessary. The cells were then centrifuged at 5000 rcf for 30 s, and the supernatant discarded. The cells were suspended in 200 µl Frozen EZ Solution 2 and frozen at –80°C or used immediately.

3.1.1.4 *Preparation of Human Repair Template*

Using primers from 3.1.1.1, each human ortholog repair template was synthesized using ORFeome clones (Rual, *et al.* 2004). PCR reaction mixture was prepared using 39.5 µl sterile diH_2O, 5 µl 10X AccuPFX reaction buffer, 2 µl template primer master mix, 2 µl DNA, and 1.5 µl AccuPFX. The following protocol was used:

Step 1: 2:00 at 95°C
Step 2: 0:15 at 95°C
Step 3: 0:20 at 58°C
Step 4: X:XX at 68°C
Step 5: GO TO Step 2 9x

Step 6: 0:15 at 95°C
Step 7: 1:25 at 68°C
Step 8: GO TO Step 6 29x
Step 9: 5:00 at 68°C
Step 10: ∞ at 10°C

Step 4 heating cycle duration was determined by confirmation PCR length at 60 s/1 kb. PCR reactions were visualized using loading dye (Thermo Scientific DNA Gel Loading Dye (6X)) on an 0.8% agarose gel run at 125 V for 25 min to confirm successful synthesis.

The PCR reaction product was then purified using a Qiagen PCR Purification Kit. Step 3.1.1.4 was repeated if the amount of repair template synthesized was not sufficient to perform the CRISPR-Cas9 transformation reactions (at least 1 µg of DNA per transformation).

3.1.2 ***Genomic Integration of Human Orthologs - CRISPR Transformation***

35–50 µl of competent cells prepared in 3.1.1.3 were used per reaction based on cell O.D. Three reactions were set up per transformation in 1.5 ml Eppendorf tubes: empty vector (EV) positive control (CRISPR vector containing a –ura marker, but no sgRNA, was provided), negative control (T^-), and the CRISPR transformation reaction (T^+). The following reagents were added:

Plate	Plasmid	Template	Frozen EZ Solution 3
EV	–sgRNA CRISPR plasmid, 100–125 ng	None	500 µl
T^-	CRISPR plasmid, 500 ng	None	500 µl
T^+	CRISPR plasmid, 500 ng	Repair template, 1 µg	500 µl

Each Eppendorf was vortexed on and off for at least 1 min to ensure reagents were thoroughly mixed, then incubated at 32°C for 45–60 min. The reactions were then centrifuged at

3000 rcf for 1 min, the supernatant discarded, and the cells resuspended in 100 µl sterile diH_2O. Each reaction was then plated onto –ura plates, then incubated at 32°C until colonies had grown.

3.1.3 *Confirmation of Genomic Integration*

3.1.3.1 *Colony Genomic DNA Preparation*

Colonies from each T^+ plate were selected for confirmation of humanized loci. Each was sampled from the topmost portion of the colony to avoid contamination from dead cells on the surface of the agar and solubilized in 78% water, 20% 1 M lithium acetate, 0.01% sodium dioctyl sulfosuccinate, total volume 100 µl in an Eppendorf 2.5 ml tube. The samples were then incubated at 80°C for 10–20 min in a heat block. 300 µl of 100% ethanol was added to each sample and mixed, then incubated at –80°C for 10–20 min. The samples were spun at high speed (20,000 rcf) for 5 minutes, then the supernatant removed. Samples were vacufuged for 20 minutes at 45°C or until completely dry. The sample was then solubilized in 100 µl of sterile deionized water. Samples were spun at high speed for 30–60 s prior to use in PCR to reduce cellular debris to the bottom of the tube. Template DNA was taken only from the top portion of the sample to avoid contamination by debris.

3.1.3.2 *Q5 Hot Start PCR*

The PCR reaction mixture to confirm humanized loci was prepared using 12.5 µl Q5 Hot Start 2X Master Mix, 2 µl template from 3.1.3.1 *Colony Genomic DNA Preparation*, 3 µl 100 mM confirmation primer master mix, and 7.5 µl sterile diH_2O. The following protocol was used:

Step 1: 0:30 at 98°C
Step 2: 0:10 at 98°C
Step 3: 0:20 at 58°C
Step 4: X:XX at 72°C
Step 5: GO TO Step 2 34x
Step 6: 2:00 at 72°C
Step 7: ∞ at 10°C

The Step 4 heating cycle duration was determined by confirmation PCR length at 30 s/1 kb. PCR reactions were visualized using loading dye (Thermo Scientific™ DNA Gel Loading Dye (6X)) on an 0.8% agarose gel run at 125 V for 20 min. Below is an example of a gel indicating successful integration of the human ortholog in place of the yeast ortholog.

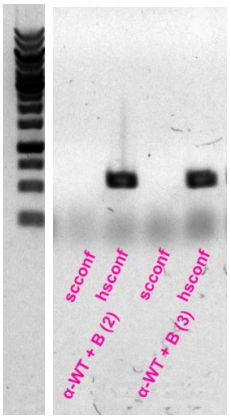

Figure 3.1.3.2: Representative gel of successful genomic replacement of the human ortholog in place of the yeast ortholog. This type of gel, which has a negative yeast PCR and positive human PCR, confirms the CRISPR-Cas9 genome editing was successful.

3.1.4 *Curing and Preparation for Iterative Humanization*

Colonies positive for locus humanization were sampled from the tip of the colony to prevent contamination from cells which did not successfully integrate the human ortholog and were cultured in 2 ml YPD overnight with shaking at 32°C, or until saturation was reached, to allow plasmid loss to occur. 5 µl of culture was streaked onto 5FOA plates and allowed to grow 2–3 days at 32°C, or until colonies were large enough to culture. This provided selection for colonies which had lost the CRISPR plasmid in preparation for iterative CRISPR-Cas9. Colonies

were cultured again in YPD and were considered "cured" of the CRISPR plasmid and ready to be humanized at other loci by repeating steps 3.1.2–3.1.4.

3.2 *Suppressor Screen to Reduce Growth Defect in Affected Populations*

3.2.1 *Passage of Cells*

In the event that a growth defect occurred with previously humanized yeast, e.g. smaller cell size, reaching saturation at lower concentration, or growing more slowly, a suppressor screen may be employed in an attempt to reduce these defects in the population.

5 µl of ancestral, or growth defective, cells were cultured in 2 ml YPD for 24 hours with shaking at 32°C. Every 24 hrs, 5 µl of cells were passaged into 2 ml of YPD until a significant increase in growth was observed. Using this method, the fastest growing cells were artificially selected.

3.2.2 *Growth Curves to Quantify Strain Growth*

Yeast strains were cultured overnight in YPD. Each strain was diluted to 0.055×10^{-7} cells in YPD in a microtiter plate, with three replicates per sample. Free cells were filled with YPD to blank the plate reader. The cells were grown for 48 hours with shaking at approximately 32°C, with OD 600 nm taken every 15 min. The data taken from the plate reader was plotted as OD 600 nm vs. time to show the cell growth over time, and could be plotted against wild-type background.

3.3 *Yeast Mating to Combine Multiple Humanized Loci*

Taking advantage of different yeast haplotypes would allow us to combine multiple humanized yeast. In this experiment, a- and α-mating types with selectable markers (–lys and –met) were used to produce multiple-humanized yeast.

3.3.1 *Preparation and Mating of Haploid Humanized Yeast*

The a- and α-mating type strains to be mated were streaked and grown on YPD plates until visible colonies had formed. a-strain yeast were streaked in parallel lines across a –lys–met plate, with enough spacing to prevent contamination from neighboring lines of yeast. α-strain yeast were streaked in parallel lines in the same manner perpendicular to the a-strain yeast to create a cross-hatch pattern. Yeast which were diploid should contain markers for both lysine and methionine, and thus should grow with selection; the converse is true for haploid yeast.

3.3.2 *Induced Homologous Recombination of Heterozygous Diploid Multiple Humanized Yeast*

Yeast which contain both the –lys and –met markers (i.e. diploid) were selected from the –lys–met plates and cultured overnight in 2 ml YPD. Using 3.1.1.3 *Preparation of Competent Cells*, competent cells were prepared for use in a CRISPR-Cas9 transformation. Step 3.1.2 *Genomic Integration of Human Orthologs - CRISPR Transformation* was performed using these competent cells, with only EV and T^- transformation reactions. Additionally, only approximately 125 ng of CRISPR plasmid was needed for the T^- reaction.

The growth on these plates was expected to differ significantly from what is usually seen with CRISPR transformation reactions attempting to genomically integrate repair template in a haploid yeast. This reaction took advantage of a gene drive, as the CRISPR plasmid provided in the T^- reaction would excise the yeast ortholog from the heterozygous locus, and the yeast would use homology directed repair to duplicate the human ortholog from the homologous chromosome in its place. Because of this, the number of colonies on the T^- and EV plates was expected to be

similar, rather than near-zero numbers on the T^- plate, as would be seen with the CRISPR-Cas9 transformation of a haploid yeast.

3.3.3 *Sporulation and Dissection of Diploid Homozygous Multiple Humanized Yeast*

Diploid humanized yeast were cultured overnight in 2 ml YPD. 1 ml culture was mixed with 1 ml sporulation media (0.1% potassium acetate and 0.005% zinc acetate in sterile diH_2O) and incubated with shaking at 25°C for 3–5 days, or until yeast were visibly sporulated under a microscope.

20–40 µl each of sporulated cells and Zymolyase were mixed in an Eppendorf and incubated at 37°C without shaking for 75–90 min. The reaction was quenched with sterile diH_2O for a final volume of 100 µl. 20 µl of sporulation mixture was plated on a YPD plate in a single line and tetrads were dissected using a tetrad dissection microscope.

4. Results

4.1 *CRISPR-Cas9 of Human Sterol Synthesis*

Using these methods, 80% of the yeast sterol pathway has been genomically replaced with human orthologs in at least one strain. Additionally, a yeast ortholog which was not previously replaceable with its human counterpart, *Hs-FDFT1*, in Kachroo, *et al*. 2015 was found to be genomically replaceable when using CRISPR-Cas9. Because of this, we can hypothesize that the other gene which was also not replaceable in the previous study might be replaceable using genomic integration. The human genes which have been genomically replaced according to the current study can be seen below in Figure 4.1.1.

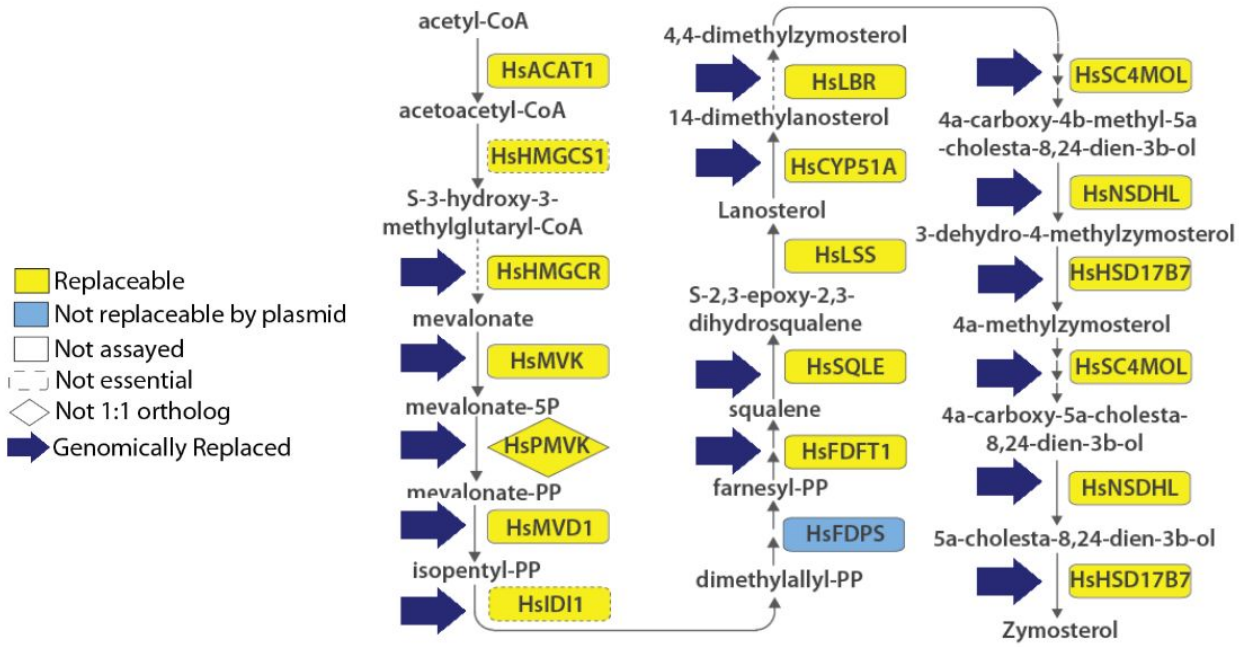

Figure 4.1.1: The humanized sterol pathway, which has had 80% of its genes genomically replaced. *Hs-FDFT1*, which was previously not replaceable, was found to be genomically replaceable when using CRISPR-Cas9 (modified from Kachroo, *et al*. 2015).

Most CRISPR transformations were attempted in both a- and α- mating types in preparation for yeast mating to combine multiple humanized loci. 63% of the sterol pathway has been replaced in a-strain yeast, and 53% has been replaced in α-strain yeast. The currently humanized strains are listed in Figure 4.1.2.

Figure 4.1.2: The set of humanized yeast strains created, by gene and mating type.

Yeast/Human Locus	a-mating type	α-mating type
scERG10/hsACAT1		
scERG13/hsHMGCS1		
scHMG1/hsHMGCR	Available	
scERG12/hsMVK	Available	
scERG8/hsPMVK	Available	
scMVD1/hsMVD		Available
scIDI1/hsIDI1	Available	Available
scERG20/hsFDPS		
scERG9/hsFDFT1		Available
scERG1/hsSQLE	Available	
scERG7/hsLSS		
scERG11/hsCYP51A		Available
scERG24/hsLBR	Available	
scERG25/hsSC4MOL	Available	Available
scERG26/hsNSDHL	Available	Available
scERG27/hsHSD17B7	Available	Available

4.2 *Iterative CRISPR-Cas9*

In addition to producing singly humanized strains, iterative CRISPR-Cas9 was used to humanize multiple sterol pathway loci in the same strain. Using this method, a strain with five humanized loci (*Hs-LBR*, *Hs-SQLE*, *Hs-IDI1*, *Hs-HMGCR*, and *Hs-PMVK*) has been confirmed *via* PCR (Figure 4.2.1), with an additional strain with three humanized loci (*Hs-SC4MOL*, *Hs-CYP51A*, and *Hs-MVD*) awaiting confirmation.

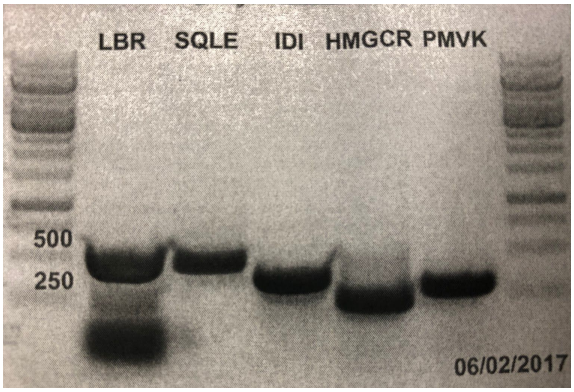

Figure 4.2.1: Gel confirmation of genomic integration of genes *Hs-LBR*, *Hs-SQLE*, *Hs-IDI1*, *Hs-HMGCR*, and *Hs-PMVK*.

4.3 *Reduction of Fitness Defects* via *Suppressor Screen*

After systematically replacing five human loci in the strain discussed in 4.2 *Iterative CRISPR-Cas9*, it was observed that the strain would repeatedly fail successive attempts at a sixth locus humanization. Growth curves plotted for this strain showed that it grows more slowly and saturates at lower cell densities. We also observed that the 5-humanized cells were generally smaller than wild type yeast cells. To combat this growth defect, we conducted a suppressor screen, which substantially rescued the growth defect, showing that the evolved strain had a growth rate more comparable to the wild-type than the ancestral strain (Figure 4.3.1).

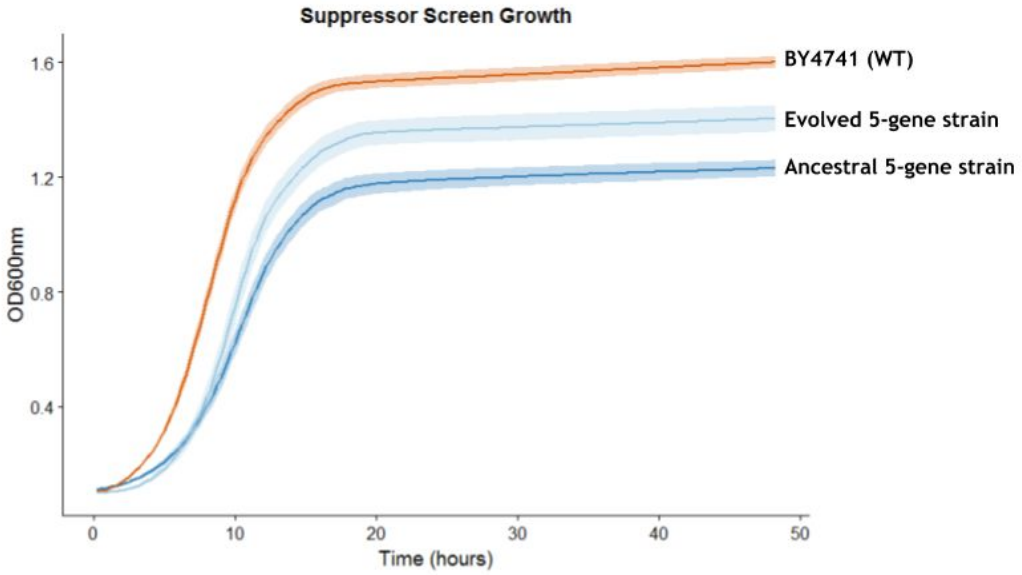

Figure 4.3.1: Use of a suppressor screen significantly rescued the growth defect for the ancestral 5-humanized gene strain.

5. Discussion

In this study, we successfully humanized 80% of the sterol pathway genes in yeast, *S. cerevisiae*, as well as produced at least one multiply humanized strain with 5 humanized loci, and significantly rescued its growth defect *via* suppressor screen. 63% of the sterol pathway has been replaced in an a-mating type background, and 53% has been replaced in an α-mating type background in preparation for yeast mating to combine multiple humanized loci into a single strain. The results of this study show promising progress towards creating a reagent for downstream research for both individualized medical care and potential drug candidate exploration.

Furthermore, a collection of paired humanizations is currently being produced *via* yeast mating which contains pairwise crosses of yeast containing only two humanized loci (e.g.

Hs-ACAT1 and *Hs-HMGCR*, *Hs-ACAT1* and *Hs-MVK*, etc.) By curating these humanized pairs, gene-gene interactions as well as their byproducts can be studied in an isolated system.

Further work is needed to completely humanize this pathway in yeast, as well as create a strain which contains all of the sterol pathway genes humanized. This will be accomplished by continuing to use the same methods being employed currently, including iterative CRISPR-Cas9 as well as yeast mating. Once these strains have been made, genomic sequencing will be used to further confirm the genomic integration of the human genes, as well as any mutations that may have occurred during these experiments or the suppressor screen. An ongoing study in the lab is working to humanize an additional four genes in this pathway following the synthesis of zymosterol in order to produce cholesterol in yeast.

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