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Coupling metabolic addiction with negative autoregulation to improve strain stability and pathway yield

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\section*{A B S T R A C T}

Metabolic addiction, an organism that is metabolically addicted with a compound to maintain its growth fitness, is an underexplored area in metabolic engineering. Microbes with heavily engineered pathways or genetic circuits tend to experience metabolic burden leading to degenerated or abortive production phenotype during long-term cultivation or scale-up. A promising solution to combat metabolic instability is to tie up the end-product with an intermediary metabolite that is essential to the growth of the producing host. Here we present a simple strategy to improve both metabolic stability and pathway yield by coupling chemical addiction with negative autoregulatory genetic circuits. Naringenin and lipids compete for the same precursor malonyl-CoA with inverted pathway yield in oleaginous yeast. Negative autoregulation of the lipogenic pathways, enabled by CRISPRi and fatty acid-inducible promoters, repartitions malonyl-CoA to favor flavonoid synthesis and increased naringenin production by 74.8%. With flavonoid-sensing transcriptional activator FdeR and yeast hybrid promoters to control leucine synthesis and cell growth fitness, this amino acid feedforward metabolic circuit confers a flavonoid addiction phenotype that selectively enrich the naringenin-producing population in the leucine auxotrophic yeast. The engineered yeast persisted 90.9% of naringenin titer up to 324 generations. Cells without flavonoid addiction regained growth fitness but lost 94.5% of the naringenin titer after cell passage beyond 300 generations. Metabolic addiction and negative autoregulation may be generalized as basic tools to eliminate metabolic heterogeneity, improve strain stability and pathway yield in long-term and large-scale bioproduction.

\section*{1. Introduction}

Metabolic heterogeneity has been found to play an essential role in determining cellular performance and pathway efficiency (Xiao et al., 2016; Ceroni et al., 2018; Rugbjerg et al., 2018). Traditional bioprocess engineering strategies, including modulating agitation, temperature, pH, dissolved oxygen (DO), dilution rate, and feeding of limiting nutrients, are largely employed nowadays to maintain metabolic homeostasis and improve the titer, yield, and productivity (TYP) of the engineered cell (Qiao et al., 2017; Xu et al., 2017). Fermentation could be viewed as an evolutionary process with productive cells constantly dividing into multiple lineages that are deviating from the parental strains (Xu, 2018). In particular, heavily engineered strains with artificial pathways or genetic circuits tend to lose the production phenotype in long-term fermentations due to metabolic burden, enzyme/cofactor imbalance, toxic chemical accumulation, and genetic instability (Wu et al., 2016). Metabolic burden drives subpopulations of cell to escape the selection pressure, regain growth fitness, propagate and dominate the producing cell line (Lv et al., 2019). Moreover, this metabolic instability is hard to comply with the high-standard of GMP guidelines in pharmaceutical and biotechnology industry (Madzak, 2015).

The decrease and even loss of production has been recently ascribed to nongenetic cell-cell variations or genetic population heterogeneity (Xiao et al., 2016; Rugbjerg et al., 2018; Rugbjerg et al., 2018). To solve this challenge, we may borrow the concept of “intelligent control” from mechanical or electrical engineering, to encode decision-making feedback functions at the population level and improve the community-level cellular performance. In such a way, the engineered cell could sense the metabolic stress and autonomously adjust cell metabolism to

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reinforce growth fitness. Indeed, dynamic feedback control has been successfully applied to various organisms to autonomously partition carbon flux and optimize cell metabolism for chemical production in recent years. The development of the metabolator (Fung et al., 2005), malonyl-CoA inverter gate (Liu et al., 2015), malonyl-CoA oscillatory switches (Xu et al., 2014; Xu et al., 2014; Johnson et al., 2017; Xu, 2019), burden-driven feedback control (Ceroni et al., 2015; Ceroni et al., 2018), have been implemented to relieve metabolic stress and boost cell's productivity in recent years. The development of the metabolator (Fung et al., 2005), a large portfolio of engineering work has been dedicated to mitigate lipogenesis or redirect lipid synthesis for heterologous expression (leucine synthesis) that is advantageous to productive cell or strain stability in long-term cultivations (Roth et al., 2009; Xu et al., 2017; Wei et al., 2019). To solve this challenge, we took advantage of the transcriptional activity of metabolite-responsive promoters (Skjoedt et al., 2016; D’Ambrosio and Jensen, 2017; Wan et al., 2019), aiming to develop an end-product addiction circuit to rewire cell metabolism in Y. lipolytica. Coupled with CRISPR-assisted ligand negative auto-regulation, the end-product (flavonoid) addiction circuit drives gene expression (leucine synthesis) that is advantageous to productive cell or deleterious to cheater cell. In such a way, we reinforced the fitness of the productive cell and enhanced the overall metabolic performance. Specifically, we have engineered a bilayer dynamic control circuit to autonomously partition metabolic flux and improve strain stability in Y. lipolytica. By coupling metabolic addiction with ligand negative autoregulation, we improved cell fitness and pathway yield, and the flavonoid-producing phenotype was sustained up to 320 generations. These results highlight the importance of applying dynamic population control and microbial cooperation to improve the community-level metabolic performance.

2. Materials and methods

2.1. Plasmids and strains

Plasmids pYLXP-Cre, pYLXP-Nluc, and pYLXP-dCas9 were frozen stocks in our lab. The dCas9 contains an SV40 nuclear localization sequence at C-terminal. Escherichia coli NEB Sa was used for plasmid construction and maintenance. Y. lipolytica Po1f-Dkm70 is a derivative of Y. lipolytica Po1f (ATCC-MYA-2613, MATA ura 3-302 leu2-270 xpr2-322 aspx2-deltaNU49 XPR2-SUC2) by removing KU70 gene. Y. lipolytica Po1f/A1R1Δ and NarPro/ASC were used as the chassis.

2.2. Molecular biology and genetic cloning

The plasmid pYLXP-URA3-loxP was constructed in the previous work (Lv et al., 2019). The site-directed integration plasmids pA1R1-loxP and pA3-core-loxP were constructed as follows. The leu2 upstream and downstream homologous arms leu2-up and leu2-down were amplified from Y. lipolytica Po1f genome DNA using primer pairs leu2-up F/leu2-up R and leu2-down F/leu2-down R, respectively. The 600-bp leu2-up fragment was assembled with AvrII digested pYLXP-URA3-loxP using Gibson Assembly, resulting in pA1R1-loxP. The 600-bp leu2-down fragment was assembled with NotI digested pA1R1-loxP using Gibson Assembly, resulting in pA3-core-loxP. The xpr2 upstream and downstream homologous arms xpr2-up and xpr2-down were amplified from Y. lipolytica Po1f genome DNA using primer pairs xpr2-up F/xpr2-up R and xpr2-down F/xpr2-down R, respectively. The 414-bp xpr2-up fragment was assembled with AvrII digested pYLXP-URA3-loxP using Gibson Assembly, resulting in pA3-core-loxP. The 552-bp xpr2-down fragment was assembled with NotI digested pA3-core-loxP using Gibson Assembly, resulting in pA3-core-loxP.

Plasmids containing fatty acid inducible promoters were constructed as follows. The 1591-bp POX2(1591) promoter was amplified from the genome DNA of Y. lipolytica Po1f using primer pair pPOX2 (1591) F/pPOX2 (1591) R. The purified PCR product was assembled with AvrII/XbaI digested pYLXP using Gibson Assembly to yield plasmid pPOX2 (1591). The 601-bp A1R1 and 438-bp A3-core promoter were amplified from Y. lipolytica Po1f genome DNA using primer pairs pPOX2 (1591) F/A1R1 R and A3 F2/pPOX2 (1591) R, respectively. The purified A1R1 and A3-core promoter fragments were assembled with AvrII/XbaI digested pYLXP using Gibson Assembly to yield plasmid pA1R1A3. The 601-bp A1R1 and 438-bp A3-core promoter were amplified from Y. lipolytica Po1f genome DNA using primer pairs pPOX2 (1591) F/A1R1 R and A3 F2/pPOX2 (1591) R, respectively. The purified A1R1 and A3-core promoter fragments were assembled with AvrII/XbaI digested pYLXP to yield plasmid (pA1R1)x2A3-Nluc. The resulting plasmids pPOX2 (1591), pA1R1A3, and p(A1R1)x2A3 will drive the transcription of gRNAs under the control of PPOX2(1591) F P(A1R1)x2A3 promoters, respectively.

To order in these inducible promoters, the luciferase encoding gene Nluc was used as reporter. Nluc was amplified from plasmid pYLXP-Nluc using primer pair Nluc F/Nluc R. The purified Nluc fragment was assembled with SnaB1 digested pPOX2 (1591), pA1R1A3, and p(A1R1)x2A3 to yield plasmid pPOX2 (1591)-Nluc, pA1R1A3-Nluc, and p(A1R1)x2A3-Nluc, respectively. The AvrII/Sall digested donor plasmids pPOX2 (1591)-Nluc, pA1R1A3-Nluc, and p(A1R1)x2A3-Nluc were ligated to SphI/Sall digested destination plasmid pA1R1A3-Nluc. The resulting plasmids were listed in Supplementary Table 2. These plasmids were used to integrate the Nluc expressing cassette to the leu2 site.

Fatty acid synthesis genes FAS1, FAS2, and FabD were used as targeting genes to auto-regulate fatty acid synthesis. To optimize the transcription efficiency, 3 gRNAs targeting different locations were designed for each gene. The gRNA sequences and targeting locations were listed in Supplementary Table 1. The 645-bp gRNA FAS1-1 was synthesized by Genewiz (Frederick, MD). Overlapping sequences were designed at 5′ and 3′ terminals for Gibson Assembly. The 5′ hammerhead ribozyme and 3′ HDV ribozyme sites flanking the gRNA were designed to generate mature gRNAs (Wong et al., 2017). gRNA FAS1-1
was assembled with XbaI/SpeI digested p (A1R1)2A3 using Gibson Assembly to yield plasmid p (A1R1)2A3-FAS1-1. The gRNA upstream xxx-up and downstream xxx-down fragments were amplified using primer pairs gRNA F/gRNA-xxx R and gRNA-xxx F/gRNA R, respectively. Fragments xxx-up and xxx-down were assembled with AvrII/Sall digested p (A1R1)2A3 using Gibson Assembly to yield plasmid p (A1R1)2A3-xxx. “xxx” here referred to gRNA. For instance, the upstream fragment FAS1-2-up and downstream fragment FAS1-2-down were amplified from plasmid p (A1R1)2A3-FAS1-1 using primer pairs gRNA F/gRNA-FAS1-2 R and gRNA-FAS1-2 F/gRNA R, respectively (primers used in this study were listed in Supplementary Table 3).

2.3. Site-directed integration

AvrII/Sall digested donor plasmid pYLXP′-dCas9 was ligated to the Nhel/Sall digested destination plasmid p (A1R1)2A3-xxx to yield p (A1R1)2A3-xxx-dCas9. The AvrII/Sall digested donor plasmid p (A1R1)2A3-xxx-dCas9 was subsequently ligated to Nhel/Sall digested destination plasmid pAvrI/2loxP to yield pAvrI/2loxP-xxx-dCas9. “xxx” here referred to gRNA(s). These plasmids were used to integrate at the leu2 site. Plasmids used in this paper were listed in Supplementary Table 2. The production addiction plasmids were constructed as follows. The hybrid promoters are composed of FdeR binding sequence FdeO and core promoters (Skojedt et al., 2016). FdeO was placed at the upstream of the TATA box of TEF(111), LEU2(78), and GAPDH(88), yielding hybrid promoters P_{TEF(111)}, P_{OLEU2(78)}, and P_{GAPDH(88)}, respectively. A 25-bp proximal motif was added between FdeO and core TEF promoter, yielding hybrid promoter P_{TEF(111)}(Hussain et al., 2016). These hybrid promoters were synthesized by Geneviz (Frederick, MD), and inserted to pYLXP′ to AvrII/XbaI site to replace the original TEF promoter, resulting in plasmids pOTEF (111), pOTEF (136), pOLEU2 (78), and pGAPDH (88). Luciferase encoding gene Nluc was amplified from pYLXP′-Nluc, and inserted into pOTEF (111), pOTEF (136), pOLEU2 (78), and pGAPDH (88) at SnaBI site using Gibson assembly, resulting in pOTEF (111)-Nluc, pOTEF (136)-Nluc, pOLEU2 (78)-Nluc, and pGAPDH (88)-Nluc, respectively. LEU2 was amplified from pYLXP′ using primer pair LEU2 F/LEU2 R, and inserted into pOTEF (111), pOTEF (136), pOLEU2 (78), and pGAPDH (88) at SnaBI site using Gibson Assembly, resulting in pOTEF (111)-LEU2, pOTEF (136)-LEU2, pOLEU2 (78)-LEU2, and pGAPDH (88)-LEU2. FdeR containing a SV40 nuclear localization sequence at C-terminal was synthesized by Geneviz (Frederick, MD), and inserted into pYLXP′, pYaliJ1, pYaliL1, pOTEF (111), pOTEF (136), pOLEU2 (78), and pGAPDH (88) at SnaBI site using Gibson assembly, resulting in pYLXP′-FdeR, pYaliJ1-FdeR, pYaliL1-FdeR, pOTEF (111)-FdeR, pOTEF (136)-FdeR, pOLEU2 (78)-FdeR, and pGAPDH (88)-FdeR, respectively (Wong et al., 2017). The recombination of these plasmids was achieved by ligating AvrII/Sall digested donor plasmid to Nhel/Sall digested destination plasmid. Plasmids used in this paper were listed in Supplementary Table 2.
combination with the CRISPRi elements to repress lipogenic pathways (Fig. 1). Previous report has validated the essential molecular components of a fatty acid inducible promoter POX2, which is composed of upstream activating sequences (A1, A2, and A3), regulatory sequences (R1 and R2), and the core promoter sequence (Fig. 2a) (Hussain et al., 2017). Both the intact POX2 promoter and its derivatives have been reported to be inducible by fatty acids (Hussain et al., 2016; Hussain et al., 2017). We tested a number of genetic configurations of the POX2 promoter to improve the dynamic output range and operational range (Fig. 2). When induced by 2% (v/v) oleic acids, the hybrid promoter (A1R1)x2A3 exhibited the highest gene expression fold change with an engineered luciferase (encoded by Nluc) as the reporter (Fig. 2b).

Considering the dynamic output and the operational range of the hybrid promoter, the hybrid promoter P(A1R1)x2A3 was chosen to drive the expression of guide RNAs (gRNAs) that were specifically designed to target the lipogenic pathways. The 5′ hammer-head ribozyme (5′ HRR) and 3′ HDV ribozyme sites flanking the gRNAs were used to generate mature gRNA (Wong et al., 2017). Due to plasmid instability in Y. lipolytica (encoded by Fas1p and Fas2p (encoded by FAS1 and FAS2) as acetyl-CoA synthetase) and malonyl-CoAs into long-chain saturated fatty acids (Schweizer et al., 1986). The Y. lipolytica homologue of malonyl-CoA-ACP [acyl-carrier protein] transacylase (encoded by FabD, GRYC ID: YAL0E18590g) was also reported to play a major role in fatty acid synthesis (Schneider et al., 1997).

For these reasons, FAS1, FAS2, and FabD were selected as endogenous CRISPRi targets to repress fatty acid synthesis. In order to achieve gradient repression levels, we tested 3 gRNAs targeting different loci for each gene (Supplementary Table 1). The hybrid promoter P(A1R1)x2A3 was used to drive the transcription of gRNAs. The mature gRNAs, which were used to guide dCas9 to the target locations, were produced by endogenous processing of the hammer-head ribozyme and 3′ HDV ribozyme sites after transcription (Supplementary 3a–c).

CRISPRi experiments showed that different gRNAs resulted in differential transcriptional repression for each gene (Fig. 3d–f). The gRNAs FAS1-2, FAS2-1, and FabD-1 showed the highest transcriptional repression efficiency, resulting in relative transcription levels of 0.55, 0.54, and 0.47, respectively, as quantified by qRT-PCR. Consistent with these transcriptional data, the gRNAs FAS1-2, FAS2-1, and FabD-1 guided dCas9 decreased fatty acid content by 24.5%, 17.2%, and 12.3% respectively (Fig. 3d–f). gRNAs targeting FabD was less efficient in decreasing fatty acid accumulation, possibly due to the fact that FabD

3.2. CRISPR interference to inhibit fatty acid synthesis

Yeast uses polysaccharide fatty acid synthetases Fas1p and Fas2p (encoded by FAS1 and FAS2) as acetyl-CoA synthetase and malonyl-CoAs into long-chain saturated fatty acids (Schweizer et al., 1986). The Y. lipolytica homologue of malonyl-CoA-ACP [acyl-carrier protein] transacylase (encoded by FabD, GRYC ID: YAL0E18590g) was also reported to play a major role in fatty acid synthesis (Schneider et al., 1997).
activity was compensated by the pentafunctional Fas1p (Schweizer et al., 1986; Kottig et al., 1991). Both gRNAs FAS1-2 and FAS2-1 were found to increase naringenin titer by 29.9%, while the gRNA FabD-1 did not have obvious effect on naringenin titer (Fig. 3g–i). These results indicated that naringenin production was improved by dCas9 interfering with fatty acid synthesis when the gRNAs were controlled by the fatty acid inducible promoter.

3.3. Fatty acid synthesis negative autoregulation by multiplexed gRNAs-CRISPRi tuning

To further divert lipogenic flux toward the flavonoid pathway, we sought to use gRNAs in combination to target FAS1, FAS2, and FabD and test whether the multiplexed gRNAs could improve naringenin production (Fig. 4a). The results showed that combinatorially repressing FAS1-2 and FAS2-1 decreased fatty acid dramatically comparing with repressing single gene, while the duplex gRNAs targeting FAS1-FabD or FAS2-FabD did not result in significant fatty acid reduction comparing with solo repression of FAS1 or FAS2 (Fig. 4b). Duplex gRNAs targeting FAS1-FAS2 increased naringenin titer by 52.8%, and triplex gRNAs targeting FAS1-FAS2-FabD increased naringenin titer by 56.5% (Fig. 4b). We further confirmed that naringenin titer was negatively correlated with fatty acid content ($R^2 = 0.98$) (Fig. 4c). These results indicated that naringenin production was improved with the multiplexed gRNAs targeting the fatty acid pathway. Specifically, the strain NarPro/ASC_Rep produced 111.4 mg/L naringenin (Fig. 4b), when triplex gRNAs (FAS1-2-FAS2-1-FabD-1) were used to repress the fatty acid pathway.
We further analyzed the time course of fatty acid and naringenin production using 250-ml shaking flask in fed-batch fermentation. Interestingly, fatty acid accumulation was reduced further in NarPro/ASC_Rep than the control strain. At the end of the fermentation, fatty acid in NarPro/ASC_Rep decreased by 37.9% (Fig. 4d). Naringenin production rate was increased substantially in the NarPro/ASC_Rep strain. At the end of the fermentation, NarPro/ASC_Rep produced 158.0 mg/L naringenin, which was 74.8% higher than that of the control (Fig. 4d). The inverse correlation between naringenin and fatty acid confirmed that the triplex FAS1-2-FAS2-1-FabD-1 gRNAs effectively guided dCas9 to divert the lipogenic carbons toward the naringenin pathway. In summary, the fatty acid-driven negative auto-regulation inverter gate provided a promising solution to mitigate precursor flux competition from lipogenesis in Y. lipolytica.

3.4. Construction of naringenin inducible promoters

Despite the improvement of naringenin production by mitigating fatty acid synthesis, we constantly observed that the engineered yeast gradually lost the production phenotype after several generations of cultivation. To solve this problem, we attempted to engineer an end-product (naringenin) addiction circuit, which will link cell growth fitness with naringenin production. This competitive growth advantage will encourage the proliferation of the high naringenin-producing populations, while suppress or eliminate the growth of the low naringenin-producing populations during long-term cultivation. To link cell fitness with the end-product, we sought to place an essential gene under the control of naringenin inducible circuit. We firstly ruled out the antibiotic resistant genes, because the antimicrobial property of both antibiotics and naringenin may make the addiction circuit difficult to validate. Because the LEU2 gene is a ready to use (the chassis is leucine-auxotrophic) and reliable selective marker, we firstly tried this non-conditionally essential gene. Cultivated with the leucine drop-out synthetic medium, genetic variants with high naringenin titer will produce more leucine and outcompete the growth of the low naringenin-producing strain. As a result, the high naringenin-producing strains will gradually dominate the cell populations and low naringenin-producing cells will be suppressed (Layer II in Fig. 1). To build the naringenin-inducible genetic circuits, we firstly sought to use the well-characterized transcriptional activator FdeR and its cognate DNA binding site Fdeo (Skjoedt et al., 2016) to control the expression of a reporter gene. A panel of synthetic promoters \( P_{O-TEF(111)}, P_{O-TEF(136)}, P_{O-LEU2(78)}, \) and \( P_{O-GAPDH(88)} \) were constructed by fusing Fdeo with the core promoters to drive the expression of the \( Nluc \) luciferase (Fig. 5a). The nuclear localization signal SV40 was fused at the C-terminal of Fdeo to facilitate nuclear transportation. With naringenin as the effector

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**Fig. 4.** Combinatory repression analysis and time course of fatty acid and naringenin production. **a** Mechanism of auto-regulating fatty acid synthesis by combinatory repressing FAS1, FAS2, and/or FabD. **b** Effects of combinatory repressing FAS1, FAS2, and/or FabD on fatty acid accumulation and naringenin titer. **c** Analysis of the tradeoff between fatty acid and naringenin synthesis. The red line refers to the linear fit to the mean values. **d** Time course of fatty acid accumulation and naringenin production. Strain NarPro/ASC, which is identical to NarPro/ASC_Rep but without the negative auto-regulation circuit, was used as control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
molecule, FdeR will bind to FdeO site and activate the transcription of the reporter gene (Fig. 5b) (Siedler et al., 2014). Experimental results demonstrated that all our constructed promoters were inducible by naringenin with an operational range from 0 mg/L to 50 mg/L (Fig. 5c). The induction reached saturation when naringenin concentration was beyond 100 mg/L. These naringenin-inducible promoters will be used to control the expression of essential gene LEU2 that dictates cell fitness.

3.5. Linking flavonoid production with cell growth fitness

One important strategy in metabolic engineering is to engineer competitive growth advantage that links cell growth with product formation. To enable naringenin inducible growth, we placed the leucine biosynthesis gene LEU2 under the control of these naringenin-inducible promoters (Supplementary Figs. 2a–d). Upon transformation into the leu2-deficient Y. lipolytica Pol1f chassis, we obtained comparable number of colonies on the plates with naringenin (CSM-Leu + Naringenin) or without naringenin (CSM-Leu) (Supplementary Fig. 2e–d), indicating that cell growth is independent of naringenin. We speculate this might be arising from the basal activity of FdeR, which was excessively expressed from the strong and constitutive TEF promoter. To overcome this limitation, we next sought to express FdeR with weak promoters POX4 and IDP2 (Wong et al., 2017). Indeed, we observed naringenin-inducible growth: FdeR expression from the weak promoters (P POX4 and P IDP2) effectively activated the leucine circuits (P TEF136-LEU2) and supported a distinguishable growth phenotype in the presence of naringenin (Fig. 6d, e, g, and h). However, when leucine circuits P TEF111-LEU2, P LEU278-LEU2, and P GAPDH88-LEU2 were tested, none of them could grow on CSM-Leu or CSM-Leu + naringenin plates, indicating an intricate interaction between FdeR and these synthetic promoters. Interestingly, FdeR expression from the naringenin-inducible promoter (P TEF136) also conferred naringenin-inducible growth (Fig. 6f and i), this possibly created a positive feedback loop that may amplify the sensor input-output relationship. We next confirmed the naringenin-inducible growth in liquid media. Indeed, Y. lipolytica strains carrying the naringenin inducible circuits grew faster in CSM-Leu + naringenin medium than those in CSM-Leu medium (Fig. 6j and l), indicating the naringenin inducible circuits conferred a competitive growth fitness for the engineered cell, albeit flavonoids have been reported as antimicrobial agents in plant signaling. These results highlighted the importance of adjusting promoter strength to achieve the desirable end-product addiction phenotype. For example, excess expression of FdeR instead led to naringenin-independent expression of LEU2, causing indistinguishable cell fitness. While too less FdeR is not sufficient to activate the LEU2 circuits and support cell growth. As for the LEU2 expression, it is important to select the naringenin-inducible promoter with broad operational range as well as minimal leaky expression.

To link cell fitness with naringenin production, we next transformed the naringenin-inducible genetic circuit into the naringenin producing strain NarPro/ASC_Rep with fatty acid negative autoregulation. The fatty acid negative autoregulatory circuits were integrated at the leu2 genomic loci. To validate naringenin-inducible growth, we observed that strains carrying the naringenin-addicting circuits grew normally on leucine drop-out plates (Supplementary Figs. 3e–h). However, the strains grew poorly or did not grow at all when the transcriptional activator FdeR was removed from the system (Supplementary Fig. 3i), indicating the tight control of the LEU2-expressing fitness circuits by FdeR. In the liquid media, strains carrying the addiction circuits enabled the cell reach 3–4 fold higher cell density (OD600) with 5 mg/L naringenin than the same strain without naringenin (Fig. 6 j, k, l).

3.6. Stabilizing naringenin-production phenotype by integrating metabolic addiction with negative autoregulatory circuits

We next coupled the metabolic addiction with negative autoregulation, and validated the long-term robustness of the engineered strains with a month-long fermentation experiment. Three naringenin addiction circuits (P TEF136-LEU2-P POX4-FdeR, P TEF136-LEU2-P IDP2-FdeR, and P TEF136-LEU2-P POX4-FdeR) were introduced to the naringenin producing strain with fatty acid negative autoregulation (NarPro/ASC_Rep chassis), which drives leucine synthesis in a naringenin-dependent manner. By incorporating the naringenin-addiction circuits, we locked the naringenin production phenotype with cell growth fitness. The NarPro/ASC_Rep chassis with blank pYLPX’ plasmid was used as control. These engineered strains were undergone serial passage in paralleled shaking flasks experiments (Rugbjerg et al., 2018). Cells harvested from 0 to 720 h (1 month, about 360 generations) were individually stocked and used as seed culture to inoculate the leucine drop-out plates (Supplementary Figs. 3e–h). For the inoculum at 648 h (which is about 324 generations), all the strains grew poorly or did not grow at all when the transcriptional activator FdeR was removed from the system (Supplementary Fig. 3i), indicating the tight control of the LEU2-expressing fitness circuits by FdeR. In the liquid media, strains carrying the addiction circuits enabled the cell reach 3–4 fold higher cell density (OD600) with 5 mg/L naringenin than the same strain without naringenin (Fig. 7 a, b, 7c and 7d), albeit the control strain (without naringenin-addiction circuit) regained growth fitness and reached relatively higher cell density (Fig. 7a). For example, the control strain and all the addiction strains produced similar amount of naringenin (80–90 mg/L) for the inoculum aging from 0 to 180 generations (2 h per generation, which is about 360 h or 15 days). After 200 generations, naringenin production declined rapidly in the control strain, compared with the strains with addiction circuits (Fig. 7b, c and 7d). For the inoculum at 648 h (which is about 324 generations), all the three strains carrying the addiction circuits maintained 90.9% of

Fig. 5. Naringenin inducible promoter analysis. a Structures of the hybrid promoters. The hybrid promoters O TEF (111), O TEF136, O LEU278, and O GAPDH88 were composed of FdeR binding site FdeO and the core promoters of TEF(111), TEF(136), LEU2(78), and GAPDH(88) respectively. b Mechanism of the naringenin inducible transcription. P hybrid refers to the hybrid promoters of P TEF111, P TEF136, P LEU278, and P GAPDH88-Nluc was used as the reporter gene. c The result of induction of the hybrid promoters. The luminescence was recoded using Cytofit 3 microtiter plate reader. The RLU was calculated by integrating the read-out data by time.
naringenin production compared with the inoculum at 0 h. On the contrary, the control strain (without the naringenin addiction circuits) almost lost all the naringenin, only produced less than 5.5% of naringenin compared with the fresh inoculum (0 h). By linking leucine synthesis with flavonoid production, our coupling strategies effectively force the cell to maintain a selective growth advantage to enrich the flavonoid-producing populations, but suppress the mutant cells that produce less naringenin. These results demonstrated that the end-product addiction circuits are invaluable tools to maintain community-level strain stability, which is a critical index to stabilize recombinant metabolite production and prevent genetic degeneration during bioprocess scale-up and long-term fermentation.

Nonetheless, the overall naringenin production is decreased after we combine the fatty acid negative autoregulatory circuit and the flavonoid addiction circuit (Fig. 7a and b). The decrease in naringenin production is possibly due to the metabolic overloading or burden effect (Wu et al., 2016) due to the expression of multiple transcriptional regulators (CRISPRi, FdeR and Por1p, and three gRNAs). From an evolutionary perspective, there is a tradeoff between metabolic stability and pathway yield. Metabolites (i.e. leucine or naringenin in this study) cross-feeding could largely elicit metabolic heterogeneity (Evans et al., 2020), for example, leucine secreted from the high naringenin-producing strain may be assimilated by the low naringenin-producing strain, therefore causing a faulted cross-talk between the sub-populations of the engineered cell. To restrict leucine cross-feeding, we may need to further delete the gene encoding leucine permease (Bap 2 homologs) to sequestrate leucine and reinforce the naringenin production phenotype. Single cell imaging and genetic analysis will be critical to help us understand the source of this metabolic heterogeneity and propose novel genetic or process engineering solutions to inhibit such metabolic heterogeneity (Hartline et al., 2020).

4. Conclusions

Metabolic heterogeneity has become a major issue that compromises the cellular performance and pathway yield. To overcome this limitation, it is important to rewire cellular logics to maintain metabolic homeostasis and improve the community-level cell performance. Social reward-punishment rules to incentivize the production cell and punish the nonproduction cell may be applied to combat this fitness loss. Such rules could be biologically implemented by conferring a selective growth advantage to the production cell, in such a way, the population of the production cell will be enriched and therefore the production phenotype might be sustained. Most of the reported strategies have focused on nongenetic cell-to-cell variations to confer competitive fitness. Genetic underpinnings that are associated with metabolic heterogeneity remain a challenging area. Single-cell analysis and microfluidic-integrated optogenetic tools might be a promising area to solve these conundrums. In this work, we have engineered lipogenic negative autoregulation with metabolite addiction to redistribute carbon flux and improve strain stability in Y. lipolytica. The flavonoid-producing phenotype was sustained for up to 320 generations. These results highlight the importance of applying dynamic population control and microbial cooperation to improve the community-level metabolic performance, which might be useful to combat metabolic heterogeneity and stabilize overproduction phenotype for long-term cultivation in industrial biotechnology settings.

Author contributions

PX and YL conceived the topic and designed the study. YL performed genetic engineering and fermentation experiments with output from YG. YL and PX wrote the manuscript. YL is a joint scientist supervised by JWZ, JLX and PX.

Declaration of competing interest

A provisional patent has been filed based on the results of this study.

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Appendix A. Supplementary data

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