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4 **Genome-wide targets identification by**
 5 **CRISPRi-Omics for high-titer production of free fatty**
 6 **acids in *Escherichia coli***

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18 **Abstract**

19 To construct a superior microbial cell factory for chemical synthesis, a major
 20 challenge is to fully exploit cell potential via identifying and engineering beneficial
 21 gene targets in the sophisticated metabolic networks. Here, we develop an approach
 22 that integrates CRISPR interference (CRISPRi) to readily modulate genes expression
 23 and omics analyses to identify potential targets in multiple cellular processes,
 24 enabling systematical discovery of beneficial chromosomal gene targets that can be
 25 engineered to optimize free fatty acids (FFAs) production in *Escherichia coli*. We
 26 identify 56 beneficial genes via synergistic CRISPRi-Omics strategy, including 46
 27 novel targets functioning in cell structure and division, and signaling transduction that
 28 efficiently facilitate FFAs production. Upon repressing *ihfA* and overexpressing *aidB*
 29 and *tesA*' in *E. coli*, the recombinant strain LihfA-OaidB results in a FFAs titer of
 30 21.6 g L⁻¹ in fed-batch fermentation, which, to our best knowledge, is the maximum
 31 FFAs titer by the recombinant *E. coli* reported to date.

32 **Introduction**

33 Microbial biosynthesis of a desired product is a sophisticated process, usually
 34 involving the coordination of multiple metabolic intermediates or reactions subject to
 35 complicated interactions^{1,2}. On the one hand, each of these intermediates flows to
 36 other metabolic processes for biosynthesis of cellular components or metabolites,
 37 acting as one of the multi-branched nodes in the topology of metabolic landscapes³.
 38 On the other hand, each reaction in the biosynthetic pathway deeply rely on many
 39 cellular processes, such as genes transcription and translation to enable enzymes
 40 function⁴⁻⁶, or cell proliferation to ensure biomass accumulation⁷. As such,
 41 manipulation of other metabolic pathways or cellular processes might lead to an
 42 unexpectedly improvement on the biosynthesis of a desired product due to distant
 43 effects of genetic modulations or unknown regulatory interactions^{8,9}. Thus, to
 44 construct a superior microbial cell factory for an enhanced product biosynthesis, it is
 45 highly desirable to genome-widely identify beneficial gene targets in the highly
 46 complicated intracellular interaction networks that can be reengineered for this
 47 purpose. As such, the interwoven linkage mechanism between product biosynthesis
 48 and other sophisticated genetic and metabolic networks can be better understood.

49 Free fatty acids (FFAs) are feedstocks for the manufacture of detergents, lubricants,
 50 cosmetics, and pharmaceutical ingredients^{10,11}. A number of metabolic engineering
 51 approaches were developed to facilitate FFAs production in *E. coli*. Overexpression of
 52 key enzymes in the FFAs biosynthetic pathway such as acetyl-coenzyme A (CoA)

53 carboxylase¹²⁻¹⁴ and fatty acyl-ACP thioesterase^{12,13,15-17}, or deletion of key genes in
 54 the beta-oxidation pathway such as *fadD*^{12,13,15-17} or *fadE*¹⁵ gene, were studied. In
 55 particular, optimally combinatorial expression of 15 crucial genes in the FFAs
 56 biosynthetic pathway with *fadD* deletion in *E. coli* BL21 resulted in a titer of 8.6 g L⁻¹
 57 of FFAs¹⁸. However, the cell potential for FFAs overproduction is still not fully
 58 unleashed via modulating the FFAs directly related metabolic pathways alone¹⁹.
 59 Besides, the cellular rewiring mechanism and the linkage between FFAs biosynthesis
 60 and other cellular processes is still largely unknown, and it remains difficult in
 61 systematically identifying beneficial gene targets using laborious and time-consuming
 62 genome engineering methods, such as homologous recombination-based gene
 63 knockout²⁰. These hurdles severely restrict the speed and scope of engineering of
 64 microbial hosts to obtain a superior overproducer.

65 Here, we employ a systems metabolic engineering strategy for rapid, systematical
 66 and effective identification of chromosomal gene targets that can be engineered for
 67 the enhanced production of FFAs in *E. coli*. This strategy makes use of both the
 68 CRISPR interference (CRISPRi) that readily down-regulates genes expression in a
 69 dose-dependent manner to rapidly identify beneficial knockdown targets, and the
 70 omics analyses of different strains that allows acquisition of comprehensive data
 71 related to mechanisms of cellular metabolism to dig potential targets in the genetic
 72 and metabolic networks. By way of this synergistic CRISPRi–Omics strategy, we
 73 identified 46 new beneficial gene targets that efficiently facilitate FFAs production,

74 which involves in cell division, signaling transduction and transport, cell structure,
 75 and other seemingly irrelevant cellular processes with FFAs biosynthesis. Upon
 76 repressing *ihfA* and overexpressing *aidB* and *tesA*', the resulting recombinant *E. coli*
 77 strain LihfA-OaidB enabled a FFAs titer of 21.6 g L⁻¹ (with productivity of 0.636 g
 78 L⁻¹ h⁻¹) in fed-batch fermentation, which, to the best of our knowledge, is the
 79 maximally reported FFAs titer and productivity by the recombinant *E. coli* to date.
 80 Our findings not only allow for enhanced biosynthesis of the desired product, but also
 81 lead to a mechanistic understanding of the underlying linkage between product
 82 biosynthesis and multiple cellular processes.

83

84 **Results**

85 **Construction of a non-toxic and functional CRISPRi system in *E. coli***
 86 **BL21(DE3).** CRISPRi system functioned through the expression of dCas9 and
 87 customized specific sgRNA²¹ (**Supplementary Fig. 1a**). However, high-level dCas9
 88 could cause a severe inhibition of cell growth and abnormal expression of genes in a
 89 strain-specific manner^{22,23}. To determine a superior dCas9 expression level in
 90 BL21(DE3), we constructed plasmids with dCas9 being expressed under the control
 91 of the promoter P_{T7}, P_{T5}, P_{Trc} and P_{BAD}, respectively (**Supplementary Fig. 2a**). The
 92 expression strengths of these promoters were P_{T7} > P_{T5} > P_{Trc} > P_{BAD}, according to our
 93 previous study²⁴. With green fluorescent protein (GFP) as a characterization signal
 94 driven by the P_{T7} promoter, fluorescence-based reporter plasmids were further

constructed (**Supplementary Fig. 2a**) and transformed into BL21(DE3), forming five strains G0 (P_{T7}-GFP), G1 (P_{T7}-GFP-P_{T7}-dCas9), G2 (P_{T7}-GFP-P_{T5}-dCas9), G3 (P_{T7}-GFP-P_{Tre}-dCas9), and G4 (P_{T7}-GFP-P_{BAD}-dCas9), respectively. We found that the OD₆₀₀ and fluorescence level of the strains G1 and G2 were greatly decreased from the strain G0, while that of G3 and G4 were little affected compared with G0 (**Supplementary Fig. 1b**). This suggested that the expression of dCas9 at relatively low level, such as under the control of the P_{Tre} or P_{BAD} promoter, did not cause overt toxicity in our system.

Then, we examined the transcriptional repression capability of CRISPRi on gene expression by co-expressing a specific sgRNA. sgRNA-*gfp* was designed to target on the non-template strand of GFP at a position ~38 nucleotides downstream of the start codon to expect a high repression efficiency²⁵. The plasmid Sg-Hgfp (**Supplementary Fig. 2b**), harboring sgRNA-*gfp* under the control of the P_R promoter, was constructed and transformed into the strains G0-G4, forming the strains g0-g4, respectively. The results showed that fluorescence levels of the strains co-expressing dCas9 and sgRNA-*gfp* (g1-g4) were all dramatically reduced in comparison to the corresponding strain expressing only dCas9 (G1-G4) (**Supplementary Fig. 1b**). It indicated that the CRISPRi system was functional for effective transcriptional repression. In addition, the strain with dCas9 regulated by the P_{Tre} promoter obtained the highest repression efficiency (93%) (**Supplementary Fig. 1b**). Thus, we selected

115 dCas9 controlled by the P_{Trc} promoter to construct the CRISPRi system in
116 BL21(DE3).

117

118 **Identification of beneficial gene targets in the pathways related to FFAs**

119 **metabolism using CRISPRi system.** Glycerol, serving as the substrate, is

120 catabolized into key intermediates glyceraldehyde-3P, pyruvate, acetyl-CoA,

121 malonyl-ACP in sequence and then converted into acyl-ACP through the fatty acid

122 biosynthetic pathway (**Fig. 1a**). However, these key intermediates also involved in

123 other metabolic pathways, resulting in diverted carbon fluxes away from FFAs. The

124 expression of truncated fatty acyl-ACP thioesterase TesA' (with leader sequence

125 deleted) could release free fatty acid intermediates from ACP in the cytosol^{26,27}.

126 Nevertheless, the synthetic FFAs are degraded naturally by the beta-oxidation process

127 (**Fig. 1a**). To channel carbon flux towards FFAs and impede products' degradation,

128 we chose 108 genes in the chromosome that related with the FFAs metabolism and

129 regulation as candidate targets for modulation by CRISPRi, and classified them into

130 five modules (upstream carbon flux diversion, downstream carbon flux diversion,

131 amino acid metabolism, beta-oxidation, and transcription factors) (**Fig. 1a**).

132 To facilitate large-scale target identification, we constructed a library of 108

133 synthetic sgRNAs that repress the expression of the 108 genes in the above five

134 modules with high silencing efficiency²¹. We introduced plasmids harboring each of

135 the 108 synthetic sgRNAs individually into the starting strain CF co-expressing TesA'

136 and dCas9 in BL21(ED3) to implement gene perturbations (**Fig. 1b**). The strain CF
137 transformed with plasmid Sg-0 that expressed sgRNA0 without target site
138 complementary sequence was named as the strain Control, which produced FFAs
139 with a titer of 631 mg L⁻¹. Any CRISPRi-engineered strain that could increase FFAs
140 titer by more than 20% compared with the strain Control was sorted out. As a result,
141 30 beneficial targets were identified from the library and the highest production of
142 FFAs reached 1,232 mg L⁻¹, 95% higher than that of the strain Control (**Fig. 2**).

143 The identified beneficial genes were almost evenly distributed in the modules of
144 upstream carbon flux diversion (**Fig. 2a**), downstream carbon flux diversion (**Fig. 2b**),
145 beta-oxidation (**Fig. 2d**), and transcription factors (**Fig. 2e**). It suggested that reducing
146 the carbon flux that flows to the by-products phosphatidylglycerol (*gpsA*, *plsY*, *pgpB*
147 and *pgpC*), D-Lactate (*dld*), acetate (*pta*, *yccX* and *eutE*) and biotin (*bioC*, *bioH* and
148 *bioD*), or impeding fatty acids degradation (*fadE*, *fadB*, *atoB* and *yqeF*) contributed to
149 the enhanced production of FFAs. Reducing the expression of a few transcription
150 factors (including *fabR*, *fadR*, *asnC*, *fnr*, *cra*, *gadW*, *glcC*, *glpR*, *ihfA*, *ihfB* and *torR*)
151 also enhanced the production of FFAs (**Fig. 2e**), which might benefit from their global
152 regulation of genes expression. However, reducing amino acids biosynthetic pathways
153 was unfavorable for FFAs production, as the engineered strains obtained decreased
154 titer of FFAs or failed to be constructed (**Fig. 2c**). The possible reason was that these
155 amino acids were necessary for cell viability, but the reduced amount of amino acids
156 upon CRISPRi perturbation was insufficient to maintain normal cell physiology. Thus,

157 in addition to the key enzymes in the FFAs biosynthetic pathway, there exist many
158 chromosomal genes seemingly unrelated to FFAs that could be beneficial to the
159 enhanced FFAs production.

160 Then, we tuned repression efficiency towards each of the 30 beneficial genes to
161 optimize their individual expression level. sgRNAs were designed to bind the
162 non-template DNA strand at the middle or terminal region to achieve medium or low
163 repression efficiency towards each target²¹ (**Fig. 3a**). The plasmids harboring each of
164 the 60 synthetic sgRNAs were thus individually introduced into the starting strain CF,
165 and the production titer of FFAs was determined, showing that 10 strains (LgpsA,
166 LpfkA, MyccX, LgabD, MgaA, MatoB, LatoB, LglpR, MihfA and LihfA) enabled
167 further improvement in FFAs production (**Fig. 3b**).

168 Among the 30 beneficial gene targets, 20 of them (*gpsA*, *plsY*, *pgpB*, *pgpC*, *dld*,
169 *yccX*, *bioC*, *bioH*, *bioD*, *gabD*, *gadA*, *asnC*, *fnr*, *cra*, *gadW*, *glcC*, *glpR*, *ihfA*, *ihfB*,
170 *torR*) were firstly engineered for FFAs production. We found three favorable
171 metabolic pathways that have not been studied or modulated for FFAs production
172 before, namely the biotin, phosphatidylglycerol, and glutamate biosynthetic pathways.
173 Even in pathways that have been modulated in previous studies, we also identified
174 novel beneficial targets, such as *dld* in the lactate biosynthetic pathway, *yccX* and
175 *eutE* in the acetate formation pathway. In particular, we found a few genes such as *pta*
176 in the acetate formation pathway that have been deleted in previous studies²⁸, which
177 however showed no promotional effect on FFAs production, while CRISPRi

178 perturbation of the *pta* expression in this study significantly enhanced FFAs
179 production. Collectively, these results demonstrate that CRISPRi is of use for
180 systematical identification of beneficial targets that can be engineered for improved
181 biosynthesis of a desired product.

182

183 **Identification of potential targets in the cellular network via proteome and**
184 **transcriptome analyses.** To explore more potential targets that could facilitate FFAs
185 production, we further focused on transcription factors in consideration of their
186 complicated regulation functions on metabolic networks. Among all the targeted
187 transcription factors, repression of *ihfA* with low efficiency (named as the strain
188 LihfA) achieved a highest FFAs production, followed by the strain HfadR that
189 repressed *fadR* with high efficiency (**Figs. 2e and 3b**). IhfA is the alpha subunit of the
190 integration host factor (IHF) in *E. coli*, which is capable of affecting transcription on a
191 genome-wide scale²⁹. We found that down-regulation of *ihfA* could enhance FFAs
192 production, and the FFAs titer was negatively correlated with the repression
193 efficiency (**Fig. 4a**). Unlike IhfA, FadR controls the expression of several genes
194 directly involved in fatty acid biosynthesis, degradation, and transport across cell
195 membranes³⁰. It is intriguing that up-regulation of FadR (named as the strain OfadR)
196 could also increase the FFAs titer as demonstrated in a previous study³¹ and this study
197 (**Fig. 4a**).

Even though the strains with FFAs overproducing phenotype have different genotypes, these strains might have convergent regulatory responses that facilitate FFAs biosynthesis. Thus, the three above engineered strains (LihfA, HfadR and OfadR, with knockdown of *ihfA*, *fadR* and overexpression of *fadR*, respectively) with high-titer FFAs and the strain Control were applied for proteome and transcriptome analyses to investigate the underlying mechanism of cellular responses to genetic modulations. Cells were sampled at the early stationary phase (24 h upon IPTG induction) (**Supplementary Fig. 3**). Hundreds of genes and proteins were differentially expressed in each pair of LihfA vs. Control, HfadR vs. Control, and OfadR vs. Control (**Supplementary Figs. 4 and 5**), suggesting the global modulation of genes in the engineered strains. To identify convergent rewiring in these high-titer strains, we plotted the differentially expressed genes (DEGs) of the three pairs in a Venn diagram (**Fig. 4b**). As a result, 24 down-regulated targets and 17 up-regulated targets were selected based on their significantly differential expression at the protein level (a and c, respectively) or at the transcript level (b and d, respectively) in at least two pairs (**Fig. 4b**) (see details in Supplementary Materials). The differential expression levels of these 41 genes are summarized in **Fig. 4c**. These potentially favorable gene targets are newly identified and seemingly irrelevant to FFAs biosynthesis in a direct fashion.

To test the function of these 41 candidate targets on FFAs production, the corresponding reverse engineering of each gene was conducted. We applied CRISPRi

219 to repress the expression of the 24 ‘down-regulated’ genes or utilized the P_{BAD}
 220 promoter to overexpress the 17 ‘up-regulated’ genes (**Fig. 5a**). The plasmids carrying
 221 the cassettes towards each candidate gene were transformed into the starting strain CF.
 222 A FFAs assay of the resulting strains showed that modulation of the 19 genes among
 223 the identified targets enhanced FFAs production by more than 20% in comparison to
 224 the strain Control (**Fig. 5b**). Considering that the selected genes were all differentially
 225 expressed in the strain pair of LihfA vs. Control (**Fig. 4c**), these targets were also
 226 manipulated in the strain LihfA to test their effects on FFAs production (**Fig. 5c**). As
 227 a result, 13 engineered strains gained further increase in FFAs production compared
 228 with the strain LihfA (**Fig. 5d**). In particular, the highest FFAs production was
 229 obtained in the strain LihfA-OaidB, in which the *ihfA* gene was repressed with low
 230 silencing efficiency and the *aidB* gene was overexpressed under the P_{BAD} promoter.
 231 The titer is up to 2,052 mg L⁻¹, which is 57% and 225% higher than that of the strains
 232 LihfA and Control, respectively (**Fig. 5d**). In summary, 26 (*norR*, *nrdE*, *npr*, *yihU*,
 233 *glpG*, *ftsQ*, *creC*, *sdhB*, *pal*, *rplW*, *tyrU*, *crsC*, *rnpB*, *ssrS*, *csrB*, *ryfA*, *sdsR*, *folk*, *aidB*,
 234 *yphF*, *deoD*, *nnr*, *rihC*, *hofP*, ECD_01466, and *ulaE*) out of the 41 newly identified
 235 genes are determined as the beneficial targets, which are validated to enhance the
 236 FFAs production by reverse engineering.

237

238 **Exploration of cellular processes facilitating FFAs production.** The newly
 239 identified beneficial targets are not in the metabolic pathways that directly related to

240 FFAs biosynthesis. To mine out the related regulatory mechanisms for FFAs
241 production, the functions of the 26 beneficial genes were analyzed according to
242 bioinformatics analysis of the database (NCBI, KEGG, UniProt and Gene Ontology)
243 and summarized into eight sets (**Fig. 6, Supplementary Table 1**). Based on the FFAs
244 titer of the corresponding engineered strains, the weight value (W value) of each set
245 was calculated to characterize their effects on the enhancement of FFAs production.
246 The eight sets were arranged in descending order of W value as follows: cell division,
247 transduction and transport, energy metabolism, cellular structure, non-coding RNAs
248 (ncRNAs), protein metabolism, nucleic acid metabolism, and cofactor metabolism
249 (**Fig. 6, Supplementary Table 1**). Surprisingly, the processes such as cell division,
250 signal transduction, protein metabolism and nucleic acid metabolism seemingly not
251 associated with FFAs biosynthesis in any previous studies that showed favorable
252 effect on FFAs production. These results indicate that the manipulation of cellular
253 processes that are seemingly irrelevant with FFAs biosynthesis can also remarkably
254 boost FFAs production.

255 Then, we further dissect the underlying connections between these enriched cellular
256 processes and FFAs production. Down-regulation of *ftsQ* or *pal* significantly
257 increased the FFAs concentration, inactivation of which resulted in deficiency in cell
258 division and formed long multi-septated cell chains^{32,33}, which might associated with
259 the increased cell volumes for storing FFAs. Down-regulation of *creC*, a sensor for
260 the early response to phosphate starvation³⁴, was favorable for enhanced FFAs

261 production, which is consistent with the promotive effects of phosphate limitation
 262 cultivations³⁵. The functioning of *npr* down-regulation and *yphF* up-regulation may
 263 be associated with the reduction of nitrogen assimilation³⁶ and the elevation of
 264 carbohydrates' uptake³⁷, indicating a regulatory link between nitrogen and carbon
 265 utilization for the efficient FFAs production. Down-regulation of ATP-consuming
 266 proteins (such as those encoded by the genes *norR*, *nrdE*, *creC* and *rplW*) indirectly
 267 increased ATP availability, a potential bottleneck for FFAs biosynthesis¹⁹. Beneficial
 268 effects from down-regulation of membrane proteins (such as those encoded by the
 269 genes *glpG*, *ftsQ*, *creC* and *pal*) might associated with microbial tolerance to FFAs³⁸.
 270 The functioning of down-regulation of *rplW* (encoding 50S ribosomal subunit protein
 271 L23), *tyrU* (encoding tRNA-Tyr) and *rnpB* (encoding M1 RNA, catalytic component
 272 of tRNA processing enzyme RNase P) was associated with the inhibition of protein
 273 synthesis, which increased partitioning of total carbon to lipids⁸. Up-regulation of *nnr*,
 274 facilitating the repair of NAD(P)H hydrates to NAD(P)H³⁹, could be an important
 275 complement strategy in enhancing NADPH availability, which plays an important
 276 role in FFAs production^{19,40}. In all, these findings provide new insights into linkages
 277 between cell functions and product biosynthesis, guiding avenues for further strain
 278 development.

279 As a result, the engineered *E. coli* strain LihfA-OaidB with simultaneous repression
 280 of *ihfA* and overexpression of *aidB* and *tesA* achieved the highest titer of FFAs
 281 production. According to the results of combined omics analyses and reverse

282 engineering, we concluded that down-regulation of *ihfA* induced comprehensive
283 adjustments of cellular processes that promoted the FFAs production. Nevertheless,
284 repression of *ihfA* might be unfavorable for cellular survival at acidic pH as IHF is
285 required for the induction of acid resistance^{41,42}. AidB, one of the DNA damage
286 repairing protein in *E. coli*, is involved in adaptive response of cells, serving to protect
287 cells from cytotoxic effects of acidified cytoplasm or alkylating agents⁴³⁻⁴⁵. Thus, we
288 speculated that up-regulation of *aidB* in the strain LihfA reconciled the stress from the
289 acidic environment posed by the produced FFAs, facilitating FFAs biosynthesis in the
290 strain LihfA-OaidB.

291

292 **Further enhancing FFAs production by fed-batch fermentation.** To further
293 enhance FFAs production of the recombinant strain LihfA-OaidB in a scaled-up
294 process, fed-batch fermentations were performed in a 5 L fermenter, using the
295 dO₂-stat feeding strategy (details described in Methods). With arabinose and IPTG
296 induction, *aidB* (DNA alkylation damage repair protein) was overexpressed by the
297 P_{BAD} promoter, *tesA'* (truncated acyl-CoA thioesterase) was overexpressed by the P_{T7}
298 promoter and *ihfA* (integration host factor subunit alpha) was repressed by CRISPRi
299 with low silencing efficiency in the strain LihfA-OaidB. The FFAs production and
300 cell density of the recombinant strain LihfA-OaidB increased along with the glycerol
301 consumption (**Fig. 7a**). When cells were cultivated for 34 h after IPTG induction,
302 LihfA-OaidB produced a titer of 21.6 g L⁻¹ FFAs, accompanied by 0.636 g L⁻¹ h⁻¹

productivity and 0.146 g FFAs per gram glycerol yield, the highest production titer and productivity in *E. coli* reported to date^{18,46}. Extracellularly secreted FFAs were clearly visible at the top of the culture medium after centrifugation (**Fig. 7b**). Moreover, floating dead cells or fatty acid particles⁴⁷ were precipitated and stuck on the fermenter inner wall and sensors over the course of the fermentation process (**Supplementary Fig. 6**), exhibiting an additional production of FFAs that is however hard to accurately quantify and not counted in the FFAs titer. Thus, the overall actual FFAs production was higher than that showed in **Fig. 7a**.

311

312 **Discussion**

Genome-scale identification of beneficial gene targets in the chromosome for improving production of a desired product is challenging due to the complicated intracellular interactions. We here developed a systems metabolic engineering approach, which took the advantage of CRISPRi to readily and rapidly down-regulate gene expression and the omics analyses to provide potential targets in the cellular interaction network, thus enabling rapid, systematical and effective identification of beneficial targets that can be engineered to optimize FFAs production in *E. coli*. This synergistic strategy integrated the ‘forward’ and ‘reverse’ metabolic engineering cycles and their interaction⁴⁸ (**Supplementary Fig. 7**). In forward metabolic engineering, CRISPRi-mediated gene down-regulation functions were pursued in this work for the identification of beneficial chromosomal targets in the competitive or

324 regulatory pathways of FFAs biosynthesis that can be repressed in high, intermediate
325 and low levels, unlike gene knockout, to enhance FFAs production. In reverse
326 metabolic engineering, proteome and transcriptome analyses of the strains with
327 differential FFAs production provide comprehensive data related to mechanisms of
328 cellular metabolism, which can be validated experimentally via CRISPRi system and
329 plasmid mediated expression system. In doing so, we identified beneficial targets that
330 even seemingly irrelevant with FFAs biosynthesis, which are generally inaccessible to
331 hypothesis-driven or random gene targeting, aiding in expanding hosts' potential of
332 enhanced products synthesis. To our knowledge, this is the first report that
333 systematically identifies beneficial target genes in the cellular interaction network for
334 enhanced FFAs biosynthesis.

335 Many new approaches, including in vitro reconstitution of enzymes in the FFAs
336 biosynthetic pathway^{49,50}, engineering the reversal of the beta-oxidation pathway⁵¹,
337 and construction of malonyl-CoA sensors^{52,53}, have been studied to enhance FFAs
338 production in *E. coli*. However, these approaches essentially focused on the
339 optimization of FFAs metabolic pathways alone, not exploiting the full potential of
340 interwoven intracellular processes. In our study, gene perturbations on the cellular
341 interaction network, including the competitive pathways of FFAs biosynthesis,
342 transcription factors and cellular processes that seemingly irrelevant with FFAs
343 metabolism, were systematically investigated to unleash the cellular potential and
344 change the cells towards a state of FFAs overproduction. As a result, the best

345 engineered strain LihfA-OaidB produced FFAs with a titer of 21.6 g L⁻¹ (0.636 g L⁻¹
 346 h⁻¹ productivity) under fed-batch conditions, which, to the best of our knowledge, is
 347 the maximally reported FFAs titer and productivity by recombinant *E. coli* to date.
 348 Furthermore, the potential of the identified beneficial targets and our constructed
 349 strains for improving FFAs production could be further integrated and amplified by
 350 combinatorial genetic perturbations⁵⁴ and exploiting nongenetic variation⁴⁶.

351 Beneficial targets that were identified by our synergistic CRISPRi-Omics strategy
 352 participate in diverse cellular processes, which support the idea that product synthesis
 353 is a complex process and that global optimization of cellular machinery is required for
 354 optimized product synthesis. Generally, these targets primarily associate with
 355 metabolic processes, other cellular processes or regulatory elements. As to metabolic
 356 processes, we identified novel beneficial targets in the common targeted pathways,
 357 such as *yccX* and *eutE* in acetate formation pathway, ATP-consuming protein *nrdE*
 358 and NAD(P)H-hydrate repair enzyme *nnr*, as well as novel pathways that have not
 359 been engineered for FFAs production such as the biotin biosynthetic pathway (*bioC*,
 360 *bioH* and *bioD*). Our target screening approach also emphasizes the importance of
 361 other cellular processes or functions in the strain improvement for optimized FFAs
 362 production, such as cell division (*ftsQ* and *pal*) related morphology, signaling
 363 transduction (*creC*) and transport (*npr* and *yphF*) related resources utilization, cellular
 364 structure (*glpG*, *ftsQ* and *pal*) related cellular tolerance, protein metabolism (*rplW*,
 365 *tyrU*, *mnpB*) related resources partition, and nucleic acid metabolism (*aidB*) related

366 adaptive response. In addition, we also identified beneficial regulatory elements that
 367 have not been engineered for FFAs production, such as the integration host factor *ihfA*,
 368 or novel ncRNAs *ryfA* and *sdsR*, which modulate FFAs production through regulation
 369 of multiple cellular processes. Thus, our findings provide insights into connections
 370 between FFAs biosynthesis and other cellular processes and expand the underlying
 371 mechanistic understanding of the regulation of FFAs production in *E. coli*.

372 Strains obtained through the identification and modulation of genes have proven to
 373 be a versatile and robust approach for improved FFAs production, which also offers
 374 avenues for engineering more potential targets for further improvement. Knowledge
 375 acquired in this work toward understanding the FFAs biosynthetic machinery can be
 376 extended to other microorganisms for FFAs production, such as yeast and oleaginous
 377 bacteria. The approach for identifying promising modulation candidates is also
 378 applicable to other products without obvious screening phenotypes.

379 In summary, the synergistic CRISPRi-Omics strategy enabling rapid, systematical
 380 and effective identification of beneficial targets in the intracellular interaction
 381 network provides novel insights and solutions for further increasing productivity of
 382 microbial cell factories and better understanding of the link between product
 383 biosynthesis and the other processes of cellular machinery.

384

385 **Methods**

386 **Experimental materials.** Strains and plasmids used in this study are listed in
 387 **Supplementary Table 2** and **Supplementary Table 3**, respectively. *E. coli*
 388 *Trans1-T1* strain was used for cloning. *E. coli* BL21(DE3) and its derived strains were
 389 used for fermentation. Plasmids pACYCDuet-1, YX210, YX212 and YX213 were
 390 used as the vector for expression of dCas9, GFP or TesA'. Plasmid Sg-S (with two
 391 BsaI restriction sites) was constructed and used as a platform vector for expression of
 392 various sgRNAs. Plasmid Sg-BAD was used as the vector for expression of
 393 chromosome genes. Plasmids, except that for sgRNAs expression, were constructed
 394 by standard enzyme digestion and ligation. Target site complementary sequences of
 395 sgRNAs were designed according to previous studies⁵⁵. Custom-designed spacers
 396 were inserted into plasmid Sg-S by one-step Golden Gate assembly^{56,57}, allowing
 397 rapid construction of plasmids for expression sgRNAs targeting any genomic locus of
 398 interest. Primers annealed to custom-designed spacers and primers used to amplify
 399 genes from *E. coli* genomic DNA were listed in **Supplementary Tables 4-7** and
 400 **Supplementary Table 8**, respectively. Codon-optimized genes were listed in
 401 **Supplementary Table 9**.

402

403 **Culturing conditions and mediums.** To assess FFAs production by different
 404 recombinant *E. coli* strains, fermentation was performed in glass tubes. Each strain
 405 was inoculated from a freshly transformed single colony in LB agar plate to 2 mL LB
 406 medium as seed culture. When cell accumulation reached to stationary phase, 1%

407 (V/V) of seed culture was re-inoculated to 5 mL modified M9 medium in glass tube.

408 The tube cultures then were induced with 1 mM IPTG when OD₆₀₀ reached ~1.0 and

409 allowed to grow for an additional 40 h at 30°C and 220 rpm. When the P_{BAD} promoter

410 was applied, 10 mM arabinose was utilized to induce gene expression at OD₆₀₀ 0.5–

411 0.6. All tube experiments were performed at least in triplicate. Flask fermentation of

412 50 mL culture broth was implemented for proteome and transcriptome analyses with

413 the identical culturing procedures of tube fermentation. M9 medium used for tube and

414 flask fermentation was as described by Schirmer et al.⁵⁸ with some modifications:

415 17.1 g L⁻¹ Na₂HPO₄·12H₂O, 3 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ NaCl, 2 g L⁻¹ NH₄Cl, 2 g L⁻¹

416 yeast extract, 3% glycerol, 0.25 g L⁻¹ MgSO₄·7H₂O, 11.1 mg L⁻¹ CaCl₂, 10 mg L⁻¹

417 thiamine and 0.1% (v/v) Triton-X100. 1 mL L⁻¹ metal trace stock solution was also

418 supplemented, which contained 27 g L⁻¹ FeCl₃·6H₂O, 2 g L⁻¹ ZnCl₂, 2 g L⁻¹

419 Na₂MoO₄·2H₂O, 1.9 g L⁻¹ CuSO₄·5H₂O, 0.5 g L⁻¹ H₃BO₃. pH was adjusted to about

420 7.2 by Tris. If necessary, 100 µg mL⁻¹ carbenicillin or 34 µg mL⁻¹ chloramphenicol

421 was supplemented.

422 For fed-batch fermentation, an overnight LB culture (2 mL) of the freshly

423 transformed single colony was sub-cultured into 200 mL modified mineral medium (6

424 g L⁻¹ NH₄Cl, 8.5 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ citrate, 5 g L⁻¹ yeast extract, 15 g L⁻¹

425 glycerol, 1 g L⁻¹ MgSO₄·7H₂O, 0.07 g L⁻¹ CaCl₂·2H₂O, 4 mL L⁻¹ metal trace stock

426 solution, 100 mg L⁻¹ thiamine)⁵⁹ with 100 µg mL⁻¹ carbenicillin and 34 µg mL⁻¹

427 chloramphenicol. When OD₆₀₀ reached to 3-4, the cultured cells were re-inoculated

428 into a 5 L bioreactor (T&J, China) with 1.8 L modified mineral medium as above.
 429 Fermentation temperature was set at 30°C and pH was controlled at 7 by feeding 6 N
 430 ammonium hydroxide via an auto pump. Air flow rate was maintained at around 2 L
 431 min⁻¹. The dissolved oxygen (dO₂) concentration was controlled above 30% by
 432 agitation cascade (300–800 r.p.m). When cell density reached about 6 and 15, 10 mM
 433 arabinose and 1 mM IPTG was added into the fermentation cell culture, respectively.
 434 Feeding medium (2.47 g L⁻¹ MgSO₄·7H₂O, 500 g L⁻¹ glycerol and 100 g L⁻¹ yeast
 435 extract) was fed to the fermentation culture after the initial 15 g L⁻¹ glycerol was
 436 almost depleted and the dissolved oxygen showed a sharp increase. The carbon source
 437 restriction strategy was carried out by dO₂ concentration, given that the dO₂ level
 438 immediately spiked once the carbon source was run out. Once the dO₂ > 60%, the
 439 feeding would automatically start at 5 mL min⁻¹ until dO₂ < 60%. Antifoam (Sigma)
 440 was added automatically as needed. Broth samples (~5 ml) were collected at a series
 441 of time points to measure cell density and stored at -20°C for further measurements
 442 of residual glycerol and FFAs production.

443
 444 **GFP Fluorescence assay.** Fluorescence signal of GFP was used to characterize
 445 repression efficiency of the CRISPRi system. Culturing procedures and induction
 446 process of the GFP reporting strains were the same as tube fermentation of the FFAs
 447 producing strains. For each tube, 200 µL of sample was diluted into the linear range
 448 of the detector with phosphate buffered saline (PBS) after IPTG induction for 20 h.

449 Fluorescence intensity (excitation at 485 nm and emission at 520 nm) and cell density
 450 (OD_{600}) were detected using 96-well polystyrene plates (black plate with a clear
 451 bottom) (Corning Incorporated 3603, USA) and a microplate reader (SpectraMax M2,
 452 Molecular Devices, USA). The relative fluorescence intensity was first normalized
 453 using OD_{600} and then subtracted that of blank *E. coli* BL21 (DE3). Experiments were
 454 performed in triplicate.

455

456 **Metabolite extraction and analysis.** FFAs titers in whole-cell culture (only FFAs
 457 was measured in this study) were quantified following previously published
 458 methods⁶⁰. Specifically, 0.5 ml of cell culture (or an appropriate volume of cell
 459 culture diluted to 0.5 ml) was acidified with 50 μ L of concentrated HCl, spiked with
 460 60 μ g of heptadecanoic acid as internal standard. The cell culture was extracted twice
 461 with 0.5 ml of ethyl acetate. The extracted FFAs were then determined using a
 462 Thermo Scientific TRACE 1300 gas chromatograph (GC) equipped with a
 463 TG-WaxMS A column (30 m $\times$ 0.32 mm $\times$ 0.25 μ m; Thermo Scientific) and a Flame
 464 Ionization Detector (FID) operating under constant flow rate of the carrier gas
 465 (nitrogen) at 1 mL min⁻¹. The following temperature program was used: hold at 50°C
 466 for 1 min, then heat to 245°C at 30°C min⁻¹ and hold at this temperature for 22.5 min.
 467 Individual fatty acid species were qualified by authentic homologous standards and
 468 quantified by comparing the peak areas with that of the internal standard using the

469 Chromeleon 7.1 software. Total concentrations of free fatty acids were calculated as
470 the sum of C₁₂ to C₁₈ (saturated and monounsaturated).

471 Glycerol concentration was determined by high-performance liquid
472 chromatography (HPLC) following Waters standard protocols. Briefly, filtered
473 culture supernatants were analyzed by a Waters HPLC system including a Waters
474 e2695 separation module, a Waters 2414 refractive index detector (RID) and an
475 Aminex HPX-87H column (Bio-Rad). The separation was performed through elution
476 with 5 mM H₂SO₄ at a flow rate of 0.6 mL min⁻¹ at 65°C for 30 min.

477

478 **Proteome analysis.** Cells were harvested by centrifugation at 5000 rpm for 5 min at
479 24 h after IPTG induction and flash frozen with liquid nitrogen. For protein
480 preparation, cells were resuspended in 600 µL lysis buffer (0.05 M Tris, pH 8, 1%
481 SDS, and 8 M Urea) and disrupted by ultrasonication for 5 min (cycles with 2s work
482 and 3s pause). After centrifugation at 13000 rpm and 4°C for 20 min, the supernatant
483 was subjected to protein precipitation by adding pre-cooled acetone with 10 mM DTT.
484 The total precipitate was collected, dried and then re-dissolved in 600 µL Urea buffer
485 (0.05 M Tris, pH 8, and 8 M Urea). The protein samples were quantitated by the
486 Bradford method and qualitated by SDS-PAGE. A total of 100 µg of each sample was
487 trypsin-digested overnight and labeled using 4-plex iTRAQ reagents (AB SCIEX) as
488 manual described. The tryptic peptides corresponding to each sample were labeled

489 with its unique iTRAQ tags and proteomics analysis was conducted using a Triple
490 TOF 6600 system (AB SCIEX). The data were analyzed by Novogene.

491

492 **Transcriptome analysis.** Cells were harvested after 24 h of IPTG induction by quick
493 centrifugation at 10000 g for 1 min and immediately frozen in liquid nitrogen. Total
494 RNA was extracted using the RNeasy pure Cell/Bacteria Kit (Qiagen) following
495 lysozyme treatment. RNA degradation and contamination were monitored on 1 %
496 agarose gels. RNA concentration was measured by Qubit[®] RNA Assay Kit in Qubit[®]
497 2.0 Fluorometer (Life Technologies, CA, USA) and RNA integrity was assessed on a
498 2100 Bioanalyzer (Agilent Technologies, CA, USA). rRNA is removed using a
499 Ribo-zero kit that leaves the mRNA. A total of 3 µg RNA was used as an input per
500 sample. Sequencing libraries were generated using a NEBNext[®] Ultra™ RNA
501 Library Prep Kit for Illumina[®] (NEB, USA) following the manufacturer's instructions,
502 and index codes were added to attribute sequences to each sample. Clustering of the
503 index-coded samples was performed on a cBot Cluster Generation System using the
504 TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's
505 instructions. After cluster generation, the library preparations were sequenced on an
506 Illumina HiSeq 4000 platform and paired-end reads were generated. After these steps,
507 the data were analyzed by Novogene.

508

509 **Data availability**

510 A reporting summary for this article is available as a Supplementary Information file.

511 The data that support the findings of this study are available from the corresponding

512 author upon request. The source data underlying Figs. 2a-e, 3b, 4a-c, 5b, 5d, 6, and 7a,

513 Supplementary Figs. 1b, 3, 4, and 5 and Supplementary Table 1 are provided as a

514 Source Data file.

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672

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Author contributions

L.F., Y.C. and H.S. conceived and designed the experiments. L.F., J.F., and C.W.
performed the plasmids construction experiments. L.F. and J.F. performed the
fed-batch fermentation experiments. L.F. and Y.C. analyzed the data. L.F. and Y.C.
and H.S. wrote the manuscript. H.S. supervised all aspects of the study.

Additional information

Competing interests: The authors declare no competing financial interests.

Figures

Fig. 1 The CRISPRi approach for identification of beneficial knockdown gene targets from cellular pathways associated with the FFAs metabolism. **a** Schematic of the metabolic or regulatory pathways related to FFAs biosynthesis in *E. coli*, which were classified into five modules (upstream carbon flux diversion, downstream carbon flux diversion, amino acid metabolism, beta-oxidation and transcription factors) and represented in blue, orange, purple, green and golden background, respectively. Genes in the above five modules are shown in italics and marked with blue, orange, purple, green and golden dot, respectively. 2854 represents gene numbered ECD_02854 in BL21(DE3). The fatty acid biosynthetic pathway is shown in red line and key metabolites glycerone-P, glyceraldehyde-3P, glycerate-3P, pyruvate, acetyl-CoA and malonyl-ACP are represented as number 1 to 6, respectively. **b** Plasmid constructs for the gene expression of *tesA*, *dCas9* and synthetic sgRNAs.

Fig. 2 Effect of genetic perturbation by CRISPRi on FFAs production. FFAs production through repression of each gene in upstream carbon flux diversion module (a), downstream carbon flux diversion module (b), amino acid metabolism module (c), beta-oxidation module (d), and transcription factors module (e) by the CRISPRi system. All target genes were repressed with high efficiency. * represents the strains with more than 20% increase in FFAs production than that of the strain Control (631 mg L⁻¹). Error bars indicate the standard deviations of three replicates. Source data are provided as a Source Data file

Fig. 3 Effect of tuning repression efficiency towards target genes on FFAs production.

a Design of sgRNA with high, medium or low repression efficiency towards target gene. **b** FFAs production under medium or low repression efficiency compared with that of the high repression efficiency. The repressed genes were screened out from those genes marked with star in Fig. 2. Error bars indicate the standard deviations of three replicates. Source data are provided as a Source Data file

718

719 **Fig. 4** Exploration of potential target genes by proteome and transcriptome analyses.

720 **a** Effect of different *ihfA* or *fadR* expression levels on FFAs production. * represents

721 the strains with high production of FFAs, and these strains were applied to the next

722 omics analyses. **b** A Venn diagram of differentially expressed genes (DEGs).

723 Common DEGs in the high-titer strains at the protein level (a, c) or at the transcript

724 level (b, d) were identified, compared with the strain Control. Green: down-regulated;

725 red: up-regulated. **c** Expression levels of the selected common DEGs in the strains

726 LihfA (1), HfadR (2), and OfadR (3) compared with the strain Control. 1466

727 represents the gene numbered ECD_01466 in BL21(DE3). Error bars indicate the
728 standard deviations of three replicates. Source data are provided as a Source Data file

Fig. 5 Reverse engineering of the selected DEGs in the strains CF and LihfA.

Schematic of cassettes used to repress or overexpress the selected DEGs in the strain

CF (a), or in the strain LihfA (c). The repression was achieved by CRISPRi and the

overexpression was enabled by the P_{BAD} promoter. $TesA'$, dCas9 and sgRNAs were

controlled by the P_{T7} , P_{TTC} and P_R promoter, respectively. y: down-regulated gene; z:

up-regulated gene. Effect of genetic perturbation of the selected DEGs on FFAs

production in the strain CF (b) or in the strain LihfA (d). * represents the strain with

FFAs titer increased >20% compared with the reference strain in each bar chart.

Green frame or column: repression; red frame or column: overexpression. Error bars

indicate the standard deviations of three replicates. Source data are provided as a

Source Data file

741

742 **Fig. 6** Function network plot of the beneficial genes derived from omics analyses. The

743 size of the node represents the number of genes within the function set. The node's

744 color represents W value of the function set. The thickness of the edge indicates the

745 number of genes overlapping two function sets. W value is calculated by averaging

746 the FFAs ratio of Hx (Ox) / Control or LiHx (LiOx) / LihfA, x referring to each gene

747 belongs to the function set. Genes and the FFAs ratio are shown in Supplementary

748 Table 1. Source data are provided as a Source Data file

749

750 **Fig. 7** Fed-batch production of FFAs in a 5 L bioreactor. **a** Growth, glucose
 751 consumption and FFAs production of the recombinant *E. coli* strain LihfA-OaidB
 752 during fed-batch fermentation. **b** Solid FFAs layer after centrifugation. After
 753 fermentation, 1 ml of sample was picked up and centrifuged at 5000 g for 10 min.
 754 Error bars indicate the standard deviations of three replicates. Source data are
 755 provided as a Source Data file