

1 A yeast-based tool for mammalian DGATs inhibitors screening

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14

15 Abstract

16

17 Dysregulation of lipid metabolism is associated with obesity, metabolic diseases but there is also
18 increasing evidences of a relationship between lipid bodies (LBs) excess and some cancer. LBs synthesis
19 requires diacylglycerol acyltransferases (DGATs) which catalyses the last step of triacylglycerol (TAG)
20 synthesis, the main storage lipid form in lipid bodies. DGATs and in particular DGAT2, are therefore
21 considered as potential therapeutic targets for the control of these pathologies. Here, the murine and
22 the human DGAT2 were overexpressed in the oleaginous yeast *Yarrowia lipolytica* deleted for all
23 DGATs activities, for evaluating the functionality of the enzymes in this heterologous host and to
24 evaluate DGAT activity inhibitors. This work provide evidence that mammalian DGATs expressed in *Y.*
25 *lipolytica* is a useful tool for screening chemical libraries to identify potential inhibitors or activators of
26 these enzymes of therapeutic interest.

27

28 Introduction

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30 The diacylglycerol acyltransferase (DGAT) enzymes catalyse the final committed step of the
31 triacylglycerol (TAG) biosynthesis by esterification of a fatty acyl moiety to a diacylglycerol. These
32 neutral lipids are stored in organelles called lipid bodies (LBs) in mammalian adipocytes tissues but also
33 in most eukaryotic cells and in some prokaryotes, as energy molecules or membrane synthesis
34 synthesis reservoir. In eukaryotes, TAGs are mainly synthesized by DGAT1 and DGAT2, belonging to
35 two different gene families. DGAT1 and DGAT2 have different roles in TAG synthesis in humans. DGAT1
36 is highly expressed in the small intestine and has a role in fat absorption while DGAT2 is expressed in
37 liver and adipose tissues and is responsible for endogenous synthesis of TAG (Cases et al. 1998; Cases
38 et al. 2001). *dgat1* knockout mice are viable with minor impact on TAG levels and are resistant to diet-
39 induced obesity (Smith et al. 2000). In contrast, *dgat2* knockout mice present severe TAG decrease and
40 die shortly after birth (Stone et al. 2004). TAGs excess in tissues is a hallmark of obesity. Therefore,
41 DGATs are considered as potential therapeutic inhibition targets for the control of obesity, but also for
42 some diseases related to lipid absorption in the intestine. Moreover, recent studies revealed that high
43 levels of LBs are also associated with breast cancer (Nisticò et al. 2021) as well as with higher tumor
44 aggressiveness and chemotherapy resistance (Tirinato et al. 2017). Interestingly DGAT2 is
45 constitutively activated in various cancers including breast cancer (Hernández-Corbacho and Obeid
46 2019). Accordingly, Nistico et al. observed that the combined effect of a DGAT2 inhibitor pre-treatment
47 (PF-06424439) and radiation enhanced radiosensitivity of MCF7 breast cancer cells (Nisticò et al. 2021).
48 In addition, importance of DGAT2-mediated regulation of TAG metabolism in triple negative breast
49 cancer has been recently highlighted (Almanza et al. 2022). Therefore, DGAT2 appears as a new
50 potential therapeutic target in the treatment of breast cancer (Hernández-Corbacho and Obeid 2019).
51 Because of the lethality of *dgat2* knockout mice model, specific inhibitors that tightly controlled
52 inhibition of DGAT2 are required. Compound libraries targeting obesity as well as cancer should be
53 evaluated in a system that could mimic human and mouse DGAT2 structure and activity with ease and
54 high throughput screening capacity.

55 Being able to express these enzymes in a simple heterologous model would provide an efficient and
56 versatile tool to characterize these enzymes and potential inhibitors. To do so, in this work, the
57 oleaginous yeast *Yarrowia lipolytica* has been used as a heterologous host. This yeast is particularly
58 valuable in this context. It has been a model for lipid metabolism for decades and has a high enzyme
59 production capacity (Nicaud 2012), it can produce large LBs and it is easy to manipulate thanks to the
60 numerous modern genetic engineering toolBox now available (Larroude et al. 2018). In particular, a
61 strain deleted for all the genes coding for enzymes with DGAT activities (Q4) is available (Beopoulos et

62 al. 2012). This strain is not able to form LBs anymore. Previous work has shown that DGAT activity in
63 *Y. lipolytica* can be easily validated, characterized and modulated by overexpression approaches in this
64 genetic background, allowing restoration of LBs formation and TAGs accumulation (Aymé et al. 2015;
65 Gajdoš et al. 2016; Gajdoš et al. 2019). Those previous works established the efficiency and versatility
66 of the heterologous expression of DGAT in this particular host.

67 In order to determine whether the heterologous constructs could potentially be used as tools to
68 measure the activity of these enzymes and thus useful for screening chemical libraries to identify
69 regulatory molecules, the murine and the human DGAT2 were overexpressed in the above mentioned
70 Q4 strain, as well as the oleaginous fungus *Umbelopsis rhamaniana* DGAT2, the first DGAT2 identified
71 and expressed in heterologous host (Lardizabal et al. 2001), and the *Y. lipolytica* DGATs for comparison.
72 MmDGAT2 and HsDGAT2 have already been expressed in heterologous systems including insect cells
73 and yeast, but mainly for *in vitro* activity assays (Cases et al. 2001; Turkish et al. 2005; Yen et al. 2005;
74 Stone et al. 2006; Kim et al. 2014). DGATs overexpression in the Q4 chassis strains were therefore first
75 evaluated for the LBs restoration phenotype and TAG accumulation to evaluate the capacity to use
76 them as *in vivo* screening tools for DGATs inhibitors candidate drugs. These strains were then exposed
77 to known inhibitory molecules of mammalian DGAT1 and DGAT2. The results showed that the DGATs
78 are functional in our chassis and that inhibitors conserved their specificities and efficacy. This work
79 provided proof of principle for using these strains as a screening system for libraries of molecules to
80 discover new inhibitors or activators of these enzymes of particular therapeutic interest.

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82 Results and discussion

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84 LBs forming phenotype complementation

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86 The Q4 chassis strain used in this study is unable to form LBs. Therefore, heterologous DGAT
87 functionality can be easily evaluated by simple observation of LBs restoration directly supporting
88 enzymatic activity in the heterologous host. The *Y. lipolytica* YIDGAT1 and YIDGAT2 overexpressed in
89 the Q4 chassis were previously validated (Beopoulos et al. 2012; Gajdoš et al. 2016) and serve as
90 controls for the Q4 strains overexpressing MmDGAT2, UrDGAT2 and HsDGAT2. All strains show a
91 complementation phenotype by formation of LBs (Fig. 1). YIDGAT2 overexpression being the highest,
92 as expected as it is the main DGAT for lipid accumulation in LBs in *Y. lipolytica* (Gajdoš et al. 2016) while
93 HsDGAT2 being the lowest in the condition tested with small LBs formation.

94

95 DGAT2 Inhibitor activity on HsDGAT2 evaluation

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97 As the HsDGAT2 is functional in *Y. lipolytica*, we therefore evaluated FP-06424439, a specific inhibitor
98 of DGAT2, for its capacity to inhibit the LBs formation complementation phenotype in yeast. Serial
99 concentration of FP-06424439 were tested against the strain Y7378 overexpressing the Human DGAT2.
100 In these experiments, we increase the C/N ratio in the medium to 30 as it increases the TAG
101 accumulation in *Y. lipolytica* (Gajdoš et al. 2016) and will consequently improve LBs visualization.
102 Accordingly, LBs appear bigger in the Y7378 strain without treatment (Fig. 2). Small inhibition of LBs
103 formation can be observed at 12.5 µg/ml and increases at 25 µg/ml, where almost no LBs can be
104 observed. At 50 µg/ml, no LBs are formed (Fig. 2). The different concentration of PF-06424439 have
105 no impact on the fitness of the strain as growth is not affected for all the concentrations tested (Fig. 3).

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107 Specificity of DGAT inhibitors on DGATs from different origins

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109 The previous experiment established a minimal concentration that inhibits LBs formation without
110 affecting growth for the DGAT2 specific inhibitor PF-06424439. This is also in the range of
111 concentrations for which LBs are reduced in MCF7 breast cancer without affecting cells viability
112 (Nisticò et al. 2021). Therefore, we tested this inhibitor at the same concentration on the six DGAT
113 overexpressing strains selected in this study to evaluate the DGAT specificity. PF-046020110, a DGAT1
114 specific inhibitor, was also evaluated as a control. PF-046020110 has no effect on the different strains
115 overexpressing DGAT even YIDGAT1 at the concentration tested (Fig. 4). PF-06424439 inhibit LBs
116 formation of the strain overexpressing the HsDGAT2 as demonstrated in Fig. 2, and has a similar effect
117 on MmDGAT2, while no inhibition was observed for the other strains overexpressing *Y. lipolytica*
118 DGATs or *U. rhamaniana* DGAT2, indicating a specificity for mammalian DGAT2 (Fig. 4). No growth
119 defects were observed for all strains with any of the compound (supplementary data).

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121 Conclusion

122

123 Here, we provide strong arguments that *Y. lipolytica* can serve as an efficient platform for the
124 expression and study of heterologous DGATs and for the screening of therapeutic molecules targeting
125 these enzymes. The mammalian heterologous DGATs cloned here are from cDNA libraries and not
126 optimized for expression in *Y. lipolytica*. Nevertheless, we show that the variants used are functional

127 and the sequences are sufficiently conserved to retain activity in the heterologous host while keeping
128 the drug inhibitor specificity. Thus, this strain platform is perfectly suited to screen compounds libraries
129 as mammalian DGAT2s react as in their natural environment. In addition, the proof of principle
130 presented here is performed in microtiterplate, which is well adapted to high throughput screening
131 thanks to the simple phenotype evaluation.

132

133 **Material and methods**

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135 **Compounds, media and culture conditions**

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137 PF-06424439 and DMSO for PF-046020110 were obtained from Sigma-Aldrich. Stock solutions were
138 prepared by resuspending the powder at 5mg/mL in sterile distilled water for PF-06424439 and in
139 pure DMSO for PF-046020110. *E. coli* strains were grown in Luria-Bertani broth medium
140 complemented with 50 µg/mL kanamycin or 100 µg/mL ampicillin when required. For yeast growth
141 and transformant selection, minimal YNB medium, composed of 0.17% (w/v) yeast nitrogen base
142 (without amino acids and ammonium sulfate), 0.5% (w/v) NH₄Cl, 50 mM phosphate buffer (pH 6.8),
143 and 2% (w/v) glucose was used. Leucine was added at a final concentration of 0.1 g/L when required.
144 For higher lipid accumulation experiments, similar YNB medium with 0.15% (w/v) NH₄Cl and 3% (w/v)
145 glucose was used, which corresponded to a carbon-to-nitrogen ratio of 30 (C/N 30). Solid media were
146 complemented with 1.6% agar.

147 For 96 wells microtiterplates, yeasts were pre-cultured in YNB overnight at 28°C, washed and diluted
148 in fresh YNB medium at an optical density at 600 nm of 0.2. 100 µl of this dilution was mixed with 100
149 µl of inhibitor solution diluted in YNB at the required concentration. Cultures were grown at 28°C under
150 constant agitation on a Biotek Synergy MX microtiterplate reader (Biotek Instruments) and monitored
151 by measuring optical density at 600 nm every 20 min for 72 h.

152

153 **Plasmid and strains construction**

154

155 *UrDGAT2* (GenBank accession number: AAK84179.1) was synthesized and codon optimized by
156 Genscript. The gene was cloned under the pTEF promoter between BamHI and AvrII cloning sites in
157 the overexpression JMP62 vector (Nicaud et al. 2002) containing the selective marker *URA3*, to
158 generate JMP2881 plasmid. *MmDGAT2* were PCR-amplified from cDNA cloned vectors (Yen et al. 2005)

159 using primers that allowed Gateway cloning by introducing attb sequences (Attb1-DGAT2_forward
160 GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAGACCCTCATCGCCGCTACTCCGGG; Attb2-
161 DGAT2_reverse GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTTCACCTCCAGCACCTCAGTCTCTG).
162 PCR fragments were cloned into the Gateway® vector pDONR207 (Invitrogen) using Gateway BP
163 clonase (Thermo Fisher Scientific) to generate plasmid JMP1783 and transferred into the *Y. lipolytica*
164 Gateway expression vector JMP1529 (Leplat et al. 2015) using Gateway LR clonase (Thermo Fisher
165 Scientific) giving rise to the plasmids JMP1785 (*pTEF-MmDGAT2-URA3ex*). The *HsDGAT2* cDNA clone
166 (NM_032564) was bought from Genscript and was amplified with the same primers as for *MmDGAT2*
167 (one nucleotide difference and no change in the amino acid sequences) to remove the C-terminal tag
168 present in the vector and introducing the attb sequences for Gateway® cloning. PCR fragments was
169 cloned into the Gateway® vector pDONR207 (Invitrogen) to generate plasmid JME4451 and transferred
170 into the *Y. lipolytica* Gateway expression vector JMP1529 (Leplat et al. 2015), giving rise to the plasmids
171 JMP4468 (*pTEF-HsDGAT2-URA3ex*). The nucleoSpin plasmid easypure Kit (Macherey-Nagel) was used
172 for plasmid purification and expression cassettes were sequence verified. Expression cassettes from
173 the *NotI*-digested plasmids JMP2881, JMP1785 and JMP4468 were used for *Y. lipolytica* transformation
174 in the Y1877 strain (the Q4 strain) which lacked the four acyltransferases (Beopoulos et al. 2012) using
175 the lithium acetate method (Le Dall et al. 1994), creating strains Y4952 (Q4-UrDGAT2), Y3137 (Q4-
176 MmDGAT2) and Y7378 (Q4-HsDGAT2) respectively. Strains Y1880 (Q4), strains Y1884 (Q4-YIDGAT1)
177 and Y1892 (Q4-YIDGAT2) were described previously (Beopoulos et al. 2012). All the plasmids and
178 strains used in this study are listed in table 1 and 2, respectively.

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180 Fluorescence microscopy

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182 For LBs staining, cells were stained at room temperature by a 10-min incubation with BODIPY 493/503
183 (Invitrogen) at 1 µg/mL. Images were taken using a Zeiss Axio Imager M2 microscope with a 100X oil
184 immersion objective, equipped with an HXP 120 C lamp.

185

186 Acknowledgments

187

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189

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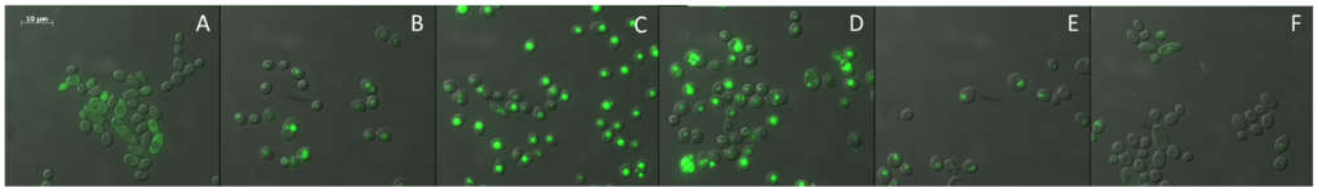
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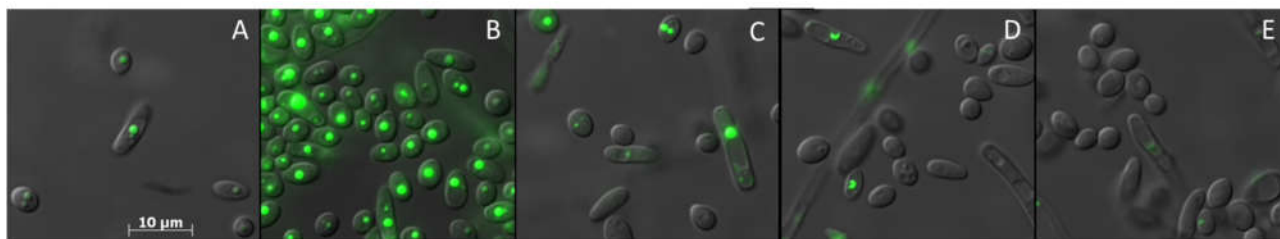


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260 Fig. 1. Phenotype complementation of strains overexpressing DGAT. A) Strain Y1880 Q4. B) Strain
261 Y1884 Q4+YIDGAT1. C) Strain Y1892 Q4+YIDGAT2. D) Strain Y3137 Q4+MmDGAT2. E) Strain Y4592
262 Q4+UrDGAT2. F) Strain Y7378 Q4+HsDGAT2.

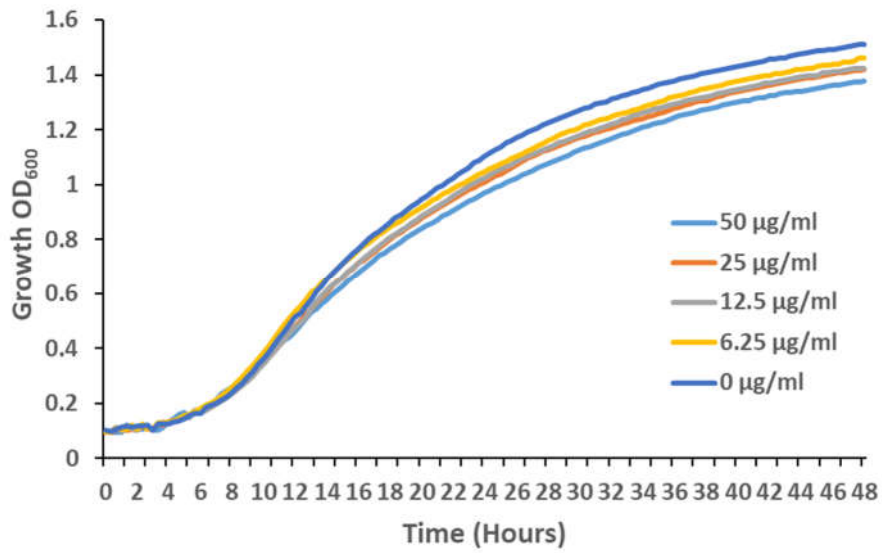
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266 Fig. 2. LBs formation inhibition evaluation in Y7378 Q4+HsDGAT2 using different concentration of PF-
267 06424439. A) 0 µg/mL., B) 6.25 µg/mL., C) 12.5 µg/mL., D) 25 µg/mL., E) 50 µg/mL.

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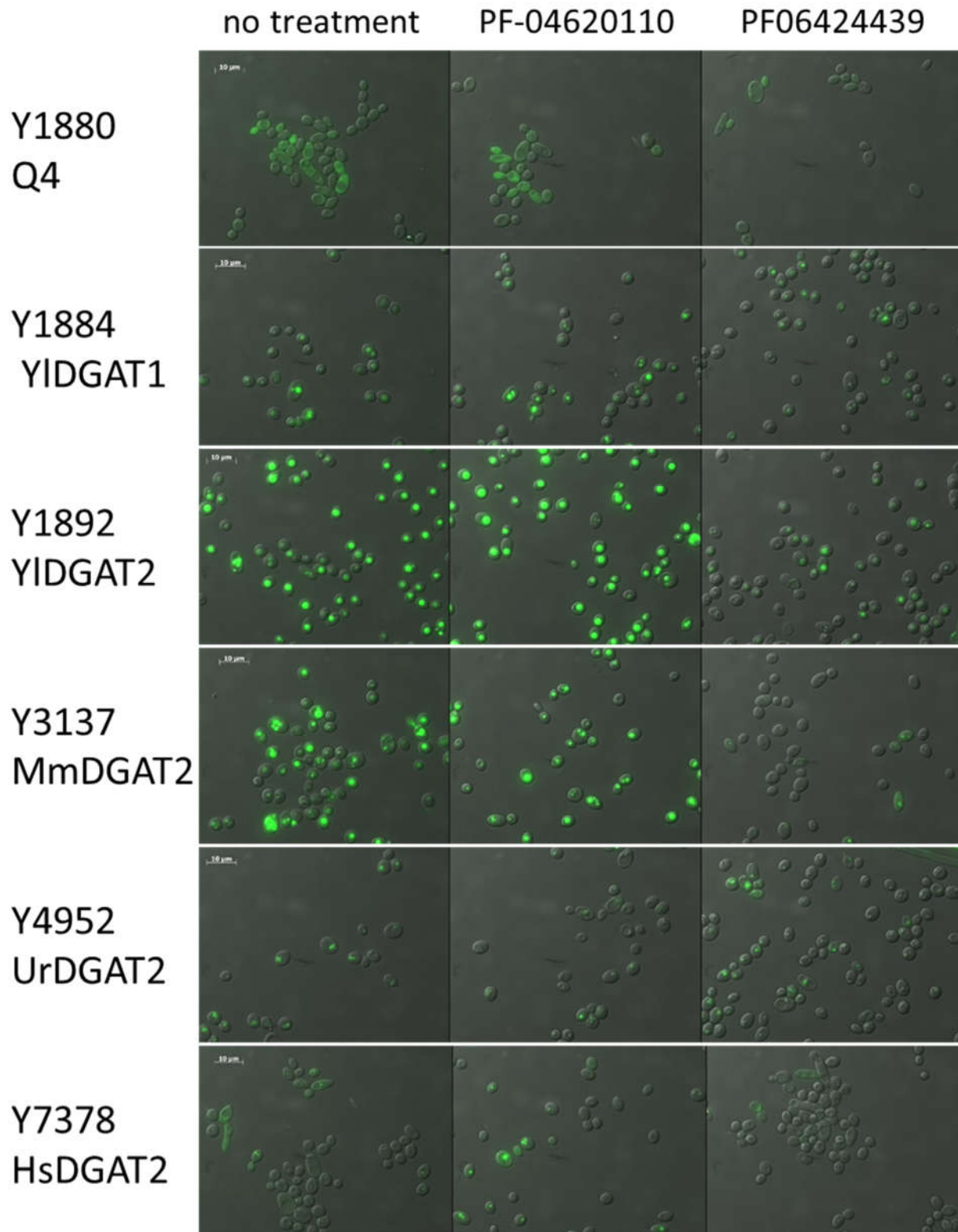


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270 Fig. 3. Y7378 (Q4+HsDGAT2) growth curve on microtiterplate with increasing concentration of PF-
271 06424439.

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275 Fig. 4. LBs formation inhibition evaluation in the strains overexpressing different DGATs using PF-
276 06424439 and PF-046020110 as inhibitors at 25µg/mL.

277

278 Table 1. List of plasmids used in this study

Plasmids	genotype	References
JMP1529	Gateway expression vector	(Leplat et al. 2015)
JMP1046	Expression vector	(Nicaud et al. 2002)
JMP2881	JME1046+UrDGAT2	This work
JMP1783	pDONR207+MmDGAT2	This work
JMP1785	JMP1529+MmDGAT2	This work
JMP4451	pDONR207-HsDGAT2	This work
JMP4468	JME1529-HsDGAT2	This work

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281 Table 2. List of *Y. lipolytica* strains used in this study

Strains	Genotype	References
Y1877 (Q4)	<i>leu2-270 ura3-302 Δdga1Δlro1Δare1Δdga2</i>	(Beopoulos et al. 2012)
Y1880	<i>leu2-270 ura3-302 Δdga1Δlro1Δare1 ::URA3 Δdga2</i>	(Beopoulos et al. 2012)
Y1884	<i>leu2-270 ura3-302 Δdga1Δlro1Δare1Δdga2 pTEF-YIDGAT1-URA3ex</i>	(Beopoulos et al. 2012)
Y1892	<i>leu2-270 ura3-302 Δdga1Δlro1Δare1Δdga2 pTEF-YIDGAT2-URA3ex</i>	(Beopoulos et al. 2012)
Y3137	<i>leu2-270 ura3-302 Δdga1Δlro1Δare1Δdga2 pTEF-MmDGAT2-URA3ex</i>	This work
Y4952	<i>leu2-270 ura3-302 Δdga1Δlro1Δare1Δdga2 pTEF-UrDGAT2-URA3ex</i>	This work
Y7378	<i>leu2-270 ura3-302 Δdga1Δlro1Δare1Δdga2 pTEF-HsDGAT2-URA3ex</i>	This work

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