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***SFH2* regulates fatty acid synthase activity in the yeast *Saccharomyces cerevisiae* and is critical to prevent saturated fatty acid accumulation in response to haem and oleic acid depletion**

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Abbreviations used : DAG, Diacylglycerol; FAS, fatty acid synthase; PtdCho, Phosphatidyl choline; PtdEtn, Phosphatidyl ethanolamine; PtdIns, Phosphatidyl inositol; PtdOH, phosphatidic acid; PtdSer, Phosphatidyl serine; SFA, saturated fatty acids; TAG, Triacylglycerol; UFA, unsaturated fatty acids;

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ABSTRACT

The yeast *Saccharomyces cerevisiae* is a facultative anaerobic organism. In anaerobiosis, sustained growth relies on the presence of exogenously supplied unsaturated fatty acids and ergosterol that yeast is unable to synthesize in the absence of oxygen or upon haem depletion. In the absence of exogenous supplementation with unsaturated fatty acid, a net accumulation of saturated fatty acid (SFA) is observed that induces significant modification of phospholipid profile [1]. Herein, we focus on the role of *SFH2/CSR1*, a hypoxic gene related to *SEC14* and its involvement in lipid metabolism upon haem depletion in the absence of oleic acid supplementation. We observed that inactivation of *SFH2* results in enhanced accumulation of SFA and phospholipid metabolism alterations. It results in premature growth arrest and leads to an exacerbated sensitivity to exogenous SFA. This phenotype is suppressed in the presence of exogenous oleic acid, or by a controlled expression of *FAS1* one of the two genes encoding FAS. We present several lines of evidence to suggest that Sfh2p and oleic acid regulate SFA synthase in yeast at different levels: whereas oleic acid acts at the transcriptional level on *FAS2*, we show that Sfh2p inhibits fatty acid synthase activity in response to haem depletion.

INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is a facultative anaerobic organism, which can alternatively utilize respiration or fermentation for its energetic requirements. Physiological adaptation to aerobiosis and anaerobiosis is accompanied by the differential expression of a large number of genes in relation to oxygen availability [2-4]. Respiration deficiency resulting from oxygen limitation has several consequences, among which unsaturated fatty acids and ergosterol deprivation are potentially sufficient to compromise growth. Indeed, the biosynthetic pathways of these essential molecules are strictly aerobic. Transcriptional induction of hypoxic genes encoding oxygen dependent enzymes including *ERG11* (for sterol biosynthesis) and *OLE1* (for fatty acid desaturation) can be a temporary mean for the cells to counteract oxygen limitation. However, long term growth in strict anaerobiosis necessitates supplementation with ergosterol and oleic acid as a source of unsaturated fatty acid.

Since haem is a cofactor of several enzymes necessary for sterol and unsaturated fatty acid synthesis, a major consequence of haem depletion in yeast is unsaturated fatty acid and sterol biosynthesis preclusion. In addition, haem depletion also results in activation of hypoxic genes through its effect on several transcriptional factors including Rox1p and Hap1p [2, 5]. Haem depletion can be easily achieved in a strain deleted for the *HEM1* gene, which encodes the δ -aminolevulinic acid synthase gene. In the presence of exogenous δ -aminolevulinic acid, a *hem1 Δ* strain is able to synthesize unsaturated fatty acid and ergosterol and hypoxic genes are repressed: this corresponds to aerobic-like growth. In contrast, a *hem1 Δ* mutant cultivated in complete medium lacking δ -aminolevulinic acid can mimic most of anaerobiosis-induced effects of oxygen deprivation, including ergosterol and oleic acid requirement for robust growth, and hypoxic gene induction [6, 7].

In this experimental system, unsaturated fatty acid starvation in haem depleted medium results in growth arrest after 5 generations. Interestingly, cells accumulate saturated fatty acids (SFA) which are not mainly stored within triacylglycerols and steryl esters but rather within specific phospholipid species, with a marked preference for PtdIns [1]. In yeast, the balance of PtdCho versus PtdIns is controlled by Sec14p, an essential protein that is involved in protein transport from the yeast Golgi complex. More specifically, it is currently proposed that this protein regulates the interface between phospholipids and proteins that participate in the traffic machinery in the secretory pathway [8]. Numerous data suggest that Sec14p controls phosphatidylcholine

(PtdCho) metabolism through the CDP-choline pathway. Indeed some of the mutants that relieve cells of Sec14p requirements (the so-called “by-pass Sec14p” mutants) inactivate structural genes for enzymes of the CDP-choline pathway (e.g., *CKII*, *CPT1*) for PtdCho biosynthesis. In addition, biochemical data indicate that Sec14p can inhibit Pct1p, an enzyme of the CDP-choline pathway [9].

Overexpression of *SFH2* was shown to suppress the thermosensitive phenotype of *sec14-1* [10]. Sfh2p belongs to a family of 5 yeast proteins which are structurally homologous to Sec14p, hence its name (*Sec Fourteen Homolog 2*). All, but Sfh1p, exhibit PtdIns but not PtdCho-transfer activity. Some experimental evidences support a role for Sfh4p at contact sites between ER and Golgi membranes to facilitate PtdSer translocation. The phenotype of *sfh3Δ* strain, and the localization of Sfh3p in lipid particle, led to propose that *SFH3* could facilitate sterol traffic between ER and lipid particles. The function of the other Sfh proteins is largely unsolved. Based on a detailed analysis of *sec14-1* suppression by *SFH2*, it was suggested that Sfh2p is the closest functional homolog of Sec14p [11]. Interestingly, *SFH2* is the only hypoxic gene within the Sec14p family [10, 12]. Here, we report on the possible role of *SFH2* in lipid and fatty acid metabolism in response to haem depletion and oleic acid starvation.

EXPERIMENTAL

Yeast strains, media, genetic techniques and culture conditions

The yeast strains used throughout this study are listed in Table 1. Strains were grown in standard minimal media (YNB) supplemented with the appropriate aminoacids and/or bases for plasmid selection. The media used for anaerobic growth were supplemented with 1% (v/v) Tween 80 (Polyethylene Sorbitan Mono-Oleate as a source of oleic acid), 80 µg.ml⁻¹ ergosterol and 1% (v/v) Tergitol NP-40/ethanol (1:1). In contrast to what is routinely used in the lab [1], the *hem1Δ* strains was supplemented with δ-aminolevulinic acid (120 µg.ml⁻¹), which achieved aerobic growth conditions until glucose depletion (*ie* stationary phase). In addition, unless otherwise stated, haem-induced lipid starved conditions were obtained by inoculating 2.10⁵ cells.ml⁻¹ of stationary phase cells in YPD medium. In these conditions, expression of hypoxic genes was routinely observed 6 hours after the shift. Alternatively, YPD medium was supplemented with 80 µg ml⁻¹ ergosterol and/or 1% Tween 80 (v/v), used as the source of unsaturated fatty acids.

Plasmid pJU1 contains the *SFH2* ORF and 220bp of upstream sequence inserted into Yep352 [13]. The insert was isolated as a *SacI* fragment from pMR270 (one of the original plasmids suppressing *sec14-1* phenotype [12]) into Yep352 ; we have confirmed that this plasmid allows full suppression of *sec14-1* mutation and complements *SFH2* deletion. Plasmid pMR306 encoding the

fusion protein MBP-SFH2 was constructed as follows. First pMR265 [12] was used to amplify the coding sequence of *SFH2* using the two primers CSRBGL2 (5'AAGATCTATTATGTCCTTTGATAGA3') and CSR3SAL (5'AGTCGACTCAAACCTTTAGAGAACC3'). The two oligonucleotides introduce a *Bgl*II and a *Sal*I restriction site immediately upstream and downstream of *SFH2* coding sequence. The PCR product was cloned into pGEMT-easy (Promega) to generate pMR303. The 1.3 kb *Bgl*II-*Sal*I fragment from pMR303 was inserted into pMALTM-c2 (New England Biolabs Inc.) digested with *Sal*I and *Bam*HI to generate pMR306. Plasmid pCF8 contains *FAS1* ORF under the control of the *tetO₇* promoter; it was constructed as follows: *FAS1* ORF was amplified by PCR from the plasmid pJS229 (kindly provided by Pr Schüller) using the two primers 5FAS1 (5'CGCTCATTATGGACGCTTAC) and 3FAS1 (5'TTAGGATTGTTTCATACTTTTC) and cloned into pGEMT-easy (Promega) to generate pGF4-S. It was digested by *Not*I and inserted into pCM185 to generate pCF8. This plasmid was used to transform a heterozygous *FAS1/fas1Δ* strain from which a haploid *fas1Δ* was isolated. Growth of this strain in the absence of doxycycline was indistinguishable from the wild type strain, but was totally inhibited with doxycycline concentrations above 1 μg.ml⁻¹ in the medium. This confirmed the functionality of the *tetO₇* promoter to control accurately *FAS1*. Plasmid pCG4GFP is an integrative vector based on Yip351 which express GFP-Sfh2p under the control of the *SFH2* promoter. It was constructed as follows: *SFH2* ORF was isolated as a *Not*I fragment and cloned into pNG188 to generate pMR314: this plasmid contains an in frame fusion between GFP and *SFH2* with a c-myc epitope in between that can be isolated as a *Spe*I restriction fragment. A 600 bp fragment containing *SFH2* promoter was amplified by PCR and inserted into Yip351 to generate Yip-PROCSR. The terminator of *PMA1* was inserted as a *Bam*HI-*Not*I fragment from plasmid pNEV-N [14]; this plasmid was named YpA. Finally, the *Spe*I fragment from pMR314 was inserted into YpA digested with *Spe*I to generate pCG4GFP. This plasmid was used to transform MRY73-6a to generate YPCG4-G9d. It was introduced into TDY36-1d by standard genetic methods.

Lipid analysis and labeling

Phospholipids and neutral lipids were analyzed using the standard procedure in use in our lab [1]. Fatty acid methyl esters obtained from bulk lipids, or each lipid class separated by TLC as described [1], were analysed by GC using a 25 m x 0.32 mm AT-1 capillary column (Alltech) with methylheptadecanoic acid as a standard. Fatty acid were quantified and used to calculate the amount of lipid in each class. The lipid content was expressed as nmol of fatty acid per 10⁹ (broken) cells.

For labeling experiments with palmitic acid, yeast cells were grown in liquid medium supplemented with δ -aminolevulinic acid ($80 \mu\text{g}.\text{ml}^{-1}$) to a cell density of $4 \times 10^7 \text{ cells}.\text{ml}^{-1}$ after which they were incubated with $1.5 \mu\text{Ci}.\text{ml}^{-1}$ of $[9,10 \text{ }^3\text{H}]$ palmitic acid ($1.11 \text{ TBq}.\text{mmol}^{-1}$, ARC Radiochemicals). After 16 hours, cells were harvested, washed with fresh non radioactive YPD medium, and chased for the indicated time (0, 4, 6, 8, 16 hours). At the end of the experiments, cells were recovered and mixed with 2×10^9 non radioactive cells. Lipids were extracted as described above. During the course of the chase experiment, there was no significant loss of radioactivity per volume of the culture within the lipid pool. The tritiated spots of individual lipid species were scraped off from the silica gel plates, transferred into scintillation counting vials and the ^3H content was determined by liquid scintillation counting.

Enzyme activities and MBP-Sfh2 purification

β -galactosidase assays were performed as described previously [1] on *hem1 Δ* cells containing a plasmid bearing the 1 kb DNA sequence immediately upstream of the *DAN1* open reading frame cloned 5' to the reporter gene *lacZ* (plasmid pMR278 [6]), grown in the appropriate medium, or with plasmid pBF16 (*ARS CEN TRP1* vector) [15] which contains *FAS2* promoter and part of the coding sequence fused in frame to *lacZ* coding sequence. Expression of the reporter gene is given as nmol of *O*-nitrophenol (measured at 420 nm).min $^{-1}.$ mg $^{-1}$ of protein, was calculated as follows: $A = (A_{420} \times 100 \times 1700) / (4.5 \times V \times c \times t)$, where A_{420} is the optical density, V is the volume of the protein extract (ml), c is the protein concentration (mg.ml $^{-1}$) and t is the incubation time at 30°C (min). FAS activity was determined according to Lynen [16] by assaying β -ketoacyl reductase-dependent oxidation of NADPH at 334 nm in the presence of acetyl-CoA and malonyl-CoA. Activity is expressed as mU.mg $^{-1}$ protein (nmol NADPH oxidised per minute and per mg of protein). Cells were broken with glass beads and cleared by a 10 minutes centrifugation at 5000 g. Depending upon the strain used, protein amounts varied between 70 μg to 130 μg to ensure an enzyme activity below 10 milliunits which guaranteed linearity between activity and enzyme amount [16]. MBP-Sfh2p was purified essentially as recommended by the manufacturer instruction (New England Biolabs) with minor modifications as described elsewhere [6].

Immunofluorescence Microscopy

Liquid cultures were grown overnight in complete medium containing δ -aminolevulinic acid. Cells were harvested and washed twice with distilled water and inoculated in complete media with or without δ -aminolevulinic acid and cultivated for 6 hours. Cells were resuspended in PBS with 0.1% (w/v) Bovine serum albumin (SIGMA, Fraction V). Staining of lipid particles with Nile Red

(Molecular Probes) was performed by incubating cells with Nile Red ($50 \mu\text{g}.\text{ml}^{-1}$) for 5 minutes, and washed in PBS 0.1% BSA. Indirect immunofluorescence was performed from TDY33-3a using the protocol described in the TRIPLES database [17] except that mounted solution contained Vectashield 10% (v/v) (Vector Lab Inc.) and that the antibodies that were used are : HA.11 polyclonal antibody (COVANCE) anti HA primary antibody (1:100) and Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Invitrogen) (1:400). Confocal microscopy was performed with the use of a Confocal Olympus FV 1000 spectral mode. Excitation wavelength for GFP and Alexa Fluor® 488 were set to 488 nm. Excitation wavelength for Nile Red was set to 543 nm. Image acquisition and conversion were done separately for red and green channel and processed with Olympus Fluoview vers1.4.

RESULTS

Lipid composition of *hem1Δ sfh2Δ* mutant in haem depleted medium

To determine whether *SFH2* is involved in lipid metabolism under haem depleted conditions a *hem1Δ sfh2Δ* mutant and the control strain (*hem1Δ*) were cultivated in complete medium supplemented with δ -aminolevulinic acid to stationary phase and inoculated into fresh medium in the absence of δ -aminolevulinic acid, ergosterol and oleate (i.e. haem-induced lipid starvation). Under these conditions, expression of hypoxic genes was detected 6 hours after the shift (see below) and growth ceased 16 hours after the shift due to sterol and oleic acid starvation [1]. The amount of phospholipids and neutral lipids were quantified in both strains before the shift and 16 hours later. Aerobic-like cultures of mutant and control strains grown to stationary phase had very similar profiles of phospholipids (Figure 1A). In contrast, noticeable differences in phospholipid composition were observed following oleic acid starvation and haem depletion: whereas a 53% decrease of PtdCho was observed in the control strain, there was a 31% increase in the mutant strain. We also noted a slight decrease of PtdIns abundance 16 hours after the shift in the mutant strain as compared to the control strain. The amount of PtdOH, a proposed key component of phospholipid metabolism [18], was also affected since haem depletion resulted in a 2 fold increase of PtdOH amount in the mutant strain; in the control strain, it remained more or less unchanged. Finally, the amount of PtdEtn and PtdSer were similar between the two strains.

Next, we examined neutral lipid composition in the mutant strain following haem-induced lipid depletion. As reported in Figure 1B, triacylglycerol (TAG) and steryl ester (SE) accumulated to the same extent in control and mutant strains cultivated to stationary phase in the presence of δ -aminolevulinic acid. We observed that SE was efficiently degraded in both strains following the shift to haem induced lipid starvation. Residual amounts of SE were still detected 16 hours after the shift. It corresponded to the amount of SE detected in normal aerobic growth during exponential phase. Surprisingly, whereas 90% of the pool of TAG was depleted 16 hours following the shift in the *hem1Δ* strain, 40% of the TAG accumulated in aerobiosis was still observed in the *hem1Δ sfh2Δ* (Figure 1B). We also noted a 61% increase in the amount of diacylglycerol (DAG) in the mutant strain whereas there was a 2.3 fold decrease of DAG in the control strain.

***Sfh2Δ* produces more saturated fatty acids upon haem-induced lipid starvation**

We have already reported that SFA preferentially accumulate within specific phospholipid species in response to haem-induced fatty acid starvation [1]. Thus, we looked more closely at the ratio of SFA within phospholipids and neutral lipids in response to haem depletion. As reported in Table 2, the increase of SFA amount did not affect all the lipid species to the same extent. We have already shown (see Figure 1A) that haem depletion does not affect the total amount of PtdSer and PtdEtn; in Table 2, it is reported that the ratio of SFA in PtdEtn was not modified either. We noted a slight decrease of SFA ratio in PtdSer in the *hem1Δ sfh2Δ* strain 16 hours after the shift as compared with the control *hem1Δ*. The fatty acid composition of PtdCho was not modified 16 hours after the shift between mutant and control (Table 2). However, bearing in mind that the amount of PtdCho had increased of 53% in the mutant and decreased to 35% in the control (see Figure 1A), it suggests that a substantial amount of SFA was contained in this phospholipid. In addition to a 2 fold increase in the amount of PtdOH (Figure 1A), the amount of SFA represented 72.4% of this lipid in the mutant and 46.3% in the control strain. Finally, although the amount of PtdIns had decreased in the mutant but increased in the control strain following the shift to haem depleted medium (Figure 1A), the proportion of SFA was increased to 71.6% in the mutant and to 60.0% in the control. Thus, similar amounts of SFA were contained in this phospholipid species. SFA were also preferentially stored in TAG in the mutant after the shift (they constitute 64.5% of total TAG in mutant 16 hours after the shift but only 29.2% in the control). The amount of DAG had increased of 61% in the mutant respective to the control (Figure 1A) but the proportion of SFA in this species was not dramatically altered (84.0 % in mutant and 81.6% in control). Finally, free SFA represented 41.4% of total free fatty acid in the mutant and 34.4% in the control.

To confirm that the *hem1Δ sfh2Δ* mutant accumulates more fatty acids than the *hem1Δ* control strain, the amount of fatty acids was compared between mutant and control, in response to haem-induced fatty acid depletion. As shown Figure 2A, and as already described, the amount of fatty acid in the cells rapidly decreased following inoculation in complete medium [1], because the cells preferentially reallocate their endogenous pool of fatty acid accumulated in stationary phase within lipid particles : indeed, 4 hours after inoculation from stationary phase, 2.5 generations had been accomplished, and the amount of fatty acids in the cells was 4 times lower suggesting that there is only a modest fatty acid biosynthesis following inoculation into fresh medium (see below).

Soon after the onset of hypoxic gene expression (6 hours), but before growth arrest of the mutant (see below, Figure 3), total fatty acids represented roughly 37.7% of the initial amount in control and 35.8% in mutant, whereas 2 hours later (i.e. 8 hours after the shift), when the mutant

had ceased growth, it was maintained at a similar level in the control strain, but it had increased to 53.4% in the mutant strain. It is interesting to observe that in both strains, the amount of unsaturated fatty acid per 10^9 cells was very similar and roughly constant during the experiment (Figure 2C). Because until 8 hours after the shift, growth of both strains was not arrested, it follows that both strains were still capable to convert newly synthesized fatty acids into unsaturated fatty acids. This is most likely the primary consequence of hypoxic induction of *OLE1* thereby allowing cells to desaturate fatty acid even though oxygen is limiting [19]. As shown in Figure 2B and 2C, the increase in total fatty acid content of the mutant described above can be solely accounted for by the steady increase of SFA amount.

***SFH2* is necessary for growth in haem depleted conditions**

To address the consequences of the lipid pattern modifications in the mutant, we compared the growth curves of the mutant strain and the wild-type in response to haem deprivation. Control *hem1Δ* and mutant *hem1Δ sfh2Δ* were cultivated to stationary phase in the presence of δ -aminolevulinate. Growth rate was monitored after a shift to fresh YPD medium without supplementation. As shown in Figure 3 and as already reported [1], haem depletion in YPD medium results in growth arrest of the control strain after about five generations (i. e. 16 hours). Hypoxic gene expression, monitored by using the *lacZ* reporter gene fused to the promoter of the hypoxic gene *DAN1*, was detected 6 hours after the shift (see arrow on Figure 3) [1]. This is probably the time period necessary to deplete the cells of their endogenous pool of δ -aminolevulinic acid. We observed that within 5 hours after the shift no effect of *SFH2* deletion could be observed. Longer incubation in the absence of lipid supplementation (oleic acid and ergosterol) results in growth arrest of the mutant: 8 hours after the shift, cell growth was totally arrested (Figure 3). In contrast, the control strain was still able to sustain growth until 15 hours after the shift. In addition, the aberrant phenotype of the *hem1Δ sfh2Δ* null mutant is relieved by supplementation of the medium with oleic acid (Figure 3) but not by ergosterol (not shown). Thus, we conclude that oleate starvation, induced by haem depletion, results in rapid growth arrest in the absence of a functional copy of the *SFH2* gene.

Neutral lipid accumulation is not responsible of *sfh2Δ* phenotype

Our results indicate that *SFH2* deletion has two major consequences on lipid metabolism in response to oleic acid starvation (*i*) a global increase in the SFA content and (*ii*) an alteration of the lipid composition including an accumulation of TAG, DAG, PtdOH and PtdCho. The accumulation of TAG in lipid particles in response to *SFH2* deletion could be interpreted in at least two ways. It

could simply result from a partial failure to hydrolyze neutral lipids from lipid particles.

Alternatively, neutral lipids might be efficiently degraded and used as acyl donors for phospholipid synthesis; *SFH2* deletion would secondarily provoke improper storage into neutral lipids. We thus questioned the significance of TAG accumulation following haem depletion in the null *SFH2* mutant.

Lipids from control *hem1Δ* and *hem1Δ sfh2Δ* mutant growing in the presence of δ -aminolevulinic acid were metabolically labeled by extensive incorporation of tritiated palmitic acid until stationary phase. Cells were then washed and inoculated in fresh YPD medium lacking δ -aminolevulinic acid and oleic acid. Radioactivity within neutral lipid species separated by TLC was quantified at various time points after the shift. As shown in Figure 4A and 4B, $35.3 \pm 2.7\%$ and $35.9 \pm 0.9\%$ of the radioactivity incorporated into lipids before the shift was recovered in TAG in the control strain and the mutant strain respectively. SE represented $6.0 \pm 0.9\%$ and $8.2 \pm 1.3\%$ of total radioactivity in control and mutant respectively. We observed that 8 hours after the shift both types of molecules were effectively hydrolyzed. In addition, we could observe a concomitant increase in the radioactivity associated to phospholipids, demonstrating that acyl chains from neutral lipids had been re allocated into newly synthesized phospholipids (not shown). However, with longer chase experiments an increase in the amount of radioactivity associated to TAG was observed in the mutant strain 16 hours after the shift. In the mutant, radioactivity incorporation into TAG constituted $28.3 \pm 1.8\%$ of total radioactivity 16 hours after the shift; in the control strain, it represented only $13.5 \pm 2.4\%$. This led us to conclude that deletion of *SFH2* leads to an improper synthesis of TAG in response to haem starvation.

To evaluate whether TAG accumulation is responsible for the premature growth arrest, the phenotype of *hem1Δ sfh2Δ* inactivation was investigated in a strain background inactivated for the genes encoding TAG and SE synthases [20]. We observed that a *hem1Δ dgal lro1 are1 are2* grew approximately 1/3 of the optical density reached by the *hem1Δ* strain (Figure 4C). This is not totally surprising since it lacked most of the fatty acids contained in the TAG and SE of the *hem1Δ* strain. Nevertheless, we observed that the growth defect resulting from *SFH2* deletion in haem and oleic acid depleted medium was still observed in this genetic background (Figure 4C).

Moreover, deletion of the TAG and SE synthases encoding genes (i.e. the quadruple mutant *dgal lro1 are1 are2*), resulted in an enhanced production of some lipid species that already accumulate in the *hem1Δ sfh2Δ* mutant (i.e. PtdOH, PtdCho and DAG accumulation) without any noticeable effect on the lack of growth of this strain in haem depleted conditions (not shown). This suggests that TAG, PtdCho, DAG and PtdOH accumulation in the *sfh2Δ* strain is not primarily responsible for the lack of residual growth upon haem deprivation.

The *sfh2Δ* mutant is hypersensitive to saturated fatty acids

The results presented so far suggest that the substantial accumulation of SFA in the mutant in response to oleic acid starvation under haem depleted growth could be responsible for the *hem1Δ sfh2Δ* phenotype. Thus, one could predict that the mutant strain would be more sensitive to exogenous SFA than the control strain under haem depleted conditions. To address this possibility, *hem1Δ sfh2Δ* and *hem1Δ* strains were cultivated to stationary phase in the presence of δ -aminolevulinic acid and shifted to complete medium containing a limited amount of Tween 80 as a source of oleic acid and variable amounts of Tween 40 as a source of palmitic acid. The optical density reached by the cultures was measured 16 hours later. An example, representative of three independent experiments, is presented in Figure 5. A limited amount of Tween 80 (1⁰/₀₀₀ v/v) could significantly increase the growth of control and mutant strains. As expected the *hem1Δ sfh2Δ* was much more sensitive to addition of saturated fatty acids than the *hem1Δ* strain: indeed, the presence of Tween 40 (0.5⁰/₀₀₀ v/v) in the medium had no detectable effect on the *hem1Δ* strain while it reduced growth of the *hem1Δ sfh2Δ* strain to 35% of what is observed in the absence of Tween 40. The amount of Tween 40 necessary to inhibit growth despite the supplementation with 1⁰/₀₀₀ (v/v) oleate was roughly 3 times less in the *hem1Δ sfh2Δ* than with the *hem1Δ* strain (2⁰/₀₀₀ and 6⁰/₀₀₀ respectively). Therefore, the growth defect of the mutant is exacerbated by SFA supplementation suggesting a deleterious effect. This confirms that growth inhibition of the *hem1Δ sfh2Δ* mutant results from endogenous accumulation of SFA.

Enhanced activity of fatty acid synthase in *sfh2Δ* *in vivo*

Thus, we tried to understand the basis of SFA accumulation in the mutant in response to oleic acid deprivation. In yeast, SFA are synthesized by a heteromultimeric $\alpha_6\beta_6$ complex encoded by the two genes *FAS1* and *FAS2* for the β and α subunits respectively [21]. Both genes are regulated similarly and appear to be down regulated by exogenous SFA in the culture medium [22]. We wondered whether oleic acid could also control FAS expression. For this purpose, we used a *lacZ* gene reporter assay to monitor *FAS2* expression in our growth conditions (plasmid pBF16 [15] kindly provided by Pr H.J. Schüller). To minimize possible interference from the genetic background, the experiments were performed in a *hem1Δ sfh2Δ* mutant transformed either with the control plasmid (YEp352) or with a plasmid expressing *SFH2* (pJU1). As reported in Figure 6, control and mutant strains behaved very similarly : addition of oleic acid in the medium in the presence of δ -aminolevulinate resulted in a 2.6 fold decrease of the reporter gene activity, a level of repression in the same range as with a SFA (palmitic acid : 2.9 fold decrease). In contrast, we observed a 1.8 fold

induction of the reporter gene under haem and oleic acid starvation. Importantly, supplementation with oleic acid in the absence of haem reduced *FAS2* expression 3.1 fold, to a level of expression below that observed in the presence of haem. Therefore, oleic acid starvation in cells lacking haem results in enhanced expression of *FAS2*.

This last observation was somewhat surprising since, although both strains regulate transcription of *FAS2* identically, the mutant only showed an increase in the amount of SFA in response to haem depletion and oleic acid starvation. Thus, we measured the activity of fatty acid synthase in the *hem1Δ sfh2Δ* and in the *hem1Δ* strains in response to haem depletion. As shown in Figure 7, fatty acid synthase (FAS) activity in cells grown to stationary phase in aerobiosis was similar in both strains (28.8 ± 2.9 mU.mg⁻¹ and 23.4 ± 3.2 mU.mg⁻¹ respectively). In the *hem1Δ*, FAS activity was maintained at a low level following oleic acid starvation in haem depleted medium: 10 hours after the shift, FAS activity was 31.9 ± 0.1 mU.mg⁻¹. In contrast, in the *hem1Δ sfh2Δ* strain, haem depletion and oleic acid starvation were associated with an increase of FAS activity (57.87 ± 4.7 mU.mg⁻¹ detected 6 hours after the shift) which was maintained at a high level during the rest of the experiment.

Suppression of *sfh2Δ* phenotype by controlled expression of *FAS1*

It has been shown that the activity of FAS holoenzyme is primarily determined by the amount of Fas1p since overexpression of *FAS1* gene results in concomitant increase of *FAS2* mRNA abundance and an enhanced activity of FAS [15]. Thus, it is possible to alter the amount of active FAS by controlling the transcriptional level of the sole *FAS1* gene. This observation provided a convenient way to test whether increased FAS activity is responsible for the phenotype of the *hem1Δ sfh2Δ* strain. Plasmid pCF8 contains an engineered *FAS1* gene whose expression is controlled by the *tetO₇-CYC1* promoter which is negatively regulated by doxycycline [23]. It was used to transform a heterozygous *FAS1/fas1Δ* strain from which a viable haploid *fas1Δ* was isolated. In this strain, expression of the episomal *FAS1* gene is driven by the *tetO₇-CYC1* promoter and consequently FAS activity is modulated by the amount of doxycycline added to the medium as demonstrated for other genes [24]: indeed, we observed that *fas1Δ* [pCF8] strain in aerobiosis is viable, and its growth is inhibited by doxycycline concentrations above 1 μg.ml⁻¹ because expression of the engineered *FAS1* gene is totally repressed and FAS activity is undetectable (not shown).

Then, we constructed *hem1Δ fas1Δ* and *hem1Δ fas1Δ sfh2Δ* strains containing plasmid pCF8; both strains were cultivated in the presence of δ-aminolevulinic acid to stationary phase and further inoculated in complete medium with variable amounts of doxycycline in the medium in

order to control FAS activity. The optical density reached by the cultures was measured 40 hours after the shift. As shown in Figure 8A, in the absence of doxycycline in the medium, or with concentration of doxycycline above $0.4\mu\text{g.ml}^{-1}$, the mean optical densities reached by the strains (0.186 ± 0.046 and 0.184 ± 0.004 for *hem1Δ fas1Δ* [pCF8] and *hem1Δ fas1Δ sfh2Δ* [pCF8] strains respectively) suggest that residual growth was limited, if not totally impaired. Indeed, the *hem1Δ sfh2Δ* mutant attained similar values under these growth conditions (not shown). In contrast, with $0.15\mu\text{g.ml}^{-1}$ of doxycycline, a significant residual growth was attained with the *hem1Δ fas1Δ*: indeed, the optical density achieved for this strain was 0.474 ± 0.02 which is only slightly lower than for a *hem1Δ* strain (0.508 ± 0.09 obtained with a similar inoculum ($2 \times 10^5 \text{ cells.ml}^{-1}$), not shown). Interestingly, a higher concentration of doxycycline was needed for the *hem1Δ fas1Δ sfh2Δ* strain to achieve a significant residual growth ($\text{OD}_{600\text{nm}}$: 0.391 ± 0.05 with doxycycline concentration of $0.30\mu\text{g.ml}^{-1}$). FAS activity was also measured in both strains after the shift (Figure 8B). We observed that, for any concentration of doxycycline, FAS activity detected in the *hem1Δ fas1Δ* [pCF8] strain was always lower than in the *hem1Δ fas1Δ sfh2Δ* [pCF8] strain. This confirms the up regulation of FAS activity in the *sfh2* null mutant. To get better insight in those activities, it is useful to compare with values obtained in *hem1Δ sfh2Δ* and *hem1Δ* strains - in which FAS activity depends on chromosomal expression of FAS genes - in response to haem depletion and oleic acid starvation (Figure 8C). FAS activity close to that obtained in the *hem1Δ* strain were observed with doxycycline concentrations compatible with significant residual growth (i.e. $0.15\mu\text{g.ml}^{-1}$ and $0.30\mu\text{g.ml}^{-1}$ for *hem1Δ fas1Δ* [pCF8] and *hem1Δ fas1Δ sfh2Δ* [pCF8] respectively). In contrast, with lower amounts of doxycycline in the medium, values of FAS activities were closer (or even higher) to values obtained in the *hem1Δ sfh2Δ*: in these growth conditions, no residual growth was observed in the corresponding strains as a consequence of overexpression of episomal *FAS1* in the *hem1Δ fas1Δ* [pCF8] strain. Thus, these experiments demonstrate that down regulating *FAS1* transcription is sufficient to suppress the phenotype of *hem1Δ sfh2Δ*. In addition, the high amount of doxycycline necessary for the *hem1Δ fas1Δ sfh2Δ* [pCF8] to achieve residual growth is consistent with an already higher FAS activity in the triple mutant strain than in the *hem1Δ fas1Δ* and confirms that residual growth necessitates a tight regulation of FAS activity by Sfh2p.

Sfh2p inhibits fatty acid synthase activity

The elevated FAS activity *in vivo* in the absence of oleic acid and *SFH2* led us to investigate the possibility that Sfh2p inhibits the activity of FAS. For this purpose, we engineered an N-terminal tagged version of Sfh2p, namely, MBP-Sfh2p. We measured the effect of adding purified recombinant Sfh2p on FAS activity *in vitro*. In this assay, cellular extracts were prepared from the

hem1Δ sfh2Δ mutant incubated for 6 hours in a δ -aminolevulinic and oleic acid free complete medium (i.e. high level of FAS activity). Various amount of recombinant MBP-Sfh2p purified from *E. coli* were incubated in the assay medium 1 minute before initiating the reaction. As shown Figure 9, recombinant Sfh2p could substantially inhibit FAS activity (up to 50% inhibition). In contrast, addition of 150 ng of purified MBP in the assay did not affect FAS activity significantly (not shown). We have already mentioned that in aerobiosis, deletion of *SFH2* resulted in a 30% increase of FAS activity. Addition of recombinant Sfh2p to cell extracts of the *sfh2Δ* mutant grown in aerobiosis restored the activity to a wild type level, suggesting that under aerobic growth, Sfh2p could also be used to regulate FAS activity (not shown).

Sfh2p is localized in lipid particles

The cellular localization of Sfh2p is still a matter of debate. It was reported to localize, at least partially, to endosomes [10]. In contrast, Schnabl *et al.* reported that Sfh2p localized mainly in the cytosol and microsomes [11]. In order to address the subcellular localization of Sfh2p, we used an integrated version of *SFH2* which expresses a GFP-tagged Sfh2p. The fluorescent tag was inserted at the N-terminus of Sfh2p and was functional as judged by the ability of the recombinant GFP-Sfh2p to rescue the phenotype of *hem1Δ sfh2Δ* mutation (not shown). The recombinant strain was grown to stationary phase in the presence of δ -aminolevulinic acid. GFP-Sfh2p localized to several punctuated structures scattered in the cytoplasm (see Figure 10 left panel). Interestingly, this pattern was similar to the one observed for the fluorescent dye Nile Red, which is specific for neutral lipids, and is used as a marker of lipid particles. Computer merge of these images confirmed the localization of GFP-Sfh2p with Nile Red structures (not shown). We estimated that more than 90% of the GFP-Sfh2p positive structures were also positive with Nile Red. Following inoculation in δ -aminolevulinic depleted medium and in the absence of oleic acid, the pattern of GFP-Sfh2p remained very similar, suggesting that the protein remained associated with lipid droplets (not shown). In contrast, in the presence of δ -aminolevulinic acid in the medium, the signal promptly faded and 6 hours after the shift, the protein was not detectable anymore (not shown), as expected for the product of a hypoxic gene whose level of expression in exponential phase in aerobiosis is low [12].

We also tried to localize Fas1p in the cell. For this purpose, we used a strain expressing a functional Fas1p-3xHA fusion protein that was kindly provided by Dr Snyder (TRIPLE database) from which a *hem1Δ* derivative was obtained. Indirect immunofluorescence using anti-HA monoclonal antibodies was performed from the strain grown in a medium supplemented with δ -aminolevulinic acid. In exponential growth, the signal was diffuse in the cell (not shown),

suggesting a cytosolic distribution as already described for this protein. In contrast, sample from stationary phase cells revealed a very different localization of Fas1p-3xHA (Figure 10B, left panel): the fluorescent signal was localized in discrete punctuate structures, which again correspond to lipid particles as judged by the co-localization with Nile Red (Figure 10B, middle panel). Inoculation of this strain in complete medium lacking δ -aminolevulinic acid and oleic acid did not modify the localization of Fas1p-3xHA (Figure 10C) which was still associated with lipid particles 6 hours after the shift. In contrast, in the presence of δ -aminolevulinic acid in the medium, the fluorescent signal was not associated with lipid droplets (Figure 10D), suggesting a cytosolic distribution of this protein. Nevertheless, under haem depletion and oleic acid starvation, both Sfh2p and Fas1p appeared to be co-localized in lipid particles. Interestingly, this localization was not modified in a *sfh2 Δ* null mutant (unpublished results). All together, genetic data and localization data support the notion that Sfh2p negatively regulates FAS.

DISCUSSION

Saccharomyces cerevisiae is only able to synthesize monounsaturated fatty acids containing a Δ -9 double bond, primarily palmitoleic acid (C16:1) and oleic acid (C18:1) which represent respectively around 45% and 25% of total fatty acids in aerobiosis. The Δ -9 desaturase responsible for the synthesis of these unsaturated fatty acids (UFA) is encoded by a single essential gene, *OLE1*. In aerobiosis, transcriptional regulation of *OLE1*, and modulation of *OLE1* mRNA stability maintain an adequate balance of UFA versus SFA [25]. Since Ole1p is strictly dependent on haem availability, hence on oxygen status, life under hypoxic or anoxic conditions has important consequences on this ratio. Accordingly, when the oxygen status is low in the cell, induction of *FAS* genes and of *OLE1* facilitates desaturation of both existing and newly synthesized fatty acids as long as oxygen is still available [26]. However in strict anaerobiosis or in haem depleted conditions, i.e. when Ole1p is not functional *FAS* genes transcription is still observed if fatty acids are not provided in the medium, and import of exogenous UFA from the medium becomes strictly necessary for optimal growth.

In this work we report a body of evidence which indicates that Sfh2p modulates FAS activity in response to haem depletion and oleate starvation and that, under this condition, this regulation is essential to prevent SFA accumulation that eventually leads to premature growth arrest.

Consistently, *sfh2 Δ* displays enhanced sensitivity to SFA in the medium whereas UFA supplementation restores wild type phenotype. In agreement with a detrimental effect of *SFH2*

deletion on FAS activity, we observed an increase of FAS activity in the mutant in response to haem depletion and UFA starvation. This is confirmed by the observation that *sfh2Δ* can be rescued by downregulation of *FAS1* gene expression while enhanced expression of *FAS1* gene generates a phenotype which is similar to *sfh2Δ* (see Figure 8). However, the mechanism of FAS inhibition by Sfh2p is still unclear. We would tend to favour a model by which Sfh2p exerts a direct effect on FAS because (i) *in vitro*, addition of purified Sfh2p inhibits FAS activity (ii) both proteins co-localize within lipid particles and, (iii) we have observed *in vitro* that Sfh2p can bind Fas1p (unpublished results). However we have not yet been able to confirm this interaction *in vivo*.

We also observed that residual growth of *sfh2Δ* mutant is impaired in genuine anaerobiosis in the absence of oleic acid supplementation (unpublished results) suggesting that Sfh2p accomplish the same function under genuine anaerobiosis as in response to haem depletion. A possible way to understand the physiological relevance of Sfh2p function in response to oxygen depletion is to examine the phenotype resulting from altered expression of FAS (Figure 8). We observed that neither high nor low FAS activity can restore residual growth in response to haem depletion and UFA starvation. This is very surprising since there is a sharp transcriptional induction of *FAS* genes in the absence of oleic acid and haem (see Figure 6). These apparent conflicting results can be reconciled bearing in mind that when oxygen becomes limited, there is transcriptional derepression of hypoxic genes, including *OLE1*, and also *FAS1* and *FAS2* if fatty acid are not present in the medium. As long as Ole1p is active (i.e. oxygen is still present in the cell), the adequate balance between saturated and unsaturated fatty acids is still guaranteed in the cell. However, as soon as haem synthesis ceases consequently to oxygen depletion, fatty acid desaturation by Ole1p is precluded whereas FAS is still abundant and active in the absence of fatty acid supplementation. We propose that the function of Sfh2p is to inhibit FAS under this condition and that this fine tuning of FAS by Sfh2p would allow the cell to prevent the deleterious accumulation of saturated fatty acid. As a Sec14p related protein, Sfh2p contains a hydrophobic pocket that can potentially bind phospholipids. It is tempting to speculate that inhibition of FAS activity by Sfh2p might be controlled by a lipid bound form. Amongst the lipids that accumulate in a *hem1Δ sfh2Δ* in response to haem depletion and UFA starvation, PtdOH fulfils several criteria to be a candidate to accomplish this function. Indeed, not only does this lipid accumulate in response to haem and UFA depletion, but it is also highly enriched in SFA (see Table2). Moreover it is produced in lipid particles [27] i.e. a compartment where Sfh2p and Fas1p are mainly localised in response to haem depletion. Further work is needed in order to test this hypothesis.

SFH2 encodes a gene which was originally identified in a screen for multicopy suppressors of the double mutant *chs5 spa2* defective in chitin synthesis and cellular morphology (Santos and Snyder, 2000). It was also observed that it can physically interact with a thiol peroxidase type II isoform (cTPx II) *in vitro* [28] although the functional significance of this interaction is unclear with the function of Sfh2p as described in this report. Most interestingly, in addition to the sequence homology between Sfh2p and Sec14p, a protein involved in the regulation of phospholipid metabolism, it was observed that *SFH2* was also a multicopy suppressor of *SEC14* loss of function [10, 12]. A decrease in FAS activity might be relevant to understand the mechanism of suppression of *sec14Δ* by *SFH2* overexpression. Indeed, we observed an increase in FAS activity in a *sec14-1* mutant at the restrictive temperature which was restored in response to *SFH2* overexpression (unpublished observations). Sec14p controls PtdCho metabolism by inhibiting Pct1p, the rate-limiting enzyme of the CDP-choline pathway for PtdCho synthesis. Recently, it was also demonstrated that Sec14p is involved in PtdCho degradation by stimulating Nte1p, a phospholipase B specific for PtdCho [29, 30]. Therefore, PtdCho level in yeast is controlled both at the level of its synthesis and at the level of its degradation. Interestingly, we observed that deletion of *SFH2* which stimulates FAS activity results in a significant accumulation of PtdCho in haem and oleic acid depleted medium (Figure 1). This suggests that FAS activity impinges on PtdCho biosynthesis and/or degradation. This view is supported by data obtained from CHO cells demonstrating that inhibition of FAS activity by the antibiotic cerulenin impairs PtdCho biosynthesis [31, 32]. These authors observed that this effect is mediated by an inhibition of Pct1p. Although it is tempting to speculate that in yeast, fatty acid synthesis inhibition, induced by Sfh2p, could lead to Pct1p inhibition and thus facilitate a by-pass Sec14p phenotype, this view is not fully supported by experimental data. Indeed, *SFH2* overexpression in the absence of *SEC14* does not seem to affect PtdCho degradation [29]. However, a significantly diminished rate of choline uptake, an accumulation of cytoplasmic choline, and an altered P-Cho:Cho ratio in the cells toward a lower amount of P-Cho versus Cho were observed in response to *SFH2* overexpression in a *sec14Δ* mutant. This suggests that the metabolic flux in the Kennedy pathway is diminished, but not through inhibition of Pct1p (which would then result in an increase of P-Cho at the expense of choline). One attractive possibility would be that this reduced flux is a consequence of the limited availability of the substrates and more specifically of reduced synthesis of DAG as a consequence of FAS inhibition. The observation that DAG also accumulates in the absence of *SFH2*, in haem and oleic acid depleted medium (see Figure 1), is consistent with this hypothesis.

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Figure Legends

Figure 1. Oleic acid starvation and haem depletion induce accumulation of specific lipids in the absence of functional *SFH2*.

Haem-induced lipid starved conditions, as described under Experimental procedures, were realized for 16 hours with the control strain MRY72 (*hem1Δ*) and the mutant strain MRY82-2 (*hem1Δ sfh2Δ*). (A) Fatty acids derived from phospholipids were quantified from control strain grown in aerobiosis to stationary phase in the presence of δ -aminolevulinic acid or 16 hours after a shift in complete medium in the absence of δ -aminolevulinic and oleic acid. (B) Same as above except that fatty acids derived from neutral lipids and free fatty acids (FFA) were quantified. For (A) and (B), data are expressed as nmol of fatty acid per 10^9 cells $\pm$ SD and represent the average of at least two independent experiments.

Figure 2. Increased amount of saturated fatty acids in haem depleted conditions and in the absence of oleate is observed in the *sfh2Δ*.

Yeast strains MRY72 (*hem1Δ*) and MRY82-2 (*hem1Δ sfh2Δ*) were cultivated in complete medium supplemented with δ -aminolevulinic acid to stationary phase. At time zero, cells were washed and re-inoculated in complete medium lacking δ -aminolevulinic and oleic acid (haem induced UFA starvation). (A) Total amount of fatty acid, (B) saturated fatty acids and (C) unsaturated fatty acids were quantified as a function of time after the shift. The data represent the average $\pm$ SD of at least two independent experiments.

Figure 3. Oleic acid starvation and haem depletion impair growth of the *hem1Δ sfh2Δ* mutant.

Yeast strains MRY72 (*hem1Δ*) (open symbols) and MRY82-2 (*hem1Δ sfh2Δ*) (filled symbols) were grown in complete medium containing δ -aminolevulinic acid to stationary phase. At time 0, they were harvested, washed with distilled water, and 10^6 cells were inoculated per ml of YPD medium with (square) or without (circle) (oleic acid). Growth was measured by optical density at 600nm. Vertical arrow indicates the time of induction of hypoxic gene expression using the promoter of *DANI* fused to *lacZ* as a reporter gene [1].

Figure 4. TAG and SE are efficiently mobilized in the absence of *SFH2*.

Yeast strains MRY72 (*hem1Δ*) and MRY82-2 (*hem1Δ sfh2Δ*) were cultivated in complete medium supplemented with δ -aminolevulinic acid to stationary phase in the presence of $1.5 \mu\text{Ci}\cdot\text{ml}^{-1}$ of [^3H] palmitic acid. At time zero, cells were washed and re-inoculated into non radioactive complete

medium lacking δ -aminolevulinic and oleic acid. **(A)** Radioactivity in TAG at time zero, 6 hours, 8 hours and 16 hours after the shift. **(B)** Radioactivity in SE at time zero, 6 hours, 8 hours and 16 hours after the shift. Data are expressed as percentage of total label within lipids at each time-point. The data represent the average $\pm$ SD of at least two independent experiments. **(C)** Yeast strains MRY72 (*hem1 Δ*), MRY82-2 (*hem1 Δ sfh2 Δ*), TDY23-e6 (*hem1 Δ are1 Δ are2 Δ dgal Δ Iro1 Δ*), TDY23-c4 (*hem1 Δ sfh2 Δ are1 Δ are2 Δ dgal Δ Iro1 Δ*) were grown in complete medium containing δ -aminolevulinic acid to stationary phase. At time 0, there were harvested, washed with distilled water, and 10^6 cells were inoculated per ml of YPD medium. Growth was measured by optical density at 600nm. Vertical arrow indicates the time of induction of hypoxic gene expression using the promoter of *DAN1* fused to *lacZ* as a reporter gene [1].

Figure 5. The mutant *hem1 Δ sfh2 Δ* is hypersensitive to SFA.

Yeast strains MRY72 (*hem1 Δ*) and MRY82-2 (*hem1 Δ sfh2 Δ*) were cultivated in complete medium supplemented with δ -aminolevulinic acid to stationary phase. Cells were washed and re-inoculated in complete medium supplemented with the indicated amount of Tween 40 (as a source of palmitic acid) or Tween 80 (as a source of oleic acid). The optical density of the culture was measured 16 hours after the shift.

Figure 6. Oleic acid mediated control of *FAS2* transcription.

Reporter plasmid pBF16 (*FAS2-lacZ ARS CEN TRP1*) was transformed into MRY82-2 (containing plasmid pJU1 (*SFH2*) or control vector (Yep352)). Transformants were inoculated in minimal medium supplemented with δ -aminolevulinic acid to stationary phase. Then, they were inoculated in complete medium with supplementation as indicated for 6 hours. Cells were collected, washed and β -galactosidase activity was measured. Data are the value $\pm$ SD of at least two experiments. Tween 80 (as a source of oleic acid) and Tween 40 (as a source of palmitic acid) were provided at a concentration of 1% (v/v).

Figure 7. The mutant *hem1 Δ sfh2 Δ* does not appropriately control fatty acid synthase activity in haem and oleic acid depleted medium.

Yeast strains MRY72 (*hem1 Δ*) and MRY82-2 (*hem1 Δ sfh2 Δ*) were processed as described in Figure 3. Specific fatty acid synthase activity was measured as a function of time following the shift in δ -aminolevulinic acid and oleic acid depleted medium. Specific activity is expressed as nmol NADPH

oxidized per minute per mg of protein ($\text{mU} \cdot \text{mg}^{-1}$). Values are the mean $\pm$ SD of at least two experiments.

Figure 8. Controlled expression of *FAS1* suppresses *sfh2Δ* phenotype.

Yeast strains TDY29-3a (*hem1Δ fas1Δ* [pCF8]), TDY29-6a (*hem1Δ fas1Δ sfh2Δ* [pCF8]), MRY72 (*hem1Δ*) and MRY82-2 (*hem1Δ sfh2Δ*) were grown in complete medium containing δ -aminolevulinic acid to stationary phase, harvested, washed with distilled water. Cells ($2 \cdot 10^5$ cells. ml^{-1}) were inoculated in complete medium containing doxycycline as indicated. (A) Optical density reached by the culture was measured 40 hours after the shift. (B) and (C) FAS activity was measured 40 hours after the shift of the corresponding strains. Values are the mean $\pm$ SD of two independent experiments.

Figure 9. Recombinant Sfh2p inhibits fatty acid synthase activity *in vitro*.

Cellular extracts were prepared from strain MRY82-2 (*hem1Δ sfh2Δ*) were processed as described in Figure 3 and incubated for 6 hours in δ -aminolevulinic acid and oleate depleted medium. Fatty acid synthase activity was measured one minute after the addition of variable amount of affinity purified recombinant MBP-Sfh2p. Activity is expressed as $\text{mU} \cdot \text{mg}^{-1}$ protein and is the mean $\pm$ SD of two independent experiments.

Figure 10. Sfh2p and Fas1p can both localize to lipid particles.

Yeast strain TDY36-1d (expressing GFP-Sfh2p and Fas1p-3xHA) was grown to stationary phase in the presence of δ -aminolevulinic acid (A, B) and processed as described in Experimental, or further inoculated and cultivated for 6 hours in complete medium in the absence of oleic acid supplementation (C), or in complete medium containing δ -aminolevulinic acid (D). Separate images of GFP fluorescence (left panel, A), or immunodecorated Fas1p-3xHA (left panel B, C and D) and Nile red fluorescence (middle panel A, B, C and D) were captured from the same set of cells. No fluorescence bleed was detected between each channel. The right panel represents transmission images of the cells.

Table legends

Table 1. Yeast strains, genotype and source

Table 2. Percentage of SFA following a shift to δ -aminolevulinic and oleic acid depleted medium of *hem1 Δ* and *hem1 Δ sfh2 Δ* .

Yeast strains MRY72 (*hem1 Δ*) and MRY82-2 (*hem1 Δ sfh2 Δ*) were grown to stationary phase in the presence of δ -aminolevulinic acid, harvested, and inoculated in complete medium lacking δ -aminolevulinic and oleic acid. Phospholipids and neutral lipids were extracted from the cells immediately after the shift (T = 0h) or 16 hours after the shift (T= 16h). The percentage of saturated fatty acids versus unsaturated fatty acids of each lipid species was obtained as described in Experimental procedures. Values are the means $\pm$ SD of at least two independent experiments.

TABLE 1.

Strains	Genotype	Source
FY1679 α	<i>MATα, leu2, his3, trp1, ura3</i>	Progeny of diploid FY1679
Y25289	<i>MATα / MATα SFH2 / sfh2::kanMX4</i> <i>his3-Δ1 / his3-Δ1 leu2-Δ0 / leu2-Δ0</i> <i>lys2-Δ0 / LYS2 MET15 / met15-Δ0</i> <i>ura3-Δ0 / ura3-Δ0</i>	[33] Euroscarf
MRY73-6b	<i>MATα his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0</i>	This study (progeny of Y25289)
MRY73-6a	<i>MATα sfh2::kanMX4 his3-Δ1 leu2-Δ0 lys2-Δ0</i> <i>ura3-Δ0</i>	This study (progeny of Y25289)
FYH04	<i>MATα hem1::LEU2 his3 leu2 trp1 ura3</i>	[34]
MRY82-2	<i>MATα sfh2::kanMX4 hem1::LEU2 his3 leu2</i> <i>lys2-Δ0 trp1 ura3 lys2-Δ0 met15-Δ0</i>	This study (progeny of MRY73-6a x FYH04)
MRY72	<i>MATα hem1::LEU2 leu2 ura3</i>	This study
H1246	<i>MATα are1-Δ::HIS3 are2-Δ::LEU2</i> <i>dga1-Δ::kanMX4 Iro1-Δ::TRP1 ura3</i> <i>leu2 his3</i>	[20]
V83B2	<i>MATα / MATα ade2-101 / ade2-101</i> <i>can1-R / CAN1 cry1R / CRY1 FAS1 / 3xHA-FAS1</i> <i>CYH2 / cyh2R HIS3 / his3-200 leu2-98 / leu2-98</i> <i>lys2-801 / lys2-801 trp1-1 / TRP1</i> <i>ura3-52 / ura3-52</i>	TRIPLES Database
Y25032	<i>MATα / MATα his3-Δ/1 his3-Δ leu2-Δ0 / leu2-Δ0</i> <i>lys2-Δ0 / LYS2 MET15/met15-Δ0</i> <i>ura3-Δ0 / ura3-Δ0 YKL182w::kanMX4/YKL182w</i>	EUROSCARF
TDY29-6a	<i>MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0</i> <i>YKL182w::kanMX4 hem1::LEU2</i>	This study
TDY29-3a	pCF8 <i>MATα his3-Δ1 leu2-Δ0 lys2-Δ0 trp-1Δ ura3-Δ0</i> <i>YKL182w::kanMX4 sfh2::KanMX4 hem1::LEU2</i>	This study
V83B2-d	pCF8 <i>MATα leu2-98 ade2-101 ura3-52 lys2-801</i> <i>trp1-1 3xHA-FAS1</i>	This study (progeny of V83B2)
TDY33-3a	<i>MATα leu2 ade2 ura3 lys2 his3 trp1 hem1::LEU2</i> <i>3xHA-FAS1</i>	This study (progeny of MRY82-2 x V83B2-d)
TDY33-2d	<i>MATα leu2 ura3 lys2 his3 trp1 hem1::LEU2</i>	This study (progeny of

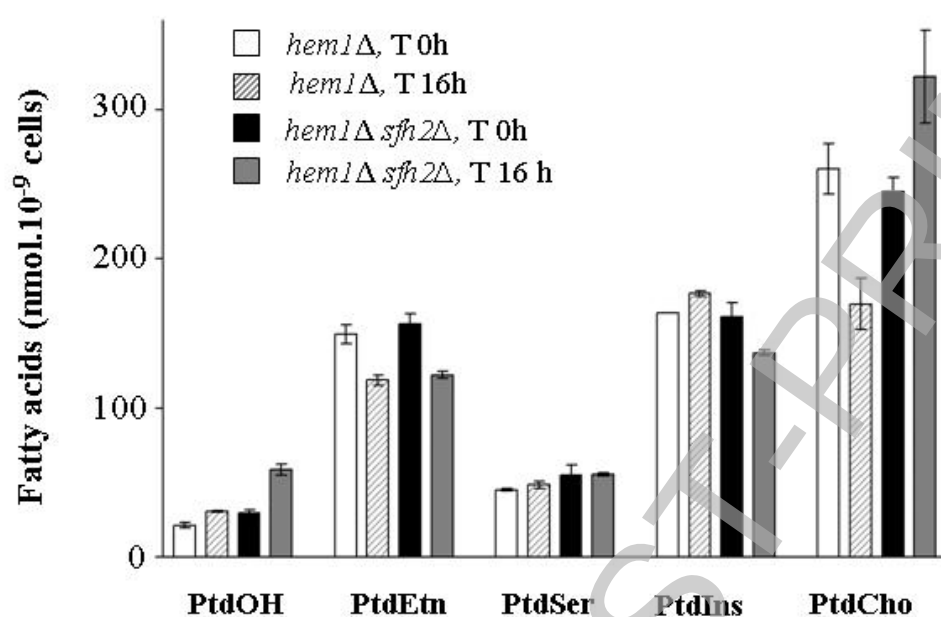
TDY36-1d	<i>3xHA-FAS1 sfh2::kanMX4</i> <i>MATa his3 leu2 lys2 ura3 trp1 sfh2::kanMX4</i>	MRY82-2 x V83B2-d) This study progeny of
TDY23-e6	<i>3xHA-FAS1 hem1::LEU2 pCG4GFP</i> <i>MATa hem1::LEU2 are1- Δ::HIS3 are2- Δ::LEU2</i> <i>dga1- Δ::kanMX4 Iro1- Δ::TRP1 ura3</i>	YPCG4-G9d x TDY33-2d This study (progeny of MRY82-2 x H1246)
TDY23-c4	<i>leu2 his3 trp1</i> <i>MATa hem1::LEU2 sfh2::kanMX4 are1- Δ::HIS3</i> <i>are2- Δ::LEU2 dga1- Δ::kanMX4 Iro1- Δ::TRP1</i>	This study (progeny of MRY82-2 x
YPCG4-G9d	<i>his3 ura3 trp1 leu2</i> <i>MATa sfh2::kanMX4 his3-Δ1 leu2-Δ0 lys2-Δ0</i> <i>ura3-Δ0 [pCG4GFP]</i>	H1246) This study

TABLE 2.

Percentage of saturated fatty acid in lipid				
	T = 0h		T = 16h	
	<i>hem1Δ</i>	<i>hem1Δ sfh2Δ</i>	<i>hem1Δ</i>	<i>hem1Δ sfh2Δ</i>
PtdCho	14.7 ± 0.3	16.2 ± 1.9	64.2 ± 5.5	68.2 ± 1.9
PtdEtn	20.0 ± 0.8	33.7 ± 1.7	50.6 ± 2.6	49.9 ± 1.1
PtdSer	32.9 ± 2.2	34.9 ± 1.1	39.6 ± 0.9	27.7 ± 2.7
PtdIns	44.1 ± 0.7	40.4 ± 3.8	60.0 ± 1.4	71.6 ± 4.7
PtdOH	35.7 ± 3.4	47.7 ± 0.4	46.3 ± 1.9	72.4 ± 0.6
DAG	29.1 ± 2.7	24.9 ± 1.1	81.6 ± 0.3	84.0 ± 0.4
TAG	29.1 ± 0.1	30.3 ± 0.8	29.2 ± 2.6	64.5 ± 2.2
FFA	45.7 ± 0.9	43.4 ± 2.5	34.4 ± 1.2	41.4 ± 1.4
SE	14.4 ± 0.5	12.2 ± 1.6	53.6 ± 0.8	68.6 ± 2.6

Figure 1

A



B

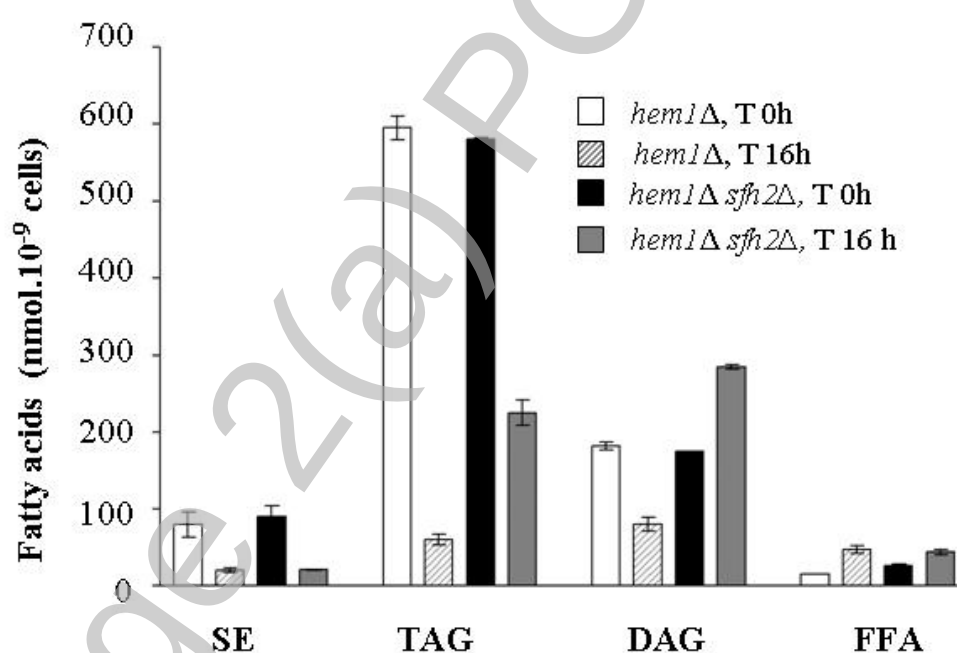


Figure 2

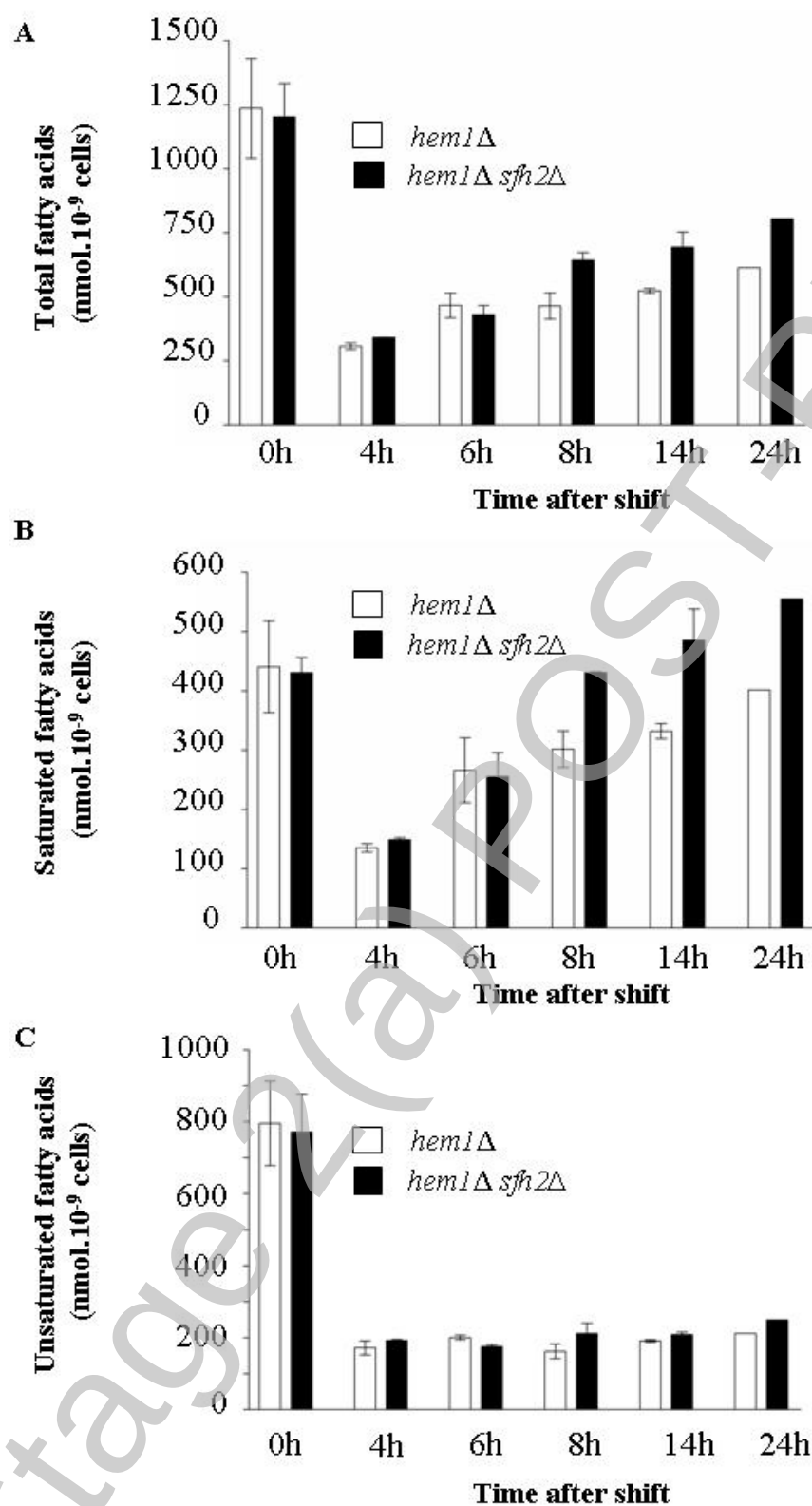


Figure 3

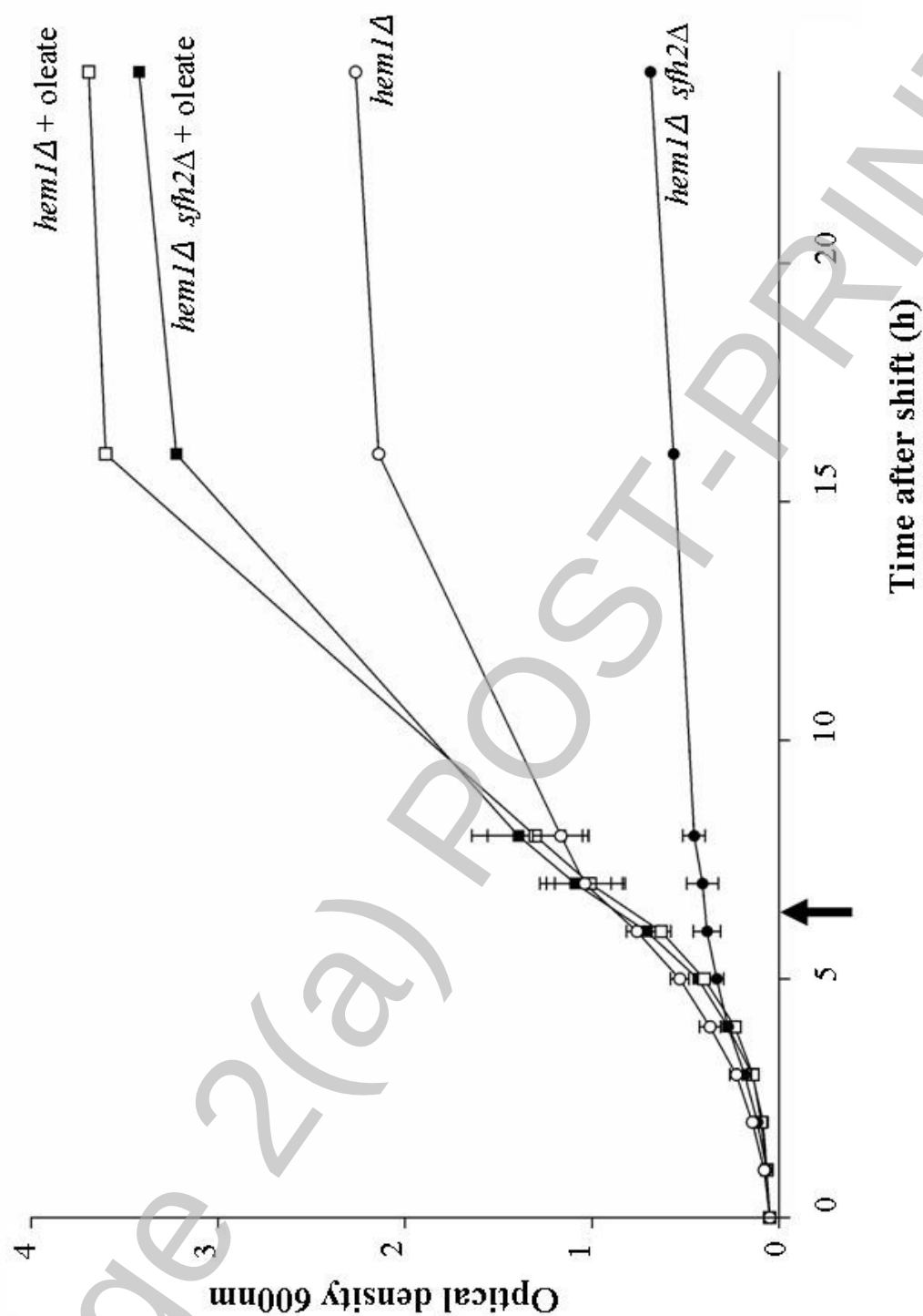


Figure 4 A, B

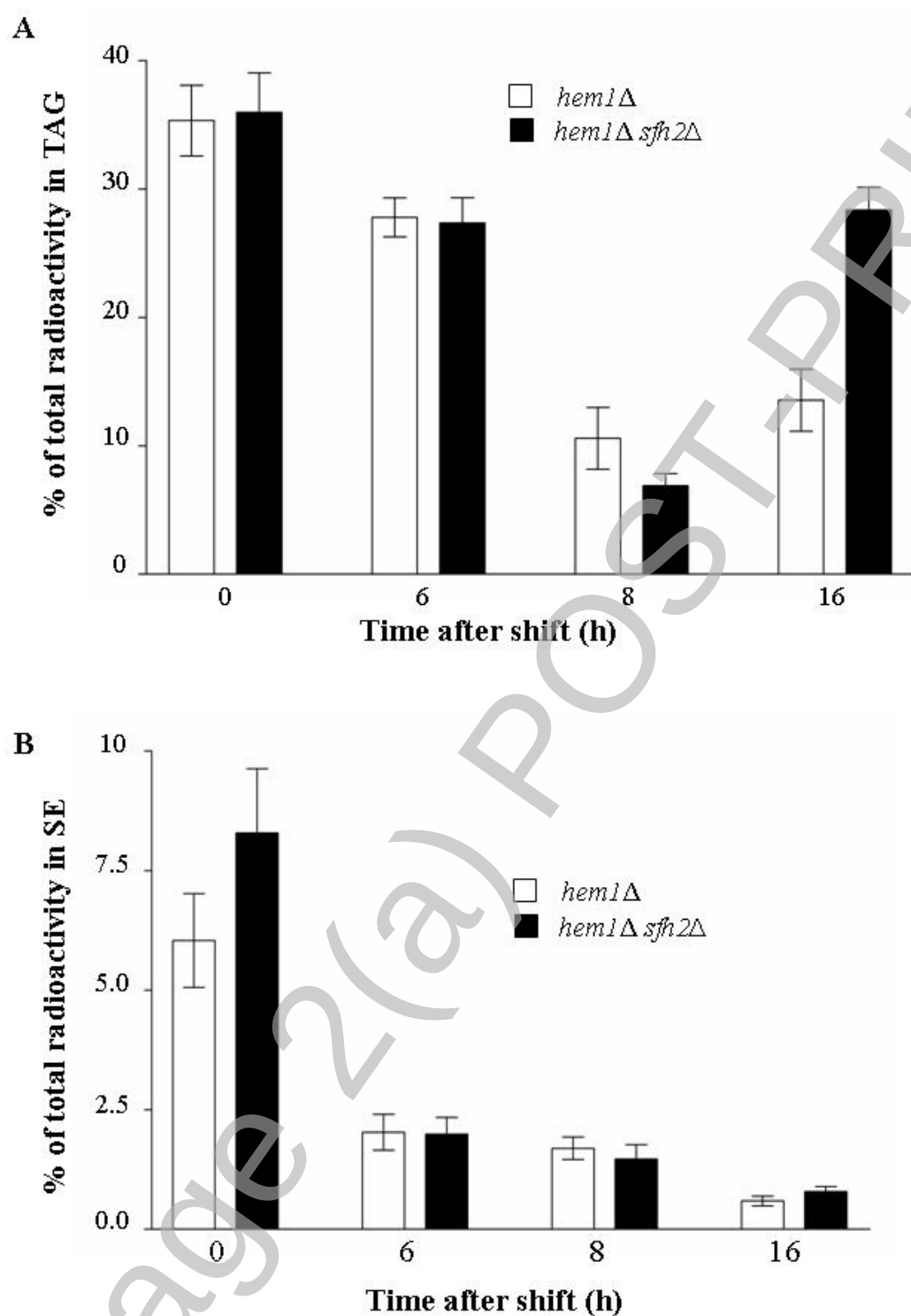


Figure 4 C

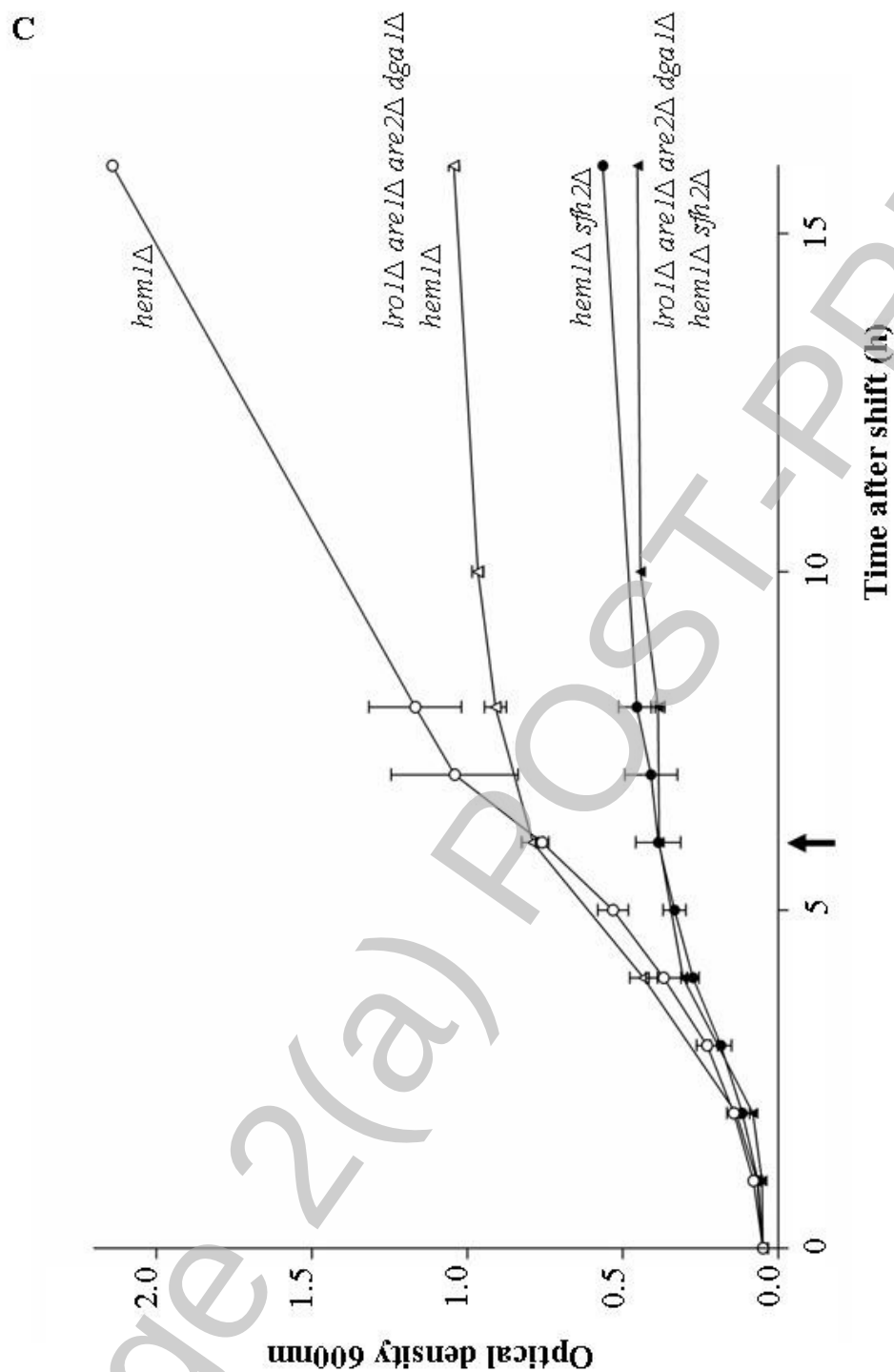


Figure 5

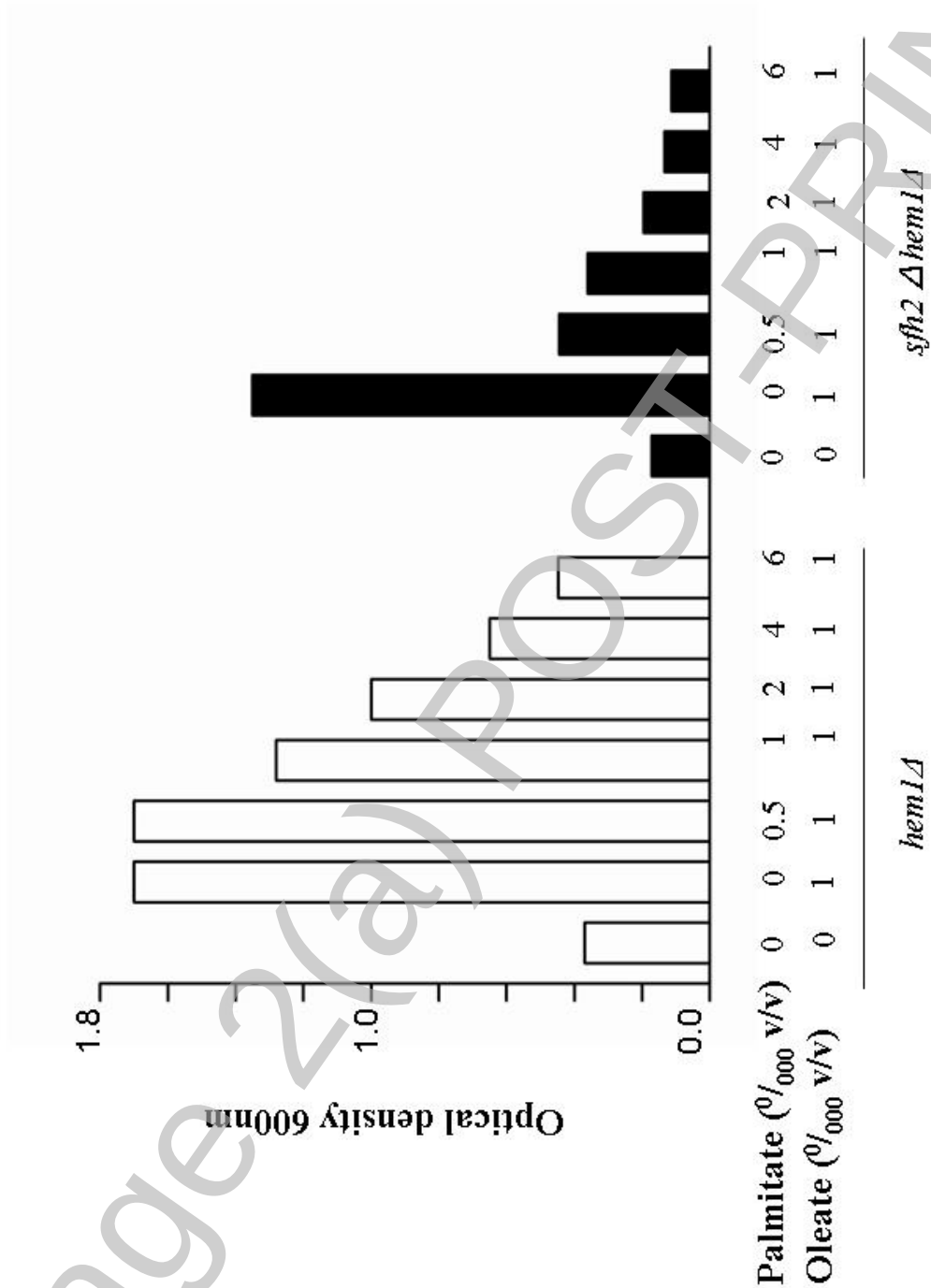


Figure 6

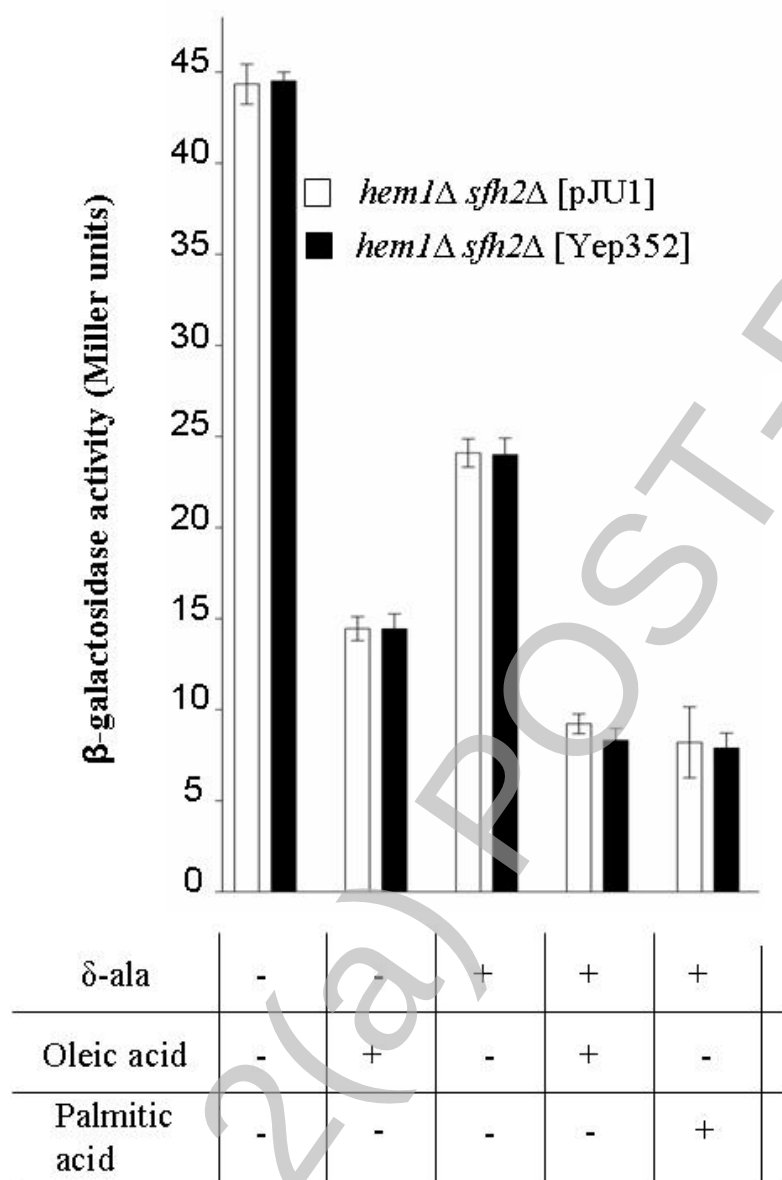


Figure 7

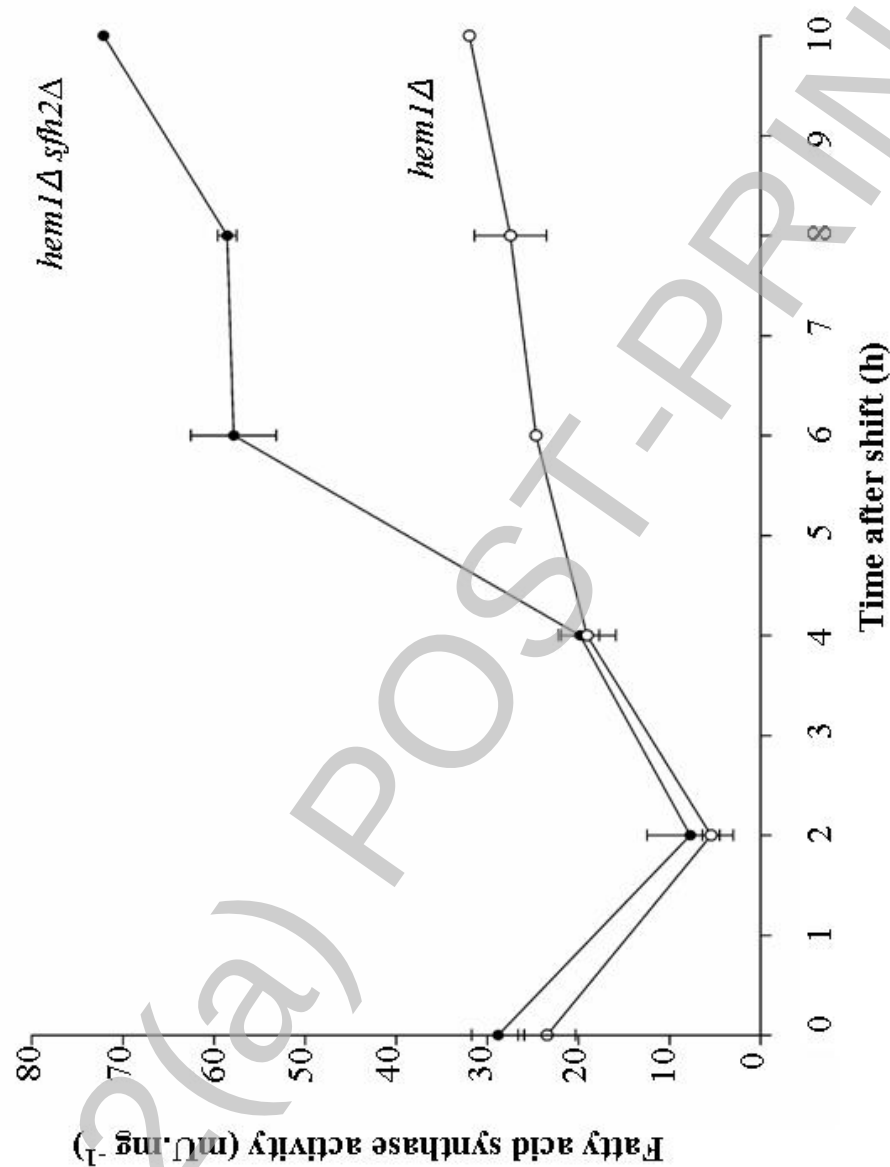


Figure 8

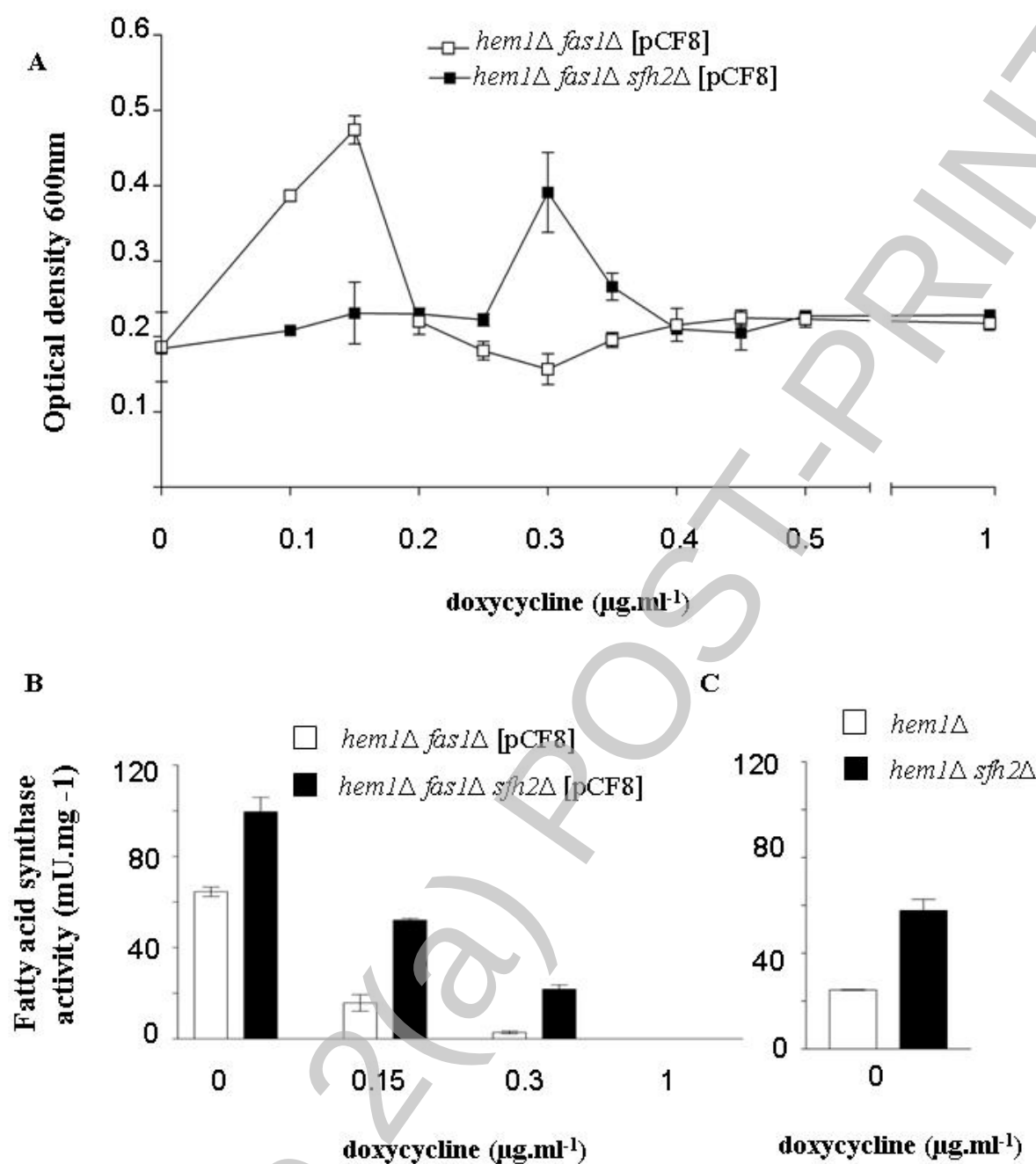


Figure 9

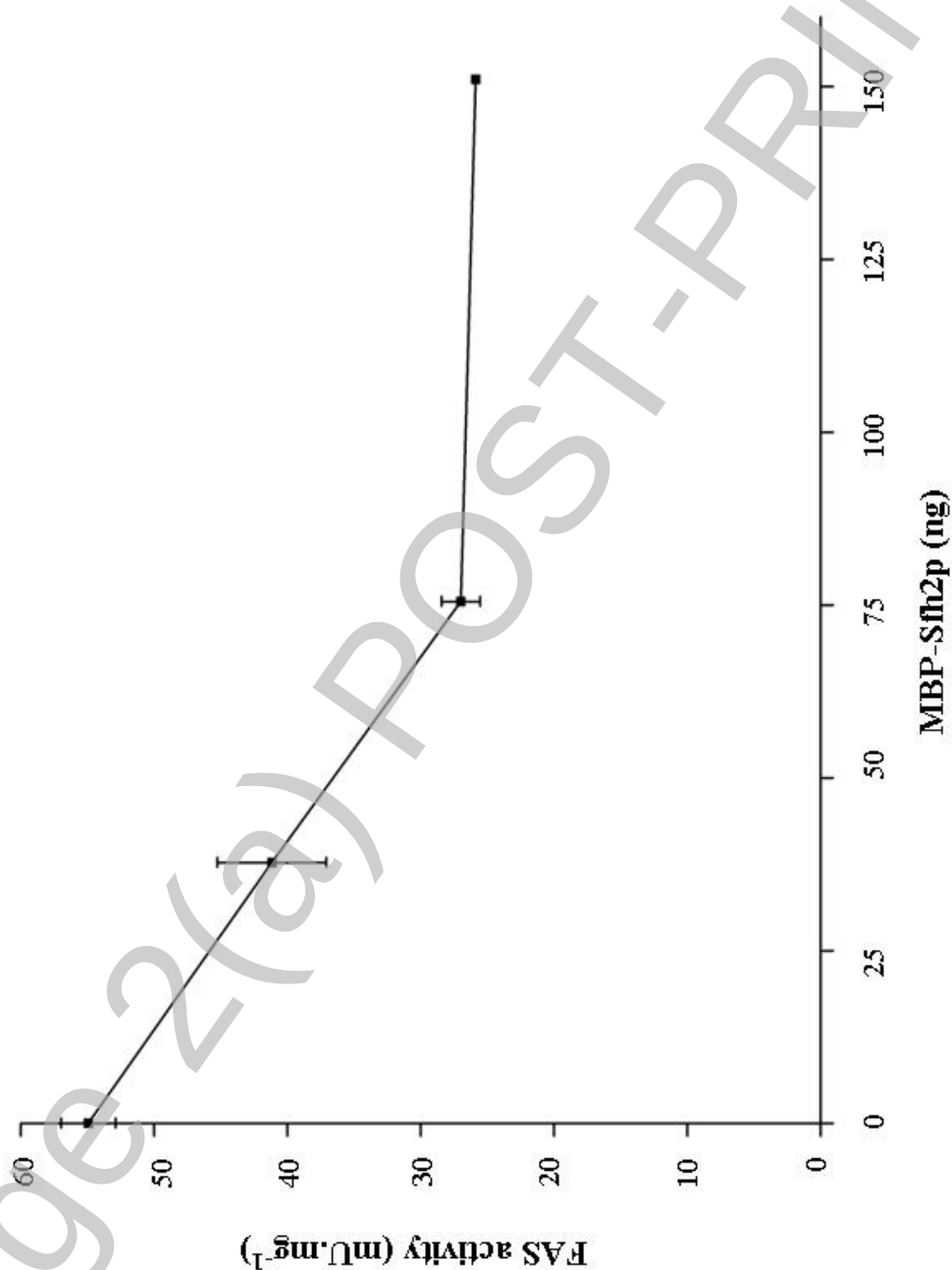


Figure 10

