Regulation of yeast fatty acid desaturase in response to iron deficiency

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Abstract

Unsaturated fatty acids (UFA) are essential components of phospholipids that greatly contribute to the biophysical properties of cellular membranes. Biosynthesis of UFAs relies on a conserved family of iron-dependent fatty acid desaturases, whose representative in the model yeast *Saccharomyces cerevisiae* is Ole1. *OLE1* expression is tightly regulated to adapt UFA biosynthesis and lipid bilayer properties to changes in temperature, and in UFA or oxygen availability. Despite iron deficiency being the most extended nutritional disorder worldwide, very little is known about the mechanisms and the biological relevance of fatty acid desaturases regulation in response to iron starvation. In this report, we show that endoplasmic reticulum-anchored transcription factor Mga2 activates *OLE1* transcription in response to nutritional and genetic iron deficiencies. Cells lacking *MGA2* display low UFA levels and do not grow under iron-limited conditions, unless UFAs are supplemented or *OLE1* is overexpressed. The proteasome, E3 ubiquitin ligase Rsp5 and the $Cdc48^{Npl4/Ufd1}$ complex are required for *OLE1* activation during iron depletion. Interestingly, Mga2 also activates the transcription of its own mRNA in response to iron deficiency, hypoxia, low temperature and low UFAs. *MGA2* up-regulation contributes to increase *OLE1* expression in these situations. These results reveal the mechanism of *OLE1* regulation when iron is scarce and identify the *MGA2* auto-regulation as a potential activation strategy in multiple stresses.

Highlights (3-5 sentences with max. 85 characters including spaces)

1) Mga2 activates *OLE1* Δ9-FA desaturase transcription in response to iron deficiency

2) *OLE1* activation by Mga2 is vital under low iron conditions unless UFAs are added

3) Defects in UFA biosynthesis end in *OLE1* activation in response to low iron conditions

4) *OLE1* up-regulation by low iron requires the proteasome, Rsp5, Npl4 and Ufd1 proteins

5) Mga2 activates its own transcription in response to low iron, temperature and oxygen

Keywords: yeast, *Saccharomyces cerevisiae*, iron deficiency, fatty acids, Ole1, Mga2, hypoxia, cold.

Abbreviations: BPS, bathophenanthroline disulfonic acid disodium; ChIP, chromatin immunoprecipitation; ER, endoplasmic reticulum; FA, fatty acid; FAS, ferrous ammonium sulfate; Pol II, polymerase II; OD, optical density; RT-qPCR, Reverse Transcription-quantitative real time Polymerase Chain Reaction; SFA; saturated fatty acid; TM, transmembrane; TR, transcription rate; UFA; unsaturated fatty acid.

1. Introduction

Cellular membranes are an essential requirement for life. In eukaryotic organisms, membranes represent selective and dynamic boundaries from the environment and between different intracellular compartments. Biological membranes are composed mostly of proteins and lipids, and glycerophospholipids have a major impact on their biophysical properties. The composition of membranes varies among organisms, cellular types and intracellular organelles to achieve defined properties and functions. Cells adjust the proportion of the saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs) of their lipid bilayers in response to multiple environmental stresses to maintain proper fluidity, lipid packing and water permeability. Defects in UFA production profoundly reorganize organelle abundance and morphology, which can lead in extreme cases to cell death. Furthermore, UFA overproduction may be harmful, limit cell division and cause necrosis (reviewed in (Ballweg and Ernst, 2017)). Therefore, UFA biosynthesis has to be tightly regulated. In humans, lipid metabolism deregulation has been linked to obesity-induced morbidity, type II diabetes, schizophrenia, Alzheimer's disease and Parkinson's disease (Aguilar and de Mendoza, 2006; Adiphatla and Hatcher, 2007).

The budding yeast *Saccharomyces cerevisiae* has been used as a reliable model organism to study multiple aspects of eukaryotic lipid biology (Singh, 2017). The yeast Ole1 Δ9-fatty acid desaturase (the denoted SCD family in mammals) is a conserved iron- and oxygen-dependent enzyme anchored to the endoplasmic reticulum (ER) membrane that catalyzes the irreversible *de novo* biosynthesis of mono-UFAs [palmitoleic (16:1) and oleic (18:1)], which represent more than 70% of total fatty acids (FAs), from the

corresponding CoA-activated SFAs [palmitic (16:0) and stearic (18:0)]. Ole1 function is essential in yeast, unless the medium is supplemented with UFAs (reviewed in (Aguilar and de Mendoza, 2006; Covino et al., 2016; Martin et al., 2007)). *OLE1* expression is highly regulated by numerous stimuli, including carbon source, extracellular FAs, temperature, oxygen levels and metal ions. Thus *OLE1* expression increases in response to low oxygen and low temperature, but is suppressed by UFA supplementation (Chellappa et al., 2001; Hoppe et al., 2000; Kwast et al., 1999; Nakagawa et al., 2002; Vasconcelles et al., 2001). Two homologous and partially redundant transcription factors, Spt23 and Mga2, regulate *OLE1* expression. They are synthesized as inactive ~120 KDa homodimeric precursors anchored to the ER membrane through their carboxy-terminal transmembrane (TM) helix domain. Proteolytic cleavage releases a ~90 KDa amino-terminal fragment that translocates to the nucleus and activates *OLE1* transcription in a response denoted as the OLE pathway (Hoppe et al., 2000; Piwko and Jentsch, 2006). Although some differences exist, the activation of both transcription factors requires specific ubiquitylation by E3 ligase Rsp5, processing by the proteasome, and mobilization from the ER membrane with the help of substrate-recruiting factor Ubx2 and segregase/chaperone complex $Cdc48^{Npl4/Ufd1}$ (Chellappa et al., 2001; Hitchcock et al., 2001; Hoppe et al., 2000; Kolawa et al., 2013; Rape et al., 2001; Shcherbik and Haines, 2007; Shcherbik et al., 2003; Surma et al., 2013). Whereas the *mga2Δ* and *spt23Δ* single mutants are viable, the *mga2Δspt23Δ* double mutant is lethal unless UFAs are added to the growth medium (Chellappa et al., 2001; Zhang et al., 1999). Although each transcription factor is sufficient for *OLE1* expression, Mga2 is the

dominant factor that activates *OLE1* in response to hypoxia, low temperature, cobalt and nickel (Chellappa et al., 2001; Jiang et al., 2002; Jiang et al., 2001; Nakagawa et al., 2002). Recent results have demonstrated that dimeric Mga2 functions as the membrane sensor for lipid saturation (Covino et al., 2016). Specifically, Mga2 uses the rotational orientation of its transmembrane helix to sense lipid packing in the ER and to control *OLE1* transcription. Thus a high proportion of ER membrane SFAs promotes an Mga2 TM helix rotational orientation that activates the OLE pathway, while an increase in UFA abundance stabilizes inactive Mga2 (Covino et al., 2016).

Budding yeast uses two partially redundant transcription factors, Aft1 and Aft2, to activate the transcription of a group of genes, collectively referred to as the iron regulon, in response to iron deficiency. The iron regulon includes metalloreductases that reduce extracellular Fe^{3+} to its more soluble Fe^{2+} form, and the high-affinity iron transport system composed of multicopper ferroxidase Fet3 and iron-permease Ftr1 (reviewed in (Kaplan and Kaplan, 2009; Sanvisens and Puig, 2011)). Yeast cells also express low-affinity iron transporters, such as Fet4 and Smf1. Despite oxo-diiron being an indispensable cofactor for the catalytic activity of Δ9-fatty acid desaturase Ole1, very little is known about the mechanisms that regulate these enzymes in response to alterations in iron bioavailability and how it affects UFA biosynthesis. We have previously reported that addition of the Fe^{2+}-specific chelator bathophenanthroline disulfonic acid disodium (BPS) to growth medium allows *OLE1* transcript levels to increase (Puig et al., 2005). In this study, we present evidence to support that Mga2 activates *OLE1* transcription in response to iron limitation. This regulatory mechanism is essential for growth under iron-deficient

conditions unless UFAs are added to the medium or *OLE1* is ectopically expressed. In mechanistic terms, ER-embedded Mga2 transcription factor activation by iron depletion seems a consequence of alterations in the relative FA composition of yeast membranes. Interestingly, we also reveal an Mga2 transcriptional auto-regulatory mechanism in response to iron deficiency, hypoxia and low temperatures or UFAs that contributes to enhance *OLE1* expression.

2. Material and Methods

2.1. Yeast strains, culture conditions, and plasmids

The yeast strains used in this study are listed in Table 1. We used the pFA6a-13Myc-KanMX6 plasmid as a template (Longtine et al., 1998), and specific oligonucleotides, to generate an integrative cassette for tagging the genomic copy of *OLE1* with the 13xMyc epitope at the carboxyl terminus (SPY901 yeast strain). Yeast precultures were incubated overnight at 30°C in liquid synthetic complete SC medium [0.17% (w/v) yeast nitrogen base without amino acids and without ammonium sulfate (Pronadisa), 0.5% (w/v) ammonium sulfate (Panreac), 2% (w/v) glucose (Panreac), and 2 g/L Kaiser drop-out (Formedium)] lacking specific requirements whenever necessary, and reinoculated at an optical density at 600 nm (OD_{600}) of 0.2. To regulate iron availability in liquid cultures, cells were incubated for 6 h at 190 rpm in SC medium (+Fe) or SC supplemented with 100 μM BPS (Sigma) (–Fe). The Fe^{2+}-specific chelator ferrozine (Sigma) was used at the indicated concentrations to limit iron bioavailability in 2% agar (Pronadisa) solid media. Fatty acids (oleic

and linoleic acids, Sigma) were added to a final 1 mM concentration and stabilized with 1% Tergitol Nonidet P-40 (Sigma). To induce P_{GAL1}-*OLE1* expression, glucose in SC was replaced with 2% galactose. For the spot assays, yeast cells were grown to the exponential phase, spotted in 10-fold serial dilutions starting at on OD_{600} of 0.1, and incubated at 30°C for 3 days. The temperature sensitive strains were cultivated in SC at the permissive temperature of 25ºC and then transferred to the non permissive temperature of 37ºC. After 1 h at 37ºC, 100 µM BPS was added, (–Fe) or not (+Fe), and cells were incubated for 5 h more. With the *npl4-1* mutant, 30ºC was used as the restrictive temperature (Hitchcock et al., 2001). For growth at low temperatures, yeast cells were collected, transferred to a pre-cold medium at 10ºC and incubated for 1 h. Hypoxia was achieved by bubbling nitrogen at a constant pressure for 5 h.

All the plasmids used in this study are listed in Table 1. To construct the pSP1039 plasmid, 470 base pairs were amplified from the promoter region of the *MGA2* gene with oligonucleotides MGA2-470F-HindIII and MGA2-lacZ-PstI-R (see Table 2), and then the PCR product was digested with restriction enzymes HindIII (Roche) and PstI (Roche). The YCp33RNR4Z plasmid, previously digested with HindIII and PstI to remove the *RNR4* promoter, was used to clone the *MGA2* promoter fused to the *lacZ* reporter gene. PCR amplifications were performed with Phusion polymerase (Finnzymes), and the cloned insert was sequenced. One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen) were used to isolate and propagate plasmids.

Table 1. List of yeast strains and plasmids used in this study.

Strain or plasmid	Description	Source
Strains		
HTLU-2832-1B	W303 *MATa HIS3, TRP1, LEU2, URA3, ADE2, can1*	F. Cross
W303-1A	*MATa, ura3-1, ade2-1, trp1-1, his3-11,15, leu2-3,112*	E. Herrero
MML1088	W303-1A *aft1Δ5 aft2::KanMX4*	E. Herrero
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Invitrogen
SPY386	BY4741 *fet3::URA3 fet4::KanMX4*	(Sanvisens et al., 20
SPY824	BY4741 *mga2::KanMX4*	Invitrogen
SPY823	BY4741 *spt23::KanMX4*	Invitrogen
YWO0607	*MATa ura3 leu2-3,112 his3-11,15 Gal*$^{+}$	D. H. Wolf
YWO0608	YWO0607 *pre1-1*	D. H. Wolf
YWO1	*MATa his3-Δ200, leu2-3,112, lys2-801, trp1-1(am), ura3-52*	(Hoppe et al., 2000)
Y0356	YWO1 *rsp5::HIS3; ura3-52::RSP5::URA3*	(Hoppe et al., 2000)
Y0358	YWO1 *rsp5::HIS3; ura3-52::rps5-2::URA3*	(Hoppe et al., 2000)
PSY580	*MATa ura3-52 leu2Δ1 trp1Δ63*	(Hitchcock et al., 200
PSY2340	PSY580 *npl4-1*	(Hitchcock et al., 200
PSY3074	*MATa his4-519, ura3-52, ade1-100, leu2-3,112 ufd1-1*	(Hitchcock et al., 200
SPY901	BY4741 *OLE1-13xMyc::KanMX6*	This study

Plasmids

pRS316-P_{GAL1}-OLE1	CEN *URA3* P_{GAL1}-*OLE1*	(Hoppe et al., 2000)
pPS2364	CEN *URA3 MGA2*	(Hitchcock et al., 200
pPS2358	CEN *URA3 MGA2-truncated*	(Hitchcock et al., 200
YEplac181-3HA-MGA2	2μ *LEU2* 3HA-*MGA2*	(Hoppe et al., 2000)
YCp33RNR4Z	CEN *URA3* P_{RNR4}-*lacZ*	R. S. Zitomer
pSP1039	CEN *URA3* P_{MGA2}-*lacZ*	This study
pFA6a-13Myc-KanMX6	Integrative *KanMX6*	(Longtine et al., 1998

2.2. RNA analyses

Total RNA extraction and cellular mRNA levels were determined by RT-qPCR as previously described (Sanvisens et al., 2014). The primers used for RT-qPCR are listed in Table 2. The *OLE1* and *MGA2* transcription rates (TRs) were determined in triplicate by the Genomic Run-On (GRO) technique as previously reported (Garcia-Martinez et al., 2004). The ArrayStat software (Imaging Research, Inc.) was used to determine reproducibility and the mean of the GRO values.

Table 2. Oligonucleotides used in this work.

Name	***Sequence (from 5' to 3')***
OLE1-qPCR-F	TCGACAAGAAGGGAAACGAA
OLE1-qPCR-R	CATGGTTGTTCGGAGATGTG
MGA2-qPCR-F	ATCTGTTCCCGTTGTCTTGG
MGA2-qPCR-R	CCTCTTCCTCATAATCCTCTTCCT
PGK1-qPCR-F	AAGCGTGTCTTCATCAGAGTTG

PGK1-qPCR-R	CGTATCTTGGGTGGTGTTCC
OLE1-ChIP-prom-287F	CTCAGACACACCTATCCCTATTGT
OLE1-ChIP-prom-186R	AGCCAGGAGCCGATGATTT
FUS1-ChIP-prom-F	CATGTGGACCCTTTCAAAAC
FUS1-ChIP-prom-R	AGACAGCGCGAAAAGTGACA
MGA2-470F-HindIII	ACTGAAGCTTCATCAAGAGCGATTGGATGACAGTT
MGA2-lacZ-PstI-R	AATACTGCAGCATAACGAAATGTTCTGTTCGCCA AAATGA
OLE1-F2	TAGTAAGAGAGGTGAAATCTACGAAACTGGTAAG TTCTTTCGGATCCCCGGGTTAATTAA
OLE1-R1	ATTTATGATTTTTCAATTTTTTTTTATGGTAGTTGC AGTTGAATTCGAGCTCGTTTAAAC
OLE1+320R	CTATTGCTCCAGGGCCCAGAA
TEF-term:135F	CGACATCATCTGCCCAGAT

2.3. Protein analyses

Total protein extracts were obtained by using the alkali method (Kushnirov, 1998). Equal amounts of protein were resolved in SDS-PAGE gels and transferred onto nitrocellulose membranes. The primary antibodies used were anti-c-myc (9E10, Roche) and anti-Pgk1 (22C5D8; Invitrogen). Immunoblots were developed with horseradish peroxidase (HRP)-labeled secondary antibodies and the ECL Select Western blotting detection kit (GE Healthcare Life Sciences). Immunoblot images were obtained with an ImageQuant LAS 4000 mini Biomolecular Imager (GE Healthcare Life Sciences). Data were processed and Ole1-13Myc/Pgk1 protein levels were quantified with ImageQuant TL analysis software (GE Healthcare Life Sciences).

Chromatin immunoprecipitation (ChIP) assays were performed to determine RNA polymerase II binding to the *OLE1* and *FUS1* promoters as

previously described (Gomar-Alba et al., 2013). Cell extracts were incubated with Dynabeads Pan Mouse IgG (Invitrogen), previously bound to a monoclonal mouse anti-Rpb1 antibody (clone 8WG16, Covance). DNA was purified with the High Pure PCR Product Purification Kit (Roche). The primers used for the *OLE1* and *FUS1* promoter RT-qPCR analyses are listed in Table 2.

2.4. Determination of total fatty acids

The fatty acid methyl ester analysis was performed following a modified version of a method previously reported, which determines total fatty acids including free and esterified fatty acids of yeast cells (Borrull et al., 2015). First 1 mL of HCl 1.25 N in methanol, 10 µL heptanoic acid (C7, 1 g L^{-1}) and 10 µL heptadecanoic acid (C17, 4 g L^{-1}) were added to glass tubes that contained a yeast pellet of around $5 \cdot 10^8$ cells. Samples were heated to 90ºC for 60 min and were then cooled to room temperature. After cooling, 1 mL of NaCl 0.9% (w/v) in water and 300 µL hexane were added. The extraction was repeated twice. Between each extraction phase, tubes were centrifuged at 3000 x g for 5 min to allow the best phase separation. Analytical GC was carried out in an Agilent 5890 connected to an HP Vectra computer with the ChemStation software (Agilent Technologies). The extract (2 µL) was injected (splitless, 0.75 min) into an FFAP-HP column of 30 m x 250 µm x 0.25 µm phase thickness (Agilent Technologies) with an automatic injector (Agilent). The temperature program shifted from 100ºC to 240ºC (5 min) at 4ºC min^{-1}. Injector and detector temperatures were 220°C and 250°C, respectively. The carrier gas was helium, applied at 1.2 mL min^{-1}. Relative amounts of the given fatty acids were calculated from their respective chromatographic peak areas after normalization with internal standards (C7 or C17).

2.5. β-galactosidase assays

β-galactosidase activity was measured as previously described (Puig et al., 2004).

2.6. Statistical analyses

To evaluate statistical significance, tailed t-student tests were applied. The asterisk (*) indicates statistically significant differences with p-value < 0.05.

3. Results

3.1. *OLE1* is transcriptionally activated in response to iron deficiency.

The expression of *OLE1*, which encodes the yeast Δ9-fatty acid desaturase, increases upon the addition of iron chelators, such as bathophenanthroline disulfonic acid disodium (BPS) and 1,10-phenanthroline ((Puig et al., 2005; Vasconcelles et al., 2001); Figure 1A). To ascertain whether *OLE1* up-regulation was due to iron deficiency and not to a secondary effect of the iron chelators, we performed the following assays. Firstly, we added an excess of ferrous ammonium sulfate (FAS) to the growth medium that contained BPS in order to restore iron bioavailability. In this case, *OLE1* mRNA levels dropped to the levels observed for the iron-sufficient conditions (Figure 1A). Secondly, *OLE1* transcript levels were determined in the yeast *fet3Δfet4Δ* mutant, which lacked the Fet3 high-affinity and Fet4 low-affinity iron transporters. As shown in Figure 1A, the *fet3Δfet4Δ* cells grown under iron-sufficient conditions displayed higher *OLE1* mRNA levels compared to the wild-type cells grown under the same conditions. Thirdly, we assessed the state of hypoxia in yeast cells treated with BPS by determining the levels of two mRNAs, *TIR3* and *HEM13*, which are up-regulated in response to low oxygen (Ter Linde and Steensma, 2002; Kwast et al., 2002). *TIR3* encodes a cell wall mannoprotein required for growth under anaerobic conditions, and *HEM13* encodes the coproporphyrinogen III oxidase within the heme biosynthetic pathway. The mRNA levels of both genes increased in response to low oxygen, but not upon iron depletion (Figure 1B). Therefore, both hypoxia markers indicated that the addition of BPS did not lead to hypoxia. Finally, to ascertain

whether the increase in *OLE1* mRNAs turned into more Ole1 protein, we epitope tagged Ole1 with 13xMyc at its carboxyl terminus, and we determined its protein levels by Western blot in iron sufficient (+Fe) and iron deficient (-Fe) conditions. As shown in Figure 1C, Ole1 protein levels increased approximately 3.6-fold upon iron depletion. These results strongly suggest that *OLE1* expression specifically increases in response to both nutritional and genetic iron deficiencies.

To assess whether the up-regulation of the *OLE1* mRNA levels by iron scarcity takes place at the transcriptional level, the Genomic Run-On approach was followed, which determines the transcription rate (TR) of specific genes (Garcia-Martinez et al., 2004). For this purpose, the wild-type cells were cultivated in the presence of 100 μM of the Fe^{2+} chelator BPS for 6 h. Both the *OLE1* steady-state mRNA levels and TR increased while iron deficiency progressed, especially after 3 and 6 h of depletion (Figure 1D). These results demonstrate that *OLE1* becomes transcriptionally activated when iron bioavailability is restricted, which increases mRNA levels.

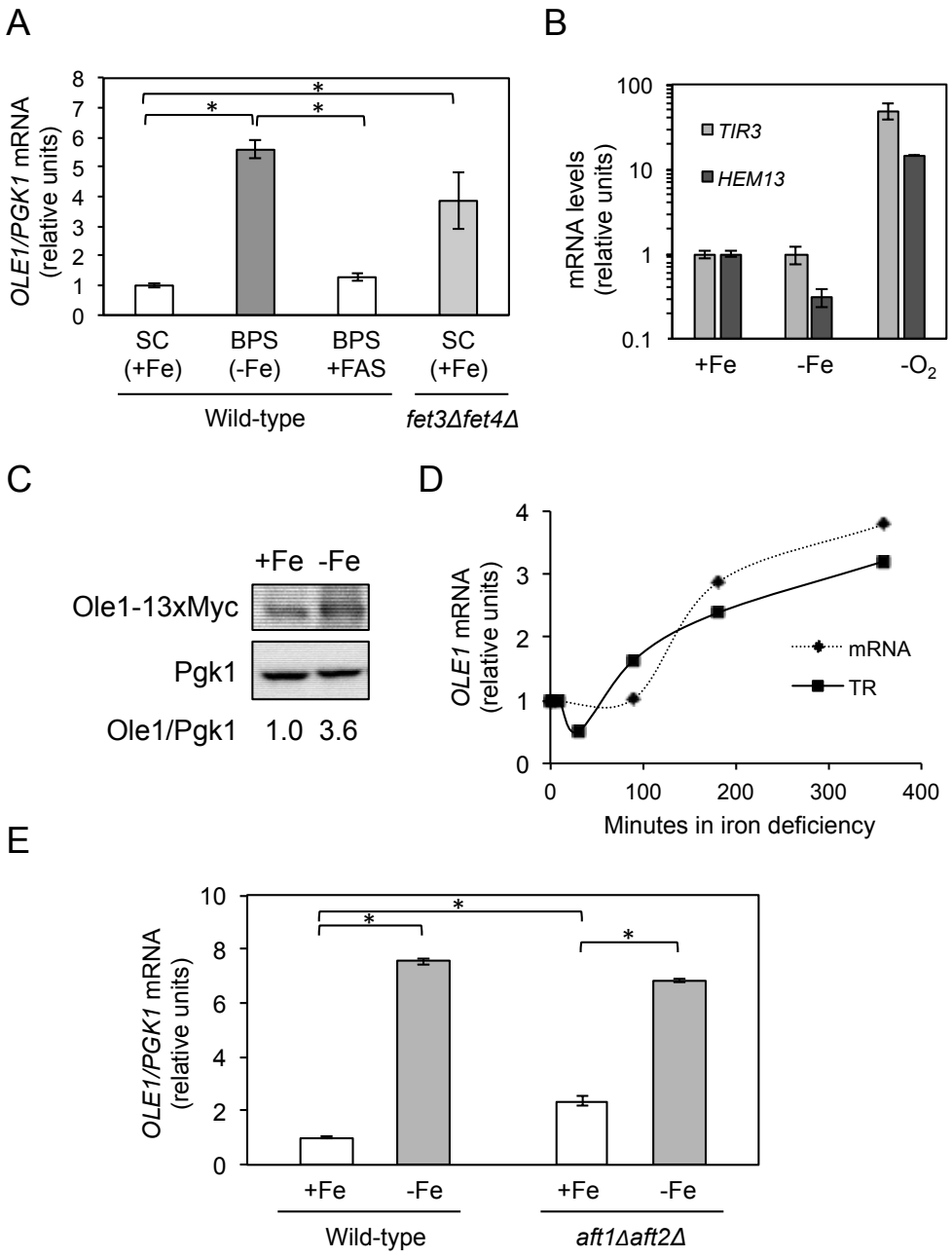

Figure 1. Regulation of the yeast *OLE1* mRNA and protein levels by iron bioavailability. (A) *OLE1* mRNA levels increased in response to nutritional and genetic iron deficiency. The wild-type (BY4741) and *fet3Δfet4Δ* (SPY386) yeast cells were grown at 30ºC for 6 h to the exponential phase in SC (+Fe), SC supplemented with 100 μM BPS (–Fe), and SC with 100 μM BPS and 300 μM ferrous ammonium sulfate or FAS (–Fe). Total RNA was extracted, and the *OLE1* mRNA levels were determined by RT-qPCR as described in Material and Methods. **(B)** The iron chelator BPS does not

activate hypoxia markers. The wild-type (BY4741) cells were grown at 30ºC to the exponential phase in SC (+Fe), SC with 100 μM BPS (–Fe), and in hypoxic conditions for 5 h ($–O_2$). Total RNA was extracted, and the *TIR3* and *HEM13* mRNA levels were determined by RT-qPCR. Transcript levels were normalized with *PGK1* mRNA. **(C)** Ole1 protein levels increase in response to iron depletion. Yeast dells expressing *OLE1-13xMyc* (SPY901) were grown to exponential phase in SC (+Fe) and SC with 100 μM BPS (–Fe). Total proteins were extracted, and Ole1-13myc and Pgk1 protein levels were determined by immunoblotting with anti-c-myc and anti-Pgk1 antibodies, respectively. Pgk1 was used as a loading control. A representative experiment of two biological samples is shown. Ole1/Pgk1 protein quantification is shown relative to the iron-sufficient conditions. **(D)** Iron deficiency increased the *OLE1* transcription rate. The wild-type (HTLU-2832-1B) cells were grown at 30ºC in SC to the early exponential phase (time zero). Then aliquots were isolated 0, 10, 30, 90, 180 and 360 min after adding 100 μM BPS. Samples were processed to determine the *OLE1* mRNA levels (mRNA) by RT-qPCR and the *OLE1* transcription rate (TR) by GRO. **(E)** Aft1 and Aft2 were not responsible for *OLE1* activation by iron depletion. The wild-type (W303-1A) and a*ft1Δaft2Δ* (MML1088) cells were grown and analyzed as in panel A. Data represent the average (and standard deviation) of three biologically independent experiments. The asterisk (*) indicates statistically significant differences (p-value < 0.05). For more details, check the Material and Methods section.

The iron-regulated Aft1 and Aft2 transcription factors activate the expression of multiple genes in response to iron deficiency. To determine whether Aft1 and Aft2 participate in *OLE1* transcriptional activation by iron depletion, the *OLE1* mRNA levels were determined in a wild-type and an

aft1Δaft2Δ mutant strain cultivated under iron-sufficient (+Fe) and iron-deficient (–Fe) conditions. We observed that in both strains, the *OLE1* mRNA levels increased when iron was limited (Figure 1E), which suggests that Aft1 and Aft2 are not the transcription factors directly responsible for *OLE1* regulation by iron. Interestingly, the *OLE1* mRNA levels augmented in the *aft1Δaft2Δ* cells compared to a wild-type strain grown under iron-sufficient conditions (Figure 1E). This observation is consistent with the genetic iron deficiency displayed by the *aft1Δaft2Δ* yeast cells, and reinforces that the Aft1- and Aft2-independent *OLE1* up-regulation is induced by iron scarcity.

3.2. Mga2 activates *OLE1* expression in response to iron deficiency.

Previous results have demonstrated that the addition of fatty acids lowers *OLE1* mRNA levels (Nakagawa et al., 2002; Vasconcelles et al., 2001). Consistently with this, addition of linoleic acid dramatically diminished *OLE1* expression under iron-sufficient conditions (Figure 2A). More importantly, iron deficiency did not activate *OLE1* expression when the medium was supplemented with linoleic acid (Figure 2A). This result suggests that changes in total fatty acid composition could influence *OLE1* regulation by iron (see below).

A drop in temperature or in the availability of oxygen or UFAs alters the fatty acid composition of membranes and promotes the release of transcription factors Mga2 and Spt23 from the ER membrane, which eventually activate *OLE1* transcription (Chellappa et al., 2001; Hoppe et al., 2000; Jiang et al., 2002; Jiang et al., 2001; Nakagawa et al., 2002). To ascertain whether they

also participate in *OLE1* regulation by iron, *OLE1* mRNA levels were determined in the wild-type, *mga2Δ*, and *spt23Δ* cells cultivated under +Fe and –Fe conditions. We observed that most of *OLE1* mRNA increase was abolished in the cells that lacked *MGA2* (Figure 2A). However, only a slight decrease in *OLE1* up-regulation was noted in the *spt23Δ* mutant (Figure 2A). We were unable to investigate the *OLE1* mRNA levels in those cells that lacked both regulators because the *mga2Δspt23Δ* double mutant is not viable (Chellappa et al., 2001; Zhang et al., 1999). Although an Spt23 secondary role cannot be ruled out, these results suggest that Mga2 is the main factor to promote *OLE1* activation when iron is scarce.

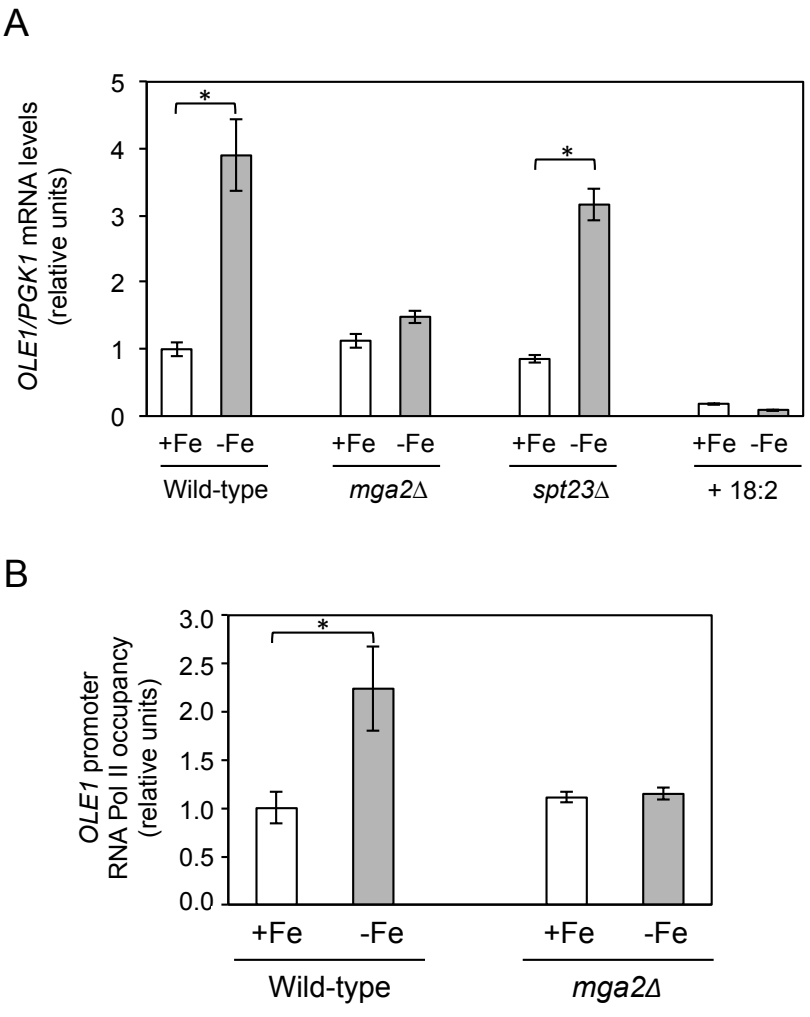

Figure 2. Mga2 activates *OLE1* expression under low iron conditions. (A) Regulation of the *OLE1* mRNA levels by iron in the *mga2Δ* and *spt23Δ* mutants. The wild-type (BY4741) cells were grown at 30ºC for 6 h in SC (+Fe) or SC+100 μM BPS (–Fe) without or with 1 mM linoleic acid (+18:2). The yeast *mga2Δ* (SPY824) and *spt23Δ* (SPY823) mutants were grown and analyzed as described in Figure 1A. **(B)** Mga2 facilitated the recruitment of RNA Pol II to the *OLE1* promoter in response to iron limitation. The wild-type (BY4741) and *mga2Δ* (SPY824) cells were grown as described in Figure 1A. Proteins were extracted and immunoprecipitated with anti-RNA Pol II monoclonal antibody, and binding to *OLE1* promoter region was determined by RT-qPCR. The results are shown in relation to the wild-type +Fe samples and were normalized to *FUS1* promoter. Data represent the average and standard deviation of three biologically independent experiments. The asterisk (*) indicates statistically significant differences (p-value < 0.05).

To further test the transcriptional regulation of *OLE1* by iron and the implication of Mga2 in this process, we studied the recruitment of RNA polymerase II (Pol II) to the *OLE1* locus by chromatin immunoprecipitation (ChIP) in both the wild-type and *mga2Δ* cells grown under +Fe and –Fe conditions. Consistently with the increase noted in *OLE1* TR obtained when iron was depleted (Figure 1B), we observed that iron deficiency stimulated the occupancy of the *OLE1* promoter by RNA Pol II (Figure 2B). It is noteworthy that RNA Pol II recruitment to the *OLE1* promoter promoted by iron depletion was not observed in an *mga2Δ* mutant (Figure 2B). These results support the notion that Mga2 activates *OLE1* transcription in response to iron deficiency by enhancing the recruitment of RNA Pol II.

3.3. Mga2 is essential for growth under low iron conditions due to defects in *OLE1* expression and unsaturated fatty acid synthesis

A large-scale research work has recently shown that yeast *mga2Δ* cells display a growth defect in the presence of the Fe^{2+} chelator Ferrozine (Samanfar et al., 2013). By adding BPS, we corroborated that *mga2Δ* exhibited complete lack of growth under iron-deficient conditions (Figure 3A). Consistently with our previous results, the *spt23Δ* mutant did not display any growth defect for the iron-deficient conditions compared to the wild-type strain (Figure 3A). To investigate the reasons that underlie the *mga2Δ* phenotype in low iron media, we attempted to rescue it by expressing *OLE1* under the control of the galactose inducible and glucose repressed *GAL1* promoter (P_{GAL1}-*OLE1* construct). Addition of galactose to the *mga2Δ* cells that expressed P_{GAL1}-*OLE1* rescued their growth defect under iron-deficient conditions (Figure 3B). To further study *mga2Δ* sensitivity to iron depletion, we ascertained whether supplementation with UFAs could also rescue growth. Indeed addition of either oleic acid (18:1) or linoleic acid (18:2), which cannot be synthesized by *S. cerevisiae*, but incorporates into the membrane lipids, recovered the growth defect displayed by the *mga2Δ* cells in low iron (Figure 3A). Collectively, these results indicate that *mga2Δ* cells possess defects in UFA synthesis under iron-deficient conditions, probably due to lack of *OLE1* induction that causes a severe growth defect.

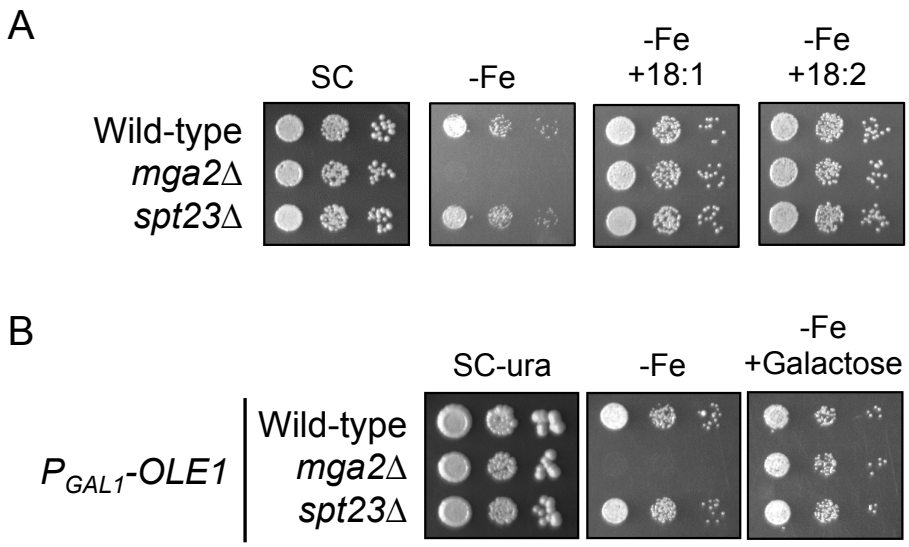

Figure 3. Requirements for growth under iron-deficient conditions. (A) Addition of UFAs rescued *mga2Δ* growth defect in low iron. The wild-type (BY4741), *mga2Δ* (SPY824) and *spt23Δ* (SPY823) yeast strains were grown to the exponential phase and spotted in 10-fold serial dilutions on SC and SC with 400 μM Ferrozine (–Fe). Oleic (+18:1) or linoleic (+18:2) acids were added to –Fe plates to a final 1 mM concentration. **(B)** The wild-type (BY4741), *mga2Δ* (SPY824) and *spt23Δ* (SPY823) cells transformed with the pRS316-P_{GAL1}-OLE1 plasmid were grown to the exponential phase and spotted in 10-fold serial dilutions on SC-ura, SC-ura with 500 μM Ferrozine (–Fe), and SC-ura without glucose, but with 2% galactose and 500 μM Ferrozine (–Fe +Galactose). Plates were incubated for 3 days at 30°C and photographed. A representative experiment of at least two independent biological replicates is shown.

As the complementation assays shown herein suggest that the *mga2Δ* mutant could suffer from defects in UFA synthesis under iron-deficient conditions, we decided to determine the total fatty acid (FA) composition of the wild-type and *mga2Δ* cells grown under +Fe and –Fe conditions. We observed

that the percentage of total UFA in relation to SFA lowered in a wild-type strain when cultivated in an iron-limited medium (Figure 4). A decrease in total UFA levels was also observed for a genetic deficiency achieved by using the *fet3Δfet4Δ* strain (Figure 4). These results suggest that UFA synthesis decreases when iron bioavailability is limited either nutritionally or genetically, probably due to a drop in Ole1 FA desaturase activity, which depends on iron. The *mga2Δ* mutant displayed a similar UFA percentage to the wild-type strain under iron-sufficient conditions (Figure 4A), which is consistent with an unaltered *OLE1* expression (Figure 2A). However, the decreased UFA abundance displayed by the *mga2Δ* mutant upon iron depletion was much more severe than that observed in a wild-type strain (Figure 4A). Probably both low iron cofactor availability and lack of *OLE1* induction contribute to the drop in the UFA levels observed in *mga2Δ* under iron-limited conditions. Collectively, these results strongly suggest that the relative UFA concentration in iron-deficient *mga2Δ* cells lowers to levels that do not allow growth.

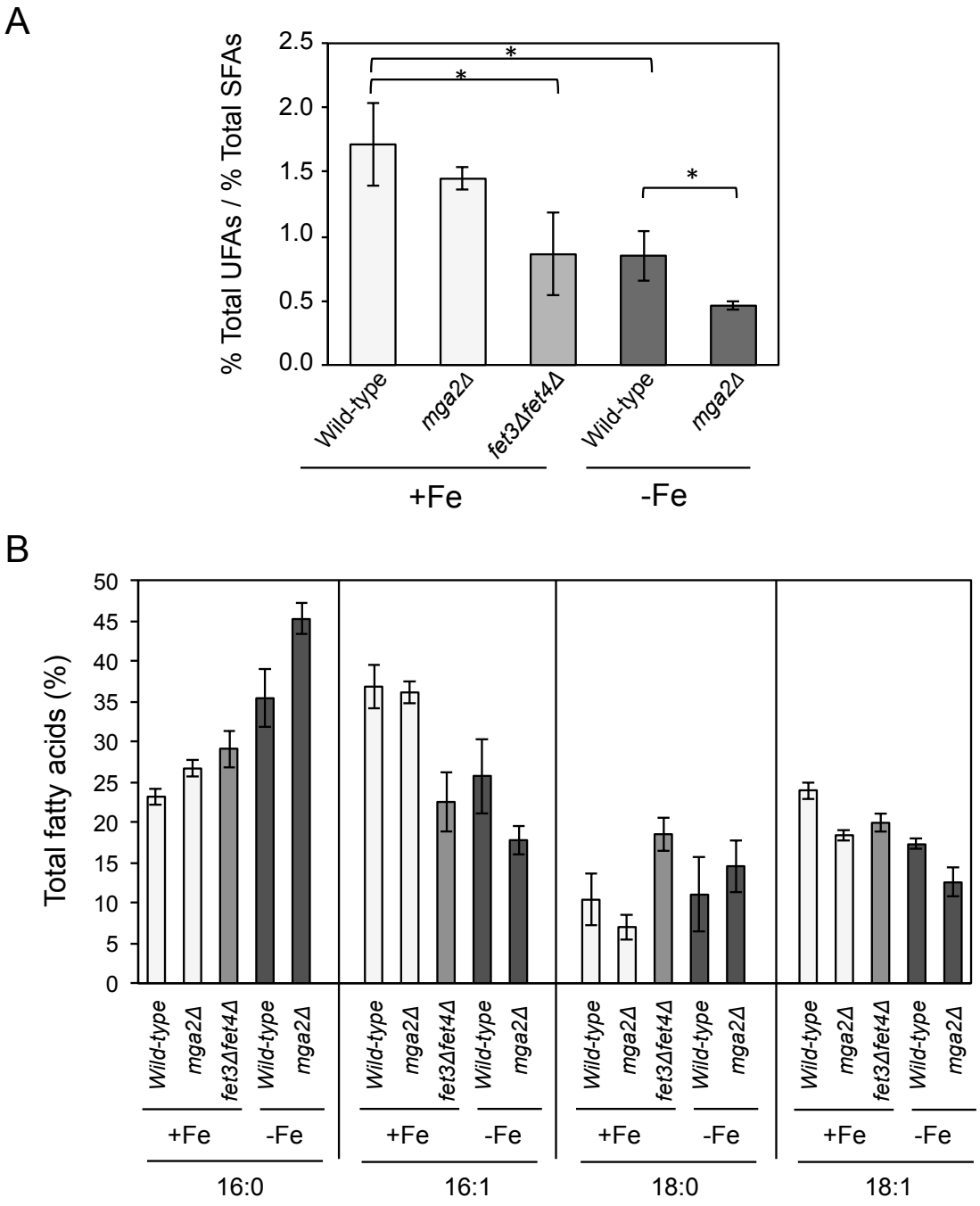

Figure 4. Effect of iron bioavailability and Mga2 on total fatty acid levels. The wild-type (BY4741) and *mga2Δ* (SPY824) cells were grown at 30ºC for 8 h to the exponential phase in SC (+Fe) or SC with 100 μM BPS (–Fe). The *fet3Δfet4Δ* (SPY386)

mutant was also grown to the exponential phase in SC (+Fe). Total FAs were extracted and quantified as detailed in Material and Methods. **(A)** Iron and Mga2 improved UFA synthesis. The percentage of UFAs (16:1 + 18:1) was represented in relation to SFAs (14:0 + 16:0 + 18:0). **(B)** The iron- and *MGA2*-deficient cells accumulated SFAs and showed diminished UFAs. Total palmitic (16:0), palmitoleic (16:1), stearic (18:0) and oleic (18:1) acids are separately represented. The results show the average and standard deviation of four biologically independent experiments. The asterisk (*) indicates statistically significant differences (p-value < 0.05).

When we itemized the different SFA and UFA, we observed that under iron-deficient conditions, both the wild-type and *mga2Δ* cells specifically accumulated palmitic acid (16:0), which is an Ole1 substrate, and displayed lower palmitoleic acid levels (16:1), which is the product of palmitic acid desaturation (Figure 4B). A similar tendency was displayed by the *fet3Δfet4Δ* strain under iron-sufficient conditions (Figure 4B). These results suggest that the bioavailability of iron limits yeast FA desaturase activity. More importantly, the iron-deficient *mga2Δ* mutants accumulated more total palmitic acid (16:0) and less palmitoleic acid (16:1) than the wild-type strain (Figure 4B). These results suggest that *mga2Δ* cells suffer a more severe FA desaturase defect than the wild-type cells when iron is not available. A similar tendency was observed when the levels of stearic (18:0) and oleic (18:1) acids were determined in the iron-sufficient and iron-deficient wild-type and *mga2Δ* cells (Figure 4B). Although multiple interpretations are plausible, we considered that these results were consistent with the possibility that alterations in the molecular

lipid packing, as a result of lowering UFA concentrations, would activate Mga2, and consequently *OLE1* expression, in response to iron deficiency.

3.4. *OLE1* activation in response to iron deficiency requires the ubiquitin ligase Rsp5, Cdc48$^{Npl4/Ufd1}$ complex and the proteasome

The Mga2 protein is tethered to the ER membrane through a carboxy-terminal transmembrane (TM) helix domain. In response to low UFA or oxygen levels, Mga2 is cleaved from the ER membrane and enters the nucleus to activate *OLE1* transcription (Hitchcock et al., 2001; Hoppe et al., 2000; Jiang et al., 2002). To inquire about the mechanism of *OLE1* regulation by iron, we decided to investigate whether processing Mga2 was necessary to activate *OLE1* upon iron limitation. We first determined the *OLE1* transcript levels in the cells that expressed an Mga2 protein that lacked its ER-anchoring TM domain (*MGA2ΔTM*). As previously reported, deletion of the Mga2 TM segment caused the *OLE1* mRNA levels to rise under normal growth conditions compared to a strain that expressed full length Mga2 (Figure 5A) (Chellappa et al., 2001). More importantly, iron depletion did not further augment *OLE1* mRNA in the cells that expressed *MGA2ΔTM* (Figure 5A). Collectively, these results are consistent with a model in which iron deficiency would alter the composition of the ER membrane, which would facilitate Mga2 release and *OLE1* transcription activation.

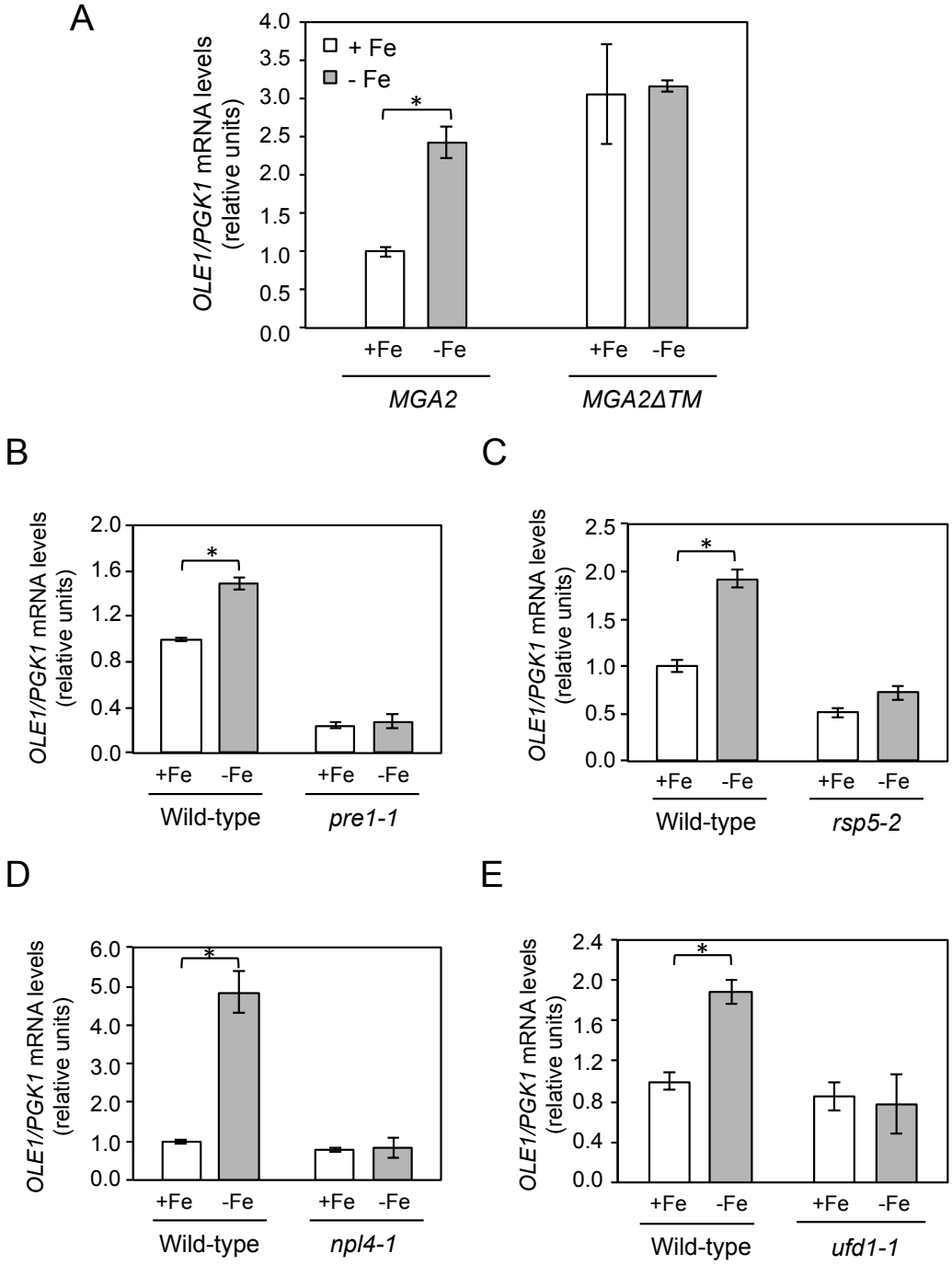

Figure 5. The proteasome, ubiquitin ligase Rsp5 and Cdc48$^{Npl4/Ufd1}$ segregase facilitate *OLE1* activation by low iron. (A) *OLE1* was not regulated by iron in the cells that expressed unanchored Mga2. The yeast *mga2Δ* (SPY824) cells transformed with MGA2 (pPS2364) or MGA2ΔTM (pPS2358) plasmids were grown and analyzed as described in Figure 1A. **(B)** The proteasome was required for *OLE1* activation by low

iron. The wild-type (YWO0607) and *pre1-1* (YWO0608) cells were grown in SC at 25ºC and then transferred to 37ºC. After 1 h at 37ºC, 100 μM BPS were added (–Fe), or not (+Fe), and cells were incubated for 5 h more. **(C)** E3 ubiquitin ligase Rsp5 was required to activate *OLE1* expression in response to low iron. The wild-type (Y0356) and *rsp5-2* (Y0358) cells were grown as described in panel B. **(D)** Npl4 facilitated *OLE1* activation by low iron. The wild-type (PSY580) and *npl4-1* (PSY2340) cells were grown in SC at 25ºC and were then transferred to 30ºC. After 1 h at this temperature, 100 μM BPS were added (–Fe), or not (+Fe), and cells were incubated for 5 h more. **(E)** Ufd1 enhanced *OLE1* expression under low iron conditions. The wild-type (PSY580) and *ufd1-1* (PSY3074) cells were grown as described in panel B. In all cases, total RNA was extracted and the *OLE1* mRNA levels normalized with *PGK1* mRNA were determined by RT-qPCR. Data indicate the average and standard deviation of three biologically independent experiments. The asterisk (*) indicates statistically significant differences (p-value < 0.05).

The release of Mga2 from the ER membrane in response to low UFA levels or low oxygen requires the proteasome, E3 ubiquitin ligase Rsp5 and segregase complex $Cdc48^{Npl4/Ufd1}$ (Hitchcock et al., 2001; Hoppe et al., 2000; Shcherbik et al., 2003). To determine the participation of these proteins in *OLE1* activation by low iron, we used the temperature-permissive mutants of *PRE1*, *RSP5*, *NPL4* and *UFD1*, which are essential genes in yeast. Whereas *OLE1* transcript abundance increased in response to iron deficiency in all the wild-type strains, no *OLE1* induction was observed for the *pre1-1*, *rsp5-2*, *npl4-1* and *ufd1-1* mutants at the non permissive temperature (Figure 5B to 5E). These

results suggest that the proteasome, Rsp5 and $Cdc48^{Npl4/Ufd1}$ are required for Mga2-mediated transcriptional *OLE1* activation by iron depletion.

3.5. Transcriptional activation of *MGA2* in response to iron deficiency favors *OLE1* expression

We decided to explore whether the expression of *MGA2* was also regulated by iron bioavailability, as previously shown for *OLE1* (Figure 1A). We observed that *MGA2* mRNA levels increased upon addition of the Fe^{2+}-chelator BPS or the deletion of *FET3* and *FET4* iron transporters (Figure 6A). Moreover, addition of excess iron to the iron-deficient medium decreased *MGA2* transcript levels (Figure 6A, BPS+FAS). We used the Genomic Run-On approach to determine whether the up-regulation of the *MGA2* mRNA by iron deficiency was mediated by changes in the transcription rate. We observed that both *MGA2* TR and mRNA levels increased progressively while iron starvation advanced (Figure 6B). These results strongly suggest that *MGA2* is transcriptionally activated in response to iron depletion.

To assess the relevance of *MGA2* up-regulation in *OLE1* desaturase activation, we compared *OLE1* transcript abundance in yeast cells with different *MGA2* expression levels. Specifically, we transformed *mga2Δ* cells with a centromeric (CEN) or a multicopy (2μ) plasmid that contained *MGA2*. We corroborated that the multicopy plasmid rose *MGA2* transcript levels would compare to the centromeric plasmid under both +Fe and –Fe conditions (Figure 6C). In both cases, *MGA2* was up-regulated when iron was depleted from the growth medium (Figure 6C). It was noteworthy that the cells which

overexpressed *MGA2* exhibited higher *OLE1* expression levels than the cells that expressed centromeric *MGA2* (Figure 6D). These results indicate that increasing *MGA2* expression favors *OLE1* up-regulation.

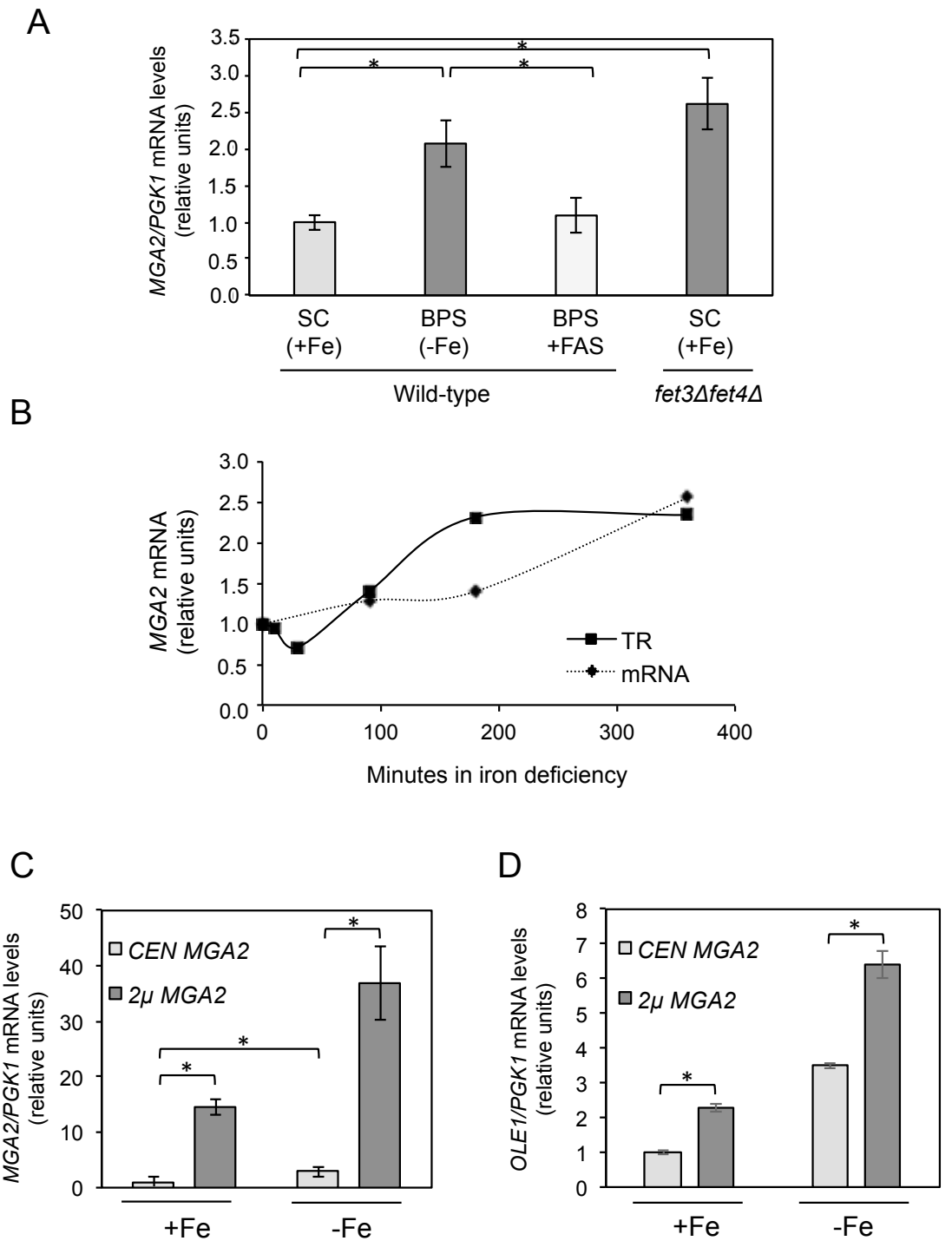

Figure 6. Regulation of the *MGA2* mRNA levels by iron bioavailability. (A) *MGA2* mRNA levels increased in response to nutritional and genetic iron deficiency. The wild-type (BY4741) and *fet3Δfet4Δ* (SPY386) cells were grown as described in Figure 1A. Total RNA was extracted, and the *MGA2* mRNA levels were determined by RT-qPCR. The *MGA2* transcript levels were normalized with *PGK1* mRNA. **(B)** Iron deficiency increased the *MGA2* transcription rate. The wild-type (HTLU-2832-1B) cells were grown as described in Figure 1B. Then samples were processed to determine the *MGA2* transcript levels (mRNA) by RT-qPCR and the *MGA2* transcription rate (TR) by GRO. **(C and D)** *MGA2* overexpression caused *OLE1* up-regulation. The yeast *mga2Δ* (SPY824) cells transformed with either a centromeric (pPS2369, CEN) or a multicopy (YEplac181-3HA-MGA2, 2μ) plasmid that expressed *MGA2* were grown for 6 h in SC (+Fe) or SC + 100 μM BPS (–Fe). Total RNA was extracted, and the *MGA2* (panel B) and *OLE1* (panel C) mRNA levels were determined by RT-qPCR. The results indicate the average and standard deviation of three biologically independent experiments. The asterisk (*) indicates statistically significant differences (p-value < 0.05).

3.6. Mga2 activates its own expression in response to iron deficiency, cold, hypoxia and a drop in unsaturated fatty acids

To investigate the mechanism of *MGA2* regulation, we ascertained whether iron-regulated transcription factors Aft1 and Aft2 were implicated in *MGA2* activation by iron depletion. We observed that *MGA2* up-regulation still occurred in an *aft1Δaft2Δ* mutant, which suggests Aft1- and Aft2-independent mechanisms (Figure 7A). As previously observed for *OLE1*, addition of linoleic acid abrogated *MGA2* regulation by iron (Figure 7B). As *MGA2* seems to be

regulated by iron and unsaturated fatty acid availability, we wondered whether this was also the case for low-oxygen and low-temperature conditions. As shown in Figure 7C, both the *OLE1* and *MGA2* mRNAs were up-regulated in response to hypoxia and cold. Since the Mga2 protein activated *OLE1* in response to iron depletion, we ascertained whether Mga2 also activated its own transcription under these conditions. To address this question, we fused the *MGA2* promoter region to the *lacZ* reporter gene (P_{MGA2}-*lacZ*) and determined β-galactosidase activity in the wild-type and *mga2Δ* cells cultivated under +Fe and –Fe conditions. As expected from the *MGA2* TR data (Figure 6A), P_{MGA2}-*lacZ* led to increased β-galactosidase activity when the wild-type cells were deprived of iron (Figure 7D). More importantly, the P_{MGA2}-*lacZ* activation by low iron did not occur in the *mga2Δ* cells (Figure 7D), which strongly suggests that Mga2 activates its own transcription upon iron limitation. To address whether *MGA2* auto-activation is a specific response to iron depletion or, instead, a more general regulatory mechanism to enhance *OLE1* activation under other conditions, we studied P_{MGA2}-*lacZ* expression in both the wild-type and *mga2Δ* cells grown at low temperature (10ºC), low oxygen or in the presence of linoleic acid (18:2). As previously shown for *OLE1* (Chellappa et al., 2001; Hoppe et al., 2000; Kwast et al., 1999; Nakagawa et al., 2002; Vasconcelles et al., 2001), P_{MGA2}-*lacZ* expression increased in response to cold and hypoxic treatments, and decreased when UFAs were added (Figures 7D and 7E). Moreover, no remarkable P_{MGA2}-*lacZ* regulation was observed in the *mga2Δ* mutant cells. Altogether these results strongly indicate that Mga2 activates the expression of both genes *MGA2* and *OLE1* in response to multiple conditions that alter the FA composition, including low UFAs, temperature, oxygen and iron (Figure 8).

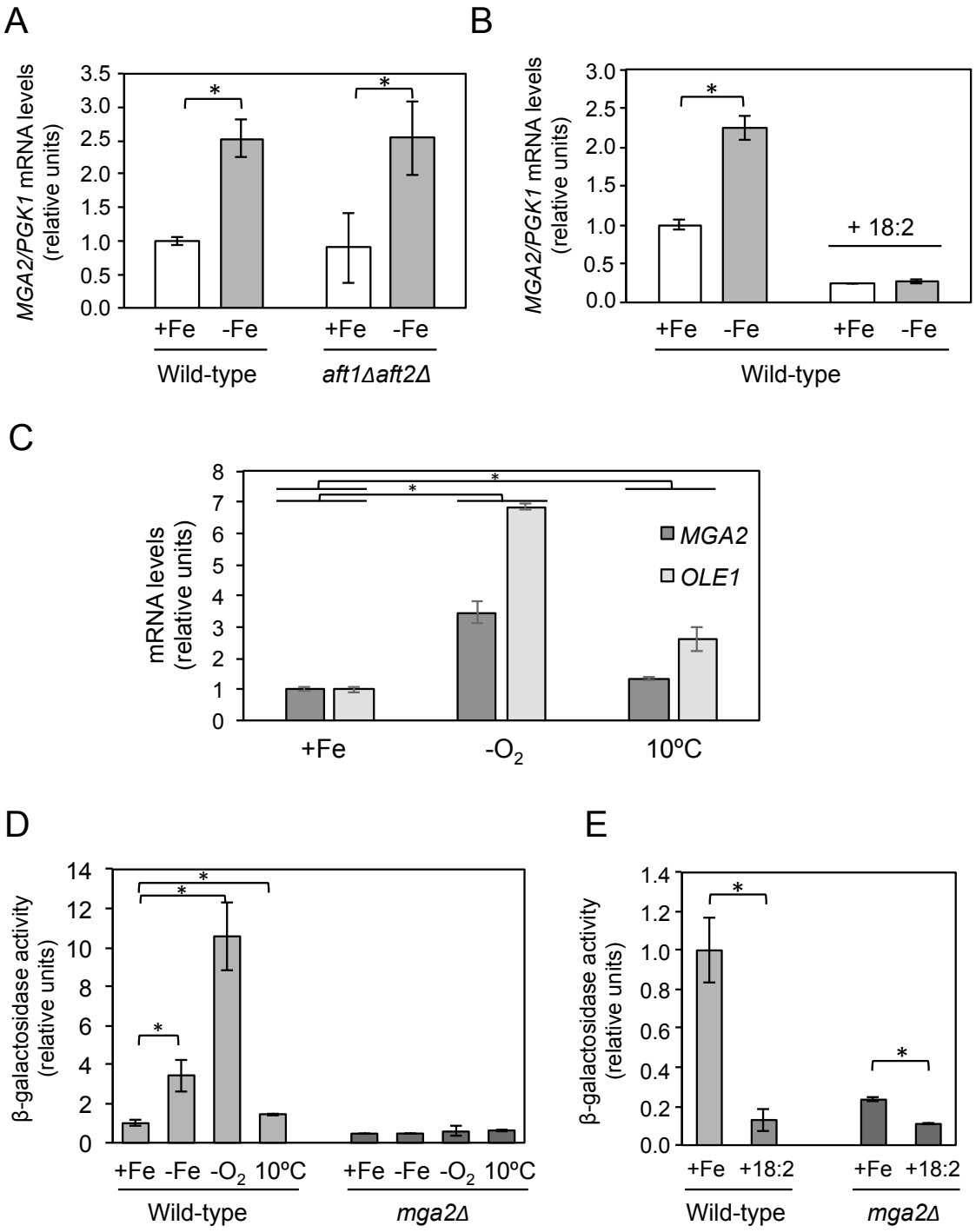

Figure 7. Mga2 activates its own expression in response to low iron, cold, hypoxia and low UFAs. (A) Aft1 and Aft2 were not responsible for *MGA2* activation by iron depletion. The wild-type (W303-1A) and a*ft1Δaft2Δ* (MML1088) cells were grown as

in Figure 1C. **(B)** Iron deficiency did not activate *MGA2* in the presence of linoleic acid. The wild-type (BY4741) cells were grown for 6 h in SC (+Fe) or SC+100 μM BPS (–Fe), without or with 1 mM linoleic acid (+18:2). **(C)** A drop in the oxygen levels or temperature activated *MGA2* expression. The wild-type (BY4741) cells were grown at 30ºC to the exponential phase in SC (+Fe) and were then transferred to hypoxia for 5 h ($-O_2$) or 10ºC for 1 h. **(D)** Hypoxia and cold activated *MGA2* expression in an Mga2-dependent manner. The wild-type (BY4741) and *mga2Δ* (SPY824) cells transformed with the pSP1039 (P_{MGA2}-*lacZ*) plasmid were grown as in panel C. (**E**) A drop in the UFA levels activated *MGA2* expression in an Mga2-dependent manner. The wild-type (BY4741) and *mga2Δ* (SPY824) cells transformed with the pSP1039 (P_{MGA2}-*lacZ*) plasmid were grown in the SC (+Fe) or SC that contained 1 mM linoleic acid for 6 h (+18:2). Either total RNA was extracted and the *MGA2* mRNA levels determined by RT-qPCR (panels A, B and C), or β-galactosidase assays were performed (panels D and E). Data represent the average and standard deviation of three biologically independent experiments. The asterisk (*) indicates statistically significant differences (p-value < 0.05).

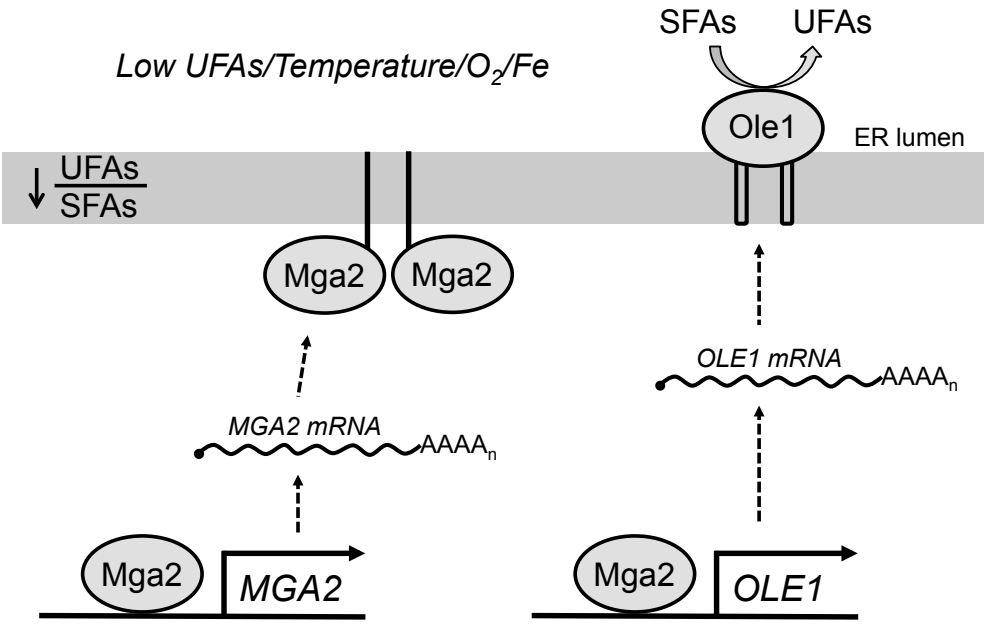

Figure 8. A model for *OLE1* transcriptional activation in response to iron deficiency. Upon iron deficiency, yeast cells diminished the availability of this cofactor for multiple iron-dependent processes, including the Ole1 Δ9-FA desaturase enzyme. As a direct consequence, the proportion of UFAs/SFAs lowered, which probably changed ER membrane fluidity and enhanced Mga2 processing. Released Mga2 translocated to the nucleus and activated *MGA2* expression, which contributed to increase the pool of the available Mga2 transcription factor. Finally, Mga2 activated the transcription of *OLE1*, which helped to increase the UFA levels.

4. Discussion

Oxygen and iron are indispensable for the catalytic activity of eukaryotic Δ9-FA desaturases. By studying yeast Ole1, multiple groups have characterized the mechanisms that activate the expression of this Δ9-FA desaturase in response to low oxygen levels (Jiang et al., 2001; Jiang et al., 2002; Nakagawa et al., 2002). More recent data support the notion that iron defects also activate *OLE1* expression. For instance, addition of Fe^{2+} chelators to the growth medium increases *OLE1* transcript abundance (Chellappa et al., 2001; Puig et al., 2005). Moreover, yeast cells that lack monothiol glutaredoxin *GRX5*, which functions in mitochondrial iron-sulfur cluster biosynthesis, up-regulate the expression of a group of genes, including the iron regulon and *OLE1* (Belli et al., 2004). Finally, it is also well-known that addition of cobalt, which induces the iron regulon, enhances *OLE1* expression (Kwast et al., 1999; Stadler and Schweyen, 2002; Vasconcelles et al., 2001). We showed here that the addition of excess iron to a medium that contained BPS decreased *OLE1* expression to basal levels (Figure 1A). A previous study indicated that the deletion of the *AFT1* transcription factor activates the expression of various genes related to oxygen and lipid metabolism probably in an Mga2-dependent manner (Lyons et al., 2004). Similarly, we used here the *fet3Δfet4Δ* and *aft1Δaft2Δ* yeast mutants to show that genetic iron deficiencies activate *OLE1* expression (Figure 1A and 1E). By using GRO, we demonstrated that *OLE1* was activated at the transcriptional level in response to iron depletion (Figure 1D). As previously reported for other conditions including hypoxia and low temperature (Chellappa et al., 2001; Jiang et al., 2001; Jiang et al., 2002; Nakagawa et al., 2002), Mga2 was also the main factor to activate *OLE1* upon

iron limitation (Figure 2). However, our data cannot ignore the contribution of Spt23 since a subtle drop in *OLE1* up-regulation by low iron was still observed in the *spt23Δ* mutants. As proposed for other stresses (Ballweg and Ernst, 2017), crosstalk between both transcription factors could occur in iron deficiency with Mga2 functioning as the main *OLE1* regulator and Spt23 being responsible for fine-tuning the response.

We determined the levels of total SFAs and UFAs in the wild-type cells grown under normal conditions and in iron-deficient cells, which were achieved either genetically (*fet3Δfet4Δ* mutant) or nutritionally (BPS) (Figure 4). Consistently with Ole1 being an iron-dependent enzyme, when the iron bioavailability of the wild-type cells was restricted the proportion of UFAs lowered. Specifically, we observed that iron deprivation led total palmitic acid to accumulate (16:0), and UFAs palmitoleic (16:1) and oleic (18:1) acid abundance to decrease. However, a previous study has not reported significant alterations in the SFAs and UFAs levels under low iron conditions (Shakoury-Elizeh et al., 2010). This discrepancy could be due to the different treatment used to limit iron availability. Our iron-deficient conditions consisted in cultivating yeast cells for 6 h in a medium supplemented with 100 μM of the Fe^{2+}-specific chelator BPS, which dramatically limits extracellular available iron. However, those authors did not add an iron chelator but, instead, they cultivated cells for 24 h in a defined-iron medium that contained 5 μM of ferrous iron (-Fe condition) compared to 300 μM of ferrous iron (+Fe conditions). In agreement with a mild iron-deficient condition, they did not observe any changes in Ole1 expression (Shakoury-Elizeh et al., 2010), whereas the more severe iron depletion that we used activated Mga2 and *OLE1* expression (Figure 1).

Despite the drop in total UFAs displayed by the wild-type cells under iron-limited conditions, yeast still synthesized sufficient UFAs for grow (Figures 3 and 4). However, deletion of *MGA2* further diminished the synthesis of UFAs under the low iron conditions to levels that blocked growth (Figure 3 and 4). These data demonstrated that the *mga2Δ* growth defect in low iron was due to the lack of UFAs as it can be recued by adding external UFAs or *OLE1* expression with a galactose-regulated promoter (Figure 3). Furthermore, these results suggested that the *OLE1* up-regulation by low iron partially compensated the drop in iron availability and, therefore, Ole1 activity.

The Mga2 and Ole1 role during the progress of iron deficiency could be divided into various stages. In an initial stage, Mga2 would not be activated and no increase in *OLE1* expression would be observed. In response to more severe iron depletion, Mga2 would activate *OLE1* expression. A two-stages situation has been described for Mga2 during the response to oxidative stress (Kelley and Ideker, 2009). By using a genome-wide approach, these authors uncovered that Mga2 transcription factor was important for the adaptive, but not the acute, response to hydrogen peroxide by activating the expression of genes implicated in the ergosterol, the FA and the zinc metabolic pathways (Kelley and Ideker, 2009). Additional evidence that connect zinc and lipid homeostasis has shown that the Mga2 transcription factor activates the expression of *IZH2* and *IZH4* in response to excess zinc (Lyons et al., 2004). Both genes display homology to vertebrate membrane steroid receptors, and are regulated by oxygen and fatty acid levels (Lyons et al., 2004).

Regarding the mechanism that activates Mga2 and, consequently *OLE1* expression, in response to iron deprivation, multiple data shown herein

suggested that changes in the proportion of FAs could cause alterations in the lipid packing of the ER membrane that promote Mga2 processing and *OLE1* transcription, as previously shown for hypoxia and low temperatures (Jiang et al., 2002; Jiang et al., 2001; Nakagawa et al., 2002). First, addition of extracellular UFAs eliminated *OLE1* activation by iron depletion (Figure 2A). Second, deletion of the TM helix, which tethered Mga2 to the ER membrane, prevented *OLE1* iron regulation (Figure 5A). Third, the proteasome, E3 ubiquitin ligase Rsp5 and the Npl4 and Ufd1 components of the $Cdc48^{Npl4/Ufd1}$ segregase complex were necessary for *OLE1* up-regulation by iron deprivation (Figure 5).

Here we have shown that yeast cells activate the transcription of *OLE1* in response to iron deprivation (Figure 1D). In the absence of *MGA2*, yeast cells are unable to enhance the recruitment of the RNA pol II to the *OLE1* promoter region and do not increase *OLE1* mRNA levels when iron is scarce (Figure 2). These results highlight the relevance of Mga2 transcriptional activity on the up-regulation of *OLE1* by iron limitation. However, post-transcriptional regulatory mechanisms could also contribute to the final Ole1 protein levels and activity. The steady-state levels of each mRNA depend directly on its TR and inversely on its decay rates. By using the GRO approach, we have simultaneously determined the experimental values for the TR and the mRNA levels of *OLE1* (Figure 1D). From these empirical data, we can estimate the decay rate of the *OLE1* mRNA at each time-point (Garcia-Martinez et al., 2004). Although both *OLE1* TR and mRNA levels increase during the progress of the iron deficiency, its pattern is not completely parallel, which suggests that a post-transcriptional contribution to the final *OLE1* transcript levels exists. Previous results have shown that Mga2 also regulates *OLE1* mRNA stability according to the

availability of fatty acids (Kadasamy et al., 2004). Mga2 seems to play a stabilization function on *OLE1* transcript in FA-free media and a destabilization effect when the medium is supplemented with UFAs (Kadasamy et al., 2004). Moreover, in response to iron depletion, yeast cells express a tandem zinc-finger protein called Cth2 that binds to mRNAs, which contain AU-rich elements within their 3'-untranslated region, and promotes their turnover (Puig et al., 2005; Pedro-Segura et al., 2008). We have previously shown that the *OLE1* transcript contains two putative AU-rich elements and its mRNA levels increase in cells lacking *CTH2* (Puig et al., 2005). These observations suggest that various regulatory factors, including Mga2 and Cth2, could control *OLE1* mRNA stability during the progress of iron deficiency.

Our data also uncovered that *MGA2* expression increased in response to multiple stresses, including changes in UFAs in the growth medium, a drop in temperature and alterations in oxygen or iron availability. Furthermore, *MGA2* up-regulation by low iron did not occur when excess UFAs were added to the growth medium (Figure 7B). These observations suggest that changes in the composition and fluidity of lipid membranes could also be responsible for *MGA2* activation. As the Mga2 transcription factor is processed and activated in response to these stresses (Hoppe et al., 2000; Piwko and Jentsch, 2006), we postulated that the Mga2 protein could activate the expression of its own mRNA in an auto-activation mechanism. Our β-galactosidase assays run with the *mga2Δ* cells that contained a P_{MGA2}-*lacZ* construct confirmed this hypothesis, and our overexpression experiments corroborated that an increasing *MGA2* expression contributed to *OLE1* activation.

The data presented herein indicated that a decrease in the bioavailability of iron caused a drop in Ole1 activity, which led to changes in the lipid composition of membranes. Consequently, Mga2 regulatory factor was released from the ER membrane, traveled to the nucleus, and activated *OLE1* transcription to compensate the unsaturated/saturated fatty acid imbalance. Furthermore, our study revealed that Mga2 activated its own expression in response to multiple inputs (Figure 8). This novel regulatory mechanism could contribute to help yeast cells adapt to hypoxia, cold, low UFA concentrations and iron deficiency.

5. Acknowledgments

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Conflict of interest: The authors declare that no conflict of interest exists.

6. References

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