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Over-expression of functional *Saccharomyces cerevisiae GUP1*, induces proliferation of intracellular membranes containing ER and Golgi resident proteins

Gianluca Bleve [a], Gian Pietro Di Sansebastiano [b], Francesco Grieco [a,*]

[a] Istituto di Scienze Delle Produzioni Alimentari del Consiglio Nazionale Delle Ricerche (ISPA), Unità di Lecce, via Provinciale Lecce, Monteroni, 73100 Lecce, Italy
[b] Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali (DiSTeBA), Università del Salento, via Prov. Lecce, Monteroni, 73100, Lecce, Italy

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ABSTRACT

High-level expression of the *GUP1* gene in *Saccharomyces cerevisiae* resulted in the formation of proliferated structures, which hosted endoplasmic reticulum (ER), Golgi and itinerant proteins. The *GUP1* over-expression enhanced ER biogenesis, as shown by the coordinated increased transcription rate of genes involved in both ER and Golgi metabolism and in phospholipids biosynthesis. The formation of Gup1-induced proliferation revealed that it depended on an intact unfolded protein response, because their assembly was reported to be lethal to yeast strains unable to initiate the UPR (Unfolded Protein Response) pathway. *GUP1* over-expression affected global ER and Golgi structure and resulted in the biogenesis of novel membrane arrays with Golgi and ER hybrid composition. In fact, a number of ER and Golgi resident proteins together with itinerant proteins that normally cycle between ER and Golgi, were localized in the proliferated stacked membranes. The described assembling of novel membrane structures was affected by the functionality of the Gup1 O-acyltransferase domain, which regulates the Gup1 protein role as remodelase in the glycosylphosphatidylinositol (GPI) anchored proteins biosynthesis. To our knowledge, we presented the first evidence of sub cellular modifications in response over-expression of a GPI-anchor remodelase in *S. cerevisiae*.

1. Introduction

Yeasts are exposed to highly variable changes in their environment, which depend on the availability and quality of nutrients, temperature, pH, radiation, oxygen and water activity. To survive in the environment, yeasts have developed strategies to react to the osmotic changes [1] involving a number of cellular responses [2]. A strategy in osmoadaptation is the increase of specific osmolytes in the cellular cytoplasm, common to many yeast species, including *Saccharomyces cerevisiae*, mostly glycerol. The accumulation in the cytoplasm of this chemically inert osmolyte can counterbalance hyper-osmotic stress [3].

To date, a number of different phenotypes were associated with *GUP1*. This gene was firstly associated with a growth defect in glycerol-based media and for interfering in glycerol/H + -symporter activity [4], it was shown to be essential for growth under anaerobic conditions [5,6] and *gup1*Δ mutant grows poorly on salt, ethanol, weak carboxylic acids [7]. Moreover, consistent evidences were recently produced that the gene *SLT1* encodes the *S. cerevisiae* glycerol/H^+ symporter [8]. An involvement in lipids metabolism was also suggested, by the finding that a deletion of *GUP1* gene resulted in an increase of triglycerides and a concomitant decrease in phospholipids synthesis [9]. Furthermore, Gup1 protein was targeted to the plasma membrane via a Sec6-dependent process and, upon treatment with glucose, it was endocytosed via *END3* and targeted for degradation in the vacuole. By immune electron microscopy assays, it was described the Gup1 protein membrane topology: the N-terminus lies in the periplasmic space, whereas its C-terminal tail has an intracellular location [10]. *GUP1* is also implicated in a wide range of crucial processes for cell preservation and functioning: membrane [11] and wall composition and integrity [7], telomere length [12], secretory/endocytic pathway [13], cytoskeleton polarization during mitosis and budding [14], bipolar bud site selection [15] and lipid metabolism in terms of sphingolipid-sterol ordered domains integrity/assembly [16]. In *S. cerevisiae* Gup1p was indicated as the enzyme performing the acylation on sn-2 position of GPI anchors [17,18] together with Cwh43p [19]. Recently, Abe et al. [20] demonstrated that the mammalian Gup1p has a completely different function, acting as a negative regulator for N-terminal palmitoylation of Sonic hedgehog protein. Considering all the above indications, the real functions of Gup1p are far from being understood.

During our study to produce the yeast Gup1 protein tagged with Gfp (green fluorescent protein), we found that its prolonged over-production induced morphological alterations such as bright patches and proliferated structures dispersed within the cytoplasm. Regulation of ER morphology and dimension has been easily observed in response to drugs or as a reaction to elevate expression of ER-resident polypeptides [21]. The newly synthesized proteins need to be

* Corresponding author. Consiglio Nazionale delle Ricerche, Istituto di Scienze delle Produzioni Alimentari, Unità Operative di Lecce, Via Prov. Lecce, Monteroni, 73100, Lecce, Italy. Fax: +39 0832422620.
E-mail address: francesco.grieco@ispa.cnr.it (F. Grieco).

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properly folded [22] and their transport to functional sites in the native conformation is supervised by a cellular mechanism denoted "ER quality control" [23]. The ER quality control system regulates retention of misfolded proteins in the ER, where they are directed to a degradation pathway denoted ER-associated protein degradation (ERAD; [24]). As a result of the above described control system, over-produced proteins can induce and localize to aberrant membrane structure of ER origin [25,26]. Several examples of morphological changes of the ER structure in response to protein over-production have been documented: stacked cisternae, denoted as karmellae [27], BiP bodies [28], enlarged ER exit loci [29,30], Russell bodies [31], proliferations of the ER-Golgi intermediate compartments (ERGIC; [32]); ER stratified aggregations [33,34]. In order to get more insight into the cellular mechanisms behind reticular proliferation in *S. cerevisiae*, we investigated the nature and the properties of the novel structures detectable in yeast cells after *GUP1* over-expression. In the present report, we showed that *GUP1* over-expression did not induce karmellae formation but a variable number of structures consisting of smooth ER-like layers, closely located near the plasma membrane. The Gup1 protein-increased production elicited the unfolded protein response (UPR) in yeast cells and resulted in a coordinated over-expression of ER- and Golgi-specific genes, affecting global ER and Golgi structure. A correlation between the biogenesis of the proliferated membranes and the *GUP1* MBOAT (membrane bound O-acyltransferase) function could be hypothesized by showing that the assembling of novel membrane structures was the result of the over-expression of an active form of *GUP1*.

2. Materials and methods

2.1. Strains and media

Yeast strains used in this study are listed in Table 1. The *Escherichia coli* strains were DH5α (F-, ϕ80d*lac*ZΔM15, Δ*lac*ZYA-*arg*F) and BL21 [*E. coli* B F^-, ompT, hsdS (r_B^-, m_B^-), gal, dcm]. Standard procedures for isolation and manipulation of bacterial cells and DNA were employed [35,36].

The strains tagged with Rfp (red fluorescent protein) for colocalization were generated by Huh et al. [37] transforming the (EY0987 strain (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) with gene-specific cassettes deriving from the plasmid pFA6a–mRFP–KanMX6, where the following genes, *ANP1*, *COP1*, *SNF7*, *SEC13*, *CHC1*, were separately cloned in C-terminal fusion with the RFP.

Yeast cells were grown in a rotary shaker at 30 °C using either a rich [YP medium = 1% (w/v) yeast extract and 2% (w/v) peptone] or a minimal medium [YNB medium = 0.67% (w/v) yeast nitrogen base, supplemented with adequate quantities of auxotrophic requirements]. Carbon sources for yeast cell growth were glucose (2%, w/v) or galactose (2%, w/v). Cultures were always harvested during the exponential phase of growth. Yeast strain maintenance was achieved by plating on to YP-glucose or YNB-glucose medium supplemented with 2% (w/v) agar. *GUP1* gene expression and localization were studied performing the growth of yeast cells under repression conditions in glucose-containing media, whereas yeast induction was obtained by incubating cells in YP or YNB medium supplemented with galactose as described in the Results section.

Table 1
Yeast strains used.

Strain	Genotype	Reference
W303-1A	*MATa leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100*	[73]
BHY54	*Isogenic to W303-1A but GUP1::His51*	[4]
JRY527	*MATa, ade2-101, his3Δ200, lys2-801, met-, ura3-52*	[74]
RWY1095	JRY527, *ire1 Δ::KanMX6*	[59]
RWY1181	JRY527, *ire1(1-1116):KanMX6*	[59]
JC104	*MATa; leu2-3,-112::LEU-UPRE-lacZ; his3-11,-15::HIS3-UPRE-lacZ; ura3-1; trp1-1; ade2-1; can1-100*	[75]
JC132	*MATa; ire1::URA3; his3-11,-15::HIS3-UPRE-lacZ; leu2-3,112; ura3-1; trp1-1; ade2-1; can1-100*	[58]
JC408	*MATa; hac1::URA3; ura3-1; leu2-3,-112::LEU-UPRE-lacZ; his3-11,-15; trp1-1; ade2-1; can1-100*	[76]

2.2. Plasmids construction

The *GUP1* gene was amplified by PCR employing the *GUPforH*/*GUPrevE* primer pair (see all primer sequences in Table 2). After digestion with *Hind*III and *Eco*RI, the obtained amplicon was ligated with T4 DNA ligase into the *Hind*III and *Eco*RI sites of pYES2 vector (Invitrogen, USA), producing the pY-*GUP*. The fusion constructs between *GUP1* and *GFP* genes were obtained as previously described [10]. To add a *6xHis* tag coding sequence to its 3'-end region, the *GUP1* gene, was PCR-amplified using the primer pair *GUPforH*/*GUPrevE-HIS*. The obtained PCR product was directionally cloned into the *Hind*III and *Eco*RI sites of the pYES2 vector and the obtained recombinant plasmid was named pY-*GUPHis*. A point mutation to change His 447 to Leu was introduced into *GUP1* gene sequence using the *GFPGUPMutF*/*GFPGUPMutR* primer pair (Table 2) and by means of the Site-directed mutagenesis Kit (Finnzymes, Finland). The plasmids pY-*GUPHis*, pY-*GUPGFP* and pY-*GFPGUP* were separately used as templates in the above site-directed mutation assay, respectively yielding pY-*GUPHis*^{H447L}, pY-*GUPGFP*^{H447L} and pY-*GFPGUP*^{H447L} recombinant plasmids.

2.3. Real time RT-PCR assay

RNA extraction has been performed as previously described [38]. Reverse transcription reactions for cDNA synthesis were carried out using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, USA). To quantify cDNA generated by reverse transcription from target RNA, real-time PCR with SYBR Green I was performed by using Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen, USA) in an Applied Biosystems 7500 Real Time PCR System 5700 sequence detection system (Applied Biosystems, USA), following the manufacturer's instructions. The 50-μl reaction mixture contained 1X Platinum SYBR Green qPCR SuperMix UDG with ROX (Applied Biosystems, USA), 0.2 μM each primer, and 100 ng of the template (reverse transcription reaction product). The following primer pair (Table 2) have been used to quantify the transcript level of a selection of marker genes: *ACT1*/*ACT2* (Actin) *ANPfor*/*ANPrev* (*ANP1*), *INOfor*/*INOrev* (*INO2*), *KARfor*/*KARrev* (*KAR2*), *OSTfor*/*OSTrev* (*OST1*), *SACfor*/*SACrev* (*SAC1*), *SEC1for*/*SEC1rev* (*SEC1*), *SEC61for*/*SEC61rev* (*SEC61*), *SEC72for*/*SEC72rev* (*SEC72*). The MicroAmp Optical 96-well reaction plates with optical caps were used, processing three replicates of each sample and the related negative and positive controls. Fluorescence was measured at the end of the annealing-extension phase of each cycle and a threshold value for the fluorescence of all samples was set manually. The reaction cycle at which the PCR product exceeded this fluorescence threshold was identified as the threshold cycle (C_T). The real-time RT-PCR results were normalized as the ratio to the level of actin mRNA.

2.4. Electron and fluorescence microscopy

Preparation of thin sections of yeast samples, immune reactions and immune-electron microscopy assay of the grids were performed as previously described [10]. To establish the number of proliferated membrane structures within each cell and the percentage of cells carrying these structures, strains were grown at mid logarithmic phase in YNB medium with 2% glucose, harvested, washed with water and induced in YNB medium with 2% galactose for at least 12 h at 30 °C and 180 rpm. Direct fluorescence microscopy was utilized for

Table 2
Oligonucleotides used.

Name	Sequence
GUPforH	GAGAAGCTTATGTCGCTGATCAGCATCCTG
GUPrevE-His	AAGGAATTCTCAGTGATGGTGATGGTGATGGCATTTTAGGTAAATTCCGTGCCT
MutGUPfor	TTCGTAGCTATATGGCTTGACATCGAACTAAAG
MutGUPrev	CTTTAGTTCGATGTCAAGCCATATAGCTACGAA
*GFP*GUPMutF	GGAAAAGACTGCTAGTGATGTCAAAACTC
*GFP*GUPMutR	TTCGTAGCTATATGGCTTGACATCGAACTAAAG
ACT1	CTGGGAYGAYATGGARAAGAT
ACT2	GYTCRGCCAGGATCTTCAT
ANPfor	AGCATTCGGTAGGCTCTCCC
ANPrev	GGCAAGCCGATGACATTGTA
GDAfor	GTCTCCACCATGCTCTTTCAATG
GDArev	TGTACGAACTAAAGAAGGTTGGTG
INOfor	GCCAACTGAGTTCACCACGA
INOrev	TCATTGCCGATAGGCTGATG
KARfor	GTCCAAGGTCGCTTATCCAATT
KARrev	CCATCAGCACCTCCGTACAAC
OSTfor	TTCACTGATTTGGTTGGCCTT
OSTrev	AATGGTCACGTTCTGCATGC
SACfor	AGTTTGAAAGTGCCGATGTCG
SACrev	CGTTGTCTTCCCAAGTGCTTC
SEC1for	CCCGCCTGATTTCCTTCTAAG
SEC1rev	GAGACTGGTTGTGCCACCG
SEC61for	GCTCTTTCTGTTGGCTCCGA
SEC61rev	TGGATGCCCCAGAACCTAAA
SEC72for	AGGAAGGCGGACTGTTTGC
SEC72rev	TTGCCCTAGCCTCTTCCCA

localization in living cells of proteins tagged with the Gfp and/or the red fluorescent protein (Rfp). Rhodamine 6G (Sigma) and DAPI staining were performed as described respectively by Terasaki et al. [39] and Bleve et al. [10]. The Zeiss LSM5 Pascal confocal laser scanning microscope (CLSM) equipped with objectives 40.0/1.0 and 63.0/1.0 oil was used to obtain confocal images. Images were analysed using the Carl Zeiss LSM5 and Adobe Photoshop (Adobe System Inc., Mountain View, CA, USA) software. Images were acquired using emission interval 505–530 nm for Gfp and 560–600 nm for Rfp or Rhodamine. Pinhole was of 1.2 μm. Qualitative fluorescence recovery after photo-bleaching assays (FRAP, Fluorescence Recovery after Photo Bleaching) were performed by photo-bleaching a region of interest at high-power laser intensity and monitoring fluorescence recovery by scanning the whole cell at low power laser intensity. Pinhole was increased to 1.8 μm to collect the total fluorescence emitted by the yeast cell.

2.5. Pulse-chase and immunoprecipitation

Pulse-chase and immunoprecipitation were performed essentially as previously described [40]. Freshly transformed yeast cells were suspended in a medium without methionine and pulse-labeled with 100 μCi/ml of an EasyTag EXPRE35S35S Protein Labeling Mix (PerkinElmer, USA) for 120 min. Chase was performed by adding unlabelled methionine to a final concentration of 1.11 mM to a final incubation volume of 1.5 ml. The zero time-point was taken by removing 200 μl of cells suspension, immediately after mixing, to a fresh, pre-cooled 1.5 ml tube containing 0.3 ml H buffer (150 mM Tris–HCl, pH 7.5; 150 mM NaCl; 2%, v/v, SDS; supplemented immediately before use with 20 μl of a protease inhibitor cocktail, (P2714; Sigma-Aldrich, USA). Further time-points samples were collected similarly after 2, 4, 6, 12 and 24 h of incubation and stored at −20 °C until use. All time-point samples were boiled for 5 minutes, freeze/thawed and incubated on ice for 15 minutes, then centrifuged at 15000 g at 4 °C for 15 min, transferring the supernatants to fresh, pre-cooled tubes in order to remove any insoluble debris. All time-point samples were brought to a total volume of 1 ml with NET-gel buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.1% (v/v) Nonidet P-40; 0.02% (w/v) NaN_3) and mixed well. Anti-GFP serum was added as appropriate, samples mixed, and incubated for 4 h at 10 °C. 100 ml of a 10% (swelled bead v/v) suspension of Protein-A Sepharose (Amersham, USA) in NET buffer was added to each sample, before incubating on a rotating wheel at 10 °C for 4 h. Beads were harvested by centrifugation at 3000 g for 1 min at 4 °C, the supernatant aspirated and the pellet suspended in 1 ml NET-gel buffer. Beads were washed in this way two times. After the removal of the last supernatant, beads were immediately prepared for separation by SDS-polyacrylamide gel electrophoresis.

3. Results

3.1. High level expression of Gup1 protein induce membrane proliferation

The *GUP1* cDNA, cloned into a multicopy expression vector tagged with *GFP* at its carboxy- and amino-terminus, was expressed in *S. cerevisiae* under control of the galactose-inducible *GAL1* promoter. *gup1*Δ strain expressing either the *GUP1–GFP* or the *GFP–GUP1* fusions were examined by electron microscope and confocal laser scanning microscopy.

The *S. cerevisiae* cells over-expressing *GUP1–GFP* or *GFP–GUP1* chimeras, induced for 16 h with galactose, were fixed and processed to IEM. Many cisternal aggregates organized in proliferated structures, were clearly detectable (Fig. 1A and B). The above membrane structures, which were observed in a number ranging from one to seven per cell, showed circular or rod-like organization, and appeared physically separated from the others. Yeast cells not over-producing Gup1 protein in N- or C-terminal fusion with Gfp and used as control did not show such marked accumulation of membranes (not shown). Ultrathin sections of cells expressing *GUP1–GFP* and *GFP–GUP1* were probed with a polyclonal antiserum raised against the Gfp. Gold particles appeared throughout the layers of membranous structures in strains that over-produced the chimerical proteins (Fig. 1A and B), whereas control sections exposed to only the gold-conjugated secondary antibody were not labeled (not shown). These results established that the chimerical proteins were present in the membrane agglomerates induced as a response to over-production

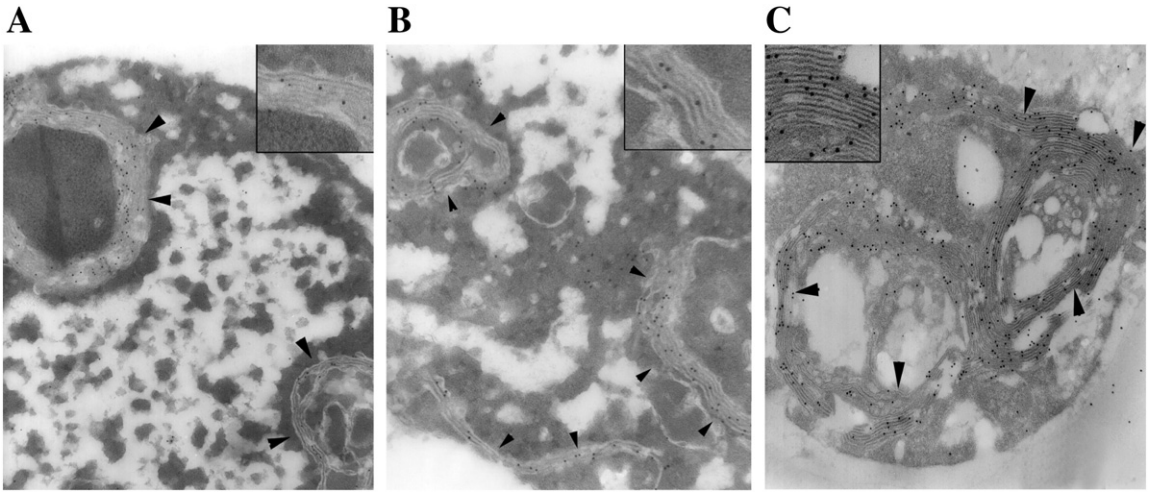

Fig. 1. Immunolocalization of Gfp in ultra-thin section of cells expressing *GUP1–GFP* (A), *GFP–GUP1* (B) and *GUP1* fused with histidine tag (C). The cells were fixed and processed for detection of the Gfp and histidine tags using the specific primary antiserum and analyzed by immunoelectron microscopy. Colloidal gold particles (arrow heads) indicate the presence of the chimerical proteins on the proliferated membrane structures. Insets, parts of newly formed membrane layers at higher magnification.

of the Gup1–Gfp and Gfp–Gup1 proteins. No gold particles were found to be associated with the nucleus or with any other organelle or throughout the cytoplasm. As shown in Fig. 1, the proliferated membranes can be assembled in stacks of paired elements similar to cisternae, which were found in various parts of the cytoplasm. Stacked cisternae labelled with gold clusters appeared to be the structure of membrane that produced the proliferations detected by CLSM (Fig. 2A, B, E). Similar results were obtained assaying, as above described, ultrathin sections of cells expressing *GUP1* tagged with the His-tag, using a monoclonal anti-His antiserum (Fig. 1C). The marked proliferation of the membrane forming stacked cisternae was immediately obvious and membranous clusters showed a similar morphology in the 70% of cells (210 out of 300 observed cells) over-producing Gup1-His protein.

After 24 h, these structures formed bright clearly defined patches and circular patterns of staining within the cytoplasm, but mainly not continuous with the nuclear envelope (Fig. 2A and B). Cells of *gup1*Δ strain transformed with the vector alone, did not show any fluorescence signal (Fig. 2C), whereas a whole cell diffused *GFP* signal could be observed when the above strain was transformed only with *GFP* gene (Fig. 2D). These observations suggested that the over-production of chimerical proteins resulted in some alterations in localization and approximately 80% (320 out of 400 observed cells) of the cells displayed the observed membrane proliferations (Fig. 3). Different microscopy areas have been observed to evaluate the distribution of proliferated membrane structures per cell in a population over-expressing the *GUP1* gene after 12 h induction. The number of proliferated membranes per cell ranged from one to seven and their distribution in a population of cells over-expressing *GUP1* was stated by observing 400 randomly chosen cells (Fig. 3). Cells commonly had 4 or fewer structures and cells with 6 or 7 of them were infrequent. In addition, no cell >8 Gup1 protein-induced proliferations was identified.

The comparison of the fluorescence from the Gfp fused to Gup1 protein with that from the DAPI-stained nuclear DNA revealed that fusion proteins were not restricted or associated with the nuclear envelope (data not shown). The bright Gfp fluorescent areas represented membrane proliferations independent from the Gfp fusion to Gup1 protein, because these areas were also revealed in cells over-expressing Gup1 protein alone and stained with the ER membrane dye rhodamine 6G (Fig. 2E). Moreover, this result indicated that both the carboxyl terminus and the amino-terminus of the Gup1 protein did not appear to be critical *in vivo* for incorporation of the protein into the newly formed membrane structures.

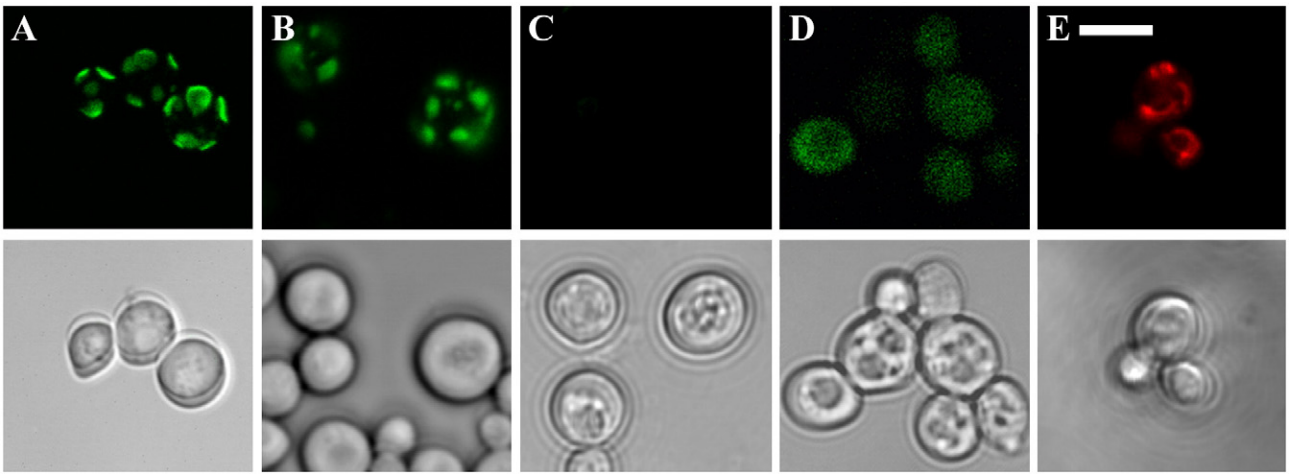

Fig. 2. Confocal laser scanning visualization, after 24 h *GAL1* promoter induction, of yeast cells over expressing *GUP1–GFP* (A), *GFP–GUP1* (B), *GFP* (D), and *GUP1* (E, staining with rhodamine 6 G) genes. Yeast cells were also transformed with the vector only, as control (C). Bar = 10 μm.

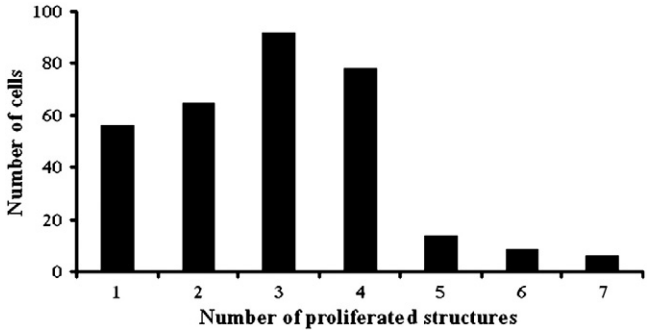

Fig. 3. The number of proliferated membrane structures per cell in a strain that over-produced the Gfp–Gup1 protein. The samples were subjected to confocal laser scanning microscopy analysis and then the number of structures was scored by analysing 320 proliferation-containing cells. For this analysis, cells in the population that did not contain proliferations were not considered. The distribution of the number of membrane proliferations per cells ranged from 1 to 7. No membrane proliferations were detected in cells transformed with the vector alone.

3.2. In vivo dynamics of membrane aggregates formation

The biogenesis of proliferated membranes produced by *GUP1* over-expression were studied by analysing during the time the accumulation pathway of the Gup1 protein either with N- or C-terminal tagging with Gfp. Time course analysis confirmed that, as previously shown (Fig. 1), both N- and C-terminal Gfp-tagged version of Gup1 protein showed an identical accumulation-kinetic pathway (not shown). We chose the Gfp–Gup1 protein for the subsequent assays. At the early stage of induction, the Gfp–Gup1 protein localized to the ER and plasma membrane (Fig. 4A, 2 h after induction). This pattern was then subjected to dramatic phenotypic variations, which appeared to be the function of the incremental induction time. In fact, after the 4 h induction, the tagged Gup1 protein appeared to accumulate less homogeneously, forming punctuate intracellular structures and, once their formation was initiated, these structures grew brighter and larger with time (Fig. 4A). By 24 h, most of the punctuate structures had disappeared and merged to form a number of larger aggregates, clearly visible as bright patches dispersed within the cytoplasm (Fig. 4A). These results suggest that the over-expression of *GUP1* alone or *GFP–GUP1* might be sufficient to induce the formation of proliferated membrane structures. The consequent questions are whether the proliferation of membrane structures induced by Gup1 protein over-production, influenced (i) the structure of the ER and the Golgi compartments and/or (ii) the lipid biosynthetic pathway related to membrane biosynthesis. Possibility (i) was assessed measuring the expression levels of genes encoding resident proteins of these two membrane systems: *OST1*, which is part of the oligosaccharyl transferase complex [41], *ANP1*, a subunit of the alpha-1,6 mannosyltransferase complex [42]. Option (ii) was tested evaluating the expression levels of *INO2*, component of the heteromeric Ino2/Ino4 proteins [43,44]. Real time RT-PCR analysis showed that, over-expression of the *GFP–GUP1* construct resulted in an up-regulation of all the three genes during the time course. In particular, *ANP1* and *OST1* showed the same trend of transcriptional enhancement, which ranged from 2-fold (4 h) to 3.5- and 3.3-fold (6 h), respectively (Fig. 4B). The transcript levels decreased to 1.7-fold for both genes after 24 h, suggesting that a steady state level had been reached for protein synthesis. In addition, *INO2* transcripts revealed a behaviour similar to *ANP1* and *OST1* during the time course (Fig. 4B), even though it was the most highly expressed, showing a higher level of induction ranging from 2.4-fold (2 h) to 18.3-fold (6 h). The *INO2* transcription level also increased to 4.1-fold after 24 h after *GFP–GUP1* induction. These results, consistent with the membrane proliferation observed by microscopy, indicate that, in response to *GFP–GUP1* over-expression, resident proteins of ER and Golgi were synthesized and new membranes were produced.

3.3. Gfp–Gup1 protein mobility within proliferated membrane structures and its diffusion in and out of these structures

A model to describe the biogenesis of proliferated membrane proteins due to over-production of Gup1 and Gfp–Gup1 proteins required the study of Gfp–Gup1 proteins mobility in and out of these structures. We wanted to test whether the bound chimerical protein detained a restricted mobility within the *de novo* membrane arrangement. To perform this investigation in *GFP–GUP1* over expressing cells, half of the area of a typical proliferated structure was photo bleached using high-power laser intensity and the fluorescence recovery was monitored using low-power laser

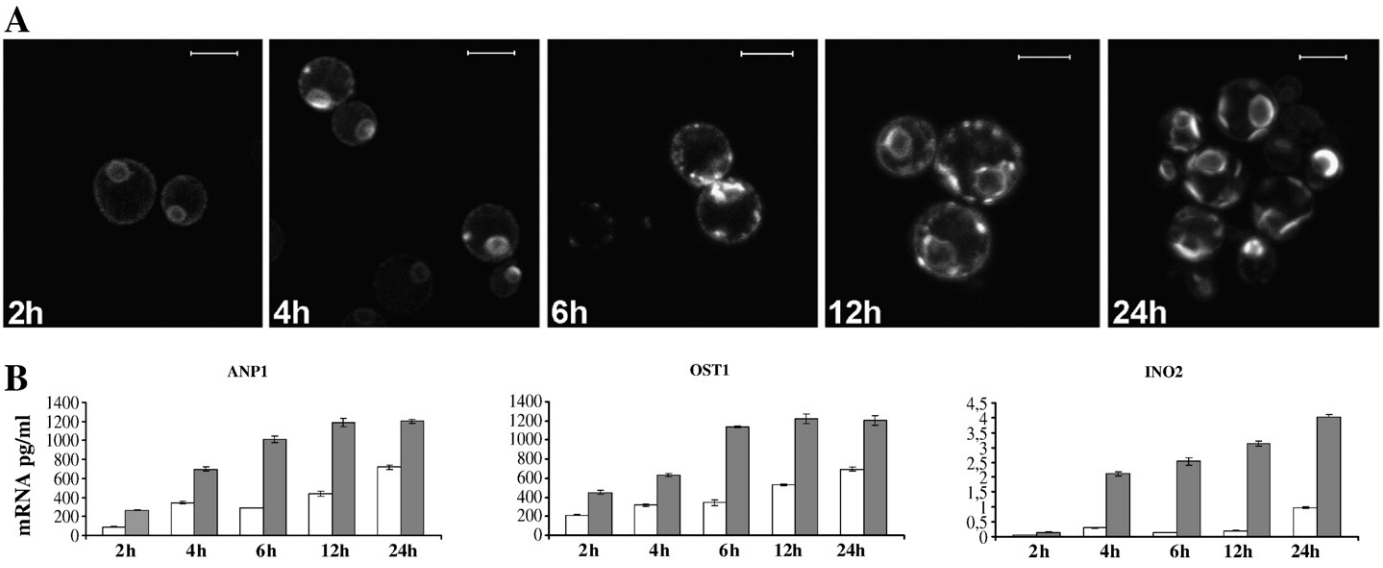

Fig. 4. Gfp–Gup1 fusion protein accumulation and transcription levels of genes selected as specific markers of cell membrane compartments in BHY54 strain transformed with pY-*GFPGUP* during 24 h induction. (A) Cells growing exponentially in YNB-glucose were harvested by centrifugation, washed twice with sterile water, and incubated in YNB-galactose. Cells were visualized by confocal laser scanning microscopy at indicated time. Bar = 10 μm. (B) Quantification of real-time RT-PCR of *OST1*, *ANP1* and *INO2* expression induced by pY-*GFPGUP* (full columns) and by pYES2 vector only (empty columns). Values have been expressed as mean ± SD.

intensity. Fluorescence recovered into the bleached area of the proliferated membrane structure (Fig. 5A). Because the pre-bleach fluorescence in the sector was fully restored, we inferred that all Gfp–Gup1 molecules were mobile within the proliferated structures, and not immobilized within proliferated structures by firm cross-linking between proteins on adjacent membranes. The same procedure was used to assess whether the chimerical proteins were able to move freely into and out of these newly synthesized structures, with the difference that an entire membrane structure (Fig. 5B), or an ER area outside of an overproduced membrane structure (Fig. 5C), were alternatively photo-bleached. The initial fluorescence pattern was recovered within 300 sec (Fig. 5B, C). These results indicate that Gfp–Gup1 protein, a membrane protein that has about predicted 10-12 trans-membrane domains, shows that it could move within the proliferated membrane structures diffusing in and out of these structures.

3.4. Increased levels of Gup1 protein affected growth of ire1Δ mutant and induced unfolded protein response

To determine whether the *IRE1*-dependent regulation of ER components synthesis was required for the formation of the aberrant membrane structures, *GUP1* was expressed in RWY1095 (*ire1Δ::KanMX6*) and the RWY1181 [*ire1(1-1116):KanMX6*] mutant strains. In fact, both the RWY1095 and RWY1181 strains were separately transformed with pYES2-derivative plasmids containing the *GUP1* gene alone (pY-*GUPHis*) and in N-terminal (pY-*GFPGUP*) or in C-terminal fusion (pY-*GUPGFP*) with the *GFP*, under the control of the galactose-inducible *GAL1* promoter. All transformed yeasts grew well on medium containing glucose. When galactose was used as carbon source (Fig. 6), the strains transformed with the empty vector or expressing *GFP* alone showed a non-affected phenotype. On the contrary, yeast carrying a complete or a partial *ire1* deletion poorly grew on galactose medium because of the over-production of the Gup1 protein alone or in fusion with Gfp. Interestingly, the over-expression of *GFP–GUP1*^{H447L}, a mutant version of *GFP–GUP1* gene (denoted *GFP–GUP1*^{H447L}) produced by site-directed mutagenesis giving a protein with the histidine residue in position 447 replaced with a leucine (L), did not induce a death phenotype in the *ire1Δ* yeast strains with JRY527 background. These results show that Gup1p-induced membrane proliferation affects negatively the growth of *ire1* mutants in the JRY527 background. Importantly, this effect is dependent on the supposed active centre of the Gup1 activity as an acyltransferase [9]. To confirm whether Gup1 protein-induced proliferations assembly provoked the UPR, we checked in cells over-expressing *GFP–GUP1* the expression levels of *KAR2*, coding the luminal HSP-70 molecular chaperone, a key protein of the UPR pathway. As shown in Fig. 7, chimerical gene over-expression resulted in an up-regulation of *KAR2*, whose transcription rate increased 24-fold compared to the amount of the correspondent mRNA in the control strain (Fig. 7), indicating that the membrane proliferations induced the UPR pathway.

3.5. GUP1 over-expression enhanced the transcription levels of key genes of the secretory pathway

In view of the above results, we decided to verify if the increased amount of ER and Golgi protein markers in the cells overexpressing *GUP1* (Fig. 4B) also corresponded to increased transcription levels of markers related to the secretory capacity of the cell. The expression levels of compartmental marker genes were evaluated by real time RT-PCR. Transcripts examined included *SEC61*, *SEC72*, and *OST1* that encode rough ER-specific proteins (Fig. 7). Results showed these genes were up-regulated to a more limited extent than *KAR2*. *SEC61*

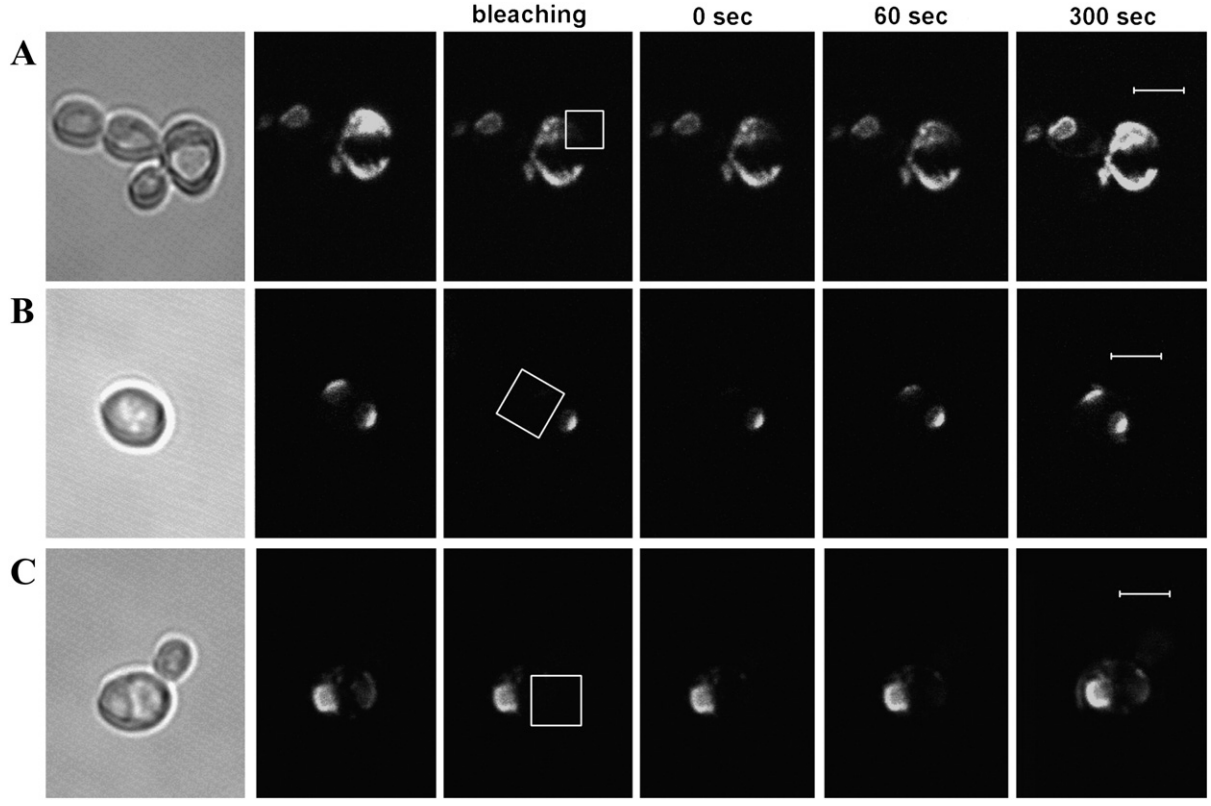

Fig. 5. Gfp–Gup1 protein mobility within and between proliferated membrane proteins and branching ER in *GFP–GUP1* over-expressing cells. Cells over-expressing *GFP–GUP1* were photo-bleached in discrete regions of interest (white outline boxes), which were then monitored for fluorescence recovery. (A) Gfp–Gup1 protein was mobile within a proliferated membrane structure; (B) when a whole proliferated membrane structure was photo-bleached, fluorescence recovery was observed; (C) Gfp–Gup1 protein was mobile within branching reticular ER and recovered rapidly. Bar = 10 μm.

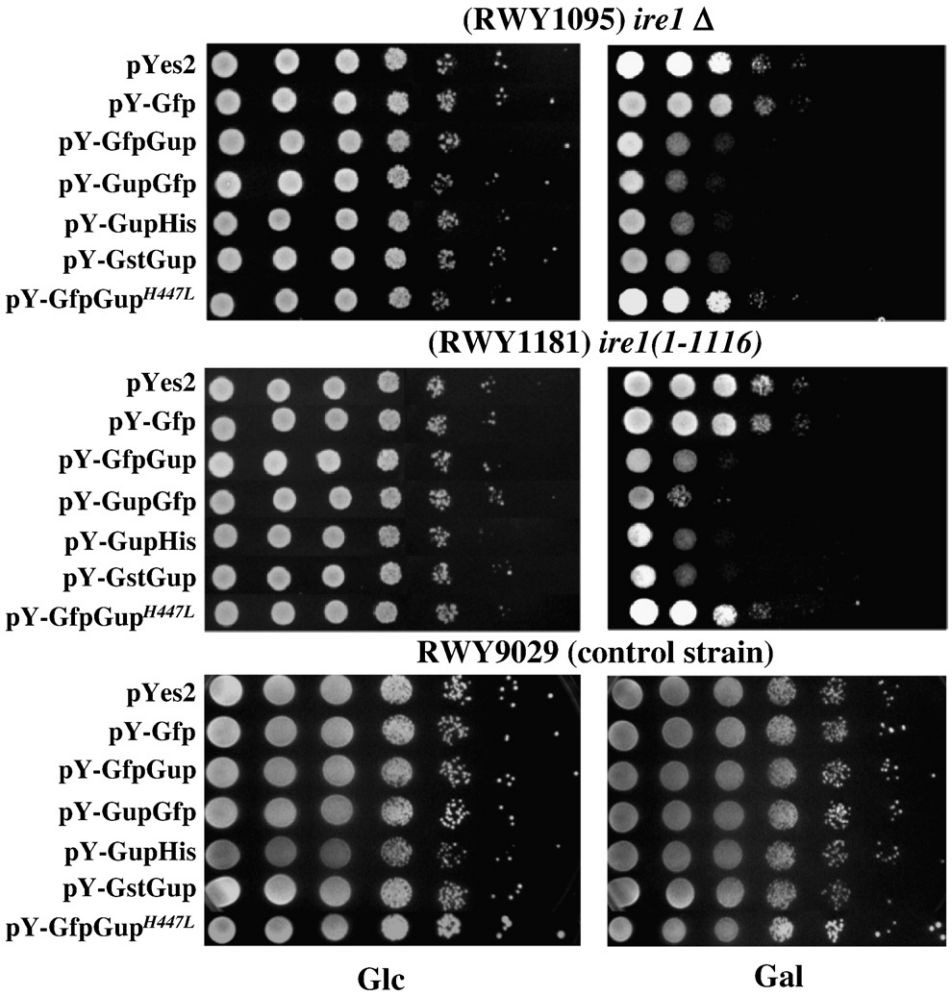

Fig. 6. Growth of *ire1Δ* mutants was inhibited by over-expression of *GUP1*. Recombinant JRY527 *ire1Δ::KanMX6* (RWY1095) and JRY527 *ire1(1–1116):KanMX6* (RWY1181) were grown on minimal medium with glucose added as carbon source. Ten microliters of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} diluted strains were spotted onto galactose medium to induce expression of *GFP–GUP1*, *GUP1–GFP*, *GUP1-His* and *GUP1*^{H447L} genes. The pYES2 vector and its derivative expressing *GFP* alone were used as control. Cells harbouring the indicated plasmids were plated either on glucose (left) or galactose (right) and incubated at 30 °C for 3 days.

was expressed at 10-fold higher levels, whereas *SEC72* and *OST1* expression, respectively, increased 6.1- and 6-fold (Fig. 7). Transcripts from the following marker genes for organelles having a post-ER role in the secretory pathway were quantified by real time RT-PCR: *SAC1* (encodes a protein localized to ER and Golgi membranes), *SEC1* (producing a cytosolic/peripheral membrane protein), and *ANP1*. Transcripts encoding the three gene products above were all produced at significantly higher levels (from 23.5- to 5.6-fold) in cells expressing *GFP–GUP1*, compared with the vector-only containing cells (Fig. 7). Even though a global analysis of all genes involved in the late secretory pathway was not carried out, the above-obtained data made it possible to hypothesize that the whole secretory pathway may be up-regulated in response to *GUP1* over-expression.

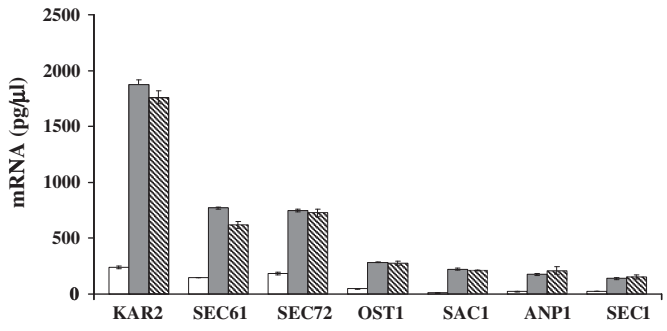

Fig. 7. Histogram representing the levels of transcripts detected in *S. cerevisiae* cells over-expressing *GFP–GUP1* (grey box) or *GUP1-His* alone (dashed box) under the control of *GAL1* promoter and in cells transformed with the empty vector (white box), as control. The following genes were analyzed: *KAR2*, *SEC61*, *SEC72*, *OST1* (rough ER-specific proteins); *SAC1* (ER and Golgi membrane protein); *ANP1* (Golgi membrane protein); *SEC1* (cytosolic/peripheral membrane protein). Values have been expressed as mean ± SD. The RNA templates were extracted after 12 h galactose induction of *S. cerevisiae* cells over-expressing *GFP–GUP1* or *GUP1-His* alone.

3.6. The proliferated membranes induced by GUP1 over-expression contain ER, Golgi and itinerant proteins

In order to investigate the nature of *de novo* formed membranaceous structure, we examined by confocal laser microscopy the colocalization of Gfp–Gup1 protein in several yeast strains, each one constitutively expressing a selected compartmental gene in fusion with the *RFP* ([37]; Table 1). The tested reference strains respectively produce as Rfp chimera the following proteins: Anp1 (Golgi marker), Cop1 (early Golgi), Sec13 (an ER to Golgi marker, component of the CopII complex) and Chc1 (Golgi/clathrin marker). Fluorescent signals produced by all Golgi marker proteins (red) were mainly superimposed on the proliferated membrane structures originated by Gfp–Gup1 over-production (green), as reported in Fig. 8. However, the typical globular localization pattern of the Sfn protein tagged with Rfp did not merge with the Gfp bright patches originated by the *GFP–GUP1* over-

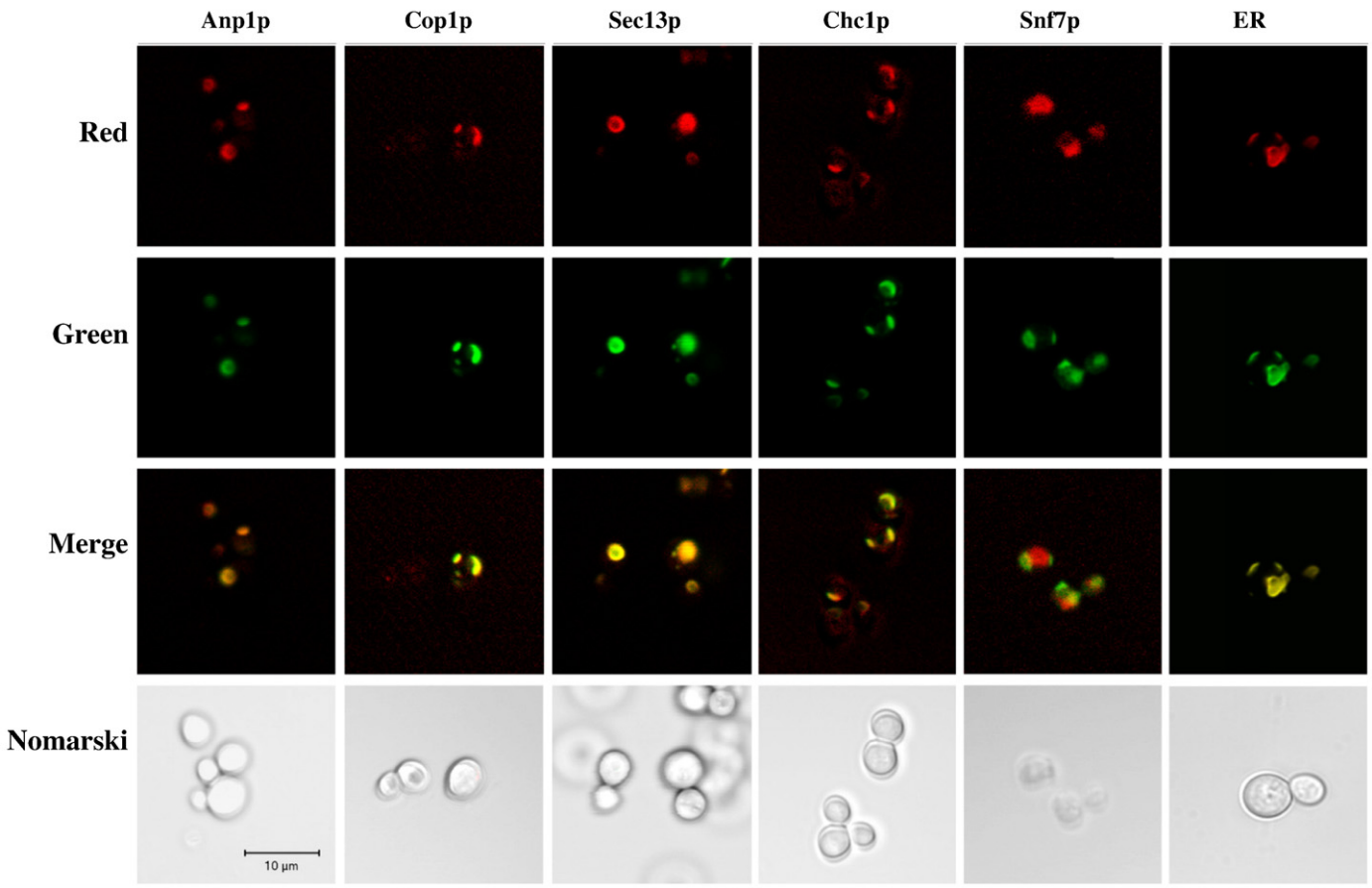

Fig. 8. Over-expression of *GUP1–GFP* in reference strains, which each one respectively produce a Golgi (Anp1p), an early Golgi (Cop1p), an ER to Golgi vesicle (Sec13p), a late Golgi/clathrin (Chc1p) and an endosome (Snf7p) resident proteins fused with the Rfp. Rhodamine 6G has been used for specific staining of ER complex. The recombinant strains were analysed by confocal laser scanning microscopy to detect Rhodamine/Rfp (Red), Gfp (Green), Rhodamine/Rfp/Gfp (Merge) fluorescent signals. Nomarski images (Nomarski) are also provided for each different experiment. Bar = 10 µm.

expression (Fig. 8). The localization pattern of the above compartmental markers in the corresponding mother strains transformed with the empty vector as control (data not shown), shown to be identical to the localization pattern previously described [37]. Quantitative analysis of individual experiments revealed that 87% of Anp1-Rfp ($n = 115$), 79 % of Cop1-Rfp ($n = 109$), 82% of Sec13-Rfp ($n = 111$) and 85% of Chc1-Rfp ($n = 108$) were colocalized with the Gfp–Gup1 protein at the level of the induced membranaceous proliferations. The Gfp fluorescent areas representing membrane proliferations also stained with the ER membrane dye Rhodamine 6G (Fig. 8). These co-localization studies confirmed that the membrane proliferations in response to *GFP–GUP1* over-expression were derived from the ER and contained also: Golgi resident proteins (Anp1 protein); proteins belonging to vesicles trafficking between the Golgi and plasma membrane and between the Golgi and endosomes (Chc1 protein), proteins responsible for retrograde transport from the Golgi to the ER (Cop1 protein) and proteins responsible for anterograde transport from the ER to the Golgi and component of the COPII coat (Sec13 protein).

3.7. Over-expression of the GFP–GUP1^{H447L} mutant gene did not induce membrane proliferation

Confocal laser scanning microscopy was used to observe the protein-induced proliferation biogenesis when *GFP–GUP1^{H447L}* gene was over-expressed in *gup1Δ* cells. These cells were monitored up to 24 h after induction and during the time course experiment the Gfp–Gup1^{H447L} chimerical protein showed a fluorescence pattern with a localization at the plasma membrane and endoplasmic reticulum (61 % of cells observed on 100 different fields) and at punctuate structures (39 % of cells) (Fig. 9A). However, the cells over-expressing the above construct did not show the proliferations described for *GFP–GUP1* gene over-expression and this behaviour was maintained also when the *GFP–GUP1^{H447L}* gene was over-expressed in the yeast wild type strain (data not shown), showing that the observed fluorescence pattern was not influenced by the yeast *gup1Δ* genotype. Yeast cells carrying *GUP1* deletion were hypersensitive to calcofluor white (CFW) [7] and this phenotype was not modified by the over expression of the *GFP–GUP1^{H447L}* gene (Fig. 9B). The real time assay, performed during a time course analysis up to 24 h, indicated that the *GFP–GUP1^{H447L}* gene detained transcription levels even higher than those showed by *GFP–GUP1* gene, harboured in the same cell line (Fig. 9C). At the same time points, comparable amounts of Gfp–Gup1 protein and Gfp–Gup1^{H447L} proteins were detected (Fig. 9D). The two last evidences indicated that the subcellular and physiological phenotypes of cells harbouring the *GFP–GUP1^{H447L}* gene were not promoted by a lower transcription or translation level of the mutant gene in the transformed yeast line under analysis. Taken together, all these findings suggested a possible role of the histidine residue in position 447 of Gup1 protein in the assembly of *de novo* membranaceous structures.

4. Discussion

In the present paper, we demonstrated that (i) high-level expression of *GUP1* gene in *S. cerevisiae* resulted in the formation of proliferated structures, harbouring ER, Golgi and itinerant proteins; (ii) the Gup1p acyltransferase activity was needed for the described membranaceous arrangements formation. As previously shown,

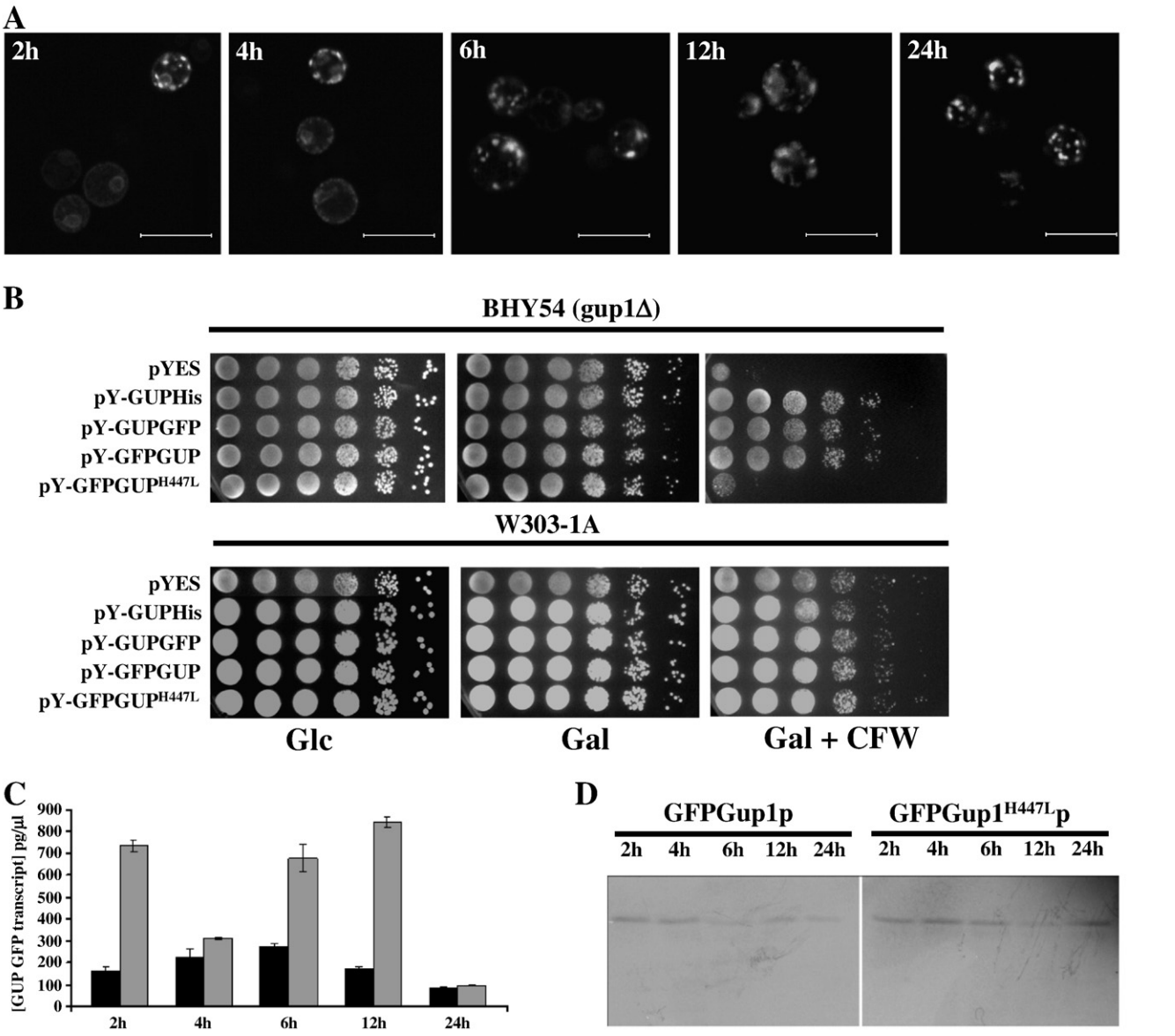

Fig. 9. (A) Confocal laser scanning visualization of cells expressing the *GFPGUP1*^{H447L} gene. Cells were exponentially grown in YNB-glucose, harvested, incubated in YNB-galactose and then sampled at the indicated times. Bar = 10 µm. (B) Yeast cells harbouring the *GFP–GUP1*^{H447L} gene are hypersensitive to calcofluor white (CFW). Complementation of the W303-1A and of the BHY54 mutant strain (gup1Δ) with *GUP1-His*, *GUP1–GFP*, *GFP–GUP1* and *GFP–GUP1*^{H447L} fusion genes. The pYES2 vector was used as control. Tenfold dilution of indicated strains harbouring the indicated was spotted onto glucose, galactose and galactose medium containing 50 µg/ml CFW. (C) Histogram representing the transcription levels of *GFP–GUP1* (black box) and *GFP1–GUP1*^{H447L} (grey box) genes detected in the transformed yeast line under analysis at indicated time. Values have been expressed as mean ± SD. (D) Accumulation of Gfp–Gup1 and Gfp1–Gup1^{H447L} proteins in the transformed yeast line under analysis. BHY54 cells harbouring *GFP–GUP1* or *GFP1–GUP1*^{H447L} genes were pulse-labelled for 2 h and chased for the indicated period of time. Proteins were visualized by autoradiography after immunoprecipitation using the anti-GFP antiserum.

when *GUP1* was expressed either by its own promoter or by *GAL1* promoter for less than 4 h, the protein localized at the plasma membrane and ER levels [10]. The membrane proliferations became detectable in the recombinant yeasts when *GUP1* was highly over-expressed for a prolonged time. As indicated by Snapp and co-workers [45], this evidence could be explained by the fact that a significant level of ER-inducing proteins in ER membranes must be achieved before proliferated structures can form within a cell. This behaviour was similar to that shown by the Hmg2 protein-induced membrane proliferations, which did not become abundant until late in the growth phase of recombinant yeast [27]. The observed formation of proliferated membrane structures in presence of Gup1 protein over-production alone or in N- or C-terminal fusion with the Gfp excluded the hypothesis that the abnormal membrane proliferation would be enhanced and/or influenced by Gfp low affinity dimer formation [45].

Two different models have been recently proposed by Fukuda et al. [46] and by Snapp and co-workers [45] to describe the Organized Smooth ER (OSER). The second model, providing that OSER-inducing proteins diffuse in and out of OSER structures and share dynamically without any cross-link among them or with other structures, could be considered more suitable to describe the biogenesis of the proliferated membrane structures produced by *GUP1* over-expression. In fact, photo-bleaching experiments, performed on the proliferated membranes described in this study, revealed that proteins responsible for these arrangements were not strongly connected among themselves and were able to diffuse in and out of structures (Fig. 5). Furthermore, considering the high transcription levels and the bright localization signals revealed for a number of tested resident proteins of ER and Golgi compartments, it could be hypothesized that Gup1 protein did not exclude other membrane proteins from the induced structures.

Vergères and co-workers hypothesized that membranes retain "sensor" capacity, which make them able to supervise their own biophysical properties, i.e. fluidity, surface charge density or protein density [47]. Since the Gup1p is involved in triacylglycerol/phospholipids anabolism [9] and in sphingolipids-sterol-ordered domains integrity and assembly [16], as well as in GPI anchors remodelling [17,48], one of these or all of these processes might be related to the membrane overproduction and to the other protein overexpression.

The arrangement of the ER membranes has a flexible configuration and it can be quickly transformed into alternative structures in reply to changed metabolic conditions, i.e. membrane protein overproduction [49]. ER could differentiate from reticular ER structures that could be arranged in different forms as: stacked cisternae originating from the outer nuclear envelope known as karmellae [27,50,51], lamellae distributed within the cell [51,52], compressed bodies of packed sinusoidal ER [53], BiP bodies [28], enlarged ER exit loci [29,30], Russell bodies [31], proliferations of the ER-Golgi intermediate compartment (ERGIC; [32]), concentric membrane whorls [51]; crystalloid ER, with hexagonal or cubic symmetry [54]. Considering their physical interaction with the main cellular compartments, the induced extra membranes could be found associated with the nuclear envelope [27,47,55], with the plasma membrane [27] or could simply be found in the cytoplasm [55]. The Gup1 protein-induced membrane proliferations could resemble the sub cellular abnormality produced by P450cm1 over-production [55], because of the absence of conserved continuity with the nuclear envelope and the lack of any stacks of packed cisternae surrounding part of the nucleus. However, the number, the morphology and the distribution of separate structures within the cell could allow differentiating proliferated structures produced by *GUP1* over-expression from the already described ER rearrangements.

In order to ascertain the formation dynamics of proliferated membrane structures, the transcription rate of marker genes of ER, Golgi and phospholipids biosynthesis had been investigated. As shown by the time course analysis of membrane proliferation biogenesis, whilst the levels of Gfp–Gup1 protein fluorescence within the cells increased due to new protein synthesis, a concomitant enhancement of ER and Golgi compartments was observed, as indicated by transcript levels of genes encoding specific resident proteins of these compartments (Fig. 4B).

INO1 transcription is activated by the heteromeric transcription activator, which binds the conserved *cis*-acting consensus sequence (ICRE; [56]). *INO2* gene encodes for a component of the above activator and it is required for derepression of phospholipids biosynthetic genes [57]. The results reported here revealed an increased *INO2* expression because of *GUP1* over-expression, thus indicating that genes involved in phospholipids biosynthesis are differentially expressed in systems where ER biosynthesis was increased. However, the obtained evidence did not allow to determine a possible direct involvement of Gup1p in phospholipids remodelling.

The signal transduction pathway of the unfolded protein response (UPR) becomes activated in response to different input signals, such as inositol depletion, accumulation of unfolded protein in the ER lumen and upon overproduction of an ER membrane protein [58]. The ability of a yeast cell to assemble Gup1 protein-induced membrane structures depended on an intact unfolded protein response because: (i) Gup1 protein-induced proliferation was inhibited in the RWY1095 and RWY1181 *ire1Δ* mutant strains, which have a non-functional UPR [58] and showed that they grow properly on galactose [59]; (ii) over-expression of *GUP1* led to increased transcription of *KAR2* gene, it being a characteristic feature of the UPR pathway activation [58,60]; (iii) Gup1 protein-induced membrane structures enhanced *INO2* transcription levels (Fig. 4B), which is correlated to the activation of the unfolded protein response (UPR) signal transduction pathway [58]. The possible influence of *GUP1* over-expression in enhancing cellular secretory capacity was verified by evaluating the transcription level of a number of genes being indicators of: ER compartment (*KAR2*, *OST1*), ER-associated protein secretion (*SEC61*, subunit of the Sec61 complex), post-translational translocation (*SEC72*, subunit of the Sec63 complex), post ER secretory pathway (*SAC1*, *ANP1*, *SEC1*). As shown for p180 over-production [34], the increase of ER and protein translocation markers indicated that functional ER membranes were produced in response to *GUP1* over-expression. This evidence indicated that the ER secretory (translational and post-translational) pathway were both induced, being *SEC61* and *SEC72* up regulated. In fact, the induction of UPR pathway proofed to be a consequence of a specific remodelling of the whole yeast secretory pathway and that a number of genes, including *SEC61* and *SEC72,* were up regulated in response to stress in the ER [61]. The post ER secretory pathway showed it was also induced by *GUP1* over-expression, as indicated by *GUP1*-dependent up regulation of *SAC1*, *ANP1* and *SEC1* genes, which all truly hallmark the cellular processes and mediate secretive passages from the endoplasmic reticulum to specific destinations inside the cell [62–64].

However, since the above genes did not act as UPR transcriptional targets [61], it could be hypothesized that the enhancement of post ER trafficking could also follow a UPR-independent mechanism. As a subsequent step, to verify if the increased transcription levels of genes involved in the yeast secretory pathway, caused by *GUP1* over-expression, coincided with possible perturbations of cellular compartments devoted to secretion activity, a morphological analysis had been performed by over-expressing *GFP–GUP1* chimerical gene in cells having single different compartmental marker genes fused with the *RFP*. The set of *in vivo* observations showed a striking redistribution to the ER of components not only belonging to the cis/medial Golgi, as could be induced by Brefeldin A treatment [65], but also of elements distinctive of the trans Golgi compartment. This redistribution, never previously observed in yeast, resembled the phenotype caused by overproduction of a number of proteins such as Rer1 [66], the ERD2-like protein ELP-1 [67] and the human KDEL-receptor [68]. Interestingly, no changes in endosome morphology and no merge between Gfp–Gup1 protein and an endosome-resident protein had been reported upon over-expression of *GUP1*, as these organelles were not able to perform a close apposition of their membranes [45]. On the other hand, it was shown that components of both COPI (Cop1 protein) and COPII (Sec13 protein) complexes localized with the membrane proliferations, thus indicating that coated vesicles were present, marking a difference from the results obtained in another similar study concerning over-production of GMAP-210 protein [69]. All the obtained results showed that the entire Golgi complex was found in the ER-proliferation, leading to suppose that the Gup1 protein-induced membranaceous structures could represent a hybrid compartment including Golgi and ER proteins.

We demonstrated that over-expression of a mutated *GUP1* version, which carried a point mutation that inactivated its MBOAT activity [17,70] prevented membrane proliferations, neither did it result in being lethal in the RWY1095 and RWY1181 *ire1Δ* strains, which cannot properly induce the UPR pathway [58]. The above evidence preliminary indicated a possible relationship between the biogenesis of the Gup1 protein-induced proliferated structures and the Gup1 MBOAT activity [11]. Most of the genes involved in GPI biosynthesis are essential for growth of *S. cerevisiae* but, on the contrary, *GUP1* is not essential for growth and its deletion mainly produces phenotypes in the cell wall [7]. Very poor knowledge is available about the effects on cellular physiology caused by up regulation of specific GPI biosynthetic genes. It could be speculated that the metabolic unbalance in GPI anchor synthesis induced by *GUP1* over-expression could affect secretion of remodelled GPI proteins [17,71]. In fact, GPI anchors are likely to act as ER exit signals, because GPI-anchored proteins and other secretory proteins come out from the ER in different vesicles [72]. *GUP1* induction could influence vesicle

trafficking in the anterograde direction from the ER to the Golgi and/or vesicle recycling from Golgi to the ER via the retrograde pathway, thus explaining the enlargement of stacked membranes with Golgi and ER hybrid composition. These effects could be facilitated by the fact that in yeast cells, no spatial segregation between ER and the Golgi apparatus exists and therefore, the pre-Golgi elements are often found next to the ER sites.

5. Conclusions

In conclusion, the studies presented here establish that high-level expression of *GUP1* gene in *S. cerevisiae* induces proliferation of membranaceous structures and results in coordinate expression of secretory pathway genes. The Gup1 protein-induced proliferations showed that they are a hybrid compartment, since they include resident ER, Golgi and itinerant proteins. The occurrence of this cellular perturbation could be due to the acyltransferase function, since it is linked to a series of lipid-related processes involved in triacylglycerol/phospholipids anabolism [9] and in sphingolipids-sterol-ordered domains integrity and assembly [16], as well as in GPI anchors remodelling. The cellular processes underlying these results are likely to relate to differentiation signaling as happens in animal cells [20].

Acknowledgments

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