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ORIGINAL PAPER

Genetic interactions between a phospholipase A_2 and the Rim101 pathway components in *S. cerevisiae* reveal a role for this pathway in response to changes in membrane composition and shape

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Abstract Modulating composition and shape of biological membranes is an emerging mode of regulation of cellular processes. We investigated the global effects that such perturbations have on a model eukaryotic cell. Phospholipases A_2 (PLA_2s), enzymes that cleave one fatty acid molecule from membrane phospholipids, exert their biological activities through affecting both membrane composition and shape. We have conducted a genome-wide analysis of cellular effects of a PLA_2 in the yeast *Saccharomyces cerevisiae* as a model system. We demonstrate functional genetic and biochemical interactions between PLA_2 activity and the Rim101 signaling pathway in *S. cerevisiae*. Our results suggest that the composition and/or the shape of the endosomal membrane affect the Rim101 pathway. We describe a genetically and functionally related network, consisting of components of the Rim101 pathway and the prefoldin, retromer and SWR1 complexes, and predict its functional relation to PLA_2 activity in a model eukaryotic cell. This study provides a list of the players involved in the global response to changes in membrane composition and shape in a model eukaryotic cell, and further studies are needed to understand the precise molecular mechanisms connecting them.

Keywords Rim101 pathway · Phospholipase A_2 · Endosomal membrane · Genetic interactions

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Introduction

Membrane curvature is emerging as an active means to create membrane domains and to organize membrane trafficking and signaling (McMahon and Gallop 2005). The aim of this study was to identify genes and biological processes that are involved in the adaptation of a model eukaryotic cell to perturbed membrane shape, since a global insight into such adaptation has not been obtained yet.

Phospholipases A_2 (PLA_2s) cleave fatty acids from the *sn*-2 position of the phospholipid molecules in biological membranes, resulting in changes of both composition and shape of the membranes. PLA_2s induce membrane curvature because of the different shape of their substrates (cylindrically shaped phospholipids) and products that

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remain inserted in the membrane (inverted cone-shaped lysophospholipids). In addition to this, fatty acids generated by PLA_2 enzymatic activity can be biologically active (Farooqui and Horrocks 2006). PLA_2s are involved in many physiological and pathological processes, such as inflammation, cell injury, tumor resistance and neurotoxicity (Kini 1997; Kudo and Murakami 2002). Probably the best studied example of the biological activity of these enzymes is that of the so-called cytosolic PLA_2s, which have long been known as inducers of the inflammatory reaction by releasing arachidonic acid (Kramer and Sharp 1997; Kita et al. 2006). This example has focused the attention mainly on fatty acids and lysophospholipids as the mediators of the biological effects of PLA_2s. However, it is clear that the increased positive membrane curvature caused by the PLA_2s' enzymatic activity could represent a further important consequence of the action of these enzymes, especially in endocytosis and other vesicular transport-related cellular processes (McMahon and Gallop 2005). Unbiased analyses that take into account all the potential modes of action of PLA_2s are therefore needed to understand the biology of this group of enzymes. For this reason, in the current study ammodytoxin (Atx) was selected, which is a PLA_2 with a high degree of phospholipolytic efficiency on phospholipid membranes of different chemical compositions (Singer et al. 2002; Petan et al. 2005).

Genome-wide analyses have recently attracted much attention because of their ability to provide a global insight into the cellular events following a perturbation, allowing identification of all the biological processes involved in these events, and generating productive hypotheses on the molecular mechanism of the perturbation. *Saccharomyces cerevisiae* is an excellent model organism in which by genome-wide screens, rational hypotheses can be generated that are subsequently tested in more physiologically relevant experimental systems. Atx, a neurotoxic PLA_2 from the long-nosed viper and a model PLA_2, is the only specific PLA_2 (i.e., without any phospholipase B activity, which results in cleavage of both fatty acids from a phospholipid molecule) that has been studied in *S. cerevisiae* cells (Petrovic et al. 2005). When expressed in yeast, Atx was active in the cytosol (Petrovic et al. 2005), and its cytosolic, rather than extracellular, activity is thought to be important for its neurotoxic effects in its physiological target, neuronal cells (Petrovic et al. 2004; Praznikar et al. 2008). In yeast, Atx expression was shown to affect control of the cell cycle, but this effect was most probably dependent on its ability to bind to 14-3-3 proteins (Petrovic et al. 2005). To obtain a broader and more complete picture of the cellular effects of PLA_2s in eukaryotic cells, we performed a genome-wide analysis to identify yeast single-gene deletion strains that are sensitive to PLA_2 activity. We report the identification of a genetic and functional interaction between PLA_2 activity and the Rim101 pathway components.

Saccharomyces cerevisiae Rim101p is a zinc finger containing transcription factor (Lamb and Mitchell 2003) that is activated by removal of its C-terminal region by a cysteine protease Rim13p (Futai et al. 1999; Penalva and Arst 2002). The signaling cascade leading to its activation, in which numerous other proteins are involved, is required for the expression of several alkaline pH-induced genes and for alkaline pH-stimulated haploid invasive growth (Su and Mitchell 1993; Lamb et al. 2001). The same pathway is also involved in processes of cell wall construction (Castrejon et al. 2006) and meiosis (Su and Mitchell 1993). A signal transduction pathway relaying information on environmental alkaline pH, leading via the endosomal sorting complex required for transport (ESCRT) and endosomal multivesicular body (MVB) formation to the Rim101 pathway, has recently been proposed (Boysen and Mitchell 2006). However, a complete picture of the functional interactions between the Rim101 pathway components and proteins involved in vesicular transport-related processes is still lacking.

In the absence of known direct molecular targets of a perturbation, analysis of genome-wide experimental data by searching for characteristics linking the identified genes is helpful. Because of the extensively annotated genome, yeast *S. cerevisiae* is an ideal model organism for such an approach (Sturgeon et al. 2006). In this study, we used BioPixie (Myers et al. 2005), a biological data integration and visualization tool that enables, based on a probabilistic system and integration of diverse genome-wide data, discovery of interaction networks in which the genes of interest participate. Annotations of the network's central genes/proteins and analysis of the actual interactions between the genes/proteins in the discovered networks then represent a basis for the generation of accurate and reliable hypotheses on the molecular mechanistic properties of the perturbation.

Using the combination of genome-wide experimental approach and bioinformatics analysis, we identified a functional interaction between the Rim101 pathway and changes in the shape and composition of cellular membranes. We were able to localize the underlying molecular events to the endosomal membrane and implicated the role of prefoldin, retromer and SWR1 complexes in a functional network including the Rim101 pathway component Rim13p.

Materials and methods

Strains and plasmids

AJY217 (S288c-derived, *MATα*, *can1Δ::MFA1pr-HIS3 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lyp1Δ*) was used to construct the starting strain for synthetic dosage lethality (SDL) analysis, MMY1001 (*MATα*, *can1Δ::MFA1pr-HIS3 mfa1Δ::GAL1pr-ATXA natMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lyp1Δ*).

MMY1001 was constructed in three steps. First, the gene coding for mature AtxA was cloned into pRD53 plasmid (pRS316 derivative containing *GAL1pr*) between *Bam*HI and *Cla*I sites. Secondly, *GAL1pr-ATXA* was inserted between *Sal*I and *Hin*dIII sites into pAG25 plasmid, which carries the *nat1* gene (*natMX* resistance cassette) from *Streptomyces noursei* encoding nourseothricin *N*-acetyl-transferase and confers resistance to the antibiotic nourseothricin (cNAT). Thirdly, the *MFA1* open reading frame (*mfa1Δ::GAL1pr-ATXA natMX*) of strain AJY217 was replaced by the *GAL1pr-ATXA natMX* cassette by integration of a PCR product generated with primers (5′-**ATCTGTAACTGTTTCTCGGATAAAACCAAAATA AGTACAAAGCCATCGAATAGAA**TTAGCATTTCT CTGACTCCT-3′) and (5′-**GGTGTAGCGGAAAAGGA AGATAAAGGAGGGAGAACAACGTTTTTGTACGC AGAAA**CACTAGTGGATCTGATATCA-3′) that contain a 55-bp sequence identical to sequences upstream and downstream of *MFA1* (bold). Transformants were selected on YPD plates containing nourseothricin (cNAT; Werner BioAgents).

For SDL, the collection of all the viable *MATa* single-gene deletion haploid strains (BY4741 derivatives) (Winzeler et al. 1999) and, for additional experiments, individual strains from the same collection were used. BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used as wild-type reference strain.

To monitor Rim101p proteolytic activation, *RIM101* allele in BY4742 strain (*MATα, his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was tagged with 3xHA inserted at codon 312 as described (Li and Mitchell 1997).

Plasmid pBJ1.0 was derived from pCM190 (*tetO*$_7$*-CYC1pr, URA3*) by cloning *ATXA* between *Bam*HI and *Not*I sites. Plasmids pBJ1.0 and pCM190 were transformed into *RIM101-HA*-tagged BY4742 using the lithium acetate method (Geitz and Woods 1994).

Media

Yeast deletion strains and the corresponding wild-type reference strain were propagated in YPD medium or on YPD plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar] containing G418 (200 mg/L, Invitrogen). Deletion strains containing the *GAL1pr-ATXA natMX* cassette were grown in YPD medium or on YPD plates containing both G418 and cNAT (100 mg/L, Werner BioAgents). For *ATXA* expression, galactose [2% (w/v)] was used as carbon source (YPgal).

Growth media and conditions for SGA analysis were as described (Tong et al. 2001). Strains transformed with plasmids were grown in synthetic minimal uracil dropout medium, YNBglc + CSM-Ura (6.7 g yeast nitrogen base, 0.67 g complete supplement mixture without uracil and 2% (w/v) glucose per 1L).

For growth curve analysis, filter-sterilized YPD and YPgal containing 100 mM HEPES at pH 3.5, 4.5 or 7.5 were used. Overnight cultures were grown in unbuffered YPraf [2% (w/v) raffinose as carbon source].

Synthetic dosage lethality (SDL) screening

The selection of haploid double-mutant strains (i.e., single-gene deletion mutants with *GAL1pr-ATXA* cassette) was performed as described in (Tong et al. 2001), with an additional step in the pinning procedure. In the last step, the expression of *ATXA* was induced by pinning the obtained double mutants on double-mutant selection plates containing galactose as sole carbon source. In parallel, as a control, double mutants were pinned on glucose-containing double-mutant selection plates. To minimize the number of false-positive results, the screen was repeated twice. For both screens, robotic manipulators were used: Virtek colony arraying and picking system (CAPS) was used for the first screen, while for the second screen a custom-made robotic manipulator with 384 floating pin replicator and with 0.787 mm diameter pins (V&P Scientific) was used. The strains that showed significantly decreased growth rate only because of *ATXA* expression were identified by comparing the growth of the double, as well as single mutant collections, both on glucose and galactose. In the first screen (CAPS pinned), the results were analyzed by visual inspection of the plates. For the analysis of the second screen, colony volume determination, normalization and growth fitness calculation were performed as described in Online Resource 1.

Growth assay

Growth curves were obtained as described in (St Onge et al. 2007) with some modifications. Deletion strains were grown overnight in YPraf. Cell cultures were then diluted to an initial OD_{600} of $\sim$0.05 in buffered YPD and YPgal and grown in 300 μL volumes in 96-well plates in a Tecan Sunrise microplate reader to stationary phase. The growth of each culture was monitored by measuring the OD_{595} every 2 min, with constant shaking between measurements.

The growth rate (K) was determined as the slope of the linear part of the growth curve during exponential phase. The growth fitness (R) of a strain was calculated as: $R = (K_{gal\Delta}/K_{galWT})/(K_{glc\Delta}/K_{glcWT})$, where $K_{gal\Delta}$ and $K_{glc\Delta}$ are the growth rates of the deletion strain on galactose or glucose, and K_{galWT} and K_{glcWT} are the growth rates of the wild-type strain on galactose or glucose.

Rim101p processing assay

To monitor Rim101p proteolytic activation, BY4742 strain with HA-tagged *RIM101*, harboring either pBJ1.0 plasmid for *ATXA* expression or pCM190 plasmid as control, was grown overnight in YNBglc + CSM-Ura. Cells were then resuspended to an OD_{600} of 0.2 in YNBglc + CSM-Ura containing 100 mM HEPES at pH 3.5, 4.5 or 7.5 and harvested in mid-logarithmic phase.

HA-tagged Rim101p was detected as described previously (Li and Mitchell 1997). Pgk1 was used as a loading control.

Endocytosis assay

The quantification of cell-associated Lucifer Yellow (LY; Lucifer Yellow CH dilithium salt, Sigma) was performed as described by Riezman (1985), with minor modifications. Cells were grown to mid-logarithmic phase in unbuffered or alkaline buffered (pH 7.4) medium, harvested and resuspended in fresh medium containing 4 mg/mL LY and 20 mM NaN_3. After 1 h incubation at 30 or 0°C, cells were washed eight times with ice-cold buffer A (50 mM Na-succinate, pH 5.0, 100 mM NaCl, 10 mM $MgCl_2$, 20 mM NaN_3) containing 10% sorbitol to prevent lysis of more fragile cells. The cells were collected by centrifugation. They were then resuspended in 1 mL of buffer B (50 mM Tris–HCl, pH 7.5, 10 mM 2-mercaptoethanol) and 100 U lyticase was added. After 1 h of incubation at 37°C, 50 µL of 10% SDS was added to the lysed cells. LY fluorescence was excited at 426 nm and emission measured at 550 nm, both with 15 nm slit widths, using a Perkin Elmer LS50B spectrofluorimeter.

Results

Genetic interactions of PLA_2 with the Rim101-signaling pathway components

In the wild-type *S. cerevisiae* cells under standard growth conditions, Atx does not affect the growth rate (Petrovic et al. 2005). Morphologically, PLA_2-expressing cells are indistinguishable from control cells and PLA_2 expression does not confer an easily detectable phenotype, such as temperature or hypo-/hyper- osmotic shock resistance (data not shown). To obtain a genome-wide picture of the effects of a PLA_2 activity on yeast cells, *ATXA* gene under the control of inducible *GAL1/10* promoter was integrated into the *MFA1* locus of all the *S. cerevisiae* strains with single deletion of non-essential genes following the method of Tong et al. (see "Materials and methods"). Two independent replicates of the experiment were performed and in the first one by visual inspection 152 mutants were identified as lethal, very sick or sick (Online Resource 2), whereas in the second screen automated analysis identified 42 mutants as having a significantly decreased growth rate, compared to the wild-type strain (Online Resource 2). In the analysis of the two gene sets, gene ontology enrichment analysis identified only one term with the P value <0.01, namely 'regulation of transcription' (P value 0.0057) from the first screen (Online Resource 2). Overlap of the two screens identified eight genes, *LGE1*, *NHP10*, *QCR6*, *RIM13*, *RIM101*, *VPS1*, *VPS5* and *YTA7* (Table 1), the deletion of which was found to increase sensitivity to induced PLA_2 expression. Six of them are not obviously related: *NHP10* codes for a component of an ATP-dependent chromatin-remodeling complex (Shen et al. 2003), *QCR6* for a subunit of the ubiquinol cytochrome *c* reductase complex (Yang and Trumpower 1994), *VPS1* for a dynamin-like GTPase (Ekena et al. 1993), *VPS5* for a nexin-1 homolog (Nothwehr and Hindes 1997), *YTA7* for an ATPase that regulates histone gene expression (Gradolatto et al. 2008) and *LGE1* for a protein of unknown function involved in histone monoubiquitination (Hwang et al. 2003). Products of two of the identified genes, however, have closely related functions: Rim13p proteolytically activates Rim101p (Futai et al. 1999), thus placing both genes in the same pathway. We therefore looked more closely at other members of this pathway, i.e., genes coding for proteins involved in activation or processing of Rim101p; (Li and Mitchell 1997; Treton et al. 2000; Xu et al. 2004; Rothfels et al. 2005), and found that nine of them (*RIM20*, *RIM8*, *RIM9*, *RIM21*, *VPS23*, *VPS25*, *VPS28*, *VPS37* and *YGR122W*) have been identified in one of our two screenings (Table S1 in Online Resource 1). This indicates that the Rim101 pathway as a whole plays a role in the sensitivity to PLA_2 expression, and we therefore focused our subsequent experiments on the effects of intracellular PLA_2 activity on this pathway.

Inhibition of growth at alkaline pH by PLA_2 activity is functionally linked to *RIM101*

The Rim101 pathway is required for alkaline pH-stimulated cellular responses (Lamb et al. 2001). To determine whether PLA_2 activity affects this function of the Rim101 pathway, we measured the growth rate of PLA_2-expressing

Table 1 Genes whose mutation conferred increased sensitivity to induced PLA_2 activity in both experiment replicates

Gene	Summary of protein function	SDL screen	
		1	2
LGE1	Protein of unknown molecular function, involved in histone methylation and regulation of cell size; homozygous diploid null mutant has delayed premeiotic DNA synthesis and its efficiency of meiotic nuclear division is reduced	s	0.13
NHP10	Probable component of the INO80 ATP-dependent chromatin-remodeling complex, related to mammalian high-mobility group proteins	s	0.09
QCR6	Subunit 6 of the ubiquinol cytochrome c reductase complex, a component of the mitochondrial electron transport chain, required for maturation of cytochrome c1	s	0.37
RIM13	Calpain-like cysteine protease that proteolytically activates Rim101p in response to alkaline pH	l	0.11
RIM101	Transcriptional repressor involved in the response to pH and in cell wall construction and required for alkaline pH-stimulated haploid invasive growth and sporulation; activated by proteolytic processing by Rim13p	l	0.46
VPS1	Dynamin-like GTPase required for vacuolar sorting, which is involved also in actin cytoskeleton organization, late Golgi retention of some proteins and regulation of peroxisome proliferation	s	0.30
VPS5	Nexin-1 homolog required for localizing membrane proteins from a prevacuolar/late endosomal compartment back to the late Golgi apparatus; structural component of the retromer complex	s	0.29
YTA7	Protein that localizes to chromatin and has a role in regulation of histone gene expression, has a bromodomain-like region that interacts with the N-terminal tail of histone H3 and an ATPase domain, and is potentially phosphorylated by Cdc28p	vs	0.24

In the two right columns are given the growth fitness values from both SDL screens, either determined by visual inspection (1) or calculated as described in Online Resource 1 (2) (relative growth fitness; R) for each of the eight genes

s Synthetic sick phenotype, *l* synthetic lethal phenotype, *vs* very synthetic sick phenotype

strains in media with different pH values. Since the PLA_2 acts in the cytosol that contains stable pH environment (Orij et al. 2009) even in the absence of the *RIM101* gene (Mira et al. 2009), the activity of the PLA_2, featuring enzymatic activity within a broad pH range (Beiboer et al. 1996), should not be affected under these conditions. While PLA_2 activity did not affect the growth of the wild-type strain at acidic pH (3.5 and 4.5), the growth rate of the PLA_2-expressing strain at pH 7.5 was 83% of that of the wild-type strain without the PLA_2 gene. Also at pH 7.5, the growth rate of the *rim101*Δ strain without the PLA_2 gene was 74% of that of the wild-type strain without the PLA_2 gene, and when PLA_2 was expressed in the *rim101*Δ strain, the growth rate was only 40% of that of the wild-type strain without the PLA_2 gene. This is significantly less than the 61% (=0.83 × 0.74), which would be expected if PLA_2 activity and deletion of *RIM101* had independent effects on the cellular response to alkaline pH. This result is in agreement with the aggravating synthetic (phenotypic enhancement) effect between PLA_2 expression and the Rim101 pathway determined in our screen.

Expression of the PLA_2-encoding gene from a multi-copy plasmid, rather than from a chromosome-integrated gene, with a consequence of at least doubled intracellular PLA_2 enzymatic activity (Petrovic et al. 2004), resulted in an even more pronounced inhibitory effect on growth at alkaline pH (i.e., growth rate was 59% of that of the wild type, compared to 83% for the integrated gene), demonstrating that the effect is gene number and, therefore, most probably concentration dependent.

PLA_2 activity increases the abundance of active Rim101p

For the induction of the Rim101 pathway under alkaline pH, Rim101p must be cleaved by the protease Rim13p (Futai et al. 1999). To investigate further the relationship between PLA_2 activity and the Rim101 pathway, we analyzed the abundance of Rim101p and its proteolytic cleavage pattern in the presence and absence of PLA_2 at different pH values (Fig. 1). At each pH, we observed the same fraction of cleaved Rim101p in the wild type as compared to PLA_2-expressing cells. All Rim101p was cleaved at basic pH in both strains and was present in similar amounts. At acidic pH, however, significantly more total Rim101p was present in the PLA_2-expressing cells, although the ratio of full-length to cleaved protein appeared similar to that in the wild-type cells. Thus, PLA_2 activity increased the abundance of the active (i.e., cleaved) form of Rim101p at acidic pH, where PLA_2 expression does not affect the growth of yeast cells (see above).

PLA_2 expression inhibits endocytosis in *S. cerevisiae*

It has recently been proposed that endocytosis of plasma membrane pH sensors is an upstream event leading to

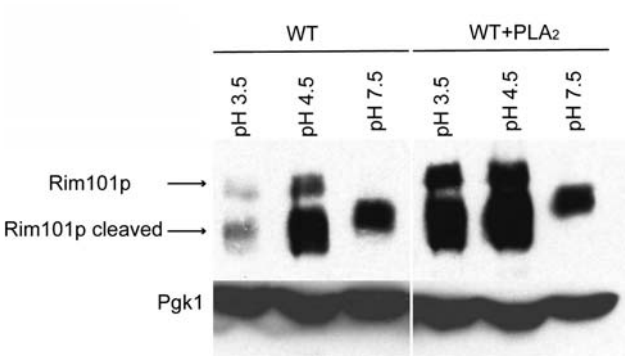

Fig. 1 Western blot showing the abundance of Rim101p and its proteolytic cleavage products in the presence and absence of PLA_2 at different pH values. The *RIM101-HA*-tagged WT (wild-type strain with empty vector) and *RIM101-HA*-tagged PLA_2-expressing strain (wild-type strain with PLA_2-expressing vector) were grown overnight in YNBglc + CSM-Ura medium and then diluted in identical medium buffered to pH 3.5, 4.5 or 7.5. The cells were collected in mid-log phase and the assay was carried out as described in "Materials and methods". Pgk1 was used as a loading control. Full-length and cleaved Rim101p are indicated. In the PLA_2-expressing strain, the ratio of cleaved to non-cleaved Rim101p under all experimental conditions is normal. The abundance of Rim101p at alkaline pH is unchanged. At acidic pH, on the other hand, significantly more Rim101p is present in PLA_2-expressing cells

Rim101p activation (Boysen and Mitchell 2006). We therefore measured LY uptake to determine the effect of PLA_2 expression on the rate of endocytosis in yeast cells. As a control, we measured the rate of endocytosis in the *end3Δ* strain (Fig. 2). The rate of endocytosis in the wild-type strain expressing PLA_2 was reduced to ~45% of

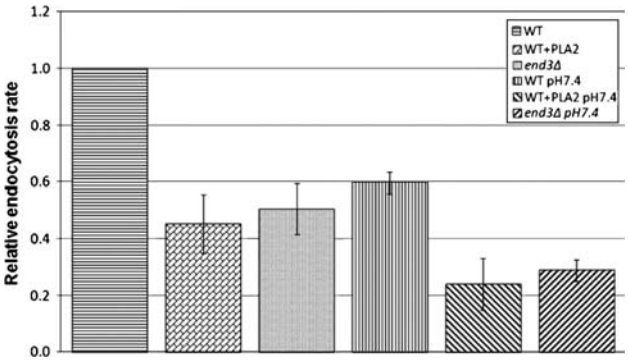

Fig. 2 PLA_2 expression inhibits endocytosis in yeast. The rate of endocytosis in the PLA_2-expressing strain (WT + PLA_2) is reduced to 45% (±10%) as compared to the control non-PLA_2-expressing wild-type strain (WT). As a positive control, we measured the rate of endocytosis in the *end3Δ* strain. Lucifer Yellow dye uptake measurement was done as described in the "Materials and methods". Represented are the relative endocytosis rates for the WT + PLA_2 and *end3Δ* strains that were calculated by normalizing to the WT measurement. The rate of endocytosis was measured for the same three strains also at alkaline pH (3 *right columns*) and normalized to the WT measurement at acidic pH (*left column*). The ratios of relative endocytosis rates are independent of pH

the control (wild-type, non-PLA_2-expressing) strain. The effect of PLA_2 on the rate of endocytosis was also observed under alkaline conditions, since also at pH 7.4 relative differences in the rate of LY uptake remained unchanged, while the absolute values were lower (Fig. 2).

PLA_2 and Rim101 pathway components are genetically linked with SWR1, retromer and prefoldin complexes

The molecular targets of PLA_2 activity in yeast cells are not known, thus it is not possible to obtain a deeper insight into the molecular effects of PLA_2s via a candidate approach. However, a global picture can be obtained, although at a lower resolution, by genome-wide analysis, relying on previously reported interactions between the yeast genes for the interpretation of results.

According to published data (Futai et al. 1999; Bensen et al. 2000; Kaeberlein and Guarente 2002; Krogan et al. 2003; Tong et al. 2004; Rothfels et al. 2005), the set of eight genes identified above can be regarded as two groups of genetically interconnected genes (*VPS1*, *VPS5*, *LGE1*, and *RIM101*, *RIM13*, *YTA7*, *NHP10*; no interactions of *QCR6* with the other seven genes have been reported) that are connected to each other by the physical interaction between Vps1p and Nhp10p (Gavin et al. 2002, 2006), (Fig. 3). These seven genes thus constitute a network that represents a context for investigating functional connection between PLA_2 activity and the Rim101 pathway.

To define indirectly related genes and proteins with PLA_2 genetic interactions, i.e., its process-specific network, we used the bioPIXIE bioinformatics tool (Myers et al. 2005) (Fig. 4). The *RIM101* and *RIM13* components of the Rim101 pathway are connected to this network primarily via interactions of *RIM13*, which has the highest confidence interactions with components of the prefoldin complex (*GIM3/4/5* and *YKE2*). Indeed, gene set enrichment analysis of the network genes revealed the highest confidence level for three molecular complexes, SWR1 (*P* value 1.03e-10), retromer (*P* value 1.12e-6) and prefoldin (*P* value 3.35e-6), which, together with *VPS1* and *LGE1* genes, are central to the gene network thus obtained (Fig. 4). SWR1 complex is involved in chromatin remodeling, specifically it is required for the incorporation of the histone variant H2AZ into chromatin (Korber and Horz 2004). Retromer complex is an evolutionarily conserved protein complex involved in retrograde transport from endosomes to the Golgi apparatus (Verges 2008). Prefoldin complex is a heterohexameric cochaperone complex that delivers unfolded proteins, such as tubulin, to cytosolic chaperonin (Lopez-Fanarraga et al. 2001). These genes/complexes share many genetic interactions (Fig. 5), strongly indicating functional relations.

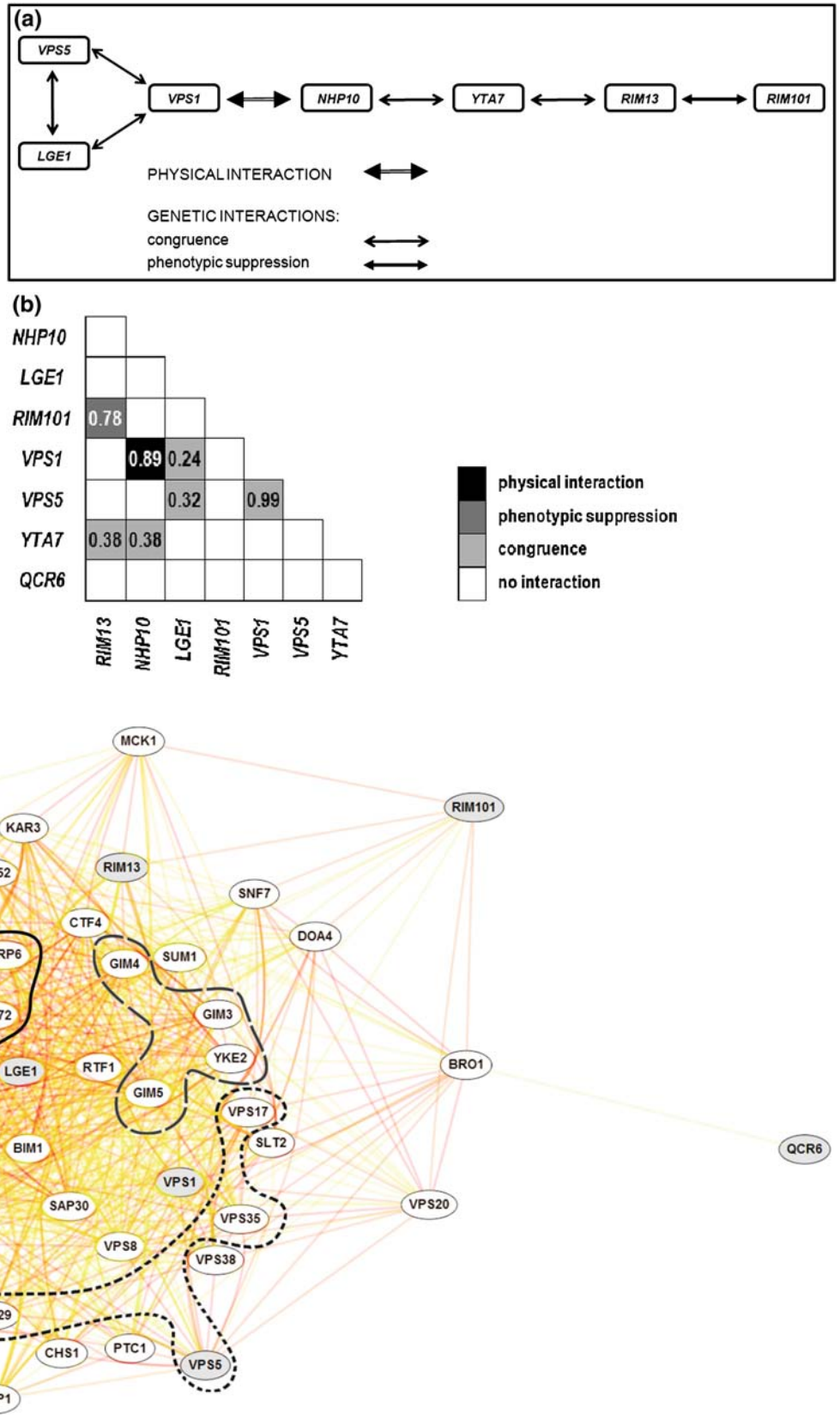

Fig. 3 Diagram representing known interactions between seven of the eight genes identified as the highest confidence PLA_2 genetic interactors (**a**) and their overall confidence of the interaction (**b**) that was obtained with the bioPIXIE tool (Myers et al. 2005, http://pixie.princeton.edu/pixie)

Fig. 4 Functional genetic network as defined by the bioPIXIE tool (Myers et al. 2005, http://pixie.princeton.edu/pixie) with genes in genetic interaction with PLA_2 expression as input. Genes in genetic interaction with PLA_2 expression are marked in *gray*. Components of the prefoldin, SWR1 and retromer complexes are *circled* with *dashed*, *full* and *dotted lines*, respectively

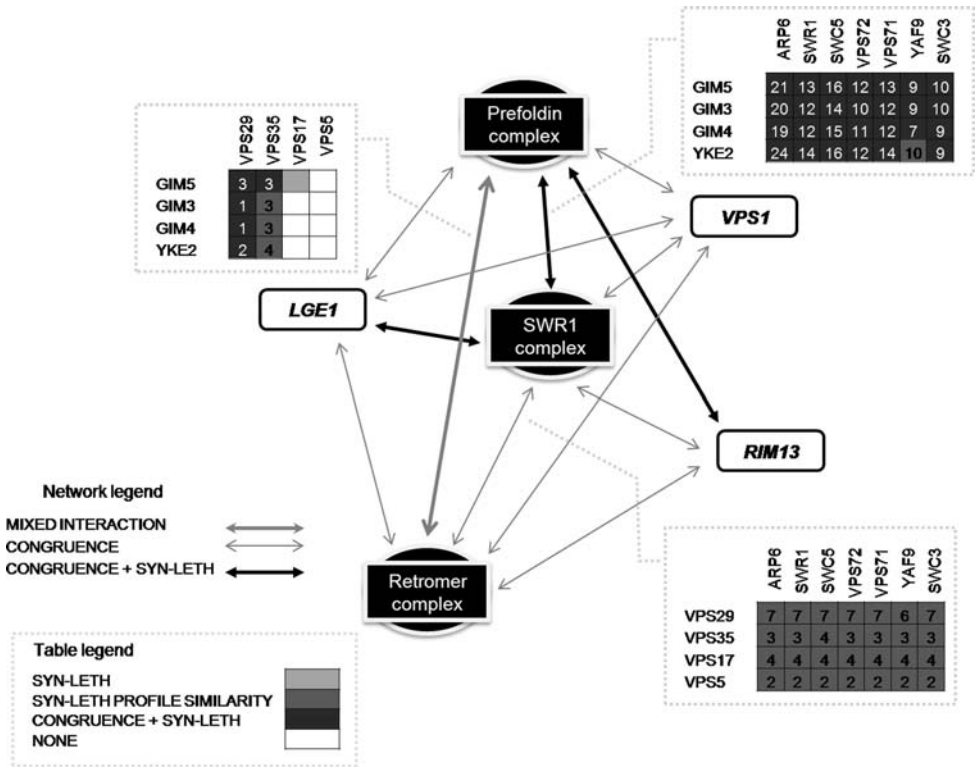

Fig. 5 Detailed representation of the interactions between the components of the gene network. The term "mixed interaction" is used to denote the presence of both types, congruent interactions and interactions whereby genes encoding the components of the complexes are both congruent and at the same time synthetically lethal, none of the types being predominant (see embedded tables for details). *Numbers* represent the raw values for synthetic lethality profile similarity (i.e., the number of genes in genetic interaction with both of the genes) and were obtained with the bioPixie tool (Myers et al. 2005, http://pixie.princeton.edu/pixie). (*SYN-LETH* synthetic lethality)

Discussion

Relationship between PLA_2 activity and the Rim101 pathway

In the set of eight genes identified in the whole genome screen for strain sensitive to heterologous expression of the PLA_2 gene, two central genes of the Rim101 pathway were identified (Table 1). Moreover, all the strains containing mutations in the genes known to be required for activation or processing of Rim101p that were included in our analysis were identified as being at least somewhat sensitive to induced PLA_2 intracellular activity in yeast cells (Table S1 in Online Resource 1), making the prediction that PLA_2 activity and the Rim101 pathway are functionally related very likely. Western blot analysis revealed increased Rim101p expression at acidic pH in PLA_2-expressing cells, where the ratio of cleaved (active) to full-length Rim101 protein resembled that in control cells (Fig. 1). Constitutive activation of Rim101p has previously been reported on mutation of ESCRT-III complex components Did4p/Vps2p, Vps4p and Vps24p, which are required for sorting of proteins into lumenal vesicles of MVBs. However, the PLA_2-mediated phenotype appears to be different, since, in the case of the *vps* mutants, increased processing of Rim101p has been observed at acidic pH, without overall increase in its amount (Hayashi et al. 2005), whereas we observed an increase in total Rim101 protein in PLA_2-expressing strains, with unaltered processing of Rim101p. Interestingly, the promoter region of the *RIM101* gene contains the oleate response element (ORE) (Karpichev and Small 1998). This DNA sequence is bound by the transcription-activating heterodimer Oaf1p/Pip2p, which modulates fatty acid-induced activation of gene expression (Luo et al. 1996). Although fatty acids generated by PLA_2 activity could explain the increased amount of Rim101p following PLA_2 expression in the assay at pH 3.5 and 4.5, this fact alone does not explain the synthetic lethal interaction between the two. Rather, the presence of ORE in the *RIM101* promoter could indicate a functional interaction between the Rim101 pathway and endogenous fatty acid-related cellular processes, in agreement with two recent reports (Ikeda et al. 2008; Yazawa et al. 2009).

It has recently been proposed that endocytosis of plasma membrane pH sensors is an upstream event leading to Rim101p activation (Boysen and Mitchell 2006). Atx, the PLA_2 used in this study, is known to inhibit endocytosis in mammalian cells when applied extracellularly (Pungercar and Krizaj 2007) and in yeast cells when expressed intracellularly (Fig. 2). Our data show that receptor-mediated endocytosis is inhibited by the PLA_2 activity in yeast cells, exemplified by the increased lifetime of Sla1-GFP-marked endocytotic patches (Mattiazzi and Drubin, manuscript in preparation). Since Rim101p activation is not inhibited in PLA_2-expressing cells (Fig. 1), inhibition of endocytosis does not explain the functional link between PLA_2 activity

and the Rim101 pathway, but from the genome-wide data hypotheses on the mechanistic link could be drawn (see below).

Genome-wide view of the gene network including PLA_2 genetic interactors

Our genome-wide analysis indicated that the major effect of PLA_2 is alteration of membrane chemistry and/or trafficking. Two of the eight genes identified in our SDL screening, *VPS1* and *VPS5*, encode membrane-bound proteins directly involved in vesicle sorting. Vps1p is a dynamin-like GTPase localized to peroxisomal and outer mitochondrial membranes, and is involved in various cellular processes such as vacuolar sorting and late Golgi retention of proteins, as well as actin and peroxisome organization and biogenesis (Hoepfner et al. 2001; Zahedi et al. 2006). Vps5p, the nexin-1 homolog, localizes to the endosome membrane and is a component of the outer shell of the retromer complex that is required for late endosome to late Golgi protein transport (Nothwehr and Hindes 1997; Seaman et al. 1998). Consistent with the finding that Vps1p and Vps5p are required for cells to counteract the effects of PLA_2, we found that PLA_2 expression reduced endocytosis rates in yeast (Fig. 2), similarly to neurotoxic PLA_2 effects in mammals (Dixon and Harris 1999; Harris et al. 2000). Membrane remodeling during fission makes lipids more accessible to PLA_2, which further alters membrane curvature and thus likely leads to the observed endocytosis defects. Our results thus indicate that additional defects in vesicle sorting induced by *VPS1* or *VPS5* mutations exacerbate the endocytosis defect caused by PLA_2 expression. Besides Vps5p, other components of the retromer complex (Vps17p, Vps35p, Vps29p and Vps26p), which consists of membrane-associated proteins involved in vesicle trafficking, were also predicted to functionally interact with PLA_2 (Fig. 4). Our genetic screen showed a consistent slight synthetic sick phenotype for all four strains (the average relative growth fitness measures (*R*) were: 0.77 for *vps17Δ*, 0.81 for *vps35Δ*, 0.81 for *vps29Δ*, and 0.82 for *vps26Δ*), which confirms this prediction.

Additionally, the retromer complex along with Ypt6p, which mediates fusion of ER-derived vesicles with Golgi and is predicted to interact with PLA_2 expression (Fig. 4), could indirectly counteract PLA_2 toxicity by activating the Rim101 pathway. MVBs are formed by invagination of the endosomal membrane and, in *A. thaliana* and in human cells, retromer complex components have been localized to MVBs (Seaman 2004; Oliviusson et al. 2006). Proteins residing in the membranes of MVBs have been shown to play a crucial role in the Rim101 pathway activation (Xu et al. 2004). Accordingly, *RIM13* was predicted to interact with the retromer complex (Fig. 4). These facts combined suggest a hypothesis that the endosomal membrane is a direct target for PLA_2 activity in yeast cells and that cleavage of endosomal membrane phospholipids, and/or modulation of the shape of this membrane, interferes with the Rim101 pathway. This hypothesis is further reinforced by the fact that two genes identified in our screen, *VPS1* and *VPS5*, are functionally linked to phosphatidylinositol 3-phosphate (Burda et al. 2002; Efe et al. 2005), which is the key endosomal signaling lipid (Schu et al. 1993).

Three out of the eight genetic interactors of PLA_2 (*LGE1*, *NHP10* and *YTA7*) are involved in chromatin modification: *LGE1* is in direct genetic interaction with SWR1 complex components (Fig. 5) and involved in histone H2B monoubiquitination (Hwang et al. 2003), Nhp10p is a component of the INO80 complex that shares components with the SWR1 complex (van Vugt et al. 2007), whereas Yta7p is involved in maintaining the barrier between heterochromatin and euchromatin and in the regulation of histone gene expression (Gradolatto et al. 2008). Interestingly, it has recently been shown that Htz1p, the Z variant of H2A histone, which is introduced by the activity of the SWR1 complex, and its chemical modification are crucially involved in fatty acid-induced transcriptional changes (Wan et al. 2009). Moreover, strains mutated in *HTZ1*, *SWR1*, *VPS71*, *ARP6* and *YAF9* genes were shown to be sensitive to the presence of free oleic acid in the medium (Lockshon et al. 2007). A hypothesis can therefore be proposed that fatty acids generated by the PLA_2 activity have similar effects on the cells, and further work will be required to test it. Congruent genetic interactions between SWR1 complex components and *RIM13* indicate a potential functional interaction also between SWR1 complex and the Rim101 pathway.

In addition to the Rim101 pathway and retromer complex, the members of which were identified by SDL screening, our computational analysis predicted that the SWR1 and prefoldin complexes also functionally interact with PLA_2 expression. These complexes are strongly connected to *VPS1* and *LGE1,* and to both the retromer complex and Rim101 pathway via *RIM13* (Figs. 4, 5). The fact that SWR1 and prefoldin complexes genes were not identified in our screen indicates a more indirect relation with PLA_2 activity, or a relation in the context of a non-essential cellular process.

What could be a physiological counterpart of Atx, i.e., an endogenous PLA_2, in yeast cells? Recently, a spore membrane bending pathway (SpoMBe), involved in spore formation/meiosis and in cell wall function, was described (Maier et al. 2008). One of SpoMBe components is also Spo1p, a structural homolog of phospholipases B and A_2. Also, the Rim101 pathway has been reported to be involved in meiosis/sporulation (Su and Mitchell 1993; Castrejon et al. 2006). Moreover, it has been proposed that

membrane remodeling/curvature could be a trigger for Rim101p processing and activation (Rothfels et al. 2005). Since the promoter of the *RIM101* gene also contains ORE and given the data presented in this study, a possibility exists that the Rim101 pathway, besides its other known functions, is involved in membrane-related cellular processes, such as membrane rearrangements during sporulation.

In this study, we identified the Rim101 pathway and the prefoldin, retromer and SWR1 complexes as players in the response of yeast cells to changes in membrane composition and shape caused by PLA_2 activity. We described a network connecting the genes involved, and further studies are needed to understand the precise molecular mechanisms connecting them.

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