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Review

Towards defining the substrate of orphan P5A-ATPases ☆

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ABSTRACT

Background: P-type ATPases are ubiquitous ion and lipid pumps found in cellular membranes. P5A-ATPases constitute a poorly characterized subfamily of P-type ATPases present in all eukaryotic organisms but for which a transported substrate remains to be identified.
Scope of review: This review aims to discuss the available evidence which could lead to identification of possible substrates of P5A-ATPases.
Major conclusions: The complex phenotypes resulting from the loss of P5A-ATPases in model organisms can be explained by a role of the P5A-ATPase in the endoplasmic reticulum (ER), where loss of function leads to broad and unspecific phenotypes related to the impairment of basic ER functions such as protein folding and processing. Genetic interactions in *Saccharomyces cerevisiae* point to a role of the endogenous P5A-ATPase Spf1p in separation of charges in the ER, in sterol metabolism, and in insertion of tail-anchored proteins in the ER membrane. A role for P5A-ATPases in vesicle formation would explain why sterol transport and distribution are affected in knock out cells, which in turn has a negative impact on the spontaneous insertion of tail-anchored proteins. It would also explain why secretory proteins destined for the Golgi and the cell wall have difficulties in reaching their final destination. Cations and phospholipids could both be transported substrates of P5A-ATPases and as each carry charges, transport of either might explain why a charge difference arises across the ER membrane.
General significance: Identification of the substrate of P5A-ATPases would throw light on an important general process in the ER that is still not fully understood. This article is part of a Special Issue entitled Structural biochemistry and biophysics of membrane proteins.

1. Background

The P-type ATPase family constitutes a large class of membrane proteins which catalyze active transport of ions and lipids across biological membranes. The family can be divided phylogenetically into five distinct subfamilies (P1–P5), each of which can again be divided into additional subgroups (A, B, etc.) [1,2]. The phylogenetic division correlates well with differences in the preferred transport substrates. P1–P3 ATPases are well-characterized ion pumps: P1A are part of bacterial K^+ transport systems, P1B are heavy metal pumps, P2A and P2B are Ca^{2+} pumps, P2C Na^+/K^+ pumps in animals, P2D are Na^+ pumps in fungi and mosses, and P3A are plasma membrane H^+ pumps found in fungi and plants. In contrast to transport of metal ions, P4 ATPases participate in lipid flipping across membranes.

The last subfamily in the superfamily, the P5-ATPases, has remained uncharacterized with respect to substrate transport and thus so far has no assigned specificity. P5-ATPases form a monophyletic group with the same phylogenetic origin as lipid flipping P4-ATPases. Their transport function, if they have one, remains elusive. No homologues have been detected in bacterial genomes even though P5-ATPases are found in every single eukaryotic genome analysed so far [3,4]. Their ubiquitous presence in eukaryotic cells indicates that they play a fundamental yet undescribed role that is not required in prokaryotes but emerged in cells at the branching point between prokaryotes and eukaryotes.

P5-ATPases can be divided into two subclasses, P5A and P5B, based on conservation of residues in the putative transport binding sites [3] and on their predicted transmembrane topology [5]. The P5A subgroup includes a negatively charged motif in the putative transport binding site in M4 (PP(D/E)LPxE) and has two extra transmembrane spanning helices predicted in the N-terminal region (Ma and Mb) (Fig. 1). On the other hand the P5B subgroup shows no conservation of charged residues in the M4 motif (PP(A/V)LPAx) and has only one extra predicted transmembrane helix in the N-terminal region (Ma). The extra transmembrane helices predicted in P5A- and P5B-ATPases show low conservation and no homology to other P-type ATPase membrane

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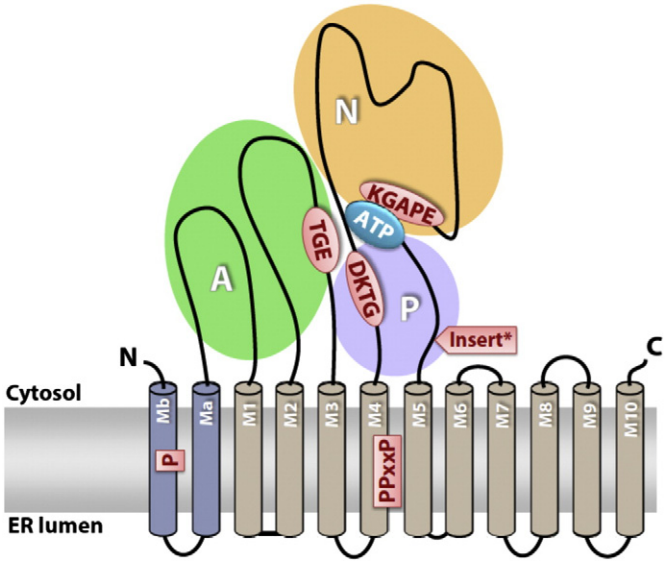

Fig. 1. Overview of P5 ATPase primary structure. P5 ATPases contains archetypical P-type motifs including the DKTG motif in the P domain, the KGAPE motif in the N domain and the TGE motif in the A domain. Whereas most P-type ATPases typically have 10 transmembrane helics, P5A-ATPases are predicted to include 12 transmembrane helices with two additional helices, Ma and Mb, in the N-terminal end [129]. The P5 specific motif PPxxP located in M4 shows conservation of charged residues in P5A-ATPases (PP(E/D)LPM(E/D)) [3]. The insert identified in P5A primary sequences between the Mg^{2+} binding site and the M5 extended stalk helix [5] (motifs shown) is marked with an asterisk.

segments. Whereas P5A-ATPases are found in all eukaryotic genomes, P5B-ATPases have been lost in some lineages of multicellular eukaryotes including land plants [3] and will not be the focus of this review.

An accumulating number of localization studies highlight that P5A-ATPases mainly associate with the endoplasmic reticulum (ER), a hallmark of eukaryotic cells. The single P5A-ATPase in *Saccharomyces cerevisiae*, Spf1p, localizes in the membrane of the ER [6,7] and in the cis-Golgi compartment [8]. Likewise the Spf1p homologues in *Schizosaccharomyces pombe* [9] and in the higher multicellular plant *Arabidopsis thaliana* [10] localize to the ER membrane. In vertebrate genomes, P5A-ATPases are encoded for by a single gene (entitled Atp13a1 and ATP13A1 in the mouse and human genomes, respectively).

2. Scope of review

Despite intense investigation and a sharp increase in genetic data from high throughput studies we still do not understand the molecular function of any P5A-ATPase. For the best studied P5A-ATPase, Spf1p, an immense amount of information has accumulated in recent years as the result mainly of genetic high throughput studies. However, we are still far from understanding the function of Spf1p. One reason is that the phenotype of *spf1* mutants is pleiotropic, that is, a mutation in the *SPF1* gene results in multiple phenotypic traits most of which seem unrelated. In this review, and following a general introduction to the structure–function relationship of P5A-ATPases, the accumulated genetic data on *SPF1* will be reviewed with the goal to identify correlations that have not been observed before and that could throw light on the molecular function of P5A-ATPases.

3. Structure–function relationship of P5A-ATPases

All P-type ATPases function by a similar reaction mechanism in which an invariant aspartate residue is phosphorylated during the catalytic cycle. A nucleotide binding domain (the N-domain) binds ATP and serves as a built-in protein kinase, which phosphorylates the P-domain. The A-domain is an intrinsic protein phosphatase, which dephosphorylates the P-domain once during each catalytic cycle. The process is tightly coupled to formation and deformation of high affinity binding sites in the transport domain by an allosteric mechanism [11].

3.1. Overall fold and signature sequences

P5A-ATPases have all the characteristic key signatures of a P-type ATPase indicating that the overall P-type domain architecture is conserved (Fig. 1). Sequence motifs shared with other P-type pumps, such as the nucleotide interaction site (KGAPE), the lysine of which interacts with the adenine ring of ATP [12], the phosphorylation site (DKTGL), which becomes phosphorylated on the aspartate residue during catalysis, and the site (TGE) in the built-in protein phosphatase, which is required for catalytic cleavage of the phosphoenzyme, are all completely conserved in P5A-ATPases. This suggests a common mechanism between these putative pumps and other P-type ATPases.

3.2. Transport binding sites

In P-type ATPase, the ligand to be transported is coordinated by functional groups in the membrane-embedded M-domain [11]. In particular, residues in transmembrane segments M4, M5, and M6 are involved in ligand binding whereas other residues in M1, M2, and M3 are part of the ligand translocation pathway. P5-ATPases can be identified by a signature PPxxP motif found in transmembrane segment M4, which has an additional proline compared to Na^+/K^+, Ca^{2+} and H^+ ATPases that have a conserved Pxxx(P/L) motif at the same site [3]. The first proline in this motif induces unwinding of transmembrane segment M4 in all solved P-type ATPase structures. This twist in turn exposes backbone carbonyl oxygens that are used to coordinate a transported cation. P5A-ATPase sequences contain a PP(E/D)xPx(E/D) motif with two conserved negatively charged residues whereas P5B-ATPase sequences are characterized by a PP(A/V)xP(A/V)x motif with two conserved hydrophobic residues at the same position. The presence and absence of two negative charges at this position should have a strong impact on the electrical field in the unwound helix region, which is likely to differentially influence the substrate specificity of the two subgroups. The two subclasses thus most likely transport different substrates although this has yet to be proven.

The ion translocation pathways so far established are centered on M1–M6 segments of the M-domain. Nevertheless several features of the core show large sequence variations among subclasses, such as the linkers of M4 and M5 to the P-domain, suggesting subclass specific modulation of the core activity. In this regard it is interesting that an insert of varying size seems to be present in the P5A ATPase primary sequence, which would separate the Mg^{2+} binding site close to the phosphorylated aspartate and the extended M5 helix involved in allosteric communication between the phosphorylation site in the P-domain and the substrate binding site in the M-domain [5].

3.3. P5A-ATPases contain an extended N-terminal region

The extra N-terminal membrane segments Ma and Mb are overall poorly conserved in P5 ATPases and display no obvious candidate residues for ion coordination [5]. Although there is no evidence that they are directly involved in establishing a high-affinity intramembranous transport binding site, an interesting topological comparison can be made with respect to the additional membrane-spanning segments Ma and Mb located in the N-terminus of the P1B-type ATPases. In the structure of the CopA Cu^+ transporter, the N-terminal segments are structurally important to establish a substrate delivery site [13,14]. The N-terminus composes a docking platform for the binding of a Cu^+ chaperone, which delivers its Cu^+ to the pump. It remains to be verified whether Cu^+ is directly delivered to the Cu^+ transport binding sites, or whether the Cu^+ ion transiently binds to the N-terminal heavy metal binding domains. In the CopA structure, Ma forms a continuous helix through the membrane while MB consists of two segmented helices—

a membrane spanning helix (Mb) and a perpendicular amphipathic helix nested at the cytoplasmic interface (Mb′), the two being separated by a highly conserved glycine. In comparison, P5A-ATPases show high conservation of a proline (Pro-31 in Spf1p) at a similar position in the first membrane spanning helix (Mb) [5] making it possible that the extended N-terminal region follows a similar fold as in the P1B heavy metal transporting ATPases. It thus seems plausible that the N-terminal region might represent a docking platform for substrate delivery, either by direct binding of the substrate to the N-terminus or by interaction with a protein partner.

Another possibility is that the N-terminal region functions as a regulator of catalytic function. In the plasma membrane Ca^{2+} ATPase, the plasma membrane H^+ ATPase and the Drs2p lipid flippase, transport activity is auto-inhibited by N- or C-terminal sequences, which can be relieved by various stimuli. In the P1B-ATPases an extended cytoplasmic tail in the N-terminal region, loaded with various numbers of heavy metal binding sites, functions as a regulator of transport function by acting either on catalytic turnover or the transport coupling ratio in the pump [15–17]. The bent MB segment in P1B-ATPases is thus likely to be involved in controlling the access of chaperones to the ion entry site of the M-domain which in turn can be auto-regulated by blockage of the heavy-metal binding domain. The human copper ATPase involved in Wilson Disease (ATP7B) is for instance phosphorylated in the N-terminal heavy metal-binding region where phosphorylation affects the interaction of the heavy metal-binding region and headpiece domains with strong influence on the catalytic properties of the enzyme [18]. So in other P-type ATPases long N- or C-terminal stretches regulate pump function. A few (mostly fungal) members of the P5-ATPases display an extended sequence stretch in either the N-terminal region (e.g. the yeast orthologue Ypk9p is equipped with a predicted cytoplasmic N-terminal tail of 293 amino acid residues). However, the general picture is that P5-ATPases lack large cytoplasmic tails positioned before or after the N- or C-terminal membrane segments, respectively.

4. Genetics of P5A-ATPase function in a model organism

Loss of P5A-ATPase gene function in both plant and yeast lead to broad unspecific phenotypes related to an inability to maintain basic secretory pathway functions. In order to understand their function in the cell we have chosen to focus on the available evidence linked to function of P5A-ATPases in a simple eukaryotic model organism, the yeast *S. cerevisiae*. As has been demonstrated in an ample number of occasions, knowledge obtained in this system is likely to be broadly applicable to other eukaryotic systems [19,20].

4.1. Spf1p of S. cerevisiae as a model P5A-ATPase

SPF1, the single gene in *S. cerevisiae* encoding a P5A-ATPase, was first identified in a screen of mutagenized cells that showed resistance to the killer toxin SMKT secreted by *Pichia farinosa*, hence the name Sensitivity to *P. farinosa* [21]. In *spf1* mutant cells, the toxin is retained at the cell wall and does not get internalized hereby representing a molecular mechanism for the resistance of mutant cells [22,23]. However, as the Spf1p protein was localized to the endoplasmic reticulum (ER) and putatively encoded an ion pump, a link between protein function and the cellular impact of its activity could not be identified.

According to the *S. cerevisiae* Genome Database (http://yeastgenome.org), at the time of the writing of this review, more than 500 experiments have now reported genetic interactions that involve *SPF1* or its encoded protein, and the list comprises 309 genes. The vast majority of these genetic interactions are negative and 17 are synthetic lethal. In genetics, two genes interact negatively if the combination of mutations in both produces a stronger effect than expected from the phenotype of two single mutants and, in the worst case, the organism does not survive the combined effect and the effect is said to be synthetic lethal. The interactors of *SPF1* fall in separate clusters (Fig. 2A) and vary tremendously both with respect to cellular location (Fig. 2B) and predicted function (Fig. 2C). Fig. 3 provides a summary of the function and cellular localization of major proteins that exert activities which are related to Spf1p. Supplemental Table 1 integrates genetic and physical interactions from high- and low throughput studies in yeast available at BioGrid (http://thebiogrid.org). Furthermore, the data from five high throughput genetic interaction yeast studies have been included and sorted by colony size to show the relative intensities of the genetic interactions within each study.

4.2. Spf1p and the unfolded protein response

What remains evident from the information gathered so far is that Spf1p is tremendously important for homeostasis of the ER. In the ER, newly synthesized proteins are folded and are subsequently processed by post-translational modifications that can involve glycosylation or addition of GPI-anchors followed by their transport to the Golgi apparatus where further modifications may occur [23–26]. Inhibition of any of these processes in this complicated machinery may affect ER homeostasis and, as a consequence, result in accumulation of unfolded proteins in the ER.

Strikingly, in a screen among deletions of non-essential genes in *S. cerevisiae* (around 4500 genes), the knockout strain that exhibited the third strongest unfolded protein response (UPR) was the *spf1* mutant [27]. This connection is conserved in other organisms as well, as induced activation of the unfolded protein response in *Aspergillus nidulans* results in increased expression of the *SPF1* ortholog, *AN3146.2* [28]. In multicellular organisms like plants, disruption of the single P5A-ATPase gene, *AtP5A1/MIA/PDR2*, also correlates with sensitizing of a subset of ER quality control responses [10,29]. The mutant also displays a changed expression pattern of genes belonging to functional groups of protein secretion, protein folding and solute transport and has an altered homeostasis of cations [10].

Unfolded proteins are recognized in the ER by the membrane-embedded protein Inositol-requiring enzyme1 (Ire1p) [30–32], which on the luminal side of the ER membrane has a receptor-domain that senses unfolded polypeptides. On the cytoplasmic side of the ER, Ire1p has a domain with RNase activity, which specifically recognizes the mRNA of the transcription factor Hac1p. The *HAC1* gene is one of the few yeast genes with an intron. Following activation of Ire1p, the RNase activity of Ire1p splices the mRNA of HAC1, and—following removal of the intron—Hac1 protein is synthesized [33,34]. The Hac1p transcription factor migrates to the nucleus where it coordinates the expression of genes, such as chaperones, that are involved in the unfolded proteins response (UPR) [32,35].

The mutant yeast strain *per9* was isolated in a screen for mutants that show a synthetic lethal interaction with *IRE1* [36]. The *PER9* gene was cloned and found to be identical to *SPF1* [7]. In a parallel approach, a mutation in *SPF1* was found to be synthetic lethal in combination with a knockout in *HAC1* [6]. The conclusion is that a mutation in *SPF1* causes such disturbance of ER homeostasis that, without Ire1p or Hac1p to initiate repair of the damage, the effect is lethal.

From an overview of all deletions of non-essential genes, which trigger the unfolded protein response, it is clear that defects in the *N*-linked glycosylation pathway and ER-associated degradation cause massive induction of UPR [27]. Similarly high inductions, however, are also found following deletion of genes involved in other processes such as membrane-insertion of tail-anchored proteins, lipid biosynthesis, glycophosphatidylinositol anchor synthesis, O-mannosylation, vesicle trafficking and ion homeostasis. Below we discuss the vital roles Spf1p carries out in membrane protein insertion, sterol biosynthesis, vesicle trafficking, protein glycosylation, GPI anchor synthesis, and cell wall formation. Lack of Spf1p in any of these processes induces the UPR system to re-establish ER homeostasis.

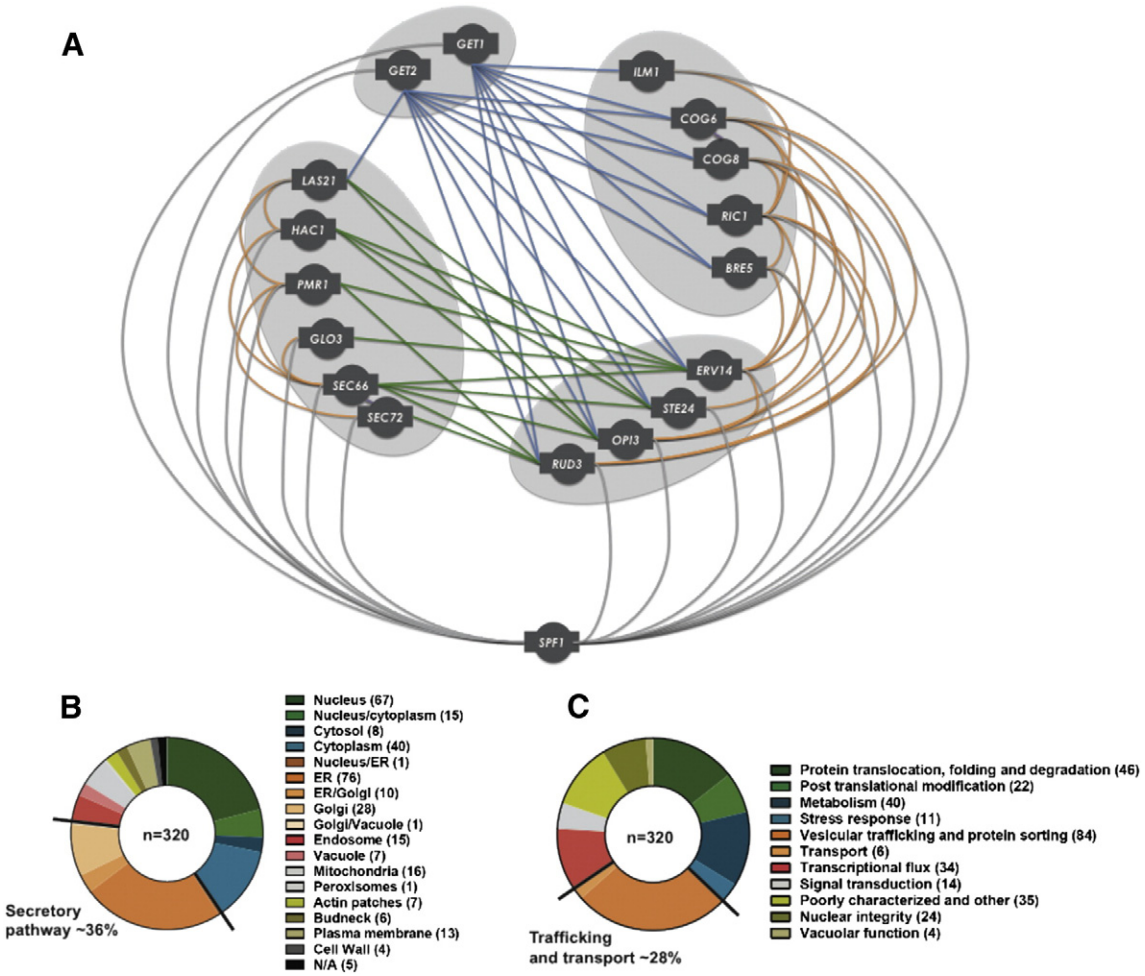

Fig. 2. Interaction data for 309 genetic interactions for SPF1 and its encoded protein identified in the *Saccharomyces cerevisiae* genome database (http://yeastgenome.org). *A*) The highest ranking interactors (identified in at least 5 independent experiments) group as clusters around members of the GET complex. *B*) Cellular location of interactors. The majority of interactors (~36%) are present in the secretory pathway. *C*) Functional role of interactors. The majority of interactors (~28%) plays a role in vesicle trafficking and transport.

4.3. Spf1p and insertion of membrane proteins into the ER membrane

Membrane proteins are inserted into the ER membrane following their synthesis and subsequently may travel to other parts of the cell via the secretory pathway. Single-pass transmembrane proteins have only a single transmembrane segment that anchors them in the ER membrane. Multi-pass transmembrane proteins have several transmembrane segments that following their synthesis have to be oriented correctly within the membrane. Membrane proteins without transmembrane segments can be anchored in the ER membrane by covalent attachment to a glycophosphatidylinositol (GPI) anchor that is embedded in the membrane. As will be evident from the following, Spf1p appears to be important for most of the processes related to insertion of membrane proteins in the ER membrane.

4.3.1. Spf1 and the topology of insertion of integral membrane proteins

All integral membrane proteins of the plasma membrane and most of the membrane proteins in intracellular membranes are first integrated in the ER before they travel to other destinations in the cells [37]. The classical co-translational pathway involves recognition of a hydrophobic segment by the signal recognition particle [38] followed by transfer to the translocon, or Sec61 complex, which inserts the hydrophobic sequence stretch into the ER membrane [37]. The major signals determining the direction of insertion of a newly synthesized transmembrane segment in the membrane is: i) the charge difference across the membrane; and ii) its total hydrophobicity [39–42]. In prokaryotes, the electric field that drives insertion of membrane proteins into the cell membrane is the result of a proton gradient, positive on the outside, negative at the cytoplasmic side of the membrane [43]. An uneven distribution of anionic phospholipids also plays an important role in creating the required electrical field [44].

A transmembrane potential gradient has not been observed across the ER membrane although a small but significant Ca^{2+} gradient is present [45]. Still, the orientation of transmembrane segments inserted into the ER follows a 'rule' in which the charge distribution in the sequence immediately preceding the first transmembrane segment determine its orientation, with the more positive portion facing the cytosol [46].

A peculiar but strong link exists between Spf1p and the way this process is taking place. In a screen devised to isolate yeast protein insertion orientation (*pio*) mutants defective in response to a strong charge difference signal, *pio1* was isolated and the corresponding gene found to be *SPF1* [47]. The altered orientation of the reporter in the ER in the *spf1* background was explained by assuming a modified electrostatic field at the ER, as in this background the positively charged sequence flanking the single transmembrane segment of the reporter has changed orientation from the cytosolic side of the ER to the lumen (likewise, the negatively charged C-terminal sequence had reverted to the cytosolic side of the membrane). In order to explain this phenotype, it would be expected that in *spf1* mutants, the luminal side of the ER is more negatively charged than in the wild-type. Several ways by which Spf1p could contribute to creating a charge difference across the ER

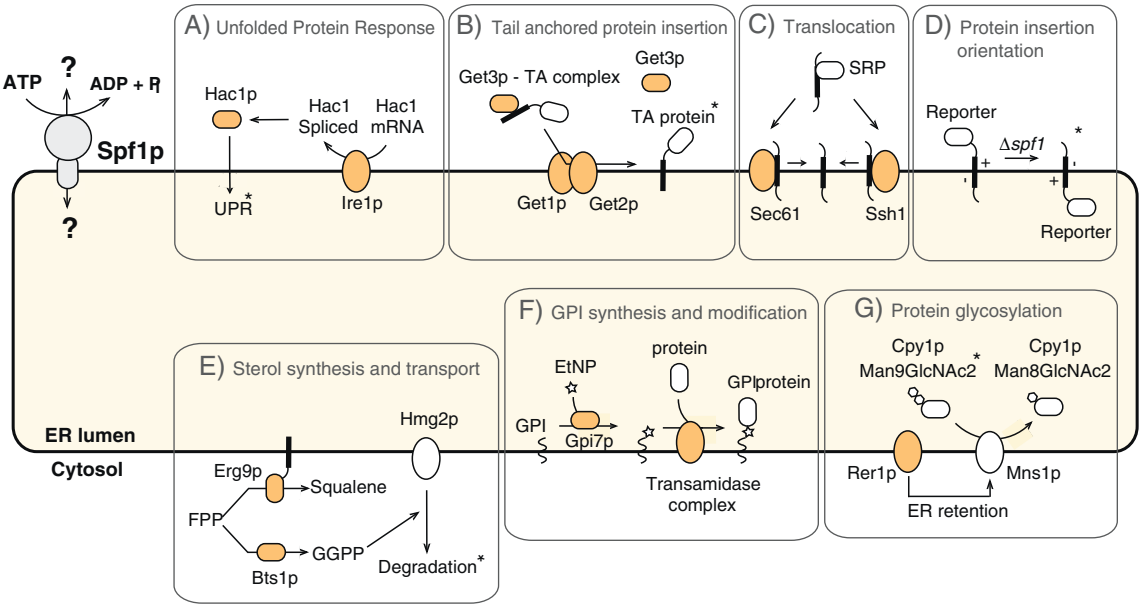

Fig. 3. Summary of the function and cellular localization of major proteins which activities are related to Spf1p. Orange colour depicts proteins encoded by genes that show genetic interaction with SPF1 (spf1p encoded in grey). The major functions includes: *A*) the unfolded protein response, *B*) tail anchored protein insertion, *C*) protein translocation, D) protein insertion orientation, *E*) sterol synthesis and transport, *F*) glycophosphatidylinositol synthesis and modification and *G*) protein glycosylation. See text for further explanations. UPR: unfolded protein response, TA: tail anchored, SRP: signal recognition particle, reporter: reporter used to study protein insertion phenotype in [47], GGPP: Geranylgeranyl pyrophosphate, FPP: farnesyl pyrophosphate, EtNP: ethanolaminephosphate, GPI: glycosylphosphatidylinositol.

membrane are possible. One obvious possibility is that Spf1p is an electrogenic pump. If so, Spf1p may contribute to charge separation by transporting either positive charge into the ER lumen or negative charge from the lumen to the cytosol.

It is worth mentioning that *STE24*, which encodes a metalloprotease integrated in the ER membrane that is involved in removal of the C-terminal AAX tripeptide from prenylated CAAX box proteins such as the a-factor [48], corresponds to *PIO2*, the second gene identified in the screen for genes of importance for protein insertion orientation [47]. Interestingly, *SPF1* and *STE24* show negative genetic interaction with each other [49–52]. In mammalian cells, ER-bound metalloproteases function as sterol-sensors that proteolytically release an ER-bound transcription factor that activates genes involved in the biosynthesis or cholesterol and fatty acids [53]. It has been suggested that Ste24p similarly is involved in the feedback control of sterol synthesis and that both Spf1p and Ste24p have in common that they indirectly contribute to control ER membrane lipid composition [47].

4.3.2. Spf1p and membrane insertion of tail-anchored proteins

A specialized pathway has evolved for insertion into the ER of a subgroup of membrane proteins with a single trans-membrane segment [37]. A relatively minor fraction (around 5%) of eukaryotic membrane proteins have a single membrane span that is situated in their carboxy-terminal end. Such 'tail-anchored' (TA) membrane proteins perform many essential processes in the cell. Once synthesis of a TA-protein is initiated on a ribosome, the carboxy-terminal transmembrane domain is recognized by a protein complex, which in yeast involves Get3p, and is subsequently directed to the ER [54]. Get1p and Get2p function as ER-localized receptors for Get3p complexed with a TA membrane protein, and the protein is released in an ATP-dependent manner while the carboxy-terminal tail is inserted in the membrane [55].

There are strong genetic links between *SPF1* and insertion of TA-proteins into the ER. First, mutations in *SPF1* lead to synthetic lethality in combination with a disruption of *GET1* (also called *MDM39*) [56]. Second, a deletion of *SPF1* is synthetic lethal in combination with a knockout in *GET2*, which encodes the second subunit of the TA-protein receptor [57]. Third, *SPF1* shows negative genetic interactions with *GET3* [49,50,52]. This implies that *SPF1* is acting genetically with all three members of the GET complex.

Tail-anchored membrane proteins destined for the mitochondrial outer membrane move into this membrane immediately after their synthesis and apparently do not require a dedicated machinery like the GET complex for membrane insertion to occur. Genes important for proper insertion of TA-proteins in the mitochondrial outer membrane were identified in a screen employing Gem1p, a model TA-protein in the mitochondrial outer membrane, which was fused to GFP and expressed in the background of all viable yeast deletion mutants. The only identified gene deletion that caused mislocalization of GFP-Gem1p to the ER was *SPF1* [58]. A closer investigation revealed that deletion of *SPF1* furthermore resulted in the mislocalization of other TA-proteins from the mitochondrial outer membrane to the ER [58].

Insertion of TA-proteins into biological membranes is strongly inhibited by ergosterol [59], and, as the mitochondrial outer membrane has the lowest sterol content of all membranes exposed to the cytosol [60], a GET complex may not be required in this membrane. As cells deleted for *SPF1* have somewhat modified ergosterol levels in the mitochondrial outer membrane [58], Spf1p may in some way regulate sterol levels and the distribution of sterols in the cell. This function may have an impact on the ability of TA-proteins to insert in the outer mitochondrial membrane and possibly also in the ER. Transfer of newly synthesized lipids including sterols into mitochondria takes place in mitochondria-associated membranes (MAMs), a region of the ER in close proximity to the mitochondrial outer membrane [61]. To our knowledge, Spf1p has not been identified in MAM structures but a role for P5A-ATPases in regulating the circuit of sterols in MAMs cannot be excluded.

4.3.3. Spf1 and translocation of secretory proteins across the ER membrane

Genetic evidence summarized below indicates that Spf1p is required to create a state of the ER in which post-translational translocation of newly synthesized polypeptides through the ER membrane is optimally functioning. Proteins to be secreted enter the lumen of the ER in an unfolded state and pass through the translocon, an intricate machinery for post-translational translocation of secretory proteins across the ER membrane [62]. Two related translocon protein channels are the so-

called Sec61 and the Ssh1 (Sec sixtyone homologue1) complexes [63]. Both are heterotrimeric complexes of three subunits ('Sec61p, Sss1p, and Sbh1p' and 'Ssh1p, Sss1p, and Sbh2p', respectively) and associate with the regulatory Sec63 complex made of four subunits (Sec62p, Sec63p, Sec71p, and Sec72p).

SPF1 shows negative genetic interactions with *SBH1*, *SBH2*, and *SSH1* of the Sec61/Ssh1 complexes [49,50,52] and with *SEC62*, *SEC63*, *SEC66* and *SEC72* of the Sec63 complex [49–52]. We can conclude that in the absence of subunits of the translocon apparatus, the negative impact this has on growth is significantly aggravated when Spf1p is lacking. The Sec61 and Ssh1 translocons both play a role in the ER associated degradation pathway (ERAD) for the retro-transport of misfolded proteins back into the cytosol where degradation in the proteasome occurs. Since Spf1p is part of the UPR and since in *spf1* mutants ERAD is malfunctioning [7], the genetic link with the translocons could be explained by a combined role in the retro-transport of misfolded proteins.

4.4. Spf1p and the biosynthesis and transport of sterols

Among the important processes taking place in the ER, the biosynthesis of sterols and isoprenoids has a profound importance for a variety of cellular processes and defects in this process have a strong impact on the UPR [27]. A number of independent observations link P5A-ATPases to the biosynthesis and transport of sterols. A knockout of *SPF1* leads to sensitivity of yeast to inhibition of ergosterol synthesis [6,10,64], to loss of control in regulated degradation of ergosterol synthesis genes [6], and to an abnormal distribution of populations of ergosterol between the ER and mitochondria [58]. Furthermore, a mammalian homologue, ATP13A1, has been identified in a screen for proteins that interact directly with cholesterol [65].

3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA-reductase) catalyzes the most important, rate-limiting step in the synthesis of sterols and isoprenoids, which is the reduction of hydroxymethylglutaryl-CoA to mevalonic acid, a step which marks the beginning of the so-called mevalonate pathway (Fig. 4). Two HMG-CoA-reductase isoenzymes, Hmg1p and Hmg2p, are responsible for HMG-CoA-reductase activity in ER membranes [66]. Hmg1p is constitutively active while Hmg2p is only active when the demand for products of the mevalonate pathway is high. Otherwise, Hmg2p is degraded in the proteasome following ubiquitinylation in response to feedback signals from the mevalonate pathway. Accordingly, when feedback signals from the sterol pathway are high, Hmg2 enters the degradation pathway.

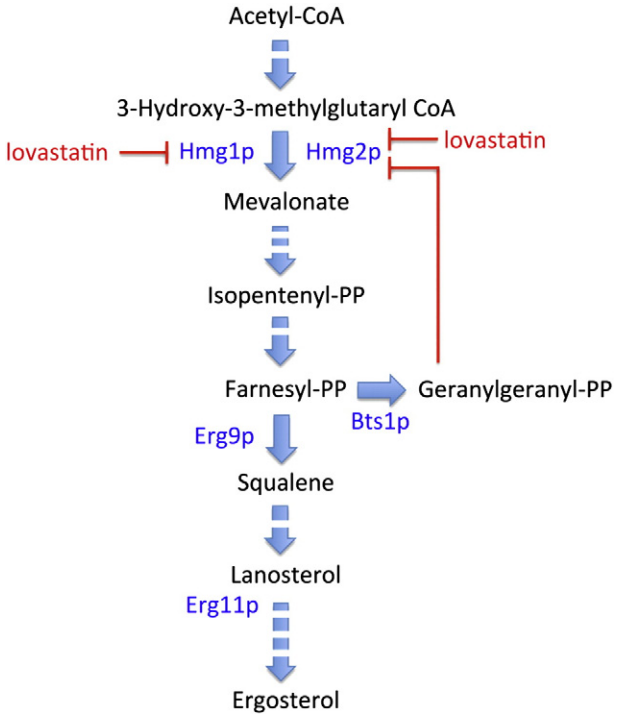

Fig. 4. Abbreviated ergosterol biosynthesis pathway. Principal intermediates and enzymes are shown. Red lines indicate inhibitory actions by inhibitory molecules.

In a screen for mutants that constitutively degrade Hmg2p under conditions where feedback signals would normally be absent, 38 mutants isolated fell into a single complementation group, *cod1* (control of HMG-CoA reductase degradation) [64]. *COD1* was cloned and proved to be identical to *SPF1* [64]. Growth of the *cod1/spf1* mutant was found to be hypersensitive to lovastatin, a HMG-CoA-reductase inhibitor, and, based on the normal response of the mutant to other inhibitors of enzymes in the mevalonate pathway, it was concluded that lovastatin hypersensitivity was specifically due to misregulation of Hmg2p [64]. This was a surprising finding, because it could indicate that an intermediate of the mevalonate pathway is accumulating in the absence of Spf1p. We now know that ergosterol levels are indeed altered in the *spf1* mutant [58]. Ergosterol by itself has only a minor effect on Hmg2p activity [67] but accumulation of this end product could influence the concentration of upstream intermediates that signal Hmg2p degradation. A deletion in *ERG11*, the enzyme in the sterol biosynthesis pathway immediately downstream of lanosterol (Fig. 4), is synthetic lethal in combination with a deletion of *SPF1* [68]. This may suggest that the Hmg2p degradation signal is formed further upstream in the pathway.

Downstream in the mevalonate pathway, the formation of farnesyl pyrophosphate is the hallmark of the biosynthesis of sterols (Fig. 4). A major feedback signal that triggers HMG-CoA-reductase degradation is geranylgeranyl pyrophosphate, rather than a sterol [69]. The enzyme that catalyzes the formation of geranylgeranyl pyrophosphate is Bts1p, which as a precursor employs farnesyl pyrophosphate. Remarkably, a deletion of *BTS1* is synthetic lethal in combination with a mutation in *SPF1* [56]. Based on this evidence, we may assume that the mevalonate pathway compound that accumulates in the absence of Spf1p leads to accumulation of geranylgeranyl pyrophosphate (via farnesyl pyrophosphate) that in turn triggers degradation of Hmg2p, and as a result further lipid biosynthesis is prevented. However, in the absence of *BTS1* no geranylgeranyl pyrophosphate can be formed, whereas in the absence of *SPF1* geranylgeranyl pyrophosphate may accumulate. How would the combined loss lead to cell toxicity? An answer may be that if Hmg2p cannot be degraded in *spf1* mutants lacking Bts1p, not only may farnesyl pyrophosphate accumulate, but also an end compound of mevalonate pathway, which in high concentrations may disturb ER functions and become lethal for the cell. Such a compound could be lanosterol and/or ergosterol (Fig. 4) as increased levels of both sterols result in rigidification of membranes [70,71]. Further, farnesyl pyrophosphate derived lipids may destabilize Hmg2p [72,73]. Taken together, it appears as if *spf1* cells somehow cannot redistribute one or more compounds in the mevalonate pathway, leading to accumulation and misregulation of feedback signals.

A closer look at the enzymes involved in the sterol biosynthesis pathway in *S. cerevisiae* reveals that squalene synthase or Erg9p, which catalyzes the first committed step in the biosynthesis of ergosterol in yeast (Fig. 4), is a tail-anchored protein [74,75]. This enzyme catalyzes the condensation of two molecules of farnesyl pyrophosphate to produce a single molecule of squalene. As a TA-protein, Erg9p might not end up in the ER membrane in the absence of Spf1p, which has strong genetic interactions with the receptor for TA-proteins (see above). It seems clear from the above discussion that in the absence of Erg9p at its correct location, farnesyl pyrophosphate and subsequently geranylgeranyl pyrophosphate are likely to accumulate, which possibly could affect Hmg2p stability.

Another possibility remains. If Spf1p is required in some way for the trafficking of ergosterol (or another end product of the mevalonate pathway) away from the ER, the absence of Spf1p would be predicted to result in the build-up of ergosterol in the ER followed by accumulation of

preceding intermediates. Accumulation of ergosterol (or another end product) may influence the properties of the ER membrane and affect negatively the spontaneous insertion of TA-proteins, a process, which is sensitive to the sterol content of the membrane (see above). What comes first in the *spf1* mutant? A deficient TA-protein insertion machinery or the accumulation of sterols? In our efforts to try and understand the genetic interaction data, we are thus faced with a 'chicken-or-the-egg' dilemma.

Statins such as lovastatin target HMG-CoA-reductase and inhibit *de novo* sterol synthesis of sterols and isoprenoids [76]. Such drugs exert multiple pleiotropic effects. Further, the mevalonate pathway is responsible for the production of numerous non-steroid isoprenoids which isoprenylate essential intracellular proteins. These proteins include small GTPases, e.g. Ras and Rho GTPase families, which are known to connect intracellular signalling processes and extracellular stimuli. Many of the pleiotropic consequences of Spf1p loss could thus be due to secondary effects of a modified content of sterols and isoprenoids in the cell. As lovastatin treatment, which should reduce farnesyl pyrophosphate and geranylgeranyl pyrophosphate accumulation, does not affect Hmg2p degradation in an *spf1*Δ mutant background [6,64], there may be alternative mechanisms for degradation of Hmg2p. In cells lacking *SPF1*, Hmg2p may be unresponsive to the in vivo degradation signal due to alterations in its structure that interfere with the degradation pathway [72,73]. Such misfolding of Hmg2p in the absence of Spf1p may have the same origin as other protein folding, glycosylation, and insertion problems described in this review.

4.5. Spf1p and post-translational modification of secreted proteins

After their synthesis and insertion in the ER, secreted proteins are often modified by addition of complex carbohydrate groups in the form of *N*-linked glycosylation, glycophosphatidylinositol anchors, or O-mannosylation. Spf1p appears to be influencing at least the first two of these processes.

4.5.1. Spf1p and synthesis of glycosylphosphatidylinotisol membrane anchors

A number of soluble proteins that are translocated into the lumen of the ER end up getting anchored to the luminal side of the ER membrane. Such proteins are typically secreted to the plasma membrane and many of them end up in the cell wall [77]. The membrane anchor, which is attached to such proteins, is glycosylphosphatidylinotisol (GPI), a glycolipid found in all eukaryotic cells. GPI-anchored proteins have in many cases been found associated with detergent-resistant membrane domains in the plasma membrane, also called lipid rafts [78]. GPI anchored proteins that end up bound to the cell wall do so as a result of a sugar group of GPI becoming covalently linked to β-glucans.

SPF1 shows strong genetic links with the last steps in the biosynthesis of GPI anchors, a process that takes place in the ER. Thus, mutations in *GPI7* (*LAS21*), which is involved in adding ethanolaminephosphate to the second mannose of GPI immediately before it is transferred to a protein, lead to synthetic lethality in combination with a disruption of *SPF1* [Ando and Suzuki, 2005]. Furthermore, *SPF1* shows negative genetic interaction with two subunits of the transamidase complex (*GPI8* and *GPI17*) [49,52], which catalyzes the transfer of the complete GPI precursor to proteins [79]. The fact that *SPF1* shows a strong negative genetic interaction with the last steps of GPI anchor synthesis suggests that Spf1p influences a process upstream in the biosynthesis that can be compensated for by other means, but in the simultaneous absence of Spf1p and downstream components the process can no longer proceed.

4.5.2. Spf1p and synthesis of N-linked oligosaccharides

Secreted proteins may be decorated in the ER by *N*-linked glycosylation and the sugar moiety is subsequently modified in the Golgi. Secreted invertase isolated from *spf1*Δ cells migrates faster through SDS-PAGE gels than does invertase secreted from wild-type cells, which is an indication that it is less glycosylated [7,21]. This was an early but strong indication that *N*-linked glycosylation is influenced by Spf1p.

CPY* is a well-characterized misfolded mutant of the secreted protein carboxypeptidase Y that following entry into the ER lumen is re-exported to the cytosol for degradation [80]. The *spf1* mutant is defective in the degradation of CPY* but not Ste6p, a misfolded version of the plasma membrane protein [7]. Characteristic for carboxypeptidase Y is that this protein is *N*-glycosylated at several sites whereas Ste6p is not, and, importantly, CPY* has to carry a mature glycosylation pattern for efficient degradation [81]. In pulse-label experiments, Vashist et al. [7] could show that glycosylation of CPY* is normal in the *spf1* mutant whereas oligosaccharide trimming from Man9GlcNAc2 to Man8GlcNAc2 is compromised. The removal of one mannose residue from a glycosylated protein, converting the modification from Man9GlcNAc to Man8GlcNAc, is a trimming process catalyzed by α-1,2-mannosidase, Mns1p, and is the last step in glycoprotein maturation in the ER and a determining step in ER protein degradation. Apparently this lack of trimming is responsible for the inability of yeast to degrade CPY* in *spf1* cells. This correlation has not been tested and no genetic interaction between *SPF1* and *MNS1* has been reported. However, there are several reports on a negative genetic interaction between *SPF1* and *RER1* [49,50,52], which encodes a protein required for retention of Msn1p in the ER membrane [82]. Mns1p itself is a single-span membrane protein with its transmembrane segment situated in the extreme N-terminus of the protein [82]. As such it does not resemble the single-span TA-proteins discussed above, and at present we cannot predict whether the lack of Spf1p influences the localization of Mns1p to the ER.

4.6. Spf1 and vesicle transport

Secreted proteins that leave the ER inside the lumen of vesicles enter the Golgi and proceeds into the trans-Golgi network, where they are sorted to proceed either to the plasma membrane or to the endosomal system [83]. However, resident proteins of the ER are directed to their correct cisternal compartments in the Golgi by the so-called conserved oligomeric Golgi (COG) complex, which mediates fusion of vesicles within the Golgi [84,85].

SPF1 show strong negative genetic interactions with several members of the COG complex, namely *COG5*, *COG6*, *COG7*, and *COG8* [49–52,86]. Together, Cog5p, Cog6p, Cog7p, and Cog8p form the so-called lobe B of the COG complex [84]. Lobe B is involved in directing late Golgi vesicle targeting and its subunits interact primarily with Rab and SNARE proteins [85]. It is difficult to explain how the activity of Spf1p is linked to that of the COG complex. It is striking, however, that mutations in *GET1* as well as in *GET2* show synthetic lethality in combination with mutants in *COG5*, *COG6*, *COG7*, or *COG8* [57]. As Get1p and Get2p form the ER receptor for TA-proteins, which somehow is dependent on Spf1p activity (see above), the COG complex may be dependent on the correct insertion of a TA-protein and this may explain its link to *SPF1*.

A number of other, seemingly unrelated, genes show strong links both to *SPF1* and genes encoding members of the GET complex. The list includes *RIC1*, *BRE5*, *ERV14*, *OPI3, and RUD3*. Among the encoded proteins, at least Ric1p, Bre5p, Erv14p, and Rud3p are involved in vesicle trafficking. Although these interactions may be relevant for our understanding of *SPF1* function, they may be indirect effects related to a dysfunctional GET complex and/or an altered content of sterol or isoprenoids in the secretory pathway. It is striking that a number of TA-proteins are involved in vesicular transport [87], such as Bos1p, Bet1p, and Sec22p, which all are SNAP receptors (SNAREs) of the ER involved in vesicular transport from the ER to the Golgi [88], and Sed5p, which is a *cis*-Golgi syntaxin that binds multiple SNARE proteins [89]. Among these, *SEC22* appear to have several genetic interactions in common with *SPF1*. As expected, *SEC22* shows genetic interactions with *GET1* and *GET2* but it also exhibits strong genetic interactions with *COG5* to *COG8*, as well as with *RUD3*, *ERV14*, and *BRE5* [27,49–52,90,91].

As Spf1p appears to be required for a functional GET complex (see discussion above) it is tempting to speculate that in the absence of Spf1p, the insertion into the ER of Sec22p and functionally related TA-proteins is compromised, which again leads to a weakening of the secretion machinery from the ER to the Golgi. For this reason, a mutation in *SPF1* in combination with mutations in genes involved in ER to Golgi transport (other than *SEC22*) would become a challenge for yeast growth. In support of this model, *SEC22* and *SPF1* both show strong genetic interactions with *GLO3* [27,49,52], which is not a TA-protein but encodes an ADP-ribosylation factor GTPase activating protein (ARF GAP) involved in vesicle transport from the ER to the Golgi [92]. Furthermore, TA-proteins constantly leak from the ER as they leave by transport vesicles heading for the Golgi and other post-ER compartments and their retrieval is dependent on vesicle transport in the opposite direction [93]. A deficiency in insertion of TA-proteins in the ER membrane may be lethal in combination with a deficiency in vesicle-mediated retrieval of lost TA-proteins. This provides an additional link between membrane insertion of TA-proteins and vesicle trafficking, both processes of which appear strongly linked to *SPF1*.

4.7. Spf1 and its influence on cell wall properties

Unfolded proteins, impaired glycosylation and reduced GPI anchors may all contribute to impaired cell wall properties [94]. As mentioned above, the initial discovery of *SPF1* was the result of a screen of yeast mutants that show tolerance of *S. cerevisiae* to the salt-mediated killer toxin (SMKT) of the halotolerant yeast *P. farinosa* [21]. Five sensitivity to *P. farinosa* toxin (*spf*) mutants were isolated and all fell into the same complementation group, named *spf1*, and subsequently the *SPF1* gene was cloned. The *spf1* mutant cells were shown to be sensitive to calcofluor white and hygromycin B [21], two compounds that amplify the effect of cell wall mutations [95,96], which suggests that Spf1p, even though the protein is resident in the ER, in some way influences the properties of the yeast cell wall.

Killer toxins are secreted proteins produced by so-called killer yeast strains that kill sensitive (nonkiller) strains. The primary receptors of all yeast killer toxins characterized so far are localized to the cell wall [97]. SMKT itself was found to interact with the cell wall [21], which would suggest that Spf1p in some way has an impact on the amount or the properties of an SMKT receptor at this position. Well-characterized killer toxins are K1 [98,99], produced by a *S. cerevisiae* killer strain, and PMKT [100] produced by *Pichia membranifaciens*. Both toxins use β-1,6-D-glucan as cell wall receptors before they enter the cell via interaction with secondary receptors, both of which are glycosylphosphatidylinositol (GPI) anchored proteins [reviewed in ref. [97]]. In many cases, genes in *S. cerevisiae* that when mutated confer resistance to K1 killer toxin encode enzymes involved in the synthesis of N-glycans, which are complex polysaccharide structures present on many secreted and membrane-bound glycoproteins [101].

P. farinosa SMKT is encoded by a single gene and is subsequently cleaved into two distinct subunits in a manner very similar to that of K1 [102]. However, SMKT kills *S. cerevisiae kre1* and *kre5* mutants being defective in β-1,6-D-glucan synthesis, which suggests that cell wall components other than β-1,6-D-glucan function as the primary receptor for SMKT [103]. As the yeast cell wall is primarily made of only four components: β-1,6-glucan, β-1,3-glucan, mannoproteins and chitin [104,105], the number of alternative candidates for primary cell wall receptors seem limited.

In this context, it is striking that a knockout of *SMI1/KNR4*, a protein involved in positive regulation of β-1,3-glucan synthesis [106], is synthetic lethal in combination with a knockout in *SPF1* [107,108]. Like *spf1* mutants, *smi1* mutants are sensitive to calcofluor white and hydromycin B and, interestingly, *smi1* mutants are also resistant to K1 killer toxin [101]. This suggests that *SPF1* and *SMI1* by independent routes are required for efficient β-1,3-glucan synthesis, which explains why in the absence of both genes the effect is lethal. However, the action of Spf1p cannot be specifically required for formation of β-1,3-glucan as a mutation in *HOC1*, which is a glycosyltransferase involved in formation of the hypermannose structure attached to mannoproteins, is also synthetic lethal in combination with a mutation in *SPF1* [108]. This hints to the possibilities that the receptor for SMKT is either β-1,3-glucan or a mannoprotein in the cell wall.

The extremely high transformation efficiency of *spf1* mutant cells further reinforces the notion that Spf1p influences the properties of the cell wall [109]. When viable deletion mutants of *S. cerevisiae* were transformed using a simplified lithium acetate method, *spf1* cells showed the highest transformation efficiency among around 5,000 mutants tested, and had a transformation efficiency and frequency almost two orders of magnitude higher than that of wildtype cells [109]. Strikingly, the transformation efficiency of *spf1* cells compared to wildtype cells disappears when the cell wall is removed enzymatically [110]. This indicates that an altered cell wall in *spf1* cells is an important determinant of the high capacity of these cells to absorb plasmid DNA. Whereas deletion of *SPF1* facilitates plasmid uptake, deletions in genes encoding proteins required for the endocytotic machinery diminishes transformation efficiency [109]. The mutant with the second lowest transformation competence was *arc18*Δ, defective in encoding a subunit of the Arp2/3 complex, which is involved in mediating actin assembly during endocytosis [109]. *SPF1* shows a negative genetic interaction with *ARC18* [27] and the null mutant is synthetic lethal in combination with null mutations in two other Arp2/3 subunits, *ARP2* and *ARC40* [108]. This suggests that the cell wall deficiency caused by disruption of *SPF1* amplifies the aggravating effect of disturbing the endocytic machinery.

5. Towards a unifying hypothesis for the function of P5A-ATPases

Is it possible from the bewildering array of phenotypes of P5A-ATPase mutants in model organisms to draw any conclusions on the function of these putative pumps? The fact that *SPF1* encodes an ER protein but influences cell wall properties, reinforces the notion that deletion of *SPF1* has pleiotropic effects. The phenotype of *spf1* mutant cells is understandable if we consider that the cell wall consists of multiple proteins and polysaccharides that originate from the ER, and have passed through the secretory pathway to reach the cell exterior. Many of the proteins in the cell wall are GPI- or tail-anchored proteins and may require other post-translational modifications by enzymes in the ER and the Golgi. Thus, disturbing ER homeostasis by mutating *SPF1* is likely to have a negative impact on the whole apparatus involved in cell wall formation. Following our analysis mainly of genetic interaction data it is possible to point to a role of Spf1p in most parts of the secretion machinery. However, some phenotypes stand out more than others and might indicate direct rather than indirect effects. First, there is a strong link between *SPF1* and the insertion of tail-anchored proteins in membranes. Second, there are many indications that Spf1p influences the biosynthesis of a product in the mevalonate pathway. Third, it appears that Spf1p influences charge separation across the ER membrane.

5.1. Could Spf1 be an ion pump?

Almost all P-type ATPases transport cations, either out of the cytosol or into the cytosol [11]. Thus, it seems an obvious hypothesis that Spf1p is an ion pump transporting positive charge into the ER lumen. Along this line, the substrate of Spf1p has been suggested to be Ca^{2+} [6,7,64] or Mn^{2+} [111].

5.1.1. Could Spf1p be a Ca^{2+} pump?

Several lines of evidence point to a connection between Spf1p and Ca^{2+} levels in the ER. First, knockout of *SPF1* in *S. cereviase* and the related P5A-ATPase genes in *S. pombe* (*CTA4*), and *C. albicans* (*SPF1*) result in an induced Ca^{2+} dyshomeostasis [6,9,112]. Second, the deletion of *SPF1* activates the Ca^{2+} dependent calcineurin pathway [6]. Third, the

negatively charged residues in the putative substrate binding sites in the M4 region, even though they only show weak similarity to Ca^{2+} binding sites in other P-type ATPases, could indeed be compatible with coordination of a cation like Ca^{2+}. Fourth, the pleiotropic phenotype of P5A-ATPase mutants is not at all incompatible with a role in regulation of Ca^{2+} homeostasis in the ER. Fifth, we note that Mns1p, an enzyme residing in the ER lumen, is dependent upon Ca^{2+} as a cofactor [113,114] and appears to be inactive in *spf1* cells (see above) as expected if luminal Ca^{2+} is decreased in this mutant.

Whereas, in yeast, a Ca^{2+} pump has never been identified in the ER, the Golgi apparatus is equipped with a Ca^{2+}/Mn^{2+} pump of the P-type ATPase family named Pmr1p [115,116]. Deletion of *PMR1* causes defects in protein-linked oligosaccharide trimming and in outer chain modification of carbohydrates in the Golgi apparatus, which closely resemble the phenotypic consequences of deleting *SPF1* [7]. It was therefore suggested that Pmr1p and Spf1p carry out identical functions but in different compartments [7]. In support of this notion is that *SPF1* in several other experiments have shown negative genetic interaction with *PMR1* [47,49,50,52].

Although it is tempting to think of Spf1p as a Ca^{2+} pump of the ER, we need to be cautious before we draw such a conclusion. The physiological effects caused by loss of Spf1p function are not necessarily primary effects but could result from Ca^{2+} related stress responses, or other indirect effects that lead to loss of the ability to control Ca^{2+} homeostasis. In fact, several lines of evidence argue against Ca^{2+} being the substrate of Spf1p.

First, knockout of *SPF1* in *C. albicans* only affect cellular Ca^{2+} levels in the presence of agents that modulate Ca^{2+} stress in which case both the cytoplasmic and total cellular calcium levels significantly increase [112]. Similarly, the total calcium content of the *SPF1* knockout in *S. cerevisiae* remains comparable to the wild type strain and a synergistic increase in total cellular calcium content is only observed when simultaneously paired with disruption of the Golgi apparatus-localized Ca^{2+} pump Pmr1p [6]. Genetic deletion of the *SPF1* homologue in *S. pombe*, *CTA4*, also leads to loss of microtubule integrity and altered nuclear Ca^{2+} homeostasis [9], which can be ascribed to altered transport of calcium across the ER membrane [117]. However, whether this effect is caused by compensatory mechanisms, for instance by increased activity of the Ca^{2+}/Mn^{2+} transporting P2-type ATPase also found in the ER of *S. pombe* [118], or if it is caused directly by loss of Cta4p function remains unclear.

Second, as Spf1p belongs to the P-type family of ATPases, which as a hallmark get phosphorylated or dephosphorylated in the catalytic cycle upon binding of the transported substrate, it is remarkable that Ca^{2+} fails to change the phosphorylation status of Spf1p [119] and only seems to affect its counterpart in plants in a non-catalytically relevant manner [120]. Neither have Ca^{2+}-dependent ATPase activity or phosphorylation of any P5A-ATPase been demonstrated, despite serious efforts on both Spf1p purified from *S. cerevisiae* [6,119] or on the plant homologue HvP5A1 from *H. vulgare* [120]. In the case of Spf1p, work was carried out on a purified and solubilized enzyme which retained ATP hydrolytic activity [6] and displayed phospho-enzyme formation [119] comparable to most other P-type ATPases. Remarkably this activity was observed in the absence of any added ligand other than Mg^{2+} and ATP and addition of Ca^{2+} showed no effect on kinetic parameters at low but physiologically relevant concentrations (>50 μM) [6,119]. Further, the fact that Ca^{2+} causes a slight inhibitory effect on ATP turnover at higher (non-physiological) concentrations (<500 μM) [6] is not in favor of Ca^{2+} as a transported ligand. In the case of HvP5A1, phosphoenzyme formation and turnover was studied in isolated intact membranes carrying the P5A-ATPase in a native lipid environment and, in agreement with Spf1p [119], the HvP5A1 phosphoenzyme formation happens spontaneously without addition of cofactors other than ATP and Mg^{2+} [120]. Among all ions required for plant growth only Ca^{2+} affects phosphoenzyme stability of HvP5A1 although this effect is only observed at high apparent concentrations of Ca^{2+} (~500 μM). Moreover, this high Ca^{2+} effect is also observed when the catalytic machinery for phosphoenzyme turnover is inactivated by mutation [20], which implies that Ca^{2+} merely affects phosphoenzyme stability rather than catalytic turnover and that the Ca^{2+} effect is unrelated to the transport function of the P5A-ATPases.

Third, the insertion of TA-proteins in the mitochondrial outer membrane is not affected by either Ca^{2+} or calcineurin [58] even though this process is strictly dependent on Spf1p activity (see discussion above).

Fourth, and not least, when yeast cells are grown on a medium containing strontium, a calcium analogue, the *SPF1* knockout accumulates significantly more Sr^{90} than wild type cells [121]. In fact, among 4,862 mutant strains tested, *spf1*Δ exhibited one of the highest degrees of Sr^{90} accumulation. In this context it is noteworthy that disruption of genes known to be involved in Ca^{2+} transport resulted in *decreased* Sr^{90} levels. This included mutations in *CCH1* and *VCX1*, encoding a plasma membrane Ca^{2+} channel and a vacuolar Ca^{2+} antiporter, respectively, and the strongest decrease was seen for a mutants in PMC1 encoding the vacuolar Ca^{2+}-pump. This illustrates that although *SPF1* contributes to the Ca^{2+} homeostasis of yeast cells, it does so in a way different from that of well characterized Ca^{2+} transporters. Taken together the available data strongly suggest that Ca^{2+} is not the transported ligand of Spf1p or any other P5A-ATPase.

Having this in mind, we might be able to provide an alternative explanation for the negative genetic interaction between *SPF1* and *PMR1*. Membrane vesicle traffic between the ER and Golgi is bi-directional and proteins involved in trafficking, as well as ER-resident proteins that have escaped the ER retention system, are continuously packaged into COPI vesicles and retrieved back to the ER [122,123]. We may assume that concentrations of ions in vesicles leaving the Golgi towards the ER are identical to those of the Golgi lumen. This implies that the activity of Pmr1p, even though it is resident in the Golgi only, might well be sufficient to provide all the Ca^{2+} and/or Mn^{2+} required for processes in the ER [45].

5.1.2. Could Spf1p be a Mn^{2+} pump?

It was recently proposed that Spf1p might transport Mn^{2+} from the cytosol into the ER lumen [111]. In support of this model, membrane vesicles isolated from *spf1*Δ cells show less Mn^{2+} than vesicles isolated from wild type cells, whereas vesicles from Spf1p overproducing cells show higher levels of Mn^{2+}. Furthermore, many proteins involved in processes in the secretory pathway that can be linked to Spf1p action are Mn^{2+} dependent enzymes [111]. Arguing against this possibility is the fact that Mn^{2+} inhibits ATP hydrolytic activity of partially purified Spf1p [119] and fails to significantly influence the phosphorylation status of the Spf1p counterpart in plants in a catalytically relevant manner [120]. A peculiar phenomenon, which appears difficult to explain by a role of Spf1p in Mn^{2+} transport but rather points to indirect effects, is that the Zn^{2+} content in microsomes derived from *spf1*Δ cells is an order of magnitude higher than that found in the wildtype whereas overexpression of Spf1p has no effect on Zn^{2+} accumulation [111].

5.1.3. Could Spf1p be a Mg^{2+} pump?

As discussed above, Mg^{2+} is the only cation required to stimulate ATP hydrolytic activity of Spf1p [119] and phosphoenzyme formation in the plant P5A-ATPase HvP5A1 [120]. All P-type ATPases require Mg^{2+} for proper ATP coordination and phosphorylation of the conserved Asp in the P-domain [11]. The kinetics of Mg^{2+} dependence on the above reactions [119,120] in both cases seems to be similar to that of other P-ATPases where Mg^{2+} acts as a cofactor. Although it cannot be ruled out that in P5A-ATPases an additional role of Mg^{2+} is to serve as a transported ligand, so far no available evidence support this conclusion.

5.2. Could Spf1 be an ER lipid flippase?

As an alternative hypothesis to Spf1p action it has been suggested that it flips a lipid species or any other organic molecule across the ER

membrane bilayer, in analogy with the phospholipid flipping P4-ATPases, that among P-type ATPases are phylogenetically most closely related to P5-ATPases [124]. The ER is the only membrane system of the secretory pathway in which lipid flippases have not been characterized. In other membrane systems of the cell the activity of P4-ATPases cause local imbalances in the number of phospholipids on each side of the membrane, and such an activity has been hypothesized to provoke a deformation of the membrane that can be stabilized by other proteins and constitute the initial formation of membrane vesicles [124,125].

Should Spf1p catalyse a flippase activity its role may not be restricted to generation of membrane vesicles. It has been hypothetized that the activities of lipid flippases in the ER are required to provide building blocks for the synthesis of *N*-linked oligosaccharides or GPI anchors [126–128]. *N*-linked glycosylation and other processes that involve carbohydrate modifications of proteins take place in the lumen of the ER and the Golgi. The carbohydrate precursors are glycolipids that likewise are integrated in the luminal leaflet of the membranes of the ER. However, biosynthesis of these glycolipid precursors is initiated by enzymes in the cytosol that only work on a substrate on the cytosolic leaflet of the ER. This implies that at some stage of their biosynthesis, glycolipid intermediates have to traverse the ER membrane by moving from one leaflet to the other (a process termed flipping).

At least three lipids required for carbohydrate modification of proteins have been hypothesized to flip across ER membranes [128]: i) $Man_5GlcNAc_2$-PP-dolichol (M5-DLO); ii) glucosaminyl-acyl-PI (GlcN-acyl-PI or GPI); and iii) mannose-phosphate-dolichol (MPD). The enzymes carrying out these flippase reactions have only been poorly characterized and no genes encoding such flippases have been identified. However, from reconstitution experiments it appears that MPD, M5-DLO and glycerophospholipid flipping are independent activities that require distinct proteins in the ER membrane [128].

There are at least two arguments against the role of Spf1p in the above described flippase activities. First, all of the mentioned flippase processes appear to be ATP independent [128] whereas Spf1p hydrolyzes ATP [119]. Second, in most eukaryotic organisms, only a single ER Spf1p homologue is present [120]. This appears to be incompatible with a specific role in at least three distinct processes. Still, we cannot rule out the possibility that Spf1p catalyzes a lipid flippase activity in the ER, which is distinct from the three mentioned above, but required in some way for vesicle generation, lipid recycling, and/or lipid distribution in the ER.

6. Major conclusions

The pleiotropic phenotype of *spf1* cells indicate that loss of its function leads to impairment of basic ER processes such as protein folding and processing. In the plant Arabidopsis thaliana, disruption of the single P5A-ATPase gene, *AtP5A1/MIA/PDR2*, also correlates with sensitizing of a subset of ER quality control responses [10,29]. Further, the knockout of *AtP5A1/MIA/PDR2* results in mutant plants with decreased fertility due to collapsed pollen grains [10], as well as inability to maintain root stem cells during limiting Pi conditions [29]. Whether this effect can be attributed to a disturbed ER equilibrium is not known.

A putative role of Spf1p in the vesicular communication between the Golgi and the ER is evident from the analysis of genetic interactions above. An important role of Spf1p in this route could explain the Ca^{2+} phenotype *spf1* in combination with *pmr1*, could explain defects in folding, sorting, glycosylation, and ergosterol distribution, and maybe, as a consequence of ergosterol accumulation in the ER, explain how it plays a role in TA-protein insertion/localization.

So why would isolated P5A-ATPase protein spontaneously hydrolyse ATP, apparently in the absence of any obvious ligand, and what would then be the transport substrate of P5A-ATPases?

First, the spontaneous formation of the phosphoenzyme corresponds to the biochemical behavior of other P-type ATPases, which do not require binding of a transport substrate to carry out pump phosphorylation. These include the phospholipid flipping P4-ATPases [129] and some heavy metal pumping P1B-1 ATPases [130]. Second, it remains possible that the coupling mechanism, which is required for communication between the transport binding site in the membrane and the nucleotide binding pocket in the cytoplasmic P domain, is lost during purification resulting in uncoupling of the pump.

It remains a possibility, however, that a substrate is already present in the reported enzyme preparations. Should this be the case, the substrate likely constitutes a component associated with the membrane itself. This could be any organic molecule, such as a lipid or small peptide fragment. The accumulation of P5A-ATPase in the E1P state would at least indicate that the substrate in the membrane preparations is limiting or that other regulatory factors are needed to activate phosphoenzyme turnover.

7. General significance

It is our hope that this review will create ideas for further studies aiming at elucidating the role of P5A-ATPases, a mysterious group of primary active pumps that still have no assigned function despite the fact that they are ubiquitously present in eukaryotic and are likely to carry out an important basic process in the machinery of the cell.

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