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Functional expression of heterologous proteins in yeast: insights into Ca^{2+} signaling and Ca^{2+}-transporting ATPases

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Ton, Van-Khue, and Rajini Rao. Functional expression of heterologous proteins in yeast: insights into Ca^{2+} signaling and Ca^{2+}-transporting ATPases. *Am J Physiol Cell Physiol* 287: C580–C589, 2004; 10.1152/ajpcell.00135.2004.— The baker's yeast *Saccharomyces cerevisiae* is a well-developed, versatile, and widely used model organism. It offers a compact and fully sequenced genome, tractable genetics, simple and inexpensive culturing conditions, and, importantly, a conservation of basic cellular machinery and signal transducing pathways with higher eukaryotes. In this review, we describe recent technical advances in the heterologous expression of proteins in yeast and illustrate their application to the study of the Ca^{2+} homeostasis machinery, with particular emphasis on Ca^{2+}-transporting ATPases. Putative Ca^{2+}-ATPases in the newly sequenced genomes of organisms such as parasites, plants, and vertebrates have been investigated by functional complementation of an engineered yeast strain lacking endogenous Ca^{2+} pumps. High-throughput screens of mutant phenotypes to identify side chains critical for ion transport and selectivity have facilitated structure-function analysis, and genomewide approaches may be used to dissect cellular pathways involved in Ca^{2+} transport and trafficking. The utility of the yeast system is demonstrated by rapid advances in the study of the emerging family of Golgi/secretory pathway Ca^{2+},Mn^{2+}-ATPases (SPCA). Functional expression of human SPCA1 in yeast has provided insight into the physiology, novel biochemical characteristics, and subcellular localization of this pump. Haploinsufficiency of SPCA1 leads to Hailey-Hailey disease (HDD), a debilitating blistering disorder of the skin. Missense mutations, identified in patients with HHD, may be conveniently assessed in yeast for loss-of-function phenotypes associated with the disease.

Saccharomyces cerevisiae; calcium ion; transporters; functional complementation

WHY YEAST?

In 1997, the haploid budding yeast *Saccharomyces cerevisiae* became the first eukaryotic organism with a completely sequenced genome. It has ~6,200 genes organized into a compact genome, essentially free of introns. These genes and their corresponding functions have been extensively annotated and catalogued in the Saccharomyces Genome Database (SGD; Stanford University, Stanford, CA) available on line (http://www.yeastgenome.org). Ninety-five percent of the yeast genome has been systematically knocked out, and the individual knockout mutants or entire collections of viable haploids (~70% of the genome), as well as the homozygous and heterozygous diploids, are commercially available for a nominal price from the American Type Culture Collection (ATCC), Research Genetics (Invitrogen), or European *Saccharomyces cerevisiae* Archives for Functional Analysis (EUROSCARF). The compact genome of yeast, combined with an ease of molecular and genetic manipulation, has made it relatively easy to perform large-scale screens for drug targets or interacting pathways. There are a growing number of online databases that catalog the results from these studies and provide an increasingly comprehensive picture of the transcriptome, proteome, or phenome. These include results from genomewide microarray studies (http://transcriptome.ens.fr/ymgv/index.php), networks of protein interactors [General Repository of Interaction Datasets (GRID) (http://biodata.mshri.on.ca/grid/servlet/Index)], and phenotypes of knockout mutants (SGD). The phenotypes range in diversity, including growth sensitivity to drugs or environmental stresses, loss of mitochondria and "petite" cell formation, and cell cycle arrest. The availability of a versatile toolkit of promoters, selectable markers, and designer strains facilitates heterologous protein expression in yeast. Thus expression levels may be controlled with *1*) promoters of varying and inducible strength; *2*) a choice of host strains, including deletion mutants of orthologous genes; and *3*) designer strains specifically tailored to eliminate functional homologs, stabilize proteolysis-susceptible proteins, or enrich for specific organelles. This review highlights the utility of the yeast model system, with emphasis on the study of Ca^{2+} transporters.

YEAST AS A MODEL FOR EUKARYOTIC CALCIUM HOMEOSTASIS

Ca^{2+} signaling cascades that have been well studied in yeast include the response to mating pheromone (38, 59), salt and environmental stresses (11, 54), nutrient sensing (1, 82), and the cell integrity response (32). These studies have shown that mechanisms of Ca^{2+} homeostasis and signaling in yeast are

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fundamentally similar to those in higher eukaryotes. The essential components of the cellular Ca^{2+} signaling machinery, or "calciome" as we name it, are conserved, including Ca^{2+} channels and transporters, Ca^{2+} sensors, and signal transducers. A major difference is that yeast lacks the redundancy of multiple isoforms and the complexity of splice variants that are characteristic of mammalian cells. Instead, there are a defined number of genes that may be deleted individually or in combination, with predictable effects. Figure 1 summarizes key pathways and proteins involved in Ca^{2+} entry, signaling, and exit from the cytosol.

Ca^{2+} channels. At the plasma membrane, high-affinity Ca^{2+} influx is mediated by a complex consisting of Cch1, a homolog of the mammalian voltage-gated α_1-subunit of Ca^{2+} channels, and Mid1, a putative stretch-activated Ca^{2+} channel (29, 37, 64). These channels have been implicated in Ca^{2+} entry associated with exposure to mating pheromone, endoplasmic reticulum (ER) stress, and depletion of Ca^{2+} stores in the ER/secretory pathway (a response similar to capacitative Ca^{2+} entry; Ref. 10). Calcineurin is known to inhibit channel activity, possibly through direct dephosphorylation. There is experimental evidence for at least one other Ca^{2+} influx pathway that remains to be identified, postulated to mediate low-affinity Ca^{2+} influx and cell-cell fusion during the late phase of the mating process (58). In yeast, the vacuole serves as a major store for Ca^{2+}, for the purposes of both detoxification and signaling. A homolog of the mammalian transient receptor potential (TRP)-like channel, Yvc1, has been shown to release Ca^{2+} from the vacuole into the cytosol in response to hyperosmotic shock and the antiarrhythmic drug amiodarone (33, 65, 98). Both mechanical activation of Yvc1 and Ca^{2+}-induced Ca^{2+} release have been reported in response to osmotic upshock (98). Interestingly, yeast inositol 1,4,5-trisphosphate (IP_3) receptor channels have not been identified, correlating with low levels of IP_3 in yeast (see *Ca^{2+} signaling pathways*), and it is not known how Ca^{2+} is released, if at all, from the ER/Golgi stores.

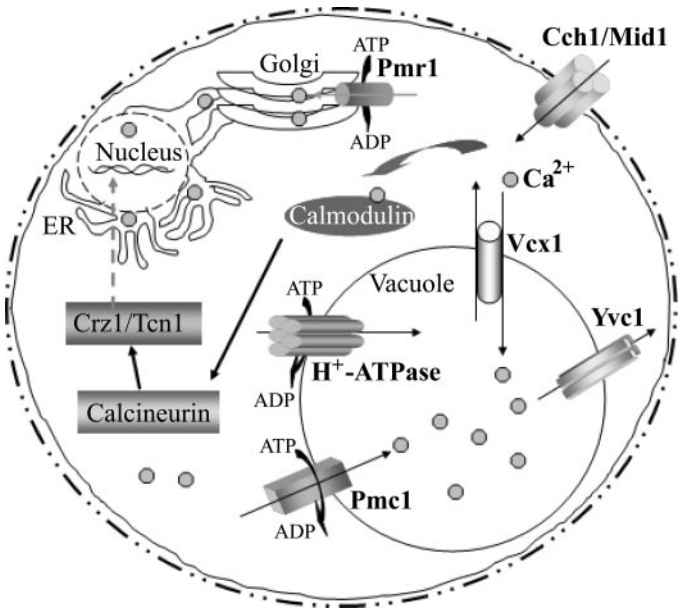

Fig. 1. Ca^{2+} signaling and transport pathways in *Saccharomyces cerevisiae*. Ca^{2+} enters the cytosol via the plasma membrane channel complex Cch1/Mid1 or the vacuolar transient receptor potential-like channel Yvc1 in response to diverse environmental cues such as endoplasmic reticulum (ER) stress, osmotic shock, or mating pheromone. The molecular pathways leading from Ca^{2+} activation of calmodulin have been well characterized. Ca^{2+} and calmodulin activate the protein phosphatase calcineurin, which in turn activates the transcription factor Crz1/Tcn1, leading to its nuclear translocation and the transcription of target genes. Ca^{2+} is primarily cleared from the cytosol by P-type ATPases in the Golgi (Pmr1) and vacuole (Pmc1), and by the vacuolar H^+/Ca^{2+} exchanger Vcx1.

Ca^{2+} pumps and transporters. In yeast and other eukaryotes, active transport of Ca^{2+} from the cytosol to intracellular stores or extracellular space occurs by ATP-driven pumps and cation-coupled exchangers. In metazoans, the well-known sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCA) sequester Ca^{2+} into the ER and play a prominent role in cytosolic Ca^{2+} homeostasis. Although there are no SERCA homologs in *S. cerevisiae*, Ca^{2+} levels in the ER are controlled by a Golgi-localized pump, Pmr1, presumably while en route to the Golgi (76). Yeast Pmr1 (for "plasma membrane ATPase related") was the first member of the family of secretory pathway Ca^{2+}-ATPases (SPCA) to be identified (71). It mediates high-affinity Ca^{2+} (and Mn^{2+}) transport under normal physiological conditions and serves a dual role in maintaining cytosolic ion homeostasis as well as in supplying the Golgi lumen with Ca^{2+} and Mn^{2+} for protein sorting, processing, and glycosylation (25). Pmr1 homologs are now known to be ubiquitous in all higher eukaryotes, with the notable exception of plants, and much of what we know about the structure and physiological role of these transporters comes from studies in yeast. More recently, these studies have extended to SPCA homologs from humans (5, 27, 83), *Caenorhabditis elegans* (13, 87), and rats (Ref. 69; reviewed in Ref. 93, 94). A more detailed review of insights into this novel class of Ca^{2+} pumps is presented later.

Plasma membrane Ca^{2+}-ATPases (PMCA) constitute a third branch of this superfamily of related pumps, represented in yeast by Pmc1. Although clearly related in sequence to the PMCA family, yeast Pmc1 lacks domains involved in regulation by calmodulin and acidic phospholipids typical of the mammalian members and localizes to the vacuolar membrane instead of the cell surface. However, detoxification of Ca^{2+} by vacuolar sequestration is topologically equivalent to transport into the extracellular space, and Pmc1-like homologs are commonly found in the acidic vacuoles of other fungi and protozoans. Transcriptional activation of *PMC1* occurs in the presence of high extracellular Ca^{2+} (19) or in the absence of Pmr1 (53) and is dependent on calcineurin. Because calcineurin-dependent transcriptional activation is a process conserved across kingdoms from fungi to mammals, it will be of interest to determine whether compensatory changes in expression levels of mammalian Ca^{2+} pumps and exchangers also occur by similar pathways.

Another major player in vacuolar Ca^{2+} sequestration is Vcx1, a Ca^{2+}/H^+ exchanger driven by the proton electrochemical gradient set up by the vacuolar H^+-ATPase (20, 55, 67). Vcx1 activity is apparently inhibited by calcineurin (20). Thus viability of a double null mutant of *PMC1* and *PMR1* can be improved by the additional deletion of the regulatory subunit of calcineurin (*CNB1*), resulting in upregulation of Vcx1 activity. The resulting triple deletion strain is devoid of endogenous Ca^{2+}-ATPases and serves as an ideal host for the heterologous expression of diverse Ca^{2+} pumps, as discussed in more detail later in this review. Mutants lacking one or more of the Ca^{2+}

transporters can also be used for assessing the contribution of different pathways involved in cellular Ca^{2+} homeostasis (33, 43).

Ca^{2+} signaling pathways. Many downstream transcriptional and translational events in yeast are controlled by the Ca^{2+}-mediated activation of calmodulin (CaM). In yeast, as in other organisms, calmodulin is essential for life (21). A yeast proteome microarray of 5,800 purified proteins has identified 6 known and 33 additional potential binding partners for calmodulin, consistent with a role for this essential protein in diverse cellular processes (99). Calmodulin participates in Ca^{2+}-dependent stress response pathways through activation of yeast CaM kinases Cmk1 and Cmk2 (57) and the phosphatase calcineurin (22). Dephosphorylation by calcineurin results in nuclear translocation of Crz1/Tcn1, analogous to mammalian NFATc, and transcriptional activation of more than 160 target genes involved in cell wall and lipid synthesis, ion and small molecule transport, vesicle trafficking, and other signaling proteins (22). Like the prototypic calmodulin, there are other EF-hand-containing Ca^{2+} regulatory proteins in yeast. Yeast frequenin (Frq1), a highly conserved ortholog of the neuronal Ca^{2+} sensor-1, functions as the Ca^{2+}-sensing subunit of Pik1, a phosphatidylinositol 4-kinase that is essential for vesicular trafficking in the late secretory pathway (75). Addition of glucose to starved cells leads to a cytosolic Ca^{2+} response within minutes that appears to be mediated by a phosphoinositide (PI)-specific phospholipase C via G protein-coupled receptors (GPCR) (80). In mammalian cells, PI turnover leads to activation of protein kinase C by diacylglycerol and intracellular Ca^{2+} release by IP_3. Curiously, in yeast, IP_3 appears to be rapidly converted to polyphosphates (IP_4, IP_5, and IP_6), and although there are no known IP_3 receptor channels, genetic disruption of IP_3 kinases enhances the Ca^{2+} signal (81).

Ca^{2+} and cell death. In keeping with its diverse cellular functions, the role of Ca^{2+} in apoptosis is like a double-edged sword. Disruption of Ca^{2+} homeostasis is a well-established signal for programmed cell death in mammalian cells, and many apoptotic effectors such as calcineurin and calpains show Ca^{2+}-dependent activation (39, 88). Prolonged cytosolic Ca^{2+} overload and/or continuous Ca^{2+} influx triggered by depletion of the ER Ca^{2+} pool can cause massive Ca^{2+} uptake into the mitochondrial matrix. This large increase in Ca^{2+} level then triggers the opening of the permeability transition pore on the outer mitochondrial membrane, resulting in the release of cytochrome *c* and proapoptotic factors into the cytosol. Conversely, Ca^{2+} also can function as an antiapoptotic signal in neurons (30, 34, 63). Although death by apoptosis is widely considered unique to metazoans, there is new evidence that unicellular organisms, including *S. cerevisiae*, also undergo programmed cell death in response to diverse insults. Not surprisingly, as in mammalian cells, Ca^{2+} can influence this process in yeast. Thus calcineurin-dependent Ca^{2+} influx in response to ER stress protects against cell death (11), whereas the novel antifungal agent amiodarone triggers massive Ca^{2+} influx from external and internal stores, leading to the appearance of apoptotic markers and, eventually, cell death (33) (unpublished observation). Markers of programmed cell death in yeast are remarkably similar to those in mammalian cells; they include loss of membrane asymmetry and phosphatidylserine externalization, reactive oxygen species generation, DNA fragmentation, loss of mitochondrial membrane potential, and cytochrome *c* release from mitochondria (50). However, the yeast apoptotic pathway appears to be less sophisticated than that of metazoans. Recently, the yeast system has been successfully exploited to study mammalian proteins involved in apoptosis, such as screening for Bax inhibitors (15) or characterization of the interaction between the proapoptotic Bax and Bid with the mitochondria (68). Given our thorough knowledge of yeast Ca^{2+} homeostasis, a genetic dissection of Ca^{2+}-mediated apoptotic pathways is now feasible.

In summary, despite some obvious differences, an overall conservation in Ca^{2+} signaling pathways and in Ca^{2+} transporters with higher eukaryotes makes yeast a simple and elegant experimental model system.

HETEROLOGOUS EXPRESSION IN YEAST

The art of heterologous protein expression in *S. cerevisiae* has been refined over many years, beginning in the 1980s. As a microbial expression system, *Saccharomyces* offers more advantages than *Escherichia coli*, since exogenous proteins may be targeted to membrane-bound compartments that are readily isolated for biochemical experiments (23, 78, 83). It also surpasses cultured insect and mammalian cell lines with simple and inexpensive growth conditions and a shorter cell cycle. High-throughput screens based on genetic features other than functional complementation are also feasible. For example, the ability to form white and red colonies on rich medium, which depends respectively on the presence or absence of *ADE1* and/or *ADE2*, genes encoding enzymes in the adenine-biosynthesis pathway, was the basis of a screen for mammalian prion inhibitors (4) and for truncation mutations in human genes (41). Other screens rely on pathway conservations between yeast and mammals. The yeast pheromone-sensing pathway is a GPCR transduction system whose homologs are abundantly represented in mammals. It is thus possible to engineer human GPCRs, particularly orphan receptors, into yeast devoid of background GPCR for high-throughput screens (17, 91).

Promoters and plasmids. One advantage of the yeast system is the availability of strong and constitutive promoters that drive the expression of proteins such as plasma membrane H^+-ATPase (*PMA1*), glyceraldehyde-3-phosphate dehydrogenase (*GPD*), phosphoglycerate kinase-1 (*PGK1*), alcohol dehydrogenase-1 (*ADH1*), and pleiotropic drug-resistant pump (*PDR5*). Of these, the *PMA1* and *PDR5* promoters are exceptionally strong and can direct high-level expression reaching up to 10% of plasma membrane proteins. In the presence of the mutant allele *pdr1–3*, encoding a constitutively active transcription factor, *PDR5* is even more heavily transcribed (61). Besides strong promoters, inducible promoters allow carefully timed expression of proteins, especially those whose presence is toxic to cell growth. These promoters include *GAL1–10* (induced by galactose), *PHO5* (induced by low extracellular inorganic phosphate), and tandem heat shock *HSE* elements (induced by temperature elevation to 37°C) (57). It is worth noting that the *GAL* promoters are also exceptionally useful to turn off gene expression following a transfer to glucose medium. Promoters that direct variable expression in response to a titratable inducer include the methionine-responsive *MET3* and *MET25* promoters and copper-dependent *CUP1* promoters. Any of these promoters may be cloned into multicopy (2μ)

or single copy (*CEN*) plasmids to give an additional level of control in expression level. The plasmids carry nutritional markers (*URA3*, *ADE3*, *HIS1*, and others) for selection in yeast and antibiotic resistance (*AMP*) for propagation in bacteria. After amplification in bacteria, plasmids are introduced into the corresponding yeast auxotrophs by methods essentially similar to bacterial transformation and can be stably maintained in dropout media.

Host strains. Not only individual genes but also members of an entire gene family, as well as the regulators that control their expression, may be systematically deleted with relative ease in yeast. This allows heterologous expression in a well-controlled system devoid of background contamination from endogenous proteins of similar function. For example, seven pleiotropic drug-resistant transporters, *YOR1*, *SNQ2*, *PDR5*, *YCF1*, *PDR10*, *PDR11*, and *PDR15*, together with their activating transcription factors, *PDR1* and *PDR3*, have been simultaneously deleted, rendering the resultant strain exquisitely sensitive to drugs (70). Mutants that change lipid composition of the plasma membrane, such as the *erg6* mutant defective in ergosterol biosynthesis, also sensitize yeast to a variety of drugs, presumably by increasing the rate of passive drug diffusion (26). Such mutants are extremely valuable in studies involving heterologous expression of multidrug resistance (MDR) proteins from human or pathogenic microorganisms and in screens for mutations conferring drug resistance. In another example, up to 18 hexose transporters responsible for glucose uptake have been knocked out (90), enabling scientists to understand the sugar-transporting network in yeast and to express any putative sugar transporter from other organisms to investigate its functions. Similarly, our laboratory has pioneered the application of a yeast triple mutant lacking the two Ca^{2+} pumps *PMR1* and *PMC1* as well as the regulatory subunit of calcineurin, *CNB1*, as host for the unambiguous biochemical analysis of plasmid-encoded Ca^{2+} pumps (74). Multiple gene deletion in yeast mostly relies on the efficiency of homologous recombination to target chromosomal loci and on the subsequent crossing of various mutants to produce the desired combination. As a result, it is possible to create an entirely new "designer strain" that serves almost any experimental need, providing that simultaneous deletion of many genes does not kill the cell.

Proteins that are highly sensitive to proteolysis can be expressed in a strain lacking the master vacuolar endopeptidase Pep4 (44), which controls the activation of other vacuolar hydrolases (92). Heterologous expression in strains carrying temperature-sensitive (*ts*) alleles of genes can be employed if the corresponding null mutant is inviable. In an innovative example of this approach, a strain containing the *ts* allele of *SEC6*, *sec6–4*, was exploited to enrich for secretory vesicles during a 2-h temperature shift to 37°C (60). The accumulated vesicles can be readily isolated in good yield and serve as a source of homogenously oriented right-side-out vesicles for H^+ transport assays. This strategy has been adapted for the expression of a variety of transport proteins in yeast, including mammalian aquaporins (18), Na^+ glucose symporter (28), multidrug transporters, and other members of the ABC superfamily (72). A similar approach could be used to enrich membranes of the ER or Golgi or to enlarge the prevacuolar compartment. There exist a multitude of mutants blocked at different, discrete steps in the secretory or endocytic pathways. For example, mutants showing defects in exit from ER (*sec18^{ts}*), Golgi (*sec7^{ts}*), or prevacuolar compartment/late endosome (Δ*vps27*) or in endocytosis (Δ*end4*) may be used to follow the biosynthetic pathway of a target protein from biogenesis to degradation.

Functional complementation. As soon as automated sequencing became cheaper and more reliable, in the late 1990s we witnessed an explosion of genome sequencing projects, resulting in the discovery of novel genes of unknown function. This is where the *S. cerevisiae* system demonstrates its most powerful feature, functional complementation: essentially the restoration to wild-type phenotypes by *YFG* (your favorite gene) in a yeast strain lacking the putative homolog. Functional complementation is also of considerable pharmaceutical interest because it allows cell-based high-throughput screens for natural or synthetic ligands, specifically small-molecule inhibitors. Another, often overlooked, advantage of functional complementation is that mutations in *YFG* may be rapidly screened for loss-of-function phenotypes in structure-function analysis. This serves to prioritize the more difficult biochemical studies by screening out mutations that have little or no effect on the biological function of the protein. Such screens are significantly harder or impossible to perform in other heterologous expression systems because of longer cell cycles, complicated growth conditions, and/or endogenous background activities. Figure 2 summarizes the process from identification of a novel gene to the use of yeast for complementation studies and beyond. When a genome sequencing or chromosomal mapping project yields a novel gene *YFG*, the first step is to search for putative homologs whose functions have been studied and/or documented in databases, in this case, the SGD. Once a putative homolog is identified on the basis of sequence similarity, *YFG* can be cloned into a yeast expression vector and transformed into the strain with a deletion in the corresponding yeast gene (Δ*scyfg*). Multiple homologs also may be deleted by homologous recombination. Most likely, Δ*scyfg* has been extensively characterized and its phenotypes in different growth conditions (nutrients, drugs, salts, or temperature) documented in the SGD. Alternatively, if *ScYFG* is essential, one of two strategies may be employed to replace it with the heterologous gene. The promoter of *ScYFG* may be replaced by the repressible *GAL1* promoter by homologous recombination. The resulting strain is viable on galactose medium and will not grow upon shift to glucose medium unless functional complementation is achieved by plasmid-encoded *YFG*. Another option is a plasmid shuffling technique that selects for loss of *ScYFG* from a *URA3* plasmid in medium supplemented with 5-fluoroortic acid (5-FOA), a toxic analog of uracil that is lethal to yeast only in the presence of the *URA3* gene. Again, viability is maintained if *YFG* can successfully replace the essential yeast gene. Functional complementation yields the first experimental clue to the biological role of a novel gene, which can be subsequently confirmed by biochemical characterization. It is important to note that there need not be sequence similarity between *YFG* and its yeast counterpart for functional complementation to be applied successfully. For example, the K^+ uptake defect of the yeast *trk1trk2* deletion mutant prevents growth in K^+-limited medium. Functional complementation of this growth limitation has been successfully exploited for expression cloning, mutagenesis, and traf-

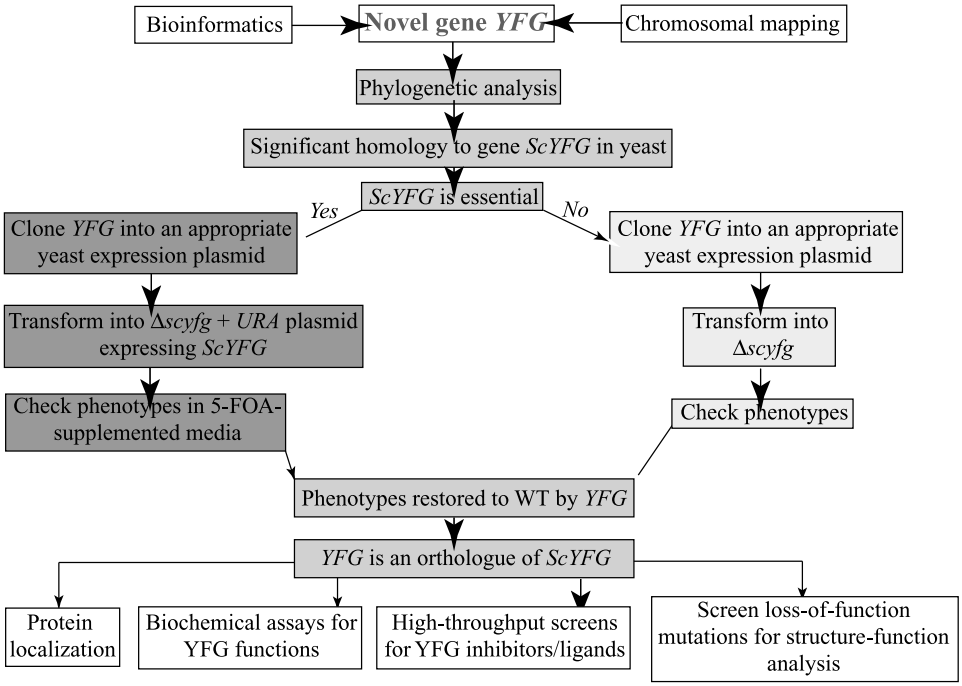

Fig. 2. Strategies in heterologous expression and characterization of a novel gene in yeast. The flow chart outlines steps leading from the identification of your favorite gene (*YFG*) to heterologous expression in yeast and subsequent functional studies. WT, wild type. See text for details.

ficking studies of a variety of unrelated K^+ channels from plants and animals (2, 6, 79).

In laboratories around the world, many types of genes from bacteria (56), protozoa (7, 12, 31), fungi (100), plants (48), and animals (83) have been expressed in and shown to complement deletion phenotypes of yeast mutants. The references given here are a very few examples of the vast number of articles published on the subject of functional complementation studies in yeast.

SECRETORY PATHWAY Ca^{2+}-ATPASE DEFECTIVE IN HAILEY-HAILEY DISEASE: LESSONS FROM YEAST

Research from several laboratories on the emerging family of Golgi/secretory pathway Ca^{2+},Mn^{2+}-ATPases, as well as on putative members of the SERCA and PMCA families, illustrate the utility of yeast in predicting the cellular role and mechanism of a novel protein.

Identification of ATP2C1 as a novel gene. Hailey-Hailey Disease (HHD), or benign familial pemphigus, is an intraepidermal blistering disorder characterized by acantholysis and impaired keratinocyte adhesion. Inherited as an autosomal dominant disease, the underlying cellular mechanism of HHD had eluded scientists for decades until simultaneous reports from two groups showed that affected individuals had mutations mapping to a novel gene, designated *ATP2C1* (36, 77). Sequence analysis revealed that the gene product of *ATP2C1* shared high identity (49%) with yeast Pmr1, the Golgi/secretory pathway Ca^{2+},Mn^{2+}-ATPase, hence the name (human) hSPCA1. Up to this point, Pmr1 was the only SPCA member to be extensively characterized. Inactivation of *PMR1* resulted in missorting and misprocessing of proteins along the secretory pathway and to N- and O-linked glycosylation defects (3, 25). These observations were consistent with depletion of luminal Golgi stores and could be separately corrected by high levels of extracellular Ca^{2+} and Mn^{2+}, respectively (25). In addition, *pmr1* mutants have elevated resting levels of Ca^{2+} in the cytosol and cannot effectively maintain cellular Ca^{2+} and Mn^{2+} homeostasis (33, 35, 62). These phenotypes are strikingly similar to observations in keratinocytes isolated from HHD patients that show higher levels of resting cytosolic Ca^{2+}, defects in recovery from a Ca^{2+} load (36), and significant decreases in luminal Ca^{2+} (5). Thus the molecular defects in HHD may be linked to either insufficient Ca^{2+} and Mn^{2+} in the Golgi or defective cytosolic Ca^{2+} clearance, or more likely, both. Inadequate glycosylation or processing of desmosomal proteins during transit through the Golgi may lead to defective cell surface adhesion. It is also known that desmosomal assembly and turnover are exquisitely dependent on Ca^{2+} such that alterations in cytosolic Ca^{2+} signaling might lead to protein internalization from plasma membrane and desmosome disruption (45). It is interesting to note that Darier disease, a related blistering disorder also characterized by loss of keratinocyte adhesion, is caused by mutations in the SERCA2 Ca^{2+} pump and, like HHD, shows autosomal dominant inheritance (73).

Putting ATP2C1 into yeast. The gene *ATP2C1* was cloned into a multicopy yeast expression vector carrying a selectable auxotrophic marker (*URA3*), under control of the promoter for phosphoglycerate kinase-1 (*PGK1*) and an NH_2-terminal His_9 epitope tag. The choice of a promoter of moderate strength was based on our earlier unpublished observation that constitutive high-level expression of yeast *PMR1* from the very strong *PMA1* promoter was lethal. The *ATP2C1* plasmid was transformed into the triple null mutant strain K616 (Δ*pmr1*Δ*pmc1*Δ*cnb1*), lacking endogenous Ca^{2+} pumps. Of the many phenotypes of *pmr1* null mutants reported, the most useful are hypersensitivity to growth in medium supplemented with BAPTA or Mn^{2+}, as we have shown that these correlate well with Ca^{2+} and Mn^{2+} transport activity, respectively. Chelation of extracellular divalent cations by BAPTA (1–2 mM) starves the secretory path-

way (most likely, the ER) of Ca^{2+} and prevents yeast growth. Expression of a high-affinity Ca^{2+} pump rescues the cells from Ca^{2+} starvation. It was observed that hSPCA1 fully complemented the BAPTA hypersensitivity of K616, as did heterologous expression of mammalian PMCA and SERCA pumps (83). Mn^{2+} hypersensitivity of the *pmr1* mutant results from the toxicity of excessive cellular Mn^{2+}; for example, Mn^{2+} is known to increase the error rate of DNA polymerases and to interfere with Mg^{2+}-binding enzymes (9). Expression of a high-affinity Mn^{2+} pump effectively compartmentalizes the toxic ion and rescues cell growth. Interestingly, hSPCA1, but not rabbit SERCA1 or human PMCA4, was seen to complement the Mn^{2+}-sensitive growth defect of the *pmr1* strain (83).

Earlier studies demonstrated a virtual absence of ATP-dependent, protonophore-insensitive $^{45}Ca^{2+}$ transport activity in Golgi vesicles purified from K616 (74); this allowed the unambiguous biochemical characterization of hSPCA1 transport activity in yeast. Similar to yeast Pmr1, hSPCA1 transported Ca^{2+} with high affinity (K_m = 0.2 μM) and was insensitive to inhibition by thapsigargin, the diagnostic inhibitor of SERCA. Mn^{2+} was an effective inhibitor of hSPCA1 but only poorly inhibited $^{45}Ca^{2+}$ transport activity of SERCA and PMCA. In conjunction with the Mn^{2+}-tolerant phenotypes described above, these data suggest that high-affinity Mn^{2+} transport is a unique property of the secretory pathway pumps. Green fluorescent protein (GFP)-tagged hSPCA1 localized to the Golgi in yeast, demonstrating the elegant simplicity yet effectiveness of functional complementation (83). Taken together, these data led us to conclude that hSPCA1 is a functional ortholog of yeast Pmr1.

Mutagenic analyses in yeast and humans. Not only is functional complementation powerful in predicting function, it is also useful in investigating how mutations affect activity. With *PMR1* itself, a large number of site-directed mutations were made and rapidly screened for hypersensitivity to BAPTA and Mn^{2+} (51, 89). Liquid growth assays in a 96-well format are amenable to testing a range of inhibitor concentrations, and growth can be monitored by using a microplate reader for high-throughput screening of mutants. Phenotype screening of Pmr1 mutants revealed residues that are critical for ion selectivity and transport in transmembrane helices M4, M5, and M6 (51, 52). Subsequent interpretation of these results in light of recent high-resolution crystal structures of the rabbit SERCA1 pump in two different conformations (84, 85) shows that side chains sensitive to substitution project into the transmembrane transport pathway. Ion selectivity mutants Q783A in M6 and V335Q in M4 appear to alter conformation-sensitive helix packing at the membrane interface (51). In summary, the results from phenotype screening of mutants are remarkably consistent with the crystal structure and constitute experimental validation of the homology models.

Figure 3 depicts the location of missense and nonsense mutations in hSPCA1 identified in HHD patients (16, 24, 36, 47, 77, 96). Although chain termination mutations are scattered throughout the length of the polypeptide, the missense mutations are strikingly clustered in regions of the proteins that are critical for function (Fig. 3*A*). These include membrane helices M4, M5, and M6, which constitute the Ca^{2+} transport pathway (Fig. 3, *B* and *C*), and the major cytoplasmic loop, which constitutes the phosphorylation and nucleotide binding domains. The carboxylic oxygen of D742 directly coordinates Ca^{2+} and has been shown to be essential for ion binding and transport in Pmr1 (52). Mutation of A304 (M4) is also likely to disrupt ion binding because the backbone carbonyl at this location coordinates Ca^{2+} in SERCA (84).

A subset of HHD mutations was introduced into hSPCA1 and assessed for functional complementation of BAPTA- and Mn^{2+}-sensitive phenotypes in K616. Mutants L341P, C411R, T570I, T709M, and D742Y failed to correct the *pmr1* growth defects (unpublished observations), consistent with the disease phenotype in humans. Surprisingly, mutant P201L fully complemented the *pmr1* null phenotypes and was indistinguishable from the wild-type hSPCA1, suggesting that P201L mutation does not disrupt pump function. Although it remains a possibility that small decreases in activity might have been masked by overexpression of the mutant, a recent biochemical analysis of this mutant expressed in COS-1 cells also failed to reveal any defects in formation of the catalytic phosphoenzyme intermediate or in subcellular localization (27). Taken together, data from yeast and mammalian cells suggest that patients with P201L harbor additional disease-causing mutations in introns or promoter regions of *ATPC1*, as suggested by Li et al. (47). Missense HHD mutations transformed into wild-type yeast did not confer hypersensitivity to BAPTA or Mn^{2+}. Therefore, we concluded that these mutants do not exert a dominant negative effect on the endogenous Pmr1 pump. Given the significant homology between hSPCA1 and Pmr1, an extension of our findings to humans would suggest that autosomal dominant inheritance of HHD likely results from haploinsufficiency of *ATP2C1* and not from a dominant negative interaction with the wild-type allele.

Other Ca^{2+} pumps in yeast. The rapid accumulation of complete genome sequences has led to an urgent need for functional data. Functional complementation in *S. cerevisiae* is the method of choice for characterization of novel genes from organisms ranging from plants to protozoans and mammals. Many *PMR1* homologs from other yeasts (40, 66, 86) were found to complement the *S. cerevisiae pmr1* mutant. In addition, newly discovered SERCA- and PMCA-type Ca^{2+} pumps from diverse organisms also restore wild-type phenotypes in yeast lacking endogenous pumps and have been biochemically characterized (23, 31, 48, 49, 78). Heterologous expression of rabbit SERCA1 in yeast has been used to produce protein for biochemical analysis of site-directed mutants (46).

CHALLENGES IN THE YEAST MODEL

Despite the advantages we describe, there remain experimental challenges to heterologous protein expression in *S. cerevisiae*. One difficulty is that protein glycosylation in yeast is different from that in mammalian cells, so in cases where glycosylation is important for function, this presents a problem. In addition, the biosynthetic quality control machinery in *Saccharomyces* is fairly stringent, so exogenous proteins may be retained in the ER, possibly due to delays or difficulties in achieving correct folding. Activation of the unfolded protein response and proteosome-mediated degradation further decreases levels of the misfolded proteins, making high-level expression impossible in such cases (42). Occasionally, accumulation of the heterologous protein is toxic, resulting in slow growth or even death of the yeast host. Fortunately, in these situations, the methylotrophic yeast *Pichia pastoris* can be

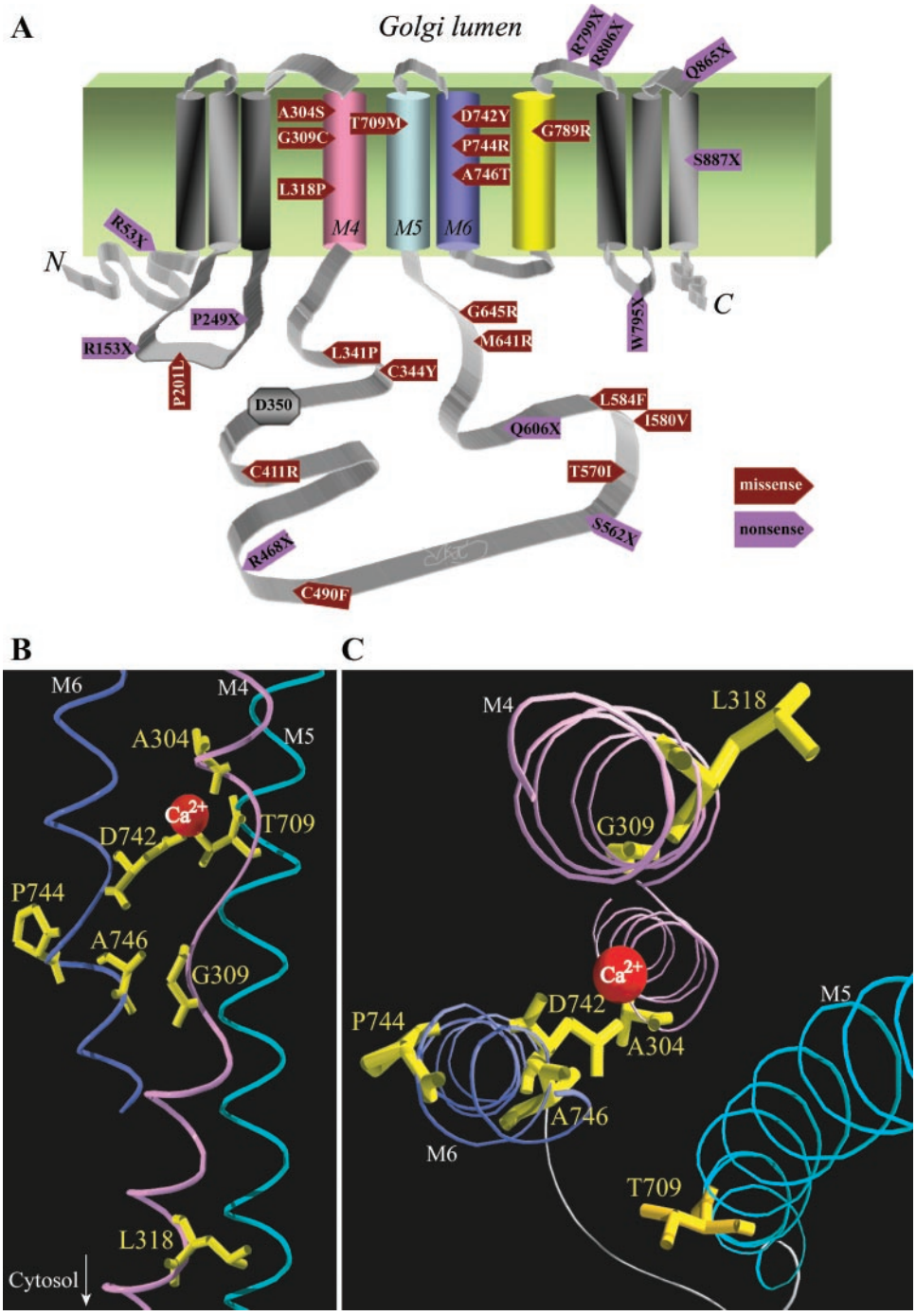

Fig. 3. Hailey-Hailey disease (HDD) mutations in human Golgi/secretory pathway Ca^{2+},Mn^{2+}-ATPase (hSPCA1). *A*: topological disposition of hSPCA1. Nonsense (chain termination) mutations identified in HHD patients are scattered throughout the polypeptide, whereas missense mutations cluster in the transmembrane helices known to be critical for transport (M4, M5, and M6) and in the central hydrophilic region constituting the phosphorylation and nucleotide binding domains. D350 is the site of catalytic phosphorylation intermediate. *B* and *C*: homology model of transmembrane helices M4, M5, and M6 of hSPCA1 based on the crystal structure of rabbit sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA1) in the Ca^{2+}-bound conformation (84). Models were generated in Deepview-SwissPDB-Viewer (GlaxoSmithKline) and rendered with Pov-Ray 3.5 and MegaPOV 1.0. The location of the single Ca^{2+} is based on information from mutagenesis of yeast Pmr1 (51) and the equivalent residues in SERCA1. Residues shown are those mutated in HHD. *B*: side view parallel to lipid bilayer. *C*: view from cytosolic face.

substituted for high-level protein expression. *Pichia* cultures grow to a phenomenally high density on methanol, and protein expression can be driven by the inducible alcohol oxidase *AOX1* promoter. Additional increases in expression levels may be achieved by using a synthetic gene in which heterologous codons are replaced with preferred yeast codons (95, 97). *Pichia* is reasonably amenable to genetic manipulation and has the ability to glycosylate proteins, albeit differently from *S. cerevisiae* (8, 14). Together, the two yeast systems can be differently exploited in multiple ways. *Pichia* may be the system of choice for generating large quantities of protein for structural studies, whereas *Saccharomyces* can be used to gain insight into protein function.

SUMMARY

Saccharomyces cerevisiae is a straightforward eukaryotic model cell for heterologous expression. It has a fully sequenced genome, deletion mutants that are readily available, and experimentally amenable genetics. Many yeast pathways are conserved across kingdoms. Bacterial, protozoan, plant, nematode, and mammalian proteins are often found to functionally complement yeast knockouts, thus providing the first

insight into function of a novel gene. As a result of its simple culturing conditions, yeast is widely used in high-throughput screens such as those that search for drug targets. Research focused on dissecting the Ca^{2+} homeostasis machinery has been facilitated by a relative simplicity in cellular design. In closing, there are many advantages of the yeast system, not the least of which is the observation (89a) that "phenomena found in higher eukaryotes acquire additional respectability when also found in yeast."

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