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PROLYL ISOMERASES IN YEAST

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1. ABSTRACT

Prolyl isomerases are enzymes that catalyze *cis-trans* isomerization of peptidyl-prolyl bonds and span three structurally unrelated protein families: the cyclophilins, FKBPs, and parvulins. The genome of the budding yeast *Saccharomyces cerevisiae* encodes eight different cyclophilins (Cpr1 to Cpr8), four FKBPs (Fpr1 to Fpr4), and a single parvulin (Ess1). Remarkably, two of these proteins, cyclophilin A and FKBP12, are conserved from yeast to humans and mediate virtually all of the intracellular actions of the immunosuppressive antifungal drugs cyclosporin A, FK506, and rapamycin. The study of prolyl isomerases in *S. cerevisiae* has proven invaluable to understand the elusive functions of these proteins, and continues to provide new insights into their diverse cellular roles. Here we review the current state of knowledge about prolyl-isomerases in this model organism.

2. INTRODUCTION

Because of its partial double-bond character, the peptide bond can adopt either of two isomeric conformations, *cis* or *trans*. In all amide peptide bonds, steric clash between the side chains of the two participating amino-acid residues hinders the *cis* conformation, and therefore favors the *trans* isomer. By contrast, the structural features of the side chain in the imino acid proline render both conformations thermodynamically equivalent in peptidyl-prolyl bonds (1,2), and approximately 7 percent of these imide bonds are found in *cis* in the native structures of proteins (3,4). Because peptide bonds are hypothesized to be synthesized exclusively in the *trans* isomeric form by the ribosome, the *cis* conformation in peptidyl-prolyl bonds must be acquired during the course of protein folding. In addition, isomerization of peptidyl-prolyl bonds might be required for protein refolding following traffic across cellular membranes, and in other processes involving protein conformational changes. Due to a relatively large energetic barrier, spontaneous peptidyl-prolyl isomerization is a slow reaction that can constitute rate-limiting steps in protein folding (for review, see 5). Prolyl isomerases (also known as peptidyl-prolyl *cis-trans* isomerases, proline isomerases, PPIases, or rotamases) are enzymes that catalyze the isomerization of peptidyl-prolyl bonds by binding to and stabilizing the transition state, which is a partially rotated imide bond (6).

Prolyl isomerases were first discovered in

mammals, and later found in a wide variety of organisms, including bacteria, fungi and plants. There are three families of structurally unrelated prolyl isomerases, namely the cyclophilins, the FK506-binding proteins (FKBPs), and the parvulins. Cyclophilins and FKBPs were identified as proteins with binding affinity for the immunosuppressive drugs cyclosporin A and FK506, respectively, and two of these proteins, cyclophilin A and FKBP12, constitute the intracellular receptors of these drugs and mediate their immunosuppressive effects (7-9). Thus, cyclophilins and FKBPs are collectively termed immunophilins. Cyclosporin A and FK506 bind to and inhibit the prolyl isomerase activity of cyclophilin A and FKBP12, respectively. Immunosuppression does not result from inhibition of prolyl isomerase activity but rather from inhibition of the protein-phosphatase calcineurin by the cyclophilin A-cyclosporin A and FKBP12-FK506 protein-drug complexes, and a subsequent block to T-lymphocyte activation (for review, see 10). The third family of prolyl isomerases, the parvulins, was discovered in bacteria, and later found to be conserved in fungi and mammals.

There is evidence that, in some cases, prolyl isomerases might serve a global role in facilitating correct protein folding in the cell. In this regard, a ribosome-associated bacterial prolyl isomerase, the so-called trigger factor (TF), has been shown to be an efficient catalyst of protein folding reactions involving isomerization of a peptidyl-prolyl bond as the rate-limiting step (11,12). TF associates with nascent polypeptide chains and participates in their stabilization in a state competent for subsequent folding, suggesting a general role for TF in protein folding (for reviews, see 13-15). Other prolyl isomerases interact physically and functionally with the conserved molecular chaperone Hsp90, supporting a role for prolyl isomerases in protein refolding by modulating the activity of Hsp90-regulated ligand receptors (16-18). However, there is also increasing evidence that native-state *cis-trans* isomerization of the peptidyl-prolyl bond in folded proteins might represent a molecular switch. In this regard, prolyl isomerases might catalyze different conformation-dependent signalling events, and in these cases involving specific proteins mediating signal transduction (19).

Prolyl isomerases are ubiquitous and in some cases highly conserved, and yet the cellular functions of these proteins remain largely undiscovered. Here we review the current knowledge about prolyl isomerases in the model organism *S. cerevisiae*. Molecular and genetic studies have revealed that none of the yeast cyclophilins or FKBPs is essential, either individually or in combination, whereas the single parvulin family member Ess1 is essential for cell growth (Figure 1). Further studies have revealed features of the endogenous cellular roles of these yeast prolyl isomerases that are the subject of this article. The roles of several of these enigmatic enzymes are being unveiled by studies of their physical and genetic interactions with other proteins of known function. The most prominent characteristics of the yeast prolyl isomerases are summarized in Figure 2 and Table 1. These and other features are detailed next.

3. CYCLOPHILINS

3.1. Cpr1

Cpr1 (Cyclosporin A-sensitive proline rotamase 1) was originally identified as a cyclosporin A-binding protein with *cis-trans* peptidyl-prolyl isomerase activity, and was found to share 65% identity with human cyclophilin A (20). In this report, Cpr1 affinity for cyclosporin A was found to be similar to that detected between this drug and human cyclophilin A, for which a dissociation constant (K_d) of 2 x 10^{-7} M has been reported (21). The newly discovered yeast cyclophilin A was soon proposed to mediate cyclosporin A toxicity in a mutant strain sensitive to this drug, because some cyclosporin A-resistant isolates obtained from this strain exhibited decreased expression of Cpr1 (22). This hypothesis was further confirmed by studies showing that deletion of the *CPR1* gene, in both the original cyclosporin A-sensitive background and other cyclosporin A-sensitive genetic backgrounds, abolished cyclosporin A toxicity (23-25).

Evidence supporting calcineurin as the target of the cyclophilin-cyclosporin A complex in yeast was established shortly thereafter. Cpr1 was found to mediate a cyclosporin A-induced defect in calcineurin-dependent recovery from alpha mating factor-G1 cell cycle arrest, strongly indicating that, as its mammalian counterpart, Cpr1 forms a complex with the immunosuppressive drug that inhibits calcineurin function (26). Calcineurin also becomes essential in yeast cells exposed to elevated concentrations of LiCl or NaCl, suggesting that this protein is required for cation homeostasis in yeast. Accordingly, cyclosporin A inhibits growth of yeast in the presence of LiCl or NaCl in a cyclophilin A-dependent manner (24,27). Cpr1 also binds to calcineurin with low affinity in the absence of cyclosporin A, suggesting that this drug may exploit a pre-existing interaction between the two proteins, although the physiological function of this drug-independent interaction remains largely unexplored (28).

Selection for yeast strains resistant to cyclosporin A led to the isolation of mutations in both cyclophilin A and the calcineurin A catalytic subunit that prevent formation of the ternary complex between the drug and these two proteins, providing valuable information about the amino acid residues participating in the physical interactions within the protein-drug-protein complex (29,30). G70S, G102A, and H90Y substitutions affect amino acid residues conserved between yeast and human cyclophilin A. These mutations map to the cyclosporin A binding pocket of cyclophilin A and confer cyclosporin A resistance by reducing Cpr1-cyclosporin A binding to calcineurin (29). When recombinant versions of these mutant proteins were purified and assayed for cyclosporin A binding *in vitro*, the G70S and G102A mutants showed a moderate decrease in binding affinity, while the H90Y mutant exhibited a more dramatic binding defect. In an *in vitro* prolyl isomerase assay, performed with the chymotrypsin-coupled cleavage reaction with a synthetic peptide substrate, the Cpr1 H90Y mutant showed reduced

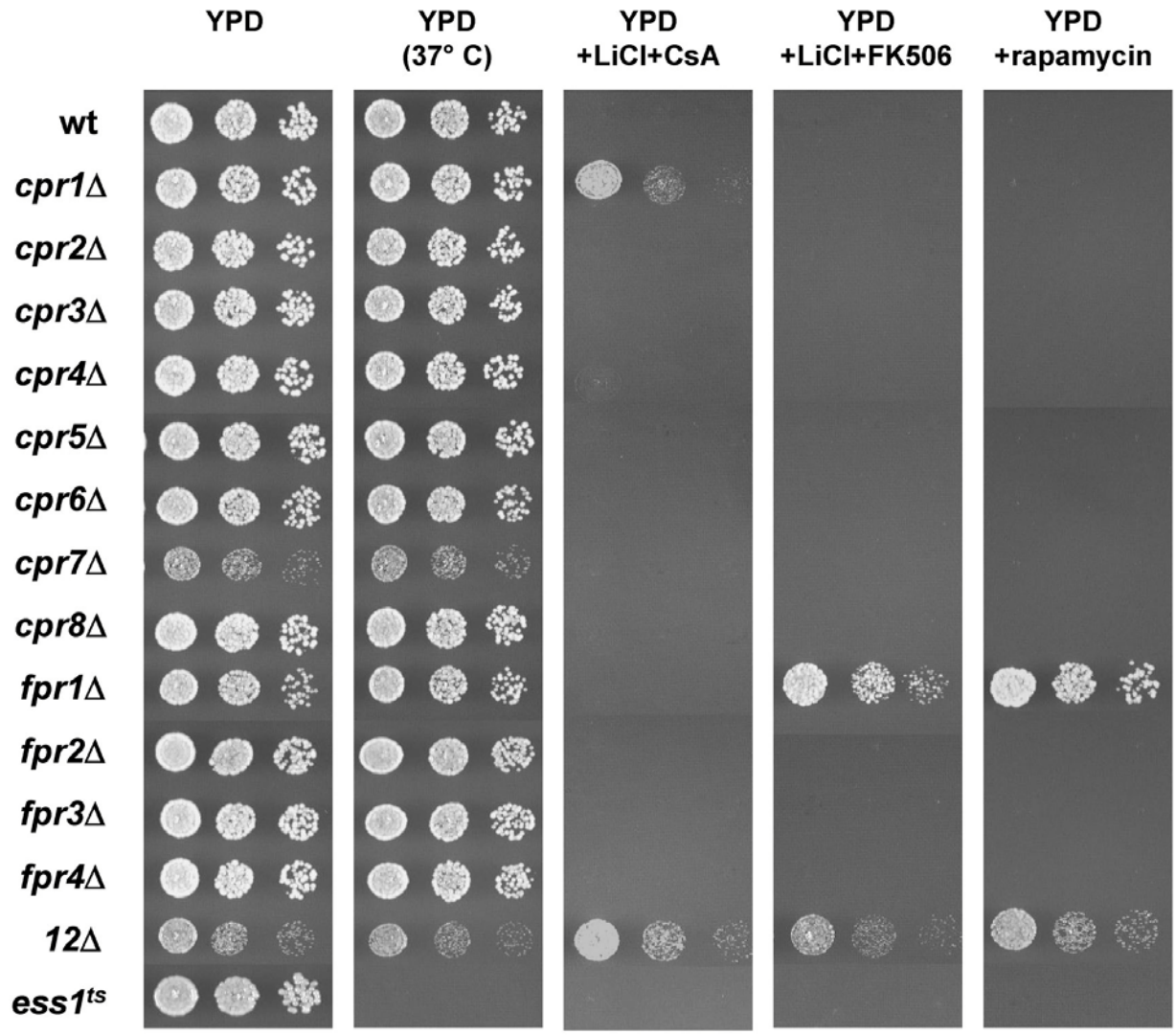

Figure 1. Yeast prolyl-isomerase mutants of *S. cerevisiae*. Cultures of yeast strains deleted for individual cyclophilin-encoding genes (*cpr1*Delta to *cpr8*Delta), or FKBP-encoding genes (*fpr1*Delta to *fpr4*Delta), or the dodecuplet mutant deleted for all cyclophilin- and FKBP-encoding genes (*12*Delta), or expressing a temperature-sensitive allele of *ess1* (*ess1*^{ts}), were serially diluted and spotted on YPD medium (222), or in YPD medium containing 0.4 M LiCl plus 100 micrograms/ml cyclosporin A (YPD+LiCl+CsA), 0.4 M LiCl plus 1 micrograms/ml FK506 (YPD+LiCl+FK506), or 0.1 micrograms/ml rapamycin (YPD+rapamycin), and incubated at 30°C or 37°C for 48 hours. A wild-type strain (wt) was included in the assays as a control.

enzymatic activity, suggesting that the amino acid at this position contributes to the cyclophilin A catalytic active site (29).

Mutations in calcineurin that block interaction with the Cpr1-cyclosporin A complex have also been identified. The amino acid substitutions T350K, T350R, and Y377F in the calcineurin A catalytic subunit Cmp1/Cna1 inhibit binding by the Cpr1-cyclosporin A complex and confer dominant cyclosporin A resistance (30). Both amino acid residues affected are located in highly conserved regions of calcineurin A. T350 lies near the junction between the phosphatase catalytic region and the carboxy-terminal unique region of calcineurin A, and is adjacent in position to a residue important in inhibitor binding to the PP1 and PP2A phosphatases (31,32). Y377 maps within the binding site for the regulatory subunit calcineurin B, supporting previous reports proposing that the target of the cyclophilin A-cyclosporin A complex is the interface between the two subunits in the heterodimeric calcineurin AB holoenzyme (33-35).

The normal cellular functions of the yeast cyclophilin A homolog Cpr1 are just beginning to be elucidated. Cpr1 expression is activated under certain stress conditions, indicating that this protein might participate in protecting the cell when exposed to stress. *CPR1* transcription is moderately induced at high temperature, and this induction is mediated by a conserved heat shock response element (HSE) located upstream of the *CPR1* ORF (36). In addition, two stress response elements (STREs) have been identified in the *CPR1* promoter region,

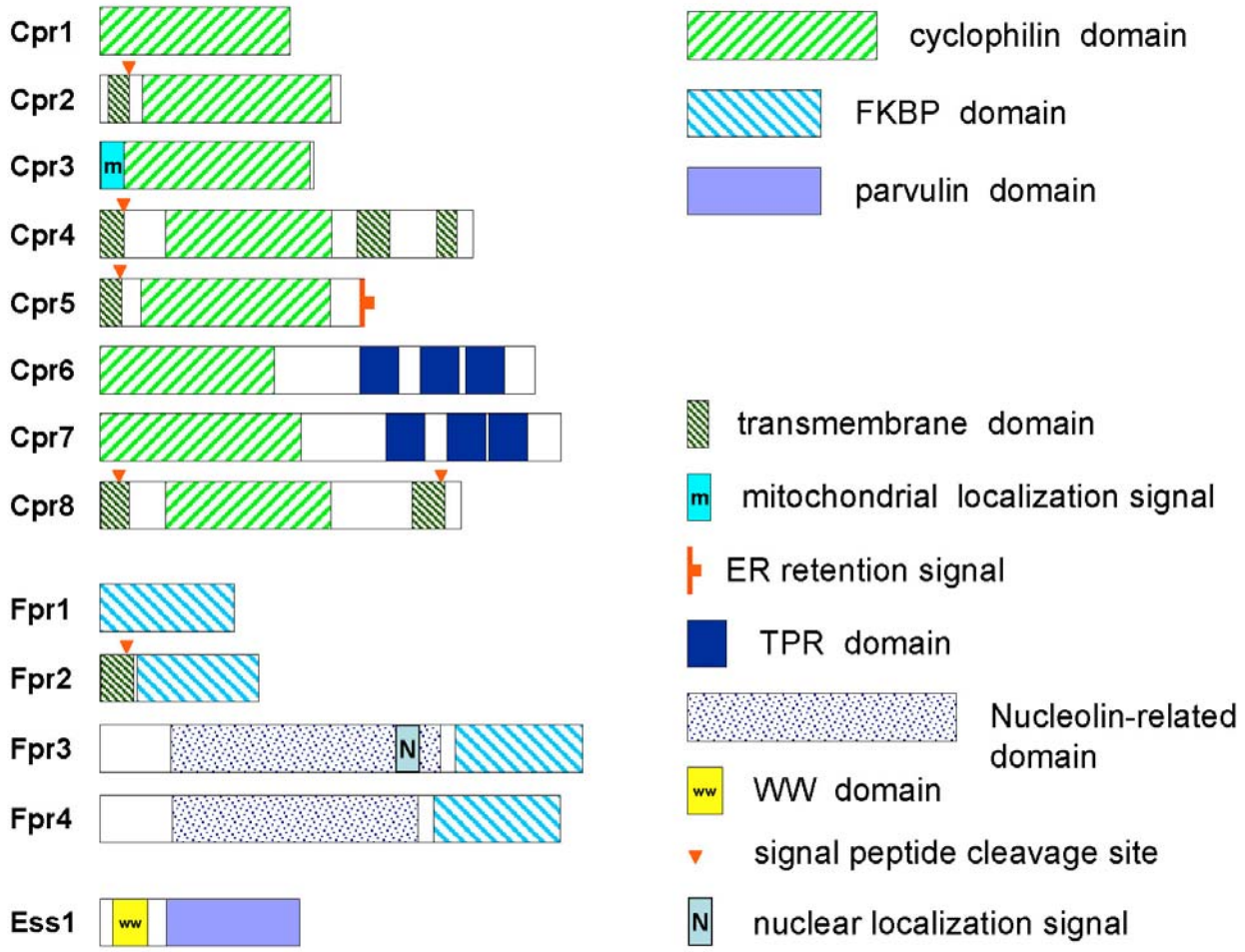

Figure 2. Domains and motifs of the yeast prolyl isomerases. Structural features found in the cyclophilins, FKBPs, and the only parvulin (Ess1) of the budding yeast *S. cerevisiae*.

indicating that expression of this gene is regulated by the Cys_2His_2 zinc finger proteins Msn2p and Msn4p and the MAP kinase Hog1; accordingly, *CPR1* transcription is activated by NaCl or sorbic acid, although not by ethanol (37). Cpr1 may play a role in heat shock survival (36), although recent studies showed no loss of viability in a *cpr1*Delta mutant exposed to high temperature (38).

Recent reports have shown that Cpr1 becomes essential in yeast cells in which the function of the Ess1 prolyl isomerase is compromised. Ess1 is a member of the parvulin family of prolyl isomerases, which are distinct from both cyclophilins and FKBPs (39-42). In these studies, *CPR1* was identified as a multi-copy suppressor of conditional temperature-sensitive *ess1* mutations (*ess1*^{ts}). Cpr1 also suppresses the lethal phenotype of an *ESS1* deletion, suggesting that Cpr1 and Ess1 share an essential function in the cell. This hypothesis is supported by the finding that *ess1*^{ts} mutations are synthetically lethal with a *cpr1*Delta mutation at permissive temperature. *ess1* suppression by Cpr1 requires Cpr1 prolyl isomerase activity, showing for the first time a cellular role for the enzymatic activity of cyclophilin A. Thus, *ess1* suppression by Cpr1 overexpression was blocked by cyclosporin A. A Cpr1 active-site mutant with reduced prolyl isomerase activity (H90Y) failed to suppress an *ess1*^{ts} mutation. Further, when a set of wild type and active site mutants of human cyclophilin A, expressed from the Cpr1 promoter in yeast high copy-number plasmids, were tested in the same assay, the level of *ess1*^{ts} suppression was proportional to the specific enzymatic activity detected with these proteins *in vitro*. The *ess1*^{ts} mutations where partially suppressed by over-expression of the related Cpr6 and Cpr7 cyclophilins, indicating an overlap in functions among Ess1, Cpr1 and these cyclophilin 40 homologs (41,42).

Cpr1 was also found to be required for *ess1* suppression by over-expression of an unrelated protein, Sap30, a member of the Sin3-Rpd3 histone deacetylase complex identified by Zhang *et al*. (43), suggesting that this complex might be a common target of Ess1 and Cpr1 functions. In support of this model, both prolyl isomerases interact physically with the Sin3-Rpd3 complex *in vitro*, and their interaction is reminiscent of that observed between the Cpr6 and Cpr7 yeast cyclophilins and the Sin3-Rpd3 complex (44). The Sin3-Rpd3 complex is a transcriptional co-repressor that can be recruited to chromatin by Ume6, a C_6 zinc cluster protein that binds to

Table 1. Main features of the yeast prolyl isomerases

Protein	Molecular mass (kDa)	Characteristics		References
Cpr1	17	Localization	Cytoplasm and nucleus	82
			Accumulates in the nucleus	Arévalo-Rodríguez and Heitman, unpublished data
		Functions	Cyclosporin A receptor	24
			Interacts functionally with the Sin3-Rpd3 histone deacetylase complex	42
			Component of the Set3 histone deacetylase complex	57
			Essential in *zpr1* mutants. Required for Zpr1 localization	38
			Required for FBPase import into Vid vesicles	60
			Regulation of meiosis	Arévalo-Rodríguez and Heitman, unpublished data
Cpr2	20	Localization	Secreted to the medium	66
		Functions	Unknown	
Cpr3	20	Localization	Mitochondria	71, 82
		Functions	Mitochondrial function at high temperature	72
			Protein folding	73
Cpr4	33	Localization	Vacuole	82
		Functions	Unknown	
Cpr5	23	Localization	Endoplasmic reticulum	71, 83
		Functions	Unknown	
Cpr6	45	Localization	Cytoplasm	82
		Functions	Interacts with Rpd3	44
			Interacts functionally with Hsp90	87, 94-96
Cpr7	45	Localization	Cytoplasm	82
		Functions	Interacts with Rpd3	44
			Interacts functionally with Hsp90	91, 93, 97-99, 103, 104
			Interacts with Hsp104	105
Cpr8	35	Localization	Vacuole	82
		Functions	Unknown	
Fpr1	12	Localization	Cytoplasm and nucleus	82
		Functions	FK506 receptor	24, 26, 27, 112, 113
			Rapamycin receptor	117, 118
			Required for feedback regulation of Hom3 aspartokinase activity	139
			Essential in *hmo1* mutants. Regulates Hmo1 self-association	146
Fpr2	12.5	Localization	Endoplasmic reticulum	82, 155
		Functions	Expression regulated by unfolded protein response (UPR). Possible role in protein folding	155
Fpr3	70	Localization	Nucleolus	82, 158
		Functions	Overexpression suppresses cell cycle defects of tom1 mutants	163, 164
Fpr4	60	Localization	Nucleolus	82
		Functions	Overexpression suppresses cell cycle defects of tom1 mutants	163, 164
Ess1	19	Localization	Cytoplasm and nucleus, predominantly in the nucleus	82
		Functions	Mutants show mitotic arrest and nuclear fragmentation	39, 40, 152
			Interacts with CTD of RNA pol II, and Sin3-Rpd3 histone deacetylase complex	41, 42, 195
			Functions in transcription initiation, elongation and termination	40, 185, 199, 197

the *cis*-acting URS1 element located upstream of a number of genes involved in meiosis and nutrient assimilation (45-49). The Sin3-Rpd3 complex also regulates transcriptional silencing at several yeast genetic loci, including the ribosomal DNA array (rDNA), the telomeres, and the silent mating-type loci HML and HMR (50-52). Over-expression of Cpr1 increased canavanine sensitivity in a strain expressing a *CAN1* marker (which encodes the canavanine permease) inserted in the ribosomal DNA array, while the presence of an *ess1*^{ts} mutation had the opposite effect. This

pattern indicates that both Ess1 and Cpr1 can regulate the activity of the Sin3-Rpd3 complex, although in opposing directions (42). In the proposed model, Cpr1 catalyzes conformational changes in the Sin3-Rpd3 complex that could assist in the recruitment of Sap30 to this complex, perhaps counteracting the adverse results of a defect in Ess1 function. Viability rescue of an *ess1*Delta mutant by overexpression of the endogenous *CPR1* gene has been recently reported by others (53).

In a search for genetic backgrounds that render Cpr1 function essential, a *cpr1*Delta allele was found to be synthetically lethal with point mutations in the *ZPR1* gene, which encodes the conserved, essential zinc finger protein Zpr1 (38). In mammals, *Schizosaccharomyces pombe*, and *S. cerevisiae*, Zpr1 redistributes between the cytoplasm and the nucleus in response to proliferation signals, and interacts with the essential eukaryotic translation elongation factor-1α (eEF-1α), encoded in yeast by the *TEF1* and *TEF2* genes (54-56). In the studies by Ansari *et al*. (38), overexpression of the *TEF1* gene suppressed the *CPR1* dependence and the cycloheximide sensitivity of a *zpr1* mutation, indicating that Zpr1 serves a role in translation. An additional suppressor screen identified the *CPR6* gene, encoding a cyclophilin 40 homolog, and the *FPR1* gene, which encodes FKBP12 (a prolyl isomerase from a different family), thereby revealing a new example of functional overlap between prolyl isomerases.

Cpr1 was found to be required for proper cellular localization of Zpr1 (38). Thus, in *cpr1*Delta cells deprived of glucose, a Zpr1-GFP fusion protein accumulated in the nucleus, whereas wild-type cells showed an even nuclear-cytoplasmic distribution of the same Zpr1-GFP fusion. These observations indicate that Cpr1 mediates nuclear export of Zpr1. Wild-type human cyclophilin A, expressed from a single-copy yeast plasmid in a *cpr1*Delta strain, restored Zpr1-GFP nuclear-cytoplasmic distribution and rescued the viability of two different *zpr1* mutants. By contrast, a human cyclophilin A active site mutant (H126Q) failed to promote Zpr1-GFP nuclear export and did not rescue either *zpr1* mutant. Zpr1-GFP mutant protein fusions were predominantly nuclearly localized even in a *CPR1* wild-type strain, indicating that Cpr1 has a reduced capacity to promote nuclear export of these Zpr1 mutant proteins. These results establish a positive correlation between cyclophilin A prolyl isomerase activity and Zpr1 nuclear export, and suggest a physical interaction between Zpr1 and Cpr1. Such an interaction could be transient, and was not detected by Ansari *et al*. (38).

Interestingly, Cpr1 itself also accumulates in the nucleus (Arévalo-Rodríguez and Heitman, unpublished data). This finding challenges the traditional view of cyclophilin A as a cytoplasmic protein, and fits well with previous observations implicating Cpr1 in a variety of nuclear functions, including chromatin modification and nuclear export. A recent report described a physical association of Cpr1 with a novel histone deacetylase complex, Set3C, which functions as a transcriptional repressor of meiosis-specific genes (57). Set3C includes two unrelated histone deacetylases: Hos2, an Rpd3 homolog (58), and Hst1, a Sir2 homolog (59). The Set3 complex controls the expression of early and middle meiotic genes. Thus, *set3*Delta/*set3*Delta or *hos2*Delta/*hos2*Delta mutant diploid strains exhibit precocious induction of *IME2* and *NDT80* gene expression and accelerated progression through meiosis, whereas an *hst1*Delta/*hst1*Delta mutant strain shows wild-type induction of these genes and normal sporulation kinetics (57). Conversely, deletion of both alleles of the *CPR1* gene in a diploid strain confers a severe sporulation defect that is associated with decreased induction of the *IME1* and *IME2* genes under sporulation conditions, by mechanisms that may involve dysregulation of repression by the Set3 complex (Arévalo-Rodríguez and Heitman, unpublished data).

Another intriguing role for Cpr1 has been reported by Brown *et al*. (60), and involves vacuolar degradation of fructose-1,6-bisphosphatase (FBPase), a process in which Cpr1 is necessary to mediate FBPase transport into Vid (vacuole import and degradation) vesicles in the presence of glucose. A defect in FBPase import into Vid vesicles in a *vid22*Delta mutant was shown to be associated with a dramatic reduction in the expression of Cpr1. Thus, FBPase import into Vid vesicles obtained from a *vid22*Delta mutant was recapitulated in an *in vitro* assay using cytosolic preparations from a wild-type strain, and further fractionation of these extracts led to the identification of Cpr1 as the complementing factor. The addition of recombinant wild-type Cpr1 expressed in bacteria stimulated FBPase import into Vid vesicles; this induction was prevented by cyclosporin A, or when the Cpr1 binding pocket mutants H90Y or G102A were used instead, suggesting that the cyclosporin A binding pocket of Cpr1 is required for Cpr1 function in FBPase import. The mechanism by which Cpr1 mediates FBPase import into Vid vesicles is still unknown, as is the manner in which Vid22, a plasma membrane protein, regulates the expression of Cpr1.

Taken together, these reports suggest that Cpr1 serves important cellular functions that can be revealed when the function of other essential proteins (like Ess1 or Zpr1), is compromised. In addition, Cpr1 has cellular roles that could become essential for survival in the yeast natural environment, such as its role in sporulation and in FBPase degradation in the absence of glucose.

Cyclophilin A has been identified in several other fungi. In the human pathogenic basidiomycete *Cryptococcus neoformans*, cyclophilin A is encoded by two homologous genes, *CPA1* and *CPA2*, which express highly related proteins with divergent functions (61). While deletion of the *CPA2* gene did not confer any phenotype under the conditions tested, deletion of the *CPA1* gene caused a growth defect at 39ºC and a reduction in virulence in rabbit and mouse models. Deletion of both the *CPA1* and *CPA2* genes caused a growth defect at 25ºC, exacerbated the temperature sensitivity and virulence defect of the *cpa1* single mutant, and conferred defects in mating and virulence factor (capsule and melanin) production. Thus, Cpa1 plays a primary role in these processes, whereas Cpa2

has an ancillary role. Reintroduction of the wild-type *CPA1* allele in a *cpa1 cpa2* double mutant restored *CPA1* function, whereas mutant *CPA1* alleles encoding active-site mutants restored growth at low but not high temperatures, indicating that Cpa1 prolyl isomerase activity is partially required for cellular function (61).

A similar role in virulence has been reported for cyclophilin A in the phytopathogenic ascomycete *Magnaporthe grisea*, in which a single gene *CYP1* encodes two cyclophilin forms, one mitochondrial and the other cytosolic (62). Deletion of the *CYP1* gene did not affect the vegetative growth of *M. grisea* in culture, but it did cause a defect in appressorium turgor-pressure generation and probably prevented cuticle penetration in rice. A similar bifunctional cyclophilin gene has been found in the phytopathogenic fungus *Botrytis cinerea*, in which a single gene, *BCP1*, encodes two proteins that differ at the amino terminus (63). The shorter ORF of *BCP1* is highly expressed and encodes a cyclophilin A homolog found to be important for virulence in *B. cinerea*.

3.2. Cpr2

The *CPR2* gene was first identified by Koser *et al*. (64, 65), and Cpr2 was proposed to be localized to the secretory pathway, based on the presence of a hypothetical transmembrane domain at its amino terminus. Cpr2 is secreted to the medium (66). As for *CPR1*, *CPR2* gene transcription is modestly induced by heat shock, and the gene is required for cell survival after heat shock (36,67). *CPR2* is also induced by tunicamycin, a glycosylation inhibitor that results in the accumulation of misfolded proteins in the ER (67).

3.3. Cpr3

Cpr3 was identified as an 18 kDa protein with prolyl isomerase activity in a soluble fraction of extract from yeast cells deleted for both the *CPR1* and *FPR1* genes (68). The predicted amino acid sequence of Cpr3 was found to be 70% and 48% identical with Cpr1 and Cpr2, respectively. Cpr3 binds cyclosporin A with a $K_d < 10^{-9}$ M (69). Like Cpr2, Cpr3 is predicted to have an amino-terminal extension that was absent from the purified protein, suggesting that this region contains a peptide signal that is cleaved as a result of subcellular localization. The predicted secondary structure of this peptide is similar to that adopted by known mitochondrial localization signal sequences, suggesting that Cpr3 localizes to mitochondria. This hypothesis is supported by the finding that Cpr3 co-fractionates with the mitochondrial protein cytochrome *c* oxidase (70), and by the results of recent immunolocalization experiments (71). Cpr3 is required for lactate metabolism at 37ºC, a mitochondrial process, indicating that Cpr3 might assist in mitochondrial protein folding under stress conditions (72).

Further support for this hypothesis comes from the work by Matouschek *et al*. (73), and from the parallel work by Rassow *et al*. (74) with the mitochondrial isoform of *Neurospora crassa* CyP20. In these studies, mitochondria isolated from yeast strains expressing or lacking Cpr3 were incubated with a fusion protein consisting of the mitochondrial matrix-targeting sequence of subunit 9 of the *N. crassa* F_1F_0-ATPase fused to mouse dihydrofolate reductase, and correct folding of the protein after import to the mitochondrial matrix was monitored by resistance to proteinase K. Mitochondria isolated from *cpr3* mutants, or from wild-type strains treated with cyclosporin A, showed a similar defect in protein refolding rate in this assay, indicating that Cpr3 participates in the folding of newly synthesized mitochondrion-targeted proteins. An active-site mutation in Cpr3 (R73A) disrupted the mitochondrial function of this protein, while another active site mutation (H144Q) did not (70). Although initial studies indicated that both of these mutant versions of Cpr3, which were virtually inactive in an *in vitro* protease-coupled peptide assay, retained a high folding activity in an assay based on the catalysis of a proline-limited folding reaction (75), further analysis showed that the folding activity detected in these assays was associated with the presence of the *Escherichia coli* prolyl isomerase SlyD, which co-purified with the recombinant Cpr3 proteins. Therefore, the R73A and H144Q mutants in fact exhibit only a very low level of prolyl isomerase activity (69).

In mammalian cells, programmed cell death involves the opening of the mitochondrial permeability transition pore, mitochondrial depolarization, and release of cytochrome *c* in a process regulated by proteins belonging to the Bcl-2 family (76). This permeability transition is inhibited by cyclosporin derivatives, suggesting that the mitochondrial permeability transition pore is regulated by a mitochondrial cyclophilin (77). Expression of the murine pro-apoptotic protein Bax in yeast induced hyperpolarization of mitochondria and cell death with no detectable release of cytochrome *c*, indicating that a programmed cell pathway is partially conserved in yeast (78). In these studies, deletion of the *CPR3* gene had a negative effect on cell death induction by Bax, suggesting that Cpr3, like its mammalian counterpart, serves a role in regulating the mitochondrial permeability transition pore.

3.4. Cpr4

The *CPR4* gene was discovered as a DNA sequence that shares sequence identity with previously described cyclophilin genes (79). *CPR4* gene expression is constitutive, and this gene encodes a protein with a putative, 20-amino acid long signal peptide at its amino terminus and two carboxy-terminal transmembrane domains, suggesting that Cpr4 is a membrane protein. Cpr4 shows high similarity with the *Drosophila melanogaster* protein ninaA, an integral membrane protein required for proper transport of the visual pigment rhodopsin Rh1 from the endoplasmic reticulum (ER) (80,81). Vacuolar localization of Cpr4 has been recently reported (82).

3.5. Cpr5

Like Cpr2 and Cpr4, Cpr5 is a cyclophilin associated with the secretory pathway. First described as cyclophilin D by Frigerio and Pelham (83), Cpr5 has an amino-terminal transmembrane domain which likely functions as a localization signal, and a carboxy-terminal HDEL sequence, a conserved motif that mediates retention of soluble proteins in the lumen of the ER. Epitope-tagged

version of Cpr5 have been localized to the ER (71, 83), and expression of the *CPR5* gene is induced by tunicamycin, suggesting that this protein plays a role in the folding of secreted proteins (67). Cpr5 homologues have been identified in *Aspergillus nidulans* and *Aspergillus niger* (84,85). *cypB* of *A. niger* localizes to the ER, and its expression is induced at high temperature or in the presence of tunicamycin or DTT (85).

3.6. Cpr6 and Cpr7

Cpr6 was first identified as a homolog of mammalian cyclophilin 40, and was found to be physically associated with Hsp82, one of the two yeast homologues of the conserved essential molecular chaperone Hsp90, which was previously found in a complex with cyclophilin 40 (86,87). Cpr6 and the related cyclophilin Cpr7 were later identified in a search for proteins interacting with the yeast histone deacetylase Rpd3 (44). In these studies, deletion of the *CPR6* gene did not have any noticeable effect on yeast, while deletion of the *CPR7* gene conferred a slow-growth phenotype (44,67). Cpr6 and Cpr7 display 38% identity, and both contain an amino-terminal cyclophilin domain followed by three copies of a degenerate 34-amino acid motif known as the tetratricopeptide repeat (TPR), which is involved in protein-protein interactions (88,89). Expression of Cpr6 is induced by heat, whereas that of Cpr7 is not (90, 91). Both proteins are monomeric and exhibit cyclosporin A-inhibitable prolyl isomerase activity *in vitro* (50% inhibitory concentration of cyclosporin A is 6×10^{-8} M for Cpr6), although the catalytic efficiency of Cpr6 is 6-fold higher than that of Cpr7, when assayed with a chymotrypsin-coupled cleavage reaction using a synthetic peptide substrate and the efficiency of Cpr6 is even higher when assayed in RNase T1 (p55) refolding experiments (90,92). The two purified proteins, Cpr6 and Cpr7, can both function as molecular chaperones *in vitro*, preventing aggregation of thermally denatured citrate synthase; interestingly, Cpr7 is a more efficient chaperone than Cpr6 in this assay (92). Recombinant GST-Cpr6 or GST-Cpr7 fusions purified from bacteria interact physically with bacterially expressed Hsp90 in the absence of other yeast proteins, and these interactions are mediated by the carboxy-terminal regions of Cpr6 and Cpr7 that contain the TPR motifs (93). While the role of Cpr6 and Cpr7 interactions with Rpd3 remains unclear, a considerable amount of data shows that both cyclophilins, similar to their mammalian counterpart cyclophilin 40, functionally interact with the Hsp90 chaperone.

The functional relationship of Cpr6 with Hsp90 is only starting to be uncovered, and recent reports suggest that Cpr6 regulates the ATPase cycle of Hsp90 (94-96). On the other hand, the role of Cpr7 in Hsp90 function is much clearer. The role of Cpr7 in Hsp90-mediated signal transduction pathways has been studied in yeast by the expression of heterologous proteins. Hormone-dependent transcriptional activity of the mammalian glucocorticoid receptor (GR), a process which requires Hsp90 function, is strongly decreased in *cpr7*Delta cells, as is the expression and activity of the oncogenic tyrosine kinase $pp60^{v\text{-}src}$, also known to be dependent on Hsp90 (93). Cpr7 is also required for signalling by the human aryl hydrocarbon (Ah) receptor, another Hsp90 client protein (97).

Roles for Cpr7 in Hsp90 endogenous functions have been unveiled as well. Together with Hsp90, Cpr7 plays a major role in transcriptionally regulating the heat shock response in *S. cerevisiae*. Thus, heat shock factor (HSF) activity is derepressed in *cpr7*Delta cells (98). In a more recent report, Cpr7 was found to be required to stabilize a novel Hsp90 client protein, the DNA-binding transcriptional activator Mal63, which is required for induction of the yeast *MAL* structural genes encoding maltose permease and maltase (99). In these studies, the half-life of Mal63 was found to be shorter in an *hsc82*Delta *cpr7*Delta double mutant; accordingly, this strain showed significant defects in maltase induction and maltose assimilation.

Synthetic enhancement of the growth defect conferred by a *cpr7*Delta mutation was observed in cells with compromised Hsp90 function. In these studies, Hsp90 function was reduced through deletion of one of the two yeast Hsp90 homolog-encoding genes (heat-inducible *HSC82*), by expression of a temperature-sensitive $hsc82^{ts}$ allele; or by exposure of *cpr7*Delta cells to the Hsp90 inhibitor geldanamycin (91,93). A similar synthetic growth defect was observed in *cpr7*Delta cells deleted for the *STI1* gene, which encodes a yeast homolog of the mammalian co-chaperone Hop. The Hop protein mediates physical interactions between Hsp90 and the essential chaperone Hsp70 via two sets of TPR domains (93,100,101). Various studies indicate that the cyclophilin domain of Cpr7 is dispensable for Cpr7 endogenous and heterologous functions, suggesting that these functions are mediated largely or entirely by the Cpr7 TPR domains. A mutant Cpr7 with an amino-acid substitution affecting a conserved residue in its putative prolyl isomerase active site complemented the growth defect of a *cpr7*Delta strain, as did over-expression of a truncated Cpr7 devoid of its entire cyclophilin domain and containing only the TPR motif units. By contrast, over-expression of the Cpr7 cyclophilin domain alone failed to complement the *cpr7*Delta phenotype (91,102). Over-expression of Cpr6 or its mammalian homolog cyclophilin 40 did not rescue the growth defect of a *cpr7*Delta mutant, indicating that Cpr6 and Cpr7, despite sharing significant identity, are not redundant (91).

The slow-growth phenotype of a *cpr7*Delta mutant was suppressed by over-expression of the Sti1 homolog Cns1 (cyclophilin seven suppressor), an essential protein with three-unit TPR motifs that is found in protein complexes with both yeast Hsp90 and Cpr7 but not with Cpr6 (91,103). Cns1 interacts directly with Hsp90, and this interaction requires the amino-terminal portion of Cns1 that contains the TPR motifs. As is the case with Cpr7, over-expression of just the Cns1 TPR domain suffices to suppress a *cns1*Delta mutation (104). A report by Tesic *et al*. (104) has revealed a number of interesting features of the functional relationships between Hsp90 and the TPR cochaperones Cpr7 and Cns1. Through the use of temperature-sensitive alleles of the *CNS1* gene, their studies confirmed that the functions of Cpr7 and Cns1

overlap, at least in part. Thus, over-expression of Cpr7 suppressed the lethal phenotype of a *cns1*^{ts} allele encoding a Cns1 protein that has reduced affinity for Hsp90 at high temperature, and a *cpr7*Delta mutation showed synthetic lethality with the *cns1*^{ts} allele. Interestingly, *cns1*^{ts} suppression by Cpr7 required the full-length cyclophilin, rather than only the TPR domain, thus revealing a novel role for the Cpr7 cyclophilin domain. However, viability or growth of a *cns1*^{ts} strain was not affected by the presence of cyclosporin A, and the R64A substitution, affecting an amino acid residue in Cpr7 conserved in the cyclophilin A active site and essential for prolyl isomerase activity, did not prevent *cns1*^{ts} suppression by Cpr7. This indicates that the cyclophilin domain of Cpr7 might serve a non-catalytic function, perhaps mediating protein-protein interactions. In support of this hypothesis, a Cpr7$^{K67P,\ N68G}$ double mutant, with substitutions corresponding to amino acid residues predicted to localize to the surface of the protein and outside of the catalytic region, was unable to suppress a *cns1*^{ts} mutation.

Although both Cpr7 and Cns1 can interact directly with Hsp90, Tesic *et al*. (104) detected a clear interaction between Cpr7 and Cns1 in cells expressing an Hsp90 mutant protein deleted for the carboxy-terminal motif EEVD, a sequence previously found to mediate interactions between TPR domains and Hsp90 (105). Thus, Cpr7 and Cns1 might exist in complexes with each other independent of Hsp90. In support of this model, Cns1 and Cpr7 were found to partially co-elute in gel filtration chromatography experiments in fractions different from those containing Hsp90 (104). Additional evidence for functional overlap between Cpr7 and Cns1 was provided by Miller and Charles (97), who showed that overexpression of Cns1 restored Ah receptor signalling activity in a *cpr7* mutant. However, the functions of Cpr7 and Cns1 are not entirely redundant, as *cns1*Delta mutants are non-viable. In addition, *cpr7*Delta mutants show dysregulation of HSF, a phenotype not observed in a *cns1*Delta mutant strain (104).

A recent report by Abbas-Terki *et al*. (105) described Cpr7 interaction with another molecular chaperone, Hsp104, which is required for stress tolerance and maintenance of [*psi*$^{+}$] and [URE3] prions in yeast (106-110). Abbas-Terki *et al*. showed that Cpr7, together with the other Hsp90 co-chaperones Sti1 and Cns1, interacts with Hsp104 under conditions associated with utilization of non-fermentable carbon sources, thus suggesting a regulated cochaperone function shared between Hsp90 and Hsp104 in response to changes in metabolic activity. Cpr7 was found to interact with Hsp104 in cells expressing a truncated form of Hsp82 devoid of its carboxy-terminal pentapeptide sequence MEEVD as the only Hsp90 source, strongly indicating that Cpr7 and Hsp104 interact in an Hsp90-independent manner. Moreover, a Cpr7-Hsp104 complex was detected in cells expressing the truncated Hsp82 protein, even under conditions that favored interaction between Cpr7 and wild-type Hsp82, suggesting that this interaction is dominant over that between Cpr7 and Hsp104, and excludes the latter during fermentative growth conditions. In support of this model, *in vitro* binding of Cpr7 to Hsp90, and its binding to Hsp104, were found to be mutually exclusive. The role of Cpr7 in Hsp104 function is presently unknown.

A cyclophilin 40 homolog, wis2^{+}, has been identified in *S. pombe*, and this protein has been implicated in regulating the cell cycle of this organism (111).

3.7. Cpr8

CPR8 encodes a 35 kDa protein with 33% identity to Cpr4. As for Cpr4, the cellular functions of Cpr8 are presently unknown. Cpr8 has been recently localized to the vacuole (82).

4. FKBPs

4.1. Fpr1

The first FK506-binding protein to be identified in *S. cerevisiae* was the homolog of the mammalian prolyl isomerase FKBP12. Yeast FKBP12 was isolated by FK506 affinity chromatography, and its coding gene was identified and denoted *FPR1*, for FK506-sensitive proline rotamase (112,113). Purified Fpr1 exhibits peptidyl-prolyl isomerase activity that is inhibited by FK506, and also by the related macrolide rapamycin, with K_d values for these drugs of 9 x 10^{-10} M and 5 x 10^{-10} M, respectively (114). The study of the molecular targets of FK506 and rapamycin in yeast established a platform by which to understand the mechanisms of action of these immunosuppressive drugs, revealing that they form toxic complexes with FKBP12 that bind to and inhibit the function of conserved proteins involved in signal transduction. Thus, yeast sensitivity to FK506 was found to be mediated by Fpr1 and, as is also the case for cyclophilin A and cyclosporin A, the FKBP12-FK506 complex was shown to target the function of calcineurin (24,26,27,112). A W430C substitution in the yeast calcineurin A subunit Cmp2/Cna2, or the equivalent W388C change in Cmp1/Cna1, blocked the interactions between the FKBP12-FK506 complex and calcineurin, and conferred dominant resistance to FK506 (30). The amino-acid residues affected in these mutations localize to the calcineurin B binding site of calcineurin A, close to residues found in the same studies to be required for interaction with the cyclophilin A-cyclosporin A complex. This provides evidence that the FKBP12-FK506 and cyclophilin A-cyclosporin A complexes have overlapping binding sites, in accord with previous results showing that the two immunophilin-drug complexes compete for calcineurin binding (115).

Like cyclophilin A, FKBP12 weakly interacts with calcineurin in the absence of any exogenous ligand, when tested *in vivo* in the two-hybrid system or *in vitro* by affinity chromatography using recombinant Fpr1 purified from bacteria; this interaction is strongly enhanced by FK506 (28). The FK506-independent FKBP12-calcineurin interaction detected in these studies was not perturbed by R49I or F94V substitutions in Fpr1, which affect surface amino acid residues previously shown to prevent binding of the FKBP12-FK506 complex to calcineurin (116). Apparently, therefore, FK506-dependent and independent FKBP12-calcineurin interactions involve different FKBP12 residues. Interestingly, while the calcineurin B regulatory

subunit was required for FK506-dependent FKBP12-calcineurin A interaction, ligand-independent FKBP12-calcineurin A interaction increased in the absence of calcineurin B, suggesting that calcineurin B and FKBP12 compete for binding to calcineurin A. In the absence of FK506, purified Fpr1 weakly inhibited the phosphatase activity of bovine calcineurin toward a synthetic phosphopeptide *in vitro* (28). In support of a possible role of yeast FKBP12 in negative regulation of calcineurin function *in vivo*, yeast *fpr1* mutants exhibited enhanced recovery from alpha factor cell cycle arrest, and also increased resistance to LiCl, when compared to a wild-type strain (28).

FKBP12 was found to mediate rapamycin toxicity in yeast, and selection for mutants resistant to this drug led to the discovery of two additional genes, *TOR1* and *TOR2* (for target of rapamycin), also required for rapamycin-induced cell cycle arrest and encoding highly similar and conserved proteins that have essential cellular functions inhibited by the FKBP12-rapamycin complex (117-123). Amino-acid substitutions blocking rapamycin sensitivity have been identified in the predicted FK506 binding pocket of Fpr1, indicating that the two macrolides share the same binding region in this protein (118,124). This lends support to previous studies showing that FK506 and rapamycin antagonize one another as T-cell activation inhibitors (8,125). Physical interactions between the Fpr1-rapamycin complex and the Tor proteins have been demonstrated in yeast, also showing that the active site mutant $Fpr1^{F43Y}$ was able to bind to these proteins in the presence of rapamycin. Thus, the formation of the FKBP12-rapamycin-Tor complex does not require FKBP12 prolyl isomerase activity (122,124,126). In these studies, amino acid substitutions in Tor2 conferring rapamycin resistance were shown to block binding to the Fpr1-rapamycin complex. The amino-acid residues affected by these substitutions, S1975, W2042, and F2049, are all conserved among Tor1, Tor2, and the mammalian homolog of these proteins, mTOR, and they map within a small region of Tor2 (residues 1886-2081), located near the carboxy-terminal phosphatidylinositol kinase-related domain of this protein and found to be sufficient to mediate interaction with the Fpr1-rapamycin complex (122,124,126).

FKBP12 homologs have been identified in the fungal pathogens *C. neoformans* (Frr1) and *Candida albicans* (Rbp1), in which they mediate toxicity by FK506 and rapamycin (127-130). FKBP12 has also been found in *S. pombe*, where it was denoted Fkh1 (131). In this organism, rapamycin does not have any apparent effect on vegetative growth, but it does inhibit mating (132). Interestingly, this rapamycin-induced mating defect does not seem to be mediated by *S. pombe* Tor proteins, but rather by Fkh1 alone, since deletion of the gene encoding this protein results in a similar developmental defect in this organism; such a finding suggests a role of its own for FKBP12 in the sexual development of *S. pombe* (131).

In *S. cerevisiae*, FKBP12 is required for appropriate expression of the mammalian P-glycoprotein (Pgp), an energy-dependent efflux pump that mediates tumor multidrug resistance by actively exporting various toxic molecules back out of the cell (133,134). In studies by Hemenway and Heitman (134), expression of murine Pgp conferred resistance to cyclosporin A, FK506, and the RNA and DNA inhibitor dactinomycin in strains sensitive to these drugs, and this Pgp-induced resistance was dependent on Fpr1. Pgp expression levels and localization, however, were not affected in the absence of Fpr1. Expression of the active-site mutant $Fpr1^{F43Y}$ restored drug resistance in an *fpr1*Delta strain expressing Pgp, indicating that Pgp function does not require Fpr1 prolyl isomerase activity. This is a finding similar to what has been reported for the FKBP12-dependent function of another large membrane protein, the ryanodine receptor, a multimeric, Ca^{2+}-release channel (135). The function of a highly similar yeast Pgp homolog, the a-factor pheromone transporter Ste6 (136,137), is independent of Fpr1, even though Pgp can functionally substitute for Ste6 in yeast (138).

In an effort to elucidate the endogenous functions of yeast FKBP12, a search for yeast proteins interacting with Fpr1 in the two-hybrid system led to the identification of the product of the *HOM3* gene (139). *HOM3* encodes aspartokinase, the enzyme catalyzing the first of three reactions in the conversion of aspartic acid into the amino acid homoserine, the common precursor for the synthesis of threonine and methionine. Hom3 aspartokinase activity is feedback-inhibited, mainly by threonine (140,141), and mutations that render this protein resistant to feedback inhibition lead to threonine overproduction in yeast, indicating that Hom3 inhibition by threonine represents the main point of control of the synthetic flux in this pathway (142-145). Alarcon and Heitman (139) found Fpr1 to be dispensable for Hom3 expression and for aspartokinase activity. Interestingly, yeast cells with a compromised Fpr1 function, either due to an *fpr1*Delta mutation, or due to FK506 inhibition, exhibited resistance to the toxic structural amino-acid analog hydroxynorvaline, a phenotype also observed in cells expressing a mutant of aspartokinase resistant to feedback inhibition (142). This suggests that Fpr1 regulates feedback inhibition in Hom3. The physical interaction between Fpr1 and Hom3 is direct and can be disrupted by either FK506 or rapamycin, or by mutations affecting amino acid residues located at or near the Fpr1 active site. Thus, aspartokinase is a relevant endogenous binding partner for FKBP12. In this respect, one possibility is that FKBP12 is required for specific conformational changes in Hom3 leading to feedback inhibition.

As a complementary approach to learn more about FKBP12 endogenous functions in yeast, we are searching for mutations in genes that result in synthetic lethality with *fpr1* mutations. One of these genetic screens has led to the identification of the *HMO1* gene (146). Hmo1 belongs to the high mobility group (HMG) proteins, a family of non-histone, chromatin-binding proteins. Hmo1 is more specifically a member of the so-called HMG1/2 class (147). Hmo1 was initially found to be a nuclear DNA-binding protein, with apparent roles in chromatin structure stabilization and plasmid maintenance (148). In addition,

mutations enhancing spontaneous and induced mutability had been previously identified and were later found to be allelic with *HMO1*. (149,150). A more recent report has identified Hmo1 as a nucleolar-localized RNA polymerase I factor (151). Thus, Hmo1 overexpression suppresses *rpa49* mutants lacking a conserved subunit of RNA polymerase I that is required for ribosomal RNA synthesis in yeast, and the double *hmo1 rpa49* mutation is synthetically lethal. Dolinski and Heitman (146) found that *fpr1* and *hmo1* individual mutants share two phenotypes, namely, slow growth and increased plasmid loss, suggesting that Fpr1 and Hmo1 participate in related cellular processes. In addition, Fpr1 and Hmo1 interact physically, and their binding is disrupted by the presence of FK506, thus revealing a probable role for the Fpr1 ligand pocket in this interaction. Hmo1 self-interaction is detected in the two-hybrid system, a result supporting previous observations indicating that Hmo1 forms dimers or oligomers (148). Interestingly, Fpr1 function regulates Hmo1 self-association. Thus, Hmo1-Hmo1 interaction increased in the presence of FK506 or as a result of deletion of *FPR1*. Further studies will be needed to elucidate the functional relationship of yeast FKBP12 with Hmo1, the first target of this prolyl-isomerase (other than calcineurin) that is known to be conserved from yeast to mammals.

More recent reports have revealed functional relationships between Fpr1 and other conserved proteins. Thus, temperature-sensitive mutations in the essential yeast gene *ESS1*, encoding a homolog of the mammalian prolyl isomerase Pin1, were found to be synthetically lethal with an *fpr1*Delta mutation at permissive temperature (42,152). In a different report, Fpr1 overexpression suppressed cyclophilin A dependence of a yeast mutant compromised for the function of the conserved essential zinc finger protein Zpr1 (38).

4.2. Fpr2

The yeast *FPR2* gene encodes a 13 kDa membrane-associated protein, homologous to human FKBP13, with prolyl isomerase activity (114,153). Fpr2 binds FK506 and rapamycin, with K_d values for these drugs of 1.8 x 10^{-8} M and 1.1 x 10^{-8} M, respectively (114). Fpr2 localizes to the ER, and its expression is transcriptionally induced when glycosylation is inhibited by tunicamycin or by mutation of the *SEC53* gene, which encodes the enzyme phosphomannomutase in yeast (82,154,155). This transcriptional activation is mediated by an unfolded protein response (UPR) element located in the promoter region of *FPR2*, suggesting that Fpr2 plays a role in protein transit in the ER under conditions that promote accumulation of these proteins as unfolded precursors. *FPR2* expression is also activated in response to heat stress (155).

4.3. Fpr3 and Fpr4

FPR3 and *FPR4* encode two related prolyl isomerases that share 46% identity. Fpr3 was identified in a search for yeast proteins interacting with a nuclear localization signal (NLS) *in vitro*, and also by affinity for the FK506-related immunosuppressive macrolide FK520 (156-158). Fpr4 was also identified as an FK520-binding protein localized to the nucleolus, and both Fpr3 and Fpr4 interact with the yeast ribosomal protein S24 in the two-hybrid assay (67,82). The amino-terminal sequence of Fpr3 is similar to that of other nucleolar proteins, and determines Fpr3 localization to the nucleolus (82,158). More recently, Fpr3 has been found in a novel ribonucleoprotein complex (159). Fpr3 is a substrate for the yeast casein kinase II protein Cka2, and for the tyrosine-specific phosphoprotein phosphatase Ptp1, indicating that these two proteins regulate the phosphorylation level of Fpr3 residue Y184 in the amino-terminal, nucleolin-related domain, perhaps affecting Fpr3 subcellular localization (160,161).

In addition, Fpr3 and Fpr4 have been identified as high copy-number suppressors of mutations in the *TOM1* (Trigger Of Mitosis 1) gene (162,163). *TOM1* encodes a protein with high similarity to the hect (homologous to E6-AP C terminus)-domain class of E3 ubiquitin ligases, and a null mutation in this gene leads to G_2 cell cycle arrest at elevated temperature (164). Tom1 is required for transcriptional regulation by the ADA/SAGA complexes (165), suggesting that the various phenotypes associated with loss of Tom1 function, including mating defects, might be indirect. Deletion of *TOM1* results in a decrease in transcription of the *ARG1* gene, and this defect is not suppressed by overexpression of Fpr4 (163). These results suggest that overexpression of Fpr4 provides a Tom1 function required for G_2–M transition at high temperature, but does not correct the transcriptional defects observed in a *tom1* mutant. However, these studies have revealed that the carboxy-terminal prolyl isomerase domains of Fpr3 and Fpr4 are dispensable for *tom1* suppression. Further studies will be necessary to elucidate the cellular functions of the prolyl isomerase activity of Fpr3 and Fpr4.

5. Ess1 --- THE ONLY PARVULIN IN YEAST

5.1. General information about the parvulin family

Parvulins, the third family of PPIases, are distinct from the cyclophilins and FKBPs in both primary sequence and in three-dimensional structure. Parvulins do not bind the immunosuppressive drugs cyclosporin A, FK506, and rapamycin, and thus are not immunophilins (166,167). In addition, some parvulins have a narrow substrate specificity and, in contrast to cyclophilins and FKBPs, are essential in some organisms.

The parvulin family is named after the prototypic enzyme, Parvulin (from Latin *parva*, meaning small), isolated from *E. coli* (166,168). Family members are defined by having a conserved parvulin-type PPIase domain approximately 82 amino acids in length. The number of parvulin family members in any given organism is very small: three in *E. coli* (parvulin, SurA, PpiD), one in yeast (Ess1), and two in humans (Pin1 and hPar14).

Unlike prokaryotic parvulins, some eukaryotic parvulins also have a WW domain (Ess1/Pin1), a proline-binding module found in a wide variety of eukaryotic proteins (169,170). Parvulin family members that contain WW domains have a narrow substrate specificity, which

may serve to limit their involvement in general protein folding. Instead, these parvulins have been implicated in specific biological processes. These include cell cycle regulation and cancer (152, 171, 172), DNA replication checkpoint regulation (173), transcription by RNA polymerase II (41, 42), signaling during embryonic development (174), genotoxic response (175, 176), and protection against age-dependent neurodegeneration (177, 178).

5.2. Ess1 in budding yeast is essential for viability

Ess1 is the only parvulin in *S. cerevisiae*. Also known as Ptf1, it was the first eukaryotic parvulin to be isolated (39,40). It is conserved in evolution and orthologs have been identified in higher organisms, such as Dodo in flies, and Pin1 in humans (152,179). All suspected orthologs tested thus far have complemented *ESS1* loss-of-function mutations in yeast (152,179-181; P. Ren, A. Rossettini, V. Chaturvedi and SDH, submitted; C. Wilcox and SDH, unpublished).

Ess1 contains two domains, an amino-terminal WW domain for substrate binding and a carboxy-terminal PPIase domain for enzyme catalysis (152). The two domains are connected by a flexible linker, as suggested by the X-ray and NMR structures of human Pin1 (182,183). This two-domain structure is conserved in all Ess1 orthologs (152,173,179-181; Ren *et al.*, submitted; Figure 3), suggesting that both domains are important for function. Consistent with this, loss of function mutations have been isolated in both domains of Ess1, and the orthologous fly protein Dodo (40,41,184).

Like Pin1, Ess1 has a unique substrate specificity. It binds phospho-Ser-Pro- or phospho-Thr-Pro-containing peptides with high affinity, and *in vitro* displays maximum catalytic activity toward these substrates (185). In fact, phosphorylation of the serine preceding proline results in a 1000-fold increase in enzyme activity (185). This specificity is due, in large part, to the WW domain, which also recognizes the phospho-Ser-Pro or phospho-Thr-Pro motif with high affinity (186).

This unique substrate specificity is crucial for the *in vivo* function of Ess1. In addition to Ess1 orthologs from fungi and metazoans, parvulins isolated from plants also show this specificity and can complement *ESS1* function when overexpressed in yeast, despite lacking a WW domain (187,188). They seem to achieve this specificity using a different sequence/structure element (188; Figure 3), and thus may have evolved via a different route than Ess1 orthologs. In contrast, hPar14, which also lacks a WW domain (Figure 3) but has a different specificity, fails to complement *ESS1* function (187,189; M. Foehr, F. Fujimori and SDH, unpublished).

Several inhibitors of the Ess1/Pin1-class enzymes have been described. Juglone, a natural compound from walnuts, irreversibly inhibits the activity of Ess1 and Pin1 by covalently altering an active-site cysteine (167). Reversible inhibitors were also reported for Pin1 (190). They were designed using variants of the optimal substrate peptides, one of which contains a phospho-D-serine in place of the normal phospho-L-serine. It is likely that these variants will inhibit the activity of Ess1 in the same manner.

Ess1 is essential for viability in *S. cerevisiae* (39). In fact, it is the only PPIase that is essential in yeast (67). It is also essential in the pathogenic fungus, *C. albicans* (180). It is not, however, essential in some other fungi, in flies or in mammals (179-181,191; Ren *et al.*, submitted). This may be due to the presence of additional Ess1-like activities, to the existence of redundant pathways, or to a different functional organization of the Ess1 pathways in higher organisms.

Ess1 is required for mitosis, since loss of *ESS1* function in yeast results in mitotic arrest and nuclear fragmentation (152). However, unlike classic cell division cycle (*cdc*) mutants, which arrest at defined steps in the cell cycle (usually during the first division; 192), yeast cells depleted for Ess1 complete two to three cell divisions before entering mitotic arrest (39,41). The slow-arrest kinetics suggest either that the Ess1 protein is very stable and supports several cell divisions, or more likely, that the function of Ess1 is not required at each cell division.

Despite the dramatic mitotic-arrest phenotype, the mechanism by which Ess1 regulates mitosis remains unclear. Although studies on *Xenopus* and human Pin1 suggest that Pin1 binds phosphoproteins required for mitotic progression (193,194), the relevance of such interactions on the function of these phosphoproteins and on cell-cycle progression remains to be established. Moreover, since Ess1 may not be required during every cell cycle, it is conceivable that Ess1 and its orthologs regulate mitosis using a different mechanism.

5.3. Ess1 plays an important role in transcription by RNA polymerase II

Perhaps the most significant breakthrough in the study of Ess1 function in yeast was the discovery of a novel link between Ess1 and transcription by RNA polymerase II (RNA pol II). Ess1 was first linked to transcription by the finding that *ESS1* was recovered in a genetic screen for mutants with defective transcription termination (*PTF1*, for processing/termination factor 1; 40,185). Ess1 was later identified as a protein that binds the phosphorylated carboxy-terminal domain (CTD) of RNA pol II (41,195). Critical support for this connection came from the result of a genetic multi-copy suppressor screen for Ess1 temperature-sensitive (*ts*)-mutants. All but one of the suppressors was known or suspected to affect the function of RNA pol II, a result that strongly suggests a role for Ess1 in transcription and that the role of Ess1 in mitosis is transcription-related (41).

The effect of Ess1 on transcription and on components of the transcription machinery has been studied primarily using genetic methods. Genetic interactions identified between *ESS1* and other RNA pol II-related genes are summarized in Table 2. For example,

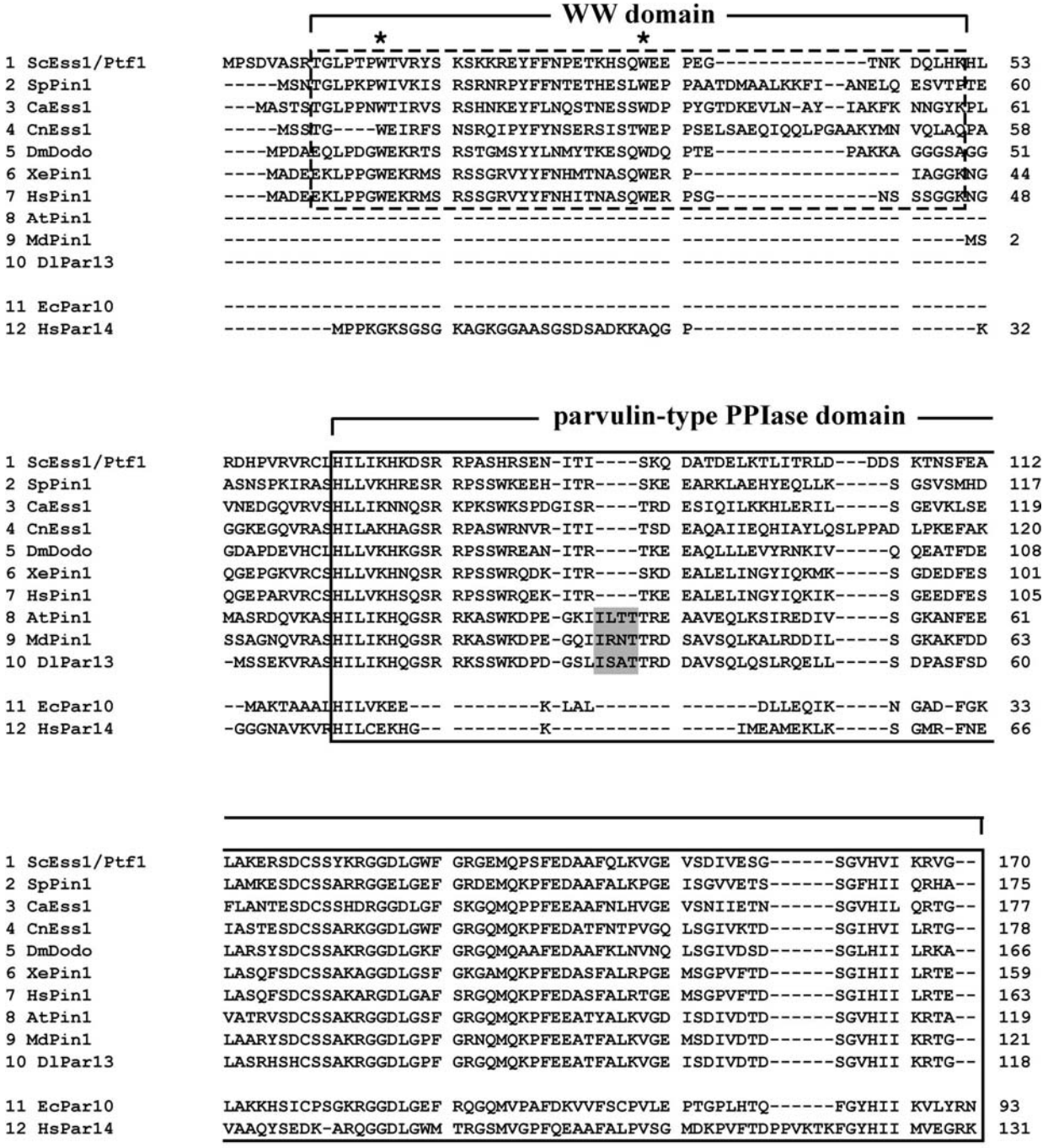

Figure 3. A multiple sequence alignment of selected Ess1 orthologs and other parvulins. All parvulins contain a PPIase domain (solid box) with similarity to the prototype enzyme, *E. coli* parvulin (EcPar10; P39159; 168). In addition, like *S. cerevisiae* Ess1 (ScEss1/Ptf1; NP_012551; 40), orthologs from *S. pombe* (SpPin1; CAA20742; 181), *C. albicans* (CaEss1; AAK00626; 180), *C. neoformans* (CnEss1; AAN03477; Ren *et al.*, submitted), *Drosophila melanogaster* (DmDodo; P54353; 179), *Xenopus laevis* (XePin1; AAF43897; 173; C. Wilcox and SDH, unpublished), and humans (HsPin1; NP_006212; 152) all contain a WW domain (dashed box) and complement loss-of-function mutations of Ess1 in yeast. Despite lacking a WW domain, parvulins from plants *Arabidopsis thaliana* (AtPin1; Q95L42; 188,223), *Malus domestica* (MdPin1; Q94G00; 188), and *Digitalis lanata* (DlPar13; Q9LEK8; 187) possess the same substrate specificity as Ess1 orthologs and complement Ess1 function in yeast. Specificity might be achieved by the four amino acid insertion (shaded in grey) unique to the plant parvulins (188). Human Par14 (HsPar14; Q94237; 187,189; M. Foehr, F. Fujimori and SDH, unpublished) has a distinct specificity due to the absence of a WW domain and fails to complement. Alignment was done using the ClustalW program (version 1.8) through the Baylor College of Medicine server. Asterisks indicate the two signature tryptophans in WW domains.

Table 2. Summary of genetic interactions between ESS1 and components of the transcription machinery

Gene	Function	Mutation	Relative growth of *ess1*^{ts} mutants at temperatures		Effect on growth defects of *ess1*^{ts} mutants	Reference
			Permissive (+++)	Restrictive (-)		
Stage-specific transcription factors						
SRB2	Mediator complex, helps initiation	Deletion	-	n.d.	Enhance	41
BYE1	TFIIS-like, possible negative elongation factor	Overexpression	+++	+++	Suppress	199
		Deletion	+	n.d.	Enhance	199
DST1	Yeast TFIIS, positive elongation factor	Overexpression	+	n.d.	Enhance	199
		Deletion	+++	+	Suppress	199
SPT5	Positive elongation factor	*spt5-194* or *spt5-242*	-	n.d.	Enhance	199
RSP5	Ubiquitin ligase, binds the CTD, degrades Rpb1 upon UV irradiation	Overexpression	+	n.d.	Enhance	205
CTD, its kinases and phosphatases						
RPB1	Largest subunit of RNA pol II, contains the CTD for accessory protein binding	Reduced level	-	n.d.	Enhance	41
		Increased level	+++	+	Suppress	205
		CTD truncations (dominant negative)	+/-	n.d.	Enhance	41
		CTD Ser5-to-Ala (1st half only)	+++	+	Suppress	197
SRB10	CTD kinase, inhibits initiation	Deletion	+++	++	Suppress	197
CTK1	CTD kinase, promotes elongation and termination	Reduced level (*ctk1*-/+)	+++	++	Suppress	197
		Overexpression	+	n.d.	Enhance	197
FCP1	CTD phosphatase, preferentially acts on Ser2	Overexpression	+++	++	Suppress	41
SSU72	CTD phosphatase, preferentially acts on Ser5	Overexpression	+++	+	Suppress	K. Shankarling & M. Hampsey, pers. comm.
Histone deacetylase complex and histone acetyl transferase						
SAP30	Sin3-Rpd3 HDAC component	Overexpression	+++	++	Suppress	41
RPD3	Histone deacetylase in the Sin3-Rpd3 HDAC	Deletion, or overexpression of dominant negative mutants	+++	++	Suppress	41
GCN5	Histone acetyl transferase	Overexpression	+++	++	Suppress	41
Other						
CTH1	Putative transcription factor, mRNA turnover	Overexpression	+++	+	Suppress	41

ESS1 interacts with *RPB1*, which encodes the largest subunit of RNA pol II; *ess1*^{ts} mutants are highly sensitive (at permissive temperature) to a reduced dosage of Rpb1, suggesting that Ess1 plays a positive role in transcription (41).

Ess1 appears to act during multiple steps in the transcription cycle. In addition to termination and 3' end processing functions (40,185), Ess1 has been linked genetically to initiation and elongation. Ess1 *ts*-mutants are lethal (at permissive temperature) when combined with a deletion of *SRB2* (41), which encodes a subunit of the mediator complex that stimulates initiation (196). In addition, *ess1* mutants are rescued by deletion of *SRB10*, which encodes a CTD kinase that inhibits initiation (197,198). These data suggest that Ess1 acts positively on initiation. Ess1 also seems to inhibit elongation (199), as first implied by the finding that one suppressor, Bye1 (bypass of Ess1), may be a negative regulator of elongation (199). Indeed, Ess1 genetically opposes the effect of the positive elongation factors Dst1 and the Spt4-Spt5 complex, and mutations in Ess1 increase resistance to the elongation inhibitor 6-azauracil (199). Importantly, the effect of Ess1 on individual steps of transcription may be independent, as for example, the termination defect in *ess1* mutant cells is not rescued by elongation-related suppressors (199).

While Ess1 appears to act on RNA pol II, its effects do not appear to be global. Ess1 affects the

expression of only a subset of genes tested (41). In microarray experiments, only about 5% of genes showed significant changes in expression. In addition, the effect of Ess1 on transcription varies; in *ess1* mutants, the expression of some genes increases whereas that of others decreases (41; XW, MA-R, JH and SDH, unpublished).

Mitotic arrest in *ess1* mutant cells is likely transcription-related. Ess1 may be required for the proper expression of genes required for progression through mitosis. Such genes, however, remain to be identified. The expression of Mih1, the yeast homolog of Cdc25, does not change in *ess1* mutant cells (41). The expression of Clb2 (cyclin B) is reduced in *ess1* mutants (41), however, since Clb2 is not essential in yeast, its reduced expression is unlikely to account for the mitotic arrest observed in *ess1* mutant cells.

5.4. Ess1 targets the CTD of RNA pol II

Ess1 has at least two known physical targets in the general transcription machinery: the CTD of RNA pol II and the Sin3-Rpd3 histone deacetylase complex (HDAC). The sequence of the CTD is a unique feature of RNA pol II, and serves as a binding platform for many accessory proteins required for transcription and mRNA processing (200-203). It consists of multiple repeats (26 to 27 in yeast) of the heptad sequence, YSPTSPS. The CTD is reversibly phosphorylated on Ser2 and Ser5 during transcription by CTD kinases and phosphatases (201,204). Phosphorylation on Ser2 or Ser5 generates Ess1 binding sites (pSer-Pro), two sites per heptad repeat. Indeed, Ess1 directly binds the phosphorylated CTD (41,195).

Ess1 also affects the function of the CTD, as supported by genetic evidence. Ess1 *ts*-mutants are more sensitive to truncations of the CTD (41). Ess1 genetically opposes Rsp5, a ubiquitin ligase that also binds the CTD via its WW domains but has a negative effect on RNA pol II transcription (205). Because the CTD serves as a platform for protein binding, Ess1 may affect the binding of various proteins to the CTD.

The current model for how Ess1 promotes transcription is as follows (Figure 4). Ess1 binds the phosphorylated form of the CTD and induces conformational changes by proline-directed isomerization. The WW domain targets Ess1 to the CTD via its high-affinity binding of pSer-Pro motifs, and the catalytic domain mediates the *cis-trans* conversion. By generating a variety of different conformers, Ess1 could change the affinity of various CTD-binding proteins for the CTD. In this way, Ess1 might control the assembly of different protein complexes on RNA pol II, and thereby play a key role in coordinating the multiple steps required during each transcription cycle. Because isomerization of the CTD is also likely to affect the binding of additional CTD kinases and phosphatases, this dependence may constitute a regulatory loop consisting of phosphorylation->isomerization->dephosphorylation. Thus, both covalent and non-covalent modification of the CTD may contribute to RNA pol II cycling.

The above model strongly predicts genetic interactions between Ess1 and CTD kinases and phosphatases. Indeed, *ESS1* is no longer essential in *srb10* deletion strains, and $ess1^{ts}$ mutants are suppressed by reducing the dosage of Ctk1, another CTD kinase (197). Thus, Ess1 may promote dephosphorylation of the CTD once these kinases have acted. Consistent with this idea, overexpression of Fcp1, a CTD phosphatase, suppresses $ess1^{ts}$ mutants, and Fcp1 enzymatic activity is necessary for suppression (41). This result suggests that Ess1-directed isomerization makes the CTD a better substrate for CTD phosphatases, and that when Fcp1 is overexpressed, the function of Ess1 is no longer required. Consistent with this idea, human Pin1 was shown to stimulate the phosphatase activity of yeast Fcp1 toward a GST-CTD fusion protein *in vitro* (206).

Which pSer-Pro motifs are being targeted by Ess1? Each CTD heptad repeat contains two pSer-Pro motifs (YSPTSPS). Ess1 *ts*-mutants are rescued by Ser5-to-Ala substitutions (intended to mimic the unphosphorylated state) in the first half of the CTD, suggesting that the role of Ess1 is to promote dephosphorylation of Ser5 (197). This function may be carried out by a newly identified CTD phosphatase, Ssu72, which shows a preference for dephosophorylating Ser5 (207; K. Shankarling and M. Hampsey, pers. comm.). Consistent with this, Ess1 mutants can be rescued by overexpression of Ssu72 (K. Shankarling and M. Hampsey, pers. comm.).

5.5. Ess1 may target other components of the transcription machinery

Another target of Ess1 in the transcription machinery is the Sin3-Rpd3 histone deacetylase complex. HDACs are recruited to the promoter of specific genes, and most often repress transcription (208). In a suppressor screen, Sap30, a component of the Sin3-Rpd3 HDAC, was found to rescue Ess1 *ts*-mutants (41). However, rescue is likely due to a decrease, rather than an increase, in HDAC activity, as *ess1* mutants are also rescued by the overexpression of Rpd3 dominant-negative mutants, or by deletion of Rpd3 (42). Consistent with this idea, *ess1* mutants are also rescued by overexpression of the histone acetyl transferase Gcn5, which presumably counteracts the effect of histone deacetylation (42,209). These genetic interactions suggest that Ess1 antagonizes the effect of HDAC. Consistent with this hypothesis, Ess1 enhances gene silencing at the rDNA array, which is also enhanced by mutations in the Sin3-Rpd3 complex (42). Affinity pull-down experiments revealed that Ess1 physically associates with the Sin3-Rpd3 complex, possibly via the Sin3 component (42). Interestingly, Sin3 contains several Ser-Pro motifs, which may serve as potential Ess1-binding sites.

Ess1 may have additional targets in transcription. One possible target is the elongation factor Spt5. Human Pin1 was reported to bind phosphorylated human Spt5 (210). This interaction may also occur in yeast, given the genetic interactions between Ess1 and Spt5 in yeast (199). In fact, Spt5 associated with Ess1 in a genome-scale protein binding study in yeast (211). However, since ySpt5 lacks the so-called CTR domains, which, in hSpt5, mediate the binding to Pin1 (210), the interaction in yeast likely occurs by a different mechanism. The relevance of this interaction

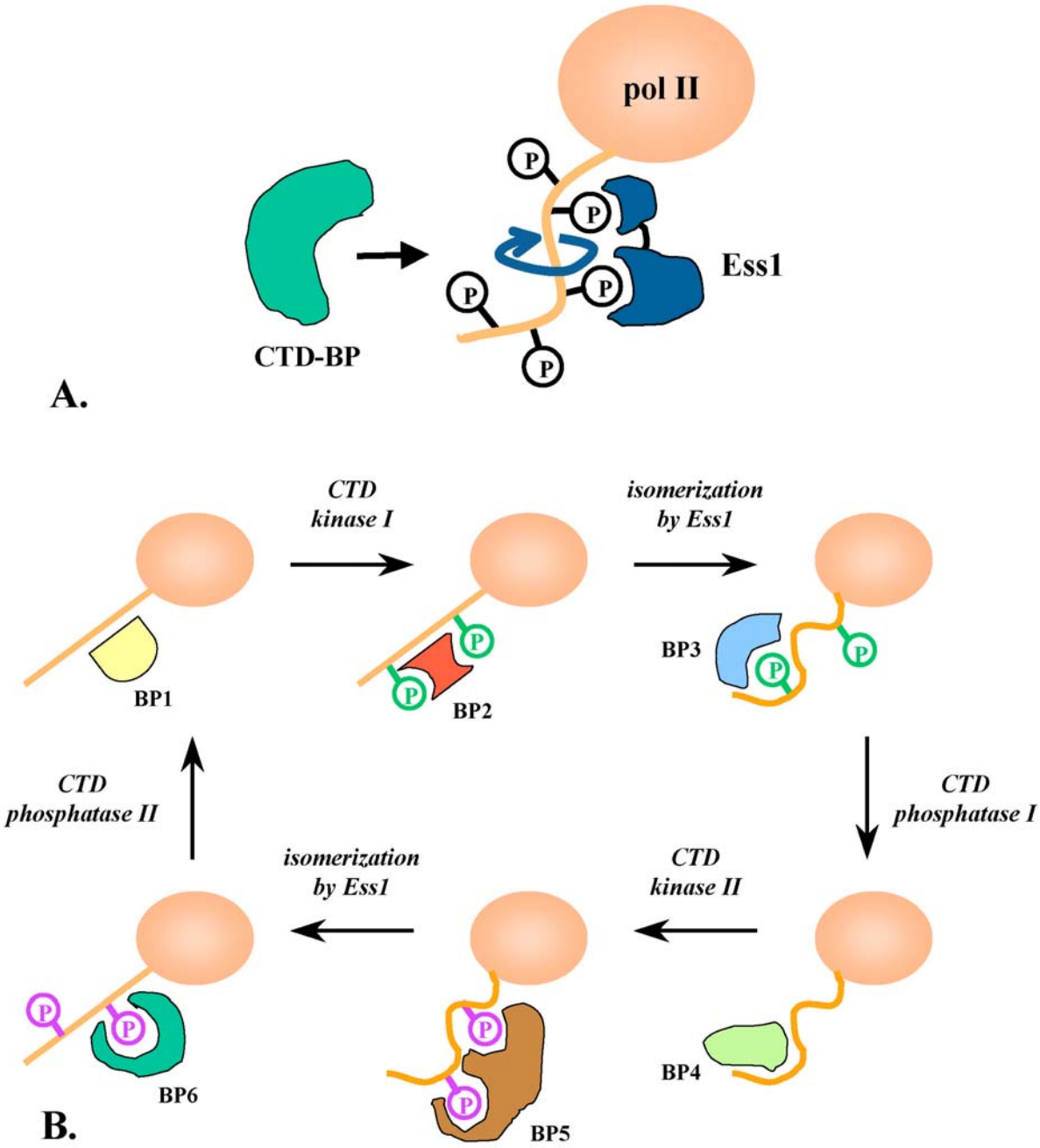

Figure 4. Model for Ess1 function in transcription by RNA polymerase II. (A) Ess1 binds to the phosphorylated form of the carboxy-terminal domain (CTD) of RNA pol II. Ess1 then catalyzes the *cis-trans* isomerization of Ser-Pro peptide bonds, causing conformational changes that signal the binding (or release) of CTD binding proteins (CTD-BP). Both domains of Ess1 (WW and PPIase) are depicted. They each bind pSer-Pro and are connected by a flexible linker. (B) Model for exchange of CTD binding proteins based on covalent modification (phosphorylation/dephosphorylation) and non-covalent modification (isomerization) of the CTD. Note that different CTD kinases (kinase I or II) may generate distinct phosphorylation patterns (*e.g.*, Ser2 *vs*. Ser5), which after isomeration by Ess1, may be dephosphorylated by different CTD phosphatases (phosphatase I or II). Exchange of CTD-binding proteins (BP) is thought to occur during all steps of the transcription cycle (not indicated) including pre-initiation, initiation, elongation, termination and mRNA processing steps.

remains to be determined.

5.6. Crosstalk between Ess1 and the cyclophilins

Cpr1, the major cyclophilin in yeast, was the only non transcription-related suppressor of *ess1* mutants identified in a multi-copy screen (41). The fact that Cpr1 overexpression substitutes for Ess1 function suggests a functional overlap (*i.e.*, a crosstalk) between two different

families of PPIase. This crosstalk extends to other cyclophilins (Cpr6 and Cpr7; see above sections), and is likely responsible for the viability of *ess1* deletion mutants in certain *S. cerevisiae* strain backgrounds (53), and in other fungi (see below). Crosstalk seems to be restricted to the cyclophilins since overexpression of Fpr1, the major FKBP in yeast, does not rescue *ess1* mutants (42). This may be because cyclophilins are relatively non-specific and thus may act upon Ess1 targets, whereas FKBPs, which have more restricted substrate specificities, cannot.

The overlapping functions between Ess1 and Cpr1 may be transcription-related. One potential common target is the Sin3-Rpd3 HDAC (see also Cpr1 section). Another potential common target is Cth1, a putative transcriptional regulator that may affect mRNA turnover (212,213). Cth1 is a multi-copy suppressor that requires the presence of Cpr1 to rescue *ess1* mutants (41,42).

5.7. Ess1 orthologs in other fungal species

Ess1 orthologs have been isolated from several other fungi, including the fission yeast *S. pombe* (SpPin1; 181), and pathogenic fungi, *C. albicans* (CaEss1; 180) and *C. neoformans* (CnEss1; Ren *et al.*, submitted). Ess1 is essential in *C. albicans* (180), but is not essential in *S. pombe* or *C. neoformans* (181; Ren *et al.*, submitted). The lack of essentiality in *S. pombe* or *C. neoformans* may be due to a functional compensation by cyclophilins, given the genetic crosstalk observed in budding yeast. Indeed, *ess1* deletion strains in these species are sensitive to the cyclophilin inhibitor cyclosporin A, indicating that they rely on the function of cyclophilins for survival (181; Ren *et al.*, submitted).

As in budding yeast, Ess1 also functions in mitosis in *S. pombe* and *C. albicans*. Although viable on its own, a deletion mutant of SpPin1 exacerbates the growth defects of cells that also carry mutations in the mitotic regulators Wee1 and Cdc25 (181). Similar to the equivalent budding yeast mutants, *C. albicans* mutants carrying a *ts*-mutation of CaEss1 arrest in mitosis at nonpermissive temperature (180). However, in contrast to *S. cerevisiae*, nuclear fragmentation was not observed in *C. albicans*, suggesting that nuclear fragmentation is not required for mitotic arrest in this organism (180). The fact that the effect of Ess1 on mitosis is conserved among various species suggests that either Ess1 serves as an important regulator of mitosis, or that mitosis is the cellular process most sensitive to the loss of Ess1.

In addition to its role in mitosis, studies in other species revealed the involvement of Ess1 in other pathways (see below). These functions cannot be easily studied in budding yeast either because the effect is masked by the mitotic-arrest phenotype or because the cellular processes involved are not present. The mechanisms of such species-specific functions are still unknown. But, given that regulated gene expression is required for these processes, the effect of Ess1 may involve its role in transcription.

In *S. pombe*, Ess1/Pin1 may function at the G_1/S transition. Overexpression of SpPin1 causes slow growth, and cells accumulate with a 1N DNA content, suggesting a G_1 delay (181). Consistent with this, deletion of SpPin1 is lethal when combined with a mutation in Cdc10, a component of a transcription complex required for the G_1/S transition (181).

In the pathogenic fungi *C. albicans* and *C. neoformans*, Ess1 functions in virulence. A decrease in Ess1 dosage in *C. albicans* (*ESS1*/*ess1*Delta) prevents the inducible formation of hyphae, indicating that CaEss1 is required for the morphogenic switch (180). Consistent with this, *ESS1*/*ess1*Delta mutant cells showed reduced virulence in mice (G. Devasahayam, V. Chaturvedi and SDH, unpublished). Similarly, *C. neoformans* Ess1 is important for the production of virulence-associated markers including melanin and urease, and Ess1 deletion strains also showed reduced virulence in mice (Ren *et al.*, submitted). Importantly, in both cases, because no significant differences in growth rates were observed between wild-type cells and the *ess1* mutants examined, the results above indicate an important role for Ess1 in virulence. Ess1 may therefore serve as a useful anti-fungal drug target. An advantage for using Ess1 as a drug target is that, because Pin1 is not essential in mice (191), it is not likely to be essential in humans. Therefore, drugs designed to inhibit Ess1 can reduce the virulence of the pathogen without harming the host.

5.8. Perspectives

The strong conservation of Ess1 through evolution and the ability of orthologs to complement in yeast, suggest that Ess1 functions by a similar mechanism in diverse organisms. Thus, the study of Ess1 in yeast provides a genetically tractable system in which to understand this mechanism and how it relates to *in vivo* function. While Ess1 orthologs have been implicated in a variety of diverse cell-signaling processes (*e.g.*, checkpoint control, apoptosis), many of these functions might ultimately be the result of changes in gene regulation. In fact, Pin1 may also control transcription via the CTD of RNA pol II in mammals; it was shown to bind the CTD and to affect its phosphorylation (214,215). In addition, the observed crosstalk between Ess1 and cyclophilins in budding yeast and other fungi may be conserved in evolution and underlie the non-essentiality of Ess1 in higher organisms.

One crucial question that remains to be further addressed is whether the PPIase activity of Ess1 is required for viability. It has been suggested that in some cases, PPIases act stoichiometrically rather than catalytically (*e.g.*, Nina A in flies and cyclophilin A in mammals; 216,217). In early attempts to address this question, *ESS1/PTF1* mutants were isolated based on defects in transcription termination and viability, and Ess1 purified from these mutants was shown to have decreased PPIase activity (185). Also, catalytically inactive mutations were engineered in Pin1 and these failed to complement *ESS1* function in yeast (152). However, conclusions from these studies were limited because other activities (*e.g.*, stoichiometric binding) were never assayed for the mutant proteins. In a more thorough study, a series of Pin1 mutants were assayed

in vitro for phospho-protein binding and PPIase activity, and were also tested for *in vivo* complementation. These analyses revealed a correlation between the PPIase activity and the ability to rescue an *ess1*^{ts} mutant, suggesting that the PPIase activity is required (218). Interestingly, the lethality of the *ess1*^{ts} mutant was also rescued by overexpression of $PP2A_1$, a phosphatase whose activity was stimulated by the PPIase activity of Pin1 *in vitro* (218), suggesting that the PPIase activity is required for isomerizing certain substrate proteins *in vivo*, resulting in enhanced dephosphorylation by phosphatases such as $PP2A_1$. Additional biochemical and genetic analysis of Ess1 mutants will be necessary to further test the hypothesis that the PPIase activity is required for viability and answer how much activity is sufficient for viability.

The essential role of Ess1 likely involves transcription. Ess1 seems to affect the expression of some, but not all, genes. Microarray analysis will be useful to identify "target" genes whose expression is Ess1-dependent. Identification of target genes will enable studies on the role of Ess1 in the expression of distinct target genes, and to dissect the precise mechanisms of Ess1 action. The set of target genes will likely include genes required for mitosis, which may be identified using genetic suppressors of *ess1* mutants, as all the suppressors rescue the mitotic lethality.

Current models envision that Ess1 functions by controlling the exchange of proteins that bind the CTD of RNA pol II. Although this model is supported by extensive genetic evidence, direct biochemical evidence is still lacking. It remains to be tested whether Ess1 controls the phosphorylation status of the CTD, and whether Ess1 controls the exchange of accessory proteins during transcription. ChIP assays (219), which allow the visualization of changes in CTD phosphorylation and accessory protein exchange during transcription *in vivo* (220,221), should allow these questions to be addressed directly. Together with studies on the requirement for the PPIase activity, the results may provide additional evidence for the hypothesis that conformational changes serve as a non-covalent signal to guide protein exchange in transcription.

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