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REVIEW

Functional diversity and pharmacological profiles of the FKBPs and their complexes with small natural ligands

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Abstract From 5 to 12 FK506-binding proteins (FKBPs) are encoded in the genomes of disparate marine organisms, which appeared at the dawn of evolutionary events giving rise to primordial multicellular organisms with elaborated internal body plan. Fifteen FKBPs, several FKBP-like proteins and some splicing variants of them are expressed in humans. Human FKBP12 and some of its paralogues bind to different macrocyclic antibiotics such as FK506 or rapamycin and their derivatives. FKBP12/(macrocyclic antibiotic) complexes induce diverse pharmacological activities such as immunosuppression in humans, anticancerous actions and as sustainers of quiescence in certain organisms. Since the FKBPs bind to various assemblies of proteins and other intracellular components, their complexes with the immunosuppressive drugs may differentially perturb miscellaneous cellular functions. Sequence–structure relationships and pharmacological profiles of diverse FKBPs and their involvement in crucial intracellular signalization pathways and modulation of cryptic intercellular communication networks were discussed.

Keywords FKBP · Tacrolimus · Sirolimus · FK506 · Rapamycin · PPIase

Abbreviations

AAC	Amino acid composition
BMP	Bone morphogenetic protein
CaN	Calcineurin
CsA	Cyclosporin A
CyP	Cyclophilin
ECM	Extracellular matrix
ER	Endoplasmic reticulum
ID	Sequence-similarity score
FKBP	FK506-binding protein
FKBD	FK506-like binding domain
MSA	Multiple sequence alignment
NFAT	Nuclear factor of activated T cells
p*I*	Piezoelectric point
RBD	Rapamycin-binding domain
Rpm	Rapamycin
TGF	Transforming growth factor
TOR	Target-of-rapamycin
TPR	Tetratricopeptide motif

Introduction

The superfamily of peptidylprolyl *cis/trans* isomerases (PPIases) consists of several discrete groups of proteins, namely cyclophilins, trigger factors, FKBPs, and Pin1 with parvulin-like proteins (see Figs. Fs1–2, supplementary material). The name "FK506-binding proteins (FKBPs)" was coined after the discovery, which has revealed that a cytosolic mammalian protein has the capacity to bind at a high affinity to the metabolites having immunosuppressive activity such as FK506 (tacrolimus) or rapamycin (sirolimus) [2, 3]. The FKBP that binds FK506 or rapamycin may be called immunophilin. The nominal mass of a monodomain

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mammalian FKBP is about 12 kDa, whereas the large multidomain FKBPs are fusions consisting from one to four consecutive FK506-like binding domains (FKBDs) and diverse sequence motifs such as calmodulin-binding, nuclear localization signal (NLS), EF-hand and Ca^{2+} binding, leucine zipper, transmembrane (TM), tetratricopeptide repeat (TPR), WH1, DNA binding, and myosin-like tail (Fig. 1) [4]. Figure Fs3 (supplementary material) shows a multiple sequence alignment (MSA) of 25 FKBDs from the human FKBPs and FKBP-like gene products. The overall sequence-similarity score (ID) calculated from the MSA25 is about 30 %, and similar values were obtained from analyses of the MSAs of the FKBPs encoded in various genomes [4]. In the often-applied nomenclature, the name FKBP is followed with the nominal mass of the protein in kDa, namely FKBP12 is a 12-kDa protein. The major drawback of this nomenclature is due to the lack of mass conservation of the FKBPs, which are expressed in various organisms. Thus, it would be more informative to correlate the FKBP with the gene encoding it and which has its orthologous forms expressed in disparate organisms [4]. For example, the abbreviation hFKBP12A is equivalent to hFKBP12, because the *fkbp12A* gene encodes it.

FKBP12, the principal intracellular binder of FK506 [2, 3], was purified from a cytoplasmic fraction of mammalian organs just several years after the macrocyclic hydrophobic antibiotic had been isolated from the strain *Streptomyces tsukubaensis* found in a soil sample from Japan [5]. Rapamycin (Rpm) is an antibiotic structurally related to FK506, which was isolated from the bacterial strain *Streptomyces hygroscopicus* found in a soil sample from Easter Island (Rapa Nui) [6]. It was shown that rapamycin has the capacity to inhibit the development and proliferation of malignant cells [7]. Since then, several structurally related molecules have been isolated from natural sources and some of them were chemically modified, namely ascomycin (FK520), purified from *Streptomyces hygroscopicus* var. *ascomyceticus* [8, 9], temsirolimus (CCI-779) [10, 11], everolimus (RAD-001) [12], a synthetic tetrazol derivative of rapamycin called zotarolimus (ABT-578) [13] (see Fig. 2), and other macrocycles [14]. Smaller-sized compounds mimicking the binding patches of FK506 or rapamycin (called peptidomimetics) have been synthesized and shown to bind to hFKBP12A but with a somewhat weaker affinity then the macrocyclic antibiotics [15, 16].

The hydrophobic macrocycles, which are shown in Fig. 2, are soluble in diverse organic solvents such as DMSO, chloroform, or ethanol. They are sparsely soluble in aqueous solution. If a soluble sample of any of these antibiotics is added to aqueous solution containing the hFKBP12A, the macrocycle is quickly sequestered by the immunophilin and the complex remains water-soluble. If either FK506 or rapamycin is bound to hFKBP12A, it rigidifies the long loop at the C-terminus [17], which contains the –AYG– triad (hFKBP12A) whose sequence variants constitute a hallmark of all the FKBPs expressed in different phyla [4]. Binding of FK506 to FKBP12A has a negative enthalpy and a positive entropy due to displacement of two

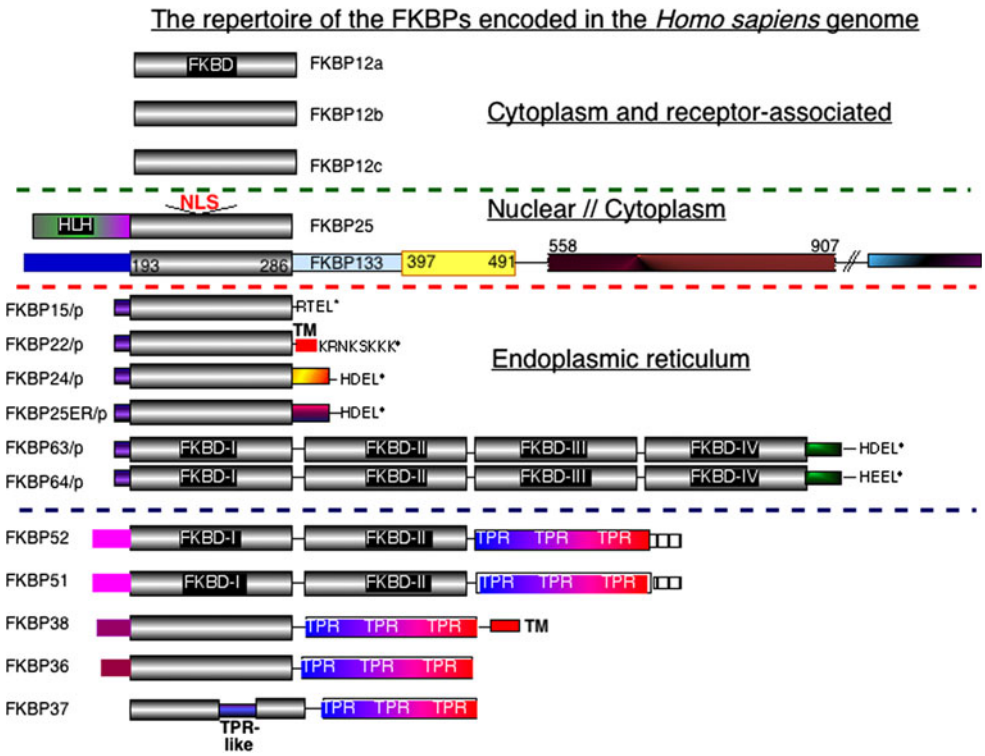
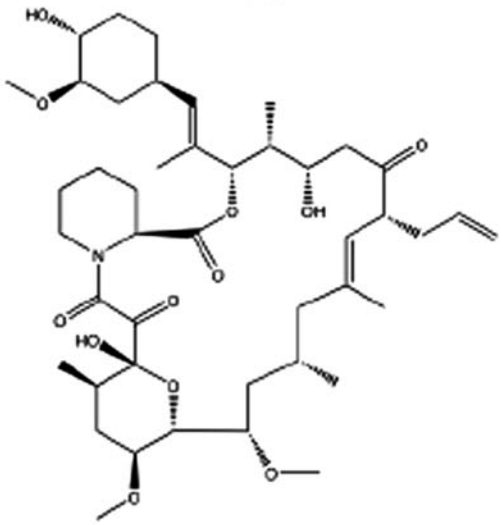
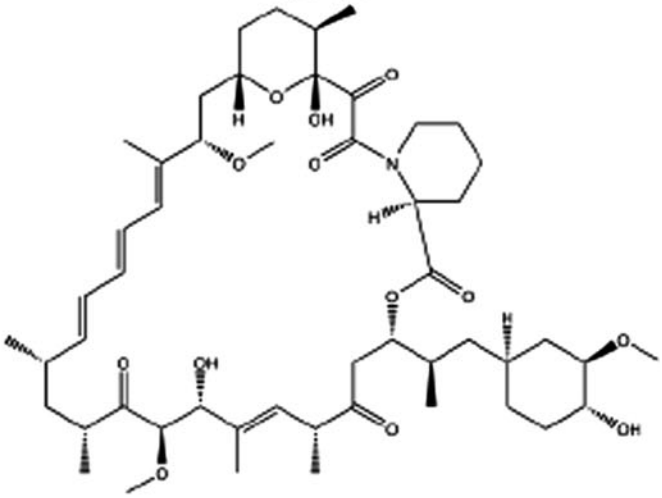


Fig. 1 Schematic domain's organization of the human FKBPs. FKBDs are indicated as *grey rectangles*; the N-terminal signal domains in the ER-anchored FKBPs are shown as *violet rectangles*; N-terminus of FKBP51, FKBP52, FKBP38, and FKBP36 are shown as *rose rectangles*; transmembrane segments in FKBP22P and FKBP38 are shown as *red rectangles*; *NLS* nuclear signalization signal, *TPR* tetratricopeptide repeat motif; the C-terminal AA-tetrads of the ER-specific FKBPs were explicitly written; domains of the FKBP133: pleckstrin homology-like (PH) domain (70–168 AAs; known as WH1 domain) is in a *blue rectangle*, DNA translocase FtsK domain (*yellow rectangle*) and chromosome segregation ATPase domain (558–907 AAs; *brown rectangle*)

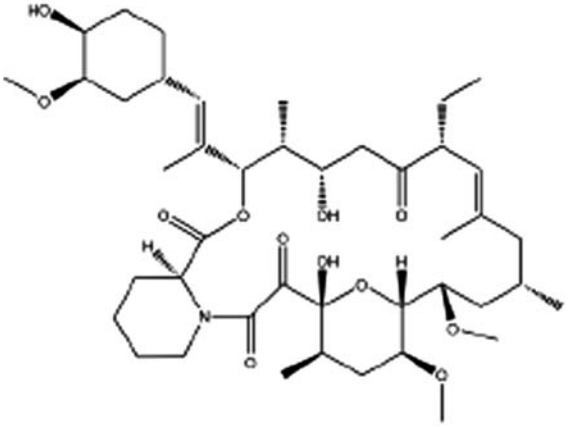
FK506



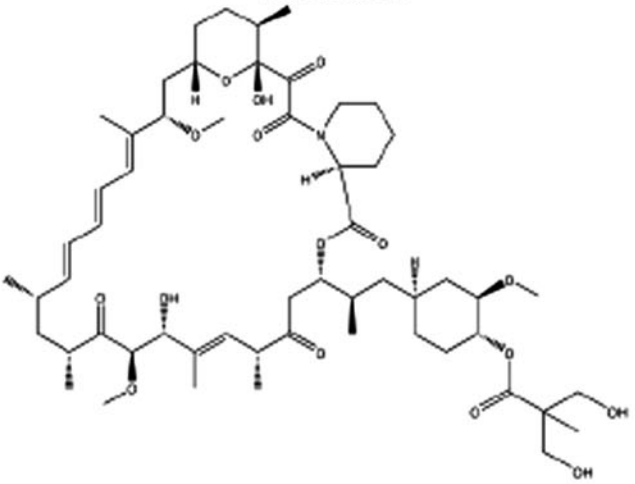
Rapamycin



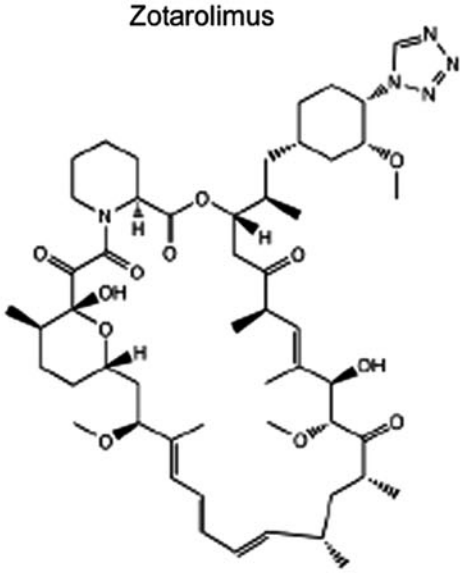
Ascomycin



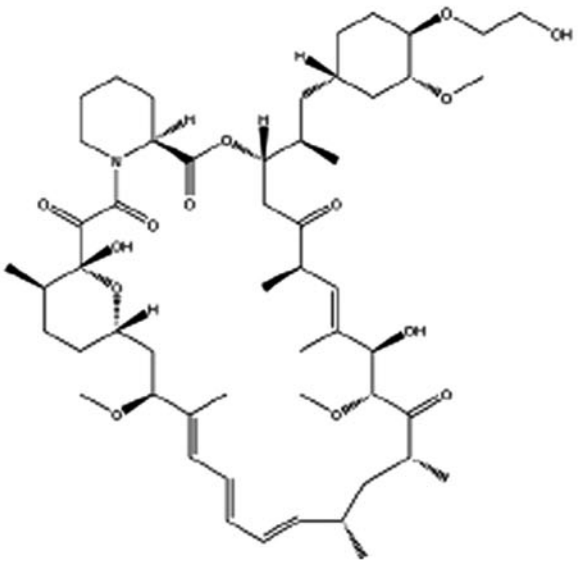
Temsirolimus



Zotarolimus



Everolimus



◀ **Fig. 2** Molecular structures of some macrocylic antibiotics, which bind to diverse FKBPs; FK506 (CAS 104987-11-3; $C_{44}H_{69}NO_{12}$, $m = 804.0182$ g mol^{-1}); rapamycin (CAS 53123-88-9, $C_{51}H_{79}NO_{13}$, $m = 914{,}1710$ g mol^{-1}); ascomycin (CAS 11011-38-4, $C_{43}H_{69}NO_{12}$, $m = 792.01$ g mol^{-1}); temsirolimus (CAS 162635-04-3, $C_{56}H_{87}NO_{16}$, $m = 1039.28$ g mol^{-1}); zotarolimus (CAS 221877-54-9, $C_{52}H_{79}N_5O_{12}$, $m = 966.21$ g mol^{-1}); everolimus (CAS, 159351-69-6, $C_{53}H_{83}NO_{14}$, $m = 958.224$ g mol^{-1})

water molecules bound to the hydroxyl of Y82 (hFKBP12A), the central residue in the –AYG– triad (Fig. Fs3); about 50 % of the surface of the antibiotic is hidden in PPIase cleft [18]. Figure 3 shows the X-ray structure of the hFKBP12A/rapamycin complex with the explicitly shown amino acid residues in PPIase cleft (AAs, displayed as spheres) that interact with the drug [19]. Sirolimus or tacrolimus bind at a high affinity to different mammalian FKBPs [1, 15], whereas human FKBP36, FKBP133, and AIP (FKBP-like protein) do not effectively bind the immunosuppressive antibiotics. The macrocycles also bind to some FKBPs from lower eukaryotes, for example the cytoplasmic FKBP12 from baker's yeasts (*Saccharomyces cerevisiae*) is a high-affinity binder of FK506 or rapamycin [1]. Even some of the prokaryotic FKBPs are strong binders of immunosuppressive drugs [1, 20].

The FKBPs and the other members of the PPIase superfamily of proteins have been found in different cellular compartments and organelles such as the cytoplasm, endoplasmic reticulum (ER), nucleus, mitochondria, polyribosomes, various large molecular assemblies such as ryanodine receptors, and other membrane-anchored entities [1]. Here we discuss only some of the principal functional and structural features of the FKBPs. Firstly, we have analyzed sequence–structure relationships in the multigene family of the FKBPs encoded in various genomes starting from lower eukaryotes, passing through marine organisms from which vertebrates sprouted, and ending on diverse functional aspects of the human repertoire of the FKBPs. Secondly, we have discussed diverse functional attributes of the FKBPs. Thirdly, we have analyzed several structural and functional aspects of the FKBP12A/(macrocyclic antibiotic) complexes, which induce crucial pharmacological effects such as immunosuppression, anti-inflammatory action in some dermatological disorders, anticancer proprieties, and as substances that maintain quiescence of some organisms. Hypotheses on the morphogen-driven signalization networks that could be under the control of the macrocyclic drugs and the FK506-induced protection of neuronal tissues from ischemia-induced oxidative stress and regeneration of neurons after ischemia have been also briefly discussed. An additional list of references grouped according to diversified functional features of the FKBPs and some of cellular activities that are controlled by them can be found in the supplementary material.

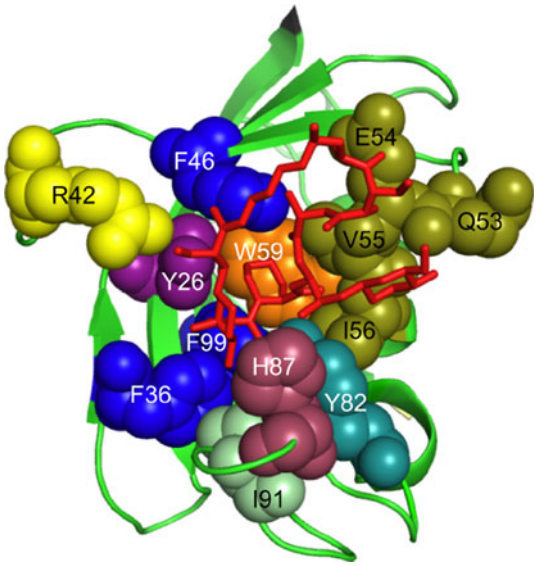


Fig. 3 X-ray structure of the hFKBP12A (*green ribbon*) bound to rapamycin (*orange sticks*; 2DG3.pdb) with the indicated AAs, which interact with the antibiotic at distances $d \leq 4.5$ Å [19]

Chimerical organization and duplication of genes coding for the FKBP family of proteins in various species

Analyses of some sequence attributes of the FKBPs

The following two simple-in-use bioinformatics tools for sequence analyses were used, namely the BLAST program [21] for analyses of various genomic databases [22] using the sequences of the FKBDs from the human FKBPs as input templates, and the poly-analysis of sequence quota (PolaSQ) algorithm, which takes into account conservation levels of several sequence attributes [23] calculated from the MSAs produced with the ClustalX program [24]. The algorithm utilizes sequence attribute clustering schema, which guides formation of discrete groups of proteins that sustain coherent functional profiles. The algorithm is especially fitted for analyses of multigene families of proteins encoded in various genomes provided that some of the sequence attributes had been conserved during evolution of species. We use the following sequence attributes for clustering of proteins' domains: (1) consensus sequence; (2) the piezoelectric point (p*I*); (3) hydrophobicity profile; (4) the amino acid composition (AAC); (5) distribution of AA bulkiness along the polypeptide chain [4, 23].

Table 1 summarizes the FKBP repertoires encoded in several different genomes starting from unicellular yeasts, passing via some marine organisms whose genomic databases are available via the National Centre of Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov)

Table 1 Repertoires of the FKBPs expressed in several organisms at different levels of development

Organism	FKBPs	Cytoplasm	Nucleus/nucleolus/ cytoplasm	Endoplasmic reticulum (foldases/chaperones)	TPR and other motifs (different cellular localizations)
Schizosaccharomyces pombe (yeast)	3	SpFKBP12 (→*fkbp1a*)–NP_595257	SpFKBP39 (*Fpr3*)–NP_596694 SpFKBP40 (*Fpr4*)–NP_594535		
Saccharomyces cerevisiae (baker's yeast)	4	ScFKBP12 (→*fkbp1a*)–NP_014264	ScFKBP43 (*Fpr3*)–NP_013637 ScFKBP45 (*Fpr4*)–NP_013554	ScFKBP13 (→*fkbp2*)–NP_010807	
Monosiga brevicollis (choanoflagellates)	5	MbFKBP49 (→*Cefkb-6*)–EDQ90188	MbFKBP23 (→*Fpr3*)–EDQ88301	MbFKBP17 (→*fkbp2*)–EDQ85159 MbFKBP21 (→*fkbp11*)–EDQ85838	MbFKBP49 (2 FKBDs; →*fkbp4*)–EDQ88213
Trichoplax adhaerens (placozoa)	10	TaFKBP12 (→*fkbp1a*)–XP_002109901	TaFKBP120 (→*fkbp15*)–XP_002109958	TaFKBP10 (→*fkbp2*)–XP_002111423 TaFKBP12 (partial, *fkbp10*)–XP_002113946 TaFKBP32– (ER-protein) XP_002119047 TaFKBP15 (*fkbp2*)–XP_002113582	TaFKBP33 (→*fkbp6*)–XP_002118278 TaFKBP37 (→*AIP*)–XP_002114988 TaFKBP48 (→*fkbp5*)–XP_002108502 TaFKBP64 (→*fkbp8*)–XP_002111046
Hydra magnipapillata (cnidarian)	13	HpFKBP11 (→*fkbp1a*)–XP_002155592	HpFKBP16 (→*fkbp3*) HpFkbp95 (→*fkbp15*)–XP_002166401	HpFKBP16 (→*fkbp2*)–XP_002154951 HpFKBP24 (*fkbp7*)–XP_002165957 HpFKBP30a (partial, *fkbp9*)–XP_002156712 HpFKBP30b (partial, *fkbp10*)–XP_002164365 HpFKBP32 (*E. coli-like* FKBP)–XP_002162221 HpFKBP43 (partial, *fkbp9*)–XP_002163230	HmFKBP22 (partial, *fkbp8*)–XP_002160252 HmFKBP23 (partial, *fkbp4*)–XP_002154321 HmFKBP46 (*fkbp5*)–XP_002164353 HmFKBP53 (*fkbp6*)–XP_002167164
Nematostella vectensis (cnidarian, eumetazoan)	13	NvFKBP10 (→*fkbp1a*)–XP_001626676	NvFKBP25 (→*fkbp3*)–XP_001625667 NvFKBP40 (→*fkbp15*)–XP_001627882	NvFKBP9 (partial, *fkbp11*)–(XP_001623432) NvFKBP14 (*fkbp2*)–XP_001632345 NvFKBP22 (*fkbp7*)–XP_001639561 NvFKBP24 (partial, *fkbp9*)–EDO44761 NvFKBP22 (XP_001626518)	NvFKBP37 (*AIP*)–EDO41295 NvFKBP45 (*fkbp6*)–XP_001636239 NvFkBP54 (*fkbp5*)–XP_001635270 NvFKBP40 (XP_001627882) NvFKBP24 (EDO44761)
Ciona intestinalis (sea squirt, urochordata, probably one of the closest invertebrate to *Homo sapiens*)	8	CiFKBP10 (→*fkbp1a*)–CAC82550	CiFKBP24 (→*fkbp3*)–XP_002128086 CiFKBP107 (→*fkbp15*)–XP_002125408	CiFKBP15 (*fkbp2*)–XP_002122987 CiFKBP22 (*fkbp7*)–XP_002127819 CiFKBP65 (*fkbp9*)–XP_002128827	CiFKBP43 (*fkbp6*)–XP_002128893 CiFKBP68 (*fkbp8*)-XP_002119960

Table 1 continued

Organism	FKBPs	Cytoplasm	Nucleus/nucleolus/ cytoplasm	Endoplasmic reticulum (foldases/chaperones)	TPR and other motifs (different cellular localizations)
Strongylocentrotus purpuratus (sea urchin, echinodermata)	9	StpFKBP8 (→*fkbp1a*)–XP_001199232	StpFKBP61 (→*Fpr3*)–XP_791717	StpFKBP15 (→*fkbp2*)–XP_785999 StpFKBP21 (*fkbp11*)–XP_001177639 StpFKBP23 (*fkbp7*)–XP_781995	StpFKBP23 (partial; →*fkbp6*)–XP_001192603 StpFKBP37 (→*AIP*)–XP_788955 StpFKBP42 (→f*kbp8*)–XP_792687 StpFKBP47 (2FKBDs; →*fkbp4*)–XP_781282
Branchiostoma floridae (lancelet, Cephalochordata, invertebrate, same basic body plan as in vertebrate)	12	BfFKBP14 (→*fkbp1*) XP_002586543 BfFKBP89 (unknown)–XP_002590072	BfFKBP47 (→*Fpr3*)–XP_002595400 BfFKBP78 (→f*kbp15*)–XP_002609450	BfFKBP15 (→*fkbp2*)–XP_002610751 BfFKBP17 (→*fkbp11*)–XP_002591360 BfFKBP27 (→fkbp7)–XP_002606030 BfFKBP31 (→*fkbp7*)–XP_002606032 BfFKBP35 (→*fkbp14*)–XP_002594856	BfFKBP30 (→*AIP*)–XP_002612027 BfFKBP35 (→*fkbp6*)–XP_002594856 BfFKBP43 (→*fkbp8*)–XP_002601637
Caenorhabditis elegans (nematode)	9	CeFKBP11 (→*fkbp1a*)–AA68610 or NP_001021722		CeFKBP15 (*fkb-1* → *fkbp2*)–NP_502056 CeFKBP29a (2 FKBDs; *fkb-5*)–NP_491258 CeFKBP29b (2 FKBDs; *fkb-3*)–NP_504835 CeFKBP29c (2 FKBDs; *fkb-4*)–NP_506197 CeFKBP32 (2 FKBDs; *fkb-8*)–NP_493256 CeFKBP33 (2 FKBDs; *fkb-8′*)–NP_493257 CeFKBP36 (1 FKBD; *fkb-7*)–NP_492792	CeFKBP48 (2 FKBDs; *fkb-6* → *fkbp4*)–NP_493256
Drosophila melanogaster (fruit fly)	8	DmFKBP12 (*FK506-bp2* → *fkbp1a*)–NP_523792	DmFKBP39 (*FK506-bp1* → *Fpr3*)–NP_524364	DmFKBP14 (→*fkbp2*)–NP_650101 DmFKBP23 (*Fkbp13* → *fkbp7*)–NP_726074	DmFKBP51 (shutdown) (*shu* → *fkbp6*)–NP_611837 DmFKBP38 (*AIP*)–NP_727574 DmFKBP44 (CG5482 → *fkbp8*)–XP_080945 DmFKBP48 (2FKBD; FKBP59 → *fkbp4*)–NP_524895

Table 1 continued

Organism	FKBPs	Cytoplasm	Nucleus/nucleolus/cytoplasm	Endoplasmic reticulum (foldases/chaperones)	TPR and other motifs (different cellular localizations)
Homo sapiens (primate)	15 + 3 FKBP-like	hFKBP12a (*fkbp1a*)–NP_000792 hFKBP12b (*fkbp1b*)–NP_004107 hFKBP12c (*fkbp1c*)–CAH71018	hFKBP25 (*fkbp3*)–NP_002004 hFKBP133 (*fkbp15*)–NP_056073	hFKBP13 (*fkbp2*)–NP_004461 hFKBP19 (*fkbp11*)–NP_057678 hFKBP22 (*fkbp14*)–NP_060416 hFKBP23 (*fkbp7*)–NP_851939 hFKBP63 (4 FKBDs; *fkbp9*)–NP_009201 hFKBP64 (4 FKBDs; *fkbp10*)–BAB20974	hFKBP36 (*fkbp6*)–NP_001128683 hFKBP38 (*fkbp8*)–NP_036313 hFKBP51 (2 FKBDs; *fkbp5*)–NP_004108 hFKBP52 (2 FKBDs; *fkbp4*)–NP_002005 FKBP-like hFKBP37 (*AIP*)–U31913 hFKBP43 (*AIPL1*)–Q9NZN9 hFKBP38L (*FKBPL*)

The repertoires of the FKBPs expressed in *H. sapiens*, *C. elegans*, *D. melanogaster*, *S. saccharomyces*, *Schizosaccharomyces pombe* were fully refined, whereas the contents of the FKBPs encoded in the other genomes may change after further refinement of the genomic databases is made; the sequences of the FKBPs were available in the NCBI server on May 3, 2012. Some FKBPs have either incomplete sequences or some inserts that are probably artifacts. The NCBI database codes given in the table and some sequence attributes can be found in the supplementary materials (MSA500.out)

server [22], and ending with the FKBPs encoded in the three reference genomes, namely the fruit fly *Drosophila melanogaster* (Dm), the nematode *Caenorhabditis elegans* (Ce), and *Homo sapiens* (h). Due to the provisional nature of the recently completed genome projects of the marine organisms [25–31], those repertoires may undergo some revisions. The organisms were ordered from the least complex at the top of Table 1 to the most developed at the bottom; the order is based on several phylogeny trees comprising the marine organisms [25–31]. An MSA of 500 FKBDs from the FKBPs expressed in various organisms ranging from prokaryotes to mammals is shown in the MSA500 file (supplementary material), whereas in Fig. Fs4 (supplementary material) is given the information entropy (I_e) graph derived from it [23]. For 108 sequence positions of the hFKBP12A used as an arbitrarily chosen reference in the MSA500, 21 positions have $I_e < 1.0$, which indicates that about 20 % positions of the highly diversified sequences of the FKBDs remain highly conserved. Those sequence positions are populated either with G residues or the AAs forming functional consensus sequence. Even if the –AYG– triad is in the long flexible loop at the C-terminal part of the protein (called 80s loop), the physical–chemical profile of the triad remains well conserved in the multigene family of the FKBPs. The triad is probably a specific recognition motif for certain endogenous targets. The overall sequence-similarity score (ID) calculated from the MSA25 comprising the human FKBDs is lower than its average (IDs 30 vs. 42 %, respectively) calculated from the MSA500. The MSA500 is biased by a large number of sequences of the monodomain FKBPs from disparate organisms, which have a lesser diversification level [4, 23] than the full set of the FKBDs from the human FKBPs. Our attempts to make phylogenetic trees using the sequences of the FKBP12s or their paralogues that are expressed in the disparate organisms listed in Table 1 failed to give congruent trees with those published in the genome sequencing papers [25–31], which is another example proving that the sequences of the multigene families of proteins are not suitable for construction of the tree of life [32].

Diversification, duplication, and loss of genes encoding the FKBPs in the genomes of disparate species

The FKBPs are encoded in the genomes of miscellaneous prokaryotes and archaea [1, 4]. For example, four FKBP-like proteins are expressed in the *Escherichia coli* [34], whereas the lower eukaryote *Schizosaccharomyces pombe* (Sp) expresses three FKBPs. Four FKBPs are expressed in *S. cerevisiae* (Sc) cells, namely a cytosolic form (ScFKBP12), a small monodomain FKBP is in the endoplasmic reticulum (ScFKBP14), and the remaining two FKBPs are in the nuclear space, such as ScFKBP43 in the

nucleolus (NP_013637, GI:6323566) and ScFKBP45 in the nucleus (NP_013554, GI:6323482) [4, 34]. The FKBPs' repertoires underwent expansion in the genomes of various organisms whose developmental level is higher than that of a unicellular yeast cell. For example, five different FKBPs are expressed in the multicellular choanoflagellate *Monosiga brevicollis* [25], which could have been an intermediary organism on the developmental pathway to species with more elaborated internal body plans. The repertoires of the FKBPs expressed in the placozoan *Trichoplax adhaerens* [26], the freshwater polyp *Hydra magnipapillata* [27], the starlet sea anemone *Nematostella vectensis* [28], the sea squirt *Ciona intestinalis* [29], the Florida lancelet *Branchiostoma floridae* [30] and the sea urchin *Strongylocentrotus purpuratus* [31] are nearly twice as large as that of the *M. brevicollis* (Mb), whereas the number of the FKBPs was tripled in genomes of vertebrates if compared to the FKBPs encoded in the choanoflagellate genome. Our analyses revealed that some rearrangements of the chimerical organization of the multidomain FKBPs and losses of their genes had taken place during the evolution of the species. For example, the numbers of the expressed FKBPs in *C. elegans* or *D. melanogaster* are smaller than those that are encoded in the genomes of the marine organisms cited in this review.

Monodomain and ER-anchored FKBPs

Genomes of eukaryotic organisms encode at least one cytosolic and one endoplasmic reticulum-anchored (ER) form of the FKBP whose PPIase cavity retains a good level of sequence conservation [1, 4]. A comparable sequence conservation level was estimated for the PPIase cavity in the cyclophilin family of proteins [35]. In both families of proteins, the cavity consists of several polar amino-acid side chains that are crucial for X-Pro *cis*/*trans* isomerization activity, and which are surrounded by a hydrophobic network of aromatic side chains. Even if the monodomain cytosolic FKBP is a hydrophilic protein, its hydrophobic PPIase cavity is a good binding site for different hydrophobic side chains of AA residues, diverse peptidomimetics, and some small-sized natural products.

One ER-anchored FKBP is expressed in *S. cerevisiae* [34] whereas among the five FKBPs encoded in the *M. brevicollis* genome, two of them reside in the ER. Likewise, two ER-embedded FKBPs are expressed in *D. melanogaster*, whereas a larger variety of the FKBPs is expressed in the ER of *C. elegans*. Monodomain ER-anchored FKBPs have a higher hydrophobicity than their small cytosolic counterparts. Some of the ER-anchored FKBPs have at their C-terminus additional sequence motifs such as EF hand or inositol-binding domain, whereas the large ER-anchored FKBPs are fusions made of two to four consecutive FKBDs [1]. Each FKBD in the ER-anchored FKBPs has two well-conserved cysteines that may form a disulfide bridge [36].

TPR motifs-containing FKBPs

TPR motif-containing FKBPs are fusions consisting of either one or two consecutive FKBDs and three TPR motifs, which are followed with an α-helical segment that, in some cases, may contain calmodulin-binding motif (CaM). TPR motif-containing FKBPs are encoded in the genomes of some fungal organisms [37], and in the genomes of all the species discussed herein. For example, one of the five FKBPs encoded in the *M. brevicollis* genome contains N-terminal FKBD followed with three consecutive TPR motifs. The PolaSQ algorithm clustered this chimerical FKBP with the fkb-6 protein expressed in *C. elegans* [38] and with the FKBDs from hFKBP52. BLAST analyses revealed that the aryl hydrocarbon receptor-associated protein (the *AIP* gene) is encoded in genomes of various invertebrates, such as *T. adhaerens* (Ta), *N. vectensis* (Nv), *S. purpuratus* (Stp), *B. floridae* (Bf), *C. elegans*, or *D. melanogaster* (Table 1). All these AIP proteins have one FKBD, which may have an extra α-helical segment inserted at the long 80s loop of the FKBD (Fig. 1) whereas three TPR motifs are at the C-terminus (see Fig. Fs5 in supplementary materials). PolaSQ analyses showed that the sequence attributes of the FKBDs in the AIP proteins, which appeared in some ancestral invertebrates, have well-conserved sequence attributes throughout the evolution of the species.

Nuclear FKBPs

The chimerical organization of the nuclear FKBPs expressed at lower eukaryotes (yeasts and alike) was retained only in some of the marine organisms analyzed here. For example, PolaSQ analyses revealed that the FKBD of MbFKBP23 has similar physical–chemical sequence attributes with the nuclear FKBPs expressed in baker's yeasts, namely that its overall p*I* is below 7, its FKBD has a basic p*I*, although its N-terminal nucleolin-like segment is shorter than those in the *Fpr3* and *Fpr4* genes of *S. cerevisiae*. The latter two encode negatively charged proteins, which have nucleolin-like motifs at their N-terminus and one basic FKBD at their C-terminus. Likewise, a 61-kDa protein from *S. purpuratus* (StpFKBP61, XP_0791717), a 47-kDa protein from *B. floridae* (XP_002595400), and a 38-kDa protein from *D. melanogaster* (DmFKBP38.3) with their nucleolin-like N-terminus, NLS signals and an FKBD at the C-terminus have analogous construction to the yeast Fpr3p and Fpr4p proteins. The proteins encoded by the *Frp3* and *Frp4* genes may function as RNA chaperones and foldases that are

involved in some events controlling the cell cycle [40–44]. It has been shown that the N-terminal nucleolin-like domain of SpFKBP39 has an in vitro histone chaperone activity and has the capacity to silence the ribosomal DNA (rDNA) locus [40]. However, mutational analyses have revealed that the C-terminal FKBD of SpFKBP39 also has a crucial contribution to silencing the rDNA locus [40]. Analyses of the genomic databases indicate that the gene coding for FKBP25 was probably generated in some marine organisms. For example, our analyses indicate that *C. intestinalis* or *N. vectensis* express the same set of the FKBPs as the mammalian organisms [4]. What gain-of-function had been achieved and which driving force had been involved in the genetic drift from the FKBPs with nucleolin-like N-terminal domain towards FKBP25-like protein that has positively charged unique N-terminal domain remain enigmatic. It is unknown whether the functional profiles of the Frp3p and Frp4p in budding yeasts and the products of the orthologous genes expressed in some invertebrates could have been retained by the FKBP25s.

A large multidomain nuclear FKBP is encoded in the *T. adhaerens*, *H. magnipapillata*, and *C. intestinalis* genomes. It is orthologous to the human *fkbp15* gene coding for the large hFKBP133 [39]. To what extent the domain structure of the *fkbp15* gene in these three marine organisms is similar to that of their human counterpart will be established when their full sequences become available. FKBP133 seems to be involved in endocytic transport and microtubule dynamics [33, 39].

Unique genomic constructs containing FKBDs

Our analyses revealed a discrete conservation of specific functional traits that were inherited by some of the marine organisms from some prokaryotic species. For example, HmFKBP23 has a C-terminal typical for the SlyD protein from *E. coli* (P0A9L1; GI:71162376) [34] and related genes in the prokaryotes, whereas MbFKBP49 is a fusion protein containing a basic FKBD and a SUI1/eIF1 RNA binding domain; the latter is also known as a density-regulated protein that appears as a separate gene in miscellaneous vertebrates. Although the *D. melanogaster* genome encodes an orthologue of the *Frp4* gene from *S. cerevisiae* [4], it is absent in the nematode *C. elegans* as well as in many other invertebrates. So far, no orthologues of the *Frp3* and *Fpr4* genes were detected in the genomes of the vertebrates.

Distribution of hydrophobicity versus p*I*s in the human FKBPs

PolaSQ analyses of several MSAs comprising the FKBDs from various species have shown an extraordinary conservation of their sequence attributes throughout the evolution of living species [4, 23]. This could imply that the genetic drift causing significant alterations of physical–chemical nature of those attributes in each group of the FKBPs had been subjected to a strong functional criterion at the dawn of evolution of multicellular species, which have been preserved up to the mammalian FKBPs [4, 23]. Figure 4 shows a distribution of the overall hydrophobicity indexes (HIs) versus the p*I*s of the FKBDs of the human FKBPs [4]. The HI indexes (expressed in %) indicate the number of AAs being in the hydrophobic segments calculated with one of the hydrophobicity scales [48]. The HIs of the FKBDs considerably differ from each other, namely an exceptionally high hydrophobicity have the FKBDs of

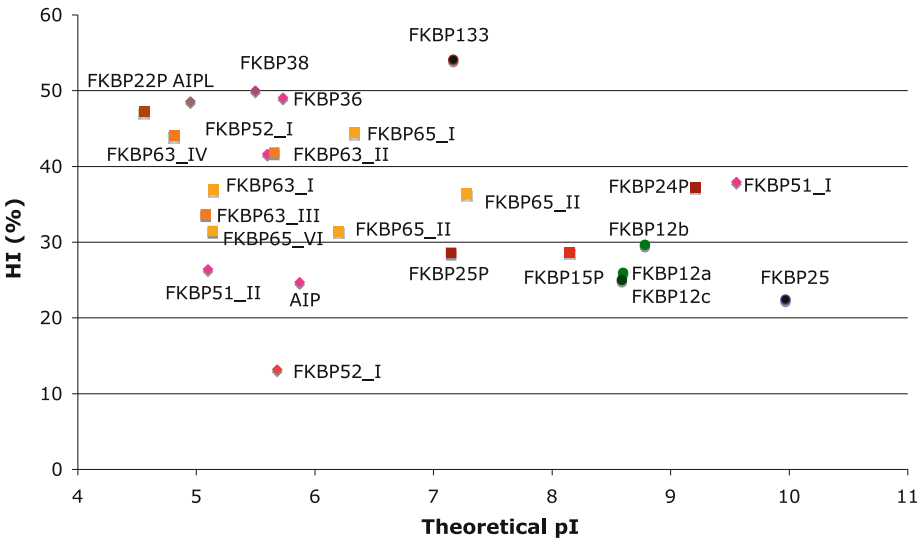


Fig. 4 Distribution of the overall hydrophobicity indexes (HI) versus the p*I*s calculated for the FKBDs of the human FKBPs; all the data are for the sequences aligned in Fig. Fs3; the HIs were calculated with a nine-residue moving frame using the [48]; the FKBDs of the ER-anchored FKBPs are shown as *squares*; the FKBDs from cytosolic and nuclear FKBPs are shown as *circles*, whereas the FKBDs from TPRs motif-containing FKBPs are shown as *diamonds*

FKBP36, AIPL1, FKBP38, and FKBP133, whereas a low value of the HI was estimated for the FKBD_I of hFKBP52 and FKBP25. FKBP25 has a dual cellular localization (nuclear/cytoplasm) and is also the most hydrophilic FKBP with the basic p*I*, whereas the other FKBPs have from moderate (HIs from 25 to 40 %) to high hydrophobicity levels (HIs $\geq$ 40 %, MSA500.out in supplementary materials) [1]. The majority of the FKBDs have acidic p*I*s (p*I*s < 7.0), whereas the FKBDs with the basic p*I*s have all the cytoplasmic form of the archetypal FKBP12, the FKBD from FKBP25, and some of the FKBDs in the ER-anchored FKBPs. Two FKBPs have a transmembrane segment (TM), namely hFKBP38 and hFKBP19. Despite the charged C-terminus of the latter, it is the most hydrophobic FKBP residing in the ER.

The canonical TPR motifs contain about 34 AA residues (AAs), which form two amphipathic α-helices [46] linked via a β-turn [47]. Such helices may contain a leucine-zipper motif, a characteristic feature having either a Leu or an Ile residue at every seventh sequence position. Six human FKBPs are fusions of one FKBD (hFKBP36, AIP, AIPL1, hFKBP38) or two FKBDs (hFKBP51, hFKBP52), all of which have three consecutive TPRs [4]. Conformational polymorphism of the TRP segments has been observed in the two crystallographic forms of bovine cyclophilin-40 [52] but it is unknown if such a phenomenon might be a general in vivo property of the TPR motifs in the FKBPs. Intramolecular interaction patterns within several consecutive TPRs (α helix bundle) are similar to those of G protein-coupled receptors (GPCRs) (Fig. Fs6, supplementary material) [48]. Physical–chemical properties of the FKBPs, which consist of one or two hydrophobic FKBDs and three amphipathic TPR motifs, might have predisposed them to target different membrane-anchored receptors, molecular channels, and other membrane-embedded entities [49–51]. For example, it has been shown that the only *C. elegans* TPR motifs-containing FKBP (CeFKBP48) has its highest expression level in the nervous system of the nematode [38], the tissue that is rich in membrane proteins. A model illustrating interaction patterns between TPR motifs-containing FKBP from the plant *Arabidopsis thaliana* (AtFKBP42; CAC00654; GI:9650631) and ABC transporter [53] would suggest that the hydrophobic domains of the AtFKBP42 interact with the transporter embedded in the membrane. The FKBP38 has been localized in the outer mitochondrial and ER membranes [54]. It has similar domains to those in the AtFKBP42.

Fine interaction patterns in the FKBDs

Structures of diverse FKBPs have been thoroughly analyzed in several papers [16, 19, 23, 55–63]. Here we compared intramolecular interaction patterns in the two FKBDs that have similar HIs but come from two different phylogenetic kingdoms, namely the N-terminal FKBD from the AtFKBP42 (2F4E.pdb) [53] and the hFKBP12A (1FKB.pdb) [55] (Fig. 5a). Intramolecular interaction networks calculated for the atoms that are not further from each other than at distances $d \leq 4.5$ Å [23] in the FKBD from the AtFKBP42 (upper triangle) and the hFKBP12A (lower triangle) are shown on a bi-triangular map (Fig. 5b). Two-dimensional intramolecular interaction maps are normally calculated for the pairs of atoms separated by distances varying from 2.7 to 4.0 Å (hydrophobic atoms) and from 2.7 to 4.5 Å for polar atoms [23]. An extension of distance computing over the limit of 4.5 Å would create geodesic-type layers of interaction clusters. Two-dimensional maps are generated from the lists of distances ordered according to the sequence alignment of the two domains (shown in the upper and lower axes of Fig. 5b). Detailed analyses of intramolecular interaction networks may be useful in discerning some hidden similarities embedded in apparently different structures of proteins [65].

Although the sequences of these two FKBDs considerably differ from each other (ID = 27 %), 2D distribution of the interaction clusters within β-strands (blue arrows) and α-helices (yellow arrows and ovals) are equivalent in both triangles, which illustrates a high level of conservation of the overall FKBD fold. In contrast, the small interaction clusters formed by the atoms that were brought close in space via the different-size loops containing β-turns and short G-type helices are dissimilar in these two folded domains. For example, the violet squares represent the interaction clusters between the –AYG– sequence hallmark, which is in spatial proximity to α-helix II, the two short sequence segments that have the highest conservation levels. These two mini-clusters are not fully equivalent with each other, which reflects some fine differences in interaction networks between these two sequence motifs. Likewise, the intramolecular interaction networks involving the hydrophobic AAs (indicated in red squares and red dashed lines) are not equivalent in these two domains, because their sequences considerably differ from each other (ID = 27 %). Moreover, the macrolide-binding cavity in the hFKBP12A is surrounded by an extensive network of aromatic/hydrophobic AAs that are only to some extent conserved in the FKBD of the AtFKBP42. As shown in Fig. 5a (left panel), the innermost part of the macrolide-binding cavity of the hFKBP12A is filled up with the side chain of W59 that is in the middle of a short α-helix, whereas the innermost PPIase cavity of the AtFKBP42 is filled up with the side-chains of L and Y residues (Fig. 5a, right panel). The sequence positions equivalent to W59 of the hFKBP12A are conserved to some extent in the other FKBPs [4, 23], namely its $I_e = 1.0$ basing on the MSA500. Some FKBDs have either an F or

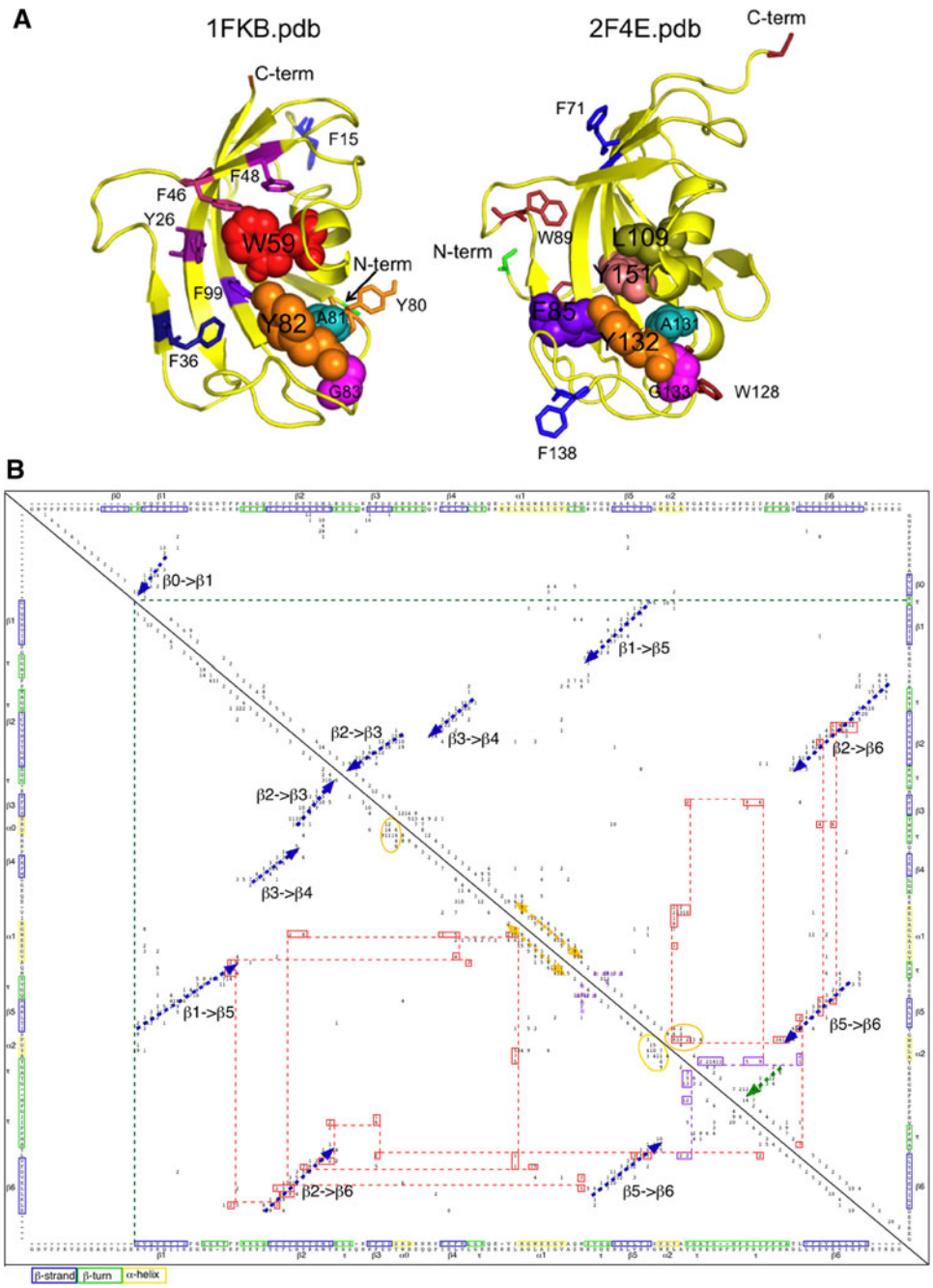


Fig. 5 **a** Crystallographic structures from which the intramolecular interaction networks were calculated: hFKBP12A (1FKB.pdb; *left panel*); the hydrophobic side chain of W59 (*red spheres*) fills in the innermost part of PPIase cleft, which is surrounded by the aromatic side chains of Y26, F36, F46, F48, Y82, and F99 [55]; the highly conserved triad A81–Y82–G83 is at the bottom of PPIase cleft; the FKBD of AtFKBP42 (2F4E.pdb; *right panel*) [53]; its "PPIase cleft" is filled in with the hydrophobic chain of L109 (*yellow spheres*) and Y151 (*rose spheres*); there is a lesser number of aromatic AAs surrounding PPIase cleft; the sequence equivalent to Y82 of the –AYG– triad in hFKBP12A is Y132 (*orange spheres*) at the bottom part to the cleft. **b** Two-dimensional distance map (made for interatomic distances $d \leq 4.5$ Å) illustrates intramolecular interaction networks in the hFKBP12A (*lower panel*) and the FKBD from the AtFKBP42 (*upper panel*). The *blue horizontal line at the upper triangle* indicates the beginning of the FKBD in the AtFKBP42. The structures were made with the MacPyMol program [64]

Y in the position equivalent to that of W59 in the hFKBP12A, which diminish their affinity towards FK506 or rapamycin. The affinity may fall by at least one order of magnitude or more as compared to the K_is of 0.2–0.4 nM established for the hFKBP12A/FK506 and hFKBP12A/Rpm complexes, respectively [1, 19].

Table 2 Some molecular and functional attributes of human FKBPs

No./protein alias	Accession code	Mass (m)	p*I*	H_I	Sequence length	PPIase domain	Chromosome	Gene	PDB codes	Drug binding	Functions or mouse phenotype	References
Cytoplasm and receptor-associated FKBPs												
1 hFKBP12A	NP_000792	11951	8.6	25.9	108	Full	20p13	*FKBP1A*	1D60	+++	Vector for FK506/Rapamycin	[1–3, 72–75]
hFKBP12									2DG3		Immunosuppression	[55–58, 79–82]
									1FKB		TGFβ signaling	[59, 60, 99, 100]
									1FKF		Binds acetylated H-ras	[93]
									1FKJ			
								Mouse(−/−)	2PPN		Cardiac and muscle function	[94, 95]
2 hFKBP12B	NP_004107	11783	8.8	29.6	108	Full	2p24.1	*FKBP1B*	1C9H	+++	RyRII	[107]
hFKBP12.6												
								Mouse(−/−)			Impairment of glucose-induced insulin secretion	[96, 97]
											Atrial fibrillation	[98]
3 hFKBP12C	CAH71018	12179	8.6	25.0	108	Full	6q12.2	*FKBP1C*		?	Unknown	[22]
Nuclear FKBPs												
4 hFKBP25	NP_002004	25177	9.7	16.5	224	109–224	14q21.3	*FKBP3*	1PBK	+++	Nuclear/cytosolic	[109–119]
									3K27			
5 hFKBP133	NP_056073	133630	5.0	25.7	1219	180–290	9q32	*FKBP15*		?	Perinuclear protein	[39]
											Microtubule dynamics	[33, 164]
ER-anchored FKBP												
6 hFKBP15P	NP_476433	15649	9.6	35.2	142	30–137	11q13.1–q13.3	*FKBP2*	2PBC	+++	ER protein folding	[1]
hFKBP13		13372	9.3	25.6	121	22–142						
7 hFKBP22P	NP_057678	22180	9.3	52.2	201	28–201	12q13.12	*FKBP11*			ER protein folding	[123]
hFKBP19		19972	9.7	46.7	180	37–144						
8 hFKBP24P	NP_060416	24172	5.7	33.2	211	20–211	7p15.1	*FKBP14*	4DIP		ER protein folding	[124]
hFKBP22		22067	5.5	26.6	192	29–135					Mutations cause Ehlers–Danlos syndrome	[125]
9 hFKBP25P*	NP_851939	25768	6.0	25.2	222	24–222	2q31.2	*FKBP7*			ER protein folding	–
hFKBP23		22971	5.4	20.1	199	36–145						
10 hFKBP63P	NP_009201	63084	4.8	39.5	570	4 FKBDs	7q11.1	*FKBP9*			ER protein folding	[126]
hFKBP60		60443	4.7	38.1	546	25–570						
11 hFKBP65P	NP_068758	64305	5.3	34.5	582	4 FKBDs	17q21.31	*FKBP10*			ER protein folding	[127–129
hFKBP61		61511	5.2	32.6	556	27–582					Mutations cause Bruck syndrome	[131, 132]
TPR motifs-containing FKBPs												
12 hFKBP36	NP_003593	37214	6.5	37.6	327	31–138	7q11.23	*FKBP6*	3B7X	–	Williams syndrome	[134]
								Mouse(−/−)			Homologous chromosome pairing/histone H2AX	[135]

Table 2 continued

No./protein alias	Accession code	Mass (m)	p*I*	H_I	Sequence length	PPIase domain	Chromosome	Gene	PDB codes	Drug binding	Functions or mouse phenotype	Reference
13 hFKBP38	NP_036313	38408	7.6	40.9	355	100–205	19p12	*FKBP8*	2AWG	+?	Hedgehog signaling	[138]
											Inhibitor of apoptosis	[136, 137]
											Cell-size regulation	[139]
											Dorsal-ventral patterning	[140]
14 hFKBP51	NP_004108	51212	5.6	26.0	457	2 FKBDs	6p21.3–21.2	*FKBP5*	1KTO	++	Chaperone/Glucocorticoid	[141, 142]
									3O5E		receptor/Different targets	
15 hFKBP52	NP_002005	51805	5.2	23.1	459	2 FKBDs	12p13.33	*FKBP4*	1N1A	++	Chaperone-HSP90	[143, 144]
									1P5Q		Galectin-1/resorption	[147]
								Mouse(−/−)			Endometriosis	[145]
											Oxidative stress regulation	[150]
											Morphogenesis	[151–153]
											Glucocorticoid receptor transcriptional activity	[154]
FKBP-like containing TPR motifs												
16 hFKBP37 AIP	NP_003968	37664	6.0	31.2	330	11–159	11q13.3	*AIP*		−	Aryl hydrocarbon receptor-associated protein	[154]
17 hFKBP43 AIPL	NP_055151	43597	5.4	22.4	384	10–158	17p13.1	*AIPL1*		−	Amaurosis syndrome	[159]
18 hFKBPL	NP_071393	38248	5.3	23.5	349	Unclear	6p21.3	*FKBPL*		−	Cancer cell associated	[160–163]

Nominal mass (m), p*I* and the overall hydrophobicity index (HI) were calculated for the sequence length as indicated using a nine-residue sliding frame and the KD hydrophobicity scale; *P* after kDa indicates signal peptide at the N-terminus; *P** an isoform of hFKBP25P is coded by NP_057189; hFKBP43 has at least two isoforms, NP_001028227 (324 AAs) and NP_001028220 (321 AAs); hFKBPL is also known as WAF-1/CIP1 stabilizing protein 39 and it has three TPR motifs (I, 210–243; II, 252–285, and III 286–319 TPR motif = [WLF]-X(2)-[LIM]-[GAS]-X(2)-[YLF]-X(8)-[ASE]-X(3)-[FYL]-X(2)-[ASL]-X(4)-[PKE]. Only a limited number of PDB codes were cited and can be downloaded from the Research Collaboratory for Structural Bioinformatics server (RCSB, http://www.rcsb.org) [165]. Drug binding: +++, $K_d \leq 1$ nM; ++, K_d 1–10 nM; +, K_d 10–500 nM; ?, unknown; –, $K_d \geq 1$ μM

Information obtained from analyses of the 2D distance maps of the FKBDs belonging to the FKBPs expressed in diverse species suggests that the overall fold remains conserved. However, AA mutations caused differentiation of intramolecular interaction networks and fine 3D geometry patterns of both, the overall fold and PPIase cavity, which in turn altered some of the fundamental functional features of the FKBPs, namely their recognition profiles, specificity, and binding affinity to different in vivo targets and small molecular mass compounds [23]. Diversified geometrical features and altered hydrophobicity context within PPIase cavity cause that some of the FKBPs lack *cis*/*trans* isomerization activity when using standard peptide-substrate-based assays and they do not bind the macrocyclic drugs.

Functional aspects of the FKBPs

Table 2 summarizes several fundamental sequence attributes of the human FKBPs together with some of their functional profiles. Although PPIase cleft has the capacity to accelerate *cis*/*trans* isomerization of X-Pro epitopes in model peptides [66], any significance of this activity for functioning of various cell phenotypes and organisms at different levels of development requires further explorations. For example, one study has shown that all eight genes encoding cyclophilin-like proteins and all four genes coding for FKBP-like proteins are individually and collectively dispensable for the viability of *S. cerevisiae* cells [67]. In contrast, the 13th PPIase gene (*ESS1*) in baker's yeast coding for a 170-amino-acid-long protein (NP_012551; GI:37362669), whose sequence is homologous to human Pin1, is essential for the viability of the cell [67].

The monodomain small FKBPs may have two major functions. Firstly, they may catalyze *cis*/*trans* isomerization of X-Pro epitopes, which is a crucial functional input into protein folding and assembly of multimolecular complexes. It is noticeable that three small and two large FKBPs are embedded in the ER of mammalian cells [4]. Secondly, the small FKBPs function as molecular chaperones, namely their binding to certain targets may alter some of the physical–chemical attributes within the complex, which in turn lead to fine-tuning of its functional or structural features [1]. The large multidomain FKBPs, however, may function as restructuring chaperones, which create novel conformational assemblies via fine spatial repositioning of domains in target complexes. This action creates unique spatial interaction networks, which thus induce a maximal functional adequacy in given molecular assembly. For example, a 33-kDa human cyclophilin (CyP33) is a two-domain protein consisting of a RNA-binding domain and a C-terminal PPIase domain [68], which is similar to the domain's structure of the FKBP25. CyP33 modifies the conformation of the mixed lineage leukemia 1 (*MLL1*) gene product, which facilitates the binding of its RNA-binding domain (RRM) to the MLL1-PHD3 (Plant Homeo Domain 3) segment [69]. Allosteric effect [70, 71] induced via binding of the multidomain PPIases to integral membrane proteins, spliceosomal complexes, polyribosomes, or mitochondria could be an indispensable control input to functional features of some components within these supramacromolecular entities.

Molecular targets of immunophilin/(immunosuppressive drug) complexes

Studies on suppression profiles of T cells, which were treated with CsA, FK506, or rapamycin, led to the proposition that the hFKBP12A is the principal intracellular carrier (immunophilin) for the immunosuppressive drugs FK506 or rapamycin with one of the following events being sufficient for inducing T-cells anergy and immunotolerance in humans [73]. Firstly, formation of the ternary complex of the serine-threonine phosphatase calcineurin consisting of the globular subunit A (CaNA), Ca^{2+}-binding calcineurin B (CaNB), and the Ca^{2+}-binding protein calmodulin [56, 57, 73] associates with the hFKBP12A/FK506 complex, which in turn blocks dephosphorylation of cytosolic form of nuclear factor of activated T cells (NFATc) and hinders its rapid transport to the nucleus where it should form a transcriptional complex with the AP1 transcription factor (Fig. Fs7, supplementary material) [78]. Similar effects can be induced by cyclosporin-A (CsA) whose complex with cyclophilin-A (CyPA) binds and inhibits calcineurin A (reviewed in [35]). Secondly, the hFKBP12A/Rpm complex binds to mammalian target-of-rapamycin (mTOR), also known as mechanistic TOR [58, 79–83] and blocks its kinase activity, which in turn breaks down signal transduction networks emanating from IL2 receptor [73]. Both of these two enzymatic entities have some vital functions in the lower eukaryotes such as *S. cerevisiae* [79, 83–85], *Cryptococcus neoformans* [86, 87], or *Candida albicans* [88], where both immunosuppressive and nonimmunosuppressive macrocycles bound to the yeast's orthologue of the hFKBP12A perturb their phosphatase and kinase activities, respectively. Yeast cells, however, do not express any homologue of the NFATc, which is a pivotal transcription factor involved in FK506- or CsA-induced immunosuppression in humans, thus the toxic effects of these drugs are transmitted via other networks of proteins [81, 82]. It has been suggested that ancestral NFAT1c was formed from a fusion of the invertebrate Rel gene with a nuclear translocation signal domain, which probably was a step among many other necessary recombination steps for the transformation of invertebrates into primordial vertebrates [89].

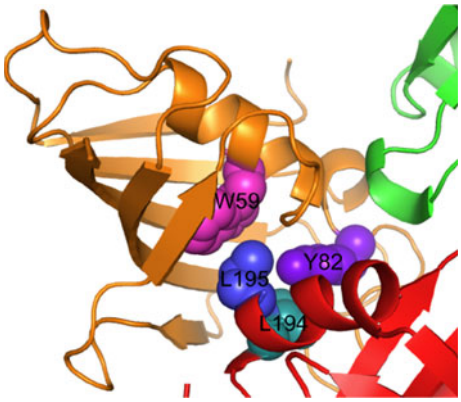


Fig. 6 The X-ray structure (3MDY.pdb) of a binary complex (hFKBP12A/BMP-RIB) [59]; some backbone of the intracellular domain of the human BMP receptor type IB, comprising GS and kinase domains, is shown as *red and green ribbons*, FKBP12A is shown as *orange ribbon* with the side chain of W59 (*rose spheres*) and Y82 (*violet spheres*) from the –AYG– triad; the interacting side chains of L194 and L195 of the BMP-RIB are shown as *blue spheres*. GS domains form a helix–loop–helix structure and are found only in type I TGFβ class of receptors; its name comes from the sequence, namely –TTSGSGSGLPLL– (human TGFβ-RI; P36897; GI:547777), which is N-terminal to intracellular kinase (ALK5 in the TGFβ-R type I) and in which Ser are phosphorylated by type II receptors

A recent study on the influence of FK506 on various cellular processes in *S. pombe* cultures has revealed that 72 deletion strains were sensitive to the drug. Various intracellular processes are controlled by FK506, such as membrane trafficking, chromatin remodeling, cytokinesis, ribosomal proteins, etc. [90]. Surprisingly, it has been shown that even if the *S. pombe* cells express TOR1 and TOR2, the strain itself was insensitive to rapamycin [91, 92]. Since the above-mentioned models of drug-induced immunosuppression were derived from the results of in vitro experiments made on T cells or their extracts [72–75], it remains to be established whether the described molecular targets and their accompanying factors remain equally operational in humans with all the competing targets for the drugs, their toxic side effects affecting different organs, and diverse immune cells' phenotypes [76], including stem and progenitor cells [77].

Is the archetypal FKBP12 involved in morphogen-driven signaling pathways?

Analyses of the repertoires of the FKBPs encoded in various genomes have shown that the gene coding for the archetypal FKBP12 had conserved its functional features throughout the evolution of disparate species [1, 4]. Moreover, it was estimated that about 80 % of genes expressed in *C. intestinalis* are homologous with their functional counterparts in *H. sapiens*. These two organisms belong to deuterostomes superphylum of animals. This high level of conservation of functional traits in evolutionary distant organisms may imply that some of the functional features of macromolecular assemblies being under the control of PPIases, which are vital for mammalian organisms, could have been already coined in some primordial multicellular marine organisms. For example, it has been shown that the hFKBP12A may control activity of mammalian TGFβ type I receptors [65, 99, 100]; the X-ray structure of the hFKBP12A bound to the intracellular part of the bone morphogenetic protein (BMP)-receptor type 1B (3MDY.pdb) is displayed in Fig. 6 [59]. It is an exquisite example illustrating that an X-Pro dipeptide (where X is any AA) is not the only sequence motif that can be accommodated within PPIase cavity. W59 (rose sphere) and Y82 (pink sphere) in the PPIase-binding cleft of the hFKBP12A (orange ribbon) are in close proximity to L194 (cyan) and L195 (violet) of the BMP-R1B intracellular domain, which is composed of a short GS sequence segment that is phosphorylated by TGFβ type II receptor and which is linked to the C-terminal kinase domain (red ribbon). Although the functional significance of interactions between the hFKBP12A and the TGFβ type I family of receptors remains controversial (SupRef.list_A in supplementary material), if such a complex had crucial in vivo input, it would imply that a vital gain of function for the archetypal immunophilin FKBP12 could have been acquired at an early evolutionary stage of multicellular organisms. It is worth mentioning that all the multicellular organisms discussed here had gained the capacity to express various activin and TGFβ family of receptors and their ligands such as inhibins, BMPs, or activins [101]. In mammalian organisms, the latent encapsulated form of the TGFβ morphogen is stored in the extracellular matrix (ECM), and if activated by the furin protease, can bind to the TGFβ superfamily of receptors, and initiate diverse morphogen-driven intracellular signalization pathways [102]. Whether binding of the archetypal FKBP12 to the GS domain of TGFβ type I receptors could have controlled complex signaling pathways and could have had a fundamental impact on development of multicellular organisms and their differentiation remain enigmatic.

Recently, it has been communicated that FK506 and CsA have the capacity to release activated factors from latent TGFβ stockpiles, which appears to be a critical step that could be responsible for kidney fibrosis in patients treated with one of these drugs [103]. Rapamycin also has an influence on the TGFβ-induced signaling pathways [104]. Those results would suggest that the immunosuppressive macrocyclic drugs, due to their high hydrophobicity, may physically perturb the storage vesicles of diverse growth hormones in the ECM and induce various cascades of pivotal intercellular signalization pathways involving networks of cytokines, proteases, and other signalization protein assemblies. In mammals, an aberrant

activation/deactivation of morphogens-driven signaling pathways may be a pivotal step leading to formation of malignant cells and solid tumors [65, 105, 106]. If the extracellular pool of the injected macrocyclic immunosuppressive drug would release diverse growth factors encapsulated in the ECM-anchored storage vesicles containing preforms of morphogens, which in turn should activate some signalization networks controlled by those morphogens, cytokine receptors, ionic channels, or GPCRs, then the integral outcome of such actions could be decisive for inducing T cell anergy or other immune responses. Some of these actions should precede the formation of "intracellular immunosuppressive complexes" containing either FK506 or rapamycin bound to the hFKBP12A.

FKBPs are associated with large molecular channels

The FKBP12A and FKBP12B (also known as FKBP12.6) were found to be associated with different large molecular assemblies such as the ryanodine receptors (RyRs), namely muscle-specific RyR1, myocardium-specific RyR2, and neuron-specific RyR3, which constitute the major intracellular factors of calcium-induced calcium release system [107]. The FKBP12 binds to the inositol 1,4,5-trisphosphate receptor [108], whereas FKBP52 was found to be associated with the Ca^{2+} transient receptor potential cation channel subfamily V member 5 (TRPV5) channel [51]. Whether some of the immunophilins are essential factors controlling physiological activity of various ionic channels and some organs such as the heart in mammals is still under debate, since some alternative models concerning molecular and functional aspects of the myocardium-specific RyR2-FKBP12.6 complex and its significance for different functions of the heart have been discussed (SupRef.list_B in supplementary materials).

Functions of the dual-compartment FKBPs

FKBP25 has a positively charged N-terminus and PPIase domain at its C-terminus [109, 110], a domain organization that is analogous to that of the small nuclear cyclophilin CyP33 [35]. Mammalian FKBP25s interact with various intracellular proteins such as casein kinase II and nucleolin [111], high-mobility group II nonchromosomal protein [112], the transcriptional silencer and enhancer YY1 belonging to GLI-Kruppel class of zinc-finger proteins, histone deacetylase [113], and MDM2 (*Murine Double Minute* oncogene) that is a crucial negative regulator of p53 tumor suppressor protein [114, 115]. All these proteins may have crucial inputs to chromatin remodeling. FKBP25 has also been detected in centrosomes [116], RNA granules [117], cellular differentiation and proliferation processes [118, 119], and some developmental stages of neurons [45]. As the mammalian FKBP25 is a strong binder of rapamycin ($K_d \leq 1$ nM) [109], thus in vivo it must compete with the hFKBP12A for the injected drug. The FKBP25/rapamycin complex may influence some cellular processes, which are not controlled by the hFKBP12A/Rpm complex.

Mammalian FKBP133 starts from N-terminal WH1 domain also known as pleckstrin homology-like (PH) domain that binds Pro-rich regions (see Fig. Fs8, supplementary materials), and is followed by several domains, namely a hydrophobic FKBD, DNA translocase unit, a chromosome segregation ATPase domain, and a myosin-like C-terminus. However, even if the FKBD from the hFKBP133 has the highest hydrophobicity level among the human FKBDs, the protein is a hydrophilic species. The hFKBP133 has been associated with myosin endocytosis [39], growth cone morphology [33], and some perinuclear functions [39].

The ER-anchored FKBPs

The ER-associated small FKBPs have an FKBD at the N-terminus, which is followed with several minidomains. A fusion of four consecutive FKBDs is a typical feature of the large ER-anchored FKBPs, which are expressed in plants and vertebrates [4]. It has been shown that the ER-associated FKBPs in the nematode *C. elegans* are vital for cold shock and knock down of their genes causes some defects in the ECM [120, 121]. The FKBP65 seems to aid the folding of collagens [127] and is overexpressed together with some ECM proteins [128], whereas its plant orthologue is crucial in some developmental processes [129]. Mutations in the sequence of FKBP65 (polymorphism of the *fkbp10* gene) may cause the recessive *osteogenesis imperfecta* and Bruck syndrome [130, 131]. The former, however, has already been correlated with an AA mutation in the *PPIB* gene coding for the ER-anchored cyclophilin-B [132]. The ER-residing FKBPs are probably essential for protein-folding fidelity and transport of folded assemblies of proteins to the Golgi apparatus and secretion vesicles. These functions could be dependent on the oxidation/reduction status of the disulfide bond that is conserved in the FKBDs of all the small and large ER-embedded FKBPs.

Diversified functions of the TPR motifs-containing FKBPs

Several different forms of the TPR motifs-containing FKBPs are expressed in vertebrates [4]. Those FKBP consist of either one FKBD or two FKBDs, which are followed with three consecutive TPRs and a C-terminal α-helix (Fig. 1). The coding exons for a 36-kDa TPR motifs-containing FKBP (hFKBP36) are in the genomic

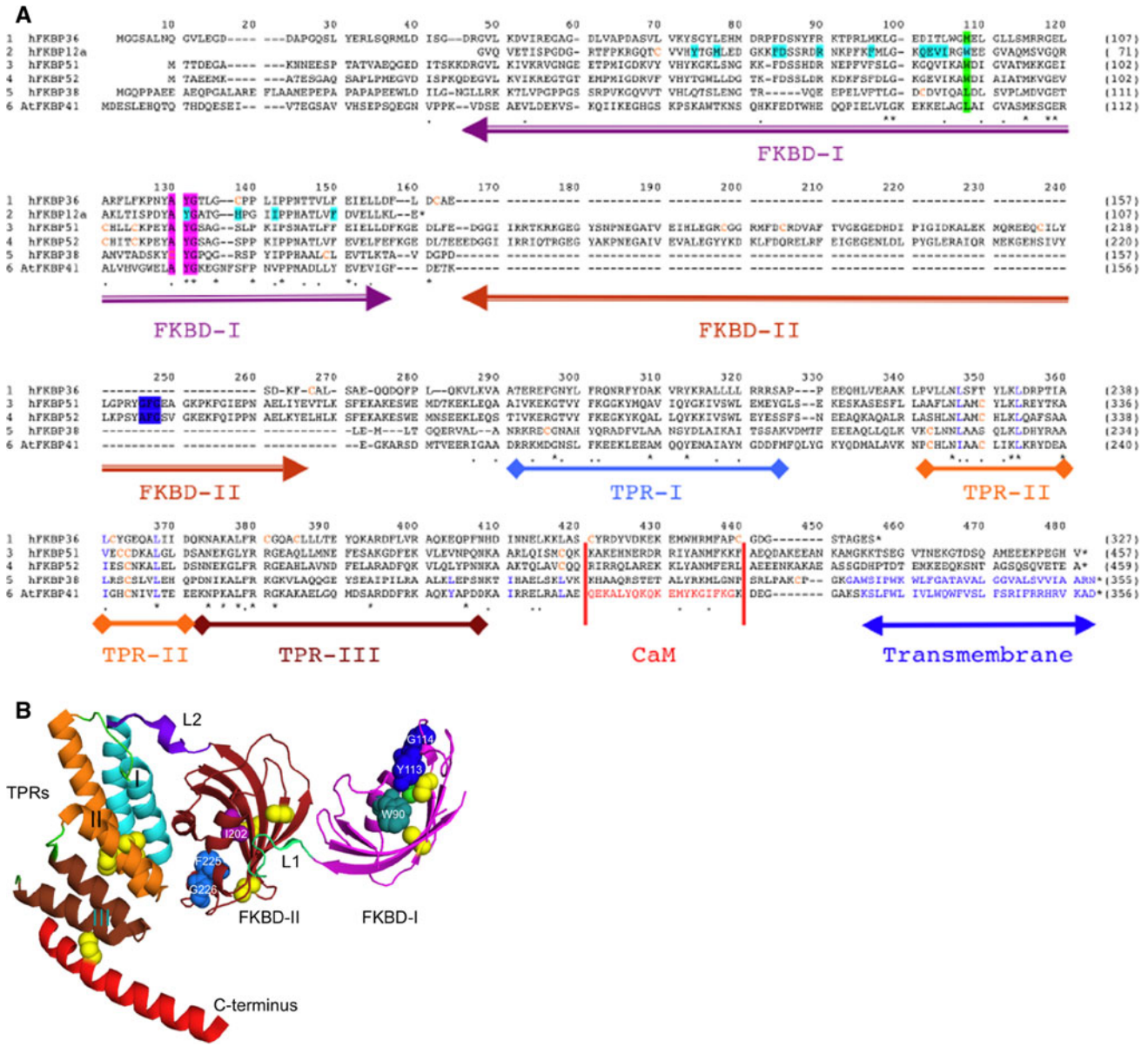


Fig. 7 **a** MSA of the TPR motifs-containing FKBPs. The AAs forming PPIase cavity in the hFKBP12A are in *cyan*, W59 (hFKBP12A) and its equivalent positions in the other sequences are in *green*, the –AYG– triad (hFKBP12A) is in *pink*. FKBD-I is indicated with a *violet arrow*, FKBD-II is as a *light brown arrow*, TPR-I (*light blue*), TPR-II (*orange*), and TPR-III (*brown*) are followed with a putative CaM-binding domain and transmembrane segment in the hFKBP38 and AtFKBP42 (*blue*); AAs participating in leucine zippers in TPR-II and TPR-III are in *blue color*. **b** X-ray structure of the human FKBP51 [62]. The cysteine residues are in *yellow*; W90 in the innermost part of PPIase cavity of FKBD-I (*cyan*) is flanked at the top with the –AYG– triad (Y113–G114 are shown in *dark blue*), whereas in the innermost cavity of FKBD-II is I202 (*pink*) that is flanked at the bottom with the –GFG– triad (F225–G226 are shown in *blue*). Linkers L1 (*green*) and L2 (*violet*) join FKBD-I with FKBD-II, and FKBD-II with the TPR motifs, respectively

segment on human chromosome 6, whose deletion gives rise to Williams syndrome [133]. FKBP38 is bound to the outer mitochondrial and ER membranes and controls the apoptotic Blc-2 protein [135, 136], whereas FKBP51 and FKBP52, which are fusions of two FKBDs with three consecutive TPR motifs, are associated to glucocorticoid receptor [140–143]. FKBP51 may have some functional inputs in the mitochondria and nucleus [141].

Our analyses show that distant orthologues of the human TPR motifs-containing FKBPs are encoded in disparate invertebrates (Table 1). A sequence alignment of the hFKBP12A with four human TPR motifs-containing FKBPs is shown in Fig 7a; the AtFKBP42 is orthologous to the hFKBP38. Putative leucine zippers in TPR-II and TPR-III with a putative CaM-binding domain were explicitly indicated. There is only about 54 % sequence

similarity for the hFKBP51/hFKBP52 pair, which may indicate that their fine functional features differ from each other despite the fact that they are co-chaperones of corticosteroid receptors. For example, in Fig. 7b is shown the X-ray structure of hFKBP51 [62]. The two SH of the Cys residues in the FKBDs-I of hFKBP51 and hFKBP52 are hidden in the hydrophobic interior of the domain and are distant from each other (hFKBP51, 1KTO.pdb, C103–C107, $d = 8.8$ Å). In contrast, one Cys out of three residues in FKBP-like domain II (FKBD-II) of hFKBP51 is solvent-exposed and might form a disulfide bridge with an accessible Cys from another protein. There is no Cys residue in the FKBD-II of hFKBP52. The FKBD-II in hFKBP51 and hFKBP52 has neither PPIase activity nor FK506 binding capacity.

A series of sophisticated gain-of-functions has been tentatively assigned to the TPR-containing FKBPs, some of which are functionally bound to glucocorticoid-driven transcription of genes and probably are crucial elements in several syndromes such as altered states within the nervous system or some testicular functions (SupRef.list_C_D_F_G in supplementary material). Whether diverse complex diseases such as bipolar disorder, Parkinson disease, or increased recurrence of depressive episodes (sections F and G in SupRef.List) are under the control of the FKBP51 or FKBP52 bound via heat shock proteins to steroid receptors need further explorations [1]. Likewise, if a genuine synergy is created by the binding of the TPR motifs-containing FKBPs to some yet-unidentified targets in neuronal tissues, which could be nullified due to polymorphism or loss of one of the genes encoding these two proteins, and in turn cause the above-mentioned syndromes still remain for further explorations. A possibility of involvement of FKBP52 and FKBP51 in neuron regeneration after ischemic shocks and related phenomena has been explored [145, 147, 148, 166], but due to the intricate nature of such processes involving cascades of various molecular events, the obtained results require further experimental refinement.

Detection of any of the TPR motif-containing FKBPs in samples obtained from either in vitro generated or genuine in vivo sources, which were subjected to such phenomena as oxidative stress, drug-suppressed or altered gene transcription, altered functions of neuronal circuits and their integrated forms [145–148], localized or systemic diseases including cancer and its metastases [155–158] may be due to their constitutive mode of expression rather than to any crucial functional input that may control these complex processes/diseases. For example, recent studies on probable origins and metastases of cancer cells have revealed that large sets of genes are involved in these processes [155]. Thus, whether a particular immunophilin, which is constitutively expressed in normal as well as in cancer cells, may have a pivotal impact on formation of primary tumor cells and their metastases to other organs needs to be scrutinized using multiple criteria. Even if a given FKBP could have a capital significance at any stage of given syndrome or diseases, its selective targeting remains problematic.

Molecular aspects of pharmacological activity of the macrocyclic drugs vectored via the FKBPs

FK506 (tacrolimus)

Probable scenarios for in vivo modus vivendi of cyclosporin A (CsA), FK506, and rapamycin have been described in many papers and reviews [1, 72–76, 79–82, 173–175]. Those far-reaching propositions and hypotheses were based on in vitro-created experimental constraints and conditions. For example, a ternary complex comprising subunit A of calcineurin (CaNA), its subunit B (CaNB), and calmodulin was shown to bind to affinity gels consisting of glutathione S transferase (GST) fusions with one of the following immunophilins bound to their respective ligands, namely GST-hFKBP12A/FK506 [72], GST-hCyPA/CsA [72], or GST-

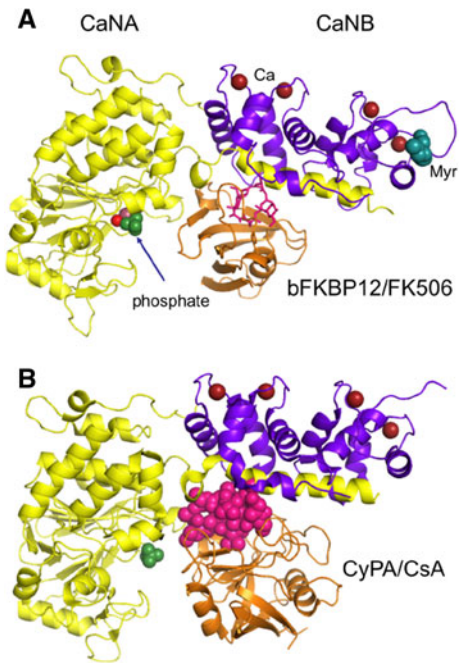


Fig. 8 X-ray structures of two ternary complexes: **a** (CaNA/CaNB)/(bFKBP12/FK506) (1TCO.pdb) [56]; the four Ca^{2+} atoms bound to CaNB (*violet ribbons*) are shown as *red-brown balls*, N-terminal myristyl group is in *blue spheres*; CaNA (*yellow ribbon*), Zn^{2+} (*red ball*), Fe^{3+} (*violet ball*) and a phosphate group (*green spheres*) bound at the active site; bFKBP12 (*orange ribbon*) and FK506 (*rose sticks*) are bound in a shallow groove formed by the CaNA–CaNB complex; **b** (CaNA–CaNB)/(CyP–CsA) (1MF8.pdb) [171]; CaNA and CaNB are colored as above, whereas cyclophilin A (*orange ribbon*) with its CsA bound to it (*rose spheres*) blocks the CaN entity in a similar fashion as it is in the bFKBP12/FK506 complex

mCyPC/CsA [168]. Since either CsA or FK506 controls an early stage of signals emanating from T cell receptor (TCR), and their respective complexes with the immunophilins inhibit phosphatase activity of calcineurin A, it seemed to be reasonable to assume that this is the step leading to T cell anergy, provided that a vital part of the intracellular pool of the CaN entity (CaNA + CaNB) is effectively blocked by "the immunosuppressive complex". Moreover, it has been suggested that a strict correlation exists between the inhibition of T cell signaling and the in vitro-observed, FK506-induced inactivation of calcineurin A [74, 75].

At least three isoforms of the CaNA are expressed in humans, namely its α-isoform (521 AAs; NP_000935.1; GI:6715568; pI = 5.5; Fig. Fs9 in supplementary materials), β-isoform (524 AAs; NP_066955.1; GI:11036640; pI = 5.6), and γ-isoform (512 AAs; NP_005596.2; GI:2136129; pI = 6.5), which have about 80 % of sequence similarity with each other. Mouse with knockout gene for the α isoform was fully immunosuppressed after treatment with either CsA or FK506 [169], which would imply that the other calcineurin's isoform was probably blocked by the immunosuppressive complex [170]. Moreover, at least two isoforms of the CaNB are expressed in the human body, namely type 1 (NP_000936.1; GI:4506025; pI = 4.5) and type 2 (NP_671709.1; GI:22212896; pI = 4.6), which have about 84 % sequence similarity with each other. In the human genome are also encoded calcineurin B homologous protein 2 (196 AAs; NP_071380.1; GI:11545811; pI = 5.8) and calcium-binding protein p22 (195 AAs; NP_009167.1; GI:6005731; pI = 4.8), which have significant sequence similarity to the above two isoforms of the CaNB as well as to calmodulin (149 AAs; NP_001734.1; GI:4502549; pI = 3.9) and various Ca^{2+}-binding proteins. It should be noted that an effective abolishment of calcineurin A activity could be only achieved if nearly all of its cellular content becomes engaged by the immunophilin/drug complex, which is far from being the case in many human cell phenotypes, which express a high level of the CaN entity.

Figure 8a and b show the X-ray structures of the (CaNA–CaNB)/(bFKBP12A-FK506) [56, 57] and (CaNA–CaNB)/(CyPA–CsA) complexes, respectively [171, 172]. A composite of several different structures comprising the CaN entity is shown in Fig. Fs10, whereas a summary of intermolecular interaction patterns in these two complexes can be found in the Dist.comp file (supplementary material).

Each of the immunophilin/immunosuppressant complexes is bound at a large and shallow space formed by the myristylated (Myr) CaNB subunit with its four Ca^{2+} cations bound (red-brown spheres), which interacts with the C-terminal α-helical segment stretching out from the globular part of the CaNA subunit, where its phosphatase active site is shown in deep blue. These two subunits have an extensive set of intermolecular interactions with each other (there are 453 atomic contacts at $d \leq 4.5$ Å, Table Ts1 and Dist.comp in supplementary materials). Likewise, numerous atoms of the bFKBP12A have van der Waals (vdW) interaction spheres with the CaN entity; at $d \leq 4.5$ Å there are 63 and 99 contacts for CaNA/bFKBP12A and CaNB/bFKBP12A, respectively. Some atoms of FK506 are in vdW distance with the AAs of the CaNA (79 contacts at $d \leq 4.5$ Å involving W352, S353, P355, F356, and E359) and with the CaNB (19 contacts at $d \leq 4.5$ Å involving M118 and V119). The solvent-exposed hydrophobic side chains of the drug are shielded in the ternary complex and form a network of intermolecular interactions with the CaN entity. Quasi-similar interaction patterns were calculated for the (CaNA–CaNB)/(CyPA–CsA) complex with a few more AA residues being engaged on the CaNA subunit (L312, Y341, W342, P344, W352, S353, and F356) and the CaNB subunit (M118, V119, N122, and L123). The above data show the CaN entity has an extensive interaction pattern with each of the immunophilins. It was not surprising that some of the FKBPs alone bind to the CaN entity, namely hFKBP51 [176], hFKBP38 [177], or ScFKBP12 [178]. The complexes of FK506 with either of the following immunophilins FKBP13, FKBP25, FKBP51, FKBP52, or FKBP38 bind to the CaN entity but at a lesser K_d than the hFKBP12A/FK506 complex [177]. For example, hFKBP13/FK506 or hFKBP25/FK506 inhibit calcineurin A at the K_ds ≥ 1 μM [167].

It has been proposed that the immunophilin/drug complex hinders access to the active site of the CaNA for some large substrates such as the phosphorylated N-terminal domain of the NFAT1c, which causes its retention in the cytoplasm of T cells treated with one of the calcineurin's inhibitors (CNI) such as CsA or FK506 [1]. Curiously, the CaNA bound to the bFKBP12A/FK506 complex is more active than its free form in cleaving the phosphate group in small organic substrates (reviewed in [173–175]). Two different sequence motifs of the NFAT1c bind to the CaNA, namely the PVIVIT peptide from NFAT1c binds to the β-strand on the globular domain of the CaNA [179–181], whereas YLAVP peptide patch and alike from the series of NFATs binds at a different site on the CaNA [182]. Figure 9a and b shows a composite of two structures roughly oriented in the same fashion, namely in the upper panel is shown the X-ray structure of the CaN entity bound to the bFKBP12/FK506 complex [56], whereas at the lower panel is shown the NMR-derived structure of GPHPVIVITGPHEE-NH_2, a sequence fragment from human NFATc1, which is bound to a fragment of the globular domain of the CaNA [183]. It illustrates that these two binding sites are entirely different, namely the bFKBP12/FK506 complex binds at the β-strand stretching out of the globular domain of the CaNA and hinders the access to its active site, whereas the peptide derived from NFATc1

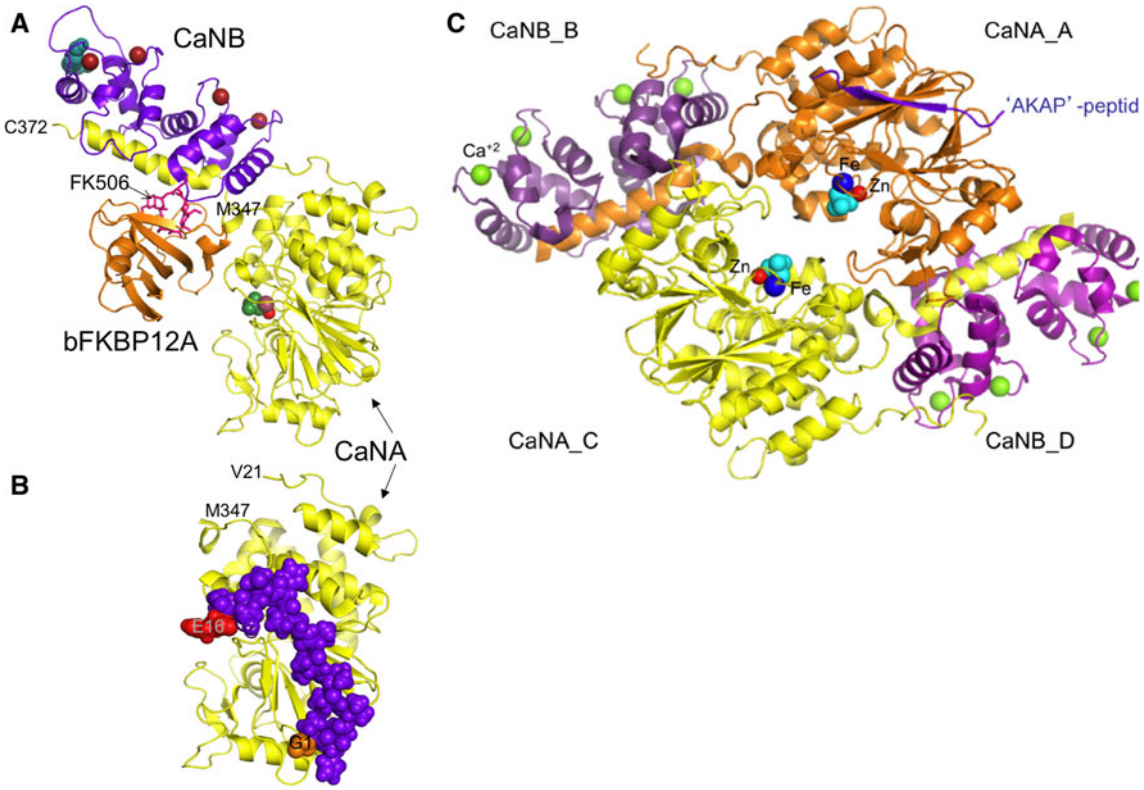


Fig. 9 **a** X-ray structure of the (CaNA–CaNB)/bFKBP12/FK506 complex [56] and **b** NMR-derived structure (2JOG.pdb) of a fragment of the CaNA (*yellow*; AAs from 21 to 347) bound to 1-GPHPVIVITGPHEELE-NH_2 peptide (*violet*) with G1 in *orange* and E16 in *red* [183]; **c** dimeric form (A + B and C + D chains) of the CaN entity with the bound peptide EPIAIIITDTE from AKAP (*violet*, AKAP5_HUMAN, AAs from 336 to 346, 3LL8.pdb) [186]. Calcineurin A (PP2BA_HUMAN, chains A and C, AAs from 14 to 70 AAs are in *yellow*/*orange*); Zn (*red ball*) and Fe (*blue ball*) are bound to the phosphate (*cyan sphere*), and calcineurin B (CANB1_HUMAN, chains B and D, AAs from 16 to 170 AAs, are in *deep violet*/*pink*) with Ca^{2+} cations indicated as *green balls*

binds to a β-strand, which is close to the active site. It is worth noting that the patches of the human NFATs that target the CaNA have a low degree of sequence conservation (Fig. Fs11, supplementary material). It has been shown that interactions between NFAT1c and CaNA can be disrupted with small molecules [184, 185]. Moreover, the cytoplasmic HOMER scaffolding proteins compete with the CaNA for binding to the NFAT1c and as such are negative regulators of T cell activation [187]. Thus, it would be interesting to explore if some peptides mimicking the patches of the NFAT1c and its homologues, which have the capacity to bind at high affinity to the CaNA, could neatly target T cells and induce immunosuppression. CaNA controls activity and translocation of a plethora of intracellular proteins. For example, the scaffold A-kinase anchoring protein (AKAP79) recruits the CaN entity to L-type of Ca^{2+} channels and creates an influx of Ca^{2+} to the cell's interior. The X-ray structure of a complex comprising a small peptide patch from the ATKP79 bound to the CaNA has been recently established [186] and is shown in Fig. 9c. The hydrophobic peptide binds to β-strand spanning from V328 to F334 of the CaNA globular domain and forms a parallel β-sheet in a similar fashion as PVIVT peptide from the NFAT1c (Fig. 9b).

Rapamycin (sirolimus)

About two decades ago, two isoforms of TOR kinase have been cloned [79] from *S. cerevisiae* budding yeast (TOR1 and TOR2) whose sequences have about ID = 39 % with the human TOR [79, 80] (Fig. Fs12, supplementary materials). In the budding yeast, TOR1 can be inhibited with the ScFKBP12/Rpm complex (TOR1, 2470 AAs; P35169.3; GI:1174744), whereas TOR2 (2474 AAs; P32600.3; GI:122066477) is insensitive to rapamycin inhibition [188]. ScTOR1 forms two different supramacromolecular assemblies [188–190], namely ScTOR1 bound to RAPTOR (mTORc complex 1) via the N-terminal HEAT (Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast PI3-kinase TOR1) repeat domains is sensitive to the hFKBP12A/Rpm complex, whereas the complex of RICTOR–MAPKAP1 bound at the same HEAT domains of ScTOR1 is rapamycin-insensitive (ScTOR1 complex 2); sequence alignment comprising

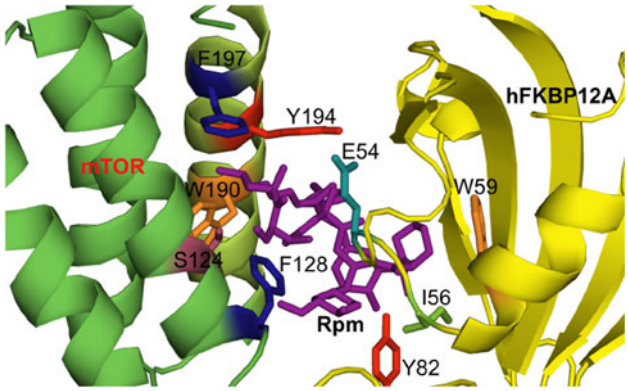


Fig. 10 Fragments of the X-ray structure (4FAP.pdb) of rapamycin-binding domain (RBD, *green ribbon*) from human TOR bound to hFKBP12A/rapamycin (*yellow ribbon*) [192]; rapamycin (Rpm) is in *deep-violet sticks* while the side chains of several AAs in close proximity to the antibiotic were explicitly shown; the pipecoline ring interacts with W59 (*orange sticks*) and Y82 (*red sticks*) from the hFKBP12A is at the *bottom* (*orange sticks*), whereas the other part of Rpm interacts with I56 (*green sticks*), and E54 (*blue sticks*) of the hFKBP12A (*yellow ribbon*); S124, F128, W190, Y194, and F197 are the side chains of the AAs from the human TOR interacting with Rpm

ScTOR1 and human TOR is shown in Fig. Fs13 (supplementary material). In mammalian cells, TOR kinase controls different processes such as autophagy, protein synthesis, cell size, and growth of muscle cells [189]. Some of the downstream and upstream targets of this vital kinase have been characterized (SupRef.list_H, supplementary material). Human TOR (2549 AAs; NP_004949.1; GI:4826730) [80, 81] and its homologue (AAC50405; NP_001175.2) [191] share about 66 % sequence similarity with each other (Fig. Fs14, supplementary material).

Figure 10 shows an X-ray structure depicting the interaction network within the rapamycin-binding domain (RBD) from the human TOR bound to the hFKBP12A/Rpm complex [192]. Rapamycin is bound to the hFKBP12A in the same fashion as in the binary complex shown in Fig. 3, whereas the part of the RBD containing the hydrophobic and aromatic AAs (L120, F128, W190, Y194, F197) and the following hydrophilic AAs (E121, S124, G129, T187, D191; Dist.comp, supplementary materials) are in van der Waals contact with the atoms of the solvent-exposed atoms of rapamycin (107 contacts at $d \leq 4.5$ Å). There are 77 atomic contacts ($d \leq 4.5$ Å) between the RBD and hFKBP12A (F46, K47, and the AAs in 80s loop). It is noticeable that the aromatic AA residues in the RBD domain are fully conserved in the human TOR, ScTOR1, and ScTOR2 (Figs. Fs11–Fs13, supplementary material). It would probably be worth to investigate in a more thorough manner the extent to which chemical modifications of rapamycin alter the inhibition profile of mTOR [193–195].

The presence of two isoforms of TOR kinase in *S. cerevisiae* and other yeasts' strains would suggest that the signalization pathways controlled by this type of kinase were coined at an early stage of eukaryotic cell development. However, the cytosolic FKBP12 could fully vector the toxic effects of rapamycin in *S. cerevisiae* via its interaction with ScTOR1 [79], provided that the remaining three FKBPs, which are expressed in baker's yeast cells, do not bind rapamycin. If the remaining three FKBPs, however, would bind rapamycin, then what impact might this cause in the cell? In contrast to yeast cells, mammalian organisms express several strong binders of rapamycin [1,

Table 3 Some pharmacological effects and clinical applications of several macrocyclic drugs, which can be vectored via their complexes with the FKBPs

Macrocycle	Cellular targets/carrier vector	Outcome/applications	Reference
FK506	hFKBP12A-mediated inhibition of the CaNA–CaNB–calmodulin complex	T cell anergy	[1, 56, 57, 73, 173–175]
Fujimycine		Immunosuppression	
Tacrolimus		Cutaneous lupus erythematosus and eczema	[212–214]
Pimecrolimus SDS ASM 381	hFKBP12A-mediated inhibition of CaNA–CaNB–calmodulin complex	Treatment of cutaneous lupus erythematosus	[212–214]
		Atopic dermatitis (eczema)	[215]
Rapamycin	hFKBP12A/rapamycin inhibition of the RBD in mTOR	T cell receptor signaling blocked, which in turn causes immunosuppression	[58, 80–82]
Sirolimus			[216, 217]
		Tuberous sclerosis	[200, 218]
		Restenosis	
Everolimus (RAD-001)	hFKBP12A-mediated inhibition of the RBD in mTOR	Immunosuppression	[12]
		Anticancer	[199, 220, 221]
Zotarolimus ABT-578	hFKBP12A-vectored inhibition of the RBD in mTOR	Prevention of restenosis	[221]
		Antiproliferative activity	[203]
Temsirolimus CCI-779	hFKBP12A-vectored inhibition of the RBD in mTOR	Anticancer	[197, 222, 223]

4, 109], and thus not all of the net effect of the drug may be assigned only to its complex with the archetypal FKBP12 and its allosteric inhibition of mTOR kinase. Moreover, it has been shown that rapamycin does not entirely inhibit mTOR complex 1 (mTORc1) [196, 197]. Whether the other members of the FKBPs bound to rapamycin may alter decisive signalization pathways in T cells that have been exclusively assigned to the hFKBP12A/Rpm complex remains to be determined. Some rapalogs bound to the FKBP12A via allosteric inhibition of mTOR kinase exhibit anticancer activity [197–199]. If kinase activity of mTOR could be blocked with a specific and safe inhibitor, then it would probably be a significant step in fighting some cancers [201–206].

Macrocyclic drugs vectored via FKBPs for treatments of various diseases

Structural diversity of macrocyclic metabolites produced by different strains (tacrolimus, sirolimus, and related rapalogs) has been explored only to a limited level of natural niches harboring diverse prokaryotes on earth [207, 208]. Some intricate functional features of the FKBPs associated with diverse targets or their complexes with small ligands could have already been coined in diverse multicellular organisms during their long evolutionary odyssey. For example, it has been suggested that subnanomolar concentrations of FK506 have neuroprotective effects during ischemia-induced oxidative stress and may induce neuronal regeneration [49, 159, 209, 210]. This extraordinary effect could have a reasonable base provided that some of the FKBPs would effectively control [59, 60] functional features of the receptors of the TGFβ family of proteins [105]. Whether a conjunction of TGFβ-driven signalization pathway with another receptor embedded in the extracellular membrane, which are controlled by the FKBPs, could be vital for the protection and regeneration of neurons, remains for further explorations [166].

Table 3 summarizes some of the pharmacological profiles of the macrocyclic drugs shown in Fig. 2, which are in phase III or pharmacological treatment of different diseases. Even if various natural and synthetic ligands of the FKBPs are applied in medical treatment of several pathologies such as *Lupus erythematous*, eczema [212–215], restenosis [200, 218], tuberous sclerosis [216, 217, 219], graft-versus-host disease [76], or antitumor actions [220–223], the full spectrum of their positive physiological actions and molecular mechanisms causing side effects remain to be established. In such a complex setting as the human body, one needs to take into consideration that both the intracellular content of the FKBPs and its extracellular counterpart, if present, must compete for the free drug that was administered in given pharmacological intervention with the final outcome being diversified due to the fact that the hydrophobic macrocycle and its hydrophilic carriers (FKBPs) control many multifactorial processes.

It has recently been suggested that at μ-molar concentrations, CCI-779 inhibits mTORc1 in an FKBP12A-independent fashion with a considerable slowing of protein synthesis on the ribosomes [198]. It has already been shown, however, that rapamycin binds to prokaryotic ribosomes [211], whereas the endogenous FKBP25 and its complex with rapamycin bind to DNA [110], but what implications may those diverse interactions have for vital cellular signalization networks? Even if the hFKBP12A is the principal hydrophilic intracellular carrier for the macrocyclic drugs, because of their very low solubility in aqueous solution and their affinity to membranes and other hydrophobic moieties, the drug being at μ-molar concentrations may be nonspecifically bound to different proteins.

Although about a quarter of a century has passed since the discovery of the immunosuppressive macrolides [5–9], the quest for novel natural and synthetic compounds that bind to the diverse FKBPs has been evolving since then [224–234]. Firstly, novel synthetic or natural compounds could have better pharmacological indexes for treatment of given disease as compared to the compounds shown in Table 3. Secondly, if given FKBP would have a unique and vital function in a specific set of cells, then its selective targeting by nonimmunosuppressive derivatives of the drugs could become of a paramount utility to fight pathologies originating from those cells. Thirdly, novel direct inhibitors of the CaNA phosphatase or the mTOR kinase could bring sizeable solutions to treatments of some diseases in humans. For example, a BLAST search of the human genomic database using the sequence of the kinase domain of the human TOR revealed that it has a low sequence similarity (BLAST max identity $\leq$ 37 %) with several other domains of different kinases such as its homologue (NP_001175.2); DNA-dependent protein kinase catalytic subunit isoform (NP_001075109.1) or Rad3-related kinase (NP_001175) (ATM.search, supplementary material). Such a low sequence similarity to the other human kinases may considerably increase the chances for the discovery of novel selective inhibitors of mTOR.

A number of direct inhibitors of phosphatase activity of the CaN entity are shown in Fig. 11a (reviewed in [234]). Likewise, small-sized synthetic inhibitors of kinase activity of mTORc1 and mTORc2 complexes [235–238] have recently been described. For example, mTOR kinase can be directly inhibited with different small compounds such as Ku-0063794, which inhibits both mTORc1 and mTORc2 at an IC_{50} of about 10 nM [235], TORKinibs [236], or WYE-125132 [237], and some others [238] (Fig. 11b). Derivatives of sirolimus that are modified at

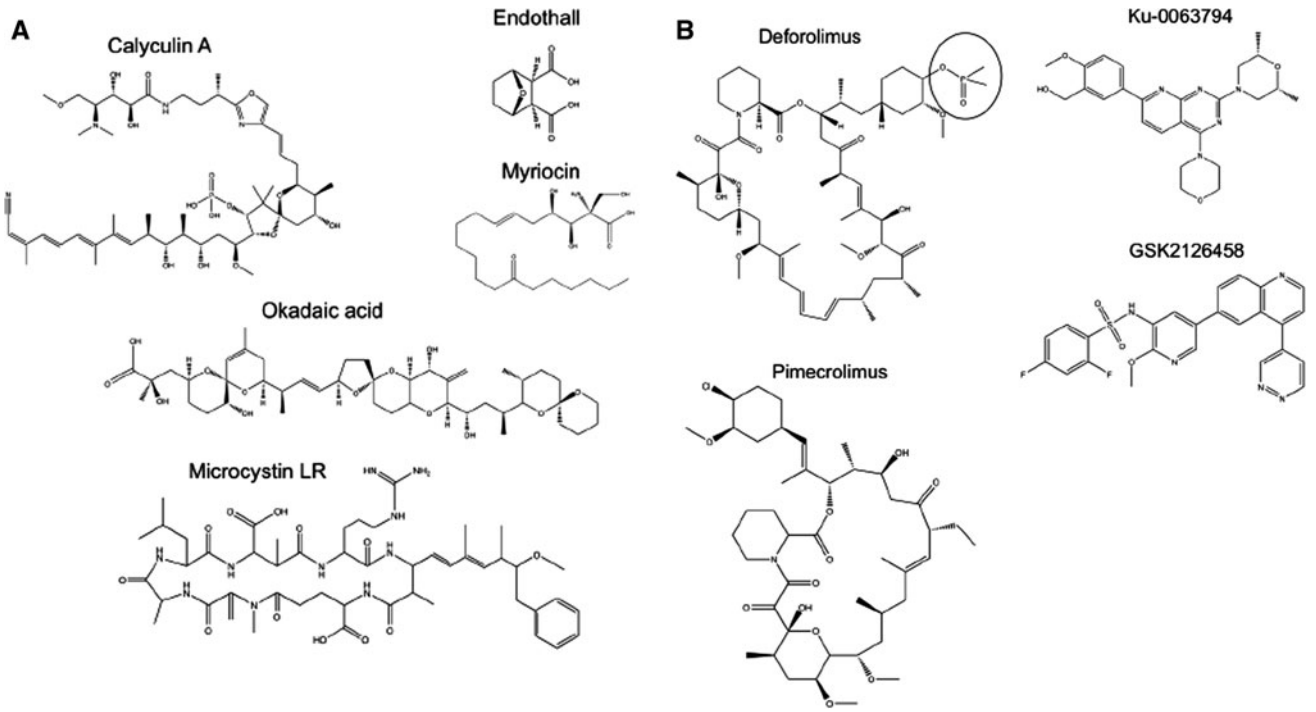


Fig. 11 **a** Chemical structures of several different molecules that inhibit the CaNA. Calyculin A is a toxin isolated from the marine sponge *Discodermia calyx* (CAS 101932-71-2, $C_{50}H_{81}N_4O_{15}P$ $m = 1{,}009.2$ g mol^{-1}); endothall is a herbicide (CAS 145-73-3, $C_8H_{10}O_5$, $m = 186.162$ g mol^{-1}); myriocin was isolated from the thermophilic fungi *Mycelia sterilia* (CAS 35891-70-4, $C_{21}H_{39}NO_6$, $m = 401.54$ g mol^{-1}); okadaic acid was isolated from the marine sponge *Halichondria okadai* (CAS 78111-17-8, $C_{44}N_{68}O_{13}$, $m = 805$ g mol^{-1}); microcystin LR was isolated from cyanobacteria (e.g., *Microcystis aeruginosa*) and is a powerful cyanotoxin (CAS 101043-37-2, $C_{49}H_{74}N_{10}O_{12}$, $m = 995.17$ g mol^{-1}). **b** Novel molecules that inhibit mTOR. Ridaforolimus (deforolimus, rapalog) with the IC_{50} of 0.2 nM for mTOR, (CAS 572924-54-0, $C_{53}H_{84}NO_{14}P$, $m = 990.21$ g mol^{-1}); pimecrolimus (CAS 137071-32-0, $C_{43}H_{68}ClNO_{11}$, $m = 810.453$ g mol^{-1}). Synthetic direct inhibitors of mTOR: Ku-0063794 with the IC_{50} ~10 nM for mTORC1 and mTORC2, respectively; (CAS: 938440-64-3, $C_{25}H_{31}N_5O_4$, $m = 465.54$ g mol^{-1}); GSK-2126458, K_i's for mTORC1 and mTORC2 are 0.18 and 0.3 nM, respectively; (CAS 1086062-66-9; $C_{25}H_{17}F_2N_5O_3S$, $m = 505.5$ g mol^{-1})

–C40–OH, such as ridaforolimus and other rapalogs modified at the same site (Figs. 2 and 11b) have better solubility indexes, which may probably decrease their side effects. If some safe and direct inhibitors of the CaN entity or mTOR kinase could effectively suppress T cells, then they could probably become useful immunosuppressive drugs.

Even if the involvement of mTOR and CaN in various cellular processes have been under investigation during the last two decades, some novel functional features of them have been recently unraveled [239–248]. For example, recent studies have shown that rapamycin has the capacity to prolong the lifespan of some organisms such as *C. elegans* or mouse [249, 250], but molecular mechanisms leading to such an extraordinary effect remain elusive. However, it has been reported that sirolimus may also accelerate the senescence of epithelial cells by shortening the telomeres [254]. Several hypotheses were proposed to link the longevity effect induced by sirolimus in mice with the suppression of kinase activity of mTORc1, which in turn would influence multiple signalization pathways in the cell. For example, it has also been suggested that sirolimus induces clearance of progerin, a protein causing aberrant mitosis and shortening of telomeres, leading to p53 expression and altered cell cycles, which would be perennial for an extension of life expectancy [251]. Those still enigmatic molecular effects would be capable of reversing senescence (aging) to quiescence (resting unchanged) of some organisms. In consequence, rapamycin-induced slowing down of senescence coupled to calorie restriction should also diminish the risk for ageing-related onset of cancers [252–260]. Quiescence of organisms [255–258] or suppression of development and metastases of cancer [100, 105, 147, 260] are complex multifactorial phenomena that cannot be solely dependent on mTORc1 and its allosteric inhibition by rapalogs vectored via FKBP12A [259], or other picomolar inhibitors of its kinase activity [235–238, 258]. Moreover, multiple clinical studies have revealed that the prolonged usage of tacrolimus, sirolimus, and their derivatives in transplant patients may cause some hazardous side effects [1, 248, 261–266].

Conclusions

Analyses of genomic databases of disparate marine organisms [25–31], yeast cells, and various prokaryotes [1, 4, 20, 34, 37] suggest that the chimeric genes coding for the large FKBPs underwent shuffling, fusion, or deletion of domains at some early evolutionary stages of multicellular species. Moreover, the repertoires of the multidomain FKBPs are not fully equivalent in the genomes of the analyzed marine organisms, which indicate that those different species inherited specific functional traits from the FKBPs encoded at lower eukaryotes and prokaryotes. The diversity of the FKBPs' repertoires expressed in the marine species probably reflects some discrete functional features that had been vital for each organism at its evolutionary niche.

Among different effects that are controlled by macrocyclic drugs, such as immunosuppression [1–3, 55–58, 72–75, 79–82, 173–175], quiescence of organisms [247, 249, 250, 257, 269, 270], anticancer activity [241–245], the morphogen-driven signalization networks [271–274], the protection of neurons from ischemia-induced oxidative stress or regeneration of neurons [275–278] might also be influenced by hidden modus vivendi of extracellular communication networks [267, 268, 271–273] and its modulation by the macrocylic drugs. In humans, little is known about the impact that the immunosuppressive drugs (Fig. 2) have on diverse intracellular signalization pathways, which are not under the control of the CaNA or mTOR.

There are more than 2,500 papers describing the isolation, physical–chemical and biochemical characterization of various FKBPs and their complexes with natural and synthetic ligands, whereas several thousands of papers describing pharmacological properties and clinical applications of sirolimus, tacrolimus, and their diverse derivatives are accessible via the PubMed server at the NCBI [22]. To date, only a small number of contradictory results have been published on the rather intricate implications of the FKBPs in diverse regulatory mechanisms of cellular processes or on physiological impacts of the FKBPs and their small ligands (SupRef.list, the supplementary material). Those apparent or real controversies could be due to the fact that the FKBPs form a multigene family of proteins starting from prokaryotes and ending with mammalian organisms, which would imply that some of their cellular functions may be seemingly common to several members of the FKBPs family. Moreover, the FKBP-driven fine-tuning of interacting interfaces in macromolecular complexes could be quasi-infinite in a sequence–structure context. This effect may have a unique impact on each of the FKBPs' targets, which in turn may cause equally unique cell-phenotype-dependent functional outputs. Thus, for the multigene family of the FKBPs, one has to precisely discern: (1) which functional contribution does given FKBP make in a particular cellular or physiological context; (2) how does the given FKBP control conformations and functions of each of its targets; and (3) to what extent may the given macrocyclic ligand alter particular cellular function that is controlled by one of the FKBPs, and whether it is dependent on its binding to this particular FKBP or it is rather a sum of outputs from the actions driven by the diverse FKBP/(immunosuppressive ligand) complexes.

Acknowledgments Due to the relatively large number of publications on the FKBPs and related subjects and even much greater number of reports on clinical applications of the drugs either vectored via the FKBPs or acting via yet-undetermined pathways and molecular mechanisms, only a small percentage of pertinent papers were cited which may, in time, not necessarily represent the most crucial contributions in this area of research and development.

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