A twist in the ABC: regulation of ABC transporter trafficking and transport by FK506-binding proteins

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Post-transcriptional regulation of ATP-binding cassette (ABC) proteins has been so far shown to encompass protein phosphorylation, maturation, and ubiquitination. Yet, recent accumulating evidence implicates FK506-binding proteins (FKBPs), a type of peptidylprolyl *cis–trans* isomerase (PPIase) proteins, in ABC transporter regulation. In this perspective article, we summarize current knowledge on ABC transporter regulation by FKBPs, which seems to be conserved over kingdoms and ABC subfamilies. We uncover striking functional similarities but also differences between regulatory FKBP-ABC modules in plants and mammals. We dissect a PPIase- and HSP90-dependent and independent impact of FKBPs on ABC biogenesis and transport activity. We propose and discuss a putative new mode of transient ABC transporter regulation by *cis–trans* isomerization of X-prolyl bonds.

Keywords: ABC transporters; ABCB; CFTR; chaperon; FKBP; FKBP38; HSP90; PPIase; TWD1

ABC transporters

ATP-binding cassette (ABC) proteins represent an evolutionarily ancient and versatile transport system operating in all living organisms [1,2]. These transport proteins constitute a large family and are grouped phylogenetically into eight subfamilies ABCA – ABCI (ABCH is not found in plants; [3]). Most ABC transporters are primary pumps, which utilize the energy of adenosine triphosphate (ATP)-dependent hydrolysis to transport various substrates across cellular membranes [4]. These include peptides, lipids, ions, sugars, hormones, sterols, alkaloids, metals, proteins, and many other structurally unrelated molecules [5].

The human genome encodes for 48 ABC transporters, with ABCB1/MDR1/PGP being one of the most prominent members of the ABC transporter family, as it promiscuously catalyzes the transmembrane export of a broad spectrum of structurally unrelated drugs and chemotherapeutics yielding in the clinically important phenomenon of *multidrug resistance* in cancer cells [6,7]. Cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) is a unique member of the ABCC/MRP family of ABC transporters, functioning as an ATP-gated chloride channel [8]. CFTR mutation is responsible for the genetic disease mucoviscidosis or cystic fibrosis [9]. This channel seems to be a rather new invention and appears in fishes during evolution [8]; however, CFTR orthologs are not found in plant genomes [3].

Plants have a particularly large number of ABC transporters [10]. The genomes of plants encode for more than 100 ABC proteins (the model plant *Arabidopsis thaliana* encodes for around 130 ABCs),

Abbreviations
ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; FKBD, FK506-binding protein domain; FKBP, FK506-binding protein; NPA, *N*-1-naphtylphtalamic acid; PPIase, peptidylprolyl *cis–trans* isomerase; TOR, target of rapamycin; TPR, tetratricopeptide repeat.

making them nearly twice as numerous in plants as in animals or microbes [10–13]. Plant ABCs were originally reported as transporters involved in detoxification processes [14,15]; however, in recent years, many have been shown to functionally participate in a wide range of essential physiological processes, such as hormone transport, pathogen resistance, and abiotic stress [16–19]. Members of the ABCB family have received special attention, as some isoforms have been shown to be implicated in the cell-to-cell movement or polar transport of the pant hormone, auxin (PAT; [20,21]). Auxin plays an important role in adaptation of plants to their surroundings, as this hormone specifies organ orientation and positioning by regulating cell growth and division in response to internal and external signals [21,22]. Differential auxin transport is thought to provide the basis for the amazing high degree of developmental plasticity of plants in comparison to animals [22].

Although much is known about the function of the ABC transporters in animals and plants, their post-translational regulation of transporter function has remained relatively uncharacterized until recently. A significant problem associated with identifying proteins that regulate ABC transporter function is the detection of interacting proteins, which is caused often by the high hydrophobicity of transmembrane ABC proteins. An investigation of the post-transcriptional regulation of ABC proteins has focused mainly on two levels: on the regulation of ABC transporter activity (mainly by protein phosphorylation) and on the control of ABC protein homeostasis (or proteostasis), a cellular key process ensuring successful development, aging and resistance to environmental stresses [23]. ABC proteostasis is guaranteed by well-controlled pathways involving biogenesis, folding, trafficking and degradation of proteins. To minimize or correct mistakes, chaperones bind to and accompany protein folding events providing constant quality control [24].

There is recent accumulating evidence that both ABC transporter activity and proteostasis might be regulated by FK506-binding proteins (FKBPs). The Heitman lab initiated this young field by showing that murine MDR3/ABCB4 transport activity in yeast depends on the presence of yeast FKBP12 [25]. Later, it was shown that auxin transport of a class of ABCBs relies on the presence of the Arabidopsis FKBP42 protein (TWISTED DWARF1, TWD1 [26–30]; ULTRACURVATA2, UCU2; Fig. 1). It has been reported that a close human ortholog of FKBP42/TWD1, FKBP38 (Fig. 1) promotes folding and ER-to-plasma membrane delivery of CFTR/ABCC7 [31,32].

FKBP-type immunophilins

FKBPs were originally discovered based on their ability to bind two different classes of immunosuppressant drugs, FK506 (Tacrolimus) and rapamycin (Sirolimus), respectively [33]. FKBPs possess usually a peptidylprolyl *cis-trans* isomerase activity (PPIase) that catalyzes the rotation of peptidylprolyl bonds (Box 1; [33,34]). PPIase activity correlates with the so-called PPIase or FK506-binding protein domain (FKBD; Fig. 1) and as such is usually inhibited by immunosuppressant drug binding to this active site. PPIase activity is not limited to FKBPs but found also for cyclophilins and parvulins, forming together the superfamily of PPIases [33]. Since cyclophilins binds the immunosuppressant drug, Cyclosporin A, they are classified as immunophilins together with FKBPs [33].

Most PPIases, like the 12 kDa FKBP12 that is composed essentially of just the prototype PPIase/FKBP domain (Fig. 1), act as molecular foldases that unlike other chaperones (such as heat-shock proteins, HSPs) do not utilize other co-factors, such as ATP [33,42]. Instead, they rather bind their client proteins using a shallow and promiscuous active site that is thought to favor proline isomerization through conformational selection [43]. Importantly, this event acts as a molecular switch during folding, activation and/or degradation of many proteins. These PPIase clients often play a key role in human diseases (e.g., cancer, neurodegeneration, and psychiatric disorders) and plant development, suggesting that PPIase inhibitors might be relevant tools as therapeutics or for controlling plant architecture [33,34,43].

In addition, some FKBPs have been demonstrated to possess an additional chaperone activity that is independent of their PPIase activity and domain and unaffected by immunosuppressant drugs [33,34]. These so-called multidomain FKBPs consist of one copy or several tandem repeats of the prototypical PPIase domain of about 100 amino acids along with additional tandem repeats of EF-hand Ca^{2+}-binding, calmodulin-binding or tetratricopeptide repeat (TPR) domains (Fig. 1; [33,34]). The latter consists of a minimum of three loosely conserved 34-amino acid TPR motifs that have been suggested to be responsible for the chaperone activity of multidomain FKBPs. Importantly, TPR domain FKBPs are often associated with HSP90 in protein heterocomplexes, where HSP90 binding is provided by the TPR domain [33]. The TPR domain of FKBPs binds to the MEEVD peptide of the HSP90 C terminus. As HSP90s themselves exhibit an ATPase-dependent chaperone activity, the specificity of the HSP90s to their client proteins is considered to be provided by the FKBPs, which

function in that respect as HSP90 co-chaperones [31,44]. Currently, it is not entirely clear if the FKBP chaperone function generally contributed to the TPR domain is provided by HSP90 or independent of HSP90 action. The latter option is supported by the finding that some multidomain FKBPs, like HsFKBP52 and HsFKBP38, participate in functional protein interactions without a known contribution of HSP90 [45]. However, in most cases the participation of individual domains of FKBPs in chaperoning is not yet analyzed in detail.

In summary, multidomain FKBPs appear to possess both PPIase and chaperone activities that roughly correlate with PPIase/FKBP and TPR domains, respectively, and as such will be discussed below separately under these names. However, it is important to keep in mind that both activities might be involved separately or together in folding processes of newly

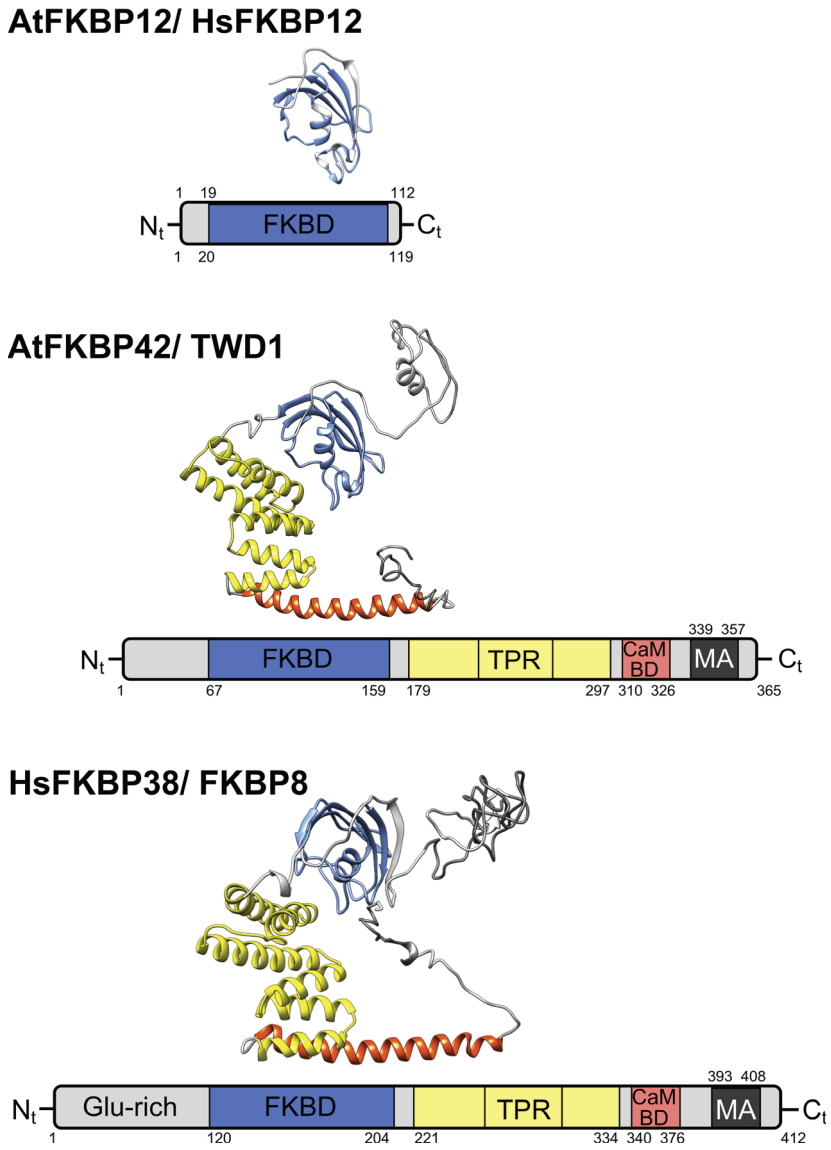
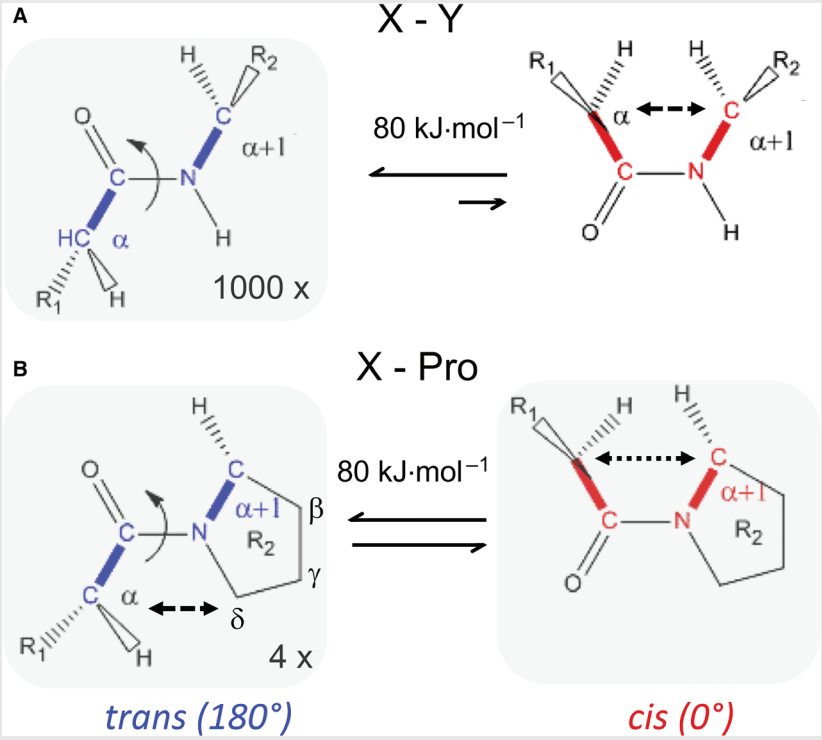


Fig. 1. Domain composition and molecular structures of human FKBP38 and *Arabidopsis thaliana* FKBP42/TWD1. Cartoon diagrams show functional domains in the two multidomain FKBPs as well as in the canonical FKBP12. Protein structures are presented as illustration and were generated using the I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER) from full-length peptide sequences without constraints. Functional domains are labeled and color-coded as follows: FKBD, FK506-binding domain (blue); TPR, tetratricopeptide repeat domain (yellow); CaM-BD, calmodulin-binding domain (orange); MA, in-plane membrane anchor (dark grey); Glu-rich, glutamine-rich domain (ERD) (light grey). Numbers indicate amino acid boundaries (FKBP12: upper and lower numbers refer to human and Arabidopsis FKBP12, respectively).

Box 1. *Cis-trans* isomerization of peptide and X-proline bonds is a rate-limiting step during protein folding

Peptide bonds in folded proteins (X-Y) are usually found in the *trans* conformation (the *trans* is 1000 times more frequent than the *cis*, due to the steric clash (dashed arrows) of the C_{α} with the $C_{\alpha+1}$ in the *cis* conformation. (A) However, the situation is different for X-Pro bonds as the unique, cyclized nature of the proline imidic peptide bond (B) positions the Cα in conflict with a carbon in both isomeric conformations, the $C_{\alpha+1}$ in *cis* or the C_{δ}, in *trans* (dashed arrows). As a result, the free enthalpies between the *cis* and *trans* conformations are very similar, and therefore, they are nearly in equilibrium in non-structured peptides or in intrinsically disordered protein regions [35–37]. In peptides dissolved in non-polar solvents, a 5% *cis* population of X-Pro was observed [35]; however, this ratio increased in polar solvents to up to 20%. When investigating a specific protein target, a 5–20% *cis* ratio was observed by NMR (average: 10%) [38]. Moreover, it is known that the type of the proceeding amino acid strongly influences the *cis-trans* ratio: aromatic residues increase the presence of the *cis* isomer (e.g., Trp-Pro 37.7%; Phe-Pro 23% [39] or 29% [40]). In summary, it appears that, depending on the amino acid proceeding the proline [39], the *cis* portion is around 5–30% (25% in average) in folded proteins. Thus, *trans* X-Pro conformations are roughly four times more frequent than *cis* conformations.

Due to the high activation energy that is required for peptide bond rotation (roughly 80 $kJ \cdot mol^{-1}$), which is caused by its partial double bond character, spontaneous peptidyl prolyl *cis-trans* isomerization is slow (on the time scale between milliseconds to seconds). Does the protein chain, however, contain non-native prolyl isomers (especially *trans* isomers that are *cis* in native states) then the protein folding can be even slowed down to minutes [41]. Thus, proline isomerization can be rate limiting and requires PPIases to alleviate this bottleneck by reducing the activation energy. Peptide bond structures were taken from https://employees.csbsju.edu/hjakubowski/classes/ch331/protstructure/PS_2C1_Main_Chain_Conf.html).



synthesized proteins, during protein trafficking and assembly of protein complexes. Additionally, this separation might be a simplification, as for some PPIases (e.g., human CypA, which consists only of the prototypical PPIase domain) a chaperone-like function has been reported [46].

However, the benefit of accessory domains, such as the TOR (target of rapamycin) domain, might lie in the fact that they create additional protein binding sites in addition to the PPIase site. Prolyl isomerization might profit from this interaction thus adding an additional layer of control for this activity. Another aspect is that multidomain FKBPs seem to act as scaffold proteins that tether different partner proteins into complexes. Finally, multivalent binding is an important feature of molecular recognition during synergistic or cooperative interactions [33].

Like the domain structure of FKBPs, also their subcellular locations and cellular functions are highly variable. While the prototype FKBP12 is cytoplasmic, additional motifs target FKBPs to different organelles or anchor them in membrane compartments (for excellent reviews on this matter, see [33,47,48]). The variability in both domain structures and locations results further in an involvement in a plethora of cellular functions. Beside classical protein folding and chaperoning activities, FKBPs are associated with receptor signaling, protein trafficking, transcription and apoptosis [48]. This high degree of flexibility can be exemplified by FKBP12 that beside as a mediator of immunosuppressant drugs through interaction with calcineurin and TOR, interacts and modulates the activity of the calcium channels, ryanodine receptor, and inositol 1,4,5 triphosphate receptor [48]. Further, FKBP12 binds to TGF-β receptor and keeps it in an inactive state [48].

Higher plants were found to be insensitive to both FK506 and rapamycin, since plant FKBP12, despite its close structural conservation (Fig. 1), has lost its capacity to bind these molecules [49]. PPIase activity is also not well-conserved in plant FKBPs, and many plant multidomain FKBPs are suggested to participate in plant development by regulation of protein-protein interactions [34,44]. Despite the important function of FKBPs and their downstream clients, the main function of FKBPs seems to lie in a fine-regulation of many cellular processes. This is underlined by the finding that deletion of all FKBPs in baker's yeast (as well as of all cyclophilins) is not lethal [50].

ABC transporter regulation by FKBPs

Murine MDR3/ABCB4 activity depends on yeast FKBP12

First insights that ABC transporters are regulated by FKBPs was provided by the Heitman lab that showed that yeast FKBP is required for murine MDR3/MDR1a function in yeast [25]. This study was motivated by the finding that immunosuppressant drugs, like cyclosporin A, FK506 and rapamycin, can partially overcome multidrug resistance (MDR) [25,51]. Although these immunosuppressant drugs reverse MDR by competitive inhibition of ABCB transport [52], the FKBP-dependent MDR3/MDR1a function raised the possibility that the targets of immunosuppressant drugs might regulate ABCB function.

Genetic deletion of yeast FKBP12 (but not of cyclophilin or calcineurin) significantly reduced MDR3's ability to confer resistance to dactinomycin or FK506/CsA in detoxification assays using yeast strains that were rendered hypersensitive toward these drugs [25]. FKBP12 deletion did not significantly alter MDR3 expression or plasma membrane location arguing for the fact that yeast FKBPs is required for MDR3 transport activity. Importantly, this regulatory effect was not dependent on FKBP12 PPIase activity, as shown by using a PPIase-deficient *FKBP12* mutant [25]. This is in analogy to regulation of the ryanodine receptor calcium channel by FKBP12 [53], suggesting that the catalytic and drug-binding site is responsible for ABC regulation. In such a model, immunosuppressant drugs would disrupt the activating ABCB-FKBD interaction, which would result in ABCB inhibition. However, up to today MDR3-FKBP12 interaction is awaiting its confirmation.

FKBP42/TWD1 functions as a chaperon for plant ABCBs

Arabidopsis ABCB1 was originally identified in a yeast two-hybrid screen using the soluble part of FKBP42/TWD1 as a bait in order to understand the drastic, pleiotropic developmental phenotype caused by loss-of *TWD1* function [30]. Functional TWD1-ABCB1 interaction was verified by co-immunoprecipitation and BRET as well as co-expression in heterologous systems [26,54]. Interestingly, also Arabidopsis ABCB1 transport activity was dependent on yeast FKBP12 [54]. This is leading most likely to the counter-intuitive effect that unlike *in vivo*, FKBP42/TWD1 acts as a negative regulator of ABCB1 in yeast most likely by competing for activation by yeast FKBP12 [54]. As for yeast FKBP12, ABCB interaction is provided by the FKBP/PPIase domain of FKBP42/TWD1. However, all attempts to demonstrate a PPIase activity for FKBP42/TWD1 failed, most likely due to the low conservation of 10 out of 14 amino acid residues, which were thought to be important for catalysis and FK506 binding [30]. Instead, a chaperone/ holdase activity for purified FKBP42/TWD1 was found that could be

most likely attributed to the TPR domain, which was also shown to bind HSP90 [55].

A key finding for an understanding of FKBP42/TWD1 functionality was that three transporters of the plant hormone auxin, ABCB1, ABCB19 and ABCB4, were retained in the ER and degraded in *fkbp42*/*twd1* loss-of-function plants [26,27] suggesting that FKBP42/TWD1 controls ABCB biogenesis. As a proof of concept, double mutants of *abcb1 abcb19* share widely overlapping phenotypes with *fkbp42*/*twd1*, including dwarfism and a helical ('twisted') disorientation of organs and epidermal layers [30]. However, apparently in the absence of TWD1, a small portion of ABCBs is able to escape ER degradation and appears on the cell surface [26]. Functional ABCB-TWD1 interaction at the plasma membrane was detected by BRET [26], suggesting the existence of at least two ABCB-TWD1 pools in the ER and in the plasma membrane, despite the fact that FKBP42/TWD1 is primarily an ER-anchored protein.

The TWD1 FKBD was shown to bind the non-competitive auxin transport inhibitor, *N*-1-naphtylphtalamic acid (NPA), and NPA binding was mapped to a small pocket of the putative active site using NMR [56]. NPA disrupts ABCB1-TWD1 interaction [57], providing a rational for the inhibitory effect of NPA in analogy to the inhibitory effect of FK506 on MDR3 transport in yeast.

Currently, it is unclear if FKBP42/ TWD1 acts only as a chaperone during ABCB biogenesis or additionally as a regulator of ABCB transport by interaction on the plasma membrane. Also, it is not known which functional domains of FKBP42/TWD1 are involved in both processes, especially if FKBP42/TWD1 owned a hidden PPIase activity similar to FKBP38 (see below). An impact of FKBP42/TWD1 in a module, where it regulates ABCB transport activity by *cis-trans* isomerization of prolyl bonds, was provided recently by the identification of a surface-exposed, diagnostic D/E-P motif in the NBD2 of ABCBs that do transport auxin [58]. Individual mutation of either E or P in ABCB1 significantly reduced auxin transport; however, the D/E-P was not essential for interaction with TWD1 [58].

An unusual feature of FKBP42/TWD1 is its 'in-plane membrane anchor' that was suggested to have perpendicular orientation to the lipid bilayer [59] and to attach FKBP42/TWD1 to the membranes of the ER, the vacuolar membrane and the plasma membrane [26,29,30,55]. Interestingly, deletion of this motif did not alter plasma membrane fixation but resulted in enhanced auxin transport and hypermorphic plants [60].

As shown for mammalian FKBPs, also FKBP42/TWD1 interacts with multiple clients: beside ABCBs, FKBP42/TWD1 was shown to functionally interact with vacuolar transporters, ABCC1 and ABCC2; however, in contrast to ABCBs, binding to ABCCs was provided by the TPR domain [29]. Additionally, FKBP42/TWD1 was shown to physically interact with the plasma membrane-embedded brassinosteroid receptor, BRI1, although the exact mode of action is far from understood [61]. Further, FKBP42/TWD1 provides direct physical connection to actin isoform 7, but modulates actin bundling in an indirect fashion [56]. As a result, ABCBs that are chaperoned by FKBP42/TWD1 are delocalized to endosomal structures in *actin7* mutants, although this action is not restricted to these ABCBs. Currently it is unclear if ABCB mistargeting in *fkbp42*/*twd1* and in *actin7* are mechanistically coupled or connected *via* previously suggested auxin-actin circuits [62].

FKBP38 controls synthesis and biogenesis of CFTR/ABCC7

FKBP38/ FKBP8 is involved in very different cellular processes that do not depend on its PPIase activity, including cell size regulation, development of the neural tube, mTOR signaling, hypoxia and viral replication (for excellent reviews on this topic, please see [31,33,48]). In contrast, FKBP38 controls also apoptosis signaling on the mitochondrial surface by inhibiting the anti-apoptotic factor, Bcl-2, in an action that requires an exclusive activation of its PPIase domain by Ca^{2+}/calmodulin [31,63,64]

Interestingly, FKBP38 associates with nascent plasma membrane ion channels, HERG (human ether-à-go-go; [65]) and CFTR on the cytoplasmic face of the ER [66–68] and apparently regulates their biogenesis. FKBP38, together with several components of an HSP70-HSP90 relay system, was initially identified as part of the CFTR interactome that was conducted in order to explore cell-surface rescue of ΔF508-CFTR [68], which is restricted to the ER and the most common disease variant [8]. Interestingly, both FKBP38 and HSP90 preferably associate with ΔF508 CFTR indicating that both play a key role in targeting folding-deficient CFTR for degradation [68]. This agrees with the original identification of CFTR as an HSP90 client by pharmacological inhibition of HSP90 activity, which dissociates HSP90 from WT CFTR leading to CFTR (and HSP90) degradation. Interestingly, both *FKBP38* silencing as well as over-expression destabilized WT CFTR consistent with an interpretation, in which the steady-state level of FKBP38 and associated

co-chaperones (the CFTR 'chaperome') contribute to the stability of nascent CFTR on the ER [68].

FKBP38 shares overall the same domain structure as FKBP42/ TWD1, however, owns additionally an N-terminal glutamate-rich domain (ERD; Fig. 1). Two independent studies were conducted to dissect the individual roles of the FKBP38 subdomains on CFTR biogenesis [32,67]: *FKBP38* knock-down was shown to increase CFTR biosynthesis but inhibited post-translational folding leading in summary to reduced steady-state levels of CFTR at the ER, decreased processing and PM expression [32]. Membrane anchoring of FKBP38 is essential for inhibition of protein biosynthesis but not for CFTR folding. Deletion of the FKBP38 PPIase domain or its inhibition by using the FKBP38-specific PPIase inhibitor, DM-CHX, promoted CFTR folding but had no significant effect on biosynthesis [32]. Strikingly, uncoupling TPR and HSP90 action by TPR mutations enhanced FKBP38 activity and resulted in increased CFTR maturation and decreased biosynthesis, suggesting that HSP90 inhibits both cellular functions of FKBP38 [32]. This is in agreement with *in vitro* studies demonstrating that HSP90 inhibits FKBP38 PPIase activity [69]. These data support an overall model in that FKBP38 regulates CFTR folding during its biogenesis primarily *via* its enzymatic PPIase activity, which is inhibited by HSP90 bound to the TPR domain but not to CFTR. FKBP38-mediated folding of CFTR would play an ancillary role.

In contrast, Hutt *et al*. [67] demonstrated that inhibition of FKBP38 PPIase activity by DM-CHX treatment resulted in a dose-dependent increase in the steady-state stability of WT and ΔF508 CFTR, although experimental setup and conditions were slightly different. These findings were obviously also in conflict with their own *FKBP38* silencing data that convincingly resulted in decreased WT CFTR channel activity. However, authors showed that FKBP38 was capable of binding CFTR independently of HSP90, supporting a model in that FKBP38 is a late acting chaperone functioning downstream of HSP90, required for ER export.

In summary, it appears that FKBP38 with its associated CFTR 'chaperome' provides the first checkpoint of CFTR quality control of nascent CFTR at the ER [66]. However, the impact of PPIase-dependent and TPR-dependent actions on CFTR has not yet been clearly dissected. The same holds true for the unclear roles of TPR-bound and CFTR-bound HSP90 pools and thus their individual roles in the ER and the PM. While most previous studies have so far focused on the regulation of ΔF508 CFTR biogenesis, the impact of FKBP38/ HSP90 on CFTR transport activity has not yet been quantified in detail. Using SRM-MS, it was shown that HSP90 binds to NBD1 of CFTR, however, that lack of HSP90 dissociation from ΔF508 CFTR prevents association of NBD1 with NBD2 leading to ERA degradation [70]. An elegant study revealed that the HSP90/ HSC70 chaperone system is not limited to ER degradation but is able to restore the channel activity of misfolded ΔF508 CFTR at the PM [71].

Cross-kingdom conservation of a regulatory ABC-FKBP/HSP90 module

A recent comparison of regulatory events of a subgroup of auxin-transporting ABCBs (ATAs) from the model plant Arabidopsis with human CFTR/ABCC7 revealed similarities in respect to their linker/R domain phosphorylation and the overlapping domain structures of interacting multidomain FKBPs, FKBP42/ TWD1 and FKBP38, respectively [72]. We here deeply extend this analysis by providing evidence that ATAs and CFTR employ an evolutionary conserved but complex regulatory mechanism, which is provided by physical interaction with FKBP-HSP90 chaperone relays. The current picture that emerges is that both FKBP38 and FKBP42/TWD1 are key regulators of ATA and CFTR biogenesis (Fig. 2), where they contribute to an early step of quality control of nascent ABCs. While for FKBP38, convincing evidence was provided that this event is mainly provided by the PPIase activity of the FKBD, which is under positive and negative control of Ca^{2+}/calmodulin and TPR-bound HSP90, respectively, this has not yet been dissected for FKBP42/ TWD1.

However, both FKBPs are apparently also important for later stages of post-ER trafficking to the PM and at least for FKBP42/TWD1 a regulatory impact on the activity of PM-resident ABCB1 was shown [26]. As such, FKBP42/TWD1 seems to act as the functional plant ortholog of human FKBP38 in respect to ABC regulation, despite the fact that actually FKBP36/FKBP6, which is best known for its interaction and regulation with GAPDH [48], is the closest human orthologous sequence to FKBP42/TWD1. On the ABC transporter side, conservation of such a regulatory module is somewhat surprising as ATAs and CFTR belong to distinct ABC subclasses, ABCBs/ PGPs and ABCCs/MRPs, respectively. Interestingly, such a regulatory circuit has not been described for non-auxin-transporting ABCBs or mammalian ABCBs. Strikingly, both ATAs and CFTR are to our knowledge the only ABC transporters that export anions, IAA^- and Cl^-, respectively. Therefore, a co-

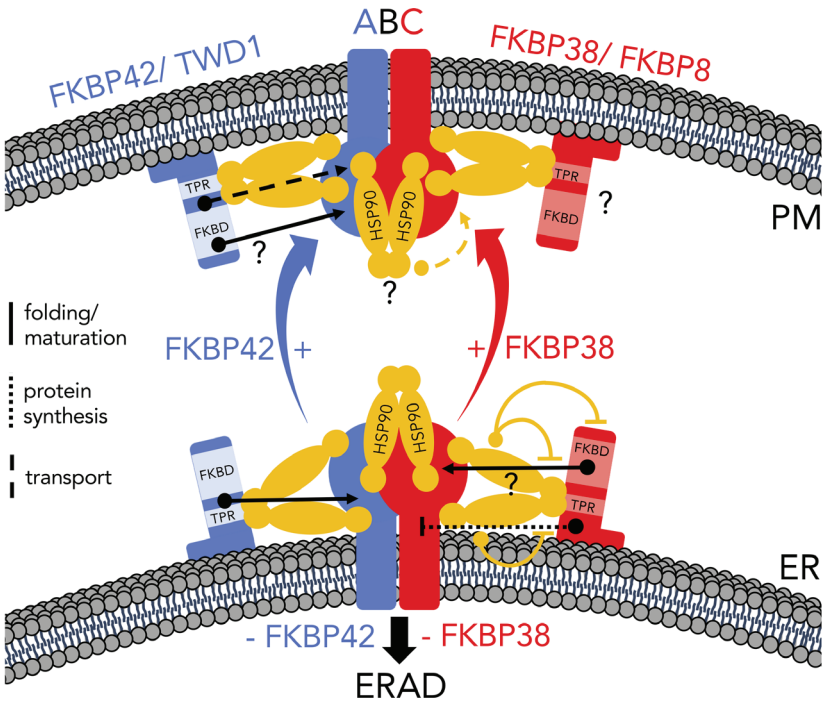


Fig. 2. Putative model of post-transcriptional regulation of plant ABCBs and human CFTR/ABCC7 by FKBP42/TWD1 and FKBP38/FKBP6. Human (red) FKBP38 and plant FKBP42/TWD1 (blue) regulate the biogenesis of CFTR/ABCC7 and auxin-transporting ABCBs (ATAs) on the ER, symbolized by ABC. In the absence of FKBP38 and FKBP42/TWD1, ABCs undergo degradation through the ERAD pathway. Additionally, FKBP38 and FKBP42/TWD1 are involved in ER-to-PM trafficking (large arrows) and probably also in events regulating ABC activities in the PM, such as transport or channel gating. It is not yet clear if ABC are HSP90 clients independent of FKBPs or if HSP90 binding to the TPR domains of FKBPs is required. Also, the distribution and roles of the ER and PM pools of HSP90 are unknown. The impact of involved functional domains (FKBD, FK506-binding domain; TPR, tetratricopeptide repeat domain) on folding, maturation or transport is specified by straight, dotted or interrupted lines; positive and negative actions are indicated by pointed and dull arrows, respectively. Uncertain findings are labeled with a question mark.

evolutionary analysis of FKBP42/TWD1-ATA and FKBP38-CFTR might be informative. Recently, co-evolution of permease-like ABC transporters with two-component histidine kinases involved in antimicrobial resistance was demonstrated [73].

For FKBP42/TWD1-ABCB1 interaction, it was shown that the FKBD interferes with the very C terminus of NBD2 [30], however, a targeted mutagenesis approach as well as several trials to extract surface residues by *in silico* docking using modeled ABCB1 [74] and the crystal structure of FKBP42/TWD1 (PDB 2IF4; [75]) failed. FKBP38-CFTR contact domains have not yet been determined. Therefore, for both interactions contacting surfaces might be predicted by using so-called *amino acid co-evolution*. This approach is based on the concept that when a pair of amino acids (one from each protein in two-protein complex) co-varies, then these two amino acids have a pressure to co-evolve because the assumed physical contact between them (intramolecular or a protein-protein interface in a complex) [76]. This approach has been successfully used to map intramolecular interaction sites in human ABCB1 and CFTR [77] and to find residue contacts between known interaction partners [78]. As an alternative approach, co-evolution of residues in contact between two interacting proteins could also be studied with Direct Coupling Analysis methods, Bayesian methods, consensus approaches or machine-learning algorithms (see [79] for details).

Still a puzzling finding is the relevance of membrane anchorage of FKBP38 to mitochondrial and ER membrane and of FKBP42/TWD1 to ER, vacuolar and plasma membrane, respectively. In general, membrane anchoring is thought to enable recruitment of cytoplasmic chaperones to membrane surfaces [33]. In light of the fact that deletion of this domain from FKBP38/FKBP42 has apparently only little influence on post-translational folding of ABCs [32,60], FKBP38 interaction with the ABC client proteins is likely sufficient to scaffold additional interacting partners.

A better understanding of FKBP42/38 functionalities toward ABC regulation is hindered by several complications: First, as explained before, multidomain FKBPs both proteins contain PPIase and chaperone activities correlating with FKBP and TPR domains that, however, can each contribute to ABC folding and maturation. While a pharmacological and mutational dissection of the PPIase domain of FKBP38 was successfully performed [32,67], this is more difficult for the chaperone function associated with the TPR domain. This holds especially true as such an inhibitor does not yet exist and mutation of the TPR domain might as well interfere with HSP90 binding.

Second, in contrast to single and other multidomain FKBPs, FKBP42/TWD1 and FKBP38 act both as chaperones and co-chaperones. For example, FKBP12 chaperones the electrophysiological properties of calcium channels [53] and FKBP52 is a co-chaperone of HSP90 action on the steroid hormone receptor [80]. While FKBP52 can bind to the steroid hormone receptor only in the presence of HSP90, the binding of FKBP38 to its client is abolished by HSP90. However, this has been shown only for Bcl-2 during onset of apoptosis [69] and can be different for ABC clients. In conclusion, one possibility is that FKBPs deliver ABC proteins to HSP90 (or *vice versa*), while a plausible alternative is that FKBPs are recruited to the ABC protein *via* HSP90 for folding of the prolyl bonds. The latter option is supported experimentally for FKBP38 [67], however, but far from being understood.

A third layer of complication is added by the fact that there might exist two independent pools of HSP90, one that binds directly to the ABCs and that is independent of the FKBPs, while another depends on interaction with the TPR domain of the FKBPs. Additionally, both HSP90 pools might vary on ER and PM, respectively, therefore a simple pharmacological disruption of HSP90 action by ansamycin inhibitors has its limitations. The TPR-bound HSP90 pool can be easily omitted by TPR mutation as shown for FKBP38. Another valid alternative might be specific drugs or peptides, like the recently discovered LB76 [81] that blocks specifically HSP90 binding to TPR domains. However, also these approaches might have their pitfalls because for FKBP38 (but not yet for FKBP42/TWD1) it was shown that TPR-bound HSP90 has an inhibitory impact on PPIase activity and thus on ABC folding. Last but not least, also an action of FKBP38/FKBP42 on ABC maturation or activity without any impact of HSP90 as described for FKBP52 [33] is imaginable but unlikely. This is indirectly supported by the fact that ATAs and CFTR but not mammalian ABCBs are HSP90 clients, indicative of the concept that client specificity might be provided by the FKBPs acting here as co-chaperones.

X-proline residues as possible molecular switches of ABC transporters

Phosphorylation of regulatory linker or R domains of ABCBs or CFTR, respectively, adds negative charges (e.g., by phosphorylation) that, by means of conformational changes, alter the functional capacities of these ABC proteins [8]. In contrast, PPIases are able to catalyze conformational changes without changing charges. Furthermore, regulation by PPIases does not require an enzyme such as a phosphatase to revert the targeted protein to its initial state, since isomerization is reversible and needs relatively low energy. Although a final demonstration is lacking, previous work suggests that CFTR channel activity is regulated by PPIases. By means of site-directed mutagenesis, proline residues in the transmembrane segments were found to be important for CFTR function: while P205 is critical for correct protein processing, P99 may contribute either directly or indirectly to the Cl- channel pore [82]. Recently, mutation of a TMH6 proline resulted in a gain-of-function phenotype [83]. Interestingly, addition of recombinant cyclophilin A or alanine mutation of three prolines in the R domain of reconstituted CFTR leads to channel opening [83]. Strikingly, CFTR opening by cyclophilin A is reversed by cyclosporin A known to inhibit its PPIase activity, providing strong evidence that ABC transport activation can depend on peptidylprolyl isomerization. This concept was recently hardened by demonstrating that transport activation of Arabidopsis ABCB1 by FKBP42/TWD1 was absent, when P1008 located at the surface of NBD2 was mutated to alanine [58]. As said before, direct evidence for any ABC transporter as a substrate for FKBP PPIase is missing and methods to identify PPIase 'hot' prolines are not available. A mapping of surface-exposed prolines on Arabidopsis and human ABCB1 and as well as on CFTR revealed that all three transporters revealed a roughly similar number of prolines that are randomly distributed over the transporter protein (Fig. 3). Excitingly, despite belonging to distinct ABC families, P1008 seems to be spatially and structurally conserved in CFTR [58]; P1175 in Fig. 3). This finding might suggest that also CFTR in analogy to AtABCB1 could prefer the recruitment of FKBP38 to the loop between TMD2 and NBD2, eventually to NBD2.

Recently, accumulating evidence was provided that prolyl *cis-trans* isomerization by PPIases can act as a

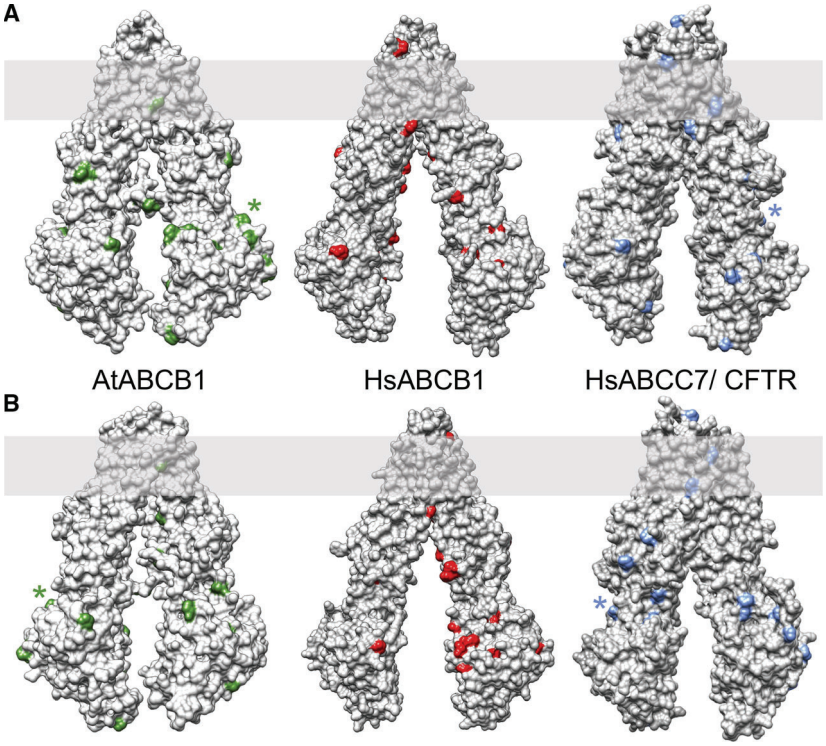


Fig. 3. Surface-exposed proline residues in Arabidopsis ABCB1, human ABCB1, and CFTR/ABCC7. Front (A) and back (B) surface models of AtABCB1, HsABCB1 (PDBID:4Q9H), and HsCFTR/ABCC7 revealing surface-exposed proline residues (in color). Arabidopsis ABCB1 (GenBank: NP_181228) modeled based on the high-resolution mouse ABCB1 structure (PDBID:3G5U) was taken from [74]. Human CFTR/ABCC7 structure (PDBID:5UAK) and the linker between TMD2 and NBD2 were modeled using the loop modeling algorithms of MODELLER (https://salilab.org/modeller). UCSF CHIMERA (https://www.cgl.ucsf.edu/chimera) was used for visualization. Conserved residues P1008 and P1175 in AtABCB1 and CFTR, respectively, are indicated by asterisks.

molecular timer to control the amplitude and duration of diverse cellular process, such as phage infection. By using high-resolution NMR spectroscopy, it was shown that a phage protein, interacting with cell surface host proteins, reveals a heterogenous mix of prolyl isomers [84]. Interestingly, the switching rate and thus the infectivity of the phage are determined by the local sequence around a critical proline [84].

However, whether specific domains of ATAs or CFTR function as molecular switches, whose states are regulated by PPIases, awaits confirmation but represents a fascinating venue. In such a scenario, internal or external signals would lead to an activation of a PPIase activity on an FKBP that would isomerize accessible prolyl residues. These would turn on ABC, here ATAs or CFTR, and transfer them into an active or hyper-active state, which would fall back automatically into its basal state after seconds to minutes. Such a mechanism can be expected to result in rhythmic activity states, which have been observed for excised plant shoot segments that reveal transport rates, which undergo oscillations with a period length of ca. 20 min after application of auxin [85].

Interestingly, there are important structural and dynamical issues associated with an activity regulation by prolyl isomerization. While, accessibility for PPIases acting during protein folding is not limited, it is crucial that the target proline residue should be accessible for the PPIase. In folded proteins, the post-translational modification sites tend to be located in intrinsically disordered regions. Importantly, even if the proline is part of a highly flexible segment, it exhibits a roughly 4-times higher preference for *trans* isomers (for details, see Box 1), which is a requirement for regulatory functions observed for proline switches. Importantly, the environment of either P1008 in Arabidopsis ABCB1 or P1175 in CFTR is likely restricted. While these residues are located between TMD2 and NBD2 corresponding to a linker region (which are usually unresolved in 3D structures) the proline is separated from the end of TMH12 only by a few amino acids. Thus, in spite of the high dynamics suggested by the invisibility of the linker region, the conformational space of these proline residues is limited by the close vicinity of more rigid regions.

However, this reasoning cannot explain how *cis-trans* isomerization of the three prolines in the middle of the disordered R domain (approx. residues between 645 and 837) can have an influential effect on CFTR function [86]. We believe that a structural background of this regulation can be imagined only in the context of intramolecular interactions, in which the middle of the R domain including the three prolines (P740, P750, and P759) forms an inhibitory intramolecular interaction with the core of CFTR. The isomerization would alter the conformation of this segment, which

consequently would dissociate from the rest of the protein or inhibiting the re-association after R domain dephosphorylation, leading to chloride channel activation. Intriguingly, all of these Pro residues are located between two important phosphorylation sites, S737 and S768. If these phosphorylation sites were mutated to alanines, CFTR exhibited an increased activity without phosphorylation [8]. Thus, these serines may serve as anchor points by stabilizing the inactive conformation *via* H-bonds thus providing the background for a two-level regulation: (a) *via* phosphorylation the H-bonds of S737 and S768 thus also the inhibitory conformation is vanished; (b) these H-bonds restrict the conformational space of this region that likely promotes a disequilibrium of Pro isomeric states thus the basis of PPIase regulation. This coincidence further supports the direct action of PPIase on these Pro residues and thus CFTR function.

Real-time NMR combining high-resolution with kinetic information of protein reactions is currently the method of choice to reveal such structural details about the interaction between FKBP-type PPIases and transient ABC transporter intermediates [87].

Conclusions and perspectives

In this perspective article, we have summarized and critically evaluated the current knowledge on ABC transporter regulation by multidomain FKBPs. We have uncovered surprising functional similarities, but also a few differences, between regulatory FKBP42/TWD1-ABCB and FKBP38-CFTR modules in plants and mammals. We have also dissected the PPIase-dependent and independent impact on ABC trafficking and activity. The picture that emerges is that multidomain FKBPs, here represented by FKBP38 and FKBP42/TWD1, are key regulators of CFTR and ATA biogenesis (Fig. 2), where they contribute to an early step of quality control of nascent ABCs. While for FKBP38-CFTR, convincing evidence shows that CFTR biogenesis mainly relies on the PPIase activity of FKBP38 [32], this has not yet been established for FKBP42/TWD1. However, both FKBPs appear to be also important for later stages of post-ER trafficking of ABC transporters to the PM and, at least for FKBP42/TWD1, a regulatory impact on the activity of PM-resident ABCB1 has been shown.

This functional conservation over kingdoms and ABC subfamilies is surprising, especially in light of the fact that such regulatory FKBP-ABC circuits have not been found to act on non-auxin-transporting ABCBs or mammalian ABCBs. Strikingly, both ATAs and CFTR are ABC transporters that provide a pathway for anions, IAA^- and Cl^-, respectively. A statistical analysis of FKBP42-ATA and FKBP38-CFTR sequence pairs may uncover co-evolution or common mechanistic details of these interactions.

As usual, any deeper analysis raises more new questions than it provided answers to already existing ones. For example, an exact dissection of PPIase/FKBD and chaperone/TPR activities for both regulatory ABC modules has not been undertaken using existing pharmacological and genetic tools. Further, HSP90-dependent and HSP90-independent actions of FKBP42/TWD1 and FKBP38 need to be separated so as to understand if they act as chaperones, co-chaperones or both. Finally, the existence and contribution of two independent pools of HSP90—one that binds directly to the ABCs and that is independent of the FKBPs and another one that depends on interaction with the TPR domain of the FKBPs—need to be investigated.

In light of the clinical importance of CFTR, it is surprising that interacting surfaces and respective prolines have not yet been mapped and/or identified. While for FKBP42/ TWD1-ABCB1 interaction it was shown that the FKBD interferes with the NBD2 containing P1008 [58], FKBP38-CFTR contacts and PPIase-relevant prolines have not yet been determined. Moreover, despite the fact that AtABCB1 P1008 seems to be conserved in the loop between TMD2 and NBD2 of CFTR [58], it will be interesting to see if the three prolines in the R domain of CFTR [86] are directly altering CFTR activity or the affinities for regulatory kinases, or both.

For both CFTR and ATAs, good evidence has been provided that beside ABC biogenesis, also transport activity can depend on peptidylprolyl isomerization provided by PPIases. In this respect, we have put forward a new mode of transient ABC transporter regulation by *cis–trans* isomerization of X-prolyl bonds that awaits future experimental confirmation. Most importantly, it needs to be clearly demonstrated that any effects of PPIase activities provided by FKBPs on ABCs are direct and not mediated through the modulation of another regulatory factor, such as a kinase. So far, evidence provided by mutational approaches, genetics, or inhibitors is only indirect, and therefore, changes in ABC structures caused by *cis-trans* isomerization need to be demonstrated *in vivo*.

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