Editorial

Immunophilins, Protein Chemistry and Cell Biology of a Promising New Class of Drug Targets – Part II

The origin of the family of proteins known as *Immunophilins* can be traced back to the year 1969, when the first specific ligand was discovered. By that time, an employee of a pharmaceutical company took with him soil samples from the Alps as part of a program of the company to analyze the presence of microorganisms able to produce new compounds of pharmaceutical interest, in particular antibiotics [1]. From those samples was isolated a fungus (*Tolypocladium inflatum*) that shows the ability to prevent the growth of other fungi. The active principle was identified as a cyclic undecapeptide, cyclosporine A [2], and its chemical synthesis was then published [3]. The studies showed that cyclosporine A has immunosuppressive effect in various experimental models [4, 5], which provided an extraordinary advance on the tissue and organ transplantation field since it was born by the end of the XIX century. At the beginning of the 80's, cyclosporin A became a sort of miracle treatment to avoid organ rejection.

After the discovery of cyclosporine A as an effective immunosuppressant, several alternative treatments were also established, including a macrolide lactone derivate known as FK506 (also called tacrolimus o fujimycin), which was isolated from *Streptomyces tsukubaensis* and firstly described in 1987 [6-8]. It was reported that FK506 is also effective in a wide variety of models of experimental transplantation and autoimmunity. Therefore, in addition to its obvious clinical importance, the discovery of FK506 yielded new insights into the mechanisms underlying the activation of T cells and its use is likely to impart even more important scientific information. The third classic drug also able to exert significant immunosuppressive actions is rapamycin, a macrolide discovered as a product of the bacterium *Streptomyces hygroscopicus* in a soil sample from Easter Island [9-11]. Soon after the discovery of these three drugs, scientists focused their studies on the identification of their intracellular molecular targets, and defined a new family of proteins known as immunophilins.

Today, the immunophilin family has been subdivided into three main categories according to the type of ligand they recognize, i.e., cyclophilins or CyPs, when they bind cyclosporine A (brand names are Gengraf, Neoral, and Sandimmune)), FKBPs or FK506-binding proteins when the bind FK506 (trade names Prograf, Advagraf, and Protopic) and also rapamycin (brand name Rapamune), and a third additional and still not well characterized CsA- and FK506-binding proteins subfamily. The two most relevant functional properties of immunophilins are the chaperone activity and the peptidyl prolyl isomerase enzymatic activity, which results inhibited upon complexing with the immunosuppressive drug. Beyond the fact that immunophilins are the cellular targets of immunosuppressive drugs, they also play several cardinal roles in the biology of the cell, which comprises various and versatile actions ranging from chaperoning client proteins for proper folding to neurotrophic or antiapoptotic actions.

The primary aim of the articles published in the present hot topic of Current Molecular Pharmacology titled *Immunophilins, a promising class of drug targets for alternative therapies,* is to contribute to a better understanding of the properties and potential uses of this important family of proteins as novel pharmacological targets. Due to the length of the articles, this issue has been split into two parts. In the part I, the first article by David LeMaster and Griselda Hernandez from the Department of Biomedical Sciences, University at Albany, analyzes the conformational dynamics of the most evolutionary and structurally similar domains to the archetypical immunophilin FKBP12, i.e. the FK1 domains of FKBP51 and FKBP52 and the low molecular weight immunophilin FKBP12.6, with the purpose to elucidate the relevance of these domains in both the therapeutic design of specific drugs and for gaining insight into how these small domains might participate in the collective transitions that occur within the signalling complexes in which they function.

Xixi Feng, Sebstian Pomplun, and Felix Hausch from the Max-Planck Institute of Psychiatry of Munich summarize recent advances in the development of FKBP ligands, which resulted in the first highly selective ligand for FKBP51 such as SAFit2, which allowed the proof-of-concept in mice for FKBP51 inhibitors as potentially novel antidepressants. Finally, the authors discuss pending issues that need to be addressed for the further development of FKBP51-directed drugs.

Amaravadhi Harikishore and Ho Sup Yoon from The Nanyang Technological University of Singapore focused their article on the molecular characteristics of canonical and non-canonical immunophilin family members from human and *Plasmodium falciparum and P. vivax*, and also analyze recent progresses on immunophilin inhibitor development, as well as future perspectives on structure-based design of non-immunosuppressive immunophilin ligands with potential pharmacological activities against infectious diseases.

Mingming Tong and Yu Jiang from The University of Pittsburgh School of Medicine provide a general comprehensive outline for the structures and diverse functions of FKBPs found in mammalian cells, including their participation in processes such as cancer neuroregeneration, neurodegenerative diseases, cell development, apoptosis, signalling cascade pathways, calcium channel regulation, etc.

Thomas Ratajczak from The University of Western Australia, analyzes the relevance of immunophilins as members of the steroid receptor-Hsp90 heterocomplex, the discovery of immunophilins, structural properties, both Hsp90-dependent and Hsp90-independent biological actions, as well as it is discussed how these immunophilins become superb candidates for diverse drug-targeting approaches in several diseases.

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In the second part of this issue, M. Lagadari, S.A. De Leo, M.F. Camisay, M.D. Galigniana and A.G. Erlejman from The University of Buenos Aires describe a novel molecular mechanism of regulation for NF-κB signalling cascade by FKBP51 and FKBP52, and postulate that the antagonistic actions of these proteins may be responsible for the pleiotropic effects of NF-κB in different cell types and tissues according to the expression balance exhibited for both immunophilins.

Naihsuan Guy, Yenni Garcia, and Marc Cox from The University of Texas at El Paso postulate the development of FKBP52-specific small molecule inhibitors as a highly targeted strategy with potential for the treatment of any disease that is dependent on given functional steroid receptor signaling pathway. They discuss that the proline-rich loop overhanging the FKBP52 FK1 catalytic domain is a key interaction surface within the receptor-chaperone complex highly attractive for a therapeutic approach to disrupt FKBP52 regulation of receptor activity in steroid hormone receptor-dependent physiology and disease.

Gabriel Fries, Nils Gassen, Ulrike Schmidt, and Theo Rein from the the Max-Planck Institute of Psychiatry of Munich analyze FKBP51 polymorphisms as emerging factors involved in stress-related mental disorders, mostly based on the inhibitory action of this immunophilin on the glucocorticoid receptor activity in the nervous system. They analyze the regulation of the feedback loops that command the biological response due to the onset of stressing and traumatic stimuli, and the relation of these events with the development and treatment of major depression syndromes.

Anna D'Angelillo, Stefania Staibano, Maria Romano and Simona Romano from The Federico II University of Naples review recent literature related to the FK506-binding protein of 51-kDa in the mechanisms that switch the TGF-β from a tumor suppressor to a pro-metastatic invader in processes that enable cancer cells to disseminate from primary tumors and spread to distant locations, therefore acquiring resistance to therapy and self-renewal capability.

Paul Lavin and Margaret Mc Gee from The University College Dublin summarize current understanding of the role of cyclophilins in cancer by reviewing the function of these immunophilins during mammalian cell division and HIV-1 infection, and highlight common processes involving members of the ESCRT (Sorting Complex Required for Transport) machinery, and Rab GTPase protein families.

Finally, Lana McClements, Stephanie Annett, Anita Yakkundi and Tracy Robson from the School of Pharmacy, Queen's University Belfast, focus on the different roles of immunophilins as therapeutic and biomarker factors for age-related vascular diseases, since many genes within this family are associated with age-related diseases such as cardiovascular diseases,, atherosclerosis, type II diabetes, chronic kidney disease, neurodegeneration, cancer and age-related macular degeneration, in addition to the ageing process itself.

In this special issue of Current Molecular Pharmacology, several aspects of the biology of immunophilins have been addressed and with the purpose of providing an updated overview of the field. It is clear that we still have more questions than answers, a state of the art that keeps feeding our thoughts proposing new hypothesis or models and, above all, stimulating us to overcome the new rising challenges shown in the course of our careers. I hope that the high enthusiasm showed by all our contributors to make this endeavor possible will be appreciated by the readers. In this regard, wish to acknowledge the valuable viewpoint of all contributing authors and hope that this assemblage of perspectives will be a valuable resource for researchers in this and other related fields.

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Mario D. Galigniana
(Guest Editor)
Instituto de Biología y Medicina Experimental-CONICET,
and Departamento de Química Biológica,
Facultad de Ciencias Exactas y Naturales,
Universidad de Buenos Aires, Buenos Aires (1428),
Argentina

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Regulation of NF-κB Signalling Cascade by Immunophilins

Mariana Lagadari[1], Sonia A. De Leo[2], María F. Camisay[2], Mario D. Galigniana[1,2] and Alejandra G. Erlejman[1,*]

[1]*Instituto de Biología y Medicina Experimental-CONICET, Buenos Aires (C1428ADN), Argentina;* [2]*Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales-IQUIBICEN, Universidad de Buenos Aires, Buenos Aires (C1428EGA), Argentina*

Alejandra G. Erlejman

Abstract: The fine regulation of signalling cascades is a key event required to maintain the appropriate functional properties of a cell when a given stimulus triggers specific biological responses. In this sense, cumulative experimental evidence during the last years has shown that high molecular weight immunophilins possess a fundamental importance in the regulation of many of these processes. It was first discovered that TPR-domain immunophilins such as FKBP51 and FKBP52 play a cardinal role, usually in an antagonistic fashion, in the regulation of several members of the steroid receptor family via its interaction with the heat-shock protein of 90-kDa, Hsp90. These Hsp90-associated cochaperones form a functional unit with the molecular chaperone influencing ligand binding capacity, receptor trafficking, and hormone-dependent transcriptional activity. Recently, it was demonstrated that the same immunophilins are also able to regulate the NF-κB signalling cascade in an Hsp90 independent manner. In this article we analize these properties and discuss the relevance of this novel regulatory pathway in the context of the pleiotropic actions managed by NF-κB in several cell types and tissues.

Keywords: FKBP51, FKBP52, Hsp90, Hsp70, FK506, p65, p50, NF-κB.

INTRODUCTION

The ability of a cell to identify the moment when it should differentiate, grow, divide or die depends on extracellular signals such as hormones, small molecules of varied nature, proteins attached to neighboring cells, or small peptides, and also how the cell process this information. Receptors are the transmitters of those extracellular signals, most likely to the nucleus, through cascades of reactions and interactions between proteins as well as metabolic reactions. Signalling cascades of the cell can be considered as a steady-state homeostatic system that results of a highly dynamic inflow and outflow of biological information. The concept of treating signalling cascades as highly dynamic steady-state systems was first introduced by Boon Chock and Earl Stadtman to the regulation of metabolic enzymes [1]. Such pioneer concept can also be applied to those biological cascades related to the various processes of the cell since most of them are also related to some type of enzymatic activity catalyzing protein modifications, *i.e.* phosphorylation, dephosphorylation, acetylation, isomerization, methylation, etc., which ultimately lead to establish a sort of steady-state condition of a whole chain of reactions able to be regulated separately at many steps [2]. These regulatory systems control the cell cycle, cell differentiation, and cell proliferation processes as a response to specific signal inputs.

*Address correspondence to this author at the Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales-IQUIBICEN, Universidad de Buenos Aires, Buenos Aires (C1428EGA), Argentina;
Tel:/Fax: +54(11) 4576-3342; E-mail: erlejman@qb.fcen.uba.ar

Molecular chaperones and their cochaperones are responsible for the interaction of multiple key components of those signalling pathways able to regulate growth, differentiation, and development. The molecular relationships between these proteins and various signalling proteins and their partners appear to be decisive for the appropriate biological action of signal transduction cascades, and the relative expression of these proteins is important for the regulation of the response since insufficient or excessive amounts could generate an aberrant control of essential cell processes such as proliferation, division, development and/or growth [3]. Both genetic and molecular interactions between regulatory proteins and the various components of the signalling pathways show us that the cross-talk between these proteins can regulate proliferation and development by preventing or enhancing cell growth and cell death as the levels of these molecular chaperones change in response to various stimuli. In this article we will focus on the recently discovered regulatory action by FKBP51 and FKP52, two Hsp90-binding chaperones belonging to the immunophilin family, on the biological properties of the transcription factor NF-κB.

IMMUNOPHILINS

Immunophilins are endogenous proteins with peptidyl-prolyl-(*cis/trans*)-isomerase (PPIase) activity, *i.e.*, the reversible *cis/trans* enzymatic interconvertion of Xaa-Pro bonds. These proteins are grouped in a common family of proteins whose signature domain is the PPIase domain. In turn, they are classified into two major subfamilies according to their capacity to bind immunosuppressant ligands [4],

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whose binding site is the PPIase domain. When immunophilins bind the cyclic undecapeptide cyclosporine A (CsA), they are called *cyclophilins* (or CyPs), and those that are able to bind the cyclic macrolide tacrolimus (or FK506) are named *FK506-binding proteins* (or FKBPs). Many members of the FKBP subfamily also bind the drug sirolimus (or rapamycin). There is a third subfamily of immunophilins referred to as *Parvulins* that show certain homology with the PPIase domain of the other subfamilies and may show enzymatic activity, but they are not able to bind immunosuppressive drugs. The most relevant parvulin in humans is Pin1, which recognizes a specific motif of a phosphorylated serine or threonine residue preceding a proline [5].

Immunophilins were first described as intracellular receptors for immunosuppressive drugs. Even though most of them bind these drugs, only the low molecular weight immunophilins FKBP12 and CyPA, the archetypal members of each subfamily, are related to the immunosuppressive effect when the FK506•FKBP12 or CsA•CyPA complex inhibits the phosphatase activity of calcineurin, a PP2B class of Ser/Thr protein-phosphatase. The inhibition of such activity prevents the dephosphorylation and subsequent activation of the transcription factor NFAT (*Nuclear Factor of Activated T cells*), which remains cytoplasmic. Therefore, the production of interleukins and interferon-γ is prevented (see [6] for a recent review).

High molecular weight immunophilins have a more complex architecture and are not related to the immunosupression process (Fig. **1**). The archetype of this subfamily is the 52-kDa FK506-binding protein, FKBP52 [7]. In addition to the active PPIase domain (also called FKBD1 or FK1 domain in FKBP proteins), which resembles the structure of the immunosuppressive factor FKBP12, there are other additional domains only present in the high molecular weight subfamily. The best studied is the TPR domain formed by sequences of 34 amino acids repeated in tandem through which they bind to Hsp90 via the MEEVD C-terminal conserved sequence of this chaperone [8] (Fig. **1**). TPR-domain immunophilins such as FKBP51 and FKBP52 are abundant and ubiquitous proteins that were first discovered associated to steroid receptors. The four more classical TPR domain immunophilins that have been relatively well characterized due to their association with these receptors are FKBP52 (gene name *FKBP4*), FKBP51 (gene name *FKBP5*), the cyclophilin CyP40 (gene name *PPID*), and the FKBP-like protein phosphatase PP5 (gene name *PPP5C*). All of them have their counter-part in plants [9], are highly ubiquitous, and are also able to form complexes (many of them still to be characterized) with several factors, although their biological functions and many aspects of their molecular mechanism of action are poorly understood.

Another important TPR-domain immunophilin is FKBP37 (gene name *AIP*), also known as XAP2/AIP. It was first discovered associated to AhR (aryl-hydrocarbon receptor, or "dioxane" receptor), where the immunophilin favors the biological actions of the receptor [10, 11]. FKBP37 is also able to interact and repress the biological activity of other member of the nuclear receptor superfamily, PPARα (peroxisome proliferator-activated receptor-α), an Hsp90-binding transcription factor [12] that modulates lipid metabolism, inflammation, and blood pressure [13].

There are two more relevant TPR-domain immunophilins whose biological roles have been elucidated more recently. One is FKBP38, which shows a mitochondrial localization signal and has been related to apoptosis (see [14] for a recent review). In spite of its almost identical three-dimensional structure of the PPIase domain with the immunosuppressive immunophilins FKBP12, FKBP38 lacks enzymatic activity and does not bind immunosuppressive drugs. However it provides a scaffold platform to facilitate protein-protein interactions. This is particularly important for the case of anti-apoptotic factors [15] such as the proto-oncogene Bcl-2 (*B-cell lymphoma 2*). This contributes to tumorigenesis and chemoresistance [16].

The other relevant TPR-domain immunophilin is FKBP36 because it is crucial to spermatogenesis since it is able to interact with components of the synaptonemal complex [17], and is also a natural inhibitor of GADPH activity [18]. GADPH is involved in the mechanism of vesicle transport from the endoplasmic reticulum to the Golgi and is also recruited by Rab2 to the vesicular-tubular clusters of the reticulum where it helps to form vesicles. Consequently, FKBP36 shows the additional potential to affect vesicle trafficking and the secretory pathways [18]. To date, there are no compounds able to recognize specifically this immunophilin.

In the mid-1990s, a 92 residue member of the parvulins was identified in *Escherichia coli,* forming the prototype of the third family of PPIases [19]. Soon after, human isoenzymes were described and the small subfamily of parvulins was born. It has only three members in humans: Pin1, Par14, and Par17 [20]. Pin1 is the best studied and its name is often used as synonym of parvulin itself. They are also able to accelerate protein folding *in vitro*, but they show unique specificity for prolines preceded by phosphorylated Ser or Thr residues. Thus, Pin1 possesses the potential to regulate several phosphorylation signalling cascades by modifying the conformation of the target protein around its phosphorylation site, making Ser or Thr residues less or more accessible for dephosphorylation (see a recent reviewed in [21]). In this sense, Pin1 may act as a molecular timer to make the first move or bring to an end signalling cascades at certain time points of the cell cycle [22]. Pin1 is prevalently overexpressed in human cancers and its expression levels correlate with poor clinical outcome [23].

Pin1 inhibitors may simultaneously block multiple oncogenic signalling pathways and thereby overcome cancer-cell resistance to inhibition of specific kinases or phosphatases [21]. The best known Pin1 inhibitors include the natural product juglone, the small molecule PiB and others of peptidic nature (see [24] for a recent review), but their specificity and potency remain a major concern, and further design and optimization of novel small molecule Pin1 inhibitors are required. Even though recent efforts have been made to obtain better compounds with higher cell membrane permeability and better affinity for this immunophilin [25, 26], the specificity of these novel small molecules still remains to be proved.

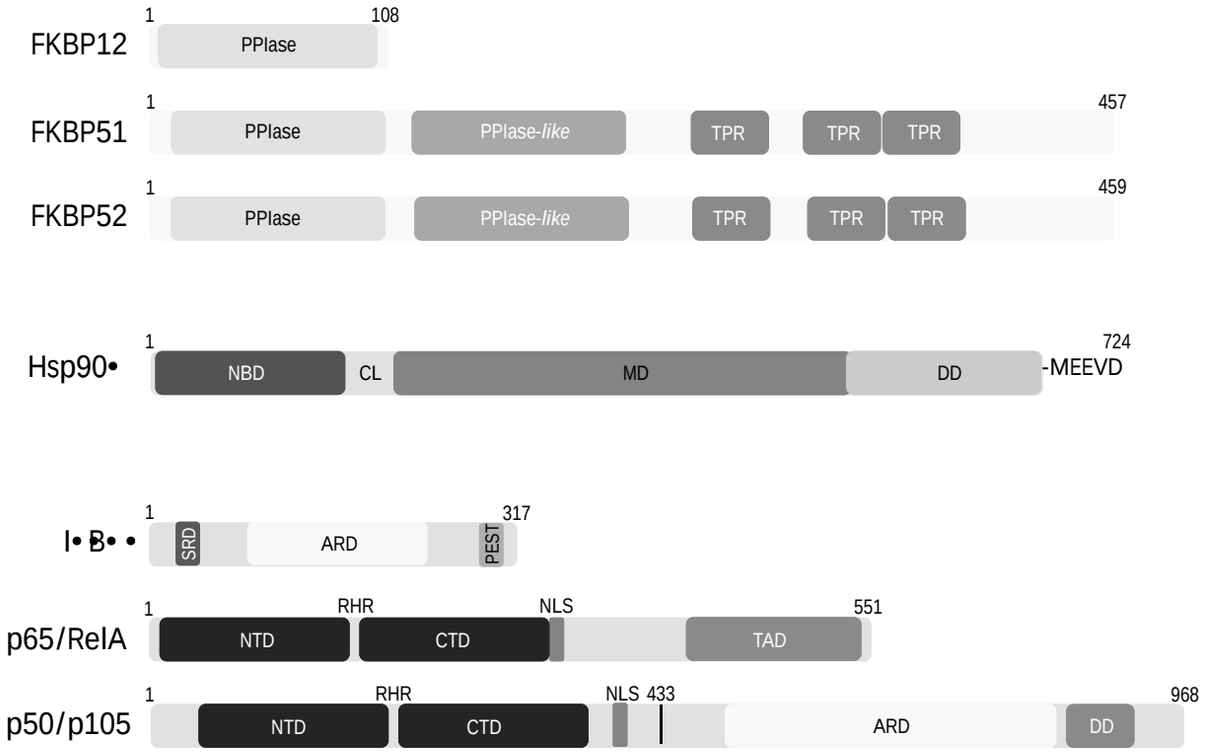

Fig. (1). Structures of FKBPs, Hsp90, IκB and NF-κB.
The upper part of the figure depicts the structural domains of FKBP51 and FKBP52 compared to FKBP12, the archetypical immunophilin responsible for immunosuppression that was the first protein well characterized of this family. The PPIase domain (also named FK1 domain) is responsible for the rotamase enzymatic activity and is also the binding site for the immunosuppressive macrolide FK506, which inhibits that activity. The PPIase-like domain (also named FK2) is the nucleotide-binding domain. The TPR domains (absents in FKBP12) are the responsible for interactions with Hsp90. This chaperone (shown in the middle of the figure) has two isoforms, α and β, the later being the active form for most signalling cascade factors. It shows four domains —NBD, nucleotide binding domain; CL, charged linker; MD, middle domain; DD, dimerization domain. The lower part of the figure depicts the structures of the two most frequent subunits of NF-κB and the domains of its inhibitor, IκB. Abbreviations are as follows: NTD, N-terminal domain; CTD, C-terminal domain; NLS, nuclear localization signal; RHR, Rel-homology region; TAD, C-terminal transactivation domain; ARD, ankyrin repeat–containing domain; DD, dimerization domain; PEST, signal–receiving domain C-terminal Pro-, Glu-, Ser-, and Thr-rich sequence; SRD, N-terminal signal–receiving domain. All these structures correspond to human proteins.

NF-κB

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) constitutes a family of highly related transcription factors able to regulate the expression of a great number of genes related to several processes such as inflammatory responses, cell growth, immune responses, cell development, synaptic plasticity, memory, cancer processes, etc [27]. This family of transcription factors belongs to the rapid-acting set of cell factors able to be activated by a large variety of signals and stressful situations; that includes cytokines, reactive oxygen species, bacterial and viral antigens, cell injuries, ionizing radiation, UV light, beta-adrenergic agonists, cocaine, etc. Since NF-κB was discovered in 1986 as a transcription factor able to bind to the enhancer element of the immunoglobulin κ light-chain of activated B cells [28], it became clear that in addition to having a crucial role in innate immunity, it is also able to regulate many other basic functions of the cell such as inflammatory responses, immune development, chronic inflammation, autoimmunity diseases, cancer promotion, cell development, programmed cell death, proliferation control, tumorigenesis, *etc*. (see [29-31] for recent comprehensive updates). NF-κB is actually regarded as a family of structurally related homologues that comprise p50 (NF-κB1), p52 (NF-κB2), p65 (Rel A), Rel B, and c-Rel. All of them share a conserved DNA-binding and dimerization domain. Potentially, they may associate in different combinations such as they can form up to fifteen types of dimers. Nonetheless, the physiological existence of all of these potential dimers has not been demonstrated to date, the p50•p65/RelA heterodimer being unquestionably the most abundant in all cell types [32]. On the other hand, the NF-κB family may be divided from the transcriptional perspective into two groups based on the occurrence of the C_T-transactivation domains, which are only present in RelA, RelB, and c-Rel [32].

NF-κB proteins bind to members of the inhibitory-κB family (or IκB) that serve as regulators of biological activity. The members of the IκB subfamily are the classical IκB proteins (IκBα, IκBβ, and IkBε), NF-κB precursor proteins (p100 and p105), and the nuclear IκBs (IκBζ, Bcl-3, and IκBNS). IκB proteins show an N_T-signal receiving domain (SRD), a central domain (ARD), and a C_T-PEST sequence

(Pro-, Glu-, Ser-, and Thr-rich domain). IκBα was originally described as disrupting factor of preformed NF-κB•DNA complexes that favors the dissociation of those complexes [33, 34]. Inasmuch as the expression of IκBα is in turn regulated by NF-κB [35]; IκB is able to regulate both NF-κB activation and inactivation.

NF-κB SIGNALLING CASCADE

In the canonical activation pathway of NF-κB (Fig. **2A**), excitatory signals activate Toll-like receptors (TLRs), tumor necrosis factor receptor (TNFR) or interleukin-1 receptors (IL-1R). Archetypal stimulating molecules are lipopolysaccharides (LPS), tumor necrosis factor α (TNFα), and interleukin-1 β (IL-1β), respectively [29]. This leads to the activation of the IκB kinase (IKK) complex, which phosphorylates IκBα. This complex is formed by IKKα and IKKβ subunits and at least one non-catalytic accessory protein, the IKKγ subunit, also known as NEMO (NF-κB Essential Modulator) [36] (Fig. **2A**). In turn, this IκB kinase complex associates to additional factors and interacts with other upstream signalling molecules and kinases. The phosphorylation of IκB favors its release from the p50•RelA/p65 dimer followed by proteasomal degradation of the inhibitor factor. Thus, the free heterodimer is retrotransported to the nucleus.

An alternative NF-κB activation pathway known as the "non-canonical pathway" (Fig. **2B**) originates from different types of receptors [37], including CD40, RANK (Receptor Activator for Nuclear Factor kappa B), BAFFR (B-cell Activation Factor), LTβR (Lymphotoxin β-Receptor) or TNFR type II. In this pathway, NF-κB is activated by the kinase NIK, which phosphorylates and activates predominantly IKKα, whose activity phosphorylates p100. This favors p100 ubiquitination and its partial degradation to generate the p52 subunit, that usually associates to RelB [38].

Besides the canonical (Fig. **2A**) and the alternative pathways (Fig. **2B**), a third manner for NF-κB activation also exists and is named the "atypical activation pathway" (Fig. **2C**). Actually, there is more than one, but the most typical is the activation of the IKK complex after genotoxic stress via the ATM kinase (Ataxia-Telangiectasia Mutated protein-kinase) leading to ubiquitination of IKKγ/NEMO [39].

In all the above-described pathways, following the liberation of the NF-κB dimers by activation of IKKs, their steady state localization is normally shifted to the nucleus and the Rel Homology Domains (RHD) (Fig. **1**) are free to bind cognate DNA-sequences in the enhancer elements of target gene promoters. Depending on the accessibility of the genome regulated by epigenetic mechanisms and the cell type, hundreds of different target genes can be transcriptionally activated and regulated by additional transcription factors. This may either enhances or reduces the NF-κB biological action. The level of complexity and crosstalks between NF-κB with other signalling pathways and transcription factors are some of the reasons that explain the pleiotropic actions of this factor.

There is general consensus that NF-κB proteins bind as a homo- or heterodimer to a 10-base-pair DNA sequence (which was first identified in the enhancer region of the immunoglobulin κ-light-chain gene of mature B cells [33, 40]). The first structure of a Rel-homology region was discovered from the structure of a p50 homodimer bound to an idealized κB target DNA sequence [41-43]. Various structures of NF-κB dimers bound to DNA have been reveled to date, showing a common pattern of structures that resemble a butterfly, where the dimer represents the wings around a cylindrical DNA structure [42]. NF-κB seems to encircle the target DNA. The analysis of different types of these structures has shown plasticity for these sequences of NF-κB homo/heterodimers. The canonical p50•RelA/p65 heterodimer recognizes the binding sequence through the p50 subunit bound to a 5-GGPyN half-site and via RelA/p65 binding to another 5-GGPyN site. Importantly, IκB is one of the proteins induced by RelA/p65, which implies the existence of a self-regulated NF-κB feedback that helps to restore the original cytoplasmic localization of NF-κB [35].

THE FKBP•NF-κB CONNECTION

As it was advanced before, all Rel factors form homodimers or heterodimers with the sole exception of Rel B, which forms only heterodimers. The relative abundance of different NF-κB proteins may vary in different tissues and cell types, whereas the p50•RelA/p65 heterodimer is highly ubiquitous and also the most frequent in most cell types and tissues [44]. In unstimulated cells, even though p50•RelA/p65 heterodimers are retained in the cytosol by IκB and translocate to the nucleus of the NF-κB via the dynein/dynactin motor complex [45] upon cell stimulation, both cytoplasmic and nuclear complexes undergo a dynamic nuclear-cytoplasmic shuttling [40, 46, 47]. This allows a low basal transcriptional activity of NF-κB given the fact that the IκB/NF-κB complex is also subject to dynamic dissociation/reassociation events. This nuclear-cytoplasmic shuttling of NF-κB resembles that observed for steroid receptors, where the inactive cytoplasmic form of these ligand-dependent transcription factors must translocate to the nucleus upon cell stimulation with steroid hormones [48-50].

In previous studies, our laboratory and others have reported that the 51-kDa and 52-kDa FK506-binding proteins FKBP51 and FKBP52 are responsible in a mutually exclusive fashion for the retrotransport mechanism of GR [51, 52] and MR [53, 54]. Both FKBPs are also regulators of the ligand-dependent transcriptional activity for those receptors [54-56] and other members of the family such as PR [57, 58], AR [59, 60] and, to a minor degree, ERα [58, 61]. These Hsp90-binding immunophilins are highly homologous and share 60% homology and 75% similarity [62]. As shown in Fig. (**1**), they are structurally characterized by the presence of two key sequences: the TPR domain, through which they bind to Hsp90, and the peptidyl-prolyl isomerase (PPIase) domain [63], where the macrolide FK506 and also the dynein/dynactin motor complex bind. Both domains are essential for the retrotransport mechanism of steroid receptors [50, 64], the first because of its interaction with the chaperone and the second due to its capacity to bind motor proteins. Nonetheless, the enzymatic activity of the PPIase domain does not appear to be essential. Upon steroid binding, FKBP51 is released from the receptor•Hsp90 heterocomplex and is replaced by FKBP52, which recruits dynein/dynactin motor proteins favoring the transport of the receptor to the nucleus on microtubule tracks [65] (Fig. **3**). While FKBP52 favors steroid binding and transcriptional

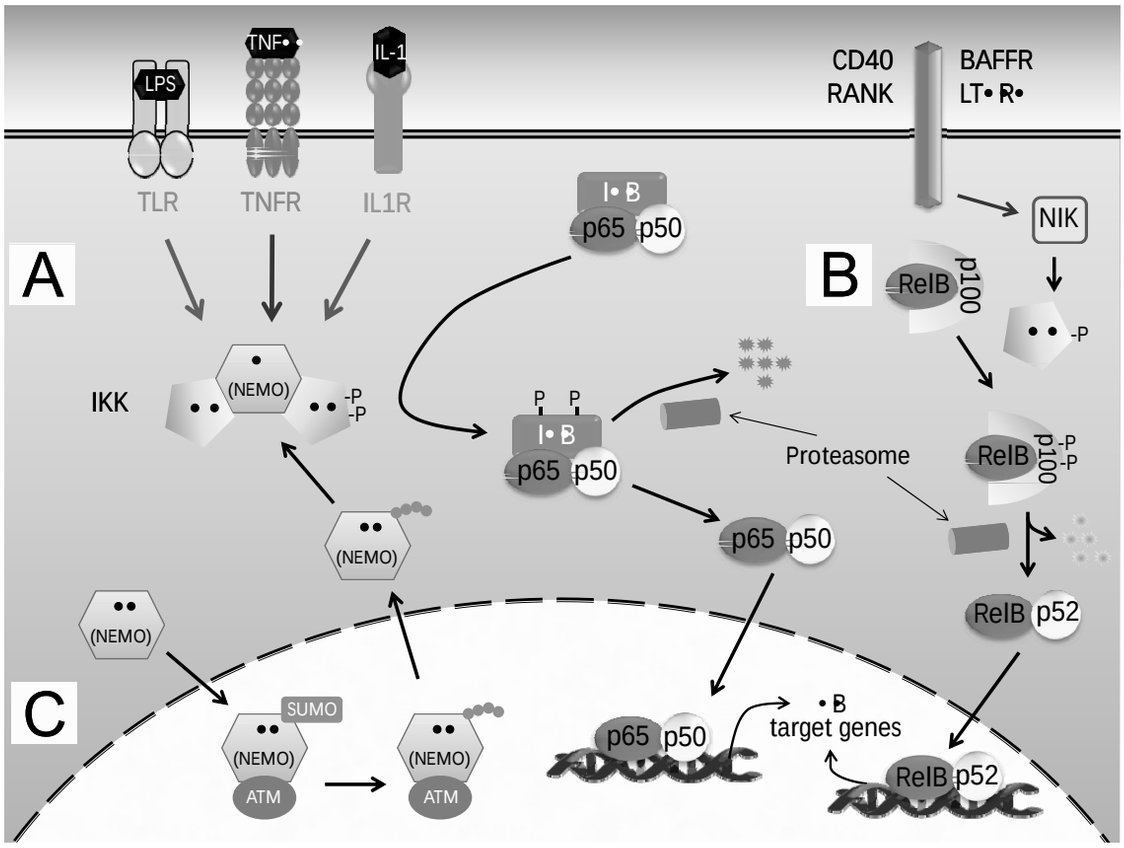

Fig. (2). Canonical, non-canonical, and atypical NF-κB signalling pathways.
A) In the canonical NF-κB signalling pathway, lipopolysaccharides (LPS), tumor necrosis factor α (TNFα) or interleukin-1 (IL-1) activate Toll-like receptors (TLRs), tumor necrosis factor receptor (TNFR), and interleukin-1 receptor (IL-1R), respectively, leading to the activation of the IKK complex. This kinase phosphorylates IκBα, a prerequisite for its subsequent polyubiquitination followed by proteasomal degradation. NF-κB homo- or heterodimers are then translocated to the nucleus and activate gene transcription. ***B)*** In the non-canonical NF-κB signalling pathway, activation of CD40, receptor activator for nuclear factor κB (RANK), B-cell activation factor (BAFFR), or lymphtoxin β-receptor (LTβR), leads to activation of IKKα by the NF-κB-inducing kinase (NIK). IKKα phosphorylates the p100 subunit leading to its polyubiquitination and subsequent partial proteosomal processing to yield the p52 subunit. p52•RelB heterodimers can then activate transcription of target genes. ***C)*** In the atypical NF-κB signalling pathway, genotoxic stress leads to a translocation of IKKγ (also called NEMO) to the nucleus, where it is sumoylated and subsequently ubiquitinated. This process is mediated by the ataxia telangiectasia mutated (ATM) checkpoint kinase. IKKγ and ATM return to the cytosol where they are able to activate IKKβ.

favors steroid binding and transcriptional activity, FKBP51 impairs both effects.

Both transcription factors, steroid receptors and NF-κB, show similar requirements for subcellular redistribution upon the onset of a given activating stimulus. A recent investigation showed that FKBP51 and FKBP52 affect the nuclear translocation of RelA/p65 and also influence the transcriptional activity of NF-κB [66]. FKBP51 delays the nuclear translocation of p50•RelA/p65 and also shows inhibitory action on transcriptional activity, an effect related to its incapacity to interact with dynein/dynactin [55], whereas FKBP52 shows a strong stimulatory effect on transcription. In contrast to steroid receptors, these biological actions for NF-κB are Hsp90-independent for NF-κB. This is confirmed by the fact that point mutants in the TPR domains of the FKBPs unable to bind Hsp90 show similar effects as the wild type immunophilins [66]. This Hsp90-independence indicates an innovative mechanism of regulation in divergence with the steroid receptors, where their ligand-dependent activation and transcriptional activity are mostly Hsp90-dependent. However Hsp90 has been associated to NF-κB activation by its role on IKK regulation, as it is described below. Importantly the PPIase activity of FKBP52 appears to be exceptional for this stimulatory action, whereas that enzymatic activity is not required for the FKBP51 inhibitory action. A similar independent effect on the PPIase activity was described for the regulation of the GR by FKBP51 [55, 67], whereas a PPIase-dependent mechanism is implicated for FKBP52 [55, 68].

One interesting extrapolation of these effects is that the biological action of NF-κB may be regulated in different tissues and cell types by the overall expression balance of both immunophilins, which could contribute in part to the pleiotropic actions of NF-κB. Moreover, our assays showed that Hsp70 is also a RelA/p65-interactor, which is in agreement with a very recent report in neurons [69] where the nuclear translocation of both RelA/p65 and Hsp70 was postulated to occur as a protein-protein complex. Interestingly, the up-regulation of Hsp70 was also reported to induce nuclear translocation of RelA/p65 in rat liver cells [70]. Never-

theless, the RelA/p65 and Hsp70 interaction not always leads to positive effects on NF-κB activation. The initial reports showed interactions that involve stress-induced situations where the effects of heat-shock proteins reduce the inflammatory responses [71]. In this scenery, it was proposed that overexpressed Hsp70 is able to interact with NF-κB, suggesting that Hsp70 may substitute IκB by anchoring NF-κB to the cytoplasm. Only increased accumulation of the chaperone could result in an inhibition complex of NF-κB [72]. It was also reported that Hsp70 shows similar effects on various intracellular immune pathways and signalling in the brain [73], although it cannot be ruled out that the biological actions may be cell- and stimulus-dependent. Also, it has been shown that Hsp70 interacts with the IKK complex to decrease NF-κB signalling [74] as well as with other members of the inflammatory signalling cascade preventing their actions [75, 76].

The IκB/NF-κB cytosolic complex is subject to dynamic dissociation/reassociation events. Experiments with IKKα knockout mice [77] demonstrated defective cell proliferation and differentiation and have also shown that IKKα is dispensable for IκB degradation. Moreover IKKα has been reported to be required for the termination of NF-κB activation [78]. A physical interaction between FKBP51 and the IKK complex has been demonstrated, most likely via the IKKα subunit bound to Hsp90 [79, 80], but the biological function of FKBP51 on IKK signalling is still unclear [80] (Fig. **3**). While down-modulation of Hsp90α and Hsp90β likewise resulted in reduced kinase activity, it has been shown that FKBP51 is not a constitutively associated component of the IKK complex [80], and its down-modulation interfered with neither TNFα-induced IKK activity nor IκBα degradation and RelA/p65 translocation. Actually, the experimental evidence shows that the prevailing complex for the IKK•Hsp90 complex to generate an activated state is the one that recruits Cdc37 rather than FKBP51 [80] (Fig. **3**), both factors being transiently associated with NF-κB [80]. Importantly, in the same study it was also reported that TNFα is unable to modify the association of IKK with those interacting factors. In short, the role of FKBP51 on NF-κB signalling cascade remained elusive to date. It is unlikely that both Hsp90-interacting factors, Cdc37 and FKBP51, are part of the same IKK complex since it has been demonstrated that the binding of a TPR protein to Hsp90 precludes the binding of Cdc37 and vice versa, perhaps due to the fact that both proteins bind to adjacent domains of the chaperone [81]. Because FKBP51 is able to associate to IKK, and because Cdc37 is essential for the maturation of *de novo* synthesized IKKs into enzymatically competent kinases, but not for assembly of an IKK holocomplex, it could be possible that the role of FKBP51 is more related to the assembly process with IKK in similar fashion as the Hsp90 co-chaperone Hop (Heat-shock organizing protein) is intermediary for GR•Hsp90 assembly, but it is not present in mature complexes [82].

Figure **3** depicts the proposed novel mechanism for the regulation of NF-κB biological actions by FKBP51 and FKBP52. These immunophilins affect NF-κB activation at different levels, *i.e.* nuclear transport, nuclear retention, and transcriptional activity. It is important to emphasize that endogenous FKBP51 was found constitutively associated to the promoter regions of an NF-κB target gene, whereas FKBP52 replaces FKBP51 in stimulated cells. Moreover, while FKBP51 represses NF-κB transcription, FKBP52 greatly enhances this activity in a mechanism that involves its PPIase enzymatic activity (and is therefore impaired by PPIase inhibitory drugs) (Fig. **3**).

A crucial nuclear mechanism for gene expression is the modification of the chromatin environment of the respective genes. It has been shown that when NF-κB is activated, histone phosphorylation can be mediated by nuclear IKKα that is recruited to the promoter sites of NF-κB-regulated genes [83, 84]. Among a number of chromatin remodelers is the PPIase protein Pin1 [85], an immunophilin-like protein that also targets RelA/p65 [86]. Because PPIase-induced conformational changes have functional effects on target proteins, the action of Pin1 on RelA/p65 is reflected in a more efficient nuclear accumulation of RelA/p65 and also a greater stability by preventing its ubiquitin-mediated proteolysis [86]. In certain types of cancer cells, Pin1 is usually up-regulated [87-89] whereas the E3-ubiquitin-ligase of RelA/p65, SOCS1 is down-regulated [90-92] or mutated [93], all of which may contribute to the constitutive activation of NF-κB in those cancers. A similar mechanism can be proposed here for the expression balance of FKBP51 and FKBP52, in particular for the latter immunophilin that shows an important stimulatory action dependent on its PPIase activity.

FUTURE PERSPECTIVES

Given the role played in the initiation and progression of cancer, the NF-κB signalling pathway is a potent node of pharmacological interference in the clinic. Because NF-κB is also an essential protein in the immunological response against cancer, there has been a reluctance to use NF-κB-targeting inhibitors for the treatment of such malignancies. Nevertheless, combining classical chemotherapeutics with inhibitors of NF-κB activation seems to result in a promising synergistic strategy [94, 95]. Elevated NF-κB activity and/or higher half-life persistence in the nucleus of cancer cells (like that observed with FKBP52) provide a survival mechanism by up-regulating anti-apoptotic genes, thereby representing a major causative factor for drug resistance [96].

The development of immunophilin ligands appears to have promising perspectives in the coming years [97]. Thus, the ability to regulate the functions of a specific protein using cell-permeable small molecules like those that bind FKBPs is an unquestionably powerful method, not only to study biological systems, but also a desired alternative to be used in therapeutic treatments. In line with this aim, it has recently been reported the synthesis of two novel compounds named SAFit1 and SAFit2, that are highly selective inhibitors of FKBP51 [98]. This new class of ligands achieves selectivity for this immunophilin by an induced-fit mechanism that is much less favorable for FKBP52. By using these ligands, it was confirmed and original report showing that the selective inhibition of FKBP51 enhances neurite elongation in neuronal cultures [99] and, even more importantly, that these drugs improve neuroendocrine feedback *in vivo* as well as stress-coping behavior [98].

Ideally, the biological function of certain nuclear factors could be regulated if we can influence the mechanisms by

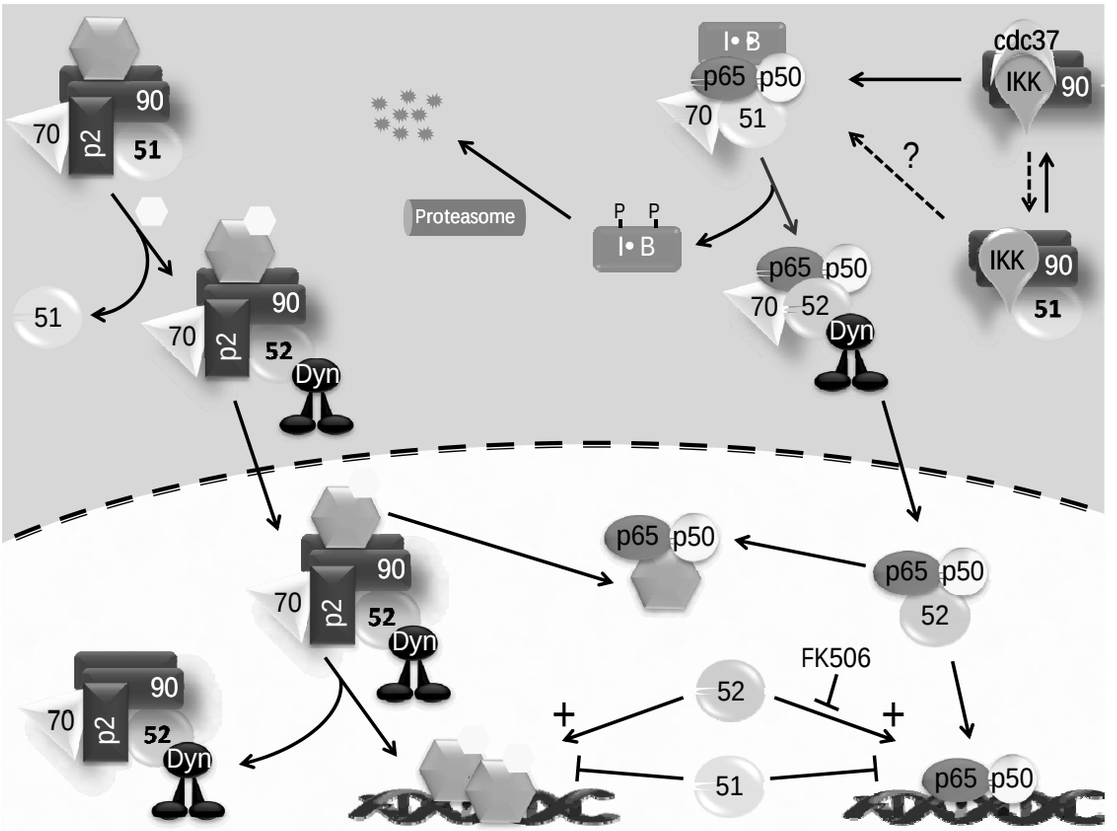

Fig. (3). Role of FKBP51 and FKBP52 on glucocorticoid receptor and NF-κB action.
Unliganded glucocorticoid receptor (GR) forms cytosolic heterocomplexes with Hsp90 dimers (90), Hsp70 (70), the cochaperone p23, and FKBP51 (51). Hormone (H) binding favors the exchange of FKBP51 by FKBP52 (52), which in turn recruits the dynein /dynactin motor complex (Dyn) able to power the GR retrotransport on cytoskeletal tracks. In the nucleus, the Hsp90-based heterocomplex dissociates and GR dimers induce the expression of target genes. NF-κB dimers also exchange FKBP51 by FKBP52 upon cell stimulation, although this mechanism is Hsp90-independent. The activation by IKK•Cdc37 complex phosphorylates IκB (which is targeted to proteosomal degradation), allowing the dynein/dynactin retrotransport of NF-κB dimers. FKBP51 shows inhibitory action on the transcriptional activity of both, GR and NF-κB, whereas FKBP52 shows stimulatory effects. FK506 prevents such stimulatory action of NF-κB indicating the relevance of the PPIase activity of FKBP52 for this effect. Note that NF-κB and GR can neutralize one another by a typical trans-repression mechanism. FKBP51 can also form complexes with the IKK•Hsp90 complex, but its role is still poorly understood, and is perhaps related to complex assembly, Cdc37 being the major factor in mature IKK complexes.

which they reach their sites of action. In this sense, because NF-κB is active in many cancer cells and its persistent localization in the nucleus strengthens or directly leads to tumor development. Therefore, based on the model shown in Fig. (**3**), it is tempting to think that targeting specifically the PPIase activity of FKBP52 (essential for its enhancement action on NF-κB effects) could be a promising novel regulatory approach to prevent NF-κB activity.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Therapeutic Targeting of the FKBP52 Co-Chaperone in Steroid Hormone Receptor-Regulated Physiology and Disease

Naihsuan C. Guy[1], Yenni A. Garcia[1] and Marc B. Cox[1,*]

[1]*Border Biomedical Research Center and Department of Biological Sciences, University of Texas at El Paso, El Paso, TX 79968, USA*

Abstract: Steroid hormone receptors are ligand-dependent transcription factors that require the dynamic, ordered assembly of multimeric chaperone complexes to reach a functional conformation. Heat shock protein (Hsp) 70 and Hsp90 serve as the central chaperones that mediate this process in conjunction with a variety of co-chaperones. Many of these co-chaperones represent potential therapeutic targets for the disruption of Hsp90 client protein function. FKBP52 is an Hsp90-associated co-chaperone that has emerged as a promising therapeutic candidate due to its functional specificity for a small subset of Hsp90 client proteins including androgen (AR), glucocorticoid (GR), and progesterone (PR) receptors. Given its Hsp90-client protein specificity, the targeting of FKBP52 should be more specific and less toxic than the Hsp90-targeting drugs. Additionally, the *fkbp52*-deficient mice display specific phenotypes related to androgen, progesterone, and glucocorticoid insensitivity suggesting minimal off-target effects. Finally, the fact that FKBP52 is already a validated target of the clinically approved immunosuppressive drug, FK506 (Tacrolimus), indicates that FKBP52 is a "druggable" protein. Thus, the development of FKBP52-specific small molecule inhibitors is predicted to be a highly targeted strategy with potential for the treatment of any disease that is dependent on a functional AR, GR, and/or PR signaling pathway. Much progress has been made in understanding the residues and domains critical for FKBP52 function. The proline-rich loop overhanging the FKBP52 FK1 catalytic domain is functionally important and likely represents an interaction surface within the receptor-chaperone complex. Thus, the targeting of FKBP52 proline-rich loop interactions is the most attractive therapeutic approach to disrupt FKBP52 regulation of receptor activity in steroid hormone receptor-dependent physiology and disease.

Keywords: Androgen receptor, FKBP4, FKBP52, glucocorticoid receptor, immunophilin, progesterone receptor, prostate cancer, steroid hormone receptor.

INTRODUCTION

The proper folding and activity of steroid hormone receptors requires no less than twelve proteins and at least three distinct chaperone/receptor complexes. Many of these chaperones and co-chaperones are attractive targets for the treatment of a variety of diseases. The heat shock protein 90 (Hsp90)-associated 52-kDa FK506-binding protein (FKBP52) is of particular interest as FKBP52 is a known positive regulator of androgen (AR), progesterone (PR), and glucocorticoid receptor (GR) activity, and serves as an attractive therapeutic target for any disease that depends on a functional AR, PR, and/or GR signaling pathway. Much progress has been made in understanding the mechanisms by which FKBP52 regulates receptor signaling and the resulting roles it plays, not only in hormone-dependent processes, but also in endocrine-independent functions, including cell architecture, neurodifferentiation, and metal transport. This review summarizes the current understanding of chaperone-dependent SHR folding, FKBP52 interactions within the receptor-chaperone complex, FKBP52 contributions to health and disease, and FKBP52's potential as a therapeutic candidate for hormone-dependent and hormone-independent diseases. Furthermore, based on the progress that has been made in understanding residues and/or domains critical for function, we discuss the most promising strategies for the therapeutic targeting of FKBP52.

*Address correspondence to this author at the Border Biomedical Research Center and Department of Biological Sciences, University of Texas at El Paso, 500 W. University Ave. El Paso, TX 79968, USA;
Tel: (915) 747-5429; Fax: (915) 747-5808; E-mail: mbcox@utep.edu

CHAPERONE-MEDIATED STEROID HORMONE RECEPTOR MATURATION

Steroid hormones are small lipophilic molecules whose functions are mediated by intracellular receptor proteins termed steroid hormone receptors (SHRs) including AR, PR, GR, mineralocorticoid (MR), and estrogen receptors (ER). These receptors are ligand-regulated transcription factors that are required to be in continuous interactions with molecular chaperones and co-chaperones to establish and maintain their functionally mature conformations necessary for hormone binding and the subsequent control of a diverse array of physiological processes and/or promotion of disease states. The activation and maturation of the SHRs depend on interactions within the Hsp90-mediated chaperoning pathway, which is an ordered, dynamic, and cooperative series of events that involves multiple chaperone and co-chaperone

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components. The heat shock proteins Hsp40, Hsp70, and Hsp90 in addition to the co-chaperones Hsp70/Hsp90 organizing protein (Hop) and p23 are minimally required for efficient SHR folding and maintenance of receptor hormone binding ability *in vitro*. Furthermore, SHRs must chronologically cycle through three distinct complexes, each with different chaperone and co-chaperone compositions, to reach their final active conformations (Fig. **1**) [1-12]. While this review focuses on FKBP52 as a therapeutic target, it is important to point out that the chaperone-dependent folding, activation and regulation of SHRs presents a variety of opportunities for therapeutic intervention. Thus, we also discuss the chaperone-dependent folding and activation pathway, and other potential targets for the disruption of SHR folding in detail below.

EARLY COMPLEX

Little is known about the SHR folding process prior to nascent chain folding as the receptors emerge from the ribosome. However, *in vitro* receptor-chaperone complex assembly studies suggest Hsp40 and Hsp70 binding as the first step in the recognition of PR and GR, respectively, in the Hsp90-dependent chaperoning pathway through binding to a single site in the receptor ligand binding domain (LBD), yet the exact binding site has not been identified [4, 13]. Regardless of the exact details, an early primary role for Hsp70 in receptor maturation is clear. The nascent SHRs are bound by Hsp70 in an ATP-dependent manner. The J-domain of Hsp40 stimulates Hsp70 ATPase activity leading to a conformational change that results in a tight association of Hsp70 with the substrate [4, 14]. Thus, the early complex of the chaperoning pathway consists of Hsp70 and Hsp40 components that prime the receptor for a second ATP-dependent interaction with Hsp90 to form the intermediate complex [4, 15]. A surveillance system in eukaryotic cells also functions at this stage in the folding cycle to modulate "protein triage" decisions that regulate the balance between protein folding and degradation for chaperone substrates [16].

PROTEIN QUALITY CONTROL: UBIQUITIN/PROTEASOMAL SYSTEM

Carboxyl terminus of Hsp70-interacting protein (CHIP) is a tetratricopeptide repeat (TPR)-containing co-chaperone that functions as a U-box dependent E3 ubiquitin ligase [17, 18]. It binds to both Hsp70 and Hsp90 through its TPR domain and inhibits the folding activity of the chaperones by

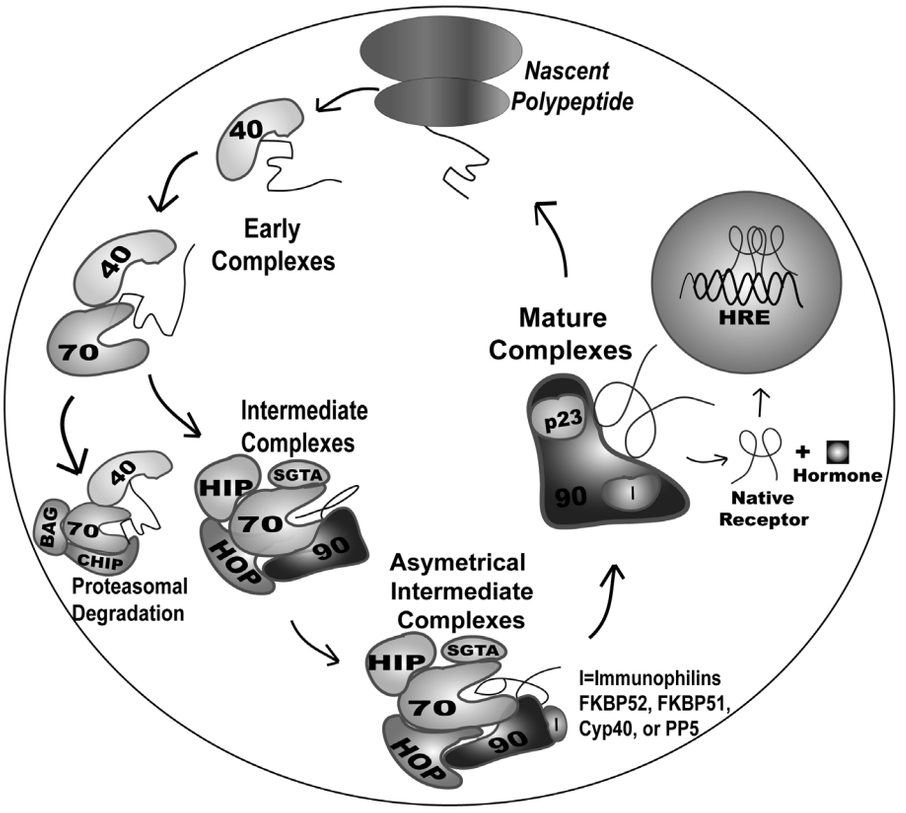

Fig. (1). Chaperone-mediated steroid hormone receptor folding. Receptors associate with chaperones and co-chaperones as they cycle through early, intermediate, asymmetric intermediate, and mature complexes offering a variety of opportunities for therapeutic intervention. Early complex assembly is initiated upon Hsp40 binding to the nascent receptor polypeptide residing in the cytosol. Hsp40 recruits Hsp70 where the fate of the nascent polypeptide is determined to proceed with the intermediate complexes or towards proteasomal degradation. The carboxyl terminus of Chip is an E3 ubiquitin ligase that, along with BAG proteins, directs misfolded receptors towards the ubiquitin and proteasomal degradation pathways. In the intermediate complex SGTA binds to Hsp70. Hsp70 then recruits HIP and HOP forming a bridge for Hsp90's binding into the complex. As the nascent polypeptide travels through the asymmetrical complex the immunophilins (I) bind in a competitive fashion to Hsp90 allowing for a conformational change. Further, the mature complex forms as HIP, HOP, and SGTA dissociate and p23 binds to stabilize the receptor-Hsp90 complex in the mature conformation to which hormone can bind with high affinity. The receptor is then able to translocate to the nucleus, dimerize and bind to hormone response elements to initiate gene transcription.

confining the chaperone in an ATP-bound conformational state [16, 18]. CHIP plays a pivotal role in the conversion of the chaperone complex from a protein-folding apparatus to a protein-degradation machine by promoting the ubiquitination of chaperone substrates and stimulates their degradation by targeting them to the ubiquitin/proteasome pathway [16, 19]. In addition, biochemical studies have demonstrated that CHIP participates in triage decisions based on stochastic sampling of chaperone-bound substrate complexes [20]. CHIP randomly samples the chaperone-bound substrates and the ones that cannot be folded efficiently and/or correctly would consequently stay in the chaperoning cycle longer and eventually be ubiquitinated and targeted for degradation [20]. In addition to CHIP, the co-chaperone Bcl-2-associated athanogene 1 (BAG-1) has also been reported to act as a coupling factor between the Hsp70 chaperone system and the protein degradation machinery [21, 22]. BAG-1 binds to Hsp70 via its C-terminal BAG domain while its N-terminal ubiquitin-like domain associates with the ATPase domain of the chaperones leading to the release of the ubiquintylated substrate and, at the same time, serves as a physical link between the Hsp70 and the 26S proteasome [21, 23-25]. Thus, BAG-1 plays a dual role, both as a scaffolding factor at the proteasome and as a substrate release factor of Hsp70. It is worth noting that CHIP cooperates with BAG-1 in targeting Hsp70 substrates to the ubiquitin/proteasome system [26]. CHIP associates with Hsp70 via its TPR domain and mediates ubiquitin attachment to the aberrantly folded substrate bound to the chaperone by recruiting and binding of the E2 ubiquitin-conjugating enzyme to its C-terminal U-box. At the same time, BAG-1 binds the Hsp70 via its BAG domain and utilizes its ubiquitin-like domain for releasing of the ubiquitylated substrate from the chaperone and targets it to the 26S proteasome where their de-ubiquitylation, unfolding, and degradation occur [26]. Interestingly, recent biochemical assays indicated that S100 proteins bind to TPR domains and interfere with CHIP/Hsp70 interactions leading to suppression of CHIP-dependent ubiquitination and degradation [27]. Therefore, the association of the S100 proteins with CHIP provides a Ca2+-dependent regulatory mechanism for the ubiquitination and degradation of intracellular proteins by the CHIP-proteasome pathway.

INTERMEDIATE COMPLEX

Those substrates deemed suitable for continued folding, as described above, are shuttled to intermediate complexes. Hsp70-interacting protein (Hip) facilitates intermediate complex formation by interacting with Hsp70 through its N-terminal TPR domain, which prevents dissociation of ADP from Hsp70. Since ADP-bound Hsp70 binds substrate with higher affinity, the binding of Hip with Hsp70 enhances the interaction of SHR with Hsp90 and Hop [1, 28-32]. Hop is another member of the TPR-containing co-chaperone family, which contains a specialized and conserved TPR-clamp domain consisting of TPR1, TPR2A, and TPR2B. Hop functions as a scaffold protein between the Hsp90 dimer and Hsp70 by their binding to its TPR2A and TPR1/ TPR2B motifs, respectively, enabling the client transfer between the chaperones [1, 6, 33, 34]. Recent studies have shown one Hop bound to Hsp90 dimer is sufficient to stabilize the dimer in an open conformation. In this intermediate complex, Hop binding to the TPR-acceptor site introduces a steric hindrance that prevents the other C-terminal TPR-acceptor site on the Hsp90 dimer to be bound.

Apart from the C-terminal TPR-acceptor motif, a novel site for TPR co-chaperone interaction near the N-terminal ATP binding domain of Hsp90 has recently been discovered [35]. This TPR-acceptor motif on the Hsp90 dimer is preferentially occupied by TPR-containing co-chaperones containing a PPIase domain and the interaction leads to the formation of an asymmetric Hsp90 intermediate complex [36-38]. GCUNC-45 is such an example of a PPIase that binds to Hsp90 via its TPR domain forming an asymmetric intermediate complex during PR chaperoning. Yeast two-hybrid analyses have revealed that GCUNC-45 directly interacts with a novel TPR-acceptor site near the N-terminus of Hsp90 [35]. The primary function of N-terminal domain (NTD) of Hsp90 is to bind ATP, which then induces an interaction between the NTDs of the Hsp90 dimer. This dimerization is further facilitated by the binding of activator of Hsp90 ATPase homologue 1 (Aha1) to the middle domain (MD) of the chaperone leading to the repositioning of a catalytic loop of this domain that interacts with the γ phosphate of ATP bound in the NTD [39, 40]. Mutational analyses have revealed that the GCUNC-45 binding motif on Hsp90 was generated by a spatial positioning of noncontiguous residues in the ATP binding domain [41]. Thus, it is suggested that binding of GCUNC-45 to the novel TPR-acceptor site near the N-terminus of Hsp90 may result in a spatial re-orientation between the NTD and MD leading to inhibition of ATPase activity of the chaperone by blocking the binding of Aha1 to MD, even though the two proteins do not share a common binding site [35, 41]. Thus, GCUNC-45 enters the chaperoning pathway at the intermediate stage forming an asymmetric intermediate complex with Hsp90/Hop intermediate complex and blocks progression of the PR complex to the next step with the purpose of allowing time for additional needed chaperoning events to occur. This confined regulation event by GCUNC-45 can be reversed in the presence of another PPIase TPR-containing co-chaperone, such as FKBP52, whose structure and function will be discussed in further detail below. PR assembly studies have demonstrated that GCUNC-45 functions upstream of FKPB52 during the chaperoning [35]. In addition, FKBP52 can reverse the confined inhibition by GCUNC-45 and promotes the progression of the PR chaperoning cycle toward the hormone binding competent mature state by competitively binding with GCUNC-45 for the novel TPR binding site near the N-terminus of Hsp90 [35, 41]. What induces the displacement of GCUNC-45 is unknown; it could be a response to specific signals and/or imbalance in intracellular homeostasis during the receptor chaperoning. It has recently been reported that S100 proteins, which are a subfamily of the EF-hand type calcium (Ca2+)-sensing proteins, compete with Hsp90 for the TPR domain of FKBP52 and cyclophilin 40 (Cyp40), which is another TPR-containing PPIase protein that is able bind to the N-terminal TPR acceptor site, in a Ca2+-dependent manner [41, 42]. Given that GCUNC-45, FKBP52, and Cyp40, but not Hop, bind to S100 suggests that only a selective subgroup of TPR co-chaperones is able to bind to the N-terminal TPR-acceptor site. S100 proteins regulate this subgroup of co-chaperones by binding to the TPR domain to competi-

tively inhibit the FKBP52-Hsp90 and Cyp40-Hsp90 interactions. Interestingly, studies have shown that S100 proteins can also regulate the Hsp70/Hop/Hsp90 intermediate complex in a Ca2+-dependent manner by binding to the TPR domains of Hop, hence disrupting the Hop-Hsp70 and Hop-Hsp90 interactions [43]. No matter what the details, it is undeniable that the presence of two Hsp90 sites for TPR protein interaction provides additional flexibility and control in modulating the Hsp90 co-chaperones and its clients during the chaperoning process.

Small glutamine-rich tetratricopeptide repeat-containing protein alpha (SGTA) is a co-chaperone that interacts directly with Hsp70, but weakly with Hsp90, via its C-terminal TPR domain and predominantly precipitates with Hsp70 from cell lysates [44, 45]. Interestingly, SGTA lacks a PPIase domain, which is a common feature among the related TPR proteins. Studies have shown that interaction with SGTA enhances Hsp70's and/or Hsp90's substrate binding affinity and the ATPase activity of the chaperones by favoring their ADP-dependent association with client molecules [44, 46, 47]. Studies by Buchanan *et al.* demonstrated that the binding of dihydrotestosterone (DHT) induces the dissociation of SGTA from the hinge region of AR, and conversely, its overexpression decreases the capacity of the hormone to mediate receptor transport to the nucleus [48]. In agreement with their experiments, our lab has reported that SGTA associates not just with AR, but also with GR and PR to regulate receptor activity. Furthermore, knockdown/deletion of SGTA enhances receptor activity, whereas the overexpression of the co-chaperone suppresses receptor activity [45]. Taken together, the data suggest that SGTA participates in the Hsp70/Hsp90-mediated intermediate complex and plays a quality control role in the chaperone-dependent receptor maturation. It suggests that SGTA enters the chaperoning pathway at the intermediate stage forming an intermediate complex and/or asymmetrical intermediate complex with Hsp70, Hsp90, Hop, and receptor substrate by interacting with the hinge region of the receptor and binding to Hsp70 via its TPR domain. However, the binding of SGTA to the hinge region affects receptor nuclear transport since the receptor nuclear targeting sequence overlaps with the SGTA binding site [49]. Thus, this putative model provides an explanation for the fact that SGTA is a negative regulator of the receptors and its overexpression suppresses receptor activity, abrogates the regulation of receptor function by FKBP52, and decreases ligand-mediated receptor transport to nucleus.

MATURE COMPLEX

Biophysical studies using fluorescence resonance energy transfer (FRET) have shown that, once the temporary inhibition of Hsp90 ATPase activity imposed during the asymmetrical intermediate complex has been removed, ATPs quickly bind and secure the nucleotides by closing the ATP lids resulting in conformational changes in Hsp90 leading to the closing of the NTDs [50, 51]. This structural modification reduces the affinity of Hop for the assembly resulting in the exiting of the adaptor protein and its associated Hsp70. At the same time, Aha1 binds the MD to facilitate the domain repositioning and interaction with NTDs [39]. The dimerized N-terminal conformation recruits the p23 co-chaperone and one of several TPR-containing PPIases (*e.g.* FKBP52) [52]. p23 is a small acidic protein containing an unstructured C-terminal tail, which is essential for its intrinsic chaperone activity [53, 54]. Additionally, it is a conformation-specific co-chaperone that binds exclusively to the closed conformation of Hsp90 [55, 56]. Furthermore, p23 facilitates the maturation of client proteins (*e.g.* SHRs) by stabilizing the closed conformation of Hsp90 [7, 52, 53]. In fact, studies have shown that the presence of p23 can partially inhibit Hsp90 ATP hydrolysis, which is indispensable for the release of the client protein, such as SHRs [10, 57-61]. It is worth noting that it is in this active Hsp90/p23/TPR-containing PPIase mature complex that the SHR is capable of high affinity hormone binding. Upon ligand binding, the receptor dimerizes and translocates to the nucleus, which then binds to the hormone response element (HRE), which in turn, recruits other co-regulators resulting in regulation of various physiological functions such as development, differentiation, metabolic homeostasis, and reproduction. Ligand binding to the receptor has long been thought to be the trigger that stimulates release of the receptor from the chaperone complex allowing receptor translocation to the nucleus. However, studies have shown that ligand-bound GR is able to undergo dynamic cycling with the chaperone machinery, which is essential for receptor trafficking to, and within, the nucleus [62, 63]. In the absence of ligand binding, the mature complex stays active until the hydrolysis of ATP followed by the dissociation of the NTDs of Hsp90 leading to the release of p23, the TPR-containing PPIase, and the folded client protein from the chaperone [37, 64]. Finally, the free hormone receptors re-enter the chaperoning cycle by binding to Hsp40 and Hsp70 for refolding.

THE FKBP52 CO-CHAPERONE

FKBP52 has been identified as one of the TPR-containing PPIase co-chaperones that are involved, together with Hsp90 and p23, in the mature SHR/chaperone complex. It is in this form of the complex that the SHRs are capable of high affinity hormone binding and consequently translocate to the nucleus to modulate transcriptional activity. While FKBP52 is not an absolute requirement for SHR hormone binding and signaling *in vitro* [9, 65, 66], it is required for efficient AR, GR, and PR hormone binding and activity at low concentrations of hormone [67, 68]. Thus, it is assumed that receptor activity *in vivo* is dependent on FKBP52 at physiological hormone concentrations. FKBP52 belongs to a family of immunophilins that is characterized by a conserved PPIase domain, which has peptidyl-prolyl cis/trans isomerase activity and also serves as a binding site for the immunosuppressive drug, FK506 [69]. Sequence data, hydrophobic cluster analysis, and crystallographic structures of overlapping FKBP52 fragments suggested the protein is composed of four distinct domains (Fig. **2a**) [70-73]. The first two consecutive FKBP domains, FK1 and FK2, are structurally similar to the PPIase domain of FK506-binding protein 12 (FKBP12); which includes a functional site for PPIase activity (FK1) and a PPIase-like domain that lacks PPIase activity (FK2) [74]. Three TPR motifs occupy the third structural domain [75] while the fourth C-terminal domain (C-Terminal Tail) contains a motif important for binding Hsp90 and putative calmodulin (CaM) binding sites [71]. In the

following sections, we will discuss the current understanding of FKBP52 structural features and how those features contribute to FKBP52 interactions and functions within the SHR/chaperone complex.

C-TERMINAL TAIL

The C-terminal 60 amino acids, more specifically a 20-amino acid consensus sequence motif within this region, play an important role for Hsp90 binding. Within this consensus sequence motif is an 11-amino acid conserved region (charge-Y motif), which can be found in other human TPR-containing Hsp90 co-chaperones. The charge-Y motif is defined by the sequence -+-+XΦYXXMF, where - represents Glu or Asp, + represent Lys or Arg, Φ represents a hydrophobic amino acid, and X represents any amino acid [76]. In addition to the charged-Y motif, the extreme C-terminal 30 amino acids also have a significant impact on Hsp90 binding [76]. Thus, the C-terminal regions outside the core TPR regions are important for optimum FKBP52 binding to Hsp90. The extreme C-terminus of FKBP52 (amino acid 400-458) also contains two predicted CaM binding sites, which enables the protein to bind to CaM-Sepharose in a Ca+2-dependent manner [71]. Amino acid sequence analysis revealed the presence of PEST sequences within the predicted sites, which are generally present in CaM-binding proteins [71, 77]. However, the biological function of these CaM binding sites is still unknown.

TPR DOMAIN

The core TPR domain (amino acids 264-400) is composed of three tandem repeats of a degenerate 34-amino acid motif. Crystallographic data have shown that each TPR motif adopts a helix-loop-helix conformation and adjacent units stack in parallel to form a saddle-shaped domain with a concave binding pocket that mediates protein-protein interactions [78-80]. It is in this conformation that the TPR domain interacts with the MEEVD sequence in the extreme C-terminus of Hsp90. Mutagenesis studies have shown that peptide bonding is mediated through electrostatic interactions by which the basic residues of the TPR domain interact with the terminal aspartate of the pentapeptide [81]. The importance of the interaction is evident by the fact that a single mutation (K354A) within the TPR can significantly reduce FKBP52 binding to Hsp90 and abolished FKBP52-mediated potentiation of receptor function. In addition, FKBP52 domain truncation mutants demonstrated the TPR domain interaction with Hsp90 alone is necessary but not sufficient for FKBP52 regulation of SHR function [82]. Thus, the core TPR domain is required for binding to Hsp90, but is inadequate for functional interaction with SHR/Hsp90 complexes [83]. In fact, additional contacts involving charged and hydrophobic residues upstream of the Hsp90 MEEVD sequence are required for enhancement of the affinity and specificity of the interaction [47, 81]. The MEEVD pentapeptide is located at the extreme C-terminus of Hsp90 is not the only interaction site for TPR domain-containing proteins. As discussed above, recent studies have identified a novel region for TPR co-chaperone interaction at the N-terminal ATP binding domain of Hsp90 [35, 41]. Mutational analyses demonstrated that an acidic motif can be generated by a spatial positioning of noncontiguous residues (E42, N46, D49, D52, L51, and D88) within and/or near the ATP binding pocket of Hsp90, which are necessary for the binding of TPR domain-containing proteins [41]. As aforementioned, recent biochemical studies have demonstrated S100 proteins compete with Hsp90 for FKBP52 TPR domain in a Ca2+-dependent manner, hence regulating the immunophilin-Hsp90 complex formation [42].

FK2 DOMAIN

A direct functional role for the FK2 domain (amino acids 167 to 253) has not been identified. It is a required domain to maintain the overall size and structure of the large FKBPs. Despite the fact that it is structurally similar to FKBP12, it only has 26% sequence identity (44% similarity) and marginal to no PPIase and drug-binding activities [74, 84]. Evolutionarily, FK2 appears to result from a duplication event of the FK domain. Mutagenesis and FKBP51/FKBP52 chimeric protein studies demonstrated that there is a unique interaction between the FK2 and TPR domains that is important for full SHR potentiating ability [85]. Furthermore, deletion of three residues (D195, H196, and D197) within the FK2 domain of FKBP51, a closely related protein that often antagonizes FKBP52-mediated functions, resulted in abnormal integration of FKBP51 into progesterone receptor (GR) complexes [84]. Thus, specific residues and/or regions likely exist within FK2 that contribute directly to receptor regulation and possibly influence interactions with the components of the receptor-chaperone complex or the receptor itself. Further studies are needed to define those critical residues and/or regions within FK2 that are required for regulation of receptor activity. Interestingly, FK2 contains a consensus ATP/GTP-binding sequence located between amino acids 199 and 222, which can be phosphorylated *in vitro* in the presence of CaM in an ATP-dependent manner [86, 87], although the ability of FKBP52 to bind and hydrolyze nucleotide has not been demonstrated. In fact, sequence and structural comparison data have demonstrated that FK2 displays structural similarity to the TPR-containing homolog FKBP38, which is PPIase-inactive under basal conditions but can be allosterically activated by CaM [88-91]. The fact that FKBP52 contains a putative CaM-binding motif at the extreme C-terminus suggests that a similar allosteric activation mechanism for the FK2 domain of FKBP52 exists.

FK LINKER

The crystal structures of FKBP52 revealed a 9-amino acid long (amino acids 138-167) flexible and solvent-accessible hinge region that connects the FK1 and the FK2 domains termed the FK linker [92]. Within this linker region there is a consensus casein kinase II (CKII) phosphorylation site (TEEED). FKBP52 is phosphorylated by CKII at T143, which is a major phosphorylation site both *in vivo* and *in vitro* [93]. *In silico* modeling and structural analyses revealed that T143 phosphorylation destabilizes the FK linker region and induces the allosteric rearrangement of the FK1 domain [92-94]. FK1, as will be discussed in more detailed in the following section, particularly the integrity of proline-rich loop that overhangs the PPIase pocket, is a functionally important interaction surface that is required for FKBP52-mediated potentiation of SHR response to hormone. Phosphorylation of T143 destabilizes the conformation of the

linker resulting in a widening of the architecture and introduces a steric hindrance by disrupting the hydrogen-bonding network within the region causing a re-orientation within the linker [94]. Subsequently, weakening the FK1-FK2 contacts introduces a remodeling of the FK1 catalytic domain by twisting a short α-helix that forms one side of the PPIase active site [94]. Interestingly, tyrosine phosphorylation of FKBP52 influences adeno-associated virus type 2 (AAV) second-strand DNA synthesis by binding to single-stranded D-sequence-binding protein within the virus' terminal repeats limiting high-efficiency transgene expression, which may have important implications for the optimal use of AAV vectors in human gene therapy [95, 96]. In addition to the T143 phosphorylation site, a conserved negatively charged motif that was predicted to be a complementary nuclear localization signal (NLS) recognition sequence also localizes within the FK linker region [67]. FKBP52 is primarily localized in the nucleus with a minority co-localizing with microtubules in the cytoplasm. Biochemical studies have shown that antibodies raised against the NLS impede the hormone-mediated translocation of glucocorticoid receptor (GR) in the nucleus [67]. This suggests that the sequence is required for anterograde movement of FKBP52 to the cytoplasm where is associates with the Hsp90 heterocomplex to facilitate the passage of the untransformed receptor through the nuclear pore.

FK1 DOMAIN

The FK1 domain is located within the first 138 amino acids in the N-terminus of FKBP52. Unlike the FK2 domain, FK1 contains a functional PPIase catalytic pocket located between amino acids 4-137 that has enzymatic activity comparable to that of FKPB12 [70, 74]. *In vitro* studies demonstrated that FKBP52 selectively potentiates hormone-dependent gene activation and hormone-binding affinity of AR, PR, and GR through interaction with the receptor LBD [82, 97, 98]. Interestingly, it is not the enzymatic activity of the FK1 that is required for receptor potentiation but the integrity of the PPIase pocket. Furthermore, gain-of-function mutagenesis studies identified a proline-rich loop that overhangs the catalytic pocket that is critically involved in receptor interactions and enhanced hormone-mediated receptor activity [85]. Structural comparison of the corresponding loop of FKBP52 and FKBP51 revealed the most divergent regions in the domains between the two proteins are the β3 bulge and β4-β5 loop (Fig. **2b**). The β3 bulge occurs when there is a discontinuity in β-strand 3. In FKBP51, P76 (K76 in FKBP52) enforces a shift of the β3 bulge toward the β2-β3 loop, which forces E75 closer to the PPIase active site compared to D75 in FKBP52, thus, compromising FK1 architecture (Fig. **2b**) [92, 99]. The same principle applies to the FD67DV double mutation, which abolishes FKBP52–dependent potentiation of GR and AR (Fig. **2b**). Substantial structural differences between FKBP51 and FKBP52 are also found at the tip of the β4-β5 loop; FKBP52 has a proline at amino acid position 119 while FKBP51 has a leucine (Fig. **2b**). P119 of FKBP52 projects outward to form a hydrophobic notch alone, while with the *trans* configuration adopted by the P119-P120 peptide bond in the β4-β5 loop forms an important functional interface that contributes to the enhancing effects of FKBP52 (Fig. **2b**) [85, 92]. On the contrary, L119 in FKBP51 projects inward and the *cis* conformation formed by the L119-P120 bond impairs the potentiation of steroid receptor activity (Fig. **2b**) [85, 92]. Gain-of-function mutagenesis studies corroborate this structural data; the FKBP51 mutation L119P conferred significant receptor potentiating ability, whereas the converse P119L mutation in FKBP52 decreased receptor potentiation. Interestingly, when a second residue, A116, in the β4-β5 loop was also mutated, the FKBP51-A116V/L119P double mutant potentiated hormone signaling similar to that of wild type FKBP52 [85]. These results all emphasize the importance of architectural integrity of the proline-rich loop in acting as a critical interface for regulating receptor interactions and activity.

FKBP52 IN STEROID HORMONE-REGULATED PHYSIOLOGY AND DISEASE

Biochemical and cellular studies demonstrated that FKBP52 associates with the SHR chaperone complex to specifically potentiate the activities of AR, GR, and PR. The physiological significance of these findings has been corroborated in *fkbp52*-deficient (52KO) mouse models as phenotypes related only to androgen, glucocorticoid and progesterone insensitivity have been characterized to date (Fig. **3**) [97, 100]. These studies firmly established FKBP52 as a relevant factor in AR, GR and PR-related physiology and disease.

PHENOTYPES IN *FKBP52*-DEFICIENT MICE

The observed reproductive phenotypes observed in the 52KO mice are attributed to the loss of steroid receptor activities. Male 52KO mice are infertile and display abnormal virilization with persistent nipples, ambiguous external genitalia, and dysgenic seminal vesicles and prostate [97, 100], which are consistent with androgen insensitivity in these tissues. Despite the androgen insensitivity, the testicular morphology, descent, histology, and spermatogenesis develop normally with unimpaired androgen production and release from the testes [97], which might suggest that testosterone levels produced locally within the testis is high enough to compensate for significantly reduced AR activity. Alternatively, it is possible that a factor present within the testis can complement for the loss of FKBP52. Despite no observable defect in spermatogenesis, sperm isolated from the organ displayed abnormal tail morphology and reduced motility, which is not androgen-dependent [101]. These findings may reflect FKBP52's ability to bind to dynein motor proteins [67].

In contrast to what is observed in 52KO male mice, 52KO females have no gross morphological abnormalities and display normal ovulation and fertilization, yet they are completely infertile [102]. The infertility is the result of failure in embryonic implantation and decidualization [98, 102-104]. The estrogen receptor (ER) and PR are critical factors mediating embryonic implantation. Interestingly, the absence of FKBP52 leads to a selective failure of receptor function resulting in female mice sterility. In fact, FKBP52 does not alter ER function in cellular studies and 52KO mice show no signs of estrogen insensitivity. Rather, the implantation and decidualization failures result from an inability of the uterus to mount a decidualization response

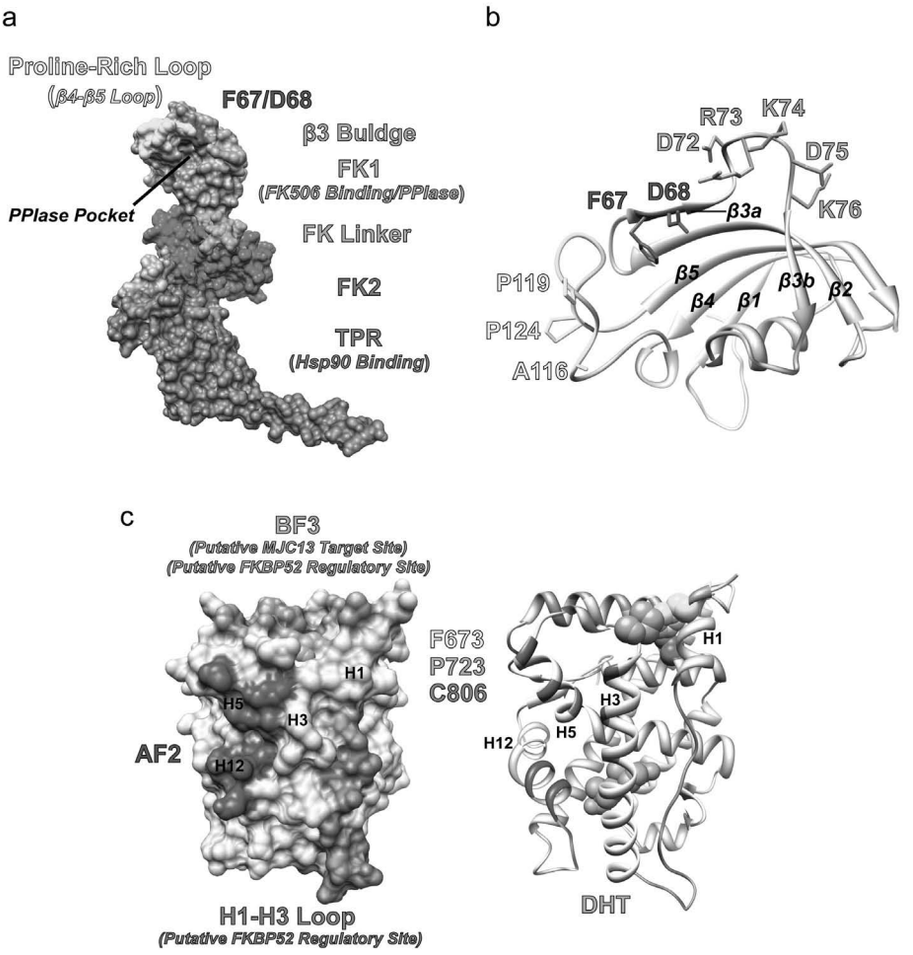

Fig. (2). Structure of FKBP52 and the putative FKBP52 regulatory surfaces on AR. **(a)** A composite of two partial structures for human FKBP52 (protein databank number 1Q1C and 1P5Q) showing the locations of the functional domains of FKBP52. The individual domains as well as regions of functional importance are individually colored. The TPR domain (green) mediates binding to Hsp90 via the MEEVD motif at the extreme C-terminus of Hsp90. The FK2 domain (red) is structurally similar to FK1, but lacks PPIase activity and the ability to bind to the immunosuppressive ligand FK506. The FK linker (teal), which connects the FK1 (blue) and FK2 (red) domains, contains a casein kinase II (CKII) phosphorylation sequence that, when phosphorylated, abrogates FKBP52 function due to the re-orientation of FK1 domain conformation. The FK1 domain (blue) is the primary regulatory domain for SHRs that displays FK506 binding and PPIase activity. FK1 is also important for FKBP52-mediated receptor potentiation. In particular, the proline-rich loop (yellow), also known as the β4-β5 loop, overhanging the PPIase pocket of the FK1 domain is crucial for receptor regulation and has been proposed to serve as a functionally important interaction surface. **(b)** A ribbon model of the FKBP52 FK1 domain is shown. The β4-β5 loop (yellow) and the β3 bulge (orange), and their respective residues (same colors), are structurally the most divergent regions at the periphery of the PPIase pocket between FKBP52 and its paralog, FKBP51. Mutational changes in the residues in and/or around the loop and bulge, such as residues F67/D68 (purple), can induce conformational changes in the pocket resulting in the obstruction of FKBP52-mediated receptor activity. **(c)** The left panel is a surface rendering of the AR ligand binding domain showing the relative locations of the putative FKBP52 regulation sites including BF3 (blue), the H1-H3 loop (green), and AF2 (purple). The right panel is a ribbon representation of the AR ligand binding domain, with dihydrotestosterone (teal) bound, showing the location of the mutated residues in relation to the BF3 surface. F673 (yellow) and P723 (orange) are within the BF3 surface and C806 (red) is buried directly below the surface. Mutations of these residues within the BF3 surface result in increased dependence on FKBP52 for function. This is also the site to which the recently characterized inhibitor of FKBP52-regulated AR activity, MJC13, is predicted to bind.

to progesterone due to progesterone insensitivity and uterine defects [102]. This implantation failure is also a result of an increased uterine oxidative stress and a reduced level of the antioxidant peroxiredoxin-6 (PRDX6) [104, 105]. Furthermore, the loss of FKBP52 promotes the growth of endometriotic lesions due to increased cell proliferation, inflammation, and angiogenesis [106]. These events are largely dependent upon progesterone actions, and, along with the corroborative data from both molecular and cellular studies, this confirms that FKBP52 is required for full PR activity *in vitro* and *in vivo*. Taken together, these data firmly establish a critical role for FKBP52 in reproductive development and success in both male and female mice, and these roles can be traced to support of AR and PR functions.

Due to the partial embryonic lethality in null 52KO mice [107], heterozygous *fkbp52*-deficient (52+/-) mice were gen-

erated to determine the *in vivo* roles for the co-chaperone in GR-mediated physiology. 52+/- mice manifested phenotypes associated with defective GR signaling including increased susceptibility to high-fight diet induced hepatic steatosis, hyperglycemia, and hyperinsulinemia. They also displayed glucocorticoid resistance and behavioral alterations under basal and chronic stress conditions [108, 109].

As previously discussed, FKBP52 does not alter ER function in cellular studies and 52KO mice do not manifest signs of estrogen insensitivity. However, studies have reported that FKBP52 expression levels are associated with ERα, which implicates FKBP52 as a potential factor in breast cancer [110]. Treatment of breast cancer cells with estradiol resulted in an increased half-life of FKBP52 mRNA, and both FKBP52 gene and protein expression have been reported to be significantly up-regulated and in ERα-positive cell lines as compared with ERα-negative cell lines [110, 111]. Furthermore, the FKBP52 gene is epigenetically silenced by methylation in ER-negative, but not in ER-positive, breast cancer cells [112]. Taken together, these studies have identified FKBP52 as a relevant factor in ERα-positive breast cancer. In addition, recent studies suggest an increased reliance on AR signaling in triple negative breast cancer [113]. Given the known roles for FKBP52 in AR signaling, these studies implicate FKBP52 as a potential target in triple negative breast cancer.

FKBP52 IN Hsp90-INDEPENDENT PHYSIOLOGY AND DISEASE

Apart from the well-established roles of FKBP52 in SHR function, FKBP52 has been identified in complex with a variety of other client-Hsp90 heterocomplexes, such as those containing kinases, aryl hydrocarbon receptor, and heat shock transcription factor to name a few. However, many of these associations are passive and transient, and have no functional impact on client activity. It is likely that Hsp90 continuously samples the available pool of TPR-containing PPIase co-chaperones and the co-chaperone that ultimately functionally interacts is dependent on the client protein present within the complex. In addition to the Hsp90-dependent client proteins, FKBP52 is also involved in various endocrine-independent processes (Table **1** and Fig. **3**). As previously discussed, FKBP52 belongs to a family of immunophilins, which can be targeted by immunosuppressive molecules. This drug-immunophilin complex then docks and inhibits the activity of calcineurin leading to immunosuppression, although FK506 binding to FKBP52 does not inhibit calcineurin [114, 115]. Over the past decade, there has been

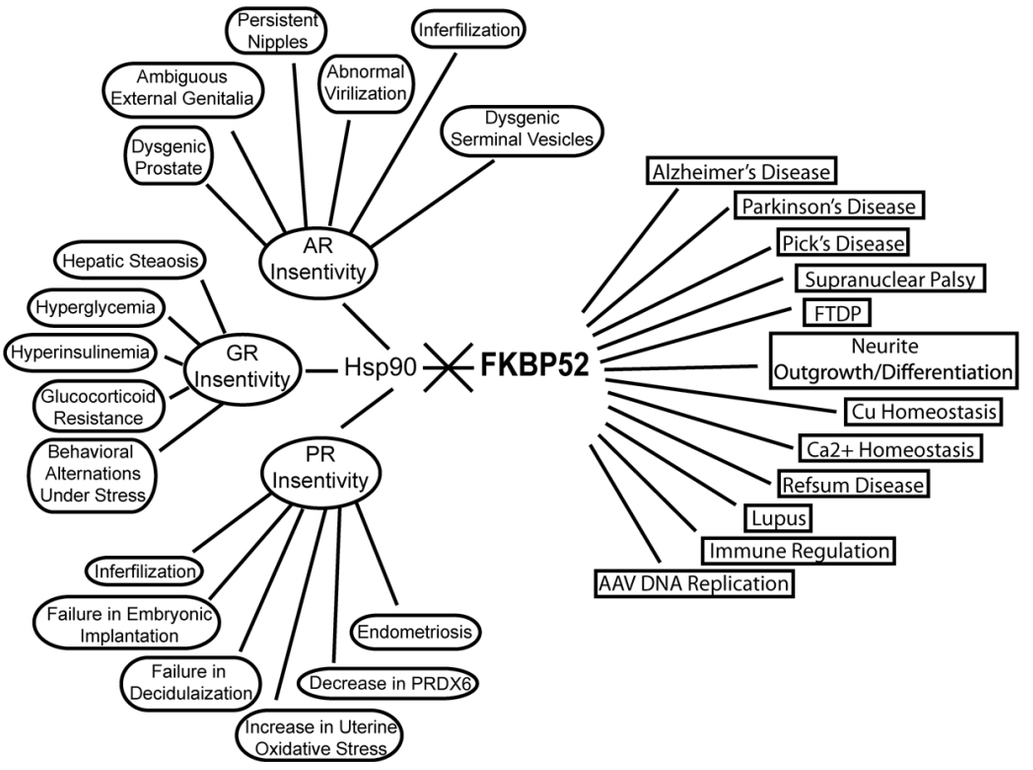

Fig. (3). FKBP52-regulated Hsp90-dependent and independent physiology and disease. The Hsp90-dependent roles for FKBP52 are largely mediated through FKBP52-Hsp90 complex regulation of AR, GR and PR signaling, which is independent of FKBP52 PPIase activity. FKBP52 is an essential player in SHR/Hsp90-regulated physiological development and reproductive success. The left side of the diagram depicts receptor-specific phenotypes that are due to defective AR, GR, and PR signaling in the absence of FKBP52. Given the positive role of FKBP52 in these receptor signaling pathways FKBP52 may also serve as an attractive therapeutic target for any disease that is dependent upon functional AR, GR, and PR signaling pathways (e.g. prostate cancer). Apart from the established roles of FKBP52 in SHR functions, the co-chaperone is also involved in various Hsp90-independent biological functions, several of which have been shown to require FKBP52 PPIase activity. The right side of the illustration shows that the absence of FKBP52 could contribute to neurodegenerative tauopathies (Alzheimer's Disease, Pick's Disease, fronto-temporal dementia and Parkinsonism linked to chromosome 17 (FTDP), progressive supranuclear palsy), disruptions in Cu and Ca2+ homeostasis and immune system, and inhibition of AAV DNA synthesis resulting in inefficient transgene expression from recombinant AAV vectors used in gene therapy.

Table 1. Alternative FKBP52 interacting proteins.

Interactors	Experimental Approach	Physiological Implications	References
Dynein	Co-IP	Intracellular trafficking of steroid receptor complexes	[68, 137]
p53	Co-IP	Cancer	[186]
HSF-1	Co-IP	Cellular stress	[187]
TRPCs	Co-IP	B- and T-cell activation; neuronal survival and growth	[136]
FAP48	Yeast two-hybrid	T-cell activation	[140, 141, 188]
PHAX	Yeast two-hybrid	Refsum disease, lupus	[189]
IRF-4	Yeast two-hybrid	Immune regulation	[138]
AAV DNA	EMSA	Gene therapy	[95]
Atox1	Yeast two-hybrid	Copper transport	[131]
Tau	Co-IP	Tauopathy	[120]
PRDX6	Co-IP	Embryonic implantation	[105]
AAP	Co-IP	Alzheimer's disease	[130]
RET51	Yeast two-hybrid	Parkinson's disease	[139]
Tubulin	Co-IP	Neuronal differentiation	[119]
S100A1 & A2	Co-IP	Ca^{2+}-dependent signaling	[42]

a growing interest to understand the role of immunophilins, including FKBP52, in the nervous system. FKBP52 is ubiquitously expressed and especially abundant in the central nervous system. Thus, it is not surprising that FKBP52 is involved in neurodegenerative tauopathies including Alzheimer's Disease (AD), Pick's Disease, fronto-temporal dementia and Parkinsonism linked to chromosome 17 (FTDP), and progressive supranuclear palsy [116, 117]. Tauopathies is defined by the neuropathological characteristic of aberrant aggregation of insoluble hyperphosphorylated microtubule-associated protein (MAP) tau within the neurons termed neurofibrillary tangles (NFTs), which are also referred to as paired helical filaments (PHF) [118]. Recent studies have reported that FKBP52 interacts directly with the hyperphosphorylated form of tau, which has antagonistic effects on tubulin polymerization and microtubule assembly [119, 120]. It is worth noting that FKBP52 regulation of microtubule assembly is likely dependent on PPIase activity, which is in contrast to that observed with FKBP52 regulation of SHR activity. The α-Synuclein (α-Syn) protein is a key player in the pathogenesis of Parkinson's disease. Knockdown of FKBP52 reduced the number of α-Syn aggregates and protected against cell death, whereas overexpression of FKBP52 accelerated both aggregation of α-Syn and cell death [121]. Finally, FKBP52 expression is enhanced in regenerating neurons, which stimulates neurite outgrowth and promotes neuronal differentiation suggesting a protective or regenerative role following injury [122, 123].

Copper (Cu) is an essential nutrient, and, as a result, cells have developed elaborated systems for Cu storage and transport. In humans, disruption of the tightly regulated cellular Cu homeostasis affects normal tissue development and leads to anemia, neutropenia, cancer, and several neurodegenerative diseases including AD [124, 125]. The amyloid precursor protein (APP) plays a central role in the development of AD through the generation of peptides called beta-amyloid (Aβ) by proteolysis of the precursor protein. Cu contributes to the neuropathology of AD by interacting with copper binding domain (CuBD) of APPs and Aβ peptides causing the formation of amyloid plaques and disrupting metal ion homeostasis [126-128]. There are several lines of evidence that have linked the protective effects of FKBP52 with intracellular Cu homeostasis. First, FKBP52, more specifically its FK1 domain, interacts directly with APP and Cu metallochaperone Atox1, which is a protein that delivers copper to the copper transporting ATPases [129, 130]. Second, mutations of FKBP52 modulate the toxic effects and level of Aβ peptides in *Drosophila* [130]. Third, mutations in the copper transport genes *Ctr1A* and *Atox1*, which directly regulate intracellular copper levels, modify Aβ-induced phenotypes in *Drosophila* [130]. Fourth, dietary fluctuation in the Cu levels influences the protective effects of FKBP52 on Aβ [130]. Finally, cells isolated from 52KO mice show increased levels of Cu compared to wild type cells and overexpression of FBP52 causes efflux of copper [131].

S100A1 and S100A2 belong to the S100 family of calcium (Ca2+)-binding proteins that are linked to regulation of various intracellular processes and are often expressed in a cell- and tissue-specific fashion [132, 133]. Cellular data has linked S100A1 to neuronal cell dysfunction/death that occurs

in AD by altering APP expression, destabilizing the intracellular Ca2+ homeostasis, and increasing sensitivity to Aβ toxicity [134]. Based on the biochemical evidence, FKBP52 is a novel target for S100A1 and S100A2. Both proteins interact with the FKBP52 TPR domain leading to dissociation of the immunophilin/Hsp90 complex in a Ca2+-dependent manner [42]. S100A1 and S100A2 proteins are not the only proteins that associate with and/or regulate FKBP52 functions in a Ca2+-dependent manner. Ca2+ homeostasis has been suggested to regulate intracellular FKBP52 functions leading to affects on the phosphorylation of tau and pathology in AD. Interestingly, a *Drosophila* orthologue of FKBP52, termed dFKBP59, was found to interact with the Ca2+ channel transient receptor potential-like (TRPL) protein in photoreceptor cells and to influence Ca2+ influx [135]. Subsequent studies revealed that FKBP52 similarly interacts with a subset of rat transient receptor potential channel (TRPC) proteins that form Ca2+ channels in the mammalian brain [136]. Although the functional importance of the CaM-binding motifs in the C-terminal tail of FKBP52 is not known [137], these roles for FKBP52 in multiple Ca2+-dependent functions suggest that the interaction of FKBP52 with CaM may be yet another CA2+-dependent mechanism by which FKBP52 could functionally affect a wide variety of CaM-dependent physiological processes including inflammation, metabolism, intracellular movement, smooth muscle contraction, and the immune response.

FKBP52 has also been found to interact directly with the interferon regulatory factor 4 (IRF-4) [138], which regulates gene expression in B and T lymphocytes; controls proto-oncogene *RET* by forming a complex with tyrosine kinase receptor RET51, which is involved in the development and maintenance of the nervous system [139]; and FKBP-associated protein 48 [140], which influences proliferation of Jurkat T cells [141]. Each of these interactions was found to be disrupted by FK506 and to target the FKBP52 PPIase domain to specific proline sites in each partner protein. Phenotypes potentially related to these interactions have not yet been assessed in 52KO mice. Not only does FKBP52 interact with proteins, but also directly binds AAV DNA and regulates replication of the viral genome [95, 142]. The relevant DNA binding site in FKBP52 has not been identified.

FKBP52 AS A NOVEL TARGET FOR PROSTATE CANCER TREATMENT

The importance of FKBP52 as a regulator for not just hormone-dependent, but also hormone-independent diseases is becoming increasingly clear. FKBP52 is often overexpressed in malignant hepatoma, T cell leukemia, ERα-positive breast cancer cell lines, pre-invasive and breast cancer tissues, and hormone-dependent cancers [141, 143-147]. Furthermore, the prostate dysgenesis observed in 52KO mice along with enhanced FKBP52 expression in several prostate cancer cell lines and prostate biopsy samples establish the protein as a critical regulator of AR-mediated prostate development [97, 100, 148, 149]. Androgens play an important regulatory role in the development and progression of prostate cancer (PCa) by binding to the hormone binding pocket in the C-terminal LBD core of AR [150, 151]. The AR LBD consists predominantly of 12 α-helices. Upon ligand binding, helix 12 is reorganized to an agonist conformation termed activation function 2 (AF2) for co-regulator binding [152, 153] (Fig. **2c**). The primary treatment for locally advanced and metastatic PCa is androgen deprivation therapy (ADT), in which the anti-androgens including Bicalutamide, Enzalutamide, and ARN-509 bind to the LBD of AR [154]. These anti-androgens inhibit AR action by competing for androgen binding and displacing helix 12 to prevent formation of a productive AF2 pocket [155]. Most tumors respond to the treatment initially. However, as the cancers progress they become resistant to the therapy, in which the condition is termed castration-resistant prostate cancer (CRPC) [156-158]. Preclinical and clinical studies suggest that acquired resistance to conventional ADT is caused by restoration of the AR pathway by AR overexpression and mutations, cross-talk between AR and other signaling pathways, and/or by-passing AR blockade through up-regulation of GR [159-164]. As a result, researchers have focused their efforts on the development of a new class of AR inhibitors termed nuclear receptor alternative-site modulators (NRAMs) targeting alternative sites on AR and receptor regulatory proteins including receptor-associated chaperones, co-chaperones (*e.g.* FKBP52), co-activators (*e.g.* β-Catenin), and AR inhibitors for which the binding sites are currently unknown [165]. In line with this idea, our lab has recently identified a small molecule termed MJC13 that specifically inhibits FKBP52 regulation of AR by blocking the hormone-dependent dissociation of the AR-Hsp90-FKBP52 heterocomplex resulting in a loss of AR nuclear translocation and an inhibition of androgen-dependent gene expression and proliferation in prostate cancer cells [166].

FKBP52-SPECIFIC TARGETING OF THE AR BF3 SURFACE

FKBP52 interacts with Hsp90, and, although the specific Hsp90 contact site on the surface of the receptor LBD has not been determined, a seven-amino acid segment located just upstream of the receptor LBD was found to be required for stable interaction with the Hsp90 MD [62]. Furthermore, the fact that FKBP52 regulation has been localized to the receptor LBD and its regulation is receptor-specific suggest that FKBP52 directly interacts with the receptor LBD within the Hsp90 heterocomplex. Thus, we propose a model in which Hsp90 brings the FKBP52 FK1 domain, more specifically the proline-rich loop, in close proximity to the receptor LBD, which leads to a direct interaction and regulation of receptor hormone binding and subcellular localization. Importantly, recent studies have identified a surface region on the AR LBD that, when mutated, displays increased functional dependence on FKBP52 and this surface overlaps with the binding function 3 (BF3) surface (Fig. **2c**) [166, 167].

BF3 is a recently characterized hydrophobic binding pocket on the AR LBD that is located near, but distinct from, the AF2, which acts as a major docking site for short hydrophobic peptide motifs featured in AR co-activators and mediates AR functional amino/carboxy (N/C)-terminal interactions (Fig. **2c**) [167-172]. The role of BF3 *in vivo* is currently unknown, however, mutational and functional analyses of the surface have confirmed its role in AR activity [173]. Small molecule docking to the BF3 surface resulted in an allosteric modification that prevents the interactions of AF2 with co-activators [167]. In fact, *in vitro* studies along with

computational molecular dynamic simulations revealed a structural connection between BF3 and AF2. A series of residues within BF3, the boundary of BF3/AF2, and AF2 are structurally interconnected and allosterically coupled [173]. Importantly, the experiments demonstrated that BF3 mutations function as allosteric elicitors of conformational changes in the AR LBD by altering AF2 propensity to reorganize into hydrophobic sub-pockets that accommodate the N-terminal domain and co-activator peptides, and inhibit co-regulator binding [173]. This induced conformation consequently may either potentiate or silence AR function. In fact, residues in the BF3 pocket have been identified as mutational target sites for PCa and/or androgen insensitivity syndrome (AIS) patients (McGill Androgen Receptor Gene Mutations Database: http://androgendb.mcgill.ca/). The importance of BF3 as a regulatory surface for AR activity was further highlighted in recent studies by Jehle *et al*. [174] in which a novel hexapeptide repeat sequence, GARRPR, was identified in the N-terminus of the co-chaperone Bag-1L that is involved in the modulation of AR activity by binding to the BF3 pocket. Thus, the AR BF3 surface may serve as a promiscuous regulatory surface for a number of co-regulators, including FKBP52.

The proline-rich loop that overhangs the FKBP52 PPIase catalytic pocket in the FK1 domain is required for receptor regulation and is hypothesized to serve as an interaction surface with the receptor LBD. Our lab has recently identified several residues (F673, P723, and C806) on the AR LBD that display increased dependence on FKBP52 (also termed FKBP52 hypersensitivity) for function when mutated. This region directly corresponds to the AR BF3 regulatory surface (Fig. **2c**) [97, 166]. The small molecule FKBP52-specific inhibitor MJC13 binds the AR LBD, but does not compete with hormone and SRC-2 binding. In addition, mutations within the BF3 surface differentially affect MJC13 activity. Thus, several lines of evidence suggest that MJC13 targets the AR BF3 surface to inhibit regulation of AR by FKBP52. This interaction prevents hormone-induced AR/Hsp90/FKBP52 heterocomplex dissociation and nuclear translocation, thus effectively blocking AR-dependent gene expression and androgen-stimulated proliferation in various human prostate cancer cell lines [166]. Taken together, our recent findings suggest that the AR BF3 surface is a putative FKBP52 regulatory and/or interaction surface and the targeting of this surface for the treatment of prostate cancer is an attractive option with fewer side effects. In fact, early preclinical studies for MJC13 suggest an excellent drug safety profile with no toxicity observed at maximum soluble concentrations, and impressive effects on tumor growth in a 22Rv1 prostate cancer xenograft model [175, 176]. Given the unique mechanism of action, MJC13 and other co-chaperone targeting drugs may be able to escape the acquired resistance that is seen with conventional ADT in subsets of patients, depending on the mechanism of resistance. MJC13 and the N-terminal Hsp90 inhibitors lack the ability to inhibit the constitutive activity of AR splice variants found to be up-regulated in CRPC [177]. This is not surprising given the fact that the splice variants lack the Hsp90-binding site in the hormone binding domain. However, MJC13 does not target FKBP52 directly, but targets the putative FKBP52 regulatory site in the AR hormone binding domain.

TARGETING FKBP52 PROLINE-RICH LOOP INTERACTIONS

Figure **4** illustrates known and predicted FKBP52 interactions and possible therapeutic targeting strategies to disrupt FKBP52 regulation of AR. While the targeting of the FKBP52 regulatory surface on AR (BF3) is a promising therapeutic strategy that allows for AR-specific targeting, the direct targeting of FKBP52 offers numerous advantages over MJC13 that would lead to a more potent and effective drug. First, the AR BF3 surface represents a less than ideal drug binding site, and, as a result, the effective MJC13 concentrations in cellular assays are in the low micromolar concentration range [166]. In contrast, the FKBP52 PPIase pocket not only represents an ideal hydrophobic drug binding pocket, but is a known 'druggable target' as the immunosuppressive drug Tacrolimus is already FDA-approved for use in the clinic. Also, given the conservation within the FKBP PPIase pocket, drugs targeting the FKBP52 PPIase pocket would likely target FKBP52 and the closely related FKBP51 protein simultaneously. While FKBP52, but not FKBP51, is largely considered the relevant steroid hormone receptor regulator, more recent evidence suggests that both FKBP51 and FKBP52 are positive regulators of AR in prostate cancer cells [178]. In addition, FKBP52 is a known positive regulator of AR, GR and PR, and the direct targeting of FKBP52 would target the activity of all three receptors simultaneously. Increasing evidence suggests that many factors (e.g. growth factors, cytokines, and angiogenic factors) implicated in prostate cancer progression are targets of the GR signaling pathway [179, 180]. In addition, recent evidence suggests that GR signaling confers resistance to current anti-androgen treatments [159]. Furthermore, recent studies by Cluning *et al.* showed that H1-H3 loop of GR LBD is not a direct interaction site for FKBP52, however, mutations within the loop can affect FKBP52-mediated receptor activities [181]. Thus, mechanistically, the H1-H3 loop acts as a regulatory surface that promotes conformational changes in BF-3, which is in close proximity and allosterically affects FKBP52-mediated receptor activities (Fig. **2c**). While very little work has been done to characterize a role for PR in prostate cancer, data suggests that PR expression is elevated in metastatic disease, and that PR antagonist are potential treatments for prostate cancer [182, 183]. Finally, FKBP52 directly regulates NFκB transcriptional activity [184] and inhibition of NFκB was recently demonstrated to restore CRPC responsiveness to ADT [185]. Thus, the direct targeting of the FKBP52 proline-rich loop with small molecules will lead to a more potent drug with the potential to simultaneously hit a variety of targets known to have, or suspected of having, a role in prostate cancer progression. Previous studies demonstrated the functional importance of the FKBP52 proline-rich loop, which establishes this site as the most attractive target site for disrupting FKBP52 interactions with the SHRs. While this surface does not represent an ideal hydrophobic drug binding pocket, the PPIase catalytic pocket does. In addition, the available co-crystal structure of FKBP12, a related family member, bound to FK506 suggests that molecules docked within the PPIase pocket could re-orient proline-rich loop conformation leading to the disruption of interactions at this surface (unpublished observations).

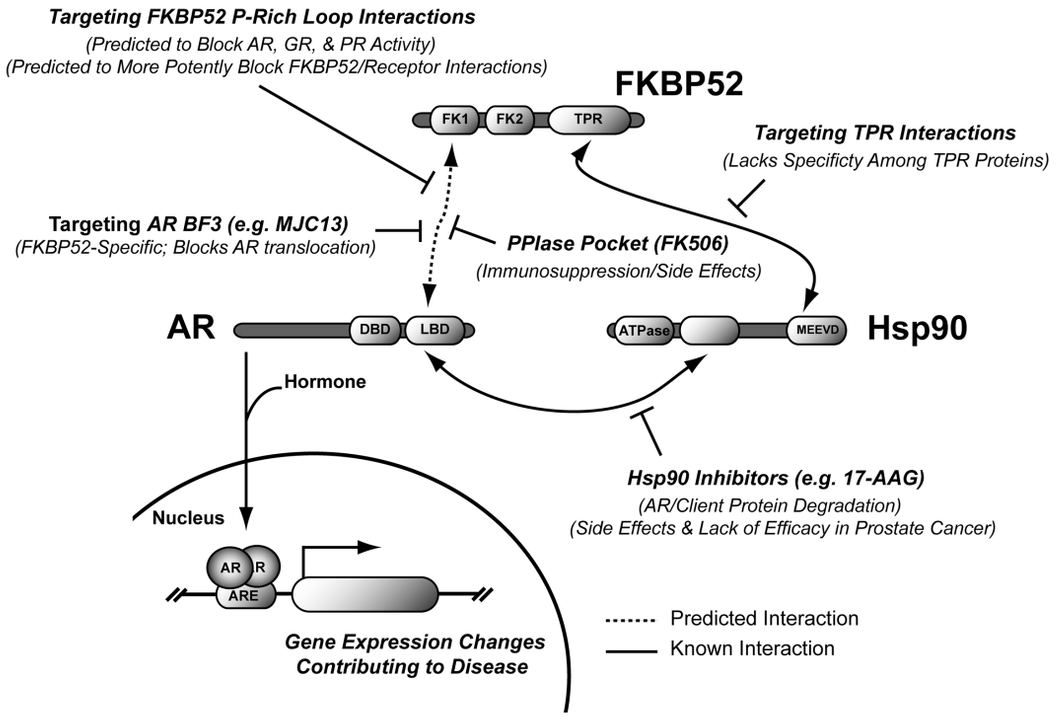

Fig. (4). FKBP52-receptor interactions and therapeutic targeting strategies. Both known (solid arrow) and predicted (dashed arrow) FKBP52-Hsp90-receptor interactions are illustrated in addition to possible strategies for therapeutically disrupting chaperone-cochaperone regulation of AR-mediated transcription for the treatment of prostate cancer. FKBP52 is a known positive regulator of AR function that associates with the EEVD motif in the C-terminus of Hsp90 by way of a TPR domain. In addition, the FKBP52 FK1 domain, the PPIase pocket and the proline-rich loop in particular, comprise a functionally important interaction surface that is predicted to interact with the AR hormone binding domain. The prevailing hypothesis is that Hsp90 brings FKBP52 in close proximity to the receptor allowing the FKBP52 FK1 domain to directly contact the receptor hormone binding domain. Our recent data suggest that this contact site is the AR BF3 surface. As detailed, several drug classes already exist for the inhibition of Hsp90 (geldanamycin and derivatives) and FKBP52 (FK506 also called Tacrolimus). Geldanamycin is currently in phase III clinical trials for the treatment of various cancers, but has proven ineffective in prostate cancer. Tacrolimus is currently used clinically to suppress the immune system during organ transplantation, and the immunosuppressive effects would be undesirable in a prostate cancer drug. However, the success of Tacrolimus in the clinic indicates that FKBP52 is a "druggable" protein. Targeting of the FKBP52 TPR domain, which would theoretically disrupt FKBP52 interactions with Hsp90, is a possible approach. However, the TPR motif is highly conserved and any molecule that targets the FKBP52 TPR would also likely target a large number of other TPR proteins. The targeting of the proposed FKBP52/β-catenin binding site on the AR hormone binding domain is also an attractive option. This approach is represented by the compound termed MJC13 and derivatives that were recently developed by our laboratory. Directly targeting the FKBP52 proline-rich loop represents the most promising approach as FKBP52 is a "druggable" protein and the proline-rich loop has been found to be critical for FKBP52 regulation of AR activity.

CONCLUSION

In conclusion, the Hsp90-associated FKBP52 co-chaperone has become increasingly associated with aberrant SHR signaling in disease. FKBP52 is a TPR-containing co-chaperone that plays a critical role in the chaperone-dependent folding of SHRs to their functionally mature conformations that are competent for hormone binding. Given the functional roles of FKBP52 in receptor-specific phenotypes, and its direct participation in the aberrant AR hyperactivity observed in prostate cancer, FKBP52 has emerged as a novel therapeutic target with the potential to treat castration-resistant PCa; thereby filling a major unmet need in PCa treatment. FKBP52 acts as a specific positive regulator of AR, PR, and GR functions through the interaction of the proline-rich loop with the LBD of the SHRs. Thus, in addition to PCa, the therapeutic targeting of FKBP52 proline-rich loop interactions represents an attractive treatment option for a number of diseases associated with the AR, GR and PR signaling pathways including benign prostatic hyperplasia, obesity/metabolic syndrome, stress and depression, and Cushing's Syndrome. In addition, drugs targeting FKBP52 regulation of SHR activity may have utility as male and/or female contraceptives.

ETHICS STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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The FKBP51-Glucocorticoid Receptor Balance in Stress-Related Mental Disorders

Gabriel R. Fries[1,2]*, Nils C. Gassen[1], Ulrike Schmidt[3] and Theo Rein[1]*

Gabriel R. Fries

[1]*Department of Translational Research in Psychiatry, Max Planck Institute of Psychiatry, Munich, Germany;* [2]*Translational Psychiatry Program, Department of Psychiatry and Behavioral Sciences, The University of Texas Health Science Center at Houston (UTHealth) Medical School, Houston, TX, USA;* [3]*Department of Clinical Research, RG Molecular Psychotraumatology, Max Planck Institute of Psychiatry, Munich, Germany*

Theo Rein

Abstract: The immunophilin FK506 binding protein 51 (FKBP51) has emerged as one of the most intensely investigated proteins in stress-related mental disorders. It was originally characterized as Hsp90 cochaperone and part of the receptor-chaperone heterocomplex that governs the activity of steroid receptors. It turned out that the presence of FKBP51 in this heterocomplex leads to diminished activity of the corticosteroid receptors. In particular, based on its inhibitory action on the glucocorticoid receptor (GR), FKBP51 was included in a candidate gene approach to discover gene polymorphisms that might be relevant for the development and treatment of major depression. The discovery that polymorphisms in the gene coding for FKBP51 were linked to the treatment response of depressed patients intensified the research on the role of FKBP51 in stress-related diseases worldwide. It has become evident that FKBP51 is not only a regulator of GR action, but also a GR target. The function of this ultrashort intracellular feedback loop is critically important for cellular and physiological stress regulation as it does not only calibrate the function of GR, but also the levels of FKBP51. Given the pleiotropic functions of FKBP51, its levels might be equally important for mental disorders as GR function and hence for the development of potential FKBP51 drug targets.

Keywords: FKBP51, glucocorticoid receptor, HPA axis, mental disorders, stress.

FKBP51 AS REGULATOR OF THE GLUCOCORTICOID RECEPTOR

About 25 years ago, FKBP51 and its close homologue FKBP52 were first discovered as components of the steroid hormone receptor (SHR)-chaperones heterocomplex [1-4]. As members of the FKBP protein family, they bind the immunosuppressant drug FK506, which inhibits their peptidyl-prolyl isomerase (PPIase) activity [5]. In addition to the eponymous FK506 binding domain, the large FKBPs contain an interaction domain for Hsp90, the so called tetratricopeptide repeat (TPR) domain [6]. This domain interacts with the N-terminal MEEVD motif of Hsp90 [7], thereby enabling them to access the Hsp90-based folding platform [8]. There is also evidence that other domains of FKBP51 and FKBP52 contribute to the interaction with steroid receptors [9, 10].

The first hint for the GR-inhibitory function of FKBP51 came from observations in Squirrel Monkeys. These neotropical primates have high circulating levels of cortisol, but they show no phenotypic signs of cortisol excess because the high cortisol levels compensate for the low affinity of their GR [11-13]. It became clear that the lower hormone binding affinity of the Squirrel Monkey GR is not caused by the changes in GR sequence but by other cellular factors [14]. After higher levels of FKBP51 were found in Squirrel Monkeys, several studies demonstrated this immunophilin to inhibit GR function [15-19]. FKBP51 reduces GR hormone binding affinity and delays nuclear translocation of GR after hormone binding [15, 16, 18]. The inhibitory action of FKBP51 on GR nuclear translocation has also been confirmed in neuronal cell cultures [20].

It is not known whether specific actions of FKBP51 in the receptor chaperone heterocomplex account for this inhibitory action or whether FKBP51 simply displaces other GR function-promoting TPR domain containing proteins from the complex such as its close homologue FKBP52 [17-19]. Likely, displacement of FKBP52 from transport complexes also accounts for the inhibitory effect of FKBP51 on nuclear translocation. FKBP52 interacts with the dynein/dynactin motor complex thereby expediting nuclear translocation of GR and also of the closely related mineralocorticoid receptor (MR) [18, 21, 22]. It has been shown that the GR heterocomplex-dynein interaction can be disrupted by overexpressing FKBP51 [18].

In addition, the immunophilins FKBP51 and FKBP52 exert functionally divergent effects in numerous other cellular processes [17-19, 23-27]. Therefore, it appears reasonable to unravel their mechanism of action by comparing their biochemical functions and structures. The PPIase activities of FKBP51 and FKBP52 are comparable [6]. Furthermore,

*Address correspondence to this author at the Department of Translational Research in Psychiatry, Max Planck Institute of Psychiatry, Munich, Germany; E-mail: theorein@mpipsykl.mpg.de; or gabrielrfries@gmail.com

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while the N-terminal PPIase domain of both FKBP51 and FKBP52 appears to be essential for their differential effects, the enzymatic activity of isomerizing peptidylprolyl bonds is not required [18, 28]. Isothermal titration calorimetry revealed a difference in the affinity of Hsp90 binding between FKBP51 and FKBP52 [6]. This difference, however, is not reflected in the abundance of the two FKBPs in Hsp90-based receptor complexes [17, 29]. As mentioned above, evidence has been provided for direct interactions between the FKBPs and their client steroid receptors [9].

Crystal structure analyses revealed a high structural similarity between FKBP51 and FKBP52 [30-32]. There are differences on the orientation of the domains that might be important for their differential functions. Interestingly, a recent study reported a differential conformational flexibility in the FK506 binding domains of FKBP51 and FKBP52, pointing to a mechanistic contribution of intramolecular movements [33]. Single amino acid differences have also been assessed for their functional contribution, including a comparison of the Squirrel Monkey FKBP51 with human FKBP51 [16, 34]. While no single amino acid alone accounts for the functional differences between FKBP51 and FKBP52, a major effect could be ascribed to the amino acids shaping the domain structure at the PPIase pocket [28]. Today, FKBP51 and FKBP52 are mostly viewed as scaffolding proteins that partly interfere with each other's function by competing for binding to several proteins.

Taken together, even though the exact mechanism remains to be elucidated, it is now firmly established that FKBP51 functions as inhibitor of GR (see Fig. **1** for a model summary).

THE GLUCOCORTICOID RECEPTOR AS REGULATOR OF FKBP51

Induction of FKBP51 mRNA by glucocorticoids had already been reported before the GR-inhibitory function of FKBP51 was established [35-37]. The glucocorticoid-responsiveness of FKBP51 has been demonstrated in numerous studies and also applies to brain regions important to stress-related mental disorders [38-40]. Glucocorticoid responsive elements (GREs) have been identified and characterized by electromobility shift and reporter gene assays in intronic regions of *Fkbp5*, the gene coding for FKBP51 [41]. Quantitative chromatin immunoprecipitation scans and analyses of enhancer activity pointed to the fact that binding of GR at about 34 kb upstream and 87 kb downstream of the transcription start site (TSS) is essential to trigger activation of the *FKBP5* locus [42]. It has further been suggested that, at these sites, the GR acts on the chromatin structure at the TSS through long distance interactions [42]. More recently, GRE-TSS long distance interactions were proven by the use of the chromatin conformation capture technique [43].

Similarly to glucocorticoids, other steroids except estrogens also activate transcription of the *FKBP5* gene [41, 44-48]. The regulation by these other steroids most likely also involves long distance interactions of regulatory factors at the DNA [45, 47]. Thus, like with several other genes, the action of GR is intertwined with the activity status of the other steroid receptors.

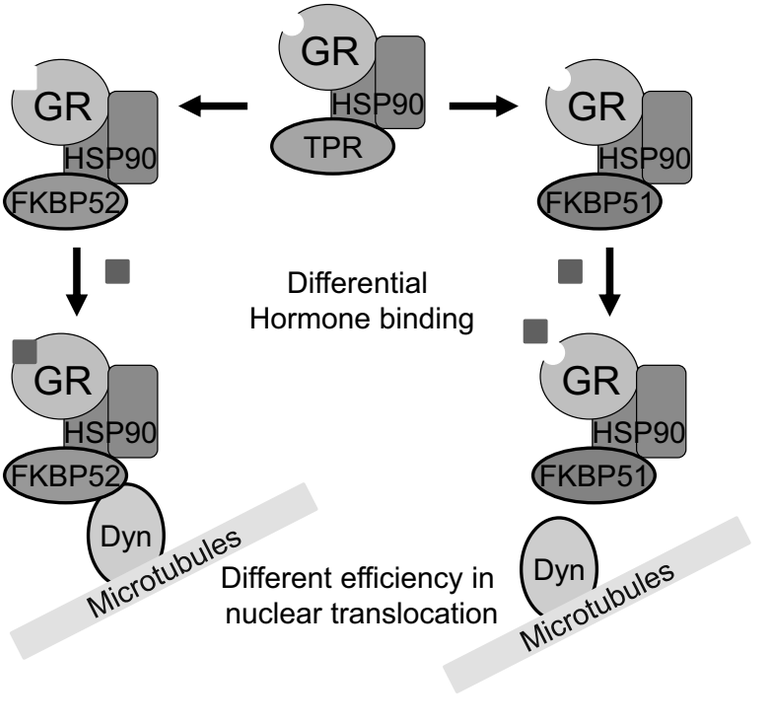

Fig. (1). *FKBP51 reduces GR's hormone binding affinity and nuclear translocation.* This model depicts a very simplified version of the heterocomplex of GR and the associated chaperones and cochaperones. The TPR acceptor site of Hsp90 can be occupied by a variety of different TPR proteins (complex top middle). In the case of FKBP52 (left hand side), the GR adopts a conformation that allows for hormone binding with high affinity. In addition, through its interaction with the dynein/dynactin transport complex, there is efficient nuclear translocation of GR in the presence of FKBP52. Conversely, when FKBP51 is at the TPR site of Hsp90, GR binds to the hormone with reduced affinity and nuclear translocation is delayed due to the lack of interaction of FKBP51 with the transport complex. GR, glucocorticoid receptor; TPR, tetratricopeptide repeat; Dyn, dynein.

Even though FKBP51 and FKBP52 are highly homologous on the protein level, most likely due to their emergence by gene duplication from a common, ancestral invertebrate gene, their genomic organization is quite different [49, 50]; FKBP52, in contrast to its functional antagonist FKBP51, appears not to be regulated by steroid hormones.

Since *FKBP5* is a GR-regulated gene, the glucocorticoid-mediated increase of its mRNA has been suggested as a marker for glucocorticoid sensitivity, potency and bioavailability [51]. In most cases, this should be quite useful, but we propose that dynamic data should be available for correct interpretation. In the absence of dynamic data, for example in case only certain tissues were assessed for expression levels at one time point, the interpretation is much more difficult. The mutual interaction of FKBP51 and GR forms an ultrashort intracellular feedback loop. Therefore, high levels of FKBP51 can obviously be interpreted either as indication of a highly active GR (FKBP51 as target of GR) or as indication of a less active GR (FKBP51 as inhibitor of GR). In any case, this feedback loop is important for regulation of the stress hormone axis, *i.e*. the hypothalamic-pituitary-adrenal (HPA) axis (Fig. **2**); the correct function of this feedback loop has been shown to be of high physiological relevance. The impact of GR on FKBP51 is regulated by disease-associated single nucleotide polymorphisms (SNPs) in interaction with environment-directed DNA methylation [43, 52]. In turn, the action of FKBP51 on GR depends on the abundance of other TPR proteins competing for access to the Hsp90-based GR-chaperone heterocomplex [17, 18] (Fig. **2**, Table **1**).

It should be mentioned that dynamic data are also preferable for other GR targets; their dynamics also reflects the history of previous stress experiences, which has been nicely exemplified in rats by two recent publications [53, 54]. The authors could show that the previous exposure to chronic restraint stress changes the transcriptional response of GR target genes to a subsequent acute challenge to glucocorticoids.

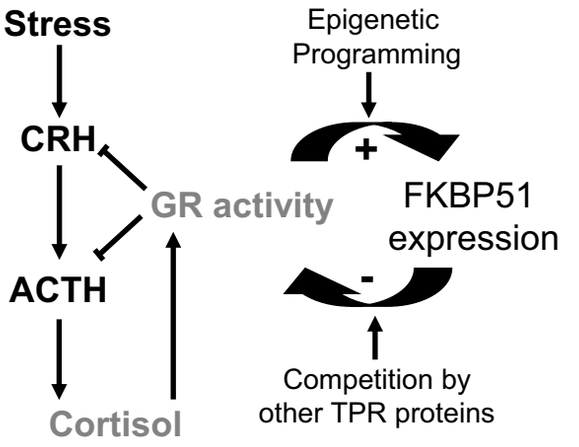

Fig. (2). *The HPA axis is intertwined with the GR-FKBP51 ultrashort feedback loop.* Upon perception of stress, CRH is released from the hypothalamic brain region, which stimulates the release of ACTH from the pituitary resulting in the release of cortisol from the adrenal glands. HPA axis activity is balanced by the negative feedback loop via GR on CRH and ACTH. The corticosteroid receptor hypothesis of depression postulates malfunction of GR as cause for dysregulated HPA axis [79], which may also apply for other stress-related disease. The activity of GR, in turn, is balanced by the ultrashort negative feedback loop with FKBP51. GR is a transcriptional activator of FKBP51, which acts as inhibitor of GR function. This feedback loop is further calibrated by genetic and epigenetic features shaping GR's effect on FKBP51 expression [52, 88, 179] and by TPR proteins competing with FKBP51 for Hsp90 binding and thus control of the GR heterocomplex [17, 18].

Table 1. ***Tetratricopeptide repeat (TPR) proteins and their effect on GR.*** **While evidence has been provided for most TPR proteins for competitive binding to Hsp90 and thus the receptor heterocomplex [17], this remains to be shown for the multi-TPR protein ANT-1.**

TPR protein	Effect on GR	Citations
carboxyl terminus of Hsc70-interacting protein (CHIP)	promotes degradation of GR	[180]
cyclophilin-40 (Cyp40)	No clear effect	[17, 19]
FK506 binding protein 51 (FKBP51)	lowers hormone binding affinity and transcriptional activity	[15, 16, 18]
FK506 binding protein 52 (FKBP52)	enhances hormone binding affinity	[19, 181]
Hsp organizer protein (HOP)	enhances heterocomplex formation	[182]
protein phosphatase 5 (PP5)	lowers GR's transcriptional activity	[183, 184]
small glutamine-rich tetratricopeptide (SGTA)	lowers GR's activity	[185]
Androgen Receptor N-terminal domain transactivating protein-1 (ANT-1)	Enhancement of GR-dependent transcription	[186]
tetratricopeptide repeat protein 2 (TPR2)	modulates GR signaling	[187]
hepatitis virus B X-associated protein 2 (XAP2)	inhibits GR mediated transcription	[188]

ANIMAL MODELS

Different laboratories developed FKBP51 as well as FKBP52 knock-out (KO) mice and initial studies did not reveal obvious defects in male or female FKBP51 KO mice [55-58]. This appeared surprising especially in the case of male fertility [59] - in light of the reported positive role of FKBP51 for the function of the androgen receptor [60-62]. It should be noted, though, that evidence has also been provided that FKBP51 might inhibit, rather than stimulate, the androgen receptor under certain experimental conditions [17]. In any case, the absence of an overt endocrine or behavioral phenotype under baseline conditions renders the animals suitable for investigating the role of FKBP51 in stress-related phenotypes.

Analysis of the HPA axis revealed no differences between FKBP51 KO and wildtype mice in their levels of corticosterone under basal conditions [63]. However, loss of FKBP51 attenuated the reactivity of the HPA axis in response to an acute stressor or in the dexamethasone (Dex) / corticotropin-releasing hormone (CRH) test [63]. These changes in HPA axis reactivity are fully consistent with the known inhibitory effect of FKBP51 on GR. Moreover, this study uncovered that deletion of FKBP51 resulted in a more active coping behavior after an acute stressor of sufficient intensity [63] which, as we showed in a recent follow-up study, was associated with prevention of stress-induced synapsin loss in the prefrontal cortex [64]. Another study assessed the vulnerability of FKBP51 KO mice to a different stressor, *i.e*. to chronic social defeat stress [65]. Analysis of physiological, neuroendocrine, behavioral and gene expression alterations revealed a lower vulnerability of FKBP51 KO mice to chronic social defeat stress in comparison to wild-type animals and a more active stress-coping behavior [65]. Thus, also this study confirms the pivotal role of FKBP51 in regulating GR sensitivity and HPA axis balance.

Since stress hormones can promote wakefulness and enhanced HPA axis overactivity has been linked to sleep disturbance [66], FKBP51 KO mice were analyzed for their sleep pattern [67]. In comparison to wild-type mice, FKBP51 KO mice displayed increased nocturnal wake periods. After stress exposure, *i.e*. social defeat or restraint stress, FKBP51 deletion led to less pronounced rebound sleep. Of note however, sleep profiles in FKBP51 KO mice were nearly opposite to those in human depressed subjects. This pro-resilience sleep phenotype in mice may result from the reduced HPA reactivity caused by FKBP51 deletion [67]. In addition, this study verified in brain tissue that loss of FKBP51 enhanced hormone binding affinity of GR, as it would have been predicted by previous cellular and biochemical studies.

Investigation of older mice (aged 11-14 months and the same mice later at the age of 18-22 months) revealed that the loss of FKBP51 modulated age-dependent anxiety determined by the elevated plus maze [68]. However, this effect did not become apparent in the light-dark maze, another behavioral paradigm assessing anxiety-related behavior [68]. Of note, a recent study found that older mice show increased FKBP51 levels and decreased *Fkbp5* methylation, suggesting that epigenetic mechanisms might underlie the glucocorticoid-mediated psychological stress-resilience processes seen in aging [69].

It should be mentioned that the FKBP51 KO mice used in these studies also carry a gene duplication of glyoxalase-1 and thus also exhibit higher levels of this enzyme [70]. Glyoxalase-1 is involved in the detoxification of methylglyoxal [71] and its dysfunction has been linked to several disease states [71, 72]. However, so far-studies did not provide evidence for a significant influence of the increased levels of glyoxalase-1 in the FKBP51 KO mice. For example, even though glyoxalase-1 has been reported to play a role in anxiety [73], no changes in anxiety-related behavior was observed in the FKBP51 KO mice under basal conditions [63, 65].

In addition to gene deletion, FKBP51 knock-down has also been used to analyze its behavioral effects in mice. FKBP51 has been identified as an important component of the neuropsin signaling pathway in the enhancement of anxiety-like behavior [74]. The authors further showed that stress-induced anxiety was blocked by silencing FKBP51 expression in the amygdala with RNAi [74].

Finally, overexpression of FKBP51 in the hypothalamus with an adeno-associated virus vector has been used to demonstrate that elevated hypothalamic FKBP51 might promote obese phenotypes [75]. Similarly, higher expression of FKBP51 in the hypothalamus has been linked to increased body weight gain [76]. These findings are also relevant for stress-related mental disorders as obesity has been reported to increase the risk for depression [77]. The results obtained from manipulating FKBP51 expression in mice are summarized in Table **2**.

STRESS-RELATED MENTAL DISORDERS

Malfunctioning of the HPA axis is one of the most consistent findings in stress-related neuropsychiatric conditions including cognitive dysfunction, posttraumatic stress disorder (PTSD), major depression, bipolar disorder (BD), schizophrenia, anxiety disorders and suicidality [78, 79]. Several studies in animal models manipulating GR expression in various ways documented the crucial role of GR function in HPA axis activity and brain function [80-87]. This plethora of findings intensified the interest in proteins potentially regulating GR activity. Based on the results of these studies, a candidate gene association study in depression was undertaken [88]. In addition to the GR and the MR, several molecular chaperones were included in this study, among them FKBP51 as a then-newly established inhibitor of GR [15, 16]. This gene association study discovered single nucleotide polymorphisms (SNPs) of *FKBP5* linked to HPA axis reactivity and to the response to antidepressant treatment. Thus, it motivated a wealth of additional clinical studies on FKBP51 which we survey in the following section.

CLINICAL EVIDENCE FOR THE ROLE OF THE GR-FKBP51 SYSTEM IN MENTAL DISORDERS

Most of the relevant clinical studies are based on genetic association findings, but evidence also extends to functional reports. Genetic variants and even epigenetic modifications of the *FKBP5* gene have been consistently correlated with FKBP5 expression and function in humans [88-91]. For example, the most studied *FKBP5* gene SNP, rs1360780, has been shown to regulate gene expression and HPA axis activ-

Table 2. Summary of the phenotypes observed upon manipulating FKBP51 expression in mice.

FKBP51 altered mice	Phenotype	Citations
FKBP5 KO	No overt phenotype under basal conditions	[55-58]
	KOs show more active coping of stress	[63-65]
	increased noctural wake	[67]
	modulated age-dependend anxiety	[68]
FKBP5 knock-down	enhancement of anxiety-like behavior	[74]
ectopic FKBP51 in the hypothalamus	obesity	[75]

ity in different populations [88, 92]. Polymorphisms in the *FKBP5* gene were also shown to modulate the recovery from psychosocial stress, in which subjects homozygous for the SNPs rs4713916, rs1360780 and rs3800737 displayed an incomplete normalization of stress-induced cortisol secretion [93]. Furthermore, a male-specific effect of the rs3800374 SNP on the response to acute psychosocial stress was found in healthy adults [94].

Moreover, a growing body of evidence suggests that *FKBP5* modifications are associated also with personality traits [95] and with the integrity and reactivity of specific brain regions, both in patients and in non-clinical human populations [96-100]. Altogether, human studies suggest a key role of genetic variants of the *FKBP5* gene in modulating HPA axis activity and the effects of stress and adversities throughout life, ultimately contributing to mediation of the susceptibility to psychiatric disorders like PTSD [43]. To increase intelligibility, we discuss the clinical evidences separately for the different types of disorders in the following sections.

Childhood Traumatic Events

One of the main findings regarding the role of the FKBP5-GR system in humans is its interaction with early-life adversities and modulation of the HPA axis and behavioral features. As recently shown, the severity of childhood maltreatment was associated with attenuated cortisol levels among carriers of the rs1360780 CC genotype [101]. In addition, carriers of the risk allele (T) of this SNP showed a more pronounced cortisol response to a social laboratory stressor [101]. Along these lines, a genetic profile that combined four stress-related genes, including *FKBP5,* positively predicted cortisol levels, and was further shown to interact with life stress to predict hippocampal and amygdala volumes [102]. Furthermore, childhood trauma was also reported to interact with different variants of the *FKBP5* gene to increase the risk for attempting suicide in later life [103]. In addition, the *FKBP5* SNP rs1360780 was not only related to cortisol reactivity, but has also been shown to interact with an insecure-resistant attachment in children, further corroborating *FKBP5*'s role in stress-linked gene-environment interactions [104]. Altogether, these data support a key role of gene vs. environment interactions in the modulation of HPA axis function, suggesting a possible role of epigenetic mechanisms in this scenario.

Accordingly, a recent study described an epigenetic mechanism mediating the combined effects of environmental exposure in early life and genetic variants of the *FKBP5* gene in the risk of developing psychiatric disorders [43]. This study showed that genetic variants in *FKBP5* can induce altered chromatin conformations resulting in a differential *FKBP5* gene expression in response to childhood abuse, ultimately altering DNA methylation and the responsiveness of FKBP5 to GR activation.

As described in the next sections, these mechanisms are suggested to mediate the risk for different stress-related psychiatric disorders such as PTSD.

Posttraumatic Stress Disorder (PTSD)

Contrary to what is observed in patients with depression, an enhanced negative feedback inhibition of cortisol along with an increased glucocorticoid sensitivity was frequently noticed in PTSD [105]. Accordingly, PTSD patients have been shown to present a reduced FKBP5 expression, consistent with an enhanced GR responsiveness [99, 106]. Moreover, low *FKBP5* mRNA expression was associated with an increased risk for a high intensity of PTSD symptoms, along with high GR number and high glucocorticoid-induced leucine zipper (GILZ) mRNA expression [107].

Of note, an up-regulation of FKBP5 expression after a traumatic event predicted the manifestation of PTSD four months later [108], thereby suggesting that *FKBP5* contributes to mediation of PTSD susceptibility. The role of *FKBP5* in PTSD is also supported by studies showing that elevated blood *FKBP5* mRNA expression is observed in response to cognitive behavior therapy in patients [99, 109], in which an increase in FKBP5 expression predicted an improvement in PTSD symptoms [99].

As with other disorders, the strongest evidence linking PTSD and *FKBP5* comes from genetic studies. Specifically, lifetime PTSD has been associated with the *FKBP5* rs9470080 polymorphism which interacts with trauma exposure and modulates the risk for PTSD [110, 111]. This particular SNP, along with other genetic markers, was later added to a clinical screening instrument to improve the accu-

racy of predicting PTSD [112]. Along these lines, other genetic variants of *FKBP5* have been assessed for their role in predicting PTSD risk [113]. The SNPs rs3800373 and rs1360780, for instance, have been associated with higher peri- and posttraumatic dissociation in children with an acute medical injury risk (risk factors for subsequent PTSD development) [107]. Moreover, polymorphisms in the *FKBP5* gene were able to propose biologically distinct subtypes of PTSD-as PTSD patients that were carriers of the risk allele of rs9296158 exhibited GR supersensitivity [89]. Recently, the *FKBP5* genotype was also associated with moderation of the long-term effectiveness of psychotherapy, in which carriers of the rs1360780 risk allele were at increased risk of symptom relapse, whereas non-carriers showed continuous symptom reduction [114].

Yehuda and colleagues [115] further explored the potential role of FKBP5 as a biomarker of PTSD and found that symptom improvement following psychotherapy was associated with a decrease in the methylation of the *FKBP5* gene exon 1. In addition, *FKBP5* promoter methylation correlated with measures of cortisol and glucocorticoid sensitivity [115] thus suggesting a role for epigenetic alterations in this locus in the pathobiology of PTSD.

Finally, consistent findings support the idea that genetic markers interact with early childhood events, thereby modulating the risk for developing PTSD. Binder and colleagues [116] reported that four SNPs of the *FKBP5* gene (rs9296158, rs3800373, rs1360780, and rs9470080) interact with the severity of child abuse and predict adult PTSD symptoms, whereas rs9470080 moderated the risk of PTSD associated with childhood abuse in African Americans, as shown in another study [117].

Depression

Among the several disorders that have been associated with the GR-FKBP5 system, mood disorders, particularly major depression, are currently placed at the top of the list. The *FKBP5* gene has been extensively analyzed regarding response to treatment, severity of depressive symptoms, and recurrence of depressive episodes. Thus, a growing body of evidence suggests that this gene is associated with depression.

In PTSD, suicide attempts, and overt aggressive behavior, variants of the *FKBP5* gene have been shown to interact with childhood adversities thereby moderating the susceptibility to depression [103, 116, 118]. Specifically, the rs1360780 TT genotype appears to interact with childhood physical abuse and was reported to increase the severity of depressive symptoms in adults [119]. In addition, patients homozygous for the minor alleles of some *FKBP5* polymorphisms have been found to be particularly sensitive to the effects of traumatic events triggering the onset of depression [120].

FKBP5 SNPs have been associated with major depressive disorder (MDD) in different populations [121-126]. In addition, a recent study has found a relationship between a *FKBP5* polymorphism and dysfunctional attitudes predisposing for depression [127]. Patients with MDD have also been shown to exhibit increased FKBP51 protein and mRNA levels in the frontal cortex [124]. In lymphocytes of depressed patients, another study also found higher levels of FKBP51 compared to controls [128]. In addition, this study determined higher levels of GR in the cytoplasm, likely reflecting FKBP51's action on GR, *i.e*. reducing hormone binding affinity and impeding nuclear translocation.

Furthermore, evidence suggests a relationship between *FKBP5* polymorphisms and the occurrence of depressive symptoms in patients with Alzheimer's disease [129], kidney transplant recipients [130], and patients with advanced gastric cancer [131]. Likewise, the rs1360780 T allele was more frequently observed in MDD patients with comorbid anxiety disorders than in those without [132]. Interestingly, depressive symptoms during pregnancy have been associated with reduced *FKBP5* mRNA expression in peripheral blood [133]. Altogether, the onset of depressive symptoms appears to be modulated by genetic polymorphisms involving the GR-FKBP5 system.

As mentioned above, *FKBP5* genetic variants have been suggested to modulate the treatment response in depressed patients. Specifically, the *FKBP5* rs1360780 minor T allele has been associated with increased intracellular FKBP51 protein expression, a rapid response to antidepressant treatment and an increased recurrence of depressive episodes [88]. This particular SNP was later associated with GR resistance in peripheral blood mononuclear cells and reduced cortisol and ACTH suppression following dexamethasone administration [134]. Of note, while the treatment-association of the *FKBP5* rs1360780 polymorphism has been replicated later [135, 136], other *FKBP5* SNPs have also been associated with antidepressant response in other populations, including the rs3800373 [136], rs352428 [137], and rs4713916 [122, 138, 139]. Accordingly, successful antidepressant treatment has been associated with a reduction in the levels of FKBP51 (-11%) in depressed patients [140]. It is also relevant to point out that other studies failed to find an association between *FKBP5* and response to treatment in depressed patients [139, 141-144], which might be explained by potential differences in linkage disequilibrium and haplotype structure in the *FKBP5* locus between different populations [142] or, possibly, by different treatment strategies. In addition, a meta-analysis suggested that this association might depend on ethnicity [145].

Bipolar Disorder (BD)

Evidence has accumulated for a role of the GR-FKBP5 system in the pathophysiology of BD. Genetic analyses involving *FKBP5* and BD are still scarce in comparison to the large body of evidence regarding the interrelation of *FKBP5* with depression and PTSD. Moreover, the results hitherto available are contradictory. An association was found between BD and the *NR3C1*, the gene encoding the GR, in an isolated northern Swedish population [146], whereas this particular study did not detect an association of BD with *FKBP5*. Accordingly, a recent study also found no association between five *FKBP5* polymorphisms (rs1360780, rs9470080, rs4713916, rs9296158 and rs9394309) and BD in a Polish sample [123]. Nonetheless, haplotypes of the *FKBP5* gene have been associated with suicidality in BD [147], and an interesting association of *FKBP5* has been described for BD in a family sample with bipolar pedigrees and

affected offspring [148]. This study reported a significant overtransmission of the major allele of the rs4713902 polymorphism in families with bipolar offsprings and suggested that genetic variation within *FKBP5* may influence attempted suicide and number of depressive episodes in BD patients [148].

Gene expression analyses indicate that the mRNA levels of *FKBP5* and *BAG1* (another co-chaperone known to impair the folding of GR [149, 150]) are increased in the prefrontal cortex of patients with BD when compared with controls, even though no differences were seen in FKBP51 protein levels [151]. This finding was also confirmed by a systematic review of genome-wide gene expression studies in BD [152].

A recent study of our group also found increased FKBP51 protein levels in peripheral blood mononuclear cells from BD patients, which were associated with reduced *ex vivo* GR responsiveness and increased methylation levels in intronic *loci* at the *FKBP5* gene [153]. Interestingly, unaffected siblings presented lower FKBP5 protein levels than patients, even though no differences were found in mRNA levels. These data collectively suggest that epigenetic modulation of the *FKBP5* gene, along with increased FKBP51 protein levels, is associated with the GR resistance and HPA axis dysregulation in BD patients [153, 154]. Thus, genetic variations of the *FKBP5* gene might influence the susceptibility to BD, but additional studies are required to substantiate the role of *FKBP5* polymorphisms in this disorder.

Psychosis

Dysregulated HPA axis has been consistently reported in patients with psychotic disorders including differences in cortisol levels and abnormalities in GR sensitivity [155]. Of note, cortisol secretion may increase dopaminergic activity in specific brain regions associated with the development of positive psychotic symptoms [155, 156]. Accordingly, *FKBP5* has been associated with psychotic disorders upon controlling for environmental exposures (*i.e.* cannabis use and parental separation) suggesting that *FKBP5*'s contribution may vary due to etiological factors [157]. An interaction was found between two SNPs in the *FKBP5* gene (rs9296158 and rs4713916) and childhood trauma on psychotic symptoms in adults which suggests *FKBP5* to moderate the psychosis-inducing effects of childhood trauma [155]. Moreover, increased *FKBP5* mRNA and protein levels were found in the prefrontal cortex of patients with schizophrenia and BD, whereas BAG1 mRNA and protein levels were decreased in both groups compared to controls [151]. These results further support a dysregulation of the glucocorticoid signaling pathway in psychotic illnesses. Of note, in comparison to other psychiatric disorders, the amount of data on the association between FKBP51 and psychosis is still very preliminary and does not allow any generalization yet. The same accounts for addiction, suicide and anxiety disorders.

Addiction

Drugs of abuse, such as cocaine and heroin, are known to stimulate several hormonal systems that are activated by stress, and addicted subjects appear to present abnormal patterns of HPA axis activity [158, 159]. Accordingly, the *FKBP5* rs1360780 SNP has been nominally associated with heroin addiction in an African American population [158]. This was replicated by another study in independent cohorts that further expanded the results to include the rs3800373 polymorphism in heroin addiction [160]. A role for *FKBP5* in alcohol addiction has also been proposed since this gene was found to be differentially expressed in *post mortem* hippocampal tissue from alcohol addicts in comparison to controls [161]. Collectively, addictive disorders seem to present alterations in the GR-FKBP5 system that deserve a deeper investigation in future studies.

Suicide

The HPA axis activity has been shown to predict future suicidal behavior [162]. This finding has prompted the assessment of the GR-FKBP5 system in suicide victims. A genetic study found an association between haplotypes in the *FKBP5* gene and completed suicide [163], and a more recent study has reported reduced gene and protein expression of FKBP5 and GR in the amygdala of suicide victims compared with controls [164]. Moreover, significant associations were found between the *FKBP5* rs1360780 T and rs3800373 G alleles and suicidal events (defined as new-onset or worsening suicidal ideation or behavior) in depressed adolescents [141]. In addition, an interaction between *FKBP5* and childhood trauma has been reported to increase the risk for attempting suicide in African-Americans [103]. Altogether, these studies suggest a role for *FKBP5* in the genetic predisposition to suicidal behavior [165].

PERSPECTIVES

FKBP52 and other TPR Proteins in Clinical Studies

A high number of cofactors regulate GR, but clinical studies largely focused on FKBP51 in the aftermath of the first clinical report linking *FKBP5* to a stress-related disorder [88]. Even though the wealth of data surveyed here document a significant contribution of FKBP51 to the pathophysiology of stress-related diseases, we propose to take also other GR regulatory proteins into consideration, in particular the TPR proteins that compete with FKBP51 for access to the Hsp90-based receptor heterocomplex. Among the TPR proteins, FKBP52 is of particular interest, as it has been shown to functionally antagonize FKBP51 in several cellular processes. Given this antagonism, it appears reasonable to speculate that the status of FKBP51 (expression level) in comparison to FKBP52 and possibly also other factors might be more informative than the status of FKBP51 by itself. For example, high levels of FKBP51 are expected to have a significant impact on GR function and stress-related pathophysiology when the levels of FKBP52 and/or maybe of other TPR cofactors are low, but only a moderate impact when these levels are also high.

FKBP51 as Relay of Glucocorticoid Action

The ultrashort feedback loop of FKBP51 and GR obviously is important for calibration of the pleiotropic actions of GR [166]. While the action of FKBP51 on GR and other

steroid receptors has been known for several years, important additional functions of FKBP51 were discovered more recently [10, 26, 167-172]. This means that, in turn, GR or in extension the HPA axis, calibrates FKBP51 activity. In principle, all of the actions of FKBP51 are potentially under the control of the GR (Fig. **3**). This has been demonstrated, for instance, for the very recently discovered role of FKBP51 in triggering autophagy [173]. In addition, evidence has been provided that synthetic glucocorticoids need FKBP51 to act on Akt1 [64, 173].

Given the adaptive nature of the ultrashort negative feedback loop intertwining GR and FKBP51, which apparently includes epigenetic programming [52], conclusions from the baseline levels of FKBP51 expression in a particular sample are hard to draw. For example, if increased levels of FKBP51 are observed in patients, are they adaptive or causal?

The two corticosteroid receptors GR and MR act in concert to shape glucocorticoid action [174]. Accordingly, their balance has been identified as crucial parameter in mental health [78, 174]. FKBP51 has been found to not only inhibit GR, but also MR [17, 27]. Thus, considering for example higher levels of FKBP51, one would expect lower binding affinities of both GR and MR, and as a consequence through HPA axis balancing higher levels of circulating glucocorticoids. In other words, it is not obvious that the balance of GR and MR activity would change, and in which direction. Nevertheless, fewer details are known for the effect of FKBP51 on MR, in particular to what degree the binding affinity is changed and what the cell type-specific effects might be. Future research should address this question.

Consideration as a Drug Target

Since FKBP51 has been proposed as a promising drug target [26, 68, 175, 176], the question of FKBP51 as friend or foe has decisive repercussions for rational drug design. For example, the corticosteroid receptor hypothesis of depression [79] would suggest to "inhibit" FKBP51's inhibitory function on GR and MR to restore receptor function. Assuming for a moment that it is possible to obtain an inhibitor of FKBP51, the question arises which of FKBP51´s molecular functions should be targeted. Strong evidence has been provided that FKBP51 promotes recovery from depression [88, 173]. Even though a bulk of data links this effect of FKBP51 to autophagy, it is currently not clear which of the pleiotropic effects of FKBP51 contribute in addition.

Thus, it should be taken into consideration that HPA axis-controlled FKBP51 activity might be equally or even more important for mental health than FKBP51-controlled HPA activity. Therefore, we hypothesize that a ligand of FKBP51 that does not affect the non-GR functions of FKBP51, but specifically blocks FKBP51's inhibitory action on GR would be a promising tool for understanding and possibly also treating mental disorders (Fig. **4**). This would enhance GR's hormone binding affinity and likely also activity in regulating transcriptional processes, which would lead to resetting of the HPA axis. Higher GR activity would lead to an enhanced production of FKBP51, thus increasing FKBP51's non-GR directed functions, which could be beneficial for treating depression [88, 173]. While this remains a hypothesis, much more research is required to pinpoint the mechanisms of action and amino acids of FKBP51 involved in its various actions. This knowledge is necessary for a rational design of FKBP51 ligands able to specifically disrupt the FKBP51-GR feedback.

Very recently, the development of a specific ligand of FKBP51 has been published [177]. This ligand binds to the FK506 binding site and inhibits the PPIase activity of FKBP51. Moreover, binding of this ligand appears to have repercussions on GR-dependent functions: the ligand exerts neuritotrophic effects in cellular assays; intriguingly, the new compound exhibits antidepressant-like effects in the forced swim test. This very promising development warrants further investigations into the additional molecular and physiological effects of FKBP51 as outlined above. In light of the still obscure, if any, physiological function of the PPIase activity of FKBP51, we speculate that protein interactions will be the key to understand the mechanism of this novel ligand.

An alternative approach might arise from the report of an Hsp90 ligand that reduces Hsp90's chaperone function, at least in part by displacing immunophilins from Hsp90, and likely from GR chaperone heterocomplexes, as well [178]. The authors further demonstrate that this novel

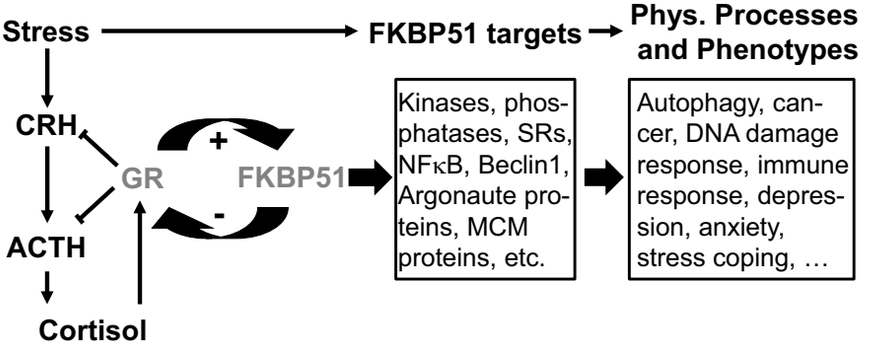

Fig. (3). *FKBP51 relays several effects of stress on pathway molecules and physiology.* Numerous non-GR binding partners of FKBP51 have been identified (box "FKBP51 targets"), and several of them also have been functionally characterized [10, 26, 167-172]. At the same time, many of these FKBP51 interactors and their physiological effects are also linked to stress. Therefore, it is possible that stress exerts at least some of its effects via FKBP51 [63, 65, 173]. In this view, the HPA axis functions to calibrate the level of FKBP51 to meet various physiological requirements. It is possible that GR or other stress factors act on targets of FKBP51 also independently of FKBP51; further research will sort out which target effects actually require FKBP51.

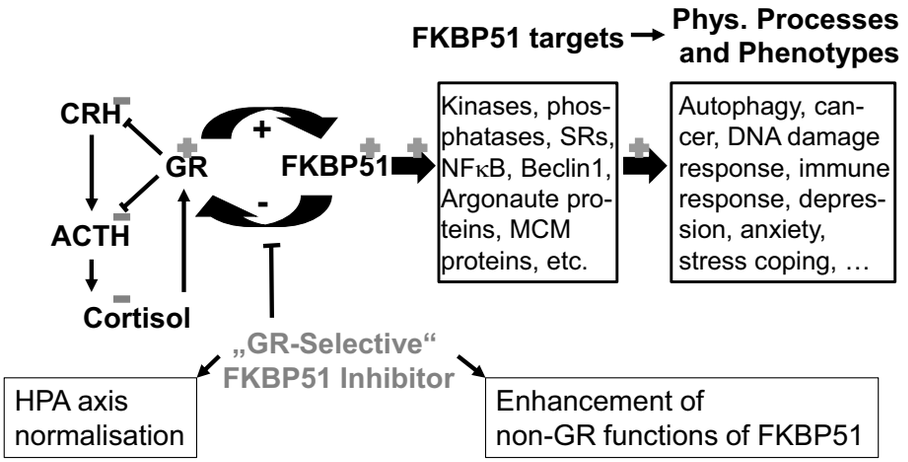

Fig. (4). *Possible effects of a putative "inhibitor" of FKBP51 selective for the action of FKBP51 on GR.* A hypothetical ligand of FKBP51 that does not affect the non-GR functions of FKBP51, but interferes with FKBP51's inhibitory action on GR, would increase the hormone binding affinity of GR, entailing higher activity of GR in the regulation of transcriptional processes. This would reset the HPA axis, and should be beneficial for treating stress-related disorders such as depression, according to the corticosteroid receptor hypothesis [79]. Higher GR activity would also lead to more FKBP51 transcription and probably also protein. This effect would enhance FKBP51's non-GR functions, some of which have been suggested to be beneficial for treating depression [88, 173]. This remains a simplified hypothesis, which could be further complicated when considering tissue-specific effects due to possibly diverging expression profiles of GR and FKBP51.

Hsp90 inhibitor does not evoke a cellular heat shock response, in contrast to other Hsp90 inhibitors. Finally, it leads to a reduction in the levels of GR, FKBP51 and FKBP52. Altogether, the net outcome on molecular stress pathways and physiology is difficult to predict and remains to be determined.

CONCLUSION

Accumulating evidence suggests a role for the GR-FKBP5 system in modulating not only the HPA axis and thereby several stress-related features, but also other physiological processes of significant clinical relevance, such as autophagy. This system is undoubtedly very complex and relies on several other protein players possibly counteracting or amplifying its function such as the closely-related FKBP52. Interestingly, GR actions on FKBP51 and FKBP51 actions on GR appear to interact with each other and add another level of complexity to this system. The effects of genetic variants on the *FKBP5* gene in modulating the susceptibility and phenotypical characteristics of stress-related disorders further suggest this system to be a major target in their treatment, which ought to be explored in future basic and clinical studies.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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LIST OF ABBREVIATIONS

ACTH	=	Adrenocorticotropic hormone
BD	=	Bipolar disorder
CRH	=	Corticotropin-releasing hormone
Dex	=	Dexamethasone
FKBP	=	FK506 binding protein
GR	=	Glucocorticoid receptor
GRE	=	Glucocorticoid responsive element
HPA	=	Hypothalamus-pituitary-adrenal
KO	=	Knock-out
MDD	=	Major depressive disorder
PPIase	=	Peptidylprolyl isomerase
PTSD	=	Post-traumatic stress disorder
SHR	=	Steroid hormone receptor
SNP	=	Single nucleotide polymorphism
TPR	=	Tetratricopeptide repeat
TSS	=	Transcriptional start site

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Molecular Aspects of FKBP51 that Enable Melanoma Dissemination

Anna D'Angelillo[1,2], Stefania Staibano[2], Michele Russo[1], Maria F. Romano[1*] and Simona Romano[1]

[1]*Department of Molecular Medicine and Medical Biotechnologies, Federico II University, Naples, Italy;* [2]*Department of Advanced Biomedical Sciences, Federico II University, Naples, Italy*

Maria F. Romano

Abstract: FKBP51 (FKBP5 Official Symbol) is large molecular weight member of the FK506 binding protein family, a subfamily of the immunophilin proteins. FKBP51 exerts multiple biological functions in the cell, including modulation of steroid hormone response, immune regulation, cell proliferation, regulation of pAkt levels and control of NF-κB activation. Several lines of evidence support a role for this protein in cancer biology, especially in resistance to chemo- and radio-therapy. Recent research studies highlighted functions of FKBP51 in promoting the epithelial to mesenchymal transition (EMT) transdifferentiation program in melanoma. This process, which is classically regulated by Transforming Growth Factor (TGF)-β, enables cancer cells to disseminate from primary tumors and spread to distant locations, acquiring resistance to therapy and self-renewal capability. This last, in turn, is crucial to their subsequent expansion at sites of dissemination. The aim of the present article is to review recent literature data that involve FKBP51 in the mechanisms that switch the TGF-β from a tumor suppressor to a pro-metastatic invader.

Keywords: Development, EMT, FKBP51, Melanoma, Metastasis, TGF-β.

INTRODUCTION

Melanoma is a dynamic and heterogeneous skin neoplasia with adverse prognosis in advanced stages [1]. The incidence of melanoma has continued to growth over the last 30 years [2]. Many factors are known to contribute to melanomagenesis, including exposure to ultra-violet radiation, skin type, the presence of dysplastic nevi and/or increased nevi number, and family history of melanoma [3]. Surgical removal is curative in the very early stages of melanoma (melanoma in situ). When the tumor invades the dermal connective tissue the complex process of metastasis begins. The treatment of metastatic melanoma remains a challenge for clinicians. Even if recent therapies targeting specific mutations such as BRAF V600E have shown a significant impact, the benefit from treatment is often short lived [4-6]. New hopes are with drugs targeting the immune response, that work through enhancing T-cell activity [7, 8]. However, to date, objective responses to immunotherapies are obtained in less than 30% of patients [9, 10]. Patients with low/no response often suffer from severe side effects. The absence of reliable predictive biomarkers makes it difficult to personalise immunotherapy to individual patients effectiveness or avoid a useless and not tolerated treatment [11]. Therefore, despite recent progress and advances in understanding of the molecular pathogenesis of melanoma [4, 5, 12-14], metastatic disease remains challenging because mechanisms underlying progression are still obscure [15].

In an attempt to find targets for chemo and radiosensitizing strategies of melanoma, we identified the large molecular weight immunophilin FKBP51 as a protein abundantly expressed in melanoma and correlated with aggressiveness and tumor progression [16, 17]. This review article aims at highlighting molecular aspects of FKBP51 that enable melanoma dissemination.

A BRIEF OVERVIEW ON FKBP51

FKBP51, encoded by FKBP5 gene (Gene ID:2289), is a 51 kDa MW protein [18] belonging to the family of immunophilins. This protein family includes cyclophilins and FKBPs. The denomination of immunophilin derives from the immunosuppressive action these proteins exert when in complex with natural compounds namely Cyclosporine A (ligand of cyclophilins), FK506 and Rapamycin (ligands of FKBPs) [19-21]. FKBP51 has, at the C-terminal, a TPR domain characterized by tandem repeats of 34 amino acids with a defined helix-alpha-helix motif. Interestingly, the structure of this domain is very similar to the structure of many co-chaperones that bind to Hsp90 [22]. At the N-terminus, FKBP51 contains two FK domains (FK1 and FK2) both involved in immunosuppressant drug binding; this interaction results in the inhibition of FKBP51's enzymatic activity. This activity, namely peptidyl-prolyl cis-trans isomerase (PPIase), is exerted only by the FK1 domain, closest to N-terminus. FK2 domain seems to not exert any enzymatic function, but it appears to have gained a protein interaction ability [23]. The PPIase activity allows the correct folding of the native structure of the proteins through the isomerization of the peptide bound cis/trans [24]; in addition, PPIases are co-regulatory subunits of molecular complexes including heat-shock proteins, steroid receptors and ion channels [25].

Among the functions of FKBP51, the immunosuppressant action is FK506-mediated, as previously mentioned, and

*Address correspondence to this author at the Department of Molecular Medicine and Medical Biotechnologies, Federico II University, Via Pansini, 5. 80131. Naples, Italy; Tel: +39/0817463125; Fax: +39/0817463205; E-mail1: mariafiammetta.romano@unina.it; romano@dbbm.unina.it

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results in calcineurin inactivation [18]. Better studied, is FKBP51's role in steroid hormone receptor (SHR) regulation. FKBP51 regulates traslocation of this receptor from the cytoplasm into the nucleus, by binding to Hsp90 in the SHR complex. FKBP51 is highly inducible by glucocorticoids, androgens and progesteron [18]. Studies in squirrel monkeys showed an inhibitory role of this protein on glucocorticoid response, due to reduced hormone receptor affinity. Similarly, in humans, FKBP51 induces glucocorticoid resistance [26]. FKBP51 is a negative regulator of SHR activity, but, has shown to be a positive regulator of androgen receptor (AR) [27]. FKBP51 stimulates androgen-dependent transcription and cell growth, and is part of a positive feedback loop involving androgen receptor and hormone [28].

In 2011, Gallo *et al*. [29] demonstrated a protective role of FKBP51 against oxidative stress. FKBP51 is a major mitochondrial factor that undergoes nuclear-mitochondrial shuttling during the stress response and exerts anti-apoptotic mechanisms.

A role for FKBP51 in NF-κB transcription factors regulation is also demonstrated. Several studies show that FKBP51 interacts with IKK complex subunits, facilitating IκB phosphorylation [30, 31]. Targeting FKBP51 with rapamycin or specific siRNAs, has shown impaired NF-κB DNA binding and transcriptional activity, in melanoma [16, 32], leukemia [33] and glioma [34]. Interestingly, NF-κB is differentially involved in the effect of 2-(4-acetoxyphenyl)-2-chloro-N-methylethylammonium chloride (CpdA), compared with fluocinolone acetonide (FA), both glucocorticoid compounds widely used in chemotherapy of hematological malignancies. The first compound exerted more prominent cytostatic and apoptotic effects on the cells because it did not induce expression of FKBP51 and did not activate NF-κB [35]. However, Erlejman *et al.*, [36] suggested an opposite action of FKBP51 on NF-κB activation. The authors showed that FKBP51 impaired the nuclear translocation rate of RelA, whereas FKBP52 favored RelA nuclear retention and association to the DNA consensus sequence. According to Erlejman, FKBP52 and not FKBP51 facilitated NF-κB transcriptional activity [36].

Another role for FKBP51 is in regulation of Akt signalling [37-39]. Recent data indicated that FKBP51 is a regulator of Akt phosphorylation through a scaffolding mechanism [37]. FKBP51 recruited the PH domain leucine-rich repeat protein phosphatase (PHLPP) and facilitated dephosphorylation of Akt [37], which, in turn, resulted in chemosensitivity of pancreatic cancer cells [38]. Fabian *et al.* [39] showed a direct interaction between FKBP51 with Akt via multiple domains, independent of their activation or phosphorylation status. Contrary to the findings by Wang *et al.* [37], the authors observed an increase, and not a reduction, in phospho-Akt, upon co-expression of FKBP51. In addition, FKBP51 inhibitors were unable to affect phosphorylation status of Akt [39]. The underlying reasons for these discrepancies remain to be established. FKBP51 can interact with several proteins of the serine-threonine kinase AGC superfamily, in addition to Akt [39]. The signaling of Akt and such kinases is highly interconnected. Interplay between AGC kinases, which is related to the cell context, can explain differences in FKBP51-regulation of pAkt levels.

EMT AND TGF-β

It is an established concept in cancer, that the acquisition of invasive and metastatic characteristics by tumor cells involves the reactivation of a developmental epithelial-to-mesenchymal transition (EMT)-like program [40, 41]. While being an integral process during development, EMT is recapitulated under pathological conditions, prominently in fibrosis and in cancer [42]. During cancer progression, the EMT phenotype is associated with tumor invasion, metastatic dissemination, and acquisition of resistance to drug treatment [43, 44]. EMT also leads to the generation of cancer cells possessing stem cell attributes of tumor-initiation [43]. EMT results from a well-orchestrated transcription program involving families of transcription factors, including the SNAIL family, ZEB family and basic helix-loop-helix (bHLH) family [45]. The Transforming Growth Factor (TGF)-β superfamily members act in concert to control this orchestrated program of transcriptional changes [45].

The human TGF-β family comprises ~40 [46-48] ligands some of which are expressed only during embryonic development and organogenesis, while others, including TGF-β itself, also play a role in the adult organism. In embryos, TGF-β drives organ morphogenesis processes, namely gastrulation and neural crest formation, lung organogenesis and specialized tissue formation such as the heart valve cushions [46, 49].

In adult tissues, TGF-β plays an important role in the control of cell proliferation, differentiation, wound healing, and immune surveillance [50-52]. Virtually, all human cell types are responsive to the cytokine, which maintains tissue homeostasis and prevents tumor formation. This inhibition of growth is exerted both directly on target cells by stimulation of cell differentiation and senescence and by cell cycle arrest and apoptosis, and indirectly, through blocking the production of mitogenic factors and inhibition of inflammation [53].

Resistance to TGF-β growth suppressor signals is a prime feature of invading cancer cell [54-57]. There are different causes of growth control failure by TGF-β. Defects in TGF-β responsiveness may result from inactivating mutations in the receptors or Smad signaling proteins. Mutations of the TGF-β receptor (TβR) I coding region are present in ovarian, esophageal, head and neck cancers and hematological malignancies [58-62]. Decreased expression of TβRI and TβRII can occur also for epigenetic alterations in lung, gastric, prostate, bladder cancers [63-67]. Smad2 and 3 mutations are infrequent in cancer, differently Smad4 is inactivated in more than half of pancreatic carcinomas [68, 69] and colorectal and esophageal cancers [70, 71]. Loss of the tumor suppressive effect of TGF-β may occur also in the absence of mutations of the signaling components. In melanoma, no genetic alterations of TGF-β signaling molecules have been identified that could explain resistance to the growth inhibitory activity of TGF-β [72, 73]. This cytokine is perfectly capable of inducing Smad signaling and Smad-dependent

transcription in melanoma cells [72], suggesting a desentization of these cancer cells to the anti-proliferative activity of the cytokine. Intrinsic plasticity of the signal by itself, which is strictly depending on Smad-interacting nuclear partners is thought to account for altered TGF-β response in aberrant melanoma cell context [74-78]. As it occurs for several normal cell types, melanocytes are very sensitive to the antiproliferative effects of TGF-β. In early-stage melanomagenesis, TGF-β still inhibits cell growth. Differently, melanoma exhibits increased resistance to growth inhibiting signals, which is proportional to tumor progression stage. Advanced melanoma may even secrete high levels of TGF-β, that feed tumor growth [72]. TGF-β overproduction correlates with increased tumor thickness and disease progression [72, 79]. In late-stage disease, TGF-β is associated with a significant decrease in survival time and suppression of the immune response [73, 80]. Recent studies involve FKBP51 in the mechanisms facilitating the pro oncogenic functions of TGF-β in melanoma.

FKBP51 ENABLES THE PRO ONCOGENIC FUNCTIONS OF TGF-β

FKBP51 expression in melanoma correlates with tumor progression [16]. Immunohistochemistry (IHC) of cutaneous melanomas from 80 patients showed that malignant melanocytes in the vertical growth phase displayed a stronger immunopositivity for FKBP51, compared to radial malignant melanocytes. A significant correlation was found between FKBP51 expression, and the thickness of the tumor lesion. Moreover, metastatic melanoma was associated with the highest FKBP51 immunoreactivity [16]. Another study performed on a series of 10 primary cutaneous melanomas and 20 brain melanoma metastases identified some IHC feature of invading melanoma cells, namely nuclear FKBP51 and membrane-cytoplasmic Nestin [81]. Notably, Nestin is an intermediate filament expressed in the cytoplasm of neuroepithelial stem cells [82]. FKBP51/Nestin double staining identified a particular cell, with cancer stem cell features, in the vertical growth phase of primary melanomas [81]. The same cell formed neoplastic emboli in dermal vessels of primary tumors and was also recognizable in vascular invasions of metastases, suggesting this particular cell had capability of extravasation and surviving within the circulation [81]. In addition, metastatic solid aggregates in the brains of melanoma patients were typically double stained in the same fashion [81]. Chromatin immunoprecipitation assays confirmed that FKBP51 exerted nuclear functions. In fact, FKBP51/p300 complexes were found bound to the promoter of the melanoma cancer stem cell marker ABCG2 [81]. This finding, together with the observation of an increased transcript level of ABCG2 and increased number of ABCG2+ cells in FKBP51 overexpressing melanoma cell line, supported a role for FKBP51 as a transcriptional coregulator [81]. It is noted that the histone acetyl transferase p300 is one of the major coactivators of Smad 2,3 [83]. Transcriptional coactivation is mediated by p300 acting as a bridge linking DNA-binding transcription factors to the basal transcriptional machinery [84, 85]. Pull down assays showed that p300/FKBP51/Smad2,3 interacted each other. This finding suggested a role for the large immunophilin in regulation of the TGF-β signal [81, 86]. Consistent with this hypothesis, TGF-β was unable to upregulate expression of VIM/Vimentin, SPARC and its downstream target SLUG [87] in condition of FKBP51 silencing [86]. VIM [87] and SPARC [88] are transcriptional target of TGF-β. It is feasible that FKBP51 enables some transcriptional activities of the cytokine, particularly, expression of factors associated with accelerated tumor growth, invasion, and poor prognosis. Interestingly, normal melanocytes, expressing very low/no FKBP51 levels, undergo EMT features, after ectopic expression of FKBP51 [86]. The observation that melanoma stem cell phenotype identified a subset of melanoma cells with the highest expression level of EMT genes, including TWIST, SNAIL, SLUG, CDH-2/N-cadherin, VIM/vimentin, SPARC [81, 86] support a strict relationship between EMT and cancer stemness in melanoma. A protein expression profile study of melanoma knockdown for FKBP51 also supported the hypothesis that FKBP51 controls EMT [81]. *In vivo* studies on a metastatic melanoma mouse model, using a FKBP51 siRNA as therapeutic agent, inequivocally showed that FKBP51-targeting dramatically reduced metastases formation [81].

Taken together, these findings support the conclusion that FKBP51 facilitates the protumoral activities of the TGF-β. A schematic representation of the proposed mechanism for FKBP51 action on TGF-β prooncogenic activities is depicted in Fig. (**1**). Concomitant loss of TGF-β tumor suppressive functions and increased FKBP51 expression, during melanoma progression, is in line with findings that involve FKBP51 in the mechanisms that switch the TGF-β from a tumor suppressor to a pro-metastatic invader [49, 89, 90].

FKBP51 INCREASES TGF-β EXPRESSION

In vitro studies, with knock in and knock down melanoma cell models, demonstrated that FKBP51 positively regulated TGF-β expression, at transcriptional and protein levels [81]. TGF-β, by itself, induces components and receptors of the TGF-β family, this creates a positive feed-back of the TGF-β signal which in turn promotes tumoral progression. Advanced melanoma is known to secrete high levels of TGF-β, that feed tumor growth [72]. A correlation between TGF-β overproduction and increased tumor thickness and disease progression is also proven [72, 79]

A positive regulation of TGF-β expression by FKBP51 has been previously observed by Komura *et al.* [91], in human idiopathic myelofibrosis (IMF). This disorder is a precancerous condition of myeloid lineage, in which the release of TGF-β1 in the bone marrow microenvironment is one of the main mechanisms leading to myelofibrosis. FKBP51 was found overexpressed in IMF $CD34^+$ cells, responsible for spontaneous growth of megakaryocyte progenitors, which is one of the biological hallmarks of this disorder [92].

Myeloid-Derived Suppressor Cells (MDSCs) are an abundant source of TGF-β production [93]. MDSCs accumulation in the tumor microenvironment promotes tumor relapse through direct effects on tumor cell survival and angiogenesis and via indirect effects on local T cell suppression

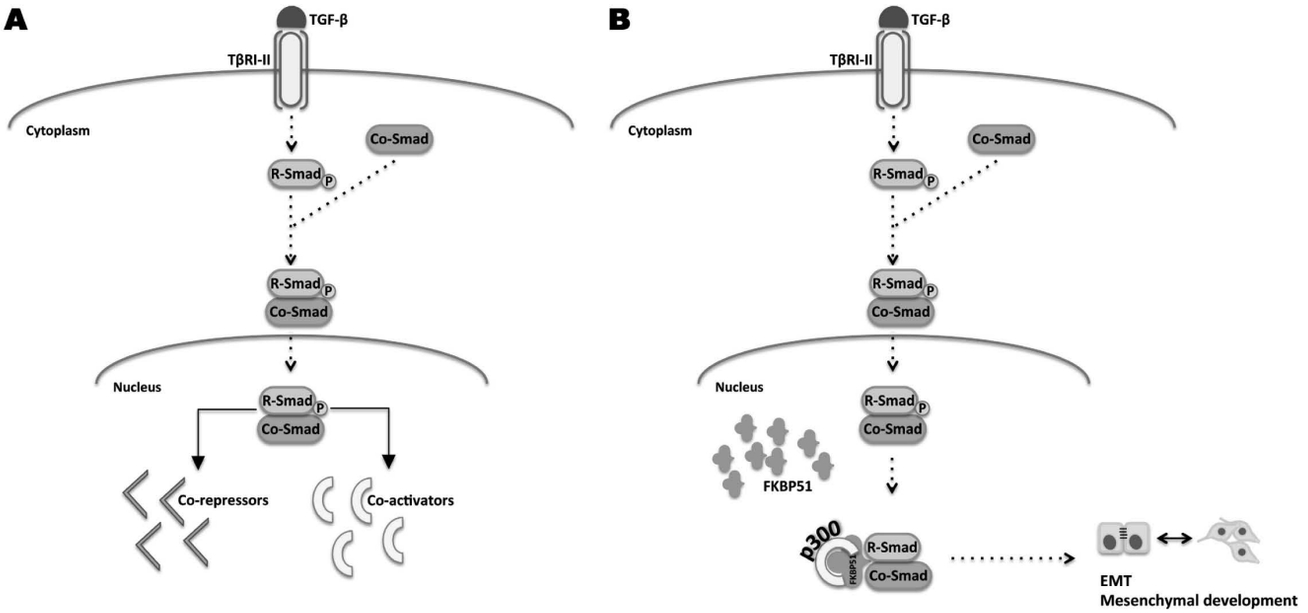

Fig. (1). *FKBP51 favours TGF-β-prooncogenic activities.* Schematic representation of the proposed mechanism for FKBP51 action on TGF-β prooncogenic activities. A Ligand binding to TGF-β receptor induces R-Smad posphorylation and activation of the Smad complex (R-Smad+Co-Smad). This latter translocates into the nucleus and associates with transcriptional co-activators or co-repressors. The nuclear context and the presence of protein adaptors influence Smad-recruitment to co-activators or co-repressors. Through this mechanism, Smads can access to separate sets of target genes, which explains the multiple, diverse and divergent actions of TGF-β. B FKBP51 promotes Smad binding to p300 transcriptional co-activator, promoting EMT or mesenchymal development.

[93]. Kim at al. [94] found an increased FKBP51 expression in MDSCs. Particularly, the authors highlighted a relevant role for FKBP51 in immune suppression activity of these cells and suggested that the immunophilin is a novel molecule that can be targeted for control of the protumoral functions of MDSCs [94].

FKBP51 IN DEVELOPMENT

FKBP51 is an important regulator of melanoma plasticity [81, 86]. The finding that FKBP51 participates in transcriptional complexes and interacts with p300 acetyltransferase is also in accordance with a role for FKBP51 in the chromatin changes required for melanoma cell reprogramming [81, 86]. To note, chromatin remodeling plays a pivotal role in developmental stages. An active participation of FKBP51 to developmental processes has been widely demonstrated both in mammals and inferior organisms. In the plant Arabidopsis, PAS-1, the FKBP51 homolog, plays a critical role in the control of cell division and plant development [95]. The PAS mutants have altered embryo, leaf and root development. Mutations in the C-terminal domain of PAS1 lead to severe developmental defects in dividing cells. PAS1 is mainly expressed in zones with high levels of mitotic activity, such as the shoot and root apical meristems [96]. In mammals, FKBP51 is among the top candidate genes expressed in mesenchymal stem cells [97], particularly during the mitotically active phase that precedes differentiation into the three mesodermal lineages, namely osteogenesis/chondrogenesis/adipogenesis [98, 99]. The temporal profile of FKBP51 mRNA accumulation showed a peak at days 2 and 4 of the differentiation program. Then, FKBP51 transcript diminished significantly by days 6 and 8, when cells had growth arrested and assumed the morphology of differentiated adipocytes. FKBP51 knockdown prevented the differentiation process. An apparently unique feature of FKBP51 is its temporal expression profile during a phase of adipocyte differentiation characterized by intense mitotic cell growth and clonal expansion. This finding suggests a specialized role for FKBP51 during the cell division cycle [98]. Quintà *et al.* [100], also showed a role for FKBP51 and its homologous FKBP52 as key genes in embryonic hippocampal neuron development. Particularly, the authors showed that neurite out-growth is favored by FKBP52 overexpression or FKBP51 knockdown, and is impaired by FKBP52 knockdown or FKBP51 overexpression, indicating that the balance, between these FKBPs plays an important role during the early mechanism of neuronal differentiation [100]. Table **1** summarizes the cell systems that find FKBP51 involved in development. The same Table highlights the functions of FKBP51 in regulation of TGF-β expression and signal.

CONCLUDING REMARKS

FKBP51 appears to be a common denominator to EMT, cancer stemness and drug resistance, in melanoma. Particularly, the involvement of FKBP51 in transcriptional complexes associated with p300 and Smad2,3 suggests important nuclear functions for this immunophilin, that allow melanoma to take advantage of TGF-β tumor-promoting activities. Through this mechanism, FKBP51 is exploited by melanoma in an opportunistic manner, to support and guarantee functions ensuring its survival, progression and regeneration.

Table 1. FKBP51 Functions Related to TGF-β and development.

Function	Cell system	Ref
DEVELOPMENT	Arabidopsis	[95]
	Mesenchymal Stem Cell	[97-99]
	Neuronal	[100]
PROMOTION OF TGF-β EXPRESSION	Idiopathic Myelofibrosis	[91, 92]
	Melanoma	[81, 86]
	MDSCs	[94]
PROMOTION OF TGF-β ONCOGENIC ACTIVITIES	Idiopathic Myelofibrosis	[91]
	Melanoma	[81, 86]

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Cyclophilin Function in Cancer; Lessons from Virus Replication

Paul T.M. Lavin and Margaret M. Mc Gee*

UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

Margaret M. Mc Gee

Abstract: Cyclophilins belong to a group of proteins that possess peptidyl prolyl isomerase activity and catalyse the *cis-trans* conversion of proline peptide bonds. Cyclophilin members play important roles in protein folding and as molecular chaperones, in addition to a well-established role as host factors required for completion of the virus life cycle. Members of the cyclophilin family are overexpressed in a range of human malignancies including hepatocellular cancer, pancreatic cancer, non-small cell lung cancer, gastric cancer, colorectal cancer and glioblastoma multiforme, however, their precise role in tumourigenesis remains unclear. In recent years, mounting evidence supports a role for prolyl isomerisation during mammalian cell division; a process with striking similarity to plasma membrane remodelling during virus replication. Here, we summarise our current understanding of the role of cyclophilins in cancer. We review the function of cyclophilins during mammalian cell division and during HIV-1 infection, and highlight common processes involving members of the ESCRT and Rab GTPase families.

Keywords: Cancer, cyclophilin, CypA, cytokinesis, ESCRT, Rab, viral.

INTRODUCTION

The interconversion of protein backbone between *cis* and *trans*, catalysed by peptidyl prolyl isomerases (PPIases), is an important event that alters protein structure and activity and regulates a wide spectrum of cellular functions in normal physiological processes. For example, isomerisation mediates the spatiotemporal control of fundamental processes including cell cycle progression, cell proliferation and cell death [1-4]. In recent years, mounting evidence implicates deregulated isomerase activity in a range of age-related pathologies including neurodegeneration, cardiovascular disease and cancer [5]. As a result, isomerases have gained significant interest as therapeutic targets in the treatment of these diseases.

The PPIase enzyme family is comprised of three structurally distinct groups; cyclophilins (Cyps), FK-506 binding proteins (FKBPs) and parvulins [1]. Although members differ in substrate specificity, all PPIases catalyse the *cis-trans* conversion of X-proline peptide bonds [1]. X-proline isomerisation is a slow rate limiting step in many reactions. PPIases accelerate the process by stabilisation of the *cis-trans* transition state [1] and as such they can assist in protein folding [6, 7] and transport [5]. Furthermore, they can function as molecular chaperones independently of their PPIase activity [8]. The parvulin Pin1 differs from the cyclophilin and FKBP families in that it specifically catalyses the isomerisation of proline peptide bonds that are immediately preceded by phosphorylated serine or threonine [9].

*Address correspondence to this author at the UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland; Tel: + 353 1 7166771; Fax: + 353 1 2837211; E-mail: margaret.mcgee@ucd.ie

The cyclophilin family is highly conserved through evolution and is comprised of 8 cyclophilin and 10 cyclophilin-like members in humans (Table **1**) that are localised in different cellular compartments including the cytosol, endoplasmic reticulum, mitochondria and nucleus [10, 11]. Cyclophilin A (CypA), the first member to be discovered [12], is a 165 amino acid protein containing a cyclophilin-type domain between amino acids 2-163. Structurally, CypA forms a right-handed β-barrel, comprised of eight antiparallel β-strands, which is flanked by an α-helix at each end. The cyclophilin domain, which is shared with all other cyclophilin members (Fig. **1**), forms a hydrophobic pocket that is the binding site for proline-containing peptides as well as the enzymatic site of the enzyme.

The FK506-binding proteins (FKBPs) are comprised of 13 human FKBPs which localise to the cytoplasm (FKBP12, 12.6, 25, 36, 38, 51, and 52), the endoplasmic reticulum (FKBP19, 22, 23, 60, and 65) and mitochondria (FKBP13) [10]. The parvulin family is composed of two members, Pin1 and Par14. Pin1 is conserved in many organisms, however Par 14 is only found in higher eukaryotes [10].

Cyclophilins and FKBPs are also designated as Immunophilins because they are the intracellular target of the chemically unrelated immunosuppressive drugs, cyclosporine A (CsA) and FK506, that are used in the suppression of graft rejection following organ transplantation [13]. CypA was first found to bind and form a ternary complex with CsA [12] and it was demonstrated that $CypA^{-/-}$ mice are resistant to CsA-mediated immunosuppression [14]. Subsequently, several other family members were found to bind CsA, including CypB [15], CypC [16] and CypD [17]. The dissociation constants for CsA binding to CypA, CypB, CypC, and CypD

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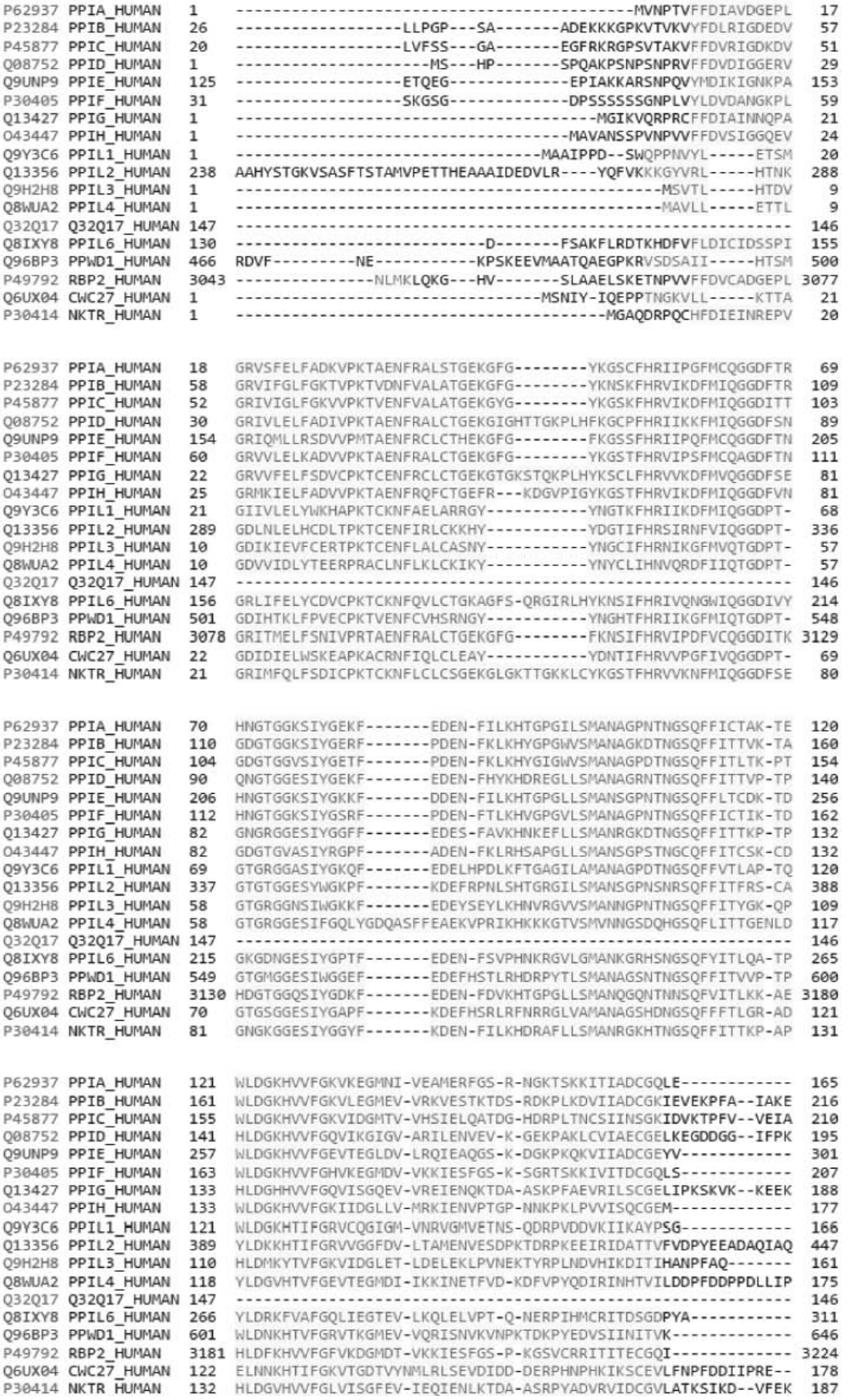


Fig. (1). Conservation of the PPIase domain (yellow) in the Cyclophilin and Cyclophilin-Like family.

Table 1. The Cyclophilin and Cyclophilin-Like Family of Proteins.

Name	UniProtKB	UniRef	Alternative Name(s)	Family
PPIA	P62937	PPIA_HUMAN	CYPA	Cyclophilin
PPIB	P23284	PPIB_HUMAN	CYPB	
PPIC	P45877	PPIC_HUMAN	CYPC	
PPID	Q08752	PPID_HUMAN	CYP40, CYPD	
PPIE	Q9UNP9	PPIE_HUMAN	CYP33	
PPIF	P30405	PPIF_HUMAN	CYP3	
PPIG	Q13427	PPIG_HUMAN		
PPIH	O43447	PPIH_HUMAN	CYP20, CYPH	
PPIL1	Q9Y3C6	PPIL1_HUMAN	CYPL1, CGI-124, UNQ2425/PRO4984	Cyclophilin-Like
PPIL2	Q13356	PPIL2_HUMAN		
PPIL3	Q9H2H8	PPIL3_HUMAN		
PPIL4	Q8WUA2	PPIL4_HUMAN		
PPIL5	Q32Q17	Q32Q17_HUMAN		
PPIL6	Q8IXY8	PPIL6_HUMAN		
PPWD1	Q96BP3	PPWD1_HUMAN	KIAA0073	
RBP2	P49792	RBP2_HUMAN	RANBP2	
CWC27	Q6UX04	CWC27_HUMAN	SDCCAG10, UNQ438/PRO871	
NKTR	P30414	NKTR_HUMAN		

are 36.8 nM [18], 9.8 nM [18], 90.8 nM [18] and 3 nM [19], respectively. CsA and FK506 bind and inhibit the PPIase activity of cyclophilins and FKBPs, respectively; however, the immunosuppressive action of each drug does not occur by PPIase inhibition. Instead, immunosuppression is achieved by a gain-of-function mechanism whereby the immunophilin-drug complex associates with and inhibits calcineurin, a protein phosphatase that dephosphorylates nuclear factor for activation of T-cells (NF-AT) [20]. Inhibition of NF-AT dephosphorylation prevents its nuclear translocation and the stimulation of cytokines that are required for T-cell proliferation [20].

Although cyclophilins have been associated with diverse cellular functions including protein folding [6, 21] and trafficking [22, 23], their precise function in normal cells remains unclear. Only a relatively small number of substrates have been identified to date, which may be explained by the spatio-temporal nature of isomerase-substrate interaction. CypA substrates include the homo-oligomeric α7 neuronal nitcotine receptor [24] and transferrin [25] which require CypA for correct protein folding. CypA binds to the cell surface receptor, CD147, and regulates its transport to the plasma membrane [26]. Consistent with this, CsA significantly reduced the surface expression of CD147. CypA also controls T-cell activation by prolyl isomerisation of interleukin-2 tyrosine kinase (Itk) which inhibits its catalytic activity [27].

In recent years, deregulated cyclophilin-mediated PPIase activity has been implicated in tumour proliferation, invasion and metastasis [28-30] and is associated with acquired chemoresistance [31-34]. Despite this, the precise signals that are regulated by each cyclophilin in tumour cells remain poorly understood. Improved understanding of cyclophilin function in cancer cells is critical to reveal strategies for therapeutic intervention. Recently, it was shown that CypA plays a role in the division of leukaemia and lymphoma cells, where it facilitates the completion of cytokinesis and the generation of two new daughter cells [35], however the precise mechanism involved is not yet established. In contrast, cyclophilin proteins have an established role as critical host factors required for viral infection [36-39]. During infection, virions target and hijack host proteins involved in topologically similar processes to facilitate assembly and release of progeny from the host cell. This review is a summary of our current understanding of the role of cyclophilins in cancer, with a focus on understanding their role in the regulation of cytokinesis and genome stability. Although much detail remains to be revealed, the review highlights interesting parallels that exist between the role of CypA in the analogous processes of cytokinesis and viral budding that may provide new insight into cyclophilin function in cancer.

CYCLOPHILIN EXPRESSION IN NORMAL AND CANCER CELLS

Members of the Cyclophilin family have been implicated in a range of cancers including lung, breast, liver, and prostate [40]. CypA was identified as a novel hepatocellular carcinoma marker that was overexpressed in patient-derived

tissue in comparison to normal and cirrhotic liver tissue [41]. It was also found that CypA is significantly overexpressed in pancreatic cancer cell lines and in human pancreatic adenocarcinoma tissue when compared to their normal counterparts, and addition of exogenous CypA significantly stimulated cancer cell proliferation [2]. Analysis of matched normal and lung cancer tissue showed overexpression of CypA in the cancer tissue [42]. Furthermore, siRNA mediated suppression of CypA in non-small cell lung tumour xenografts resulted in reduced cell growth [28]. In addition, CypA was found to be overexpressed in metastatic melanoma [43] and gastric adenocarcinoma [44] when compared to their normal counterparts. CypA was also found to be overexpressed in esophageal cancer cell lines [45] and in clinical endometrial carcinoma specimens [46]. Reduction of CypA levels in the endometrial carcinoma cells using RNAi technology significantly suppressed cell growth and induced apoptosis [46].

CypB is implicated in the proliferation and survival of breast, liver, brain, and myeloma cancer. siRNA mediated repression of CypB expression in ductal breast epithelial tumour cells decreased cell growth, proliferation and motility [47]. CypB interacts with the transcription factor STAT3 in HepG2 liver cells which mediates the interleukin-6 family of cytokines [48]. Inhibition of CypB in STAT3-dependent human myeloma cell lines resulted in apoptosis, suggesting that CypB acts as a pro-survival protein in these cells [48]. Furthermore, CypB is overexpressed in malignant glioma tissue and suppression of CypB resulted in reduced cell growth and survival *in vitro* and *in vivo* [49].

CypC was identified as a novel gene marker for detecting circulating tumour cells in patients with ovarian cancer, and increased CypC expression in circulating tumour cells after chemotherapy is associated with poor patient survival [50]. CypD is also significantly up regulated in ovarian cancer [51], breast cancer [51], uterus cancer [51] and prostate cancer [52]. Cyp33 is significantly up regulated in glioblastoma compared to non-neoplastic brain tissue [53]. PPIL1 is overexpressed in patient-derived colon cancer tissue, and siRNA mediated suppression of PPIL1 in the human colon cell line SNUC4 suppressed cell growth [54]. PPIL3 mRNA is overexpressed in human glioma tissues [55]. Gene mutations of RANBP2 have also been identified in colorectal cancer tissue [56] while a RANBP2-ALK fusion gene has been detected in acute myelomonocytic leukaemia [57] and inflammatory myofibrolastic tumour [58]. Furthermore, RANBP2 is up regulated in multiple myeloma [59]. In contrast, NKTR was found to be down regulated in cancer-associated prostatic fibroblast tissue when compared to matched normal fibroblast tissue [60].

CYCLOPHILINS AND CHEMORESISTANCE

Cyclophilin family members are associated with cancer chemoresistance. Overexpression of CypA is associated with resistance of prostate cancer cells to cisplatin-induced cell death and it is proposed that CypA suppresses cisplatin-induced ROS production and the loss of mitochondrial membrane potential [34]. Consistent with that, loss of CypA expression increased mitochondrial membrane depolarization and reduced survival following H_2O_2 treatment [34]. Furthermore, there is evidence that CypA is transcriptionally regulated by the hypoxia inducible factor (HIF)-1α under hypoxic conditions [34, 61] and it is suggested that up-regulated CypA desensitises cells to hypoxia-induced cell death [34].

CypA is down regulated in melphalan-resistant MCF7 breast cancer cells when compared to non-resistant cells [62], and it is proposed that downregulation of CypA allows evasion of apoptosis by inhibition of apoptosis-inducing factor (AIF) [63]. Furthermore, CypA-overexpressing endothelial liver cells display resistance to doxorubicin and vincristine, which was accompanied by up-regulation of cytokines such as interleukins and chemokines [64]. CypA is also overexpressed in paclitaxel resistant endometrial cancer cells, HEC-1-B/TAX and AN3CA/TAX. Knockdown of CypA with siRNA significantly inhibited cell proliferation and invasion when exposed to paclitaxel, which was accompanied by reduced phosphorylation of Akt and the MAPK ERK1/2, p38 and JNK suggesting that overexpression of CypA allows for enhanced MAPK activity [45].

Other cyclophilins have also been implicated in chemoresistance. Under hypoxic conditions HIF-1α upregulates CypB in human hepatocellular carcinoma and CypB knockdown significantly reduced cell survival when subjected to hypoxia, cisplatin, or H_2O_2 treatment [32]. This suggests that CypB may play a similar role to CypA [34] in cisplatin resistance by protecting the cells against ROS induced stress. Furthermore, overexpression of another family member, RANBP2, was detected in cisplatin resistant ovarian carcinoma [65].

Collectively these studies confirm that upregulation of cyclophilin protein is associated with a range of cancers (Table **2**), and supports a role for cyclophilins in tumourigenesis and acquired chemoresistance. Thus, cyclophilins may represent valuable biomarkers for chemoresistance and potential therapeutic targets to sensitise cancer cells to chemotherapy. In support of this, treatment of cisplatin-resistant hepatocellular carcinomas with the cyclophilin inhibitors, CsA or sanglifehrin A (SFA), in combination with cisplatin, synergistically enhanced apoptosis [33]. It is only in recent years that the role of cyclophilin proteins, and other PPIases, in cancer has begun to be explored. To date, reports suggest that PPIases are associated with an increase in cell proliferation, migration and invasion [2, 23, 30].

THE MAMMALIAN CELL CYCLE

The cell cycle involves the co-ordinated division of a cell into two genetically identical daughter cells (Fig. **2A**). Progression through the cell cycle is regulated by cyclin-dependant kinases (Cdks); a family of serine/threonine protein kinases that require binding to cyclin regulatory subunits for biological activity. Thus, the synthesis and degradation of the cyclin proteins during the cell cycle ultimately drives cell cycle progression [66]. Transition through the cell cycle is also controlled by checkpoints that are switch-like transitions that regulate S phase entry, mitotic entry and mitotic exit and are active at the G1/S boundary, G2/M boundary and at the metaphase/anaphase boundary respectively. Progression through the cell cycle can be halted at these checkpoints if conditions for successful cell division are not met [67].

Mitosis (M) phase of the cell cycle is divided into prophase, metaphase, anaphase, telophase and cytokinesis (Fig. **2B**). During prophase the nuclear membrane breaks down,

and duplicated chromosomes, which are comprised of two sister chromatids, condense while the centrosomes nucleate the mitotic spindles [68]. During metaphase the spindle microtubules attach to the sister chromatids via their kinetochores, and they align at the centre of the mitotic spindle. During anaphase the sister chromatids are separated and move to opposite poles of the mitotic spindle. Separation of sister chromatids should take place only when all chromosomes are attached to the bipolar mitotic spindle via their kinetochores. Separation is promoted by the anaphase-promoting complex (APC) [68], an E3 ubiquitin ligase that targets the degradation of securin, which is an inhibitor of the protease separase. Degradation of securin liberates active separase, which is free to degrade the cohesion proteins that hold sister chromatids together [68]. APC also targets cyclin B for degradation, which culminates in inactivation of Cdk1 and marks mitotic exit [66]. During telophase, the two sets of daughter chromosomes arrive at the spindle poles, where they decondense and a new nuclear envelope is formed. Finally, cytokinesis involves the separation of the cytoplasm, organelles and DNA of a dividing cell into two new daughter cells [69].

Table 2. Cyclophilin and Cyclophilin-Like proteins that are overexpressed (↑) or down regulated (↓) in cancer.

Cyp Member	Cancer Type	Altered Expression in Cancer	Reference
CypA	Liver	↑	[29, 41, 64]
	Gastric	↑	[44]
	Pancreatic	↑	[2, 107, 179]
	Non-Small Cell Lung	↑	[42, 180]
	Breast	↑	[62, 181]
	Esophageal	↑	[182]
	Endometrial	↑	[45, 182, 183]
	Melanoma	↑	[43]
CypB	Breast	↑	[47]
	Liver	↑	[32, 48, 184]
	Brain	↑	[49]
	Myeloma	↑	[48, 185]
CypC	Ovarian	↑	[50]
CypD	Breast	↑	[51]
	Uterus	↑	
	Ovarian	↑	
	Liver	↑	[186]
	Prostate	↑	[52]
Cyp33	Brain	↑	[53]
PPIL1	Colorectal	↑	[54]
PPIL3	Brain	↑	[55]
RANBP2	Colorectal	↑	[56]
	Ovarian	↑	[65]
	Myeloma	↑	[59]
	Leukemia	↑	[58]
NKTR	Prostate	↓	[60]

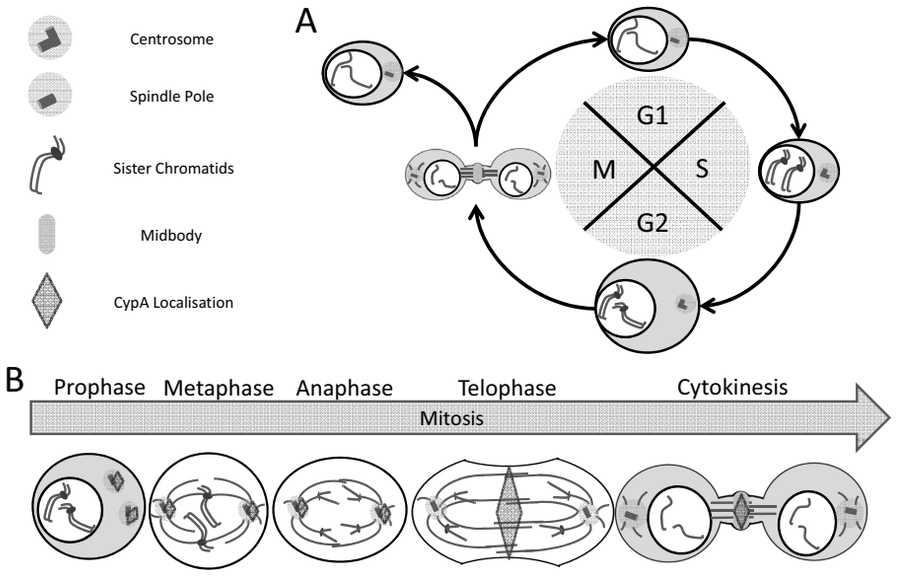


Fig. (2). Overview of the cell cycle. (**A**) The phases of the cell cycle including Gap 1 (G1), Synthesis (S), Gap 2 (G2) and Mitosis (M). (**B**) The stages of mitosis showing CypA localisation at each stage. The DNA condenses and the nuclear envelope is degraded during prophase. The chromosomes align at the centre of the dividing cell and the spindle microtubules attach to the chromosomes during metaphase. At anaphase the chromosomes are separated and move towards the spindle poles. During cytokinesis, cytoplasmic division is mediated by the formation of a contractile ring that condenses midzone microtubules to form the midbody. The midbody acts as a platform for the recruitment of proteins required for final abscission event that gives rise to two genetically identical daughter cells.

Cytokinesis and Abscission

The first event of cytokinesis is the formation of the central spindle, a narrow zone of bundled overlapping non-kinetochore microtubules at the midzone between separating chromosomes. Central spindle formation requires the microtubule binding and bundling protein PRC1, and the motor protein KIF4 that blocks microtubule growth thereby promoting normal midzone architecture [68]. Activation of PRC1 causes microtubule bundling to form the midzone which serves as a platform for the localisation of other critical components of the spindle, including centralspindlin and Chromosomal Passenger Complex (CPC) [70] (Fig. **3**). Centralspindlin is a heterotetramer comprised of two molecules of MKLP1 and two molecules of CYK4 which contains a GTPase-activating protein (GAP) domain [71]. The CPC is composed of Aurora B and three non-enzymatic proteins required for Aurora B regulation, INCENP, Survivn, and Borealin [72]. Aurora B phosphorylates MKLP1, promoting the recruitment of centralspindlin to the midzone, which tethers the central spindle to the plasma membrane [73]. PRC1 and MKLP2 recruit Plk1 to the central spindle where it phosphorylates the centralspindlin subunit CYK4, generating a binding site for Ect2, a guanine nucleotide exchange factor for Rho GTPases [68]. Ect2 binds and is directed towards the plasma membrane around the centre of the dividing cell. Ect2 activates RhoA by conversion of RhoA-GDP into RhoA-GTP, which promotes contractile ring assembly at the equatorial membrane [74]. As the actiomyosin ring is constricted, the central spindles at the midzone become densely packed to form the midbody, located at the centre of the intercellular bridge. The midbody acts as a protein scaffold for components required for the final stage of cytokinesis known as abscission. The midbody recruitment of Centrosome protein of 55 kDa (Cep55) is essential for abscission. During early mitosis Erk2/Cdk1 phosphorylates Cep55 at Ser425 and Ser428 which results in Cep55 disassociation from the spindle poles [75]. Phosphorylation of Cep55 at Ser425 and Ser428 allows for the recruitment and phosphorylation by Plk1 on S436 within a C-terminal region containing the binding site for MKLP1 [75-77]. While there is general agreement that Plk1 phosphorylation controls Cep55 localisation during cytokinesis, Plk1 phosphorylation is reported to act as a positive and negative regulator of Cep55 function [75-77]. One model proposes that Plk1 phosphorylation is required for Cep55 localisation and function at the midbody [75], whereas the other model proposes that Plk1 prevents Cep55 from interacting with the central spindle and midbody protein MKLP1 during anaphase. The progressive degradation of Plk1 during mitotic exit exposes the MKLP1 binding site which allows Cep55 recruitment to the midbody [77].

A recent discovery was that the Endosomal Sorting Complex Required for Transport (ESCRT) machinery, which are known for their role in membrane scission during viral budding and the formation of multivesicular bodies, also play a key role in abscission [68, 78, 79] (Fig. **4**). The protein family, which is comprised of 4 complexes (0, I, II, III) and VPS4, are involved in membrane remodelling during cytokinesis and abscission [68, 80]. Cep55 is the key protein directing ESCRT recruitment. It recruits the ESCRT-I subunit Tsg101, and ALIX to the midbody which allows for recruitment of ESCRT-III complex to the flanking midbody ring [79]. ESCRT-III accumulates at the abscission site and promotes membrane severing by the formation of constrict-

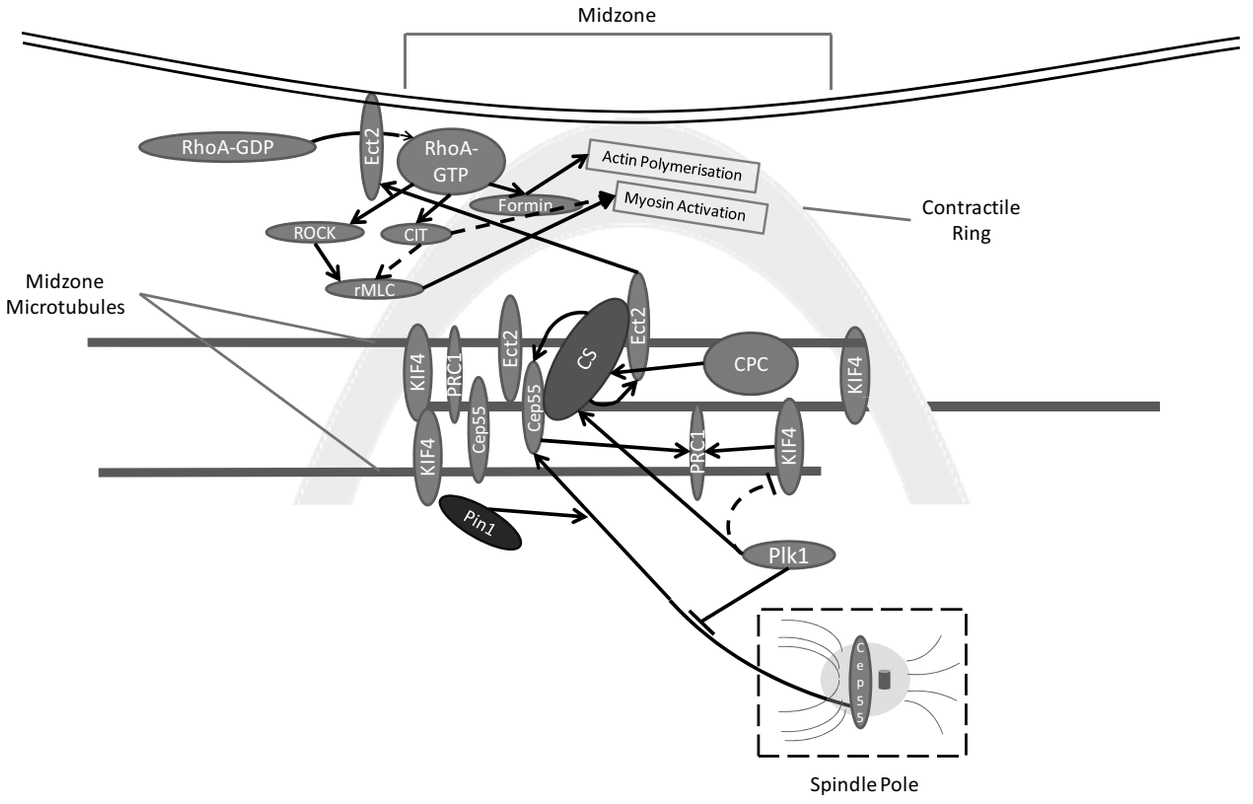
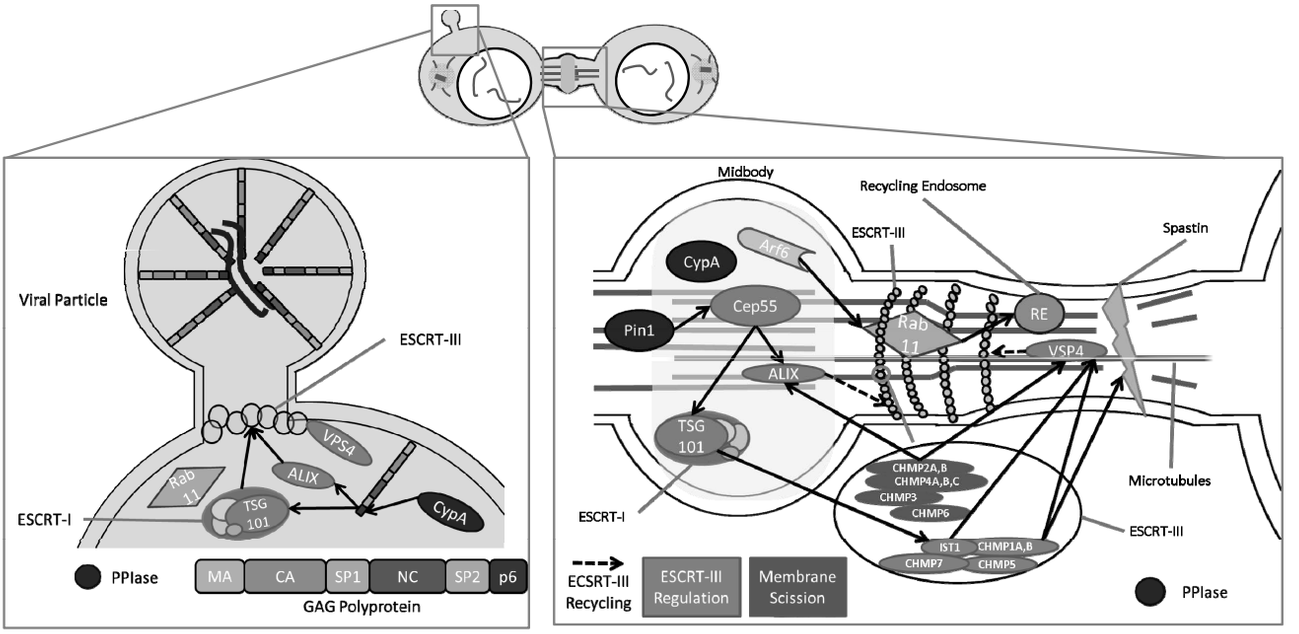


Fig. (3). Schematic showing the formation of the contractile ring and central spindles during telophase. PRC1 interacts with the motor protein KIF4 which directs KIF4 to the antiparallel microtubules, PRC1 bundles the midzone microtubules by cross linking antiparallel microtubules while KIF4 caps the midzone microtubules which limit their growth. Centralspindlin (CS) is recruited to the midbody by the chromosomal passenger complex (CPC) and it tethers the central spindles to the plasma membrane. Plk1 and Pin1 control the translocation of Cep55 from the spindle poles to the centralspindlin complex. Centralspindlin also recruits Ect2 to the plasma membrane where is activates RhoA by conversion from RhoA-GDP to RhoA-GTP. Active RhoA induces contractile ring formation by activating pathways for actin and myosin formation. (Dashed lines, non-direct interaction).

ing helical oligomers and the loss of the midbody microtubules at the intercellular bridge [68]. This is coupled with ESCRT-III recruitment of the ATPase spastin which displays microtubule severing activity and is believed to be responsible for the final scission and separation of the daughter cells (Fig. **4**) [68]. ESCRT-III also recruits the disassembly factor AAA-ATPase VPS4 which releases the ESCRT-III from the membrane, allowing it to bind elsewhere [68]. Additional membrane-trafficking proteins localise to the intercellular bridge and play a role in the final stages of cytokinesis including dynamin, soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins (SNAREs), Rab proteins and exocyst components [80, 81]. However, while many of the proteins that are important for abscission have been identified, their precise mechanism is still unclear.

CYTOKINESIS FAILURE AND CANCER

The majority of cancer cells contain large-scale alterations in chromosome structure and number [82-86]. Loss or gain of whole chromosomes, known as aneuploidy, has long been associated with cancer, however, there has been much debate surrounding how aneuploid cells arise and whether they are a cause or consequence of cancer [82-86]. The cumulative evidence to date suggests that aneuploidy promotes tumourigenesis [82-86]. In that context, a long standing hypothesis is that failure of cell division generates unstable tetraploid cells that represent the first step to aneuploidy and tumourigenesis [84]. In support of this, mutation of adenomatous polyposis coli (APC) in colorectal cancer inhibits spindle anchoring during anaphase and results in cytokinesis failure with the generation of tetraploid genomes *in vivo* [87]. Transient inhibition of cytokinesis generates genetically unstable tetraploid cells that promote tumourigenesis in $p53^{-/-}$ mouse mammary epithelial cells [88]. Furthermore, tetraploidy and chromosome instability are early events detected in human cervical cancer tissue [89]. Mouse ovarian surface epithelial cells derived from an intermediate tetraploid cell, generated through chromosome mis-segregation during mitosis, exhibited aneuploidy and were tumourigenic *in vivo* [85]. Overexpression of Aurora B in murine epithelial cells gives rise to tetraploid cells that formed tumours when injected into mice [90]. Genomic analysis of the tumours revealed widespread genomic reorganisation including trisomies, amplifications, and deletions [90]. Impaired integrin traffic, which is critical for cell adhesion, induces aneuploidy via a near-tetraploid intermediate and results in oncogenic transformation *in vitro* and *in vivo* [91]. In addition, loss of the Breast Cancer Susceptibility Gene, BRCA2 [92] or the LATS1 tumour suppressor [93] is accompanied by cytokinesis defects, suggesting a role for these tumour suppressors in cytokinesis. Aneuploid cells that arise from tetraploidy are



Protein	HIV-1 Budding		Cytokinesis		Protein	HIV-1 Budding		Cytokinesis	
ALIX	✓	[137,139]	✓	[79]	CHMP7	✓	[137]	✓	[79]
Cep55			✓	[75,76,79]	CypA	✓	[129]	✓	[35]
CHMP1A,B	✓	[137]	✓	[79]	Gag	✓	[137,139]		
CHMP2A,B	✓	[137]	✓	[79]	Pin1			✓	[112]
CHMP3	✓	[137]	✓	[79]	Rab11	✓	[164]	✓	[147]
CHMP4A,B,C	✓	[137]	✓	[79]	Spastin			✓	[68]
CHMP5	✓	[137]	✓	[79]	Tsg101	✓	[137]	✓	[79]
CHMP6	✓	[137]	✓	[79]	VPS4	✓	[137]	✓	[68]

Fig. (4). Schematic showing proteins involved in membrane re-modelling during the analogous processes of HIV-1 viral budding and cytokinesis. HIV-1 Gag regulates viral budding by binding the ESCRT-I component, Tsg101, and ALIX to L-domains within p6, which in turn recruits the ESCRT-III complex that is required for resolution of the membrane stalk connecting the virions to the host cell. During cytokinesis Cep55 recruits Tsg101 and ALIX to the midbody where they recruit ESCRT-III. The ESCRT-III complex depolymerises the microtubules and mediates abscission by plasma membrane remodelling along with recycling endosomes that are directed to the abscission site by Rab11 and Arf6 binding. CypA interacts with HIV-1 Gag p6 and acts as a general catalyst for *cis-trans* proline isomerisation. CypA and Pin1 mediated *cis-trans* isomerisation is also required at the midbody for the timely completion of mammalian cytokinesis.

most likely generated due to multiple centrosomes and multipolar mitoses, which can lead to gain or loss of whole chromosomes and to chromosome breaks. Collectively, these studies support the 100 year old hypothesis proposed by Theodor Boveri [94] that abnormal mitosis promotes genome instability and cancer.

The mitotic checkpoint is the major regulator of chromosome segregation during mitosis. Mitotic checkpoint components including Mad1, Mad2, Bub1, BubR1, Bub3 and CENP-E are recruited to kinetochores on chromosomes and they act to inhibit anaphase onset and mitotic exit by inhibiting the APC [95, 96]. Defects in mitotic checkpoint genes results in numerical aneuploidy. In recent years a number of animal models that exhibit aneuploidy and/or chromosome instability due to mutation in mitotic checkpoint genes have been tested for their tumour forming ability. Results indicate that $Mad^{+/-}$ and $Cdc20^{+/AAA}$ mice as well as those that overexpress Bub1 or Mad2 promote tumourigenesis. On the otherhand, mutations that suppress tumourigenesis have also been reported including $securin^{-/-}$, while others had no effect on tumour incidence including inactivated Bub1 or $Bub3^{+/-}$, while mutations such as $Bub1^{-/H}$, $BubR1^{+/-}$ and $Mad2^{+/-}$ either promote or suppress tumourigenesis depending on the context. Thus, overall, the tumour promoting ability of aneuploidy and chromosome instability is dependent on the context and the specific genes involved (for review see [97]). Interestingly, data from mouse models and patients indicates that low and intermediate rates of chromosome instability can promote tumours, whereas, high rates of chromosome instability is incompatible with viability leading to cell death and tumour suppression, and correlates with improved patient outcomes [97]. Thus, a better understanding of the role of aneuploidy and genome instability in tumourigenesis is important in order to exploit the process therapeutically.

THE ROLE OF CYCLOPHILIN AND CYCLOPHILIN-LIKE PROTEINS IN THE CELL CYCLE

ESS1, a homolog of Pin1, was first identified in *Saccharomyces cerevisiae* as a novel protein required for growth [98]. Studies in yeast found that loss of ESS1 led to defective cytokinesis revealing a role in cell division. Moreover, the CypA homolog, CYP1, was sufficient to rescue ESS1 loss in yeast, and CYP1 becomes essential for growth in stable ESS1 null yeast [99, 100]. Thus, it was proposed that ESS1 regulates the expression of genes required for cell growth [101]. ESS1 binds directly to the Sin3-Rpd3 histone deacetylase complex and the Set3C histone deacetylase complex and modulates their deacetylase activity [100, 102]. CYP1 also interacts and regulates Sin3-Rpd3 histone in yeast [103]. These findings suggest that ESS1 and CYP1 act within the same pathway to control transcription of cell cycle genes in yeast.

A number of studies report a role for cyclophilin proteins during progression of the mammalian cell cycle. Fragile histidine triad (FHIT) acts as a tumour suppressor and down regulation is associated with tumour growth [104]. Loss of FHIT expression results in increased CypA levels in mouse lung tissue, mouse kidney cells and in the human lung cancer cells. Treatment of FHIT negative cells with extracellular recombinant CypA led to up regulation of cyclin D1 and activation of Cdk4 permitting transition from G1 to S phase of the cell cycle [104].

CypD has also been implicated in cell cycle progression. Knockdown or deletion of CypD in glioblastoma cells and mouse embryonic fibroblasts enhanced cell proliferation via accelerated entry into S phase [105]. Increased STAT3 transcription and activity were detected in CypD null MEF cells [105]. While the connection between CypD expression and STAT3 is unclear, STAT3 plays a role in G1 to S phase transition by up regulation of cyclin D or inhibition of cell cycle repressors such as p21 [106]. Thus, it is proposed that by its interaction with STAT3, CypD can regulate the G1 to S phase transition. Furthermore, recent work has demonstrated that CypA stimulates phosphorylation of STAT3 which results in increased protein levels of cyclin D1 and survivin [107].

The phosphorylation-dependent PPIase, Pin1, is overexpressed in a large number of tumours [108] and is associated with poor survival [109]. Pin1 plays an important role in the cell cycle by regulating cyclin D1 and influencing the G1 and S phase transition [110] as well as regulating numerous mitotic substrates including NIMA, Wee1, Cdc25, Plk1, CENP-F (for review see [111]). In recent years, several studies have provided a link between PPIase localisation at the centrosome and a role in cytokinesis. Pin1 is localised to the centrosome and undergoes mitosis-specific phosphorylation dependent relocalisation to the midbody [112, 113]. The midbody protein Cep55 must be tightly regulated to ensure proper execution of cytokinesis. Pin1 interacts with Cep55 *in vitro* and *in vivo* and it is suggested that Pin1 isomerisation of Cep55 at the midbody enhances Plk1 phosphorylation of Cep55 on Ser436, an essential step for the timely execution of cytokinesis [113]. Consistent with this, deregulated Pin1 function, as a result of overexpression or depletion, causes cytokinesis defects. For example, overexpression of Pin1 in mouse mammary glands induced malignant mammary tumours that was accompanied by centrosome amplification and aneuploidy [112]. In addition, Pin1 knockout mouse embryonic fibroblasts (MEFs) and HeLa cells display cytokinesis defects [113].

Pin1 also mediates the final separation of daughter cells via interaction with the septin family member, SEPT9 [114]. Specifically, SEPT9 is phosphorylated by Cdk1 at Thr24 which acts as a site for Pin1 isomerisation, and is required for abscission [114]. In addition to SEPT9, a number of septins are implicated in the earlier stages of cytokinesis including SEPT2, SEPT7, and SEPT11 [115] however, the role of isomerisation in the regulation of their activity remains unknown.

CypA localises to the centrosome during interphase in a range of human tumour cells including haematopoietic cells, lung cancer cells and HeLa cervical carcinoma cells [35]. CypA forms part of the spindle poles during early mitosis and relocalises to the midzone and midbody during telophase and cytokinesis respectively. Moreover, while CypA PPIase activity is not required for centrosome or midbody localisation, it is required for the timely completion of cytokinesis [35]. Bannon *et al*., demonstrated that expression of wild-type CypA rescued the defect whereas an isomerase defective mutant did not. Furthermore, loss of CypA expression, by homozygous deletion or RNAi, led to supernumerary centrosomes and a tetraploid genotype indicative of cytokinesis failure. Loss of CypA also significantly reduced clonogenic potential indicating that CypA expression confers a growth advantage to cancer cells [35]. These findings provide a mechanistic link between aberrant expression of CypA and cancer.

More recently it was discovered that RANBP2 localises to the centrosome in mammalian cells [116] and in *Drosophilia* [117]. In addition, RANBP2 was detected at the spindle pole and kinetochore during mitosis in HeLa cells, which was controlled by binding to importin [116]. Downregulation of RANBP2 induces chromosome missegregation and aneuploidy, leading to mitotic catastrophe and cell death [116]. This suggests that RANBP2 plays a crucial role in faithful chromosome segregation, which may provide mechanistic insight into previously reported phenotypes associated with disrupted RANBP2 expression including tumour growth and embryonic lethality in knockout mice [116]. Mice with low levels of RANBP2 were highly sensitive to tumour formation, which is likely due to defective chromosome segregation [118]. Collectively these reports support a role for prolyl isomerases in the regulation of cell cycle progression and highlight how their deregulation can contribute to tumourigenesis.

CypA is required for viral budding, a process that is topologically similar to cytokinesis. In addition, members of the ESCRT protein family play key roles during these analogous processes and are involved in signalling pathways that are regulated by isomerisation. Similarly, Rab GTPase play important roles during viral budding and cytokinesis and recent evidence supports a role for isomerases in their regulation. The following section will review the role of cyclophilin proteins during viral infection, and highlight common targets

and pathways shared with ESCRT proteins and Rab GTPases during viral budding and cytokinesis.

THE ROLE OF CYCLOPHILINS IN THE VIRUS LIFE CYCLE

CypA is involved in the lifecycle of several viruses, including human immunodeficiency virus type 1 (HIV-1), influenza virus, hepatitis C virus (HCV), hepatitis B virus (HBV), vaccinia virus (VV), human cytomegalovirus, human papillomavirus, coronavirus, vesicular stomatitis virus (VSV), and rotavirus [119-128]. A large body of evidence indicates that CypA is an important host factor for successful viral infection and CypA is also incorporated into several enveloped virus particles, such as HIV-1, influenza virus [129-131], however, the function of CypA in virus particles is still unclear.

HIV-1 UNCOATING

The physiological relevance of cyclophilins in HIV infection was revealed when cells containing a disrupted CypA gene displayed defective HIV replication [129]. The first evidence that cyclophilin proteins function as viral co-factors was reported for CypA in HIV infection [36], and mounting data suggest a multifunctional role for isomerases in the virus life cycle [37, 132-136]. An early and critical event in HIV life cycle is uncoating of the viral core that precedes genomic RNA release, reverse transcription, and nuclear import. HIV assembly and disassembly is controlled largely by the Gag-CA protein and changes in the timing of viral uncoating blocks viral infection highlighting the important timing of these events [137]. CypA binds to CA and blocks access to the cellular restriction factor, TRIM5α, which blocks uncoating during the early stage of the HIV life cycle [134, 138].

HIV-1 ASSEMBLY

Assembly of progeny virions and their release from the virus producing host cells are well co-ordinated processes that are required to complete the virus life cycle. Virion assembly and release are driven by the viral Gag protein and are dependent on host cellular factors. The Gag protein is the major structural protein of the virus capsid and is a 55kDa polyprotein comprising of four subdomains - Matrix (MA), Capsid (CA), Nucleocapsid (NC) and p6, which are interspaced by SP1 and SP2 (Fig. **4**) [137, 139]. It is widely accepted that HIV-1 Gag assembly and budding occur predominantly on the plasma membrane, where the N-terminal myristoylated MA domain mediates membrane anchoring and assembly of Gag. MA membrane binding is coupled with Gag multimerisation at the plasma membrane and is required for particle formation and viral infectivity, however, MA itself is not required for Gag-Gag interactions and particle formation [140-143]. Similarly, p6 recruits components required for viral budding, but does not make Gag-Gag interactions. Critical lateral Gag-Gag interactions that facilitate lattice formation are initiated primarily by CA and SP1, and to a lesser extent NC domains [141].

HIV-1 MATURATION AND BUDDING

The formation of mature virions requires Gag polypeptide processing into its component fragments, together with morphogenesis of a spherical particle into the viral envelop that is lined with MA, and the CA containing conical core harbouring mature dimeric viral NC/RNA complex. Packaging of CypA into HIV-1 virions is essential for efficient replication [119]. Incorporation is mediated through binding to the Gly-89-Pro-90 peptide bond of the N-terminal domain of newly assembled HIV-1 capsid (CA) [144].

p6 is a small protein that does not play a structural role in virion maturation, but it is required for virion incorporation of the viral accessory protein R (Vpr). CypA binds to the N-terminal heptapeptide motif RHFP^{35}RIW of Vpr centred at Pro-35 and it catalyses the prolyl *cis-trans* interconversion of the highly conserved proline residues (Pro-5, -10, -14 and 35) of Vpr [132]. Mutation of Pro-35 disrupts the interaction of Vpr with CypA [136]. It was subsequently found that CypA binds to a non-proline containing 16 residue region of C-terminal Vpr ^{75}GCRHSRIGVTRQRRAR^{90} with similar affinity as full length Vpr and Arg-80 was identified as a key residue in the C-terminal binding domain [38]. Although the biological significance of the binding of Vpr to CypA remains elusive, several key functions of Vpr are associated with the identified N and C-terminal CypA binding domains. These include G2 cell cycle arrest and apoptosis [38, 136, 145], suggesting a role for isomerisation in the regulation of these virus-induced processes.

The p6 Gag protein also regulates the final abscission step and budding of nascent virions from the cell membrane by the action of two late assembly (L) domains, PTAP and YPXnL (where X is any amino acid and n=1 to 3 residues) motifs, located within p6 [137, 139, 141]. The 52 amino acid p6 peptide binds two cellular budding factors; the conserved PTAP motif binds Tsg101 (a component of host ESCRT I complex), whereas the YPXnL motif binds Alix (Apoptosis-linked gene 2 -interacting protein X) [137, 139]. The p6 binding to Tsg101 allows recruitment of the core ESCRT-I heterotetramer complex, which facilitates ESCRT-III recruitment. On the other hand, Alix is a Bro domain protein containing a proline rich C-terminal residue that harbours binding sites for interaction partners including the human ESCRT-III protein, CHMP4 [137]. Thus, components of host ESCRT pathway, Tsg101 and Alix, play important roles linking Gag protein to viral budding by recruitment of human ESCRT-III and resolution of the membrane stalk that connects the virion to the host cell. As such, Gag p6 acts as an adaptor protein for the host cell machinery to promote budding. A striking feature of the p6 protein is the high relative content of proline residues located at positions 5, 7, 10, 11, 24, 30, 37, and 49. Recently, CypA was shown to interact with p6 and act as a general catalyst for *cis-trans* isomerisation of all proline residues in the full length protein [37]. Crucially, the binding motifs of p6 to Tsg101, Alix and Vpr coincide with binding regions and catalytic sites of p6 to CypA [37]. Based on this, it is possible that CypA binding and isomerisation represents an important molecular switch

that mediates the interaction of p6 with cellular factors such as Tsg101 at the p6 N-terminal or Alix at the C-terminal.

THE ROLE OF GTPase DURING CYTOKINESIS AND VIRAL BUDDING

The processes of cytokinesis and viral budding also require specific membrane trafficking events to deliver new membrane to the growing furrow and viral bud respectively. The small GTPase superfamily, which is comprised of Ras, Rho/Rac, Rab, Arf and Ran, control diverse events including cell proliferation and differentiation, as well as the regulation of actin cytoskeleton, membrane trafficking and nuclear transport [146]. In particular, the Rab GTPases represent a large family of over 60 small guanosine triphosphate (GTP)-binding proteins, and localise to distinct membrane compartments where they act as master regulators of vesicular membrane transport on both the endocytic and exocytic, and transcytic pathways [146].

Rab proteins are implicated in the completion of cell division [147]. Completion of cytokinesis in *C. elegans* requires Rab11 dependent membrane trafficking events to deliver new membrane to the furrow and for abscission, and depletion of Rab11 leads to cytokinesis defects including furrow regression and abnormal scission, confirming that endosomes provide membrane for cytokinesis [148]. Consistent with this, Rab11 and its binding partner FIP3 localise to the cleavage furrow during cell division in *C. elegans* and in mammalian cells [149, 150].

Two additional GTPases, Arf1 and Arf6, are also implicated in cytokinesis. Arf6 localises to the midbody during telophase and is responsible for the recruitment of Rab11/FIP3 and Rab11/FIP4 complexes [149]. Rab35, which is involved in the early endocytic recycling pathway, plays a crucial role during cytokinesis by mediating the localisation of phosphatidylinositol 4,5, bisphosphate (PIP2) lipid and the septin, SEPT2, to the growing cytokinetic bridge [151]. Thus, the essential role of Rab11 and Rab35, which control distinct endocytic recycling pathways, implies that multiple endocytic routes are essential for cytokinesis. Furthermore, the transport of Rab8 positive vesicles to the midbody during cytokinesis via a dynein-dependent manner implicates the retrograde pathway in the completion of cytokinesis [152]. CypA associates *in vitro* and *in vivo*, via its PPIase domain, with the dynein/dynactin motor protein complex suggesting a role for CypA PPIase activity in cargo binding for retrograde movement along microtubules [153].

Rab21 plays a role in cell division by integrin targeting to the growing cleavage furrow, and loss of Rab21 leads to cytokinesis failure, aneuploidy and tumourigenesis *in vivo* [91]. RABL6A a novel RAB-like protein, plays a role in centrosome regulation and maintenance of chromosome stability in non-transformed cells, key processes that ensure genomic integrity and prevent tumourigenesis [154]. Rab24 is an atypical member of the GTPase family and its function remains largely unknown. It was previously shown that Rab24 and CypA co-localise at the perinucleus in COS cells, implying that they may co-operate to function in a signalling pathway at that position or elsewhere [155]. Importantly, it was recently demonstrated that, like CypA [35], Rab24 is a centrosome protein that is redistributed to the mitotic spindle and midbody during mitotic progression, where is regulates multiple events including chromosome segregation and cytokinesis [156]. Depletion of Rab24 in COS-7 cells increased chromosome segregation errors and cytokinesis errors implicating Rab24 in the completion of normal cell division [156]. Thus, although there is no evidence to date that Rab function is regulated by isomerisation, it is possible that CypA isomerisation controls the distribution of Rab24, and other Rabs such as Rab11 and Rab35, to mitotic structures during the cell cycle.

Recent publications show that Rab-controlled trafficking pathways are altered during tumourigenesis. Certain members including Rab25 acts as both an oncogene and a tumour-suppressor gene [157, 158]. Accelerated cell migration induced by upregulated Rab11 and Rab25 is associated with increased vesicular transport efficiency in the inner cell compartment and at the plasma membrane [159]. Furthermore, Rab 25 and chloride intracellular channel 3 (CLIC3) drives invasiveness of pancreatic and ovarian cancer by regulating the recycling of α5β1 integrin from late endosomes to the plasma membrane [160]. In contrast, Rab37 suppresses tumour metastasis by exocytosis of the tissue inhibitor of metalloproteinase 1 (TIMP1), thereby inhibiting matrix metalloproteinase 9 (MMP9) [161]. Finally, Rab18 is highly expressed in Hepatitis B virus (HBV)-associated HCC tissue and upregulated Rab18 is mediated by HBV X protein, which promotes hepatoma cell proliferation [162].

Increasing evidence supports a role for Rab proteins in the viral life cycle. For example Rab7A, a major regulator of the late endocytic pathway, is required in the late stages of the HIV-1 replication cycle [163], whereas Rab11-FIP1C and Rab14 direct plasma membrane sorting and particle incorporation of the HIV-1 envelope glycoprotein complex [164]. Furthermore, Rab6 and Vps53 implicate the retrograde pathway in viral entry [165].

TARGETING CYCLOPHILINS IN ANTI-VIRAL AND ANTI-CANCER THERAPY

The pleiotropic involvement of CypA in the lifecycle of various viruses opened the way for the development of broad range antiviral compounds. However, given the growing body of evidence linking deregulated cyclophilin expression with tumour development, metastasis and chemoresistance, it is not surprising that the family has recently gained interest as potential anti-cancer targets. The cyclophilins are already a target for immunosuppression, with Cyclosporine A (CsA), an eleven amino acid cyclic nonribosomal peptide, used to inhibit the immune response in organ transplant patients [13]. The immunosuppressive effect of the CsA-CypA complex, is independent of isomerase inhibition, and occurs by inhibition of the phosphatase calcineurin, which is required for T cell activation [166]. However, off-target effects associated with the CsA scaffold and their high molecular size, has limited their clinical use. In recent years, much effort has focussed on the development of non-immunosuppressant derivatives of CsA with applications as anti-viral agents. Overall,

a number of studies report that chemical inhibition of CypA by CsA and non-immunosuppressive CsA analogues can inhibit HIV-1 replication [167-169]. Alisporivir (Debio-025) is a first-in-class non-immunosuppressive cyclophilin inhibitor to enter clinical trial and has shown promise in the inhibition of HIV-1 [167, 170] and HCV [171-173].

Emerging data implicating cyclophilin proteins in tumourigenesis provides a rationale to investigate the anti-tumour effect of cyclophilin inhibitors. Early work using the non-immunosuppressive analogue of CsA, O-acetyl cyclosporin A showed a two-fold increase in sensitivity of lung cancer cells compared to CsA [174] however, little work has been carried out on O-acetyl cyclosporin A since. Interestingly, NIM811 induced apoptotic cell death in human melanoma cells. Moreover, studies in an *in vivo* murine model of melanoma showed that NIM811 retards tumour progression and significantly decreases tumour volume after intratumoral application [175].

CONCLUSION AND FUTURE PERSPECTIVE

It is intriguing that normal cells harbour relatively high cyclophilin levels [5], yet, knockout studies in mice indicate that CypA is not essential for mammalian cell viability [176]. In contrast, depletion of CypA causes cytokinesis defects and reduces the proliferation of tumour cells, supporting the view that CypA provides a growth advantage to tumour cells. Thus, due to its requirement during cytokinesis of rapidly dividing cancer cells, CypA represents a novel anti-mitotic drug target. The finding that CypA is not required for normal cell viability provides a therapeutic window to selectively kill cancer cells and to reverse acquired chemoresistance, without having detrimental effects on normal cells. However, not all cyclophilin proteins are involved in mitosis, yet, cyclophilin inhibitors developed to date block the activity of numerous family members. For instance, NIM811 inhibits CypA, CypC and CypD [177, 178]. Therefore, a major challenge is the development of compounds that can inhibit individual family members. Such compounds may have utility in the treatment of cancer and may sensitise resistant cancer subtypes to chemotherapy.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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The Role of Peptidyl Prolyl Isomerases in Ageing and Vascular Diseases

Lana McClements, Stephanie Annett, Anita Yakkundi and Tracy Robson*

School of Pharmacy, Queen's University Belfast, Belfast, United Kingdom

Tracy Robson

Abstract: Peptidyl prolyl isomerases (PPIases) are proteins belonging to the immunophilin family and are characterised by their cis-trans isomerization activity at the X-Pro peptide bond, in addition to their tetratricopeptide repeat (TPR) domain, important for interaction with the molecular chaperone, Hsp90. Due to this unique structure these proteins are able to facilitate protein-protein interactions which can impact significantly on a range of cellular processes such as cell signalling, differentiation, cell cycle progression, metabolic activity and apoptosis. Malfunction and/or dysregulation of most members of this class of proteins promotes cellular damage and tissue/organ failure, predisposing to ageing and age-related diseases. Many individual genes within the PPIase family are associated with several age-related diseases including cardiovascular diseases (CVDs), atherosclerosis, type II diabetes mellitus (T2D), chronic kidney disease (CDK), neurodegeneration, cancer and age-related macular degeneration (AMD), in addition to the ageing process itself. This review will focus on the different roles of PPIases, and their therapeutic/biomarker potential in these age-related vascular diseases.

Keywords: Ageing, age-related diseases, CypA, FKBPs, Pin1, PPIases, vascular.

INTRODUCTION

With an increase in life expectancy, the biggest challenge facing healthcare organisations is the management of age-related diseases. Age is the most strongly associated risk factor for diseases such as CVDs, cancer, T2D, CKD, neurodegenerative diseases (ND), AMD and atherosclerosis [1]. Therefore finding a way of slowing down ageing and delaying or preventing these age-related diseases will lead to longer life expectancy, healthy ageing, and a better quality of life, thus reducing the financial burden on healthcare systems.

Twin studies have shown that for cohorts born about 100 years ago, approximately 25% of the variation in population lifespan is determined by genetic differences and that the genetic influence on lifespan and age-related diseases in particular, becomes relevant in those people who survive to 60 years [2]. There have been major successes in the identification of new genetic variants involved in important age-related disorders including: cancer (in particular, prostate, breast and colon [3-5]); CVDs [6, 7] and CKD [8]. However, many of these genetic variants, individually or combined, explain only a small component of the heritability of each disease. This modest contribution does not match with the high recurrence risks of age-related disorders in families. This apparent paradox may in part be explained by the contribution of low frequency variants, unrecognized single nucleotide polymorphism (SNP) epistasis, gene-environment interactions, epigenetic and gene expression changes. Epigenetic data is particularly valuable to help interpret genome wide association studies (GWAS) by adding biological/mechanistic information [9, 10]. One of the major challenges over the next few decades will be to unravel the interactions between genetic variants and environmental factors. GWAS have shown that SNPs linked to multiple age-associated diseases are generally clustered on chromosome 6, in particular the Major Histocompatibility (MHC) locus within 6p21, in addition to the INK4/ARF (CDKN2a/b) tumour suppressor locus on chromosome 9p21.3. These SNPs accounted for almost a third of all the diseases analysed by GWAS [11].

Aberrantly activated pathways in ageing identified by association studies using long-lived cohorts include the insulin/insulin growth factor-1 (IGF-1), antioxidant, inflammatory, sirtuin, lipid metabolism, stress resistance and the mammalian target of rapamycin (mTOR) pathways [12-18]. The main targets of the insulin and IGF-1 pathway are the FOXO transcription factors which have important roles in stress resistance, immunity and metabolism [19, 20]. The sirtuin and mTOR pathways are nutrient-sensing pathways and these pathways are linked to longevity (high sirtuin and low mTOR levels) because of their ability to mediate the effects of nutrients and insulin. Since the mTOR pathway is a strongly implicated pathway, it represents a viable target for prevention of ageing and age-related disease. Peptidyl prolyl isomerases (PPIases) also known as immunophillins, are a family of proteins that bind to rapamycin-mTOR complexes and regulate the mTOR signaling pathway. As such, these proteins play a significant role in ageing and age-related diseases [21]. Therefore, the focus of this review will be on the role of PPIase in ageing and age-related diseases: CVDs, T2D, CKD, ND, AMD and cancer.

*Address correspondence to this author at the School of Pharmacy, Queen's University Belfast, 97 Lisburn Road, Belfast, UK, BT9 7BL;
Tel: +44/02890972360; Fax: +44/02890247794; E-mail: t.robson@qub.ac.uk

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PPIases

The PPIase family are important determinants of ageing and disease. Many individual genes within the PPIase family are associated with several age-related diseases, in addition to the ageing process itself. Peptidyl prolyl isomerases (PPIases) are proteins belonging to the immunophilin family and are characterised by their cis-trans isomerization activity at the X-Pro peptide bond. The term immunophilin is derived from the ability of these proteins to bind immunosupressive drugs; cyclophilins (18 members, 17 genes) bind to cyclosporine A and FKBPs (FK506 binding proteins; 17 members, 17 genes) bind to the macrolide, FK506. A third subfamily, parvulins (3 members, 2 genes), contain the PPIase domain but do not bind immunosuppressive drugs [22]. Immunosuppression is generally associated with the smaller PPIase-complexes and the larger PPIases lack this effect but they contain the tetratricopeptide repeat (TPR) domain facilitating protein-protein interactions, significantly impacting many essential cellular processes. Therefore, aberrant function of these proteins can lead to tissue damage and predisposition to ageing and age-related disease [23, 24].

Ageing

Ageing, in terms of endothelial system changes, encompasses molecular and functional modifications such as shortening of telomeres, structurally and functionally altered endothelial cells, increased levels of vasoconstrictive, pro-inflammatory, proliferative and pro-coagulatory substances, reduced nitric oxide (NO) bioactivity and apoptosis [25]. These processes lead to an increase in blood pressure, a reduction in the glomerular filtration rate, atherosclerosis and therefore to age-related diseases.

More recently, cellular senescence and changes in immune system surveillance have been identified as being the most significant processes in ageing due to their ability to activate pro-inflammatory pathways [26-28]. Other aberrant ageing processes include protein aggregation, DNA damage, mitochondrial damage and accumulation of reactive oxygen species (ROS; Fig. **1**). More recently, research has focused on the role of ageing stem cells on age-related diseases and the ageing process itself. Due to their long lifespan, stem cells are more prone to cellular damage as they accumulate ROS, damaged proteins, DNA damage, epigenetic alterations and mitochondrial dysfunction [29]. All of these aberrant changes can lead to stem cell apoptosis, senescence, dysfunction and thus the inability of stem cells to orchestrate tissue regeneration and proliferation.

The Role of PPIases in Ageing

The role of many individual PPIases in ageing has been studied. PPIases play a significant role by binding to and regulating the mTOR signalling pathway which has very well characterised roles in ageing and age-related diseases [21]. For example, CypA expression increases with ageing [30-32] while suppression of CypB induces cellular senescence [33] and its expression decreases in ageing rats [34]. Likewise, CypC [35, 36]; CypD [37-39]; CypJ [40] have all demonstrated significant roles in animal models of ageing. FKBPL, a divergent member of the FKBP group of immunophilins, resides on the gene loci, 6p21.3 which is within a significant peak of age-related disease association [11, 41].

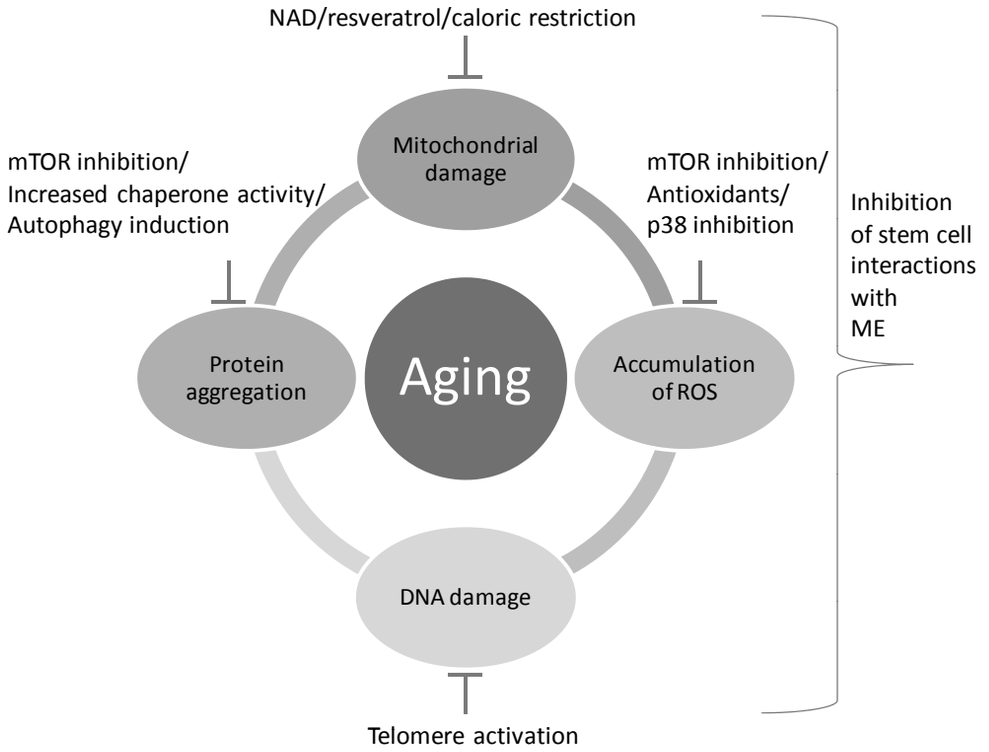

Fig. (1). Different mechanisms involved in ageing and the associated targeting strategies. *ME – microenvironment; NAD - nicotaminamide* adenine dinucleotide *(the sirtuin pathway activator).*

Importantly, Pin1 has the strongest link to ageing and is indeed a critical regulator of ageing; Pin1-/- mice develop normally but show pronounced and premature ageing, with reduced body size and bone density as well as atrophy of the skin, testis and breast [42]. Pin1 appears to control ageing by telomere shortening, via TRF1 phosphorylation and stability [43], and also regulates senescence, via the p53-BTG2 pathway [44].

CARDIOVASCULAR DISEASES

CVDs are the most common of all age-related diseases and are the leading cause of death in people over the age of 65 [45]. Considering that the ageing process leads to an overproduction of pro-inflammatory, pro-coagulatory, vasoconstrictive and other related factors, these process can brittle heart walls, leaky/thickened heart valves and deterioration in the heart muscle, leading to poorer ability to pump blood efficiently around the body [46]. Therefore, these changes together with the age-related changes in the endothelial system mentioned above, can lead to atherosclerosis, angina, atrial fibrillation and orthotropic hypertension, potentially causing myocardial infarction and stroke [47]. The key signalling pathways associated with CVDs include the insulin and IGF-1, sirtuin and mTOR pathways. The IGF-1 pathway appears to have a protective mechanism against atherosclerosis in humans whereas in mice it led to an increase in life span [48, 49]. The role of the sirtuin pathway in CVD is unclear due to a lack of consistency in the published data to suggest a strong role for this pathway in the development of CVDs; further research is therefore required [50]. On the other hand, the inhibition of the mTOR pathway, has demonstrated a role in longevity [51]; rapamycin can alleviate cardiac hypertrophy, T2D, adipogenesis and lipogenesis as such has a vital role in ageing and CVDs [47, 52]. Furthermore, the AMPK (AMP-protein activated kinase) signalling pathway, which negatively regulates mTOR, is also involved in CVDs; aberrant expression of AMPK in simple organisms, mice and humans has been implicated CVDs and ageing [53-55].

The Role of PPIases in CVDs

FKBP12, a cytoplasmic FKBP, has a well-established interaction with the ryanodine receptors, RyRs, resulting in the stability of this channel. FKBP12 knockdown results in the opening of the RyRs channel and augments calcium release into a wide range of tissues [56, 57]. Therefore, FKBP12 and FKBP12.6 have an important role in cardiac regulation and deficiency in these proteins contributes to the pathogenesis of hypertension. In murine models, FKBP12 knockout (KO) resulted in cardiac defects and altered RyRs function [58-61]. Therefore the treatment with FK506 and rapamycin may contribute to vascular dysfunction and hypertension by inducing intracellular leakage of calcium ions in endothelial cells [56, 62]. A novel antiarrhythmic compound, K201 (JTV– 519), which binds to FKBP12.6, thus stabilising RyRs channels and decreasing spontaneous calcium release, is currently in clinical trials [63].

The most abundant member of the cyclophilin family, CypA, is secreted exogenously in response to inflammatory stimuli and is able to increase ROS formation in endothelial cells, macrophages and vascular smooth muscle cells [64-67]. Therefore, CypA is a critical regulator of CVDs. In terms of vascular remodeling, CypA KO mice had significantly less thickened arteries when compared to the wild type (WT) mice and therefore are less likely to develop cardiac/vascular hypertrophy or myocardial ischaemia which can lead to myocardial injury [68-70]. CypA's involvement in ROS generation and cardiac fibroblast proliferation and migration, renders it responsible for the development of cardiac hypertrophy, the basis of most of the CVDs [70, 71]. Interestingly serum levels of CypA were significantly higher in patients with acute coronary syndrome (ACS) when compared to healthy patients or patients with stable angina and the levels also correlated with the severity of ACS, potentially suggesting a role for CypA as a biomarker to predict the severity of ACS [72]. Furthermore, CypA has a well-established role in atherosclerosis and the mechanisms involve an increase in the uptake of low-density lipoproteins by the vessel wall due to CypA-mediated overexpression of the scavenger receptors, pro-inflammatory and endothelial cell activation of vascular cell adhesion molecule-1 (VCAM-1) and a decrease of the endothelial nitric oxide synthase [32].

Another cyclophilin with a role in CVDs is CypD. Interestingly, it has a cytoprotective role during ischaemia-reperfusion injury as a regulator of the mitochondrial permeability transition pore (mPTP) complex formation [73, 74].

Finally, Pin1, the most extensively researched member of the parvulins subgroup (Pin1-3), has a significant role in cardiac hypertrophy. The loss of Pin1 attenuates cardiac hypertrophic responses following severe vasoconstriction by binding to Akt, mitogen activated protein kinase (MEK) and Raf-1; all essential components of the cardiac hypertrophy [75].

Type II Diabetes Mellitus

T2D is an age-associated disease, more specifically related to accelerated ageing. Most T2D patients are between the age of 65 and 74 [76]. T2D is more prevalent in men within this age group and its incidence decreases above 75 years of age [77]. The pathophysiology of T2D is very closely linked to the dysfunction of pancreatic islet β-cells in addition to insulin resistance [78]. The pancreatic β-cells appear to lose their proliferative, secretory and regenerative function as part of ageing, mainly due to cellular senescence [79]. Furthermore, the proliferative and apoptotic ability of the pancreatic β-cells seem to be the most apparent change in ageing, obese and diabetic patients. These cells are also able to adjust their proliferative activity in metabolic distress e.g. in metabolic syndrome, by increasing their self-renewal capacity to manage the increasing demand for glucose utilisation [79]. Interestingly, the pancreatic β-cells display similar characteristics to stem cells such as low proliferative profile and a very long lifespan [80]. The proliferative and regenerative capacity of β-cells might be diminished with age as a result of accumulation of DNA damage during their long lifespan or or senescence or apoptosis as a result of age-mediated shortening of the telomeres and/or activation of p53 and/or $p16^{INK4A}$ [81, 82].

Aberrant molecular mechanisms involved in the induction of cellular senescence of the pancreatic β-cells include telomere shortening and cycle-dependent kinase inhibition by p53 and $p16^{INK4A}$, which are also tumour suppressor genes [83]. Other pathways involved in the dysfunction of the pancreatic β-cells include the mTOR, sirtuin and IGF-1 pathways, also strongly associated with ageing and other age-related diseases [84]. Furthermore, the negative regulator of the mTOR pathway, AMPK, has also been significantly implicated in the metabolic disorders and T2D; aberrant expression of AMPK in both mice and humans leads to insulin resistance [54].

The Role of PPIases in T2D

The role of PPIases in T2D is still in its infancy however some interesting data has been recently reported to suggest an important role for this group of proteins in T2D. For example, FKBP51 SNPs were found to be associated with T2D phenotypes in large population studies [85]. Also, the change in FKBP51 gene expression was demonstrated in response to stress and diet therefore indicating a correlation between FKBP51 levels and higher food intake. Similarly, in mice, FKBP51 KO demonstrated a leaner phenotype when compared to the WT mice [86]. Furthermore, in conjunction with insulin resistance markers, FKBP51, as a steroid hormone responsive and regulatory gene, demonstrated an increase in the expression, following dexamethasone exposure [85].

Moreover, CypA has a role in T2D and vascular complications of T2D due to its pro-inflammatory role; patients with T2D were reported to have lower levels of CypA in high glucose-primed monocytes but high plasma levels of CypA when compared to healthy volunteers therefore suggesting a role for CypA as a biomarker of inflammation in T2D patients [87]. Moreover, PPIases in general have a varied role in the regulation of vascular function, suggesting possible involvement in T2D-mediated vascular abnormalities [56, 68-70, 73, 88].

Chronic Kidney Disease

CKD is a leading cause of morbidity and mortality. Epidemiological studies demonstrated around 13% prevalence worldwide [89, 90]. CKD arises from complete progressive destruction of nephrons resulting in the intact nephrons having to manage an increased load [8, 91]. Despite research efforts, the pathophysiology of CKD is still not fully understood, although vascular, glomerular and tubular events are implicated in the disease [92, 93]. Furthermore, podocytes or visceral epithelial cells within the Bowman's capsule have a role in preventing protein escape into the urine and therefore the loss of podocytes has been associated with the development of diabetic nephropathy [94, 95]. Aberrant mTOR activation is associated with this process and its inhibition by drugs such as rapamycin may be of a potential clinical benefit [94, 96]. Similarly, the mTOR pathway is involved in aldosterone mediated signalling through the mineralocorticoid receptor within renal tubular epithelial cells of distal nephrons; important for the regulation of fluid homeostasis [97, 98]. The activation of the mineralocorticoid receptor and its target genes including some of the PPIases, has been linked to tissue inflammation and fibrosis leading to CKD [99-101].

The Role of PPIases in CKD

In relation to CKD, FKBP12 exhibits an inhibitory activity on calcium oxylate crystal deposition and may prevent nephrolithiasis [102]. Nephrolithiasis is often perceived as a relatively minor acute illness, but increasing evidence suggests that it can lead to CKD [103-105]. Furthermore, the pathogenesis of the condition shares overlapping features of many diseases of ageing such as hypertension, CVD and T2D [104, 106, 107]. Recently, using a GWAS population analysis approach, FKBP51 has shown significant differences in DNA methylation in CKD patients [108]. Aldosterone plays a significant role in the development of CKD and evidence suggests that FKBP51 protein and mRNA expression are induced by aldosterone in the kidney and intestinal tissues [109-111]. On the other hand, CypA has a role in renal acidosis [112], diabetic nephropathy [113] and renal cell carcinoma [114]. Furthermore, Pin1 inhibition affects CKD associated with secondary parathyroidism [115].

Neurodegeneration

Neurodegeneration is the umbrella term for the progressive failure of neuronal networks leading to neuron death; many of these diseases share similarities at the sub-cellular level [116, 117]. Ageing is the main risk factor for the development of these diseases and the accumulation of atypical proteins, abnormal tangles and network dysfunction are classic hallmarks of these diseases [118].

Protein aggregation is a well-known feature of these diseases; however, the role of this process is not fully understood. Post-mortem examination of deceased brains have revealed that amyloid plaques in Alzheimer's disease and Lewy bodies in Parkinson's disease can be present even in asymptomatic patients and the extent of plaques present does not correlate to the severity of the disease at the time of death [119].

Sustained activation of neuronal PI3K/Akt/mTOR signalling has been noted in early Alzheimer's disease [120]. In the temporal lobes of Alzheimer's patients, Akt activation leads to mTOR and tau phosphorylation and a decrease in cyclin-dependent kinase inhibitor 1 [121]. Furthermore, the aberrant activation of the Akt pathway has been linked to disrupted clearance of Aβ and tau resulting in synaptic loss and cognitive decline [120]. Nevertheless, the cause of Alzheimer's disease is still largely unknown however the most prevalent genetic risk factor is the presence of ε4 allele of the apolipoprotein E (APOE) and it is expressed in half of sporadic Alzheimer's disease cases [122, 123].

On the other hand, Parkinson's disease is the second most common ND after Alzheimer's diseases. It is a degenerative disorder resulting from the death of the dopamine producing cells in the substantia nigra (SN) [124-126]. Age-related mitochondrial dysfunction and alterations in protein degradation are more detrimental to the neurons in the SN than in any other regions of the brain [127]. The classic hallmark of this disease is the presence of the protein alpha synuclein which binds to ubiquitin in damaged cells forming

eosinophilic cytoplasmic inclusions called Lewy bodies [125, 126, 128]. In Parkinson's disease, the PI3K/Akt/mTOR pathway is dysregulated in a different manner than in Alzheimer's disease. The dopaminergic neurons from Parkinson's patients display downregulation of phosphorylated Akt and supressed mTOR signalling resulting in neuronal death [129]. Furthermore, rapamycin, the inhibitor of mTOR, has a neuroprotective effect by protecting phosphorylated Akt at a critical site for cell survival [130].

The Role of PPIases in Neurodegeneration

Calcium dysregulation contributes to unhealthy brain ageing by reducing neural excitability and impairing memory. Disruption of FKBP12 in the hippocampal neurons destabilised calcium and *in vivo* FKBP12 knockdown is associated with an upregulation of RyR2 and mTOR protein expression [131]. FKBP12 has been shown to bind to the intracellular domain of the amyloid precursor pathway and shift APP processing to the amyloidogenic pathway [132, 133]. Moreover, the FKBP12 gene expression is downregulated in the hippocampus of ageing rats and in early stage Alzheimer's patients [134]. When FK506 is used as an immunosuppressant agent, it appears to have neuroprotective effects [135].

FKBP38 is a well-known inhibitor of apoptosis through a reduction in mitochondrial Bcl-2 [136, 137]. Hsp90 can inhibit the apoptotic function of FKBP38 by interfering with the FKBP38/calmodulin/calcium complex which regulates the anti-apoptotic protein, Bcl-2 [138]. This property of FKBP38 protein has been exploited for the treatment of ND [137, 139].

FKBP51's PPIase activity has a role in microtubule stabilisation through Hsp90-mediated dephoshorylation of tau [140, 141]. On the other hand, FKBP52 is ubiquitously expressed at high levels and has been associated with microtubule destabilisation and tubulin depolymerisation [141-143]. FKBP51/FKBP52 bound to heat shock proteins may have a role in neurodegeneration by modulating protein folding and aggregation [24]. FKBP51 siRNA knockdown reduced tau levels in HeLa cells and FKBP51 overexpression increased levels of tau. [140]. In addition, knockdown of Hsp90 also reduced levels [144]. In contrast, FKBP52 overexpression downregulated tau protein levels and knockdown resulted in increased tau binding to microtubules, resulting in longer projections [132, 143]. Cao and Konsolaki proposed that the opposing effects of FKBP51 and FKBP52 could be due to the differences in PPIase activity as tau contains a high percentage of proline residues [132]. Furthermore, FKBP52 is upregulated after injury in regenerating neurons and Alzheimer's patients have a lower expression of FKBP52 in the temporal lobe and hippocampus [132]. FKBP52 is involved in the regulation of intracellular copper and this may cause FKBP52 to have an effect on Aβ levels [145-147]. Furthermore, Conejero-Goldberg and colleagues demonstrated that FKBPL was one of the key genes differentially expressed in the brain tissue, where it appeared to act in a protective role, in young individuals at high risk of Alzheimers disease preselected by the APO4 signature [148].

The role of CypA in Alzheimer's disease has also been reported, possibly due to its ability to activate pro-inflammatory pathways, NF-κB and MMP-9; these pathways in brain capillary pericytes regulate the release of neurotoxins. This whole process is initiated by APO4 within astrocytes [149]. CypD's involvement in the mPTP complex has also found application in Alzheimer's disease due to recent reports which suggest that Aβ proteins influence mPTP formation when in a complex with CypD [150]. Conversely, the loss of Pin1 expression is correlated with Alzheimer's disease and neurodegeneration due to Pin1's important role in the stabilisation and regulation of tau and Aβ proteins [42]. Tau protein hyperphosphorylates in the absence of Pin1 leads to its dysfunction and inability to regulate microtubule stabilisation in the neurons [151].

Age-related Macular Degeneration

AMD is a leading cause of blindness worldwide and old age is the major risk factor with an incidence of 10% in individuals over 80 years of age [152, 153]. It results from degeneration of the macular region of the retina, a central part of the retina and AMD susceptibility is increased by age, environmental (e.g. smoking) and genetic factors [153, 154]. Many different genetic factors have been implicated in AMD including SNPs within some of the proteins involved in the mTOR pathway [155].

The Role of PPIases in AMD

In AMD, GWAS detected the presence of a SNP on chromosome 6p21 in the FKBPL region, therefore suggesting a potential role for FKBPL as an AMD susceptible gene [156]. This study was carried out using two cohorts of advanced AMD patients against matched controls to validate the findings and it also indicated Notch4 as a potential AMD susceptible gene. Our own lab has generated data to suggest that in addition to the well-established FKBPL's regulatory role of the CD44 pathway, it is also involved in the regulation of the Notch pathway (unpublished data).

Cancer

Cancer is defined as the development of 'abnormal cells' due to genetic and epigenetic changes in oncogenes and tumour suppressors [157]. These genetic changes can be inherited, acquired by various DNA damaging agents or certain types of viruses. There are a few theories of carcinogenesis nevertheless it is considered a multistep process involving genetic instabilities which drive normal cells to malignant, cancer cells. More recently, a subgroup of cancer cells, termed cancer stem cells (CSCs) or tumour initiating cells, have been characterised as a group of cells carrying the oncogenic and tumour suppressor mutated genes responsible for tumour initiation and progression [158].

Numerous cellular and intracellular pathways regulating tumourigenesis have been implicated in the development of cancer. A pathway readily activated as a result of a loss of the main tumour suppressor genes, p53 or PTEN, is PI3K-Akt survival pathway [159]. This pathway regulates the mTOR pathway and once the mTOR pathway is activated, negative feedback results in PI3K inhibition. Therefore when the mTOR pathway is inhibited by rapamycin, for example,

the mutated or lost negative feedback loops, commonly present within cancer cells, activate the PI3K-Akt pathway instead of inhibiting it, thereby preventing the anti-proliferative effect of the mTOR pathway inhibition [160, 161].

The Role of PPIases in Cancer

The roles of PPIases in cancer have been studied extensively. Some members appear to have oncogenic activity whilst others behave as tumour suppressors. FKBP12 is overexpressed in benign and malignant endothelial-lined vasculature and as a natural ligand of TGF-β receptor I is subsequently involved in regulating cancer invasion [162]. Knockdown of FKBP12 results in the cell cycle arrest at the G1 phase by downregulation of TGF-β signalling [163]. Furthermore, FKBP12 activates TGF-β receptor I kinase thus triggering apoptosis by a mitochondrial dependent pathway [164]. In addition, it is a regulator of H-Ras trafficking by promoting depalmitoylation through its PPIase activity [165]. Disruption of the interaction between FKBP12 and calcineurin signalling leads to potent anti-angiogenic effects and tumour growth inhibition in breast cancer [166].

FKBP38 is capable of potentiating the biological function of Bcl-2 protein leading to tumourigenesis and chemoresistance [137, 167]. Furthermore, Bcl–2 overexpression has been associated with the cancer stem cell phenotype and it may contribute to chemoresistance within these cells [168].

FKBP51 expression is hormone related and its overexpression has been associated with leukaemia, breast, prostate and brain tumours [169, 170]. FKBP51 is a negative regulator of the Akt pathway and regulates cell response to chemotherapy [171]. Furthermore, FKBP51 regulates the NF-κB pathway which is implicated in apoptosis and radioresistance in melanoma cells [172, 173]. More recently, the role of FKBP51 in stemness and metastasis in melanoma was demonstrated by Romano *et al.* (2013), where FKBP51 was overexpressed and associated with tumour aggressiveness and treatment resistance by stimulation of the EMT process, migration and invasion via the TGF-β pathway [174]. Furthermore, androgens upregulate FKBP51 by initiating direct binding between FKBP51 and the androgen receptor (AR) [175]. In murine xenograft models it was demonstrated that FKBP51 is a direct regulator of cell growth and may have a role in the highly invasive androgen-independent type of prostate cancer [175, 176]. The FKBP51/AR interaction is mediated by Hsp90, and Hsp90 inhibitors such as geldanamycin are currently in clinical trials in a variety of cancers [177, 178]. In pancreatic cancer, FKBP51 acts as a scaffolding protein to the phosphatase PHLPP resulting in upregulation of the pro-survival Akt pathway and reducing sensitivity to the chemotherapy [171]. Conversely, in colorectal adenocarcinoma, FKBP51 suppresses proliferation through its action on the glucocorticoid receptor [179].

Less is known about the role of FKBP52 in cancer, although its inhibition has been shown to block AR dependent gene expression and prostate cancer cell proliferation [180]. Moreover, FKBP52 is highly expressed in hormone-positive cancers such as oestrogen receptor positive (ER+) breast cancer; its expression in pre-invasive breast cancer was also much higher than the surrounding normal breast tissue speculating its role in breast cancer initiation [181, 182]. FKBP52 is not a functional regulator of the ER but interestingly, it is upregulated in breast tumours and FKBP52 gene methylation only occurs in ER negative breast cancer cells [183]. Furthermore, FKBP52 auto-antibodies may be a useful biomarker for early diagnosis and monitoring of breast cancer [184].

FKBP65 is highly expressed in early benign lesions in the colon, compared to normal mucosa [185]. This suggests that FKBP65 may be involved in colorectal carcinogenesis and could be a novel colorectal biomarker [184]. FKBP65 is strongly expressed in normal and benign ovarian epithelium but a low expression in high grade serous carcinoma (HGSC) is probably due to frequent loss of chromosome 17 [186, 187]. This indicates a tumour suppressor function for FKBP65 in ovarian carcinomas.

FKBPL has a well-established role in cancer and whilst most FKBPs are positive regulators of cancer growth, FKBPL, as a divergent member of this family, is not. FKBPL acts as a co-chaperone protein in a complex with Hsp90 where it has a regulatory role in steroid receptor signalling (ER) [188]; (AR) [189]; (GR) [190]. Due to this negative regulatory effect of the steroid receptors, overexpression of FKBPL demonstrated inhibition of cancer cell growth in ER+ breast cancers [188]; in lymphoma this inhibitory effect was associated with a FKBPL, Hsp90 and p21 complex [191]. Furthermore, in ER+ breast cancer, high FKBPL levels improved the response to endocrine therapy such as tamoxifen and fulvestrant and sensitised cells to oestrogen deprivation and was also prognostic for survival [188]. Other relevant roles of endogenous FKBPL in a complex with Hsp90, p21 and GTSE-1 (G2 and S phase expressed protein 1) include, chemo- and radiosensitivity via regulation of the cell cycle protein, $p21^{CIP1/WAF1}$, and a reduction in the DNA repair [192, 193]. All of these FKBPL-related roles are associated with intracellular FKBPL however more recently, an extracellular role for FKBPL was identified. This extracellular role was associated with a potent anti-angiogenic and anti-CSC function which is initiated following binding of FKBPL to the CD44 cell surface receptor [194-196]. The region responsible for this interaction is the N-terminal region of FKBPL, which is unique and not homologous to other FKBPs. Based on this anti-angiogenic domain, a clinical candidate 23-amino acid therapeutic peptide, ALM201, was designed in collaboration with Almac Discovery which will enter clinical trials this year [197]. Therefore, FKBPL as a divergent member of the FKBPs appears to be involved in similar biological processes to other FKBPs whilst exerting an opposite function as an anti-cancer or tumour suppressor protein.

In cancer CypA is significantly upregulated and as such is involved in malignant transformation, tumour growth, invasion, metastasis and the inhibition of apoptosis [198-201]. This is not surprising considering CypA has a role in the stimulation of endothelial cell migration which is important for tumour growth and invasion [69]. Furthermore, CypA is transcriptionally regulated by p53 and hypoxia in-

ducible factor-1α (HIF-1α) both factors commonly mutated in cancer [202]. CypA seems also responsible for paclitaxel-induced resistance in endometrial cancer and overexpression of CypA can reduce cisplatin and hypoxia-induced apoptosis [203, 204]. On the other hand, CypA has also an important role in the cytokinesis where it relocalises from its original position, in the centrosome, to the midbody; the loss of CypD leads to defective cytokinesis which can increase genomic instability associated with cancer [205]. Other members of the Cyp family group CypB, CypC and CypD in addition to CypA also appear to be upregulated at the transcriptional levels in various cancers [202]. CypB and CypC are associated with the ER and as such form various complexes with other oestrogen-related chaperones and CypB, in particular, protects cells from ER stress-induced death [206, 207]. However, overexpression of CypB has been linked to tumour progression because it regulates various hormone receptors and their downstream targets [208]. Also, CypB could be a useful target for delivery of anti-cancer vaccines due to its two antigenic epitopes identifiable by the cytotoxic T-lymphocytes [209]. Moreover, Cyp40 mRNA levels were reported to be high in response to stress in breast and prostate cancer cell lines when compared to normal breast and prostate cell lines [210]. CypD, as mentioned above, is involved in mPTP complex formation and as such has a role in the resistance to mPTP-induced cancer cell death. This is mediated by other co-chaperone proteins such as Hsp90 and TRAP which are highly expressed in cancer cell mitochondria and their ability to inhibit CypD therefore disabling mPTP formation and its apoptotic effects [211]. Also, mitochondrial CypD knockdown is associated with STAT3 activation which leads to an increase in cell proliferation, by accelerating entry into S-phase, and migration, via the chemokine network, CXCL12-CXCR4 [212]. Both of these phenotypes are closely linked to cancer progression and metastasis. Furthermore, $CypD^{-/-}$ mice exhibit similar phenotype to $Pin1^{-/-}$ mice in terms of abnormalities observed in retina and breast development which could potentially lead to malignant transformations in these organs [213, 214]. Also, transcriptional and posttranslational regulation of CypD by Pin1 was demonstrated in the more recent study therefore explaining the phenotypical similarities observed in $CypD^{-/-}$ and $Pin1^{-/-}$ mice [214].

Pin1 has been researched extensively for its oncogenic role in cancer [215]. Pin1 is also important for tumourigenesis and for the regulation of CSCs via the Notch pathway [216]. In fact, deletion of Pin1 in mice prevented oncogenic activation of Neu and Ha-Ras which abrogated breast cancer [217]. In p53-KO mice Pin1 deletion was able to completely abrogate tumour development but had adverse effects including thymic hyperplasia mediated via the Notch pathway [218]. Nevertheless, Pin1 does not affect the p53 tumour suppressor activity [219]. Other cancer-associated processes that Pin1 affects include regulation of cell cycle, DNA damage, cell signaling, transcription and splicing [220]. In terms of cell-cycle regulation, Pin1 also has a role in cytokinesis by binding to the crucial centrosome protein, Cep55, which further explains its role in tumourigenesis [221].

PPIases as Targets to Prevent Ageing or to Treat Age-related Diseases

An advantage of characterising this gene family is that they are targetable and various drugs targeting these proteins have been reported, including FK506, sirolimus/rapamycin, cyclosporine, and tacrolimus [22]. Ligands of these proteins, although first approved as immunosuppressive agents, for the prevention of allograft rejection, are effective against age-related diseases. Several FKBP-binding macrocyclic drugs, everolimus, zotarolimus and temsirolimus are in phase III trials as targets for cell proliferation, immunosuppression and anti-cancer effects [23]. Recent evidence has also identified rapamycin/sirolimus as being the first drug to extend lifespan in a range of species from yeast to mammals [222, 223], highlighting the potential for drug targeting within this gene family to alleviate the ageing process. Importantly, recent studies have also shown that FK506-binding proteins can modulate Akt-mTOR signalling in the absence of rapamycin [21].

One of the problems associated with PPIase inhibitors however, is their off-target effects, particularly and not surprisingly, immunosuppression. However, more recently there has been a concerted effort to generate compounds that lack immunosuppressive activity, with various levels of success. Examples of such compounds include non-immunosuppressive analogues of cyclosporine A which may have applications in multiple therapeutic areas e.g. Alisporivir (Debio 025) and NIM811 [224, 225]. Similarly, the development of cell impermeable, non-immunosuppressive analogues of cyclosporine A has permitted the inhibition of extracellular CypA in mouse models of inflammation [226]. Such drugs have huge potential in the treatment of ageing disease in which CypA is involved.

The novel FKBPL-based therapeutic, ALM201, unlike other PPIases, appears to be protective of age-related diseases [148, 155, 188, 194, 195, 197]; ALM201 is a peptide mimetic of FKBPL and could essentially correct a deficiency in FKBPL in a number of diseases. It has already completed preclinical evaluation for imminent phase I/II clinical trials in cancer patients [197].

CONCLUSION

In conclusion, there are many different significant roles of PPIases in age-related processes and diseases as indicated above (Table **1**; Fig. **2**). Even though these proteins belong to the same family group, their roles are quite diverse and in some instances opposite. Therefore, it is of a paramount importance to elucidate the mechanisms involved in the interactive regulation of this gene family. This may allow the development of a genetic signature which could stratify patients with higher predisposition to unhealthy ageing therefore enabling early treatment to delay or prevent these age-related vascular diseases and their complications. Members of this family of proteins are therefore excellent targets for interventions as well as biomarkers of ageing and age-related diseases. Because many of the PPIase family members are secreted

Table 1. Summary of the roles of PPIases in the age-related diseases. ROS – reactive oxygen species; ACS – acute coronary syndrome; MEK - mitogen activated protein kinase; mPTP - mitochondrial permeability transition pore; APP – amyloid precursor protein; T2D – type II diabetes; RyRs - ryanodine receptors; AR-androgen receptor; HIF-1α – hypoxia inducible factor 1 α; ER – oestrogen receptor; CSCs – cancer stem cells.

Age-Related Diseases	PPIase	Mechanisms	References
Cardiovascular diseases	FKBP12	Calcium augmentation; RyRs channel regulation; hypertension	[56-63]
	Cyclophilin A	Pro-inflammatory; generation of ROS; atherosclerosis, ACS biomarker	[67-72]
	Cyclophilin D	Stabilisation of the mPTP complex;	[73, 74]
	Pin1	Binds to Akt, MEK and Raf-1	[75]
Type II diabetes	FKBP51	Associated with T2D phenotype; gene expression changes in response to stress and diet	[85, 86]
	Cyclophilin A	Biomarker of inflammation in T2D and vascular complications	[70, 87]
Chronic Kidney Disease	FKBP12	Calcium oxylate crystal deposition	[102]
	FKBP51	DNA methylation; expression induced by aldosterone	[109-111]
	Cyclophilin A	Association with renal acidosis, diabetic nephropathy, renal cell carcinoma	[112-114]
	Pin1	Downregulation in secondary parathyroidism, complication of CKD	[115]
Neurodegeneration	FKBP12	Augmentation of calcium and APP processing; downregulation in Alzheimer's disease	[131-135]
	FKBP38	Inhibition of mitochondrial Bcl-2 in the brain	[136-139]
	FKBP51	Microtubule stabilisation through Hsp90 dephosphorylation of tau	[140-142]
	FKBP52	Microtubule destabilisation and tubulin depolymerisation	[133, 142-147]
	FKBPL	Protective role in Alzheimer's disease	[148]
	Cyclophilin A	Regulation of inflammatory pathways, NF-κB and MMP-9; release of neurotoxins	[149]
	Cyclophilin D	Stabilisation of mPTP complex; regulation of Aβ protein activity within mPTP complex	[150]
	Pin1	Stabilisation and regulation of tau and Aβ	[42, 151]
Age-related macular degeneration	FKBPL	AMD susceptibility gene	[155, 156]
Cancer	FKBP12	Apoptosis via TGF-β mitochondrial pathway	[162-166]
	FKBP38	Chemoresistance via Bcl-2	[167-169]
	FKBP51	Regulation of Akt, NF-κB pathways and AR; chemoresistance, apoptosis, stemness	[170-179]
	FKBP52	AR-dependent gene expression in prostate cancer; novel breast cancer biomarker; DNA methylation in ER- breast cancer	[180-184]
	FKBP65	Malignant transformation in colorectal cancer; downregulation in ovarian carcinoma	[185-187]
	FKBPL	Anti-angiogenic and anti-cancer stem cell properties via CD44 dependent mechanism; steroid receptor and cell cycle regulation	[188-197]
	Cyclophilin A	Regulated by p53 and HIF-1α; stimulation of endothelial cell migration; apoptosis, invasion, metastases chemoresistance	[114, 198-205]
	Cyclophilin B	Regulates ER complexes; protection against ER stress induced death, tumour progression in breast cancer	[206-209]
	Cyclophilin 40	mRNA levels regulated in response to stress in breast and prostate cancer	[210]
	Cyclophilin D	mPTP complex formation; apoptosis resistance	[211-214]
	Pin1	Oncogenic potential; regulation of CSCs through the Notch pathway	[214-221]

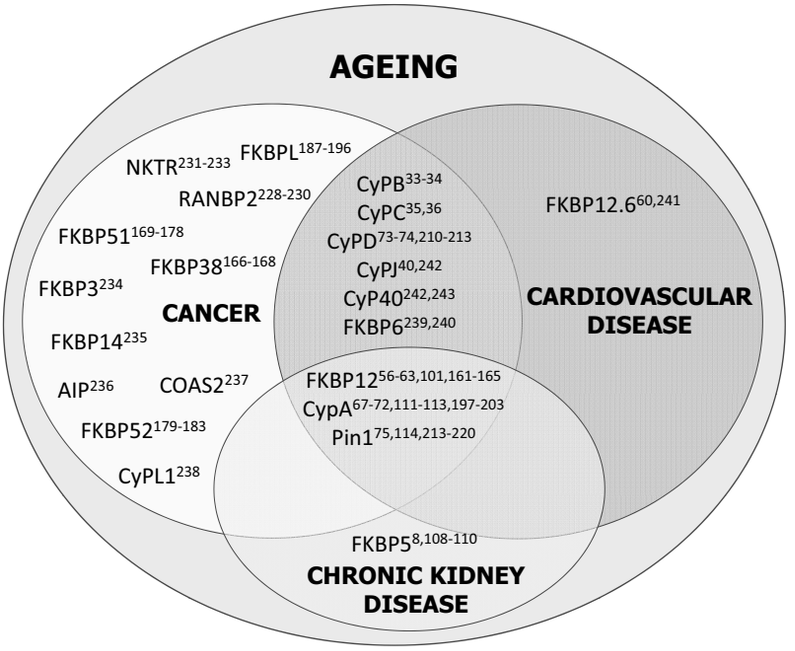

Fig. (2). PPIases in ageing and age-related diseases (cancer, cardiovascular disease and chronic kidney disease).

[227, 228], monitoring them within ageing populations will be minimally invasive and therefore practical for routine clinical use or home test.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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