Nuclear Receptor Research
Vol. 1 (2014), Article ID 101094, 17 pages
doi:10.11131/2014/101094

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Review Article

The Emerging Role of TPR-Domain Immunophilins in the Mechanism of Action of Steroid Receptors

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Recieved 26 May 2014; Accepted 28 August 2014

Editor: Susana Castro-Obregon

Abstract. In the absence of ligand, some members of nuclear receptor family such as corticosteroid receptors are primarily located in the cytoplasm, and they rapidly accumulate in the nucleus upon ligand-binding. Other members of the family such as the estrogen receptor are mostly nuclear. Regardless of their primary location, these oligomeric proteins undergo a dynamic nuclear-cytoplasmic shuttling, and their transport through the cytoplasmic compartment has always been assumed to occur in a stochastic manner by simple diffusion. Although heuristic, this oversimplified model has never been demonstrated. Moreover, it has always been assumed that the first step related to receptor activation is the dissociation of the Hsp90-based heterocomplex, a process referred to as 'transformation.' Nonetheless, recent experimental evidence indicates that the chaperone machinery is required for the retrotransport of the receptor throughout the cytoplasm and facilitates its active passage through the nuclear pore. Therefore, transformation is actually a nuclear event. A group of Hsp90-binding cochaperones belonging to the immunophilin family plays a cardinal role not only in the mechanism for receptor movement, but also in nuclear events leading to interactions with nuclear sites of action and the regulation of transcriptional activity. In this article we analyze the importance of molecular chaperones and TPR-domain immunophilins in the molecular mechanism of action of steroid receptors.

Keywords: Steroid receptor, TPR-domain immunophilins, Hsp90, Dynein, Trafficking

1. Introduction

Protein transport is a fundamental mechanism for the regulation of protein localization and, consequently, protein function. Thus, it is hardly surprising that several pathologies are related to mislocalization and altered function of a variety of proteins, which may lead to cell death, cell proliferation, or initiation and progression of cancer [1–8]. It is currently accepted that soluble proteins are not confined to the cytoplasm or the nucleus in a static manner but are capable of shuttling dynamically through the nuclear pore [5, 9–11]. This property is particularly relevant for members of the nuclear receptor family since they may exist in the cytoplasm as transcriptionally inactive proteins that must translocate to the nucleus upon ligand activation to become transcriptionally active factors. Moreover, the structural reorganization and subcellular redistribution of nuclear receptor proteins is an essential step to acquire certain functions and/or repress others.

For years, an unsolved question that pertains to all signalling pathways that act via effects on gene transcription relates to how soluble factors move throughout the cytoplasm to reach the nuclear compartment. The same unsolved problem is also valid for the intranuclear movement of these factors. Actually, our knowledge about the latter mechanism is even more limited. It has always been assumed that the driving force commanding steroid receptor movement throughout the cytoplasm was a passive, simple diffusion mechanism. According to the classic model, this is triggered by dissociation of the Hsp90-based chaperone complex and, subsequently, the nuclear localisation signal (NLS) becomes 'exposed' and is recognized by the specific nuclear import machinery formed by importins-RanGTP proteins [12–18]. Such stochastic mechanism of movement implies that random collisions occur between soluble receptors and cell structures. After an effective collision, signaling proteins become trapped at their sites of action by protein-protein or protein-nucleic acid interactions. If the mechanism were this, it would be difficult to explain how each protein exerts specific effects when a given cascade is activated since the responsible protein for triggering the process would freely spread throughout one or more cell compartments. Moreover, a mechanism based solely on free diffusion collides with a basic biological concept; that is, work is highly compartmentalized in the cell. Inasmuch as proteins normally occupy the entire cell compartment upon their activation, additional mechanisms, which may include specific protein-protein interactions, must regulate protein movement to focus those proteins to specific targets.

Cryoelectron tomography images demonstrated that the cytoplasm has highly packed assembles of organized filaments and macromolecules forming interconnected functional structures rather than freely diffusing and colliding soluble complexes [19]. Even though this organization should permit the transport of soluble proteins by simple diffusion, it clearly makes the delivery of signaling factors less efficient. When the efficiency of this transport is low, soluble proteins are usually targeted to degradation [20].

Steroid receptors are a good experimental model to analyze the molecular mechanisms involved in the transport of soluble proteins due to the fact that the subcellular localization can be manipulated in a very simple manner. Some of these ligand-activated transcription factors are primarily located in the cytoplasm in the absence of hormone. This is the case for glucocorticoid receptor (GR) [21, 22], mineralocorticoid receptor (MR) [23, 24], androgen receptor (AR) [25], dioxin receptor (AhR) [26], or vitamin D receptor (DR) [27]. Upon ligand-binding, these receptors rapidly move (minutes) towards the nucleus, whereas they cycle-back to the cytoplasm in a slower manner (several hours) upon ligand withdrawal [28]. Other members of the family such as ER [29] or PR [30] are primarily nuclear in the absence of ligand. Of course there are exceptions to the general rule according to the cell type and physiologic condition. For example, in the absence of steroid, the MR is usually more nuclear in COS- 7 cells [31] and CHO cells [32] than in mouse fibroblasts and rabbit duct cells [24], whereas it is entirely nuclear in cardiomyocytes [33]. Surprisingly, GR is fully nuclear in WCL2 cells [34], and PR is cytoplasmic rather than nuclear in endometrial cancer cells [35]. Regardless of their primary localization, however, these receptors (and other nuclear factors) undergo a permanent and dynamic nucleocytoplasmic shuttling. Clearly, such diversity is most likely related to a dissimilar import/export balance, which could be due to the expression balance of TPR-domain immunophilins [36, 37], as we will discuss later in this article. The biological relevance of the nucleocytoplasmic shuttling is implied by the fact that the intersection of the predicted interactome for the Hsp90/Hsp70 chaperone machinery and the interactome of steroid receptors represents ˜20% of genes whose products are related to intracellular transport and/or nucleocytoplasmic shuttling [38].

It was first postulated [39] that GR activation proceeded until an equilibrium between Hsp90-free and Hsp90-bound receptors is reached, such that Hsp90 release (a process referred to as 'transformation') consisted of a simple change in the conformation of the receptor molecule induced by steroid binding. This triggers a series of conformational and structural changes that result in dissociation of the chaperone complex unmasking a nuclear localisation sequence that allows receptor trafficking to the nucleus via classical import mechanism. This classic model for the mechanism of action of steroid receptors was posited several years ago [39, 40] and endured for decades [41]. It supported the heuristic notion that the receptor•chaperone heterocomplex must be dissociated immediately after steroid binding. This transformation permits the simple diffusion of the receptor towards the nucleus. GR and MR are the members of the steroid receptor family that show the highest cytoplasmic to nuclear localization ratio in the absence of steroid. Therefore, most of the recent advances to elucidate the putative transport mechanism for steroid receptors were reached in studies where these two ligand-activated transcription factors have been used as experimental model. The current evidence indicates that the cytoplasmic movement of receptors is not passive and requires the assistance of the molecular chaperones associated to receptors.

2. Molecular Chaperones and TPR-Domain Immunophilins

The classic concept of molecular chaperone sustains that they are able to recognize structural elements of unfolded or partially denatured polypeptides preventing or rescuing the incorrect intermolecular association of improperly folded or unfolded proteins, a situation that ultimately leads to their aggregation and/or proteasome degradation [42]. They are induced by several stimuli such as heat, cold, radiation,

UV light, metals, toxics, reactive oxygen species, organic solvents, and any situation of stress. These chaperons show highly flexible conformation such that they can adapt to different environmental conditions and interact with several client proteins.

Conformational changes are triggered by slight modifications of temperature, and the expression of their genes is greatly and efficiently induced. It is accepted that nearly 50 to 200 genes are induced from archaea to human [43] and that the leading group across the species in terms of induction level are the heat-shock proteins (Hsps). Among them, Hsp90 is a distinctive Hsp because, in addition of showing all the properties that define a molecular chaperone, its principal role in the cell is to provide biological activity to properly folded client proteins that show a preserved tertiary structure [44, 45]. In other words, Hsp90 works as a delicate and refined sensor of protein function rather than a mere folding factor. This is particularly important for steroid receptors because Hsp90 is the protein that provides them ligand-binding capacity [46].

A particular group of chaperones has been classified into a particular group, the immunophilins (IMMs). They are comprised of a family of intracellular proteins with peptidyl-prolyl-*cis/trans*-isomerase (PPIase) activity, that is, *cis*↔*trans* interconvertion of Xaa-Pro bonds. In turn, they are subclassified by their ability to bind immunosuppressant drugs –c*yclophilins* bind cyclosporine A and *FKBPs* (*FK506- binding proteins*) bind FK506 [46–48]. The signature domain of the family is the PPIase domain. Only the low molecular weight IMMs FKBP12 and CyPA are related to the immunosuppressive effect when the drug•immunophilin complex inhibits the Ser/Thr-phosphatase activity of PP2B/calcineurin [49]. High molecular weight IMMs have three additional domains –the nucleotide-binding domain, where ATP binds, the calmodulin-binding domain, a poorly characterized domain able to interact with calmodulin, and TPR domains, sequences of 34 amino acids repeated in tandems through which they bind to Hsp90 [50]. TPR proteins may show tandem arrays from 3 to 16 motifs [51], which usually fold together to produce a single and linear solenoid domain. Not all TPR proteins are able to interact with Hsp90, but when the association is possible, Hsp90 forms dimers that limit only one TPR acceptor site per dimer [52]; that is, TPR-domain proteins are able to associate to Hsp90 when they compete one another for the only one TPR acceptor site generated per Hsp90 dimer. This shows important consequences from the biological point of view. In this regard, the most frequent competitive binding showing opposite biological effects takes place between two TPR-domain IMMs that share 60% identity and 75% similarity in their amino acid sequences, FKBP51 and FKBP52 [53]. Both proteins are highly homologous not only because of their amino acid sequences, but also due to their domain organization and three-dimensional structures [54].

3. The Hsp90•FKBP52•dynein/dynactin Molecular Machinery of Transport

Steroid receptors form heterocomplexes with molecular chaperones. The final heterocomplex undergoes a process of maturation (Figure 1) where the TPR-domain protein Hop/p60 plays an initial key role bringing together dimers of Hsp90 with the Hsp70/Hsp40 complex. Hop/p60 is critical because it stabilizes the open conformation of Hsp90 dimers and prevents its intrinsic ATPase activity and Hsp90 interaction with p23 [55]. The oligomeric complex is transferred to the receptor in an ATP-dependent manner, favoring the recruitment of a stabilizer of the complex, the small acidic cochaperone p23. As a consequence, Hsp90 closes its open conformation weakening Hop/p60 binding, which is released in a BAG-1/Hip-assisted mechanism **[55, 56]**. The empty TPR acceptor site is then occupied by another TPR-domain protein, a high molecular weight IMM. To date, the IMMs that have been recovered with nuclear receptors are FKBP51, FKBP52, and CyP40, as well as the immunophilin-like proteins PP5, XAP2/AIP, and FKBPL/WisP39 [57]. The latter group also possesses both the TPR and PPIase domains, but their members lack enzymatic activity of prolyl-isomerase.

The stoichiometry of the final receptor complex includes a dimer of Hsp90, one molecule of Hsp70, one molecule of the cochaperone p23, and one molecule of a TPR-domain protein [58, 59] (Figure 1). While the TPR-domain cochaperone Hop/p60 is only present during the maturation cycle and is not part of mature heterocomplexes, most steroid receptors recruit other TPR factors such as FKBP51, FKBP52, PP5, FKBPL/WisP39, or CyP40. Among them, FKBP51 is the only IMM unable to interact with dynein/dynactin complexes [60–62]. Even though the biological function of these IMMs remains poorly understood, it is accepted that they are not related to immunosuppression, a property that concerns to the smallest members of the family, CyPA and FKBP12 (see [48] for a recent update). Importantly, it was demonstrated that the intermediate chain of the motor protein dynein coimmunoprecipitates with the Hsp90•FKBP52 complex bound to the GR [21, 61, 63] and MR [36] suggesting that the motor protein powers the retrograde movement of the receptor. Actually, the use of inhibitors of the ATPase activity of dynein and/or the disruption of the complex impairs the retrotransport of the receptor [36, 64]. Figure 2-A shows an integrated scheme of such proposed molecular machinery of transport and the points where the machinery can be interfered. Disruption of the Hsp90 function with the ansamycin geldanamycin (GA) slows the nuclear translocation rate of receptors by an order of magnitude (from t0.5 = 4-5 min to 45-60 min) [21]. Similar results were obtained by blockage of the Hsp90•FKBP52 or FKBP52•dynein interactions with an excess of TPR domain or the PPIase domain of the IMM, respectively, or by disruption of dynein/dynactin function

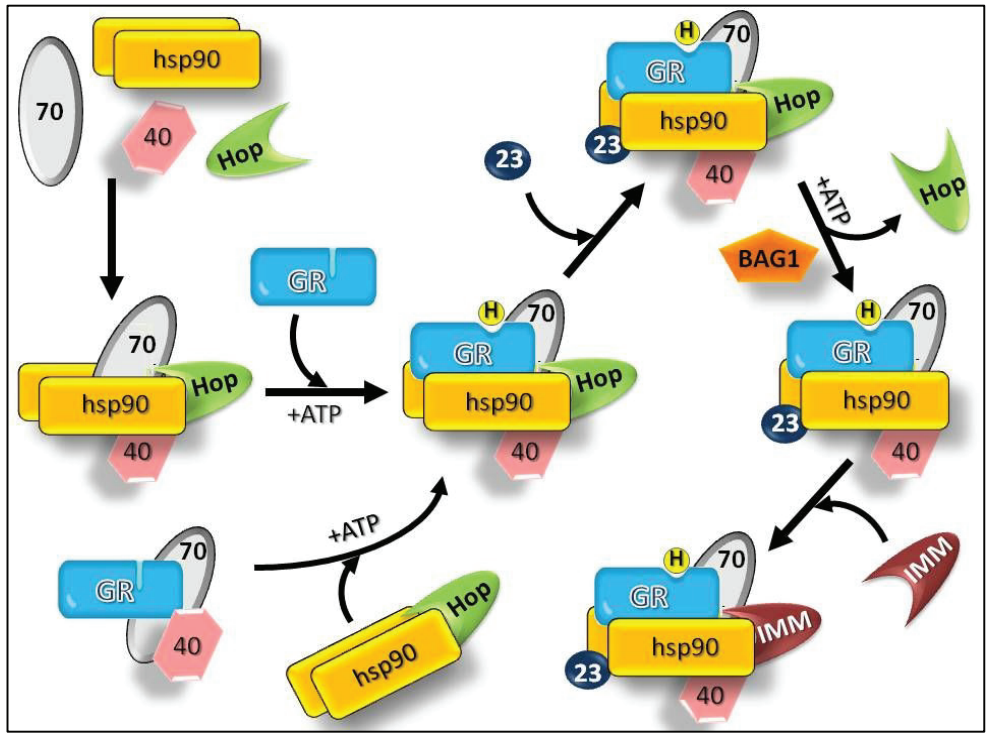

Figure 1: **Steroid Receptor•Hsp90 Assembly.** The glucocorticoid receptor (GR) is shown as a standard model. The chaperones Hsp90 and Hsp70 (and their associated cochaperone Hsp40) are assembled thanks to the presence of Hop/p60 (heat-shock organizing protein, formerly called p60). Hop/p60 has tetratricopeptide repeats (TPR) and is absolutely required to bring the two master chaperones together. This assembly can be reached spontaneously by mixing all proteins in buffer. When this basic complex is not associated to the GR, the receptor cannot bind hormone (H, yellow sphere) because its ligand-binding domain is collapsed, but the transference of the chaperone complex to the receptor in an ATP- and K+-dependent manner opens the receptors cleft that can be accessed by hormone. The small acidic protein p23 stabilizes the complex when it is bound to Hsp90 dimers. Even though the chaperone complex can be transferred as a block to the GR, it can also be primed by Hsp70•Hsp40, and then the Hsp90•p23 complex is recruited. When the GR is properly folded and able to bind steroid, Hop/p60 is released from the complex leaving the TPR acceptor site on the Hsp90 dimer available, which is occupied by a TPR-domain immunophilin (IMM) to form the 'mature' final complex. BAG-1 (Bcl-2- associated gene product-1), an Hsp70-binding protein, promotes the release of Hop/p60 from the complex without inhibiting GR•Hsp90 heterocomplex assembly. The release of Hop/p60 can be prevented by Hip (Hsp70-interacting protein), a BAG-1 antagonistic cochaperone. Neither BAG-1 nor Hip are essential for the final folding of the heterocomplex and are not present in the mature form of the GR•Hsp90 complex, but they play regulatory roles on the dynamic assembly of the heterocomplex and the termination of the transcriptional activity by GR.

after the overexpression of the p50/dynactin-2 subunit of dynactin [36]. In all these cases, the nuclear localization of the receptor was not fully inhibited, but it was only impaired (Figure 2-B). This suggests the existence of two mechanisms of transport, a rapid Hsp90•FKBP52•dynein complex-dependent mechanism (Figure 2-B, blue continuous line) and an alternative, slower and heterocomplex-independent mechanism (Figure 2B, red dotted line), perhaps due to simple diffusion. Importantly, when the nuclear translocation rate of these receptors is impaired, they are highly sensitive to proteasomal degradation [20, 36]. The same Hsp90•FKBP52•dynein complex constitutes the molecular machinery responsible for the retrotransport of the proapoptotic factor p53 [65], suggesting that it may play a general role in the retrotransport mechanism of a number of Hsp90-associated factors.

A number of publications have demonstrated that this transport mechanism first proposed for the GR is used by several other factors such as the AAV-2 (adeno-associated virus-2) [66], poly-glutamine aggregated proteins in Kennedy disease cells [67], the brain specific protein PAHX-AP1 [68], the proapoptotic factor p53 [65], the cell cycle arresting protein p21 [69], the mineralocorticoid receptor (MR) [36], FKBPL/DIR1/WisP39 client proteins [62], the transcription factor RAC3 [70], the ecdysone receptor [71], and so on. Moreover, the interaction between dynein and IMMs has also been found in plants [72], suggesting that the functional role of this complex has been preserved during the evolution. In all these cases, the disruption of Hsp90 function was critical, which is not surprising if we consider that this chaperone is the gravity center of the transport molecular machinery. It is tempting to justify the inhibitory action of Hsp90 inhibitors

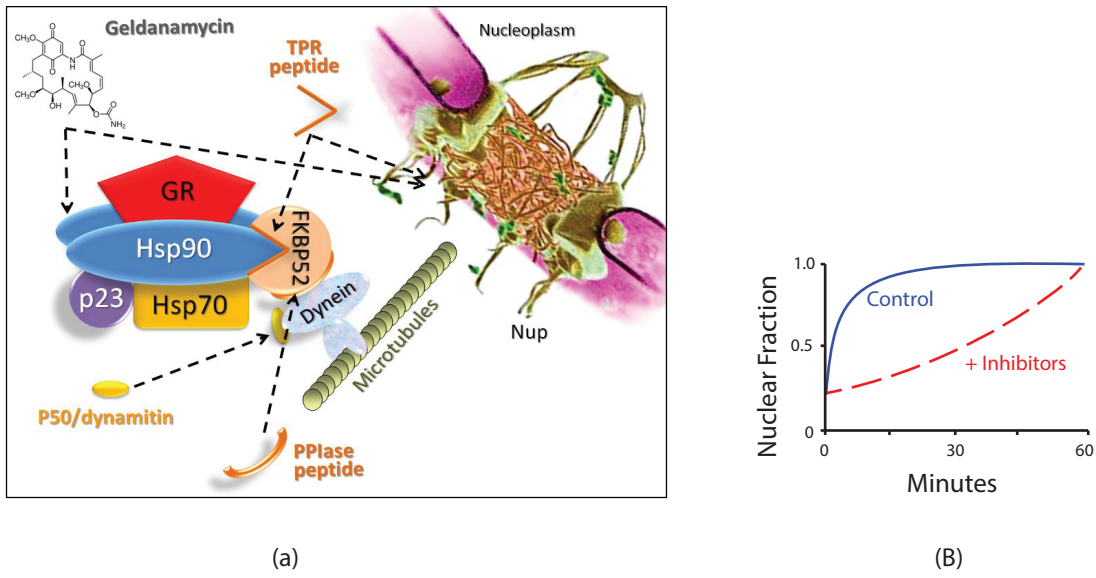

Figure 2: **The GR•Hsp90•FKBP52 Molecular Machinery of Movement.** (A) The glucocorticoid receptor (GR) is shown as a standard model. GR is associated to a dimer of Hsp90 and one molecule of Hsp70, p23, and a TPR-domain immunophilin. The inactive isoforms of the heterocomplex are primarily cytoplasmic and FKBP51 is recovered bound to the chaperone complex. Upon steroid binding, FKBP51 is exchanged by FKBP52, a highly homologous IMM that is able to interact with dynein/dynactin motor proteins. This molecular complex retrotransports GR to the nuclear compartment using microtubule tracks. Arrows show the points where the complex has been experimentally disrupted impairing the GR retrotransport, that is, by using the Hsp90 inhibitor geldanamycin, by overexpression of the TPR domain peptide that prevents the Hsp90•IMM interaction, or by overexpression of the PPIase domain peptide that prevents the IMM•dynein interaction. Also, the overexpression of p50/dynamitin (*Dyt*) subunit of dynactin interferes with the proper assembly of the dynein/dynactin motor complex. Both geldanamycin and overexpression of the TPR peptide interfere with the association of the oligomeric complex with structures of the nuclear pore making the nuclear accumulation of the receptor slower. (B) Nuclear translocation rate after the addition of steroid at zero time. The dotted red line represents the translocation rate measured when the transport machinery is disrupted in the points shown in panel A.

such as geldanamycin or radicicol due to their inhibitory action on the ATPase activity of Hsp90. Even though it is correct that the ADP-bound isoform of Hsp90 shows low affinity for client proteins, Hsp90 does not dissociate immediately from the receptor in intact cells. Significant dissociation starts after 2-3 h of cell treatment with the drug [73]. Because of the alternative retrotransport mechanism is slow, but still able to move the receptor towards the nucleus (making it fully nuclear after 45-60 min), it is implied that such alternative mechanism is less efficient because Hsp90 is stabilized in its less active ADP conformation.

The regulation of steroid receptor retrotransport was one of the first biological roles discovered for FKBP51 and FKBP52. Nonetheless their functions are more extensive. For example, IMMs play important roles in the nucleus as transcriptional regulators [74] and protein-protein linkers with other nuclear factors and structures [75].

4. The Hsp90-Based Heterocomplex Interacts with the Nuclear Pore

The modern model for steroid receptor action predicts that transformation should be a nuclear event, and it also raises the concept that the chaperone system could interact with the structures of the nuclear pore complex (NPC). The NPC is a macromolecular structure of ˜125-MDa embedded in the nuclear envelope [76]. While small molecules are able to diffuse freely through this structure, molecules larger than ˜40-kDa require an active passage mediated by adapter receptors, the importins [77, 78]. Proteins possessing a classic nuclear localization signals (NLS) such as the case of the SV40T antigen, nucleoplasmin or steroid receptors, utilize importin-α, a protein that binds the NLS of the substrate and forms a trimeric complex with importin-ß, a factor known as the transport receptor that favors the passage of many cargoes through the NPC [79].

Intuitively, the Hsp90•FKBP52-dependent model for steroid receptor retrotransport opened the possibility that the heterocomplex could interact with structures of the NPC such as nucleoporins (Nups). *In silico* analyses for protein-protein interactions of the GR•Hsp90•TPR-domain IMM complex yielded a number of potential interactors related to proteins associated with the cytoskeleton, motor proteins, and factors belonging to the nuclear import/export system [80]. This led to analyze the potential interaction of GR•Hsp90 complex with importin-ß and Nups. Proteins belonging to the untransformed receptor (Hsp90, Hsp70, p23, and TPR-domain cochaperones such as FKBP52 and

PP5) were recovered associated to the integral nuclear pore glycoprotein Nup62. Interestingly, importin-ß1 was also recovered associated to GR and Hsp90 [80].

There are reports showing that the GR also associates to importin-α [81–83]. It appears, however, that importin-α can bind to the NLS in the presence and absence of steroid [24]. It has also been reported that the GR binds to importin-7 and importin-8 in a steroid-independent manner [82], all of which imply that additional factors are required for the hormonal regulation of GR localization. Importin-7 alone and the α/β importin heterodimer were able to import an NLS-containing fragment of GR in an *in vitro* assay where permeabilized cells were used in the presence of Ran•GDP and ATP, whereas they failed to import purified full-length GR unless cell cytosol was added [82]. It is unlikely that such diluted extract was simply providing additional importins for the reaction since they were in great excess in the mixture. Therefore, the need of other factors (for example, soluble chaperones and cochaperones) could be inferred. The recent demonstration that chaperones and TPR-domain proteins are associated to structures of the NPC is in line with this speculation [80, 84, 85]. Interestingly, it has recently been shown that the combined functional chaperone activity of DNA-J/Hsp40-like protein and Hsp70 is required for the formation of structures in the nuclear envelope that appear to emerge from membranes next to specific subsets of NPCs, which results in the coordinate influx of membranes into the nucleus [84]. These atypical NPCs are attached to the outer nuclear membrane only and generate double-membrane structures called DNAJ-associated nuclear globular structures, whose content and biological function remain to be elucidated to date.

It is possible that in the absence of hormone, the GR is tethered to the Hsp90•FKBP52 heterocomplex, which may also be required for the proper function of some components of the NPC. In a recent study, it was shown that the GR binds to both importin-β and Nup62 [80]. Studies of reconstitution of the heterocomplex using purified proteins and reticulocyte lysate as a source of chaperones demonstrated the interaction between GR and Nups is strengthened when both factors are chaperoned. This observation results reasonable from the perspective that the novel model for nuclear import of steroid receptors proposes that transformation should be nuclear. On the other hand, the discovery that Nups are chaperone-interacting proteins suggests a potential regulatory role of the chaperones for the nuclear import process in addition of acting as facilitators of the protein-protein interactions required for the cargo passage through the pore. It is known that the affinity of a protein cargo for its cognate importin adaptor influences its nucleocytoplasmic transport efficiency and represents a subtle effector of transport regulation [86]. There is a correlation between the binding affinity of a NLS cargo for the NLS receptor, importin-α, and the nuclear import rate for this cargo. This correlation, however, is not maintained for cargoes that bind to the NLS receptor with very weak or very strong affinity. Similarly, the interaction of the GR with Nups may also impair the efficient delivery of the receptor into the nucleus. In this sense, the strong association found between GR and Nup62 (in their respective chaperoned complexes) was weakened by the presence of cytosolic factors [80], suggesting that soluble cytosolic factors may affect the interaction and the import rate of cargoes. Among them, there is evidence that importin-α is cointernalized with the GR [83], whereas importin-β is not. Nonetheless, the knock-down of importin-β significantly delayed GR nuclear import [80]. In this sense, it has been reported that many importins including importin-β do not only mediate active transport through NPCs, but also effectively suppress the aggregation of cargoes [87], which enhances the potential role of Hsp90 associated to this protein. The antiaggregation activity of importins involves shielding of basic patches on the cargo and predicts a precise match between cargo and receptor. However, it is hard to explain how a single type of factor could shield each of thousands of different protein-, RNA-, and DNA-binding domains that are import substrates. Therefore, it may be envisioned that the presence of chaperones and cochaperones associated to importin, Nups, and the cargo itself may act as a whole cooperative system to prevent the aggregation of cargoes when relatively hydrophobic domains are exposed during the translocation step. This may explain why when the GR and Nup62 are properly folded with the Hsp90 complex, there is a more efficient interaction compared to the naked proteins. It is likely that this could favor the translocation step. On the other hand, when these complexes are disrupted by Hsp90 inhibitors such as radicicol or geldanamycin, the nuclear translocation rate of GR [21], MR [88], and AR [89] undergoes a substantial delay (see scheme in Figure 2-A).

Interestingly, it has been reported that proteins carrying NLS bound to the α/β-importin complex dissociate slowly, whereas the release of the cargo in the nuclear basket structure facing the nucleoplasm milieu is faster. Consequently, it was postulated that the rate-limiting step in the α/β- importin- and Nups-mediated import pathway is the dynamic assembly and disassembly of the importin•cargo complex rather than the translocation process *per se* [90]. Recent studies on the role of FG Nups (nucleoporins containing a high number of Phe-Gly repeats) as functional elements of the NPC permeability barrier showed that these proteins are highly flexible and devoid of an ordered secondary structure [91], but those related to the NPC center are able to bind each other via hydrophobic attractions generating a sort of cohesive meshwork that may model the architecture of the pore [92] (Figure 2-A). If integral Nups such as Nup62 are chaperoned by Hsp90, Hsp70, p23, and/or TPR-domain IMMs, it would be entirely possible that the putative permeability barrier may be regulated by protein-protein interactions allowing (or not) the passage of certain cargoes. In line with this original hypothesis, more recent studies have confirmed that Hsc/Hsp70 complexes localize in the nuclear pore and are

able to recruit other proteins and cause effects on the nuclear translocation [84, 93].

Association of TPR-domain IMMs such as FKBP52 and PP5 to Nup62 seems to be Hsp90- dependent, as it was shown by the almost complete dissociation of these IMMs from Nup62 in the presence of radicicol [80]. However, indirect immunofluorescence assays performed in intact cells treated with radicicol still show the presence of both IMMs in the perinuclear ring, suggesting that these TPR proteins may also bind to perinuclear structures of the NPC (other Nups?) in an Hsp90- independent manner. Nonetheless, competition experiments with the TPR domain overexpressed in intact cells showed that the perinuclear signal of FKBP52 was totally abolished, indicating that most, if not all, types of associations of this IMM with any structure of the nuclear envelope require the TPR domain. This fact is shown in the model of Figure 2-A. It should be noted that FKBP51 was not recovered, associated to cytoplasmic structures of the NPC, although very recent evidence demonstrated that FKBP51 is able to interact with lamin B in the inner face of the nuclear envelope [94, 95].

The chaperone Hsp70 and the cochaperone p23, both regular components of the GR•Hsp90 heterocomplex, are also Nup62-associated proteins. In contrast with Hsp90, this association is constitutive and suggests that both proteins are required for the proper architecture of Nup62. Inasmuch as Hsp70 uses its ATPase cycle to control substrate binding and release [96, 97], likewise, substrate binding to importins is also coupled to Ran•GTP cycles. However, for some receptor-substrate pairs, the presence of Ran•GTP in not sufficient for cargo release; instead, an appropriate binding site for the cargo is also required. Therefore, it would be possible that Hsp70 may be related to the substrate binding-release equilibrium in the NPC. In this sense, it is noteworthy to emphasize that Hsp70 has been involved in the nuclear export mechanism of importins depending on its ATPase activity [98].

Inasmuch as steroid receptors are constantly shuttling between cytoplasm and nucleus, it is likely to speculate that GR•importins and GR•nucleoporins complexes form and disassemble constantly in a highly dynamic manner, even in the absence of hormone. There are several situations where cells broadly could alter nuclear translocation of steroid receptors and many other nuclear factors, but the mechanisms by which this occurs are not well defined to date.

5. Nuclear Events

Steroid receptors must dimerize to become transcriptionally active. Dimerization interfaces have been well characterized in both ligand-binding domain and DNA-binding domain [99]. Nonetheless, the classic model for steroid receptor action does not explain when and where this essential step of receptor activation takes place. The course of DNA-protein assembly has been discussed for a number of transcription factors, and the consensus is that there are three possible mechanisms. One mechanism implies that receptor dimerization could take place in the cytoplasm before its nuclear translocation [100, 101]. The dimer pathway model sustains that transcription factors must dimerize on the nuclear environment to permit DNA binding, and the monomer pathway postulates that two monomers can bind sequentially to promoter sequences and protein assembly takes place during the *in situ* dimerization [102, 103]. Also, it has been suggested that the level of receptor expression affects the formation of homodimers [101]. Nevertheless, it is still unknown whether steroid receptor homodimers form before or as a result of binding to hormone-responsive elements because some studies have showed some evidence favoring the monomer pathway model [104, 105], whereas others support the dimer pathway model [106–108]. According to the observation that Hsp90•TPR- domain factor is required for the retrotransport of steroid receptors, the modern model predicts that dimerization is likely to occur in the nucleus rather than in the cytoplasm. Consequently, after the dissociation of the chaperone complex, the dimerization domains are uncovered and monomers can interact. If this event would take place in the cytoplasm, the movement of receptors towards the nucleus would become inefficient due to the disassembly of the molecular machinery of retrotransport. In line with this prediction, very recent experimental evidence suggested that receptor transformation is indeed a nuclear event. For example, native heterocomplexes cross-linked with GR [80] or MR [36] are able to reach the nuclear compartment in a steroid-dependent manner, indicating that Hsp90 dissociation is not required for the nuclear accumulation of steroid receptors. Moreover, immunoprecipitation assays of native receptors present in the nucleoplasm ˜5 min after the addition of steroid have shown the presence of Hsp90, p23, FKBP52, and dynein. Accordingly, the receptor is poorly associated to chromatin during these early events and is fully bound to the insoluble chromatin fraction 10 min after the addition of steroid [36]. At this point, no receptor is recovered in the nucleoplasm [36].

Sucrose density gradients also demonstrated the association of the Hsp90•FKBP52 complex in the nuclear pool of receptors during the first steps of nuclear translocation. These observations were confirmed recently [109] by using extended bioluminescence resonance energy transfer (eBRET) and fluorescence resonance energy transfer (FRET) techniques. All these evidences univocally prove that receptor transformation and receptor homodimerization are a nuclear process. Interestingly, cell treatment with the Hsp90-disrupting agent geldanamycin shows that homodimerization takes place even in the absence of ligand because Hsp90 is dissociated from the receptor in the cytoplasm [109]. However, such Hsp90 inhibition prevents the nuclear translocation of the receptor and inhibits receptor binding to DNA. This inhibition is less efficient for GR than for MR [109], a dissimilarity that could contribute to the mechanism by which

MR differs from GR in those cells where both receptors recognize equal hormone-response elements. Importantly, only homodimers formed in the nucleus regulate gene expression, whereas those formed in the cytoplasm do not possess the ability to translocate to the nucleus and consequently are unable to influence transactivation.

Very recent findings from our laboratory also demonstrated that the NF-κB transport towards the nucleus is also regulated by the expression balance of the TPR-domain IMMs FKBP52 and FKBP51 [110]. Interestingly, NF-κB is not chaperoned by Hsp90, which assigns a cardinal role to these TPR proteins *per se*. In both cases, steroid receptors and NF-κB, the overexpression of FKBP52 favors the nuclear retention time and nuclear anchorage of the transcription factor, whereas the overexpression of FKBP51 favors their cytoplasmic localization. Similarly, FKBP52 favors transcriptional activity and FKBP51 is regarded as an inhibitory factor, except for the case of the androgenic response [111, 112].

The first studies performed decades ago where the nuclear distribution of steroid receptors was analyzed by microscopy evidenced a dispersed localization throughout the nucleus with a clear exclusion from nucleoli [113, 114]. The subsequent development of more sophisticated techniques such as confocal laser scanning microscopy revealed that the nuclear pool of steroid receptors was indeed located in multiple discrete foci disseminated throughout the nonnucleolar space, whereas the absence of ligand makes that punctuated nuclear signal observed in the presence of steroid diffuse [10, 30, 115–118].

There is strong evidence that steroid receptors occupy their nuclear sites in a transient manner relying on a hit-and-run mechanism [119], a phenomenon that is shared by several other nuclear factors [120]. Studies have revealed rapid cycling processes during transcription, which emphasize the central role of time-dependent events in the mechanism of gene regulation. Thus, after the proper stimulus nuclear proteins are recruited to promoters in an ordered manner on a time scale that may vary from minutes to hours (see [121] and references therein for a very recent update). During the development of this response, the nuclear factors that are able to interact with chromatin may cycle on and off the promoter site multiple times, and those factors belonging to functional complexes often exchange very rapidly (seconds). This fast exchange of molecules within a given complex takes place independently of long-term cycling on chromatin. These processes count with the active participation of the same molecular chaperones that form heterocomplexes with steroid receptors. It was shown that the GR released from chromatin recycles to chromatin upon rebinding hormone without exiting the nucleus [122]. When the steroid is washed out from the culture medium, the GR release from chromatin is inhibited by the Hsp90-disrupting agent geldanamycin [123], suggesting a role for the Hsp90-based chaperone complex in the termination of transcriptional activation as free hormone levels decline. A direct evidence for the role of Hsps and TPR-domain cochaperones in nuclear mobility of steroid receptors was provided by the ATP-dependent recovery of nuclear mobility of GR and PR on incubation with various combinations of purified chaperone and/or cochaperone proteins [124]. The nuclear presence of FKBP51 increased GR mobility., and more recently, it was demonstrated that the expression balance of the Hsp90-cochaperones FKBP51 and FKBP52 determines the amount of corticosteroid receptors accumulated in the nucleus in the absence of ligand [36], this effect being related to ability of FKBP52 to attach receptors to the nuclear matrix.

The association of steroid receptors to specific hormone-responsive elements results in a localized chromatin transition at these sites, which depends on the formation of a complex between the receptor and the ATP-dependent Swi/Snf coregulator complex by altering nucleosomal structure and increasing the accessibility of proteins to specific sequences [125]. These Swi/Snf complexes interact with Hsp90 and are rapidly recruited to the chaperone upon the onset of heat shock [126]. In turn, they are also counted among the SmyD•Hsp90 substrates implicated in chromatin remodeling, such that they are upregulated by SmyD. Interestingly, Hsp90 interacts with a TPR domain present at the C-terminal end of SmyD and induces a gain-of-function conformational change [127]. It is likely that other TPR-domain proteins such as FKBP51 and FKBP52 may also regulate these events in similar fashion. It is tempting to hypothesize that the regulatory action of both TPR-domain IMMs on transcription may lie on mechanisms where they interact with coregulators, although this is uncertain to date.

In addition to the combined effects of heat-shock proteins, TPR-domain IMMs, and chromatin remodeling, the residence times of steroid receptors at the promoter binding sites are also dependent on proteasomal activity [128, 129]. Proteasome modulates steroid receptor function by regulation of receptor bioavailability and also by interfering with its intranuclear trafficking [129–131]. Accordingly, FRAP assays have shown that the presence of proteasomal inhibitors reduces the mobility of the GR [124, 128], an effect where the role of nuclear molecular chaperones has been involved. Thus, GR nuclear mobility assayed in digitonin-permeabilized cells was fully restored on incubation with a mixture containing purified FKBP51, Hsp90, p23, and the E3 ubiquitin ligase of the GR machinery of proteasomal degradation, CHIP (carboxyl terminus of Hsc70-interacting protein) [124]. One possible explanation for these observations is that molecular chaperones may disengage the receptor from nuclear anchoring sites due to heterocomplex reassembly. Thus, it has been shown that Hsp90 and p23 are both recruited to glucocorticoid-responsive elements upon steroid activation of the GR [132]. The need of FKBP51 is in agreement with the effect of this IMM on the nuclear retention of nuclear factors by competition with the anchoring effect of FKBP52 [36, 110]. A similar effect is

observed in FKBP52 KO cells and due to the overexpression of the TPR peptide, which abolishes the nuclear pool of steroid receptors in the nucleus due to a 'dominant-negative' effect on receptor anchorage to nuclear structures [36].

Interestingly, it has been shown that the proteasome is also required for GR removal from DNA, such that proteasome inhibition decreases receptor mobility in the nucleus by inducing nuclear matrix binding [133, 134]. The proteasome could regulate receptor function due to two possible mechanisms, by decreasing receptor stability by proteolysis or by affecting receptor motility in the nucleus. It has been shown that the average residence time of the GR on GRE sequences depends on the proteasome activity in an ATP-dependent fashion [128], such that the disruption of either the proteasome pathway or Hsp90 function by geldanamycin has opposing effects on the exchange rate of GR [128]. This suggests that a balance between both the chaperone- and proteasome-dependent mechanisms needs to be in place for proper nuclear recycling of GR. Proteasome inhibition also results in immobilization of polyubiquitinated forms of estradiol-bound hERα within the nuclear matrix [135], and a similar mechanism was also suggested for the accumulation of polyubiquitinated forms of p53 [136]. It appears that the nuclear transcription factors are moved from the promoter site to active sites of degradation, the nuclear matrix being the scaffold structure that provides a key role in this movement since it is the site of proteasome action.

Clusterin is a molecular chaperone whose expression level is stress-induced via HSF-1 [137]. It is highly expressed in various cancer types, including prostate cancer. Recently, it was shown that the proteasomal degradation of AR is increased in prostate cancer cells when clusterin is knocked down by a mechanism that involves the expression of the TPR domain IMM FKBP52 [138]. Casually, one of the downstream effectors of clusterin is FKBP52, such that AR proteasomal degradation was prevented by overexpression of FKBP52 and the expression of prostate-specific antigen was restored. This demonstrates that the effects of clusterin on AR stability by the proteasome are mediated by FKBP52. Unfortunately, it was not studied whether the PPIase activity of this IMM is required for such action as to target FKBP52 with therapeutic purposes.

6. TPR-Domain Immunophilins in Cancer

Among the TPR-domain family members, many of them have been described to have a potential role in cancer development and chemoresistance. Not surprisingly due to its role in steroid receptor action, FKBP52 was found overexpressed in many hormone-dependent cancers, particularly in ER-positive breast cancer cells and preinvasive breast cancer tissues [139, 140]. Also, FKBP52 shows high level of expression in hepatocellular carcinomas [141] and prostate cancer cells and has been proposed as a biomarker for the latter pathology [142]. Recently, we demonstrated that FKBP52 greatly enhances NF-κB biological response [110], a transcription factor that is linked to chronic inflammation processes and progression of multiple diseases, including cancer, where NF-kB is related to tumor promotion and progression, as well as chemotherapy and radiotherapy resistance [143, 144].

As it was commented above, FKBP51 is overexpressed in a number of tumor cells and cancer tissues. One of the first evidences connecting FKBP51 with malignant pathologies was the observation that this TPR-domain IMM is overexpressed in idiopathic myelofibrosis [145], a known chronic myeloproliferative disorder characterized by bone marrow fibrosis and megakaryocyte hyperplasia. The overexpression of FKBP51 affects the regulation of the growth factor independence of megakaryocyte progenitors and induces resistance to apoptosis. Overexpression of FKBP51 has also been documented in several human cancers such as lymphomas, gliomas, melanoma, prostate cancer, and so forth [146], but it is downregulated in pancreatic cancer [147]. Interestingly, FKBP51 binding to Hsp90 favors the recruitment of the cochaperone p23 and positively regulates AR signaling [148] and is associated with chemoresistance and radioresistance [147, 149]. Actually, AR is the exception among members of the steroid receptor family because FKBP51 is regarded as a negative regulator for most of them [54].

FKBPL/WisP39 is a TPR-domain IMM that shares the same structural properties as the other members of the FKBP family. Nonetheless, it is an IMM-like protein because its PPIase domain lacks enzymatic activity [150, 151]. FKBPL/WisP39 was originally found during screening for genes that were protective against ionizing radiation [150, 152]. It is most closely related to FKBP52 and also shows the ability to interact with Hsp90 in steroid receptor complexes, sharing with FKBP52 exactly the same properties for the cytoplasmic retrotransport of the GR [62, 153]. Also, FKBPL/WisP39 stabilizes newly synthesized p21 preventing its degradation [154, 155]. There is conflicting data on FKBPL regarding its role in conferring radiation resistance. It was first reported that, in response to radiation, the FKBPL/Hsp90/p21 heterocomplex favored the stabilization of p21 leading to a pro-survival effect by G2 cell cycle arrest [155]. Recently, it was shown that after radiatio n there is p21 downregulation and that such decrease of p21 is the relevant action involved in the pro-survival effect [150, 156, 157]. In addition to radiation resistance, FKBPL/WisP39 plays a significant role in tumor progression [150, 152, 155, 157]. In tumor cells, FKBPL/WisP39 favors the tumor growth and it is also related to the sensitivity of the tumor to chemotherapeutic compounds [158].

Importantly, FKBPL/WisP39 interacts with the ER•Hsp90 heterocomplex [159], and the expression of this IMM is regulated by estrogens. Increased FKBPL/WisP39 levels of expression lead to decreased ER expression [159, 160], and this is associated with increased survival of

untreated breast cancer patients by sensitization of cancer cells to the antiproliferative effect of tamoxifen [161, 162]. Recently, it was also demonstrated that FKBPL possesses antiangiogenic properties [163].

CyP40 is another TPR-domain IMM able to form heterocomplexes with steroid receptors via Hsp90. In contrast to the FKBP subfamily that bind the immunosuppressive macrolide FK506, CyP40 belongs to the cyclophilin subfamily and binds the cyclic undecapeptide cyclosporine A. CyP40 is not recovered with native GR and MR, but with PR and ER [37, 164, 165]. Because CyP40 is also known to form complexes with dynein/dynactin [60] and associates to the cytoskeleton [166], it is entirely possible that this TPR-domain IMM plays a redundant role with FKBP52 and FKBPL/WisP39 in receptor trafficking. It has been shown that CyP40 is overexpressed in breast cancer tissues when it is compared to normal breast tissue [139] and also that the breast cancer cell line MCF-7 shows 75-fold higher level of Cyp40 mRNA expression in response to high temperature stress and a marked redistribution of Cyp40 protein from a predominantly nucleolar location to nuclear accumulation [167]. In the same cell type, estradiol increases protein expression and also the average half-life of its mRNA [168], and oxidative stress increases Cyp40 expression at higher levels than in normal cells, a property that was also observed in prostate cancer cell lines [169]. Interestingly, breast cancer cells respond similarly for both IMMs, CyP40 and FKBP52, and such upregulation in response to the mitogenic action of estradiol in breast cancer cells is consistent with a possible wider role for both TPR-domain IMMs in cell proliferation.

7. Summary

Essential to understanding cellular signalling mechanisms is the ultimate comprehension of how soluble proteins involved in signalling cascades move throughout the cellular milieu of subcellular compartments to reach their sites of action. The first discoveries focused the interest on the nature of the signals present in the travelling proteins, for example, those conserved amino acid sequences known as nuclear localization signals or nuclear export signals. Nowadays most studies are trying to understand the mechanisms of signalling protein movement within both the cytoplasmic compartment and the nuclear compartment. Most of the advances in this field were reached studying the properties of steroid receptors, perhaps due to the fact that the members of this subfamily of the nuclear receptor superfamily are highly versatile factors whose distribution can be easily manipulated by the operator by adding or withdrawing the ligand from the medium. As a consequence of this, there is considerable evidence that the dynamic assembly of some transcription factors with the Hsp90•FKBP52-based heterocomplex is involved in the movement of them within the cytoplasm and the nuclear compartment.

It is still uncertain whether Hsp90•TPR complex assembly is related to the subcellular relocalization of a limited number of transcription factors or whether the chaperone machinery also affects long- range movement and local mobility of a wider range of signalling protein solutes. In this regard, the number of Hsp90 client proteins is nearly 400 proteins, a large number of them belonging to the protein-kinase family [44, 170–172]. Even so, a direct role of proteins such as FKBP51 and FKBP52 cannot be ruled out since these IMMs could also act *per se* in the subcellular distribution of nuclear factors in an Hsp90-independent manner. Due to technical reasons, our lack of capability to examine in more detail molecular events at high time resolution in living cells has veiled the dynamic complexity of transport mechanisms. This is true for cytoplasmic events, but it is even more dramatic for our understanding of those mechanisms responsible for the intranuclear transport of soluble factors.

Ideally, we will be able to regulate the subcellular localization of nuclear factors (and consequently their biological actions) when we understand the mechanism of action for that trafficking. For example, NF-κB is constitutively active in many cancer cells [173] and persistent localization in the nucleus has been implicated in tumor development. On the other hand, p53 activation promotes cell-cycle arrest and apoptotic cell death, and p53 mislocalization in the cytoplasm is responsible for tumor development [174]. Unlike NF-κB, localizing p53 to the nucleus would be desirable for the control of cell survival. Similarly, nuclear localization is essential for steroid receptors to trans-activate their target genes, but it should also be thought that these receptors also have nongenomic functions in the cytoplasm. Therefore, their nucleocytoplasmic trafficking becomes an essential mechanism able to contribute to the regulation of their biological actions and also to integrate nuclear transcription with signalling actions in the cytoplasm. Accordingly, an unbalanced cytoplasmic localization of the α- isoform of the ER is known to enhance the nongenomic actions of ERα, which has been proposed to contribute to tumorigenesis as well as antiestrogen resistance of breast cancer cells [175, 176]. Strikingly, during the progression of the prostate cancer disease, the AR acquires the ability to undergo androgen-independent nuclear import and androgen-independent transactivation [177]. Importantly, AR is not mutated, indicating there is a gain-of-function in critical aspects of the AR import and transactivation pathways. The androgen-independent mechanism that controls AR localization is currently unknown, although the involvement of MAP kinase pathways has been suggested [178].

Nuclear retention of steroid receptors can also be affected by other adapter factors, such as 14-3-3 proteins [179] and p160 co-activators [180]. Interestingly, TPR-domain proteins and 14-3-3 proteins share similar structural and functional properties [181]. 14-3-3 proteins show a TPR-like domain and are able to interact with GR thereby favoring its cytoplasmic localization, perhaps through the 14-3-3 export

signal [179], by anchoring the receptor to the cytoskeleton [182], or simply due to interference with the Hsp90•TPR protein retrotransport. This may play a key role in prostate cancer where GR behaves as a counter-balance factor of the oncogenic AR (Mazaira G.I. & Galigniana M.D. et al., unpublished observations). Similarly, the expression levels of the TPR-domain Ser/Thr phosphatase PP5 affect the subcellular localization of the GR, increasing nuclear accumulation, interaction with GRE sequences, and increasing GR response in the absence of steroid [183, 184].

The proper and efficient intracellular localization of steroid receptors plays an essential role in maintaining major functions in the cell, such that the manipulation of protein shuttling could be used for treating diseases [185, 186]. In this regard, targeting TPR-domain proteins IMMs, IMM-like factors such as the Ser/Thr-phosphatase PP5, TPR-like proteins such as 14-3-3, and TPR-containing cochaperones such as Hop/p60, all of them able to associate to transcription factors, can certainly affect the final biological response. As we decode the particulars of real-time mechanisms for protein trafficking, as well as protein-protein and protein-DNA interactions involved at the chromatin- transcription factor interface, we will be able to move towards the design of drugs and/or therapeutic strategies to manipulate events that are critical for the regulation of gene expression and the consequent biological responses.

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