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Paxillin Actions in the Nucleus

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Abstract

Paxillin is a group III LIM domain protein that is best characterized as a cytoplasmic scaffold/adaptor protein that functions primarily as a mediator of focal adhesion. However, emerging studies indicate that paxillin's function s are far broader. Not only does paxillin appear to regulate cytoplasmic kinase signaling, but it also cycles between the cytoplasm and nucleus, and may be an important regulator of mRNA trafficking and subsequent translation. Herein, we provide some insights suggesting that paxillin, like its relative Hic-5, has nuclear binding partners and mediates critical processes within the nucleus, at least in part functioning as coregulator of nuclear receptors and nuclear kinases to mediate genomic signaling.

Keywords

Paxillin; Nuclear; Androgen; Coregulator; Cancer

1. Introduction

Paxillin, first identified as a vinculin-binding focal adhesion protein, demonstrates versatile functions at the plasma membrane and within the cytoplasm. Being a major substrate of Src tyrosine kinase, paxillin plays a critical role in regulating focal adhesion assembly and organization [1, 2]. Within focal adhesion complexes, paxillin serves as a bridge that connects integrins with Focal Adhesion Kinase(FAK), mediating integrin bidirectional signaling that then allows cells to sense and respond to extracellular stimuli. Besides its function in focal adhesions, paxillin also serves as a scaffold protein that regulates spatial and temporal organization of cytoskeleton and cytoplasmic signalosomes.

Given its importance in maintaining cellular structure and interactions, paxillin would be expected to be important for normal organ development and function. Emerging evidence indicates that paxillin participates in many developmental and physiological processes.

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Paxillin, which is known to mediate fibronectin receptor signaling, is a critical modulator of the development of several mesodermal-derived organs. For example, the paxillin knockout mouse is embryonic lethal very early in embryogenesis due to poor early development of the heart and somites [3]. Furthermore, recent studies in zebrafish suggest that double mutants of two paxillin genes, *pxna* and *pxnb,* leads to defects in axial and skeletal muscle development as well as in the cardiovascular system. Specifically, paxillin together with its binding partner FAK are critical players in the maintenance of cardiac contractility, with failing of this orchestrated interplay resulting in heart failure [4]. In addition to its effects in the heart, paxillin is part of myosin regulatory light chain signaling in response to neurostimulation by force development in tracheal smooth muscle. Paxillin is also important for skin fibroblast morphology, with its levels declining during skin aging [5, 6]. Finally, paxillin has been implicated in various diseases, including Alzheimer's disease [7] as well as many kinds of cancers [8–10].

While paxillin's roles in the aforementioned processes have been primarily linked to its function in the membrane and cytoplasm, emerging evidence indicates that paxillin may also signal in the nucleus to mediate important processes. This review will focus on paxillin's role as a liaison that connects extranuclear and nuclear signaling, as well as its actions in the nucleus to regulate genomic signaling in a variety of models.

2. Paxillin structure

2.1 LIM domains

As a multiple domain adaptor protein, paxillin has two major sets of motifs: four LIM domains in the carboxyl terminal half of the protein and 5 LD domains close to the amino terminus (Fig. 1). LIM domains are cysteine-rich protein regions that contain two contiguous zinc-fingers, separated by a two amino acids spacer.

Unlike metalloproteinases or helix–loop–helix transcription factors, which also contain zinc finger motifs, LIM domain-containing proteins are not classified by similar functions, but are instead separated by the subtype of domain structure. There are 14 types of LIM domain proteins falling into four groups. Some are LIM domain only (LMO) proteins, whose functions are considered to be primarily transcriptional within the nucleus [11]. In contrast, the remaining LIM proteins are composed of other functional domains such as PDZ or LD regions in addition to LIM domains. These more complex proteins are thought to be primarily cytoplasmic [12]. Paxillin belongs to the group III LIM proteins, together with several other members, including zyxin and testin [13].

Most LIM domain-containing proteins have essential functions in cytoskeletal organization, cell fate determination, and differentiation through their interactions with other adaptor proteins or with DNA. In paxillin, the LIM2 and LIM3 domains have been identified as focal adhesion targeting motifs, and phosphorylation of these LIM domains is specific and critical for paxillin localization to focal adhesions [14]. Notably, in other LIM domain-containing proteins, the zinc finger motifs can mediate DNA binding of many transcription factors. As mentioned, group I LIM family members, LIM Homeobox (LHX) and LMO proteins, are well known to be localized in the nucleus and to participate in tissue specific

gene regulation. LHX3, a neuroendocrine transcription factor, is expressed in nuclei of adult human pituitary cells, where it induces transcription of the glycoprotein alpha-subunit promoter to promote expression of pituitary-derived glycopeptides [15]. LMO1/2, engages with a large array of proteins, including LIM domain-binding protein 1 (LDB1), stem cell leukemia protein (SCL), and E-protein, to form a transcriptional complex that plays roles in the transcriptional regulation of normal and malignant hematopoiesis [16]. Zyxin, one of the LIM group III proteins, is an important component of focal adhesion plaques, like paxillin, but has also been shown to shuttle between the cytoplasm and nucleus. Although there is no conventional nuclear localization sequence identified, *in vitro* evidence suggests that zyxin interacts with several nuclear proteins, including transcription factors, to regulate gene expression [17, 18]. Taken together, it is intriguing to speculate that many if not all LIM domain-containing proteins may, in addition to their extranuclear effects, modulate gene transcription in the nucleus.

2.2 LD motif

Near the amino terminus of paxillin, there are five tandem LD motifs (Fig.1). LD motifs contain sequences rich in leucine and aspartate. The LD motif is a major targeting sequence for many protein-protein interactions, forming a scaffolding surface that can coordinate large sets of enzymatic reactions between interacting molecules within a protein complex. LD motifs contain many phosphorylation sites that are crucial for protein activation and signal transduction. For example, one major focal adhesion molecule, Focal Adhesion Kinase (FAK), while interacting with paxillin through paxillin's LD2 domain, also binds with Crk-associated substrate (p130Cas) to regulate its downstream effects. In this situation, paxillin is therefore serving as both kinase and scaffold protein in regulating focal adhesion assembly [19]. More specifically, the Focal Adhesion Targeting (FAT) homology domain in FAK binds hydrophobically through its HP1 (Hydrophobic patch 1) and HP2 (Hydrophobic patch 2) sites to paxillin LD motifs, LD2 and LD4, under normal conditions [20, 21]. However, in disease conditions such as lung cancer, paxillin can be mutated such that it exhibits a disordered intra-molecular regulatory region that results in masking of one of the LD motifs and therefore preferential binding of FAT with the other LD domain, leading to signaling and adhesion changes that may promote tumor growth [22]. Using similar binding machinery, Cerebral Cavernous Malformations 3 (CCM3), which is a frequently mutated protein in cerebral cavernous malformation disease, binds to paxillin via its LD1, LD2, and LD4 motifs to colocalize in mouse cerebral pericytes and possibly regulate cell adhesion [23].

Furthermore, the LD1 motif of paxillin is sufficient to bind to the mitogen-activated protein kinase kinase (MEKK2) amino terminal region, thus relieving MEKK2 auto-inhibition and triggering its activation [24]. Lastly, a recent study suggests that FAK binding to LD domains of paxillin plays a key role in paxillin shuttling between cytoplasm and nucleus [25]. While some interactions with LD1, LD2, and LD4 are characterized, the binding partners of LD3 and LD5 are not well known.

2.3 Nuclear Export Sequence(NES)

As we will discuss, paxillin appears to have nuclear as well as extranuclear actions. However, despite reports of paxillin located in the nucleus, no apparent nuclear localization signal has been reported. Instead, a nuclear export signal (NES) was identified. Initial evidence suggesting the existence of an NES in paxillin was discovered in fibroblasts treated with the nuclear export inhibitor leptomycin B, a treatment that led to paxillin retention within the nucleus [26]. A study by Dong et al proposed that the LD4 motif may consist of a potential leucine-rich NES sequence. Specifically, phosphorylation of the Ser272 within the LD4 motif is critical for blocking paxillin nuclear export, as well as for reducing G protein-coupled receptor kinase-interacting protein (GIT1) binding, without altering FAK1 affinity [27]. While these studies were suggestive, identification of the protein crystallography structure of the paxillin NES sequence (264RELDELMASLSDFKFMAQ281) together with the nuclear export protein CRM1/XPO1 provided the final proof that the NES in paxillin is utilized for paxillin shuttling through nuclear pore [28]. Again, the lack of a conventional NLS (nuclear localization signal) within paxillin protein suggests that paxillin may initially enter the nucleus via a non-conventional NLS or by association with other NLS-containing proteins. For instance, cell adhesion kinase beta/proline-rich tyrosine kinase 2, which is a non-receptor tyrosine kinase member of FAK family that is localized at the perinuclear region and shuttles between cytoplasm and nucleus, has been shown to bind with paxillin's relative Hic-5 and facilitates its nuclear transportation [29].

3. The paxillin superfamily and their functions in nucleus

The paxillin superfamily includes three main members: paxillin, Hic-5 and Leupaxin. Similar to paxillin, Hic-5 is a group III LIM protein that consists of four LD motifs and four LIM domains that are highly conserved within the paxillin superfamily. Hic-5 localizes and functions in both focal adhesions and in the nucleus. At focal adhesions, it acts as a scaffold/adaptor protein that appears to participate in skin fibroblast contractility, hypertrophic scar tissue formation, platelet aggregation and breast stromal extracellular matrix remodeling [30–32]. Interestingly, in these focal adhesions, Hic-5 shares some binding partners and has some overlapping functions with paxillin. However, unlike the global paxillin knockout mice, Hic-5 deficient mice are viable with no obvious histological abnormalities and only minor vascular defects [33] that includes altered vasculature recovery after injury and enhanced stretch induced vascular smooth muscle cell apoptosis. Together, these observations suggest that Hic-5 may play a less critical role than paxillin in organ development, or that the deficiency of Hic-5 may be compensated by other members in the paxillin family. In the nucleus, Hic-5 was initially characterized as a glucocorticoid receptor (GR) coactivator that binds with GR at its tau2 transcriptional activation domain [34]. Hic-5 has been shown to selectively regulate certain sets of GR target gene expression, perhaps in part by inhibiting GR interaction with several chromatin remodeling enzymes such as chromodomain-helicase DNA-binding protein 9 (CHD9) and Braham homologue (BRM), which ultimately leads to chromatin remodeling and selective GR binding to DNA [35]. In addition to GR, Hic-5 also interacts with androgen receptor (AR) to modulate AR actions. As a stromal specific coactivator of AR, Hic-5 affects androgen-induced keratinocyte growth

factor expression in prostate stromal cells, which then alters the stromal microenvironment to favor tumor growth [36].

Leupaxin, another member of paxillin family, is enriched in cells of leukocyte lineage, but is also broadly distributed in other tissues. A recent study in hepatocellular carcinoma suggests that leupaxin serves as a coactivator of beta-catenin by assisting in the recruitment of the coactivator complex consisting of steroid receptor coactivator 1 (SRC-1) and P300 to enhance beta-catenin's transcriptional activity [37]. Evidence also suggests that leupaxin shuttles between cytoplasm and nucleus, perhaps interacting with the AR in a ligand-dependent pattern to regulate AR-dependent transcription in prostate cancer cells [38].

4. Paxillin actions in the nucleus

Base on the nuclear actions of other paxillin superfamily proteins, as well as on reports that paxillin can be found in the nucleus, it is not surprising that recent studies strongly support a role for paxillin itself in nuclear signaling.

The first evidence that paxillin cycles between cytoplasm and nucleus in a physiologically relevant scenario is from a study of paxillin interactions with an mRNA binding protein, polyadenylation binding protein1 (PABP1), in fibroblast cells [39]. Paxillin directly associates with PABP1 through the amino-terminal, LD-domain-rich region (residues 54–313), co-localizing in the endoplasmic reticulum and in the nucleus, as well as at the tips of lamellipodia. This association is necessary for efficient nuclear export of PABP1, and facilitates transport of mRNA from nucleus to sites of protein synthesis that are occurring at or near the leading edge during cell migration [40]. A recent study suggests that the paxillin protein binds with embryonic PolyAdenylation Binding Protein (ePABP) on polyadenylated *Mos* (a germ cell specific Raf) mRNA upon androgen stimulation, which induces Mos protein translation and subsequent oocyte maturation (meiotic re-entry) in *Xenopus laevis* model [41]. Notably, this paxillin-ePABP interaction appears to be enhanced by phosphorylation of serine residues also located within the amino-terminal half of paxillin (Fig.1).

Besides its function as a binding partner of PABP, paxillin also interacts with nuclear receptors within the nucleus and functions as an AR and GR coactivator similar to its family member Hic-5 [42], in prostate cancer cell lines and prostate tissue. Studies from the DeFranco group have revealed that both Hic-5 and paxillin interact with steroid receptors using their carboxyl-terminal LIM domains. However, paxillin appears to potentiate AR and GR transactivation through the same carboxyl-terminal domain, whereas the receptor coactivation domain of Hic-5 seems to be located in its amino-terminal region [34, 43].

With these studies in mind, our group then demonstrated that paxillin regulates both cytoplasmic kinase signaling as well as nuclear transcription. In fact, we found that paxillin serves as a liaison between non-genomic steroid signaling in the cytoplasm and genomic steroid signaling in the nucleus. This pathway was first discovered in the aforementioned study of oocyte maturation (meiotic resumption) in *Xenopus laevis*, where we showed that paxillin is an essential regulator of meiosis in *Xenopus laevis* oocytes. Specifically, we

found that paxillin functions to enhance androgen-induced translation of the Mos protein in Xenopus oocytes, which then leads to activation of the MAPK cascade and subsequent meiotic resumption. Once extracellular signal-regulated kinase (Erk) is activated, it regulates the phosphorylation of serine residues on paxillin, which then acts in a positive feedback mechanism (likely through interactions with PABP) to enhance MAPK signaling and eventually oocyte maturation [41, 44]. Interestingly, we went on to demonstrate that paxillin similarly plays an important role in extranuclear androgen-mediated MPAK activation in somatic cells. In prostate cancer cells, androgen binds to membrane-localized ARs to promote the MMP-mediated release of membrane-bound EGF receptor (EGFR) ligands, which then transactivate the EGFR. Activated EGFR further induces extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling via Src-mediated tyrosines 31/118 phosphorylation on paxillin [45]. ERK1/2, which is still complexed with paxillin, then mediates phosphorylation of serines 83/126/130 on paxillin. These results are reminiscent of studies by Ishibe and colleagues, who demonstrated that hepatocyte growth factor (HGF) receptor signaling relies on a similar mechanism to regulate cell spreading, migration and tubulogenesis [46, 47]. Furthermore, these protein complex formations with EGFR, paxillin, and ERK1/2, were confirmed to be occurring in living cells using a novel method of fluorescence photolithography [48]. Interestingly, we found that, once phosphorylated on serines 83/126/130, phosphoserine paxillin is then able to translocate into the nucleus, where it can enhance AR and ERK-mediated transcription. In fact, Chromatin Immunoprecipitation (ChIP) studies using an anti-paxillin antibody demonstrated that, upon androgen or ERK activation, paxillin was targeted to the promoter regions of AR and ERK-dependent genes, respectively. In sum, our studies suggest that extranuclear androgen actions via the AR are inextricably linked to nuclear AR actions in a serial fashion, with paxillin serving as a mediator of both processes (Fig. 2). Both extranuclear and nuclear actions of paxillin appear to be involved in growth, migration, and invasion of prostate cancer cells both *in-vitro* and in mouse xenografts [49]. Finally, expression of paxillin and nuclear paxillin are increased in human prostate cancer versus benign prostate tissue, confirming that paxillin may be important in cancer and may also serve as a biomarker of prostate cancer [49].

Tying many of the aforementioned actions together, we have also recently shown that paxillin similarly regulates AR signaling in granulosa cells within ovary, where it mediates androgen-induced suppression of pro-apoptotic protein expression, as well as mediates androgen-triggered translation of follicle stimulation hormone (FSH) receptor protein, both of which ultimately lead to enhanced ovarian follicle growth and development [50]. It is intriguing to postulate that paxillin and PABP1 are working together to promote androgen-induced FSH receptor expression, much like they work together to enhance translation in other models mentioned above.

Besides mediating nuclear effects of AR and ERK in the prostate and ovary, paxillin also plays critical roles in the nucleus of other cell types and disease processes. In a model of pulmonary hypertension, hypoxic exposure and platelet derived growth factor (PDGF)-BB induce Y13 and Y118 phosphorylation of paxillin and its subsequent localization into the nucleus in a time dependent fashion, leading to increased proliferation and decreased apoptosis of pulmonary arterial smooth muscle cells [51]. In addition, paxillin's nuclear actions modulate expression of the parental imprint genes H19 and IGF2. In this model,

paxillin promotes an interaction between an enhancer region and the *IGF2* promoter, leading to increased *IGF2* expression. In the meantime, paxillin suppresses an enhancer/promoter interaction within the *H19* gene, leading to decreased gene expression. Ultimately, these changes may play a role in cell proliferation and fetal development [27, 52].

Taken together, the aforementioned studies, plus others, demonstrate that paxillin exhibits versatile functions in the nucleus, ranging from nuclear receptor coactivation to facilitation of mRNA translocation, all contributing to enhanced genomic signaling and downstream physiological processes.

5. Therapeutic potentials by disruption of nuclear targeting of paxillin

Paxillin overexpression or dysregulation has been implicated in numerous cancers and other diseases. However, most of the studies have focused on the focal adhesion or scaffolding functions of paxillin in the cytoplasm. Since little has been known about its nuclear actions until recent years, effort at targeting only its nuclear actions have been limited. As mentioned, multiple studies now suggest that paxillin's nuclear functions are critical for cell proliferation, as well as steroid-dependent cancer progression. For instance, depletion of paxillin by shRNA leads to decreased S phase cell population and increased early apoptosis in colorectal carcinoma cells [53]. Additionally, the paxillin-ERK1/2-cyclin D1 pathway is essential for the PDGF-dependent pulmonary artery smooth muscle cell proliferation and vascular remodeling underlying pulmonary hypertension [51]. Moreover, as mentioned, our group has shown that paxillin modulates AR and ERK dependent gene expression in prostate cancer cells and promotes prostate cancer xenograft growth *in vivo*. In addition, paxillin expression is elevated in human prostate cancer tissue compared to normal prostate [49]. Likewise, in breast cancer patients, paxillin's expression is upregulated and correlates with HER2 overexpression [54].

With this in mind, it is intriguing to speculate that specifically targeting nuclear actions of paxillin, while sparing its important structural functions outside the nucleus, might be a useful approach toward slowing proliferation in cancer cells – especially hormone related malignancies such as prostate cancer and breast cancer, which compose a large subset of life threatening diseases all over the world. Although hormone deprivation therapy is usually used as the first line treatment in the advanced disease group, in many cases, tumors gradually evolve to the resistance subtype, resulting in more rapid disease progression. With emerging studies on steroid receptors characteristics, especially the relationship and significance of extranuclear and intranuclear signaling [55] (Fig.2), potential therapies that target the nuclear portion of steroid receptors, prevent steroid receptors nuclear import, or block the activation of coactivator/corepressor, are gaining more attention.

Presence of certain nuclear specific proteins are often related to poor prognosis and drug resistance. One frequently mutated gene, the truncated form of Erb2, is present in the nucleus and leads to ErB2 kinase inhibitor resistance in breast cancer [56]. Likewise, a major AR splicing variant- AR-V7, which lacks the ligand binding domain and is constitutively activated in the nucleus, has been implicated as a biomarker of enzalutamide resistance and poor prognosis among prostate cancer patients [57]. These evidences suggest

that discovering efficient methods that target not only the steroid receptors directly, but also the nuclear coregulators, will be critical for next generation of therapies.

Although paxillin's potential as drug target has been studied for years, the currently available inhibitors are either Src inhibitors or pan-tyrosine kinase inhibitors, most of which have limitations of specificity and efficacy. For instance, Imatinib, the first targeted tyrosine kinase inhibitor that is used in treatment of Philadelphia chromosome (Ph+)-positive chronic myelogenous leukemia, actually enhances tyrosine phosphorylation of p130Cas, FAK, and paxillin in glioblastoma multiforme tumor cells. Imatinib also induces cell migration and invasion *in vitro* [58]. Thus, a paxillin-specific inhibitor may be a helpful addition when using this or other kinase inhibitors. A pharmacological study from the Ginsburg group using large scale library screening identified one paxillin inhibitor that blocked alpha4 integrin-paxillin binding and reduced mononuclear leukocytes accumulation in inflammation sites [59]. More recently, a study from the Yates group discovered a small molecule inhibitor, JP-153, that abrogates the interaction between paxillin and the FAT domain in FAK, thus breaking down their extranuclear complex formation and paxillin activation, and possibly paxillin nuclear translocation. This compound exhibits inhibitory effects on VEGF induced retinal angiogenesis [60]. Interestingly, a transcriptomic study revealed that a *lncRNA-PXN-AS1* is present in hepatocellular carcinoma cells. This natural paxillin anti-sense molecule is alternatively spliced by oncofetal splicing factor, MBNL3, which results in a splice variant that upregulates paxillin expression [61]. Studies of *lncRNA-PXN-AS1* and MBNL3 may open new venues for paxillin targeting therapy.

As a key component in the focal adhesion complex, the normal function of paxillin in regulating focal adhesion should be taken into consideration during drug design. Thus, targeting the tyrosine residues critical for paxillin initial activation, for example pY118, may hinder its focal adhesive function. In contrast, designing inhibitors that could prevent serine phosphorylation of paxillin and therefore nuclear entry and subsequent DNA or transcription factor binding, may serve as a more specific target, and therefore might be more beneficial for patients with advanced hormone related cancers.

Conclusions

Paxillin and its family members are complex proteins that play major roles in modulating signaling throughout cells. When paxillin is outside the nucleus, it functions to modulate cell-cell interactions, regulate cytoskeletal changes, and modulate kinase signaling. Changing in the latter process then leads to serine phosphorylation of paxillin, allowing it to enter the nucleus where it can regulate transcription, translocation of mRNAs from the nucleus back into the cytoplasm, and subsequent translation of mRNAs into proteins. Some evidence suggests that paxillin expression, activation, and nuclear localization are upregulated in cancer cells, which may play a critical role in tumor progression. If so, then nuclear paxillin, may serve as an important biomarker for diagnosis, prognosis, and treatment of some cancers.

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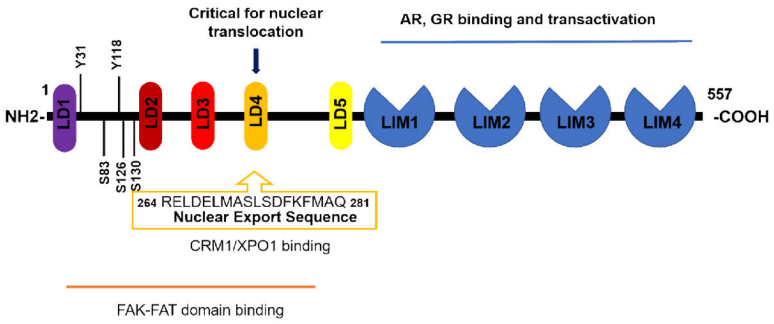

Figure 1. Schematic structure of paxillin with highlights of nuclear function related domains

Paxillin contains five LD domains on the N-terminal half and four LIM domains on the C-terminal half of the protein. The shown N-terminal domains are critical for FAK binding and nuclear translocation, with highlights of critical serine/tyrosine phosphorylation sites as well as the NES sequence within LD4 domain. The LIM domains on the C terminal half are related to paxillin's binding and transactivating of AR and GR.

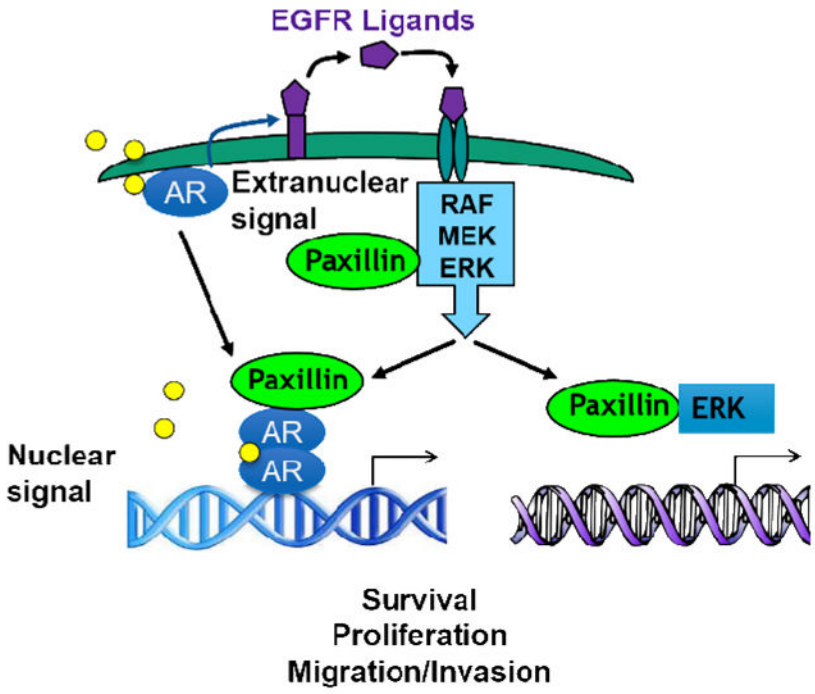

Figure 2. Model of paxillin mediated steroid signaling

In somatic cells, androgen binds to membrane-localized ARs to promote the MMP-mediated release of membrane-bound EGF receptor (EGFR) ligands, which then transactivate the EGFR. Activated EGFR further induces Erk1/2 signaling, which then regulates serine phosphorylation of paxillin. Phosphorylated paxillin is then able to translate into the nucleus, where it either binds with AR or associate with ERK to induce gene expression ultimately results in cell survival, proliferation, migration and invasion.