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Tyrosyl Phosphorylated Serine-Threonine Kinase PAK1 is a Novel Regulator of Prolactin-Dependent Breast Cancer Cell Motility and Invasion

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Abstract

Despite efforts to discover the cellular pathways regulating breast cancer metastasis, little is known as to how prolactin (PRL) cooperates with extracellular environment and cytoskeletal proteins to regulate breast cancer cell motility and invasion. We implicated serine-threonine kinase p21-activated kinase 1 (PAK1) as a novel target for PRL-activated Janus-kinase 2 (JAK2). JAK2-dependent PAK1 tyrosyl phosphorylation plays a critical role in regulation of both PAK1 kinase activity and scaffolding properties of PAK1. Tyrosyl phosphorylated PAK1 facilitates PRL-dependent motility via at least two mechanisms: formation of paxillin/GIT1/ β PIX/pTyr-PAK1 complexes resulting in increased adhesion turnover and phosphorylation of actin-binding protein filamin A. Increased adhesion turnover is the basis for cell migration and phosphorylated filamin A stimulates the kinase activity of PAK1 and increases actin-regulating activity to facilitate cell motility. Tyrosyl phosphorylated PAK1 also stimulates invasion of breast cancer cells in response to PRL and three-dimensional (3D) collagen IV via transcription and secretion of MMP-1 and MMP-3 in a MAPK-dependent manner. These data illustrate the complex interaction between PRL and the cell microenvironment in breast cancer cells and suggest a pivotal role for PRL/PAK1 signaling in breast cancer metastasis.

5.1 Role of Prolactin in Regulation of Breast Cancer Cell Motility

Prolactin (PRL) is a peptide hormone secreted from the anterior pituitary and was originally discovered in the early twentieth century as a hormone that regulates milk production in mammals [1, 2]. In addition to lactation, PRL was also implicated in mammary gland growth and development [3–6]. Significant progress was made in determining PRL-mediated signaling pathways upon the characterization of the prolactin receptor (PRLR) in the 1980s [7]. The PRLR is a transmembrane protein that belongs to the cytokine receptor superfamily and is expressed in variety of tissues, most notably the mammary epithelium [8]. The PRLR has no intrinsic kinase activity and relies on nonreceptor tyrosine kinases to facilitate PRL-mediated downstream signaling pathways. The most well characterized mediator of PRL signaling is the nonreceptor tyrosine kinase Janus-kinase 2 (JAK2) [9–11]. Upon PRL binding to its receptor, PRLRs dimerize, resulting in the activation of JAK2, as

characterized by autophosphorylation of Tyr1007/1008, and promoting tyrosyl phosphorylation of the PRLR [12–14]. PRL signaling induces the activation of several signaling cascades, including the signal transducers and activators of transcription (STATs), mitogen-activated protein kinases (MAPKs), protein kinase C, and phosphatidylinositol 3-kinase (PI3K) [15–21]. Since then, PRL signaling has been shown to regulate a variety of normal and pathological cell processes, one of which is cell motility.

Cell migration is critical for many vital biological functions, including embryonic development, the inflammatory immune response, wound repair, tumor formation and metastasis, and tissue remodeling and growth. The actin cytoskeleton provides both the protrusive and contractile forces required for cell migration via a combination of actin polymerization and depolymerization, actin filament cross-linking, and the interaction of myosin-based motors with actin filaments [22]. The complexity of cell motility and the fact that it is regulated by many hormones, cytokines, and growth factors suggest that multiple signaling mechanisms exist to regulate this process.

Little is known about the mechanisms that underlie the process of PRL-induced cell motility and its putative role in breast cancer metastasis. PRL was previously shown to act as a chemoattractant for human breast carcinoma [23]. Actin-based structures are most commonly controlled by small Rho-GTPases Rac1, Cdc42, and RhoA and these proteins are activated by guanine nucleotide exchange factors (GEFs) and repressed by GTPase-activating proteins (GAPs). PRL can activate Rac1 and several pathways have been implicated in this Rac-dependent regulation [24–26]. The first pathway has been shown to depend on PRL-induced activation of tyrosine kinase Tec which associates with and enhances activity of Vav1, the GEF factor for Rac1 [24]. According to the second proposed mechanism, PRL induces activity of serine-threonine kinase Nek3 (NIMA-related kinase 3) followed by activation of Vav1/Vav2 and subsequent activation of Rac1 [27, 28]. In addition, PRL stimulation also induces an interaction between Nek3 and focal adhesion protein paxillin and significantly increases paxillin serine phosphorylation [28]. In addition to Rac, PRL also activates another small GTPase Cdc42 that plays an important role in development and differentiation of mammary epithelia [25]. We have recently proposed two novel mechanisms to regulate PRL-dependent breast cancer cell motility: (1) through a serine-threonine kinase p21-activated kinase 1 (PAK1) and its substrate, the actin-binding protein filamin A and (2) through regulation of adhesion turnover ([29]; see below).

Cell migration depends on optimal levels of cell adhesion. The mechanisms that regulate focal adhesion assembly, maturation, and turnover are not well understood and have become a critical area of emerging interest. Over 180 proteins are found in adhesions, many of which exhibit multiple protein–protein interactions [30]. Cell adhesion regulated by both PRL- and extracellular matrix (ECM)/integrin-dependent pathways is essential for all aspects of normal mammary gland development and function (reviewed in [31–33]). PRL also regulates activation of numerous proteins participating in breast cancer cell adhesion. Thus, in early studies it has been noticed that PRL dramatically changes adhesiveness of breast cancer cells [34]. PRL activates focal adhesion kinase (FAK) and eventually induces phosphorylation of paxillin, an event that is essential to the rapid turnover of adhesions during cell motility [35]. FAK is a nonreceptor tyrosine kinase that mediates integrin

signaling and regulates focal adhesion assembly and maturation during cell spreading and migration through phosphorylation of various adhesion proteins (reviewed in [36]). PRL causes tyrosyl phosphorylation of paxillin in an Src/FAK-dependent manner and serine phosphorylation of paxillin by serine-threonine kinase Nek3 ([28, 37]). In addition, transmembrane glycoprotein signal regulatory protein- α (SIRP α) has been implicated in the PRL- and integrin-activated cross talk in breast cancer cells [38]. We will discuss the role of PRL-activated serine-threonine kinase PAK1 in the regulation of breast cancer cell adhesion (see below). Thus, PRL has evidently been shown to increase cell motility in breast cancer cells.

Epidemiologic studies also linked elevated level of circulating PRL to breast cancer metastases [39–41]. In addition, PRLR expression has been found in patients with colorectal cancer, with high concordance between primary tumors and corresponding metastases [42]. These data, combined with animal studies reporting increased metastases with PRL administration [43], suggest that PRL is involved in the development of metastasis and tumor progression.

We have previously found that the serine-threonine kinase PAK1, a downstream effector for both Cdc42 and Rac1, participates in PRL-dependent signaling. We have shown that PAK1 is a novel substrate of the JAK2 tyrosine kinase and that PRL-activated JAK2 phosphorylates PAK1 in vivo and in vitro. PAK1 tyrosines 153, 201, and 285 were identified as sites of JAK2 tyrosyl phosphorylation by mass spectrometry and two-dimensional (2D) peptide mapping [44].

The aim of this review is to introduce tyrosyl phosphorylated PAK1 as a novel player in the field of PRL signaling and to discuss several mechanisms of pTyr-PAK1-dependent regulation of breast cancer cell motility, adhesion, and invasion.

5.2 p21-Activated Kinase 1 (PAK1)

5.2.1 PAK1 Structure and Activation

The PAKs are an evolutionarily conserved six member family of serine/threonine kinases and can be categorized into two groups based on structure and function: Group I (PAKs 1–3), which are activated in a GTPase-dependent or independent manner in response to extracellular signals, and Group II (PAKs 4–6), which are generally not regulated by Rho-GTPases but most likely through intramolecular mechanisms (reviewed in [45]). PAK1, a Group I member, is the most well-studied representative of the six PAK family members and is widely expressed in a variety of tissues. PAK1 plays a pivotal role in a range of cellular processes including cell proliferation, survival, motility, and invasion. PAK1 consists of an N-terminal regulatory domain containing a GTPase binding domain (GBD) that is partially overlapped with an autoinhibitory domain (AID). PAK1 enzymatic activity derives from its C-terminal serine/threonine kinase domain. The N-terminal regulatory domain of PAK1 has additional sites of protein–protein interaction that can mediate PAK1 activation and localization, including five classical proline-rich regions (PXXP), two of which facilitate binding to adaptor proteins Nck and Grb2. PAK1 also contains a nonclassical proline-rich region (PXP) that mediates interaction with the p21-interacting

exchange factor PIX. In addition, there are three nuclear localization signals (NLS) and multiple phosphorylation sites, seven of which (serines 21, 57, 144, 149, 199, 204, and threonine 423) are sites of PAK1 autophosphorylation. PAK1 activation and localization are dependent on protein–protein interactions and both autophosphorylation and direct phosphorylation of PAK1 by other kinases (Fig. 5.1).

PAK1 was initially discovered as an effector protein for two members of the Rho-family of small GTPases, Cdc42, and Rac [46]. These Rho-family GTPases serve as activators of PAK1 kinase activity. Inactive PAK1 resides in the cytoplasm as a homodimer, where the AID of one PAK1 molecule is obstructing the kinase domain of the other and vice versa (Fig. 5.2; [47]). Binding of Cdc42 or Rac1 to PAK1's GBD induces a PAK1 conformational change that allows autoinhibitory relief and autophosphorylation of several sites on PAK1, keeping it in an open and active conformation. Recent studies suggest that membrane localization of inactive PAK1 via the adaptor proteins Nck and Grb2 promotes a semi-open conformation of PAK1 which facilitates autophosphorylation of several serines, including Ser199 and Ser204, and promotes initial kinase activation of PAK1. This semi-open/semi-active PAK1 is more susceptible to interaction with Rac1 or Cdc42. Rho-GTPase/PAK1 binding facilitates a fully open PAK1 conformation and allows autophosphorylation of Ser144 in the GBD and Thr423, the major PAK1 autophosphorylation site mediating PAK1 kinase activity, in the kinase domain [48].

PAK1 activation is not solely dependent on GTPases, since the interaction of PAK1 with a variety of different proteins can regulate PAK1 kinase activity. Membrane localized PAK1 can be activated by direct phosphorylation on the critical Thr423 by phosphoinositide-dependent kinase 1 (PDK1) [49]. Similarly, certain lipids at the plasma membrane, like phosphatidic acid and sphingosine, can bind to the regulatory domain of PAK1 and induce kinase activation and subsequent autophosphorylation to the same extent as GTPase-activation of PAK1 [50]. Also at the plasma membrane, the actin cross-linking protein filamin A (FlnA) can mediate PAK1 kinase activity in two ways: by binding directly to the PAK1 GBD, therefore stimulating PAK1 activation, and facilitating the interaction of PAK1 with lipids [51, 52]. Akt (protein kinase B) can directly activate PAK1 and phosphorylate Ser21 on PAK1 which negatively mediates Nck/Grb binding and membrane localization [53, 54]. Some proteins, such as the guanine exchange factor (GEF) PIX, can induce PAK1 activation in both a GTPase-dependent and independent manner. The binding of PAK1 to PIX localizes PAK1 to cell–matrix adhesions and can directly mediate PAK1 activation, and since PIX is a Rac1-specific GEF, PIX can indirectly activate PAK1 through activation of Rac1 [55, 56]. Interestingly, PAK1 can also be activated upon tyrosyl phosphorylation. The nonreceptor tyrosine kinase Etk/BMX can tyrosyl phosphorylate PAK1 and induce PAK1 kinase activation; however, the sites for Etk-induced phosphorylation have not been mapped [57]. The nonreceptor tyrosine kinase JAK2 can also tyrosyl phosphorylate and activate PAK1, and we will review this activation and downstream effects on PAK1 signaling in this chapter [44]. Downregulation of PAK1 kinase activity is also important, since hyperactivation of PAK1 can induce mammary gland tumor growth [58]. PAK1 enzymatic activation and localization to focal adhesions can be inhibited when the tumor suppressor protein Merlin is bound to the PAK1 GBD [59]. Likewise, the integrin-binding protein Nischarin can bind to the kinase domain of activated PAK1, greatly reducing PAK1 kinase

activity [60]. Cystein-rich protein CRIPak has also been identified as an inhibitor of PAK1 [61]. Human PAK1-interacting-protein 1 (hPIP) binds to first 70 amino acids of PAK1 and blocks kinase activity [62]. P35/Cdk5 phosphorylates PAK1 and inhibits kinase activity while phosphatases POPX1 and POPX2 dephosphorylate threonine 423 of PAK1 and also inhibit it [63, 64]. Protein kinase p110C binds to amino acids 210–332 of PAK1 and inhibits it [65].

The diverse means in which PAK1 is regulated lends PAK1 to participate in a variety of fundamentally different cellular processes (Fig. 5.3).

5.2.2 PAK1 Acts as a Scaffold

While PAK1 kinase activity plays a major role in PAK1 downstream signaling events, PAK1 can also act independent of its kinase activity as a molecular scaffold to facilitate the interaction between different proteins. Thus, PAK1 can regulate the actin cytoskeleton in both kinase-dependent and independent ways. PAK1 mutants with a modified N-terminus have dramatic effects on the actin cytoskeleton regardless of the presence of an active kinase domain [46, 66]. PAK1 overexpression has been shown to increase random cell movement irrespective of its kinase activity [67]. Also, an SH3 domain of PIX protein binds to a noncanonical proline-rich region on PAK1 independently of PAK1 kinase activity, and through association with GIT1, localizes PAK1 to the adhesion protein paxillin to regulate cell adhesion [55, 68]. These data, in combination with the fact that overexpression of kinase-dead PAK1 facilitates the formation of focal adhesions and stabilizes stress-fibers [69], suggest a role for the scaffolding abilities of PAK1 in regulation of cytoskeletal and adhesion dynamics.

PAK1 has also been shown to act as a scaffold in coordinating signaling between Raf-1, MEK, and ERK proteins upon cell adhesion to fibronectin or treatment with PDGF [70]. Overexpression of a kinase-dead PAK1 increased phosphorylation of MEK and ERK in kinase-independent way [71]. In addition to the scaffolding function, PAK1 phosphorylates both MEK and Raf-1 to amplify the ERK signaling [72]. The MAPK pathway is not the only pathway that benefits from the nonenzymatic activity of PAK1. Akt, a major regulator of several cell survival pathways, is activated upon phosphorylation by PDK1 and the plasma membrane localization of Akt is important for the activation [73, 74]. Higuchi et al. demonstrated that upon growth factor stimulation Akt binds to the C-terminal domain of PAK1 and is consequently targeted to the plasma membrane. At the plasma membrane, PAK1 can also bind to PDK1, bringing together both Akt and PDK1 thereby facilitating Akt stimulation by PDK1. These findings confirm scaffolding, kinase-independent functions of PAK1 [75]. There is also evidence of kinase-independent roles for PAK1 in regulation of the cell cycle. Overexpression of the kinase inhibitory domain of PAK1 (AID) induces cell cycle arrest and decreases cyclin D1 and D2 expression independently of PAK1 kinase activity [76]. We have previously demonstrated that three tyrosines on PAK1 molecules and PAK1-Nck interaction play a critical role in PAK1-dependent regulation of cyclin D1 promoter activity in response to PRL and proposed that Nck-PAK1 complex (formation of which does not depend on PAK1 kinase activity) can sequester PAK1 in cytoplasm to

prevent PAK1 nuclear shuttling thereby inhibiting PAK1-dependent activation of cyclin D1 promoter [77].

The multifunctionality of PAK1 as both a kinase and a scaffolding protein allow PAK1 to modulate a diverse array of cell processes, such as cell proliferation, survival, motility, and invasion.

5.2.3 PAK1 Regulates Cell Proliferation

PAK1 has been shown to stimulate cell proliferation (Fig. 5.3). Thus, highly proliferating human breast cancer cell lines and tumor tissues have been shown to contain hyperactive PAK1 and its upstream regulator Rac3 [78]. Tyrosyl phosphorylation of PAK1 by nonreceptor tyrosine kinase Etk/Bmx leads to increased proliferation of human breast cancer MCF-7 cells [57]. In addition, expression of kinase-active T423E PAK1 mutant in mammary glands induces hyperplasia in the mammary epithelium [79]. One of the first conclusive evidence that PAK1 has a role in cell cycle regulation was the finding that overexpression of activated PAK1 in human breast cancer cells leads to the abnormal accumulation of centrosomes and aberrant mitoses [80]. Furthermore, PAK1 is present at histone complexes, centrosomes, and at mitotic spindles during mitosis [81]. During the early stages of mitosis, DNA must be tightly packed into chromosomes to allow for proper gene segregation through a process called chromosome condensation. This process is highly regulated by various posttranslational modifications to the DNA-bound histone protein complexes. One such event is the phosphorylation of Ser10 on histone H3 that is necessary for the initiation of chromosome condensation [82, 83]. Li et al. reported that active PAK1 can translocate into the nucleus (via the PAK1 NLSs, Fig. 5.1) where it can directly bind to and phosphorylate histone H3 on Ser10, promoting chromosome condensation and aiding in the progression of metaphase to anaphase during mitosis [81]. PAK1 can also mediate proper formation of the mitotic spindle and microtubule dynamics during mitosis. Tight regulation of microtubule dynamics is absolutely required for proper spindle formation and chromosomal segregation. Tubulin cofactor B (TCoB) assists in the assembly of α and β -tubulin and is localized at the centrosomes. PAK1 has been implicated in TCoB activity during mitosis. PAK1 colocalizes with TCoB at the centrosome and phosphorylates two serine residues on TCoB, encouraging the microtubule polymerization activity of TCoB [84]. PAK1 can also regulate the formation of the mitotic spindle at the centrosome through Aurora A. Aurora A is a serine/threonine kinase that is present at the centrosome throughout mitosis and is responsible for the recruitment of several microtubule-associated proteins required for proper spindle formation. Regulation of Aurora A is important, since knockdown of Aurora A leads to abnormal maturation of the centrosome [85]. Through interaction with PIX/GIT1 complex, PAK1 is localized to the centrosome where it directly induces Aurora A activation by phosphorylating Thr288 and Ser342 [86]. These data combined with the fact that hyperactive PAK1 in cells leads to aneuploidy [80] suggest an important role for PAK1 in chromosome segregation and microtubule regulation during mitosis. PAK1 can also induce expression of cyclin D1, one of the key mediators of cell cycle progression. Overexpression of active PAK1 increases cyclin D1 expression in breast cancer cells while knockdown of PAK1 significantly reduces cyclin D1 expression [87]. Our lab demonstrated that PRL-mediated activation of PAK1 increased nuclear localization of

PAK1 and activation of cyclin D1 promoter and that PAK1/Nck binding inhibited PAK1 nuclear localization and cyclin D1 promoter activity [77]. Balasenthil et al. proposed that PAK1 can increase cyclin D1 transcription through two independent pathways—the NF κ B pathway and phosphorylation of S305 of estrogen receptor alpha (ER α) [87, 88]. PAK1 has been previously shown to directly phosphorylate ER α at Ser305 and promote its transactivation functions [79]. Interestingly, PAK1 itself is activated by estrogen suggesting a positive feedback loop [89]. Lastly, PAK1 can regulate cell proliferation through activation of the Ras/ERK pathway [90–92]. The regulation of the ERK pathway by PAK1 requires both kinase-dependent and independent functions of PAK1 as stated earlier. Thus, these data describe a multifunctional role for PAK1 in the regulation of cell proliferation and cell cycle progression.

5.2.4 PAK1 Regulates Cell Survival

PAK1 also plays a role in cell survival (Fig. 5.3). PAK1 inhibits the release of pro-apoptotic factors from the mitochondria. BAD is a proapoptotic protein that binds and inhibits the prosurvival proteins Bcl-2 and Bcl-X. Phosphorylation of BAD at Ser112 and Ser136 blocks BAD binding to Bcl-XL and promotes cell survival [93]. PAK1 promotes cell survival by directly phosphorylating these serines on BAD [94]. Also, we had previously mentioned that PAK1 can act as a scaffold for Raf-1, MEK, and ERK, promoting the activation of the MAPK pathway in the regulation of cell proliferation; however, PAK-mediated phosphorylation of Raf-1 can also mediate cell survival. Raf-1 phosphorylation at Ser338 by PAK1 can induce the translocation of Raf-1 to the mitochondria where it binds to Bcl-2 and phosphorylates BAD at Ser112, thus providing an additional mechanism in which PAK1 can regulate BAD activity and prevent apoptosis [95]. PAK1-mediated BAD phosphorylation was described as a critical event in survival signaling induced by the HIV viral Nef protein [96]. In addition to directly regulating BAD activity, PAK1 induces the degradation of proapoptotic proteins such as BimL [97]. Typically, dynein light chain 1 (DLC1) is bound to BimL, preventing BimL from inactivating Bcl-2, thus promoting cell survival, that is until BimL is released upon proapoptotic signals [98, 99]. PAK1 can phosphorylate both DLC1 and BimL leading to DLC1/BimL degradation and therefore promoting cell survival [97]. Another mechanism in which PAK1 can regulate cell survival is through activation of the NF κ B pathway [100–102]. PAK1 mediates NF κ B activation by Ras, Raf-1, and Rac1 and expression of active PAK1 can stimulate NF κ B on its own without activation of the inhibitor of κ B kinases [100]. Friedland et al. discovered that PAK1-induced NF κ B activation prevented apoptosis in three-dimensional (3D) cultures of mammary epithelial cells [102]. During *Helicobacter pylori* infection of human epithelial cells, PAK1 activates NF κ B via activation of upstream regulatory kinase NIK (NF κ B-inducing kinase) [103]. Furthermore, PAK1 also inhibits apoptosis by phosphorylating and inactivating cell survival forkhead transcription factor, FKHR [89]. Hence, PAK1 has both direct and indirect roles to play in the regulation of cell survival.

5.2.5 PAK1 Regulates the Actin Cytoskeleton

The first described and most well-understood function of PAK1 is the role for PAK1 in the regulation of the actin cytoskeleton and cell motility (Fig. 5.3). Active PAK1 is localized to areas of actin remodeling, such as filopodia and lamellipodia of motile cells, membrane

ruffles, and pinocytosis vesicles [46, 104, 105]. Overexpression of kinase-active PAK1 induces the formation of lamellipodia and membrane ruffles [46, 104]. PAK1 phosphorylates a variety of different actin cytoskeleton proteins such as Lim Kinase 1 (LIMK1), p41-Arc, filamin A, myosin light chain (MLC) and myosin light chain kinase (MLCK). LIMK1 is a kinase that upon activation can phosphorylate and inactivate cofilin, an actin-binding protein. Cofilin depolymerizes actin fibers in ruffles and lamellipodia, promoting actin recycling and retrograde flow [106, 107]. PAK1 can directly phosphorylate and activate LIMK1, resulting in downstream inactivation of cofilin and subsequent stabilization of actin filaments [108, 109]. Actin filaments stabilization allows for the efficient formation of protrusive structures, such as lamellipodia and filopodia during cell motility [109]. Proper protrusion formation also relies on the creation of a branched actin network. The Arp2/3 complex is a complex of proteins that facilitates branching actin filaments by binding to existing actin fibers and providing nucleation sites for new actin filaments at a 70° angle from the original filament. The nucleation property of the Arp2/3 complex requires p41-Arc protein, which can be regulated by PAK1. Phosphorylation of p41-Arc on Thr21 by PAK1 induces the localization of p41-Arc to the Arp2/3 complex, facilitating actin nucleation and branching during cell motility, while blocking PAK1-mediated phosphorylation of p41-Arc inhibits cell motility [110]. Similarly, PAK1 can bind to the actin-cross-linking protein filamin A. Serine phosphorylation of FlnA by PAK1 at Ser2152 results in PAK1-dependent membrane ruffling [51]. FlnA in turn activates PAK1, furthering PAK1 downstream actin-modulating signals. Actin stress-fibers are anchored by focal adhesions to provide support and bind to nonmuscle myosins that regulate tension and contraction. PAK1 can modify actin–myosin binding and focal adhesion assembly by interacting and phosphorylating MLC and MLCK. MLCK typically phosphorylates MLC at Ser19, promoting actin–myosin binding and increased contractility [111]. PAK1, however, can phosphorylate Ser439 and Ser991 of MLCK and inhibit MLCK [112]. Inhibition of MLCK by PAK1 reduces stress fiber formation and leads to the disassembly of focal adhesions, both processes that are necessary to promote cell motility. PAK1 can directly phosphorylate MLC at Ser19, promoting myosin–actin binding which may regulate the contraction of the trailing edge of motile cells [67, 113]. Furthermore, PAK1 facilitates integrin-mediated cell adhesion [114–116], as activation of PAK1 promoted disassembly of actin stress, abolishment of focal adhesion, and reduction of cell attachment, while PAK1 silencing enhanced cell adhesion and/or spreading and led to increased size and number of mature focal adhesion [117–122].

5.2.6 Role of PAK1 in EMT

Epithelial-mesenchymal transition (EMT) is a process where tightly adhered, non-motile epithelial cells lose their epithelial characteristics and display the loose adherence and motile phenotypes of mesenchymal cells. EMT was first described in the context of embryogenesis, where it leads to the generation of mesenchymal cells. Epithelial cells undergoing EMT acquire a morphology that is appropriate for migration through the extracellular environment, and for settlement in areas of new organ formation. In recent years, EMT-like processes have been the focus of active research on their potential role as determinants of cancer cell invasion and metastasis. Certain proteins can be used as markers for pathogenic EMT, including E-cadherin, N-cadherin, and vimentin. EMT is characterized by the

reduction of E-cadherin expression and an increase in both N-cadherin and vimentin expression. E-cadherin transcription is controlled by various transcription factors (TFs), one of which is Snail [123, 124]. The Snail superfamily of TFs is composed of two families; the Snail family (Snail, SNAILP, and SLUG), and the Scratch family (SCRATCH1 and SCRATCH2) (reviewed in [125]). Members of the Snail family have been shown to regulate EMT [124, 126].

PAK1 has been implicated in regulation of EMT by findings that E-cadherin expression in MCF-7 cells was downregulated upon transfection of PAK1, and conversely, E-cadherin expression in MDA-MB-435 cells was upregulated through inhibition of PAK1 expression [127]. A similar effect of PAK1 has been also demonstrated in keratinocytes [128]. On the other side, PAK1 was also shown to be required for the stabilization of adherent junctions through a recently discovered target of PAK1 Ajuba, an actin-binding protein that colocalizes with cadherins [129]. The role of PAK1 in the stabilization of E-cadherin cell-cell junction has also been shown during zebrafish epiboly [130].

PAK1 has been shown to regulate E-cadherin expression through Snail [127]. Yang et al. demonstrated that PAK1 phosphorylates Ser246 on Snail. Serine phosphorylation of Snail facilitates the accumulation of Snail in the nucleus and promotes transcriptional repression of E-cadherin. Knockdown of PAK1, or mutation of serine 246 on Snail to an alanine, leads to increased cytoplasmic Snail and a reduction of Snail repressor activity [127]. In contrast, the same lab had previously demonstrated that PAK1 phosphorylates corepressor CtBP (C-terminal binding protein 1) that leads to translocation of CtBP from the nucleus into the cytoplasm and relieves its corepressor activity toward the E-cadherin promoter. They demonstrated that CtBP-mediated repression of the E-cadherin promoter was relieved by transfection of PAK1 [131]. If so, the effect of PAK1 on E-cadherin expression may be either stimulating through inhibition of CtBP or repressive through activation of Snail.

PAK1 also interacts with β -catenin and promotes β -catenin activation in gastric epithelial cells [132]. Phosphorylation of β -catenin at Ser675 by PAK1 increases the stability and transcriptional activity of β -catenin in colorectal cells [133]. PAK1 knockdown in human colorectal cell lines inhibits β -catenin expression, β -catenin transcriptional activity, and the expression of c-Myc and suppresses the tumor growth and metastasis in mouse model [134].

Recently, PAK1 has been shown to activate β -catenin transcriptional activities and promote EMT in podocytes [135].

In addition, PAK1 can mediate peroxisome proliferator-activated receptor gamma (PPAR γ)-induced EMT of intestinal epithelial cells through activation of the ERK1/2 pathway [136].

5.2.7 Role of PAK1 in Breast Cancer

PAK1 plays an important role in such vital processes like cell proliferation, survival, cell motility and EMT, therefore it is no surprise that misregulation of PAK1 activity is present in many cancers. Altered expression and/or activation of PAK1 is evident in various cancers, including brain, pancreas, colon, bladder, ovarian, hepatocellular, urinary tract, renal cell

carcinoma, thyroid, and breast cancers ([137–146], reviewed in [147]). Of these cancers, the role for PAK1 in breast cancer has been studied to the most extent ([58, 80, 87, 148–152], reviewed in [147, 153]). PAK1 is overexpressed or upregulated in some breast cancers. The PAK1 gene is localized within the 11q13 region, and 11q13.5 → q14 amplifications involving the PAK1 locus are found in 17 % of breast cancer [154, 155]. Overexpression of PAK1 was observed in 34 of 60 breast tumor specimens [87] and expression of PAK1 in human breast tumors correlates with tumor histologic grade [80, 150]. PAK1 expression and activity were higher in human breast tumors as compared to their adjacent controls [156]. Furthermore, expression of PAK1 in human breast tumors correlates with tamoxifen resistance [150]. PAK1 kinase activity can also be increased in human breast tissue by the upregulation of Rac3 activity or Rac1 expression [78, 157]. In a transgenic mouse model, PAK1 hyperactivation (PAK1 T423E mutant) leads to the formation of mammary gland tumors [58]. Of particular interest, PAK1 plays a critical role in premalignant progression of MCF10 series of human breast epithelial cell lines grown in 3D reconstituted basal membrane overlay cultures [158]. It has been demonstrated that expression of a kinase-dead PAK1 mutant in highly invasive breast cancer cell lines led to reduced invasiveness [69]. Conversely, hyperactivation of the PAK1 pathway in the noninvasive breast cancer cell line MCF-7 promotes cell migration and anchorage-independent growth [80]. As we described above, PAK1 phosphorylates several transcription factors, among them CtBP1 and Snail both of which are important for EMT [127, 131]. Another possible mechanism of PAK1-mediated malignant transformation is the enhancement of PAK1-regulated cell motility because PAK1 kinase activity participates in directional motility and PAK1 directly phosphorylates cytoskeletal proteins as we discussed above. For example, depletion of PAK1 has been shown to contribute to breast cancer cell invasion through cofilin-dependent mechanism [159]. Thus, PAK1 has become one of the focal points in the investigation into the mechanism and onset of human breast cancer. Recently, our lab has demonstrated a role for PRL-mediated tyrosyl phosphorylation of PAK1 in breast cancer cell motility, adhesion and invasion.

5.3 PRL Regulates Breast Cancer Cell Motility Through Tyrosyl Phosphorylated PAK1

5.3.1 JAK2 Tyrosyl Phosphorylates and Activates PAK1 in Response to PRL

In 2007, we demonstrated that PAK1 is a novel substrate of the JAK2 tyrosine kinase and that PRL-activated JAK2 phosphorylates PAK1 *in vivo*. PAK1 tyrosines 153, 201, and 285 were identified as sites of JAK2 tyrosyl phosphorylation by mass spectrometry and 2D peptide mapping. Our findings indicated that this phosphorylation plays an important role in cell survival and in the regulation of cyclin D1 promoter activity [44, 77].

In an attempt to understand the mechanism of JAK2-dependent activation of PAK1, we first focused on testing PAK1 kinase activity in an *in vitro* kinase assay with P³²-ATP and exogenous H4 histone as a substrate. Indeed, PAK1 kinase activity was increased in the presence of overexpressed activated JAK2 but not kinase dead JAK K882E. Active JAK2 had no effect on the kinase activity of the PAK1 Y3F mutant in which the three JAK2 phosphorylation sites (Tyr(s) 153, 201 and 285) were mutated to phenylalanine [44]. PRL

treatment activated both PAK1 WT and PAK1 Y3F (which is catalytically active). However, in the presence of PRL, the kinase activity of PAK1 WT was significantly stronger than PAK1 Y3F in MCF-7, T47D and TMX2–28 breast cancer cell lines (Fig. 5.4; [29, 160]). Heregulin (HRG), a ligand for HER3 (human epidermal growth factor receptor-3) and HER4 (human epidermal growth factor receptor-4), activates both PAK1 WT and PAK1 Y3F to the similar extent confirming that PAK1 Y3F retains its kinase activity. How does PRL activate JAK2-phosphorylation-deficient mutant PAK1 Y3F? Presumably, it works through Rac1. Indeed, both PAK1 WT and PAK1 Y3F were similarly activated by either activated Rac1 V12 or by activated Cdc42 L61 [160]. This Rac1/Cdc42-dependent activation is pTyr-PAK1-independent, therefore PAK1 Y3F mutant exhibits some kinase activity in response to PRL (black bars for PAK1 Y3F in Fig. 5.4).

However, PAK1 is activated by GTPase-independent mechanisms as well. As we discussed above, membrane recruitment of PAK1 by Nck and Grb2 adapter proteins results in the stimulation of PAK1 kinase activity through interaction with lipids such as sphingosine or phosphatidic acid [50]. Membrane-localized PAK1 can also be activated by PDK1 [49]. The nonreceptor tyrosine kinase Etk/BMX can tyrosyl phosphorylate PAK1 and induce PAK1 kinase activation [57]. In addition, PAK1 can be directly activated by Akt [54] and FLNa [51]. Which mechanism acts in response to PRL? It has been demonstrated that PAK1 phosphorylates FLNa on Ser 2152 and FLNa activates PAK1 in a positive feedback loop [51]. We have shown that Ser-phosphorylation of FLNa was increased when FLNa was coexpressed with PAK1 and constitutively active JAK2 V617F as compared to coexpression of FLNa with kinase inactive JAK2 mutant K882E. Furthermore, we have demonstrated that PAK1 phosphorylates Ser 2152 of FLNa to a greater extent when PAK1 is tyrosyl-phosphorylated by JAK2 in response to PRL [29]. Thus, PRL can induce PAK1 kinase activity in two ways: first in a GTPase-dependent manner, activating both PAK1 WT and PAK1 Y3F, but also through pathway(s) that are pTyr-PAK1-dependent, further activating PAK1 WT but not PAK1 Y3F, for example, through filamin A (Fig. 5.5).

Moreover, PAK1 has a dual activity. First, PAK1 is a serine-threonine kinase and this activity depends on activation of the PAK1 kinase domain. Second, as we discussed above, PAK1 acts as a scaffold for many proteins, for example, for Raf-1, MEK and Erk [70] and this PAK1 activity depends on its ability to initiate protein–protein interactions. One might speculate that tyrosyl phosphorylated PAK1 may create additional docking sites to recruit SH2-domain containing proteins to facilitate local activation of recruited proteins and amplify PRL-dependent signaling. In such case, pTyr-PAK1 will be able to recruit additional proteins to function as a scaffold to locally amplify PRL signaling.

Thus, PRL may stimulate both PAK1 activities: kinase activity and scaffolding ability of PAK1 through JAK2-dependent tyrosyl phosphorylation of Tyr(s) 153, 201, and 285.

In our search for scaffolding activities of pTyr-PAK1, we focused on β PIX/GIT1 proteins. A proline-rich motif of PAK1 (residues 182–203) binds directly to the SH3 domain of GEF β PIX [55]. The PIX proteins associate with G protein-coupled receptor kinase-interacting target 1 (GIT1), a GTPase activating protein (GAP) for Arf, that targets adhesion complexes by binding to paxillin [68]. β PIX and GIT1 can homodimerize and form large aggregates in

the cell [161, 162]. This oligomerization is essential for localization to sites of adhesion since mutations that disrupt either GIT- β PIX association or β PIX homodimerization result in diffuse cytoplasmic localization of both proteins [163, 164]. PAK1 is an important component of this complex and formation of the four-molecule PAK1/ β PIX/GIT1/paxillin signaling module transiently targets PAK1 to the sites of adhesion [165–168]. To provide insight into whether tyrosyl phosphorylation of PAK1 increases the ability of PAK1 to bind β PIX and GIT1, we immunoprecipitated PAK1 from the lysates of PAK1 WT and PAK1 Y3F cells treated with PRL over a time course and assessed these immunoprecipitates for endogenous β PIX. The quantification of PAK1 and β PIX bands in the immunoprecipitates showed that PRL increased association of β PIX with PAK1 WT about 8.5-fold. The amount of endogenous β PIX bound to PAK1 Y3F was left unchanged during PRL treatment. Next, we also assessed GIT1 associated with PAK1 WT and PAK1 Y3F upon PRL treatment. We demonstrated that threefold more GIT1 was associated with tyrosyl phosphorylated PAK1 WT than with PAK1 Y3F. These data demonstrate that tyrosyl phosphorylation of PAK1 regulates its binding activity toward β PIX and GIT1. We hypothesize that phosphorylation at position Y285 may affect the interaction with β PIX by inducing a conformational change that makes the proline-rich motif of PAK1 more accessible to β PIX (Hammer et.al. unpublished).

Overall, PAK1 WT and PAK1 Y3F have similar kinase activity in response to active Rac1/Cdc42. PRL stimulates both PAK1 WT and Y3F through Rac1 and additionally activates PAK1 WT (but not PAK1 Y3F) by Rac1-independent mechanism(s) which depends on tyrosyl phosphorylation (for example, by local activation by filamin A). Furthermore, pTyr-PAK1 is able to recruit additional proteins to function as a scaffold to locally amplify PRL signaling further (for example, via β PIX/GIT1 recruitment). These two activities are interconnected because high local concentration of PAK1 in PAK1/ β PIX/GIT1/paxillin complexes permits autophosphorylation, stimulating the kinase activity of PAK1 (Fig. 5.5).

5.3.2 Tyrosyl Phosphorylated PAK1 Regulates the Actin Cytoskeleton and Cell Motility in Response to PRL Through Filamin A

PAK1 is major regulator of actin cytoskeleton dynamics and can bind a variety of different actin-modulating proteins. We have implicated tyrosyl phosphorylation of PAK1 in the regulation of unstimulated phagocytosis, which is a combination of two processes that are dependent upon changes in the actin cytoskeleton: cellular movement and phagocytosis [44]. Later, we have demonstrated that overexpression of WT PAK1 enhanced the ability of PRL to induce cell ruffling. In contrast, overexpression of PAK1 Y3F failed to increase ruffling [169]. Membrane ruffling has been observed in many cell types in response to certain extracellular factors, and on motile cells where they are believed to be required for directed cell migration. Thus, the formation of membrane ruffles may be considered as a sign of increased response to external stimuli and of elevated cell migration (for review, [170, 171]). We extended our findings and demonstrated that overexpression of PAK1 WT strongly enhances cell migration in response to PRL in both cell wounding and Boyden chamber assays [29]. In an attempt to understand the mechanism of the amplifying effect of tyrosyl phosphorylated PAK1 on cell motility, we focused on filamin A for several reasons. First, the actin-binding protein FLNa is a binding partner of PAK1 [51]. Second, we have

previously implicated FLNa in PRL-dependent signaling through adapter protein SH2B1 β [169]. Filamin A is a 280 kDa actin cross-linking protein containing an N-terminal actin-binding domain and a rod region containing 24 immunoglobulin-like repeats (Fig. 5.6; reviewed in [172]). The last repeat of the rod region enables the FLNa molecules to dimerize, allowing for a flexible structure mediating the actin gelation activity of filamins. Filamins have > 90 interacting partners, including adapter proteins, small GTPases, transmembrane receptors, and membrane channels [173]. FLNa participates in the activation of various kinases as well as being regulated by kinases itself. FLNa binding to PAK1 enhances the kinase activity of PAK1, which subsequently phosphorylates FLNa at Ser 2152, resulting in PAK1-dependent membrane ruffling [51]. FLNa also stimulates PAK1 by interacting with sphingosine kinase 1, which phosphorylates sphingosine, leading to the direct activation of PAK1 [52]. As a potent actin cross-linking protein, FLNa regulates cell migration, although the role of FLNa in this process is controversial. Thus, multiple studies have demonstrated a positive impact of FLNa on the migration of different cell types (for example, [174–177]). One of the first noted defects of FLNa-deficient melanoma cells (M2 cells) was the inability to migrate due to inefficient polarization and continuous blebbing, which was rescued once FLNa was stably reexpressed (A7 cells) [174]. In contrast, FLNa overexpression inhibits neuronal migration [178] and downregulation of FLNa stimulates cancer cell migration, invasion, and metastatic formation [179]. In support of the latter finding, we demonstrated that the depletion of FLNa increased basal nonstimulated migration of T47D cells. However, PRL-induced cell migration was suppressed by FLNa knock-down. These previously published and our current results suggest that at normal expression levels, FLNa activity should be strongly regulated to coordinate cell migration. In addition, we show that PAK1 phosphorylates Ser 2152 of the actin-binding protein filamin A to a greater extent when PAK1 is tyrosyl-phosphorylated by JAK2 in response to PRL. Downregulation of PAK1 or filamin A abolishes the effect of PRL on cell migration. Thus, these data bring some insight into the mechanism of PRL-stimulated motility of breast cancer cells [29].

We have proposed a model for PRL-dependent regulation of the actin cytoskeleton (Fig. 5.7; [29, 169]). According to this model, upon ligation of PRLR and activation of JAK2, adapter protein SH2B1 β translocates to activated PRLR-JAK2 complexes, where it cross-links actin filaments via its two actin-binding domains and binds to FLNa [169, 180]. PRL-activation of JAK2 also leads to tyrosyl phosphorylation of PAK1, thereby increasing PAK1's activities (both the kinase and scaffolding activities) and stimulating phosphorylation of FLNa. FLNa, in turn, activates PAK1, binds to SH2B1 β and relocates more SH2B1 β to the JAK2/PAK1/FLNa complex. Because SH2B1 β enhances the tyrosine kinase activity of JAK2 [181], the formation of this multiprotein complex results in enhancement of JAK2 activation and further activation of the JAK2/PAK1/FLNa-actin complex, leading to actin cytoskeleton reorganization.

5.3.3 PRL-Mediated pTy-PAK1 Regulation of Adhesion Turnover

Cell adhesion is the basis for cell migration. Dynamic changes in cell–matrix adhesions are necessary for both cell spreading and cell motility. The rapid assembly and disassembly of adhesions during cell migration is called adhesion turnover. Upon contact with the ECM, or

in response to external stimuli, there is clustering and activation of the cell-surface proteins integrins, the chief proteins regulating cell–matrix adhesion. Integrin clustering induces autophosphorylation of FAK on Tyr397, enhancing FAK kinase activity and recruits the adhesion scaffolding protein paxillin ([182, 183], reviewed in [184]). Localized FAK and paxillin, along with the actin-binding protein talin, at integrin clusters form small adhesion complexes, called nascent adhesions, at the distal edge of the lamellipodium [185]. Nascent adhesions are unstable and can either immediately disassemble, or mature into larger focal complexes. Nascent adhesion maturation requires FAK-mediated phosphorylation of paxillin on two tyrosines, Tyr31 and Tyr118 [182]. This tyrosyl phosphorylation of paxillin increases the affinity of paxillin for FAK, recruits a variety of kinases, scaffolding proteins, and regulators of GTPase activity, and induces the maturation of the nascent adhesion into a focal complex [185–187]. Focal complexes can then either further mature into larger focal adhesions, or disassemble (turnover), a process that occurs rapidly during cell motility. Modulation of adhesion dynamics is both tightly regulated and highly complex.

PAK1 activity has been implicated in regulating cell–matrix adhesion dynamics. PAK1 is localized at adhesions, where it regulates both adhesion assembly and disassembly [117, 119, 188, 189]. One of the first observations was that overexpressed PAK1 WT and kinase-dead PAK1 localized to the focal adhesions and caused the accumulation of focal points [117]. These data were conformed later by demonstration that overexpression of kinase-dead PAK1 facilitates the formation of focal adhesions [66, 69]. Several years later, PAK1 was shown to be directly involved in mediating adhesion turnover, a process that could be reversed upon expression of the AID domain, suggesting that PAK1 kinase activity was necessary for proper adhesion turnover [119, 189]. It turns out that both PAK1 kinase activity and scaffolding properties are required to modulate adhesion turnover in motile cells. In order for PAK1 to be localized to adhesion complexes, PAK1 must bind to the β PIX protein as well as the GIT1 protein, however this interaction is completely independent of β PIX's GEF activity and GIT1's GAP activity [55, 167]. β PIX binds to noncanonical proline-rich motif on PAK1 (Fig. 5.8) and this interaction is negatively regulated by autophosphorylation of PAK1 at Ser199/Ser204 [55, 165]. As mentioned previously, PAK1/ β PIX binding does increase PAK1 activation and PAK1 can subsequently phosphorylate β PIX at Ser340; however, this phosphorylation does not regulate PAK1/ β PIX interaction and the physiological relevance of this event has yet to be uncovered [190, 191]. The complex formation of PAK1 and β PIX is not sufficient to locate PAK1 at adhesion complexes. However, β PIX binds to GIT1 and GIT1 binds to paxillin thereby targeting this trimolecular complex to adhesions (Fig. 5.8; [168]). Once the GIT1/ β PIX/PAK1 complex arrives at the adhesion complex, PAK1 can phosphorylate Ser273 on paxillin, increasing the affinity of GIT1 for paxillin and recruiting more GIT1/ β PIX/PAK1 complexes to the adhesion [189]. At the same time, serine phosphorylation of paxillin reduces the affinity of FAK to paxillin, setting the stage for adhesion disassembly yet freeing FAK to facilitate the formation of new nascent adhesions [189]. Thus, PAK1 can facilitate adhesion turnover by means of its scaffolding and enzymatic activity.

We have recently shown that PRL-mediated tyrosyl phosphorylation of PAK1 regulates adhesion turnover. When breast cancer cells stably overexpressing either PAK1 WT or PAK1 Y3F were plated on collagen IV in the presence of PRL, PAK1 WT cells displayed a

It is now well documented that the interaction of cells with 2D substrates is significantly different than the more natural 3D environments that cells are embedded within in vivo [202–204]. Furthermore, the molecular composition of the ECM, specific association of multiple growth factors and cytokines with the matrix, along with the aforementioned “dimensionality” play roles in the responses of cells to their local matrix microenvironment [192]. Cells embedded in 3D matrix have higher amounts of ligated matrix-receptors as compared to the cells grown on the top of a thin film of matrix. Collagen receptors, such as integrins and discoidin domain receptors (DDRs), are signal transducing receptors. Integrin clustering initiates an array of signaling cascades, including activation of the Rho family of small GTPases, MAPKs, and PI3-kinases [205], and any of them can lead to regulation of matrix metalloproteinases (MMP) expression.

MMPs are a family of Zn^{2+} -dependent enzymes composing 23 members. MMP-1 (collagenase 1) is a major proteinase of the MMP family that specifically degrades type I collagen, a major component of the ECM. It also degrades other fibrillar collagens of types II, III, VII, VIII, X, XI as well as gelatins, aggrecan, entactin, tenascin, and perlecan [206–208]. As these collagen types are the most abundant proteins in the body, MMP-1 is critical for the modeling and remodeling of the ECM [209]. In clinical studies, increased MMP-1 expression is associated with the incidence or invasiveness of several cancers: colorectal, esophageal, pancreatic, gastric, breast, and malignant melanoma [210–215]. Increased MMP-1 expression is also associated with advanced stages of breast cancer and may be a predictive marker for the development of invasive disease [216]. MMP-3, or stromelysin 1, can degrade a variety of ECM substrates, such as type III, IV, V, VII, and IX–XI collagens, laminins, fibronectin, osteopontin, and proteoglycans. MMP-3 is expressed by stromal cells during normal mammary gland development and is strongly upregulated during postlactational mammary involution when considerable ECM remodeling and alveolar apoptosis occur. MMP-3 is upregulated in many breast tumors and contributes to cancer development. Indeed, mice overexpressing MMP-3 show excessive side branching and eventual tumor formation in the mammary gland ([217–219]; reviewed in [220–222]). MMP-3 induces EMT in mammary cells through cleavage of E-cadherin, expression of Rac1b and transcriptional factor Snail [223]. MMP-2 and MMP-9 are both type IV collagenases that contribute to tumor invasion in vitro because of their ability to break down basement membrane, degrading collagen IV in particular [224, 225]. Elevated circulating MMP-9 levels have been demonstrated in patients with breast cancer and MMP-2 and/or MMP-9 release has been associated with tumor invasion and metastasis ([226, 227]; reviewed in [228–231]). The expression of MMPs is regulated at the transcriptional and posttranscriptional levels (including the stability of mRNA and protein as well as the release and activation of protein) by a number of hormones, growth factors, and cytokines [232]. Despite efforts to discover the cellular pathways regulating MMPs, little is known as to how different cytokines cooperate with cytoskeletal proteins to regulate MMP expression.

PRL regulation of MMP expression and breast cancer cell invasiveness is complex. It has been shown that PRL-induced activation of MAPK/AP-1 pathway is inversely related to PRL-induced STAT5 activation [233–235]. Thus, PRL together with IGF-I promotes MMP-2 expression and cell invasion (AP-1 targets MMP-2 gene) [236]. In contrast, WT STAT5a overexpression inhibits MMP-2 transcription/activity while reduction of STAT5 by

siRNA or inhibition of STAT5 activity increases the PRL-dependent transcription/activity of MMP-2 and invasiveness [235, 237]. T47 cells overexpressing degradation-resistant PRLR demonstrated increased proliferation and invasiveness while silencing of PRLR dramatically reduced the cell invasion and MMP-9 secretion [238]. In vivo, murine PRL-induced mammary carcinomas with lower level of pSTAT5 demonstrate higher level of MMP-9 expression [234]. Recently, these findings have been linked to the ECM stiffness [233]. Thus, in compliant 3D collagen I, PRL signaling has been shown to be mediated predominantly through the STAT5 pathway. This pathway results in prodifferentiation outcome with no MMP2 expression and absences of invasion. In contrast, in stiff collagen I, PRL signaling is mediated by Src/FAK and pERK 1/2 pathways resulting in MMP2 expression and enhanced invasion. Thus, increased stiffness of the ECM switches the signal in breast cancer cells from differentiation toward enhanced tumorigenic processes [233]. PAK1 also plays a pivotal role in the regulation of cell transformation, invasion, and MMP secretion. Role of PAK1 in the EMT and breast and other cancers has been discussed above. Of particular interest, PAK1 plays a critical role in premalignant progression of MCF10 series of human breast epithelial cell lines grown in 3D reconstituted basal membrane overlay cultures [158]. Thus, PAK1 expression and activity increased with premalignant progression from normal mammary cells through hyperplasia, atypical hyperplasia, to ductal carcinoma while dominant-negative PAK1 or knock-down PAK1 reduced cell proliferation, migration, invasion, and pericellular proteolysis of collagen IV in these 3D cultures [158]. PAK1 has been also implicated in MMP regulation. TNF α -induced MMP-9 is mediated through PAK1 and JNK activation [239]. PAK1 regulated IL-1 β -induced production and activity of MMP-13 and MMP-14 (MT1-MMP) in synoviocytes during inflammatory joint disease [240] as well as MMP-2 activity in ovarian cancer cells [241]. In addition, inhibition of Rac1 reduces MMP-1 expression [242].

We have recently implicated both PRL and PAK1 together with 3D collagen IV in the regulation of breast cancer invasion. PAK1 stimulates the PRL-dependent invasion of TMX2–28 cells (highly invasive ER-negative clone of MCF-7 cells) through Matrigel. We have shown that TMX2–28 cells stably overexpressing PAK1 WT have upregulated expression and secretion of MMP-1, -2, and -3 when they grow in 3D collagen IV, which makes up 31 % of Matrigel protein composition. PRL-induced PAK1 tyrosyl phosphorylation leads to a further increase in MMP-1 and MMP-3 expression and cell invasion in MAPK-dependent manner.

Why have we seen these effects only in 3D collagen IV? Different collagens regulate expression of different MMPs. Collagen I induces expression/secretion of MMP-1 and MMP-9 ([239, 243–247], while collagen IV upregulates expression of MMPs 2 and 9 [248]. MMP-2 is often constitutively expressed and controlled through a unique mechanism of enzyme activation. MT1-MMP (MMP-14)-mediated activation of pro-MMP-2 is upregulated by 3D collagen I in endothelial cells [249–251], fibroblasts [252–254], and cancer cell lines [255–259].

Embedding cells in a 3D matrix can amplify signals from ligated integrins/DDRs and lead to MMP expression. Furthermore, there are numerous reports of “cross talk” and “synergy”

between signaling by ECM receptors and by various growth factors and cytokines. Such cross talk involves cooperation in the downstream signal transduction pathways.

Another possible explanation of how 3D collagen results in elevated expression of MMPs as compared to 2D collagen relates to the physical properties of 3D matrixes. ECM physical properties often refer to rigidity, porosity, insolubility, topography, and other characteristics that are essential for its scaffolding role in supporting tissue structure and integrity, and for its role in migration and anchorage of the cell (reviewed in [205, 208]). The aforementioned recent paper from Dr. Schuller's lab has evidently demonstrated the role of 3D collagen I stiffness in the production of MMP-2 [233].

The actin cytoskeleton appears to be the major cellular system for transduction of force generated by the external network. Cytoskeletal stretching correlates with the recruitment of adhesion complex proteins and triggers signals resulting in the induction of a matrix-degrading protease (reviewed in [260, 261]). This may explain our data demonstrated that 3D collagen I induces expression of only MMP-9 while 3D collagen IV upregulates expression of MMP-1, 2, and 3 but not MMP-9 [160]. Collagen I is a fibril-forming collagen while collagen IV is a network forming collagen. We can speculate that cells embedded in the network formed by 3D collagen IV, but not collagen I, can sense geometry and the external force generated by this network. We speculate that, in addition to the ligation of different receptors, physical properties of the 3D collagen IV network activate cytoskeletal-triggered signaling pathways that are distinct from those activated by 3D fibrillar collagen I that results in induction of distinct MMPs.

Another possible explanation of how 3D collagens can induce MMPs expression is the observation that the ECM acts as a "sink" or "reservoir" for growth factors/cytokines. Indeed, the ECM is essential for shaping the concentration gradient for many growth factors, including bone morphogenetic protein, fibroblasts growth factor, Hedgehog, and Wnts [208, 262, 263]. We can speculate that the 3D collagen IV network retains PRL to a better extent than 3D fibrillar collagen I or 2D collagens, therefore leading to amplified PRL signal which leads to MMP-1 and -3 productions.

We have hypothesized that contact with 3D collagen IV may be an important invasive stimulus for breast cancer cells (Fig. 5.10). Mammary cells are normally surrounded by basement membrane, comprised mostly of type IV collagen. In normal cells, signals from collagen IV do not induce MMP expression. In contrast, in breast cancer cells PRL initiates the JAK2-dependent tyrosyl phosphorylation of PAK1, increasing PAK1 signaling. Importantly, PAK1 expression is also elevated in breast cancer [87]. Filamin A can serve as a bridge between activated integrins and pTyr-PAK1 to integrate signals from cytokines (PRL) and the ECM (collagen IV). PAK1 activates Erk 1/2, p38 MAPK, and JNK 1/2, each of which can activate AP-1. Genes encoding MMP-1 and -3 have an AP-1 binding site supporting the transcription of these MMPs after induction by PAK1. MMP-1 degrades type I collagen, which is a major component of the ECM and MMP-3 degrades collagen IV which is a main component of basement membrane. We have also shown that secretion of MMP-1 and -3 is required for PRL-dependent invasion [160]. Given the complexity of these

signaling cascades it is likely that additional signaling molecules are also involved in the modulation of MMP expression.

5.4 Conclusion and Future Directions

PRL binding to the PRLR induces receptor dimerization and activation of the non-receptor tyrosine kinase JAK2. Activated JAK2 phosphorylates the serine/threonine kinase PAK1 on three tyrosines 153, 201, and 285. This tyrosyl phosphorylation of PAK1 enhances such important PAK1 functions as kinase activity and the ability to form protein–protein interactions. Both of these PAK1 activities are important for adhesion, motility, and invasion of breast cancer cells in response to PRL. During cell adhesion, PRL promotes formation of the GIT1/ β PIX/pTyr285-PAK1 complex. This complex localizes to small adhesion sites (adhesion complexes), the amount of which is increased by PRL treatment. In these small adhesion complexes at cell periphery PAK1 phosphorylates serine 273 on paxillin that results in enhanced adhesion turnover. Enhanced adhesion turnover facilitates cell motility and, indeed, PRL stimulates breast cancer cell motility.

In addition to GIT1/ β PIX/pTyr285 PAK1-dependent mechanism, we implicated actin-binding protein filamin A in the regulation of cell motility. Tyrosyl phosphorylation of PAK1 in response to PRL increases PAK1/FLNa interaction, and subsequent serine phosphorylation and activation of FLNa. Phosphorylated FLNa stimulates the kinase activity of PAK1 and has increased actin-regulating activity. FLNa directly binds to adapter protein SH2B1 β (which is tyrosyl phosphorylated by JAK2 in response to PRL), relocates SH2B1 β to the JAK2-PAK1-FLNa complex. Since SH2B1 β is the enhancer of the kinase activity of JAK2, the formation of the complex results in enhancement of JAK2 activation and further activation of the JAK2-PAK1-FLNa complex that leads to actin cytoskeleton reorganization via actin-regulating proteins PAK1, FLNa, and SH2B1 β , which has two actin-binding sites and cross-links actin filaments [169, 180].

PRL-induced pTyr-PAK1 also activates MAPK pathways, leading to the expression and secretion of MMP-1 and MMP-3 in response to 3D collagen IV microenvironment. MMP-1 degrades type I collagen, which is a major component of the ECM and MMP-3 degrades collagen IV which is a main component of basement membrane resulting in increased invasion of breast cancer cells in response to PRL.

PAK1 is important for a variety of fundamentally different cellular processes therefore it is critical to understand how PAK1 functions are controlled. The role of PAK1 tyrosyl phosphorylation is incompletely understood and there are only a few publications in this field although PAK1 is ubiquitously expressed, subject to growth factors, cytokine and hormone regulation and participates in various cellular functions. Fundamental questions whether PRL-dependent regulation of PAK1 also plays a critical role in normal mammary gland development, growth, and differentiation also remain (Fig. 5.11).

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p21-Activated Kinase 1 (PAK1)

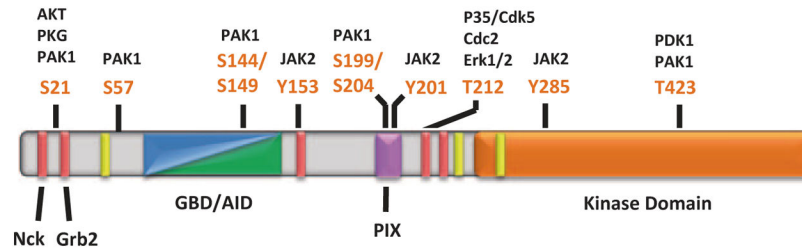


Fig. 5.1.

PAK1 domain structure and phosphorylation sites. The N-terminal regulatory region of PAK1 is composed of overlapping GBD/AID domains (aa 70-149, *blue/green*), five proline-rich regions (*bright red*), one nonclassical proline-rich region (aa 182-203, *pink*), and three nuclear localization signals (*yellow*). The C-terminal kinase domain (aa 249-545) is represented by the *bright orange* region. The two most N-terminal proline-rich regions (aa 12-18 and aa 40-47) mediate Nck/Grb2 binding, respectively, and subsequent PAK1 membrane localization. The non-classical proline-rich region regulates PIX/PAK1 binding and subsequent localization of PAK1 to adhesion complexes as well as facilitates PAK1 kinase activity. There are seven PAK1 autophosphorylation sites (S21, S57, S144, S149, S199, S204, and T423) that modulate PAK1 kinase activity, in addition to other sites phosphorylated by protein kinases that mediate PAK1 activity and localization. (Modified from Bokoch 2003)

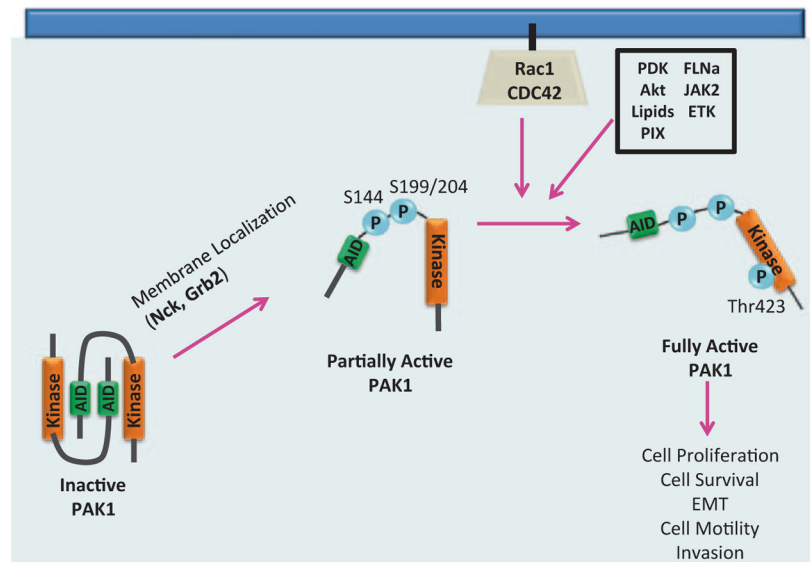


Fig. 5.2.

Model for PAK1 activation. Inactive PAK1 is localized to the cytoplasm as a homodimer. Upon binding to Nck or Grb2, PAK1 is localized to the plasma membrane and undergoes a slight conformational change that facilitates partial autophosphorylation. PAK1 is now more susceptible to Rac1/Cdc42 binding, leading to a further conformational change, autophosphorylation at T423, and fully active PAK1 kinase activity. PAK1 can also be activated by interaction with Akt, lipids, PIX, FLNa, ETK, JAK2 or by direct T423 phosphorylation by PDK1. (Modified from Parrini et al. 2009)

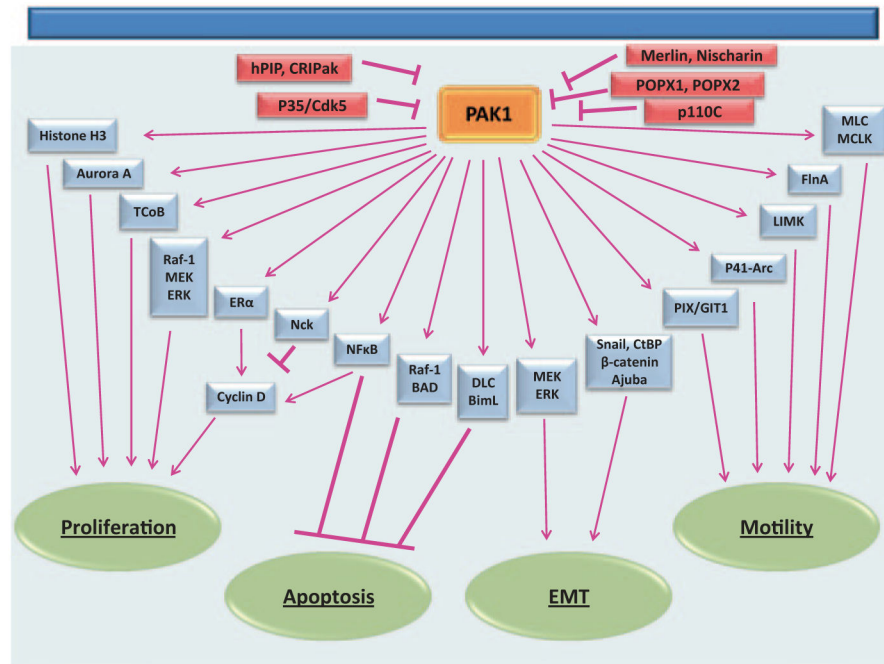


Fig. 5.3. PAK1 regulates different cellular functions, including cell proliferation, survival, cell motility, and EMT. PAK1 kinase activity and/or interaction with various proteins mediate PAK1s variable functions within the cell

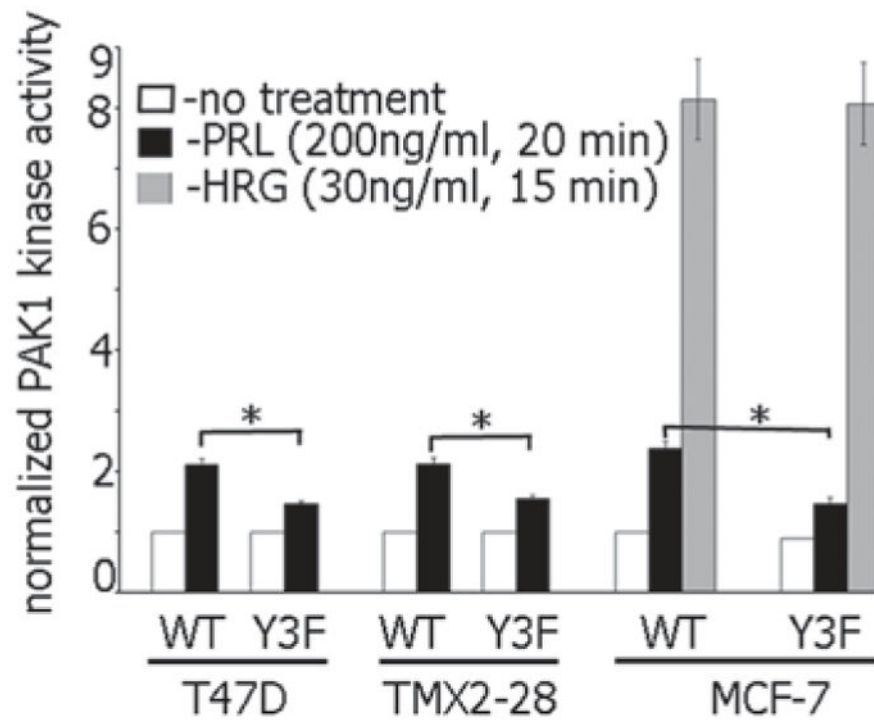


Fig. 5.4.

Tyrosyl phosphorylation of Tyr 153, 201, and 285 is required for maximal PAK1 kinase activity in response to PRL but not heregulin. Indicated cell lines stably overexpressing PAK1 WT or PAK1 Y3F were deprived of serum and treated with or without prolactin (*PRL*) or heregulin (*HRG*). Myc-PAK1 was IP'd and subjected to an in vitro kinase assay with H4 histone as a substrate. Relative PAK1 kinase activity was then normalized by the amount of IP'd PAK1 for each lane and plotted. *Bar* represent mean \pm S.E., *, $p < 0.05$ compared with the same cells without treatment ($n = 3$)

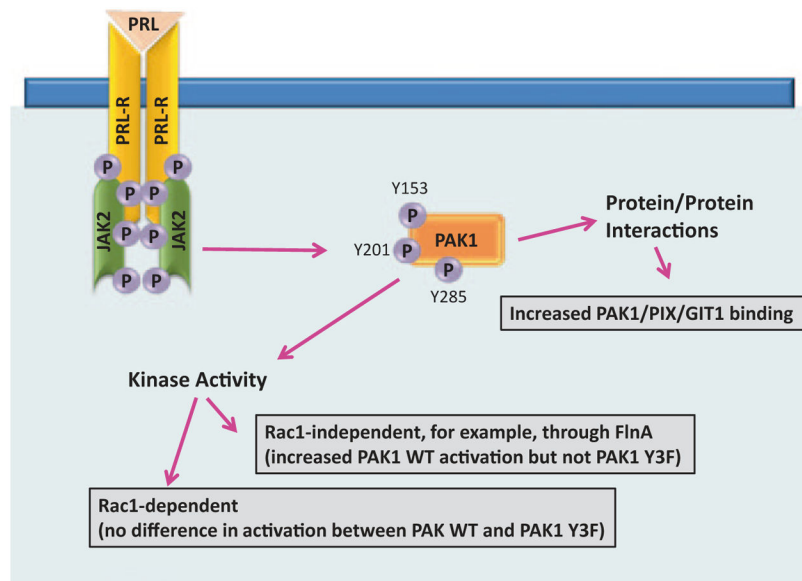


Fig. 5.5. PRL stimulates kinase activity of PAK1 and PAK1 ability to form protein–protein interaction. PRL binding induces dimerization of the PRLR and subsequent activation of JAK2. JAK2 phosphorylates PAK1 on three tyrosines, Tyr 153, 201 and 285. Tyrosyl phosphorylation of PAK1 enhances both a Rac-dependent (Rider et al. 2013) and Rac-independent PAK1 kinase activity (Hammer et al. 2013), and also the ability for PAK1 to act as a molecular scaffold (Hammer et al., unpublished)

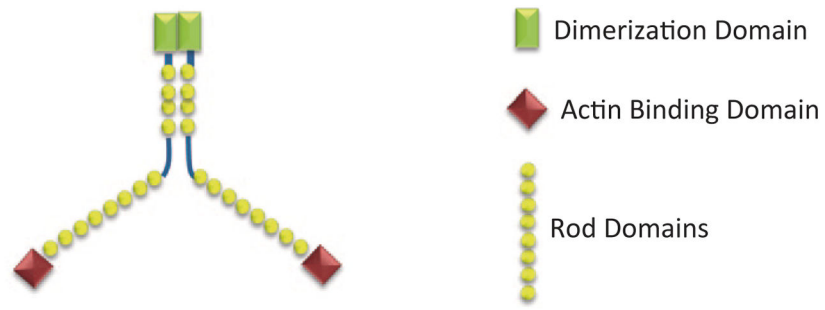


Fig. 5.6. Schematic diagram of FLNa dimer. FLNa consists of an N-terminal actin-binding domain, a *rod* domain containing 24 IgG-like repeats and C-terminal dimerization domain. (modified from Cukier et al. 2007)

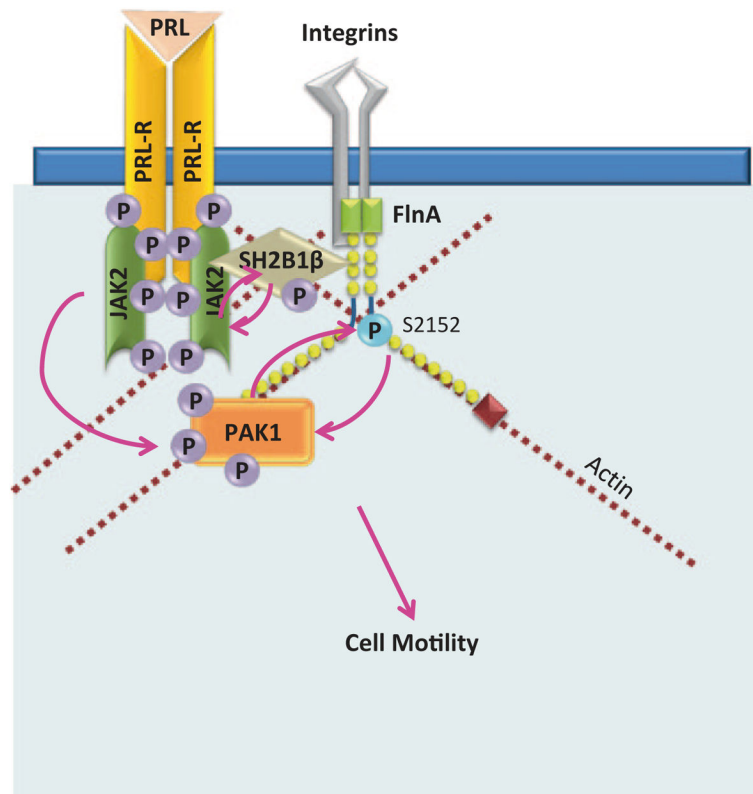


Fig. 5.7.

Tyrosyl phosphorylated PAK1 regulates cell motility in response to PRL through filamin A. PRL-activated JAK2 tyrosyl phosphorylates PAK1 increasing PAK1 kinase activity and scaffolding ability of PAK1. pTyr-PAK1 has increased FlnA interaction and phosphorylates FlnA on Ser2152. FlnA activates PAK1 in a positive feedback loop. JAK2 also phosphorylates SH2B1 β , which binds to FlnA and cross-links actin filaments. Positive feedback from FlnA to PAK1 and SH2B1 β to JAK2 facilitates the formation of more JAK2/SH2B1 β /FlnA/pTyr-PAK1 complexes that regulate actin remodeling during enhanced cell motility in response to PRL

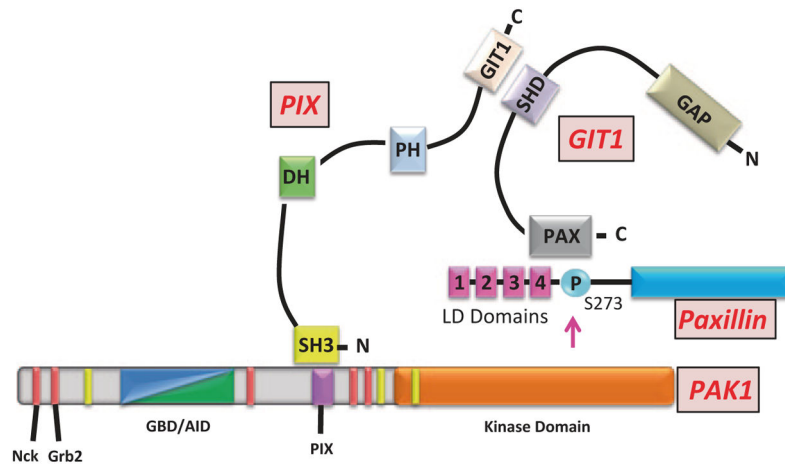


Fig. 5.8.

GIT1/βPIX/PAK1/paxillin complex. PAK1 binds to the N-terminal SH3-domain of βPIX. βPIX has a DBL-homology (*DH*) domain and a plextrin homology (*PH*) domain, and binds to GIT1 through a C-terminal GIT1-binding domain. GIT1 has a C-terminal paxillin-binding domain (*PAX*) that binds to the LD4 domain of paxillin and SHD domain that binds to βPIX. PAK1 can phosphorylate Ser273 of paxillin at adhesion complexes

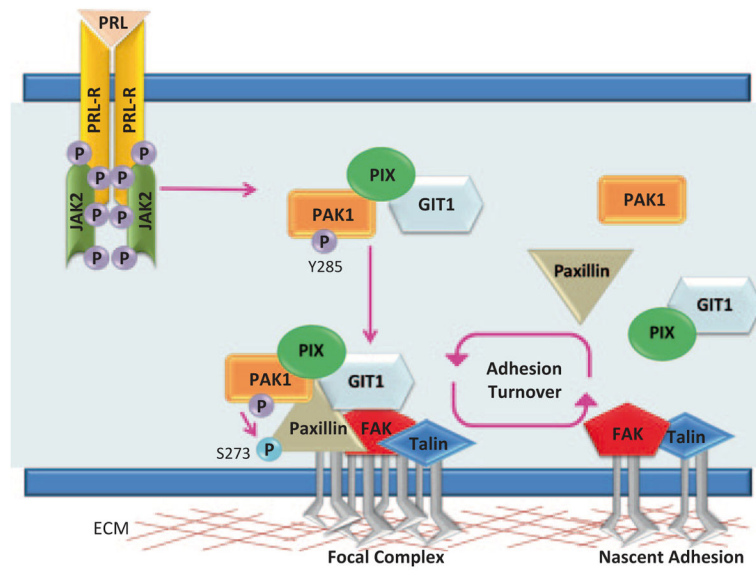


Fig. 5.9.

PRL-dependent tyrosyl phosphorylation of PAK1 regulates adhesion turnover. PRL-activated JAK2 phosphorylates PAK1 at Tyr285 and stimulates both PAK1 activities: kinase activity and ability of PAK1 to form the GIT1/ β PIX/pTyr285 PAK1 complex. This complex localizes to small adhesion complexes, the amount of which is increased by PRL treatment. Increased GIT1/ β PIX/pTyr-285PAK1 association leads to enhanced phosphorylation of paxillin on Ser 273 that results in enhanced adhesion turnover and finally to increased cell motility

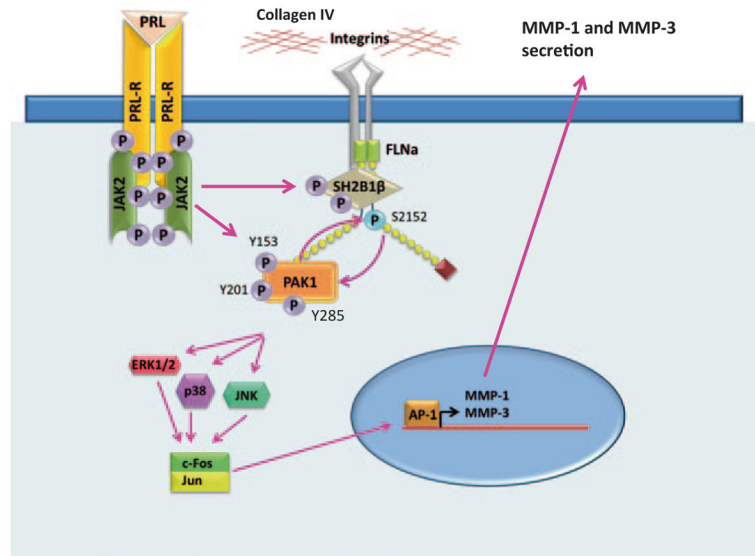


Fig. 5.10.

PRL-dependent tyrosyl phosphorylated PAK1 and three-dimensional (3D) collagen IV regulate MMP-1 and MMP-3 production and invasion via MAPK pathways. PRL-activation of JAK2 leads to tyrosyl phosphorylation of PAK1 on tyrosines 153, 201, and 285, thereby increasing PAK1 activities and stimulating phosphorylation of FLNa. Phosphorylated FLNa stimulates the kinase activity of PAK1 in a positive feedback loop. In turn, FLNa binds to β -integrin and transduces signals from surrounding matrix to inside of a cell. 3D collagen IV-induced signals, in combination with pTyr-PAK1, produce intense synergistic increases in MMP-1 and MMP-3 production via MAPK pathways. MMP-1 degrades type I collagen, which is a major component of the ECM and MMP-3 degrades collagen IV which is a main component of basement membrane resulting in increased invasion of breast cancer cells in response to PRL.

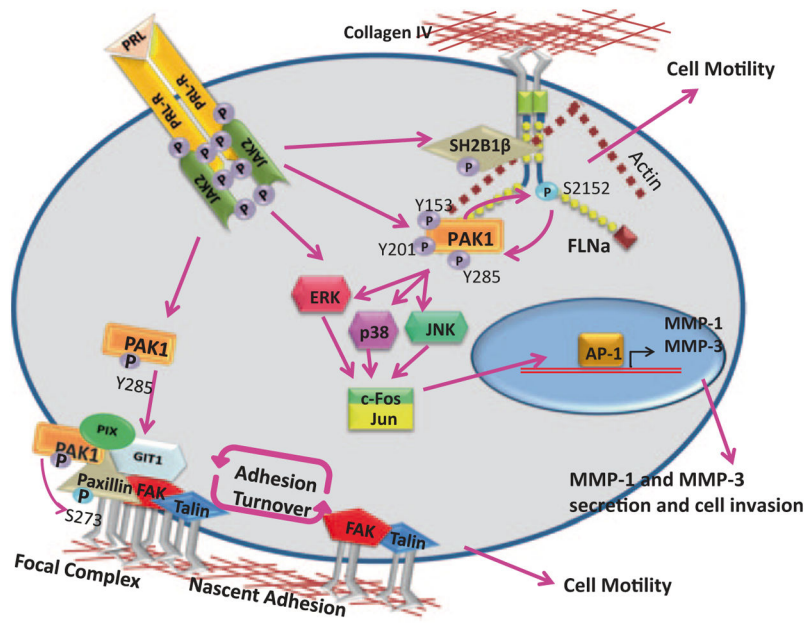


Fig. 5.11. PRL-dependent tyrosyl phosphorylated PAK1 regulates breast cancer cell motility and invasion