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“CaMKIIγ: un substrato nuevo de PAK1 en líneas celulares de cáncer de mama ErbB2/HER2+

T E S I S

Que presenta

M.C. Héctor Iván Saldívar Cerón

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Directores de tesis:

Dra. Rosaura Hernández Rivas

Dr. Luis Enrique Arias Romero

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DEPARTMENT OF MOLECULAR BIOMEDICINE

"CaMKIIγ: a new substrate of PAK1 in ErbB2/HER2+ breast cancer cell lines

T H E S I S

Presented by

M.D. Héctor Iván Saldívar Cerón

TO OBTAIN THE MASTER IN SCIENCES DEGREE IN THE SPECIALTY OF MOLECULAR BIOMEDICINE

Thesis Directors

Dra. Rosaura Hernández Rivas

Dr. Luis Enrique Arias Romero

Mexico, Mexico City August, 2017

SUPERVISORS

Dr. Leopoldo Santos Argumedo

Principal Investigator at the Department of Molecular Biomedicine

CINVESTAV-IPN

Dr. Nicolás Villegas Sepúlveda

Principal Investigator at the Department of Molecular Biomedicine

CINVESTAV-IPN

Dr. José Vázquez Prado

Principal Investigator at the Department of Pharmacology

CINVESTAV-IPN

This work was carried out in the laboratory of Dr. Luis Enrique Arias Romero, Assistant Professor of the Biomedicine Unit of FES Iztacala, UNAM and in the laboratory of Dr. Rosaura Hernández Rivas, Principal Investigator of the Department of Molecular Biomedicine of the Center for Research and of Advanced Studies of IPN.

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II. ABREVIATIONS

°C	Celsius Degrees
aa	Amino acids
Amp	Ampicillin
ATP	Adenosine triphosphate
bp	Base pair
CAM	Ca^{2+}/calmodulin
CaMKIIɣ	Calcium/Calmodulin dependent protein kinase II gamma
cDNA	Complementary DNA
CK	Cytokeratins
DEPC	Diethyl pyrocarbonate
DTT	Dithiothreitol
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ErbB2/HER2	Epidermal Growth Factor Receptor 2
GST	Glutathione-S-transferases
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton
LB medium	Luria-Bertani medium
MAPK	Mitogen-Activated Kinase
mM	Millimol
ng	Nanogram
ORF	Open reading frame
PAK	P2-activated kinases
PBS	Phosphate-buffered saline
PI3K	Phosphatidyl inositol 3 kinase
PR	Progesterone receptor
RTKs	Receptor tyrosine kinases
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TKI	Tyrosine-kinase inhibitors
µg	Microgram
µL	Microliter

III. LIST OF TABLES

Table 1. Oligonucleotides used for the generation of GST-CaMKIIγ-212-317aa recombinant fragment. In red are indicated the BamHI and EcoRI restriction sites for cloning.

Name	Sense oligonucleotide	Position	Anti-sense oligonucleotide	Position
GST-CaMKIIγ 212-317aa	CaMKIIγ BamHI 5'cgcggatccGCGCCTCCCTCCTGGGAT 3'	+764 to +782	CaMKIIγ EcoRI 5' ccggaattcCGGTGAGAAGTTCCTGGA 3'	+1082 to +1100

Table 2. Oligonucleotides used for plasmid sequencing.

Name	Plasmid to be sequenced	Oligonucleotide
5'-pGEX Forward	pGEX-6P1	5'GGGCTGGCAAGCCACGTTTGGTG 3'

Table 3. Oligonucleotides used for site directed mutagenesis. Bold, underline and italic letters indicated the single point mutation.

Name	Sense oligonucleotide	Anti-sense oligonucleotide
GST-CaMKIIγ Thr277Ala	5'GTCTGTCAACGATCC***g***CGGTGGCATCCATGATG 3'	5'CATCATGGATGCCACC***g***CGGATCGTTGACAGAC 3'
GST-CaMKIIγ Thr287Ala	5'ATGCATCGTCAGGAG***a***CTGTGGAGTGTTTGCGC 3'	5'GCGCAAACACTCCACA***g***CCTCCTGACGATGCAT 3'

IV. ABSTRACT

p21-activated kinases (PAKs) are Cdc42/Rac–activated serine-threonine protein kinases that play an important role in physiological processes such as motility, survival, mitosis, and apoptosis. *PAK1* is amplified and/or overexpressed in about 25-30% of breast cancers, and understanding how it signals could have therapeutic implications. Previously, a phospho-antibody array assay showed that several signaling molecules involved in breast cancer initiation and/or progression are hypophosphorilated in *PAK1* deficient breast cancer cells, including some well characterized PAK1 substrates and also some other proteins that could be regulated directly or indirectly by PAK1. Here, we show that CaMKIIγ, a Calcium/Calmodulin-dependent Protein Kinase (which recently has been associated to breast cancer initiation and progression) is phosphorylated both *in silico* and *in vitro* by PAK1. An *in silico* analysis using the GPS 3.0 software suggested that at least two threonine residues at the positions 277 and 287 are potential PAK1 phosphorylation sites. *In vitro* kinase assays using a GST tagged CaMKIIγ fragment corresponding to amino acids 212-317, showed that only threonine 277 is phosphorylated by recombinant PAK1. However, CaMKIIγ mutants lacking both threonine residues are still phosphorylated by PAK1, suggesting that additional PAK1 phosphorylation sites are still present in this CaMKIIγ fragment. Therefore, further studies are needed in order to identify the additional residues in CaMKIIγ sequence, which are phosphorylated by PAK1, and the role of PAK1 mediated phosphorylation in the activity of CaMKIIγ.

V. RESUMEN

Las proteínas cinasas activadas por p21 (PAKs) son proteínas cinasas de serinas y treoninas activadas por Cdc42/Rac que juegan un papel importante en procesos fisiológicos tales como motilidad, supervivencia, mitosis y apoptosis. El gen *PAK1* se encuentra amplificado y/o sobreexpresado en alrededor del 25-30% de los cánceres de mama, y la comprensión de cómo funciona esta vía de señalización podría tener implicaciones terapéuticas. Previamente, un ensayo de microarreglos con fosfo-anticuerpos mostró que varias moléculas de señalización involucradas en el inicio y/o progresión del cáncer de mama son hipofosforiladas en células de cáncer de mama deficientes en *PAK1*, incluyendo algunos sustratos de PAK1 bien caracterizados y también algunas otras proteínas que podrían ser reguladas directa o indirectamente por PAK1. En este trabajo nosotros mostramos que la proteína cinasa dependiente de calcio/calmodulina gamma, CaMKIIγ, (que recientemente se ha asociado en el inicio y la progresión del cáncer de mama) es fosforilada tanto *in silico* como *in vitro* por PAK1. Un análisis *in silico* usando el software GPS 3.0 sugirió que al menos dos residuos de treonina en las posiciones 277 y 287 son potenciales sitios de fosforilación mediados por PAK1. Los ensayos de cinasa *in vitro* usando un fragmento de CaMKIIγ correspondiente a los aminoácidos 212-317 fusionado con la proteína GST, mostraron que solo la treonina 277 es fosforilada por PAK1. Sin embargo, la forma mutante de CaMKIIγ que carece de ambos residuos de treonina continua siendo fosforilada por PAK1 *in vitro*, lo que sugiere que existen otros sitios de fosforilación mediados por PAK1 en este fragmento de CaMKIIγ. Por lo tanto, se requieren estudios adicionales para identificar los otros residuos en la secuencia de CaMKIIγ que son fosforilados por PAK1 y el papel de la fosforilación mediada por PAK1 en la actividad de CaMKIIγ.

1. INTRODUCTION

1.1 CANCER

Cancer is defined as a group of diseases in which malignant cells proliferate in an uncontrolled way, and in some cases spread to distant organs and tissues [1].

1.2 CANCER EPIDEMIOLOGY

It is estimated that more than 600,000 Americans will die from cancer in 2017, which is equivalent to 1,650 deaths per day. The types of cancer with the highest mortality rate are lung and bronchus, colon and rectum and prostate in men, and lung and bronchus, breast and colon and rectum in women (Fig. 1). These four cancers account for about 50% of all cancer deaths [2].

Breast cancer is the most common type of cancer among women in the world, which accounts for about 16% of all cancer diagnoses in the female population and it is estimated that during 2004, more than 500,000 women die due to this disease [3]. In Mexico breast cancer has occupied the first place in incidence and mortality due to malignant neoplasms in women over 25 years old. During 2008 breast cancer mortality rate was 4.5 per 100,000 women, followed by cervical (3.8) and stomach cancers (2.4) [4].

Estimated Deaths

Males				Females		
Lung & bronchus	84,590	27%		Lung & bronchus	71,280	25%
Colon & rectum	27,150	9%		Breast	40,610	14%
Prostate	26,730	8%		Colon & rectum	23,110	8%
Pancreas	22,300	7%		Pancreas	20,790	7%
Liver & intrahepatic bile duct	19,610	6%		Ovary	14,080	5%
Leukemia	14,300	4%		Uterine corpus	10,920	4%
Esophagus	12,720	4%		Leukemia	10,200	4%
Urinary bladder	12,240	4%		Liver & intrahepatic bile duct	9,310	3%
Non-Hodgkin lymphoma	11,450	4%		Non-Hodgkin lymphoma	8,690	3%
Brain & other nervous system	9,620	3%		Brain & other nervous system	7,080	3%
All Sites	**318,420**	**100%**		**All Sites**	**282,500**	**100%**

Figure 1. Cancer epidemiology in the United States, 2017. (Reproduced from 2, Siegel, 2017)

1.3 BREAST CANCER

Breast cancer is a highly heterogeneous and complex disease comprising a large number of tumor entities related to histological patterns, biological characteristics and distinct clinical behavior. Recently, high-throughput microarray-based technologies have been used to unravel the molecular characteristics of breast cancer that control lymph node infiltration and metastasis [5]. However, high heterogeneity can not only be explained by clinical data such as tumor size, lymph node infiltration, histological grade, age or by biomarkers such as the expression of estrogen receptor (ER), progesterone receptor (PR) or epidermal growth factor receptor 2 (ErbB2/HER2) [6].

To analyze the molecular profile of breast cancers, several groups have performed gene expression microarrays in order to compare the transcriptional signature of several tumors in a single experiment [7]. In 2000, Perou and colleagues published the first paper classifying breast cancer into intrinsic subtypes based on gene expression profiles and was the first to provide a molecular classification for breast cancer [7]. Using a cDNA microarray of 38 breast cancer cases, the group defined a list of “intrinsic” genes [6]. According to the hierarchical clustering analysis, breast cancer was grouped into four molecular subtypes: luminal, ErbB2/HER2-enriched, basal-like and normal breast. Subsequently the authors expanded the study using a

larger cohort, showing that the luminal subtype could be divided into 2 groups (luminal A and B), which had different clinical prognoses. This classification was later validated by independent groups, establishing that this analysis allowed to group tumors according to their biological characteristics regardless of their clinical or prognostic variables (Fig. 2) [6, 8].

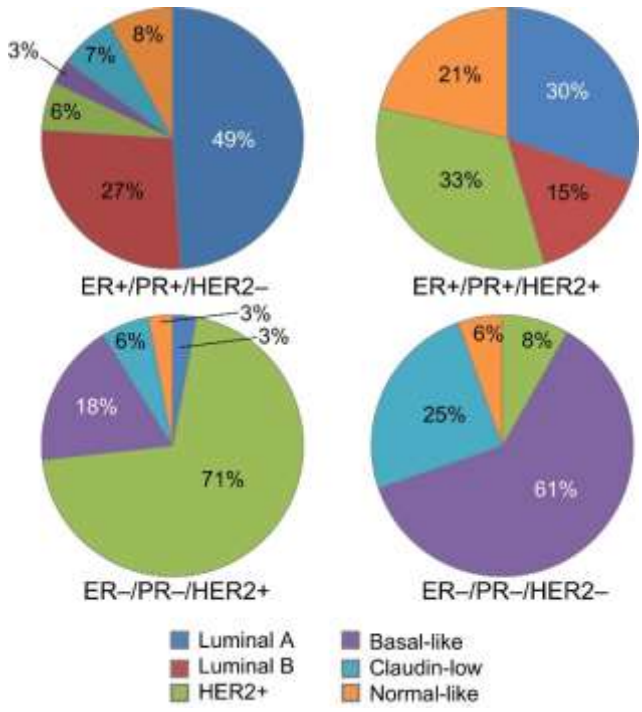


Figure 2. Correlation between immunohistochemical and molecular classification of breast cancer (Reproduced from 8, Rivenbark, 2013).

1.4 BREAST CANCER INTRINSIC SUBTYPES

1.4.1 LUMINAL A

Luminal A, is the most common subtype of breast cancer, which accounts for about 50% of all breast cancer cases, this subtype is characterized by the expression of genes activated by ER, which is normally expressed in the epithelium of the mammary gland, and a reduction in the expression of genes associated with cell proliferation [7]. The reported immunohistochemical profile of this subtype is characterized by the expression of ER, PR, Bcl-2 and cytokeratins (CK) 8 and 18, and by the lack of expression of ErbB2/HER2 and Ki67, these tumors also have a low histological grade. Patients with this subtype of tumors have shown to have a good prognosis with a rate and time of relapse of 28% and 2.2 years respectively [6]. This tumor subtype is susceptible to treatment with selective estrogen receptor modulators (SERM), such as tamoxifen, selective aromatase inhibitors and pure selective regulators of ER like fulvestrant [9].

1.4.2. LUMINAL B

The luminal B subtype constitutes about 20% of the tumors and, compared to the luminal A, is more aggressive with a higher histological grade, a higher proliferation rate and a worse prognosis. The survival from the moment of a reported relapse is 1,6 years compared to the 2.2 of the luminal A [10]. Although both luminal subtypes of breast cancer express ER, their prognosis is very different, for this reason, several researchers have looked for differences between these subtypes that could serve as clinical biomarkers. The main biological difference between these two subtypes, is that luminal B tumors display an increased expression of proliferation-related genes such as Ki67 and cyclin B1 in addition to often express the ErbB2/HER2 receptor [8]. Luminal B tumors have a worse prognosis and appear to be unresponsive to tamoxifen and aromatase inhibitors (AI), unlike luminal A, but these tumors have a better response to neoadjuvant chemotherapy, achieving a complete response in 17% of patients vs 7% of the luminal subtype A [11].

1.4.3 ERBB2/HER2 ENRICHED

The ErbB2/HER2-enriched subtype accounts for 15 to 20% of breast tumors, this subtype is characterized by the presence of an amplicon located on chromosome 17q12, known as ErbB2/HER2 amplicon, the overexpression of other genes associated with the ErbB2/HER2 pathway, and/or the co-amplification of at least other 14 genes located on the ErbB2/HER2 amplicon [8, 12]. This subtype exhibits a high expression of genes associated with cell proliferation, morphologically this subtype is highly proliferative, with a high histological grade and more than 40% of the tumors have mutations in the *p53* gene. The immunohistochemical profile for these tumors is ER- ErbB2/HER2+, which does not correspond perfectly with the intrinsic subtype, since only 70% of the tumors bearing the amplicon, display ErbB2/HER2+ overexpression at protein level [13, 14]. Clinically, this subtype has a poor prognosis. However, in the last ten years the treatment with anti-ErbB2/HER2 targeted therapies has improved significantly the survival not only for patients with metastasis but also at early stages [15, 16]. The ErbB2/HER2-enriched subtype has a high chemosensitivity with higher response rates than the luminal subtypes [13].

1.4.4 BASAL-LIKE

The base-like type subtype represents between 10% and 20% of breast cancer tumors. This term was coined due to the expression of genes exclusively expressed in normal myoepithelial breast cells, including high molecular weight cytokeratins such as CK5 and CK17, P-cadherin, caveolin 1 and 2. It also shares features with other subtypes such as CK8/18 expression present in the luminal subtype but with a lower expression [6]. From the clinical point of view, this subtype is characterized by its appearance at an early age, they are commonly larger in size, accompanied by a high histological degree and infiltration to lymph nodes and metastasis [17]. Basal-like tumors are infiltrating ductal carcinomas of high mitotic rate, presence of tumor necrosis, with margins of expansion and a lymphocytic stromal response [18]. They present a very aggressive growth pattern with high metastatic relapses, preferentially to visceral organs like lungs, the central nervous system and lymph nodes [19]. This subtype is characterized by the lack of expression of the 3 target

receptors in breast cancer: ER, PR and ErbB2/HER2 (Fig. 2). Several research groups have attempted to identify an immunohistochemical profile of the basal-like subtype leading to the selection of 5 biomarkers: ERB, PR, ErbB2/HER2, EGFR and CK5/6, achieving a specificity of 100% and a sensitivity of 76% [20]. Unlike the rest of the subtypes, the basal-like subtype has a worse prognosis with a high relapse rate in the first 3 years. However, this intrinsic subtype has a better response to chemotherapy. Therefore, it is fundamental to identify new therapeutic targets and personalized treatments [6, 21].

1.5 EGFR/ERBB FAMILY

The ErbB family of receptor tyrosine kinases (RTKs) or subclass I RTKs consist of 4 members: ErbB1/HER1 (also called epidermal growth factor receptor EGFR), ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. These four proteins are transmembrane receptors tyrosine kinases, which are activated by dimerization and transphosphorylation after ligand binding to their extracellular ligand binding domain. [22,23]. These receptors have a wide range of ligands, some of which may have receptor specificity (EGF, TGF-α, AR and epigen bind to EGFR) or bind to one or more related receptors, for example neuroregulins 1-4 and bind to ErbB3/HER3 and ErbB4/HER4, whereas HB-EGF, epiregulin and β-cellulin activate EGFR and ErbB4/HER4. ErbB2/HER2 is an orphan receptor that does not bind to any known ligand, but heterodimerizes with other ErbB receptors. In addition, some reports show that its three-dimensional structure model mimics an activated state which allows its dimerization (Fig. 3.) [22].

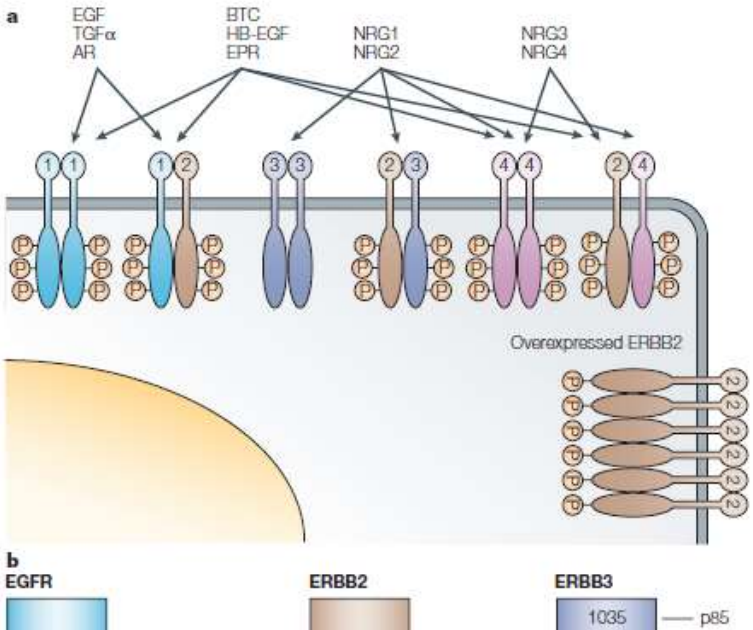


Figure 3. Schematic representation of the ErbB protein family, ligands, dimers and downstream signaling pathways (Reproduced from 28, Hynes, 2005).

The physiological role of the EGFR/ErbB family during organogenesis and its precise function is not totally understood. Transgenic overexpression of EGFR/ErbB family members under the transcriptional control of endogenous promoters has been unsuccessful, suggesting that increasing the signaling emanating from these receptors is lethal during development. The most important experiments demonstrating the *in vivo* functions of EGFR were obtained from knock-out mice [23].

Several research groups have generated knock-out mice for different members of the ErbB/HER family and some of their ligands. However, *ErbB2/HER2, 3, 4* or *heregulin* (ErbB3/4 ligand) knockout mice were found to be lethal, due to deficient cardiac function and the abnormal development of the nervous system [24-26]. In the embryos of *ErbB2/HER2-/-* mice, the normal development of the sensory ganglia from the neural crest is affected, and the development of the motor nerves is compromised. In contrast, embryos of *ErbB4/HER4-/-* mice show clear alterations in the innervation of the posterior brain and central nervous system. Mice lacking *ErbB2/HER2* or *ErbB3/HER3* are devoid of Schwann cells. As a consequence, both motor and sensory neurons undergo apoptosis in the later stages of embryonic development [27].

EGFR/ErbB receptors play important roles in human cancer. In particular, the expression or activation of ErbB2/HER2 is altered in many epithelial tumors. Clinical

studies clearly demonstrate that these receptors play a critical role in oncogenesis [28].

1.6 ERBB2/HER2 AND BREAST CANCER

The first insights about the critical role of ErbB2/HER2 for cancer development, were inferred when Neu, the rat ortholog of human ErbB2/HER2 isolated form chemically induced neuroblastomas, was able to induce transformation of NIH 3T3 fibroblasts [29]. Further studies confirmed the nucleic acid sequence homology between *Neu* and *ErbB/HER*-related genes, and the frequent amplification of *ErbB2/HER2* in human mammary tumors [30]. When genetic engineering of mice became possible, several transgenic mouse models expressing the *Neu* oncogene under the control of mammary specific promoters such as the mouse mammary tumor virus (MMTV) and the whey acidic protein (WAP) promoters were generated in various genetic backgrounds [31, 32]. Female mice of these strains developed spontaneous mammary tumors that better recapitulate the human pathology in addition to present lungs and bone metastasis [33, 34]. These animal models and several well characterized human breast cancer cell lines, have been a very useful tool, which has facilitated a better understanding of the activities and mechanisms downstream this receptor, and have helped to elucidate key signaling pathways triggered by over-active ErbB2/HER2 responsible for tumorigenesis. For instance, it is well documented that ErbB2/HER2 signaling promotes Ras-mediated stimulation of several downstream protein kinase cascades, including the RAF/MEK/ERK and PI3K/Akt pathways, known to affect both tumor cell growth and migration [35].

1.7 THERAPEUTIC TARGETING OF ERBB2/HER2 AND RESISTANCE

Since the ErbB2/HER2 receptor plays a critical role in tumor progression, it has become a very attractive therapeutic target for breast cancer treatments [36]. The ErbB receptors have an extracellular region, which is divided, into 4 domains; two domains are rich in leucine (I and III) and the other two in regions rich in cysteines (II and IV), a transmembrane region and finally an intracellular domain with tyrosine kinase activity. In the intracellular region there is a tail with phosphorylation sites that allow the recruitment and activation of downstream molecules (Fig 4) [29].

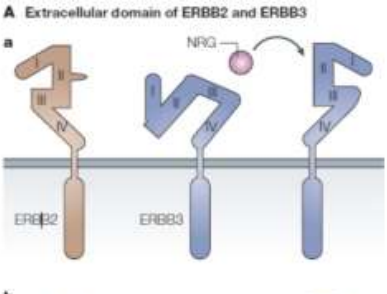
1.7.1 TRASTUZUMAB

One of the first drugs designed to block the activity of the ErbB2/HER2 receptor was Trastuzumab (Herceptin®), which is a humanized monoclonal antibody that binds specifically to the IV domain of the extracellular region. The proposed mechanism of action of Trastuzuman is: “(1) inhibition of ErbB2/HER2 shedding, (2) inhibition of PI3K-Akt pathway, (3) attenuation of cell signaling, (4) antibody-dependent cellular cytotoxicity, and (5) inhibition of tumor angiogenesis” (Fig. 4) [37, 38].

The use of Trastuzumab was approved as adjuvant therapy along with the classic regimen: doxorubicin, cyclophosphamide and paclitaxel in women with metastatic ErbB2/HER2-enriched breast cancer. Treatment approval was based on phase I through III clinical trials that showed a significant increase in disease-free survival compared to the classic scheme without Trastuzumab. Clinical trials demonstrated that the introduction of trastuzumab produced a significant increase in disease-free survival and in overall survival [39].

1.7.2 PERTUZUMAB

Another targeted therapy directed against ErbB2/HER2 is Pertuzumab, a humanized monoclonal antibody that blocks the activation of the ErbB2/HER2 receptor by a different mechanism of action than Trastuzumab. Pertuzumab binds to domain II in the extracellular region of the receptor, hindering its dimerization. It has been approved in combination with Trastuzumab and docetaxel in patients with metastatic ErbB2/HER2-enriched breast cancer without previous treatment with hormone therapy or chemotherapy. The approval of Pertuzumab was based on the results of the Pertuzumab and Trastuzumab Clinical Assessment (CLEOPATRA) trial. This trial compared Trastuzumab plus docetaxel (plus placebo) with Trastuzumab plus docetaxel plus Pertuzumab in ErbB2/HER2-enriched metastatic breast cancer patients. The results of the study showed an increase in disease-free survival of 6.1 months in patients receiving Pertuzumab plus Trastuzumab and docetaxel [39, 40].



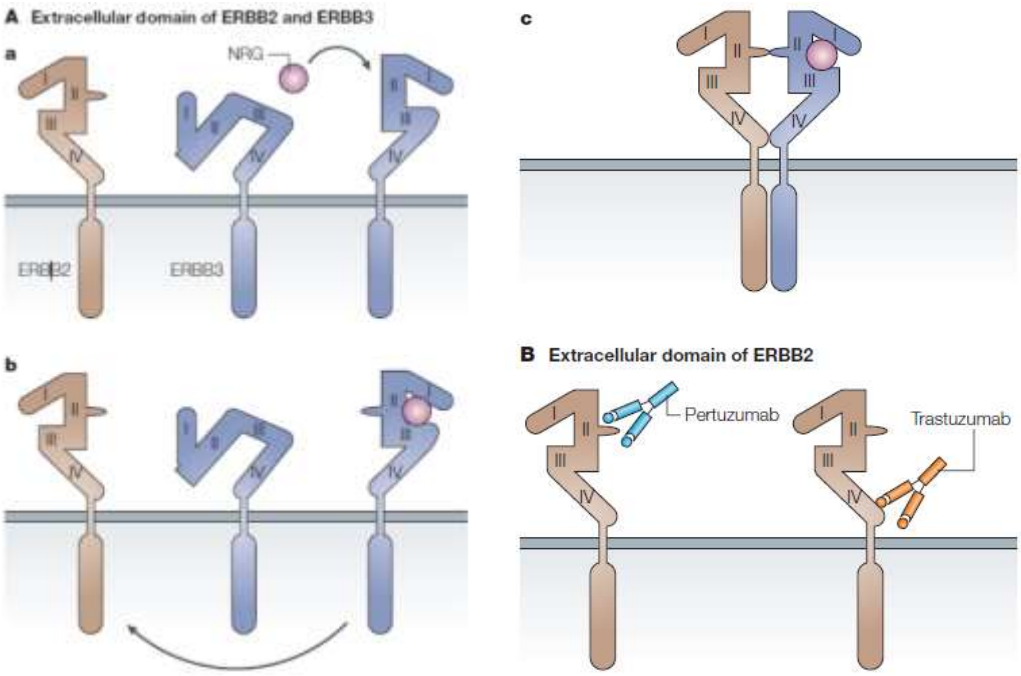


Figure 4. ErbB/HER-receptor ectodomain structures. A) The extracellular region of the ERBB receptor. In the absence of ligand, ERBB3 assume a tethered structure (a). The domains I and III are involved in neuregulin (NRG) binding and, following this, the dimerization arm in domain II is exposed (b) this binding promotes receptor−receptor interaction (c). However the ERBB2/HER2 has a fixed conformation that resembles the ligand-activated state of EGFR and ERBB3. B) The ERBB2/HER2-directed antibodies trastuzumab and pertuzumab bind domains IV and II, respectively (Reproduced from 28, Hynes, 2005).

1.7.3. RESISTANCE TO ERBB-DIRECTED THERAPEUTICS

During the oncogenic process, transformed cells accumulate multiple mutations, which contribute and are necessary for a complete transformation. Therefore, the pharmacological inhibition of a single therapeutic target unlikely would be sufficient to kill all the tumor cells. For instance, in ErbB2/HER2-overexpressing metastatic breast cancer patients treated with Trastuzumab, response rates are approximately of 35%. Several factors may account for the ineffectiveness of therapy, including resistance to targeted therapy or the mutational activation of phosphorylation cascades downstream ErbB2/HER2 [28].

Regarding to the involvement of compensatory pathways to confer resistance to anti-ERBB/HER drugs, it is known that such resistance is not only limited to antibodies targeting ErbB2/HER2, but to small molecule tyrosine kinase inhibitors that compete with ATP binding at the catalytic kinase domain of ErbB2/HER2, thus blocking autophosphorylation and activation of downstream signaling pathways involved in proliferation and survival [41].

Some of the proposed mechanisms by which ErbB2/HER2 breast tumor cells circumvent the effects of targeted therapy are the expression of a truncated form of ErbB2/HER2 lacking the Trastuzumab binding site; and the activation of pro-survival signaling pathways downstream the receptor [42, 43]. Among the most commonly deregulated signaling pathways downstream ErbB2/HER2 in breast cancer are the Ras/RAF/MEK/ERK and PI3K/Akt/mTOR cascades, known to affect both tumor cell growth and migration. Signaling through these two pathways can be influenced by Group I p21-activated kinases (PAKs), a family of effectors of the Rho GTPases Rac1 and Cdc42. In the recent years, these kinases have been recognized as plausible targets for cancer therapeutics due to their role as drivers of many of the cellular processes that are the hallmarks of cancer [43]. Of special interest is the mechanisms by which PAK1 contributes to ErbB2/HER2 signaling in breast epithelial transformation. In order to determine if PAK signaling is relevant in the various cellular processes (i.e. tumorigenic cell behaviors like increased migration and proliferation or inhibition of apoptosis), that are known to be altered during tumor initiation and progression

1.8 PAK FAMILY

“The p21 activated kinase (PAKs) were the first Rho GTPase-activated kinases to be identified” [44]. These proteins play essential roles in a wide range of cellular processes, including cell morphology, survival, gene transcription, cell cycle progression, motility, apoptosis and hormone signaling among others; and are activated by extracellular signals through GTPase-dependent and -independent mechanisms [45]. Therefore, the aberrant activity of these enzymes has been linked with various diseases, including cancer [46]. Six PAK family members have been

identified in mammals, and on the basis of biochemical and structural features have been divided into two groups: Group I comprises PAK1, PAK2 and PAK3 and Group II comprises PAK4, PAK5 and PAK6 (Fig. 5) [47, 48].

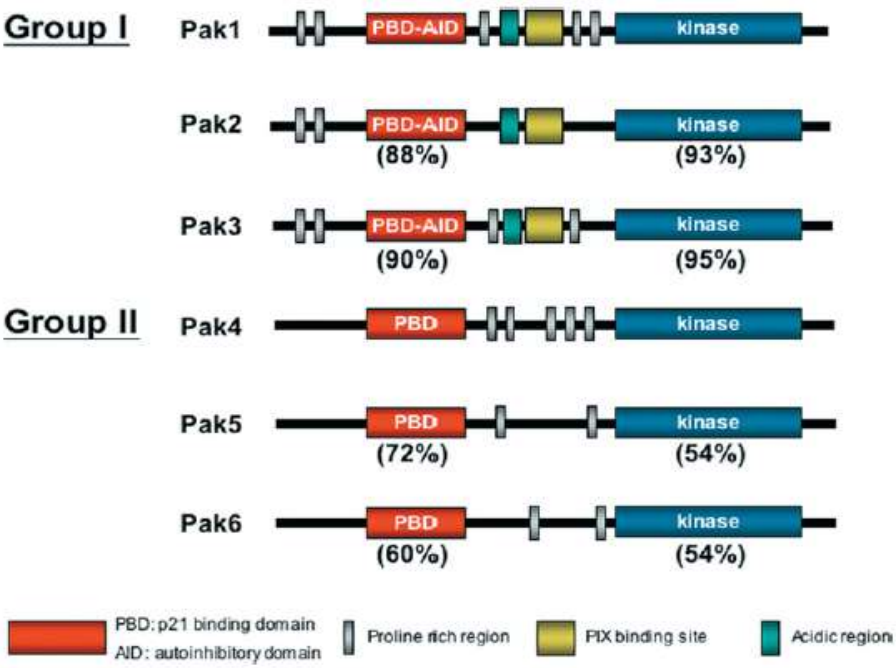


Figure 5. Schematic representation of the PAK family members. PBD, p21-binding domain; AID, autoinhibitor domain (Reproduced from 47, Arias, 2008).

1.8.1 MECHANISM OF ACTION

The Group I PAKs are characterized by an N-terminal region that includes a conserved CRIB (Cdc42/Rac1 Interacting Binding) domain [also known as PBD (p21-Binding Domain) or GBD (GTPase Binding Domain], which overlaps with an AID (Auto Inhibitory Domain), and a C-terminal kinase domain. Additional features present in the N-terminal region of Group I PAKs include the presence of poly proline rich motifs (PXXP), two canonical SH3 (Src homology 3)-binding motifs and a conserved non-classical SH3-binding site for the guanine-nucleotide-exchange factor β-PIX (PAK Interacting Exchange Factor). The first canonical SH3 site has the capability to bind to the adaptor protein Nck, while the second SH3 site binds to Grb2 for cell membrane recruitment. Group I PAKs exist in an inactive conformation as

antiparallel homodimers wherein the kinase domain of one monomer binds to an autoinhibitory AID of the other monomer [44, 45, 47].

Structural and biochemical studies, have shown that the binding of active GTPases to the CRIB domain of Group I PAKs disrupts dimerization, leading to conformational changes which destabilize the folded structure of the AID, inducing its dissociation from the catalytic domain and allowing autophosphorylation that is required for full kinase activity [49].

Once activated, PAK kinases control cellular functions by phosphorylating their downstream targets and/or serving as scaffold proteins for the indirect phosphorylation of other effectors. The importance of PAKs activation in tumor cells is a reflection of its ability to control a range of upstream signals and not necessarily dependent on Rho GTPases. Therefore, the ability of PAKs to influence regulatory processes in cancer cells can be attributed to the non-dependence of GTPase activation on its kinase activity [47, 49].

Although Group I and Group II PAKs share structural features; the two groups have overlapping and non-overlapping functions, and are activated by distinct mechanisms. As opposed to Group I PAKs, Group II PAKs are monomeric kinases, which lack the PXXP motifs and the β-PIX binding site found in Group I PAKs. Until recently, it was thought that these enzymes were constitutively activated, that they lacked an AID, and that interactions with Rho GTPases were important to determine subcellular localization [44, 45, 47]. However, recent reports have demonstrated that PAK4 possess an AID in the N-terminus that inactivates its catalytic domain in *cis* until Cdc42 binds to the CRIB domain and allows activation [50].

An alternative activation model proposes that PAK4 is inhibited by the interaction of its catalytic domain with a pseudosubstrate sequence (PS) located in PAK4 N-terminus. In this model, the binding of SH3 domain-containing proteins to the PS destabilizes such interaction and promotes the release of the catalytic domain activating the kinase. It is thought that PAK5 and PAK6 also follow this activation model [51].

1.9 PAKS AND CANCER

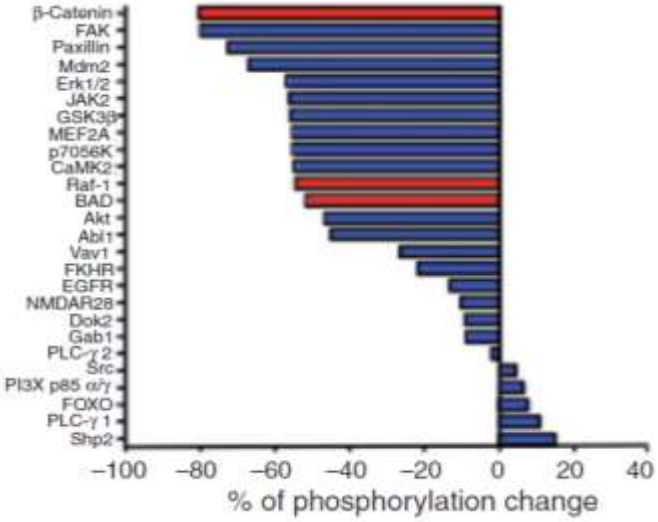
Several studies indicate that PAK is aberrantly activated in a number of human cancers, and generally is correlated with a high histologic grade and a decrease in patient survival [52]. Mechanisms that explain the hyperactivity of this family of kinases involve *PAK1* gene amplification on chromosome 11q13 or *PAK4* on chromosome 19q13 [53-55]. However, PAKs may also be overexpressed in the absence of gene amplification due to activating mutations in the *PAK4* and *PAK5* genes or by mutations in their upstream activators [56-58].

The observation that PAK genes are frequently amplified (*PAK1* and *PAK4*), or mutated in human tumors (*PAK4* and *PAK5*) is in line with their well defined role as promoters of cell cycle progression, suggesting that these kinases play a fundamental role in oncogenesis [59-62]. Increasing evidence, indicates that PAK1 signaling is important for the fully activation of the Ras/RAF/MEK/ERK, PI3K/Akt/mTOR and Wnt signaling pathways, which control cell cycle progression, cell survival, differentiation and proliferation [63]. In the MAPK pathway, PAK1 phosphorylates c-RAF in Ser338 and MEK1 in Ser298, which allows the efficient activation of ERK and subsequently the expression of Cyclin D1, although there are still some unknown aspects about how PAK1 regulates this signaling pathway [64]. For example, it has been reported that overexpression of an inactive form of PAK1 has the ability to phosphorylate c-RAF, suggesting that PAK1 functions as a scaffold protein facilitating the interaction of c-RAF with MEK, indicating that the scaffold functions of the protein contribute to the transduction of proliferative signals [64]. These observations suggested that PAK1 contributes to MAPK activation not only by kinase dependent, but also by kinase independent mechanisms. PAK1 also has been reported to function as a scaffold protein for the PI3K/Akt/mTOR pathway, in this case, PAK1 scaffolding activity allows the formation of a PAK1/PKPK1 complex (3-Phosphoinositide-Dependent Protein Kinase 1), which subsequently promotes the recruitment of Akt to the plasma membrane for its activation [65].

Recently, PAK1 has been implicated in breast cancer for several reasons: 1) it is frequently amplified in mammary tumors, and its presence is associated with resistance to tamoxifen; 2) the transgenic expression of constitutively active PAK1 under a specific mammary gland promoter induces the formation of mammary tumors with increased levels of MAPK and p38 activity [66-68]; and 3) in a 3D cell culture model of mammary epithelial cells transgenetically expressing the ErbB2/HER2 receptor, *PAK1* knockdown downregulates ERK and Akt signaling, resulting in the restoration of the normal acinar architecture. However, the silencing of the closely related *PAK2* gene failed to restore normal acinar morphology, although it also compromised the activation of ERK and Akt. These results suggested that, PAK1, but not PAK2 signaling plays an important role in ErbB2/HER2 driven transformation. Furthermore this phenomenon does not involve the ERK and Akt pathways [69]. These unexpected observations prompted to carry out a phosphoproteomics analysis, which unveiled the participation of several signaling molecules. These studies revealed that PAK1 phosphorylates β-catenin on Ser663 and Ser675, and that these phosphorylation events stabilize β-catenin promoting its relocalization into the nucleus and the transcription of direct Wnt target genes such as *c-Myc* and *Cyclin D1* [62]. Remarkably, the knockdown of *PAK2* has no effect on β-catenin expression levels or phosphorylation in breast epithelial cells. Suggesting that an ErbB2/HER2-PAK1-β-catenin axis is essential for transformation of mammary epithelial cells [69, 70].

All the evidence mentioned above, indicates that PAKs occupy a central position in oncogenic signaling, driving several processes that are the hallmarks of cancer initiation, growth, and spread. In proliferative signaling, PAK activity is required for efficient activation of ERK, Akt, and β-catenin in many tissues. With respect to decrypting the role of PAKs in cancer, much progress has been made during the last years, but certain basic questions remain unsolved, for example, the identities of the most relevant substrates, and whether these are unique to individual members of the PAK family [48]. Efforts in this direction have already begun, employing diverse technologies such as phospho antibodies microarray assays. By using these methodologies, Arias *et al.* showed that in PAK1 deficient breast cancer cells, a large

number of proteins lost their phosphorylation, one these molecules is the



Calcium/Calmodulin-dependent Protein Kinase II (CaMKII) suggesting that it could be a novel PAK1 substrate (Fig. 6) [69].

Figure 6. Phospho-antibody microarray showing changes in protein activity in the ErbB2/HER2 signaling pathway. Bars in red represent characterized PAK1 substrates (Reproduced from 69, Arias, 2010).

1.10 CALCIUM/CALMODULIN DEPENDENT PROTEIN KINASE II

"The Ca^{2+}/calmodulin (CaM)-dependent protein kinases (CaMKs) are multifunctional serine/threonine kinases whose activity is regulated through Ca^{2+} signaling" [71]. CaMKII is a multimeric complex, comprising between 12 and 14 subunits [72]. Each of these monomers has a catalytic domain at the N-terminus, followed by a regulatory domain overlapping with a Calcium/Calmodulin binding domain and finally an association domain at the C-terminus responsible for multimerization (Fig. 7). Like other kinases, the catalytic domain of CaMKII has an ATP binding pocket that creates a microenvironment to decrease the energy required to hydrolyze ATP, this allows the increase of the transfer rate of the ATP γ-phosphate to a residue of Ser or Thr in the target protein [73]. The regulatory domain may undergo post-translational modifications such as phosphorylation, O-linked N-acetylglucosamine of serines, oxidation of methionines, which are essential for the regulation of the function of this kinase [74-78].

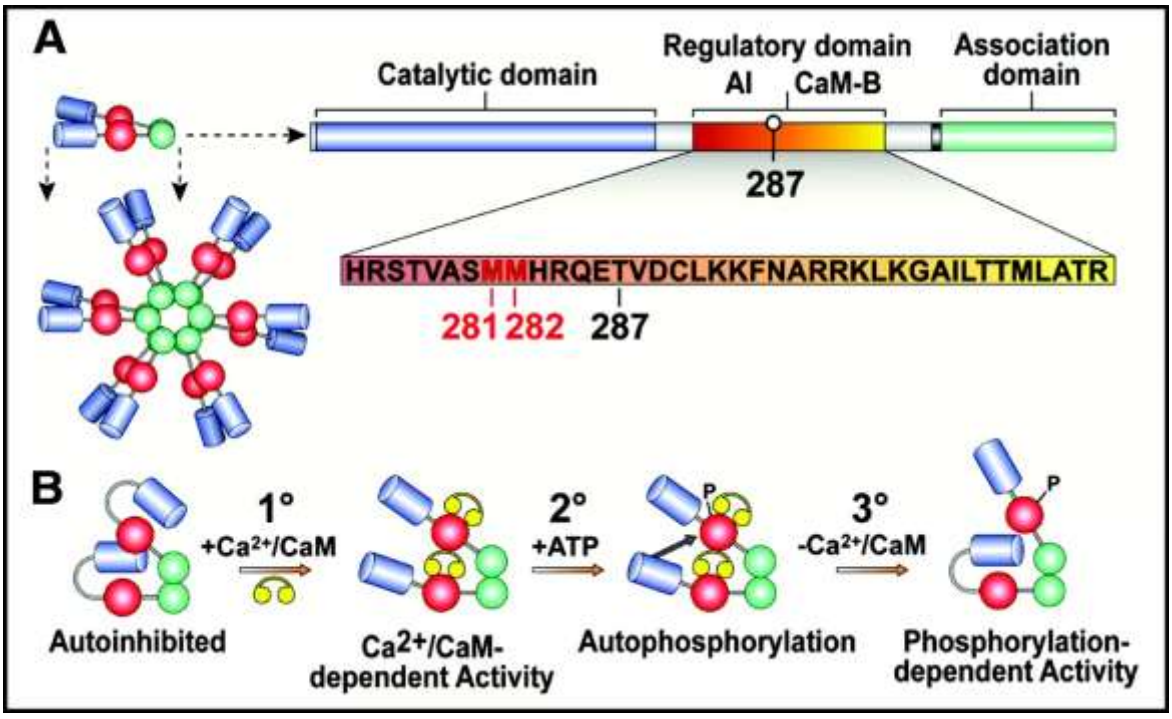


Figure 7. Structure and function of CaMKII. CaMKII formed by 12 to 14 monomers arranged as a hexameric ring (A). The association domain directs multimeric assembly, the catalytic domain performs kinase function, and the regulatory domain containing the calmodulin-binding region that modulates CaMKII function via ligand binding and biochemical modification. CaMKII: calmodulin kinase II; AI: autoinhibitory region; CaM-B: calmodulin-binding region (Reproduced from 75, Swaminathan, 2011).

Four different genes encoding different CaMKII proteins (α, β, γ and δ) have been identified. All of these CaMKII proteins share a common regulatory mechanisms and similar substrates, but they differ in tissue distribution and subcellular localization. Under conditions of low activity, the catalytic domain is bound to the regulatory domain of the adjacent monomer in the multimeter this allows the kinase to be inactive. When intracellular calcium levels rise, calcium-bound Calmodulin binds to the regulatory domain of CaMKII, which releases the catalytic domain of the adjacent monomer allowing autophosphorylation in Thr286 (for CaMKIIα) or in Thr287 (for CaMKIIβ, γ and δ) for the fully activation of this kinase. This phosphorylation has two effects, 1) increases more than 1000 times the binding affinity of the CaM complex for the CaMKII regulatory domain, and 2) blocks the reassociation of the catalytic and regulatory domains preventing self-inhibition even if calcium levels decrease. This autonomous calcium/calmodulin activity will persist until the phosphate group is eliminated by the protein phosphatase 2A (PP2A) [75, 76]. The regulatory domain may also undergo oxidations (Cys280/Met281 on CaMKIIα, Met281/Met282 on CaMKIIβ, γ and δ) due to elevated levels of reactive oxygen species (ROS) that positively regulate CaMKII by preventing the binding of the catalytic domain to regulatory domain and therefore its inactivation [77]. Oxidation further increases the sensitivity of CaMKII to the calcium/calmodulin complex even in the absence of the autophosphorylation of Thr287, which causes insensitivity to phosphatases. In other conditions, such as hyperglycemia and diabetes, the addition of a modification of O-GlcNAC CaMKII in Ser279 allows the activation of CaMKII autonomously creating a molecular memory, even after the Ca^{2+} levels decrease (Fig. 8) (Fig. 8) [73, 74, 78, 79].

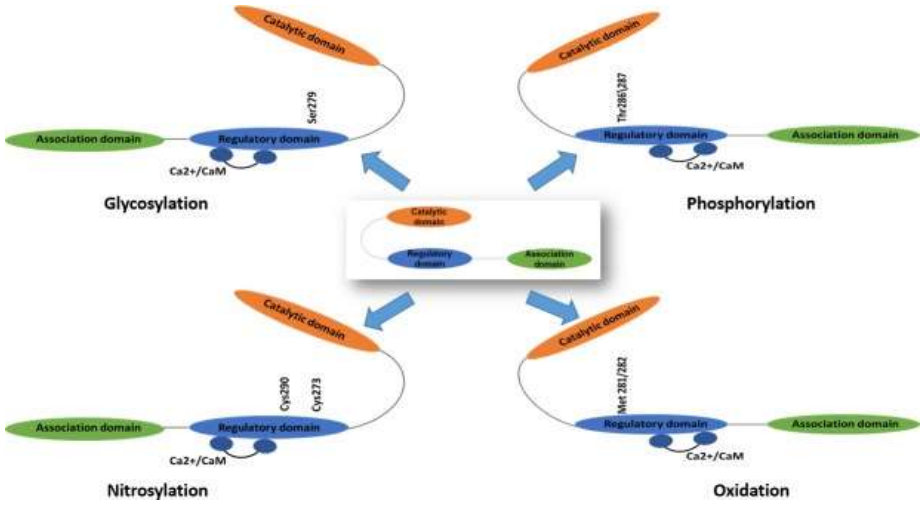


Figure 8. The activation mechanisms of $Ca^{2+/}$calmodulin dependent protein kinase II (CaMKII). CaMKII could be activated by phosphorylation, oxidation and glycosylation (Reproduced from 73, Wang, 2015)

1.11 CAMKII AND CANCER

Recent studies have implicated CaMKII as an important component in the proliferation of cancer cells. The use of CaMKII pharmacological inhibitors, such as MG-63 and KN-93 (which prevent the binding of the calcium/calmodulin complex and its subsequent activation), in the human osteosarcoma cell line 143B resulted in a dramatic decrease in proliferation. In addition, *in vivo* administration of KN-93 to xenografted mice significantly reduced intratibial and subcutaneous tumor growth. Subsequently, the effect of CaMKII inhibition on cell proliferation was associated with increased gene expression and expression of the p21cip/KIP protein, and decreased phosphorylation of the retinoblastoma (Rb) protein, and the E2F transactivator [80]. CaMKII inhibitors are also efficient in liquid tumors as reported by Si *et al.*, who observed suppression in the proliferation of myeloid leukemia cells and its effect was related to several signaling pathways [81]. Also, the cell proliferation effects of CaMKII are dependent of the phosphorylation at specific sites reported by Hoffman *et al.* In this study they showed that the overexpression of wildtype or a Thr286 phosphomymetic form of CaMKII increased the proliferation rates of neuroblastoma and breast cancer cells, whereas overexpression of a Thr253 phosphomymetic form, significantly reduced the proliferation rates of these cells due to cell arrest and apoptosis [82]. A recent study has linked ErbB activity to CaMKII. In this study, Cohen *et al.* showed that downregulation of ErbB could suppress the CaMKII

signaling, which is coincident with the induction of apoptosis in breast and prostate cancer cells [83].

In the last years, several research groups have focused on ErbB2/Her2 signaling in breast cancer, in particular, on identifying downstream signaling elements that might serve as the basis for new therapeutics. One such element, the Rac/PAK signaling axis, seems particularly promising. Recent findings made by our research group, clearly establish to PAK1 as a new target in ErbB2/HER2-driven breast cancer and define a new mechanism of action conducted by the downregulation of several signaling pathways involved in cell survival, migration, proliferation and cell cycle progression among others. The phospho-antibody array assay previously described, allowed the identification of CaMKII as a potential PAK1 substrate in ErbB2/HER2-driven breast cancer cells. For all these reasons, the main goal of this work was determine if CaMKIIγ is an actual PAK1 substrate.

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2. JUSTIFICATION

Breast cancer is one of the leading causes of death worldwide among the female population. In some countries it is the most important because of its high incidence rates, while in other places it occurs more sporadically. In Mexico, breast cancer causes more deaths than cervical cancer since 2006 [84]. It is the second leading cause of death among women aged 30 to 54 years and affects all socioeconomic levels. The data on its detection, although underreported, show 6,000 new cases in 1990, and an expected increase in more than 16,500 a year by 2020. In addition, most cases are self-detected and only 10% are identified in early stages.

Currently, in developed countries, biopsies obtained from the patient are used to study the mechanisms of tumor progression and metastasis at the molecular level. In some tumors, these advances are already being incorporated into clinical practice. It has recently been observed that in certain tumors, abnormalities of several genes are correlated with the prognosis of each patient. By means of the determination of different marker macromolecules, molecular biology laboratories can provide important prognostic and assistance information in the selection of therapeutic strategies.

Different research groups have reported that in approximately 25-30% of breast cancer samples there is an amplification of the 11q13 chromosomal region which is where the *PAK1* gene is located [54, 85]. The protein encoded by this gene is a regulator of different transduction pathways involved in cytoskeletal reorganization, cell migration and proliferation, etc. For this reason, the study of the molecular mechanisms through which this enzyme controls such processes is of particular importance to better understand the pathophysiology of breast cancer. Recently, it has been reported that PAK1 might be involved in breast cancer progression and in the regulation of hormone response through the interaction and phosphorylation of the receptor estrogen alpha (ERα) on the serine residue 305 [86]. Also has been proposed that the inhibition of PAK1 may be beneficial in patients with ErbB2/HER2 positive breast tumors and who are insensitive to treatment with tamoxifen [87]. In addition, results obtained in phosphoproteomic experiments have allowed the

identification of different signaling molecules whose activity is affected by the inhibition of PAK1. An example is the protein tyrosine kinase C-ABL [88], which in its active form promotes the development of leukemia [89], and has recently been linked to the metastatic potential of breast tumors [90, 91]. Another example is the CaMKII kinase, which has recently been associated with tumor progression in breast carcinomas [92].

These properties make PAK1 an interesting therapeutic target, since in theory its inhibition may be beneficial for the treatment of different types of cancer, especially in the breast tumors in which the PAK1 gene is amplified or overexpressed. In recent years, cancer has registered a higher incidence in our country, so it is necessary to carry out studies that potentially can be transferred to the clinic. In this case, a detailed study of the molecular mechanisms by which PAK1 promotes cell transformation, as well as the effects of its inhibition on cellular and animal models, could potentially help to improve the design of combined therapies for cancer treatment in a more effective way.

3. HYPOTHESIS

The Calcium/Calmodulin-dependent Kinase II gamma protein, CaMKIIγ, is phosphorylated by the oncoprotein PAK1 *in vitro*.

4. GENERAL OBJECTIVE

Determine if CaMKIIγ is a substrate of PAK1 *in vitro*.

4.1 SPECIFIC AIMS

1. Identify the potential PAK1-mediated CaMKIIγ phosphorylation sites by *in silico* analysis.
2. Determine if CaMKIIγ is a direct substrate of PAK1 *in vitro*.
3. Determine the CaMKIIγ phosphorylation sites by PAK1 *in vitro*.

5. MATERIALS AND METHODS

5.1 Bioinformatic: kinase-specific phosphorylation site prediction GPS 3.0

The potential serine and threonine phosphorylation sites of CaMKIIγ by the kinase PAK1 were predicted by the kinase-specific phosphorylation site prediction GPS 3.0. (http://gps.biocuckoo.org/ and subsequently oligonucleotides were designed to make point mutations for threonine-to-alanine substitution [93].

5.2 Cell Culture

5.2.1. HEK293T

The HEK293T human embryonic kidney cell line was cultured in DMEM medium (GIBCO). This medium was supplemented with 10% fetal bovine serum (GIBCO), 2mM of L-glutamine (GIBCO), and 1X of 100X penicillin-streptomycin (10,000U/mL, BIOWEST). The cell cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO_2/95% air.

5.2.2 BT-474

The BT474 ErbB2/HER2+ breast cancer cell line was cultured in DMEM-F12 medium (BIOWEST). This medium was supplemented with 10% fetal bovine serum (GIBCO) and 2mM of L-glutamine (GIBCO), and 1X of 100X penicillin-streptomycin (10,000U/mL, BIOWEST). The cell cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO_2/95% air.

5.3 PAK1 recombinant protein

To generate the PAK1 recombinant protein we used the pCMV-6M-PAK1-L107F vector, which was a gift from Jonathan Chernoff (Addgene plasmid # 12209), this plamid contains a DNA fragment encoding myc-tagged (in the N-terminal) constitutively active PAK1 [94].

5.4 Transfection of pCMV-6M-PAK1-L107F

DNA transfection into HEK293T cells, the lipofection method was used under the following protocol: $5x10^6$ cells were seeded into T-75 flask (70% confluent) and

transfected with 50µL of Lipofectamine®LTX reagent (Invitrogen) which was diluted in 700µL of Opti-MEM® (GIBCO) and combined with 10µg of the plasmid (pCMV-6M-PAK1-L107F) diluted with 720µL of Opti-MEM® and 10uL of PLUS™ Reagent then incubated for 15 min at room temperature. The DNA-lipid complex was then added to the cells with 10mL of Opti-MEM® and placed in an incubator (37°C) for 48h prior to confirmation of transfection [95].

Cells were allowed to express the protein for 48h post-transfection, washed in phosphate-buffered saline (PBS, 137mM NaCl, 2.7mM KCl, 10mM NaH2PO4, 2mM KH2PO4, pH 7.4) and scraped into 1000µL of 1X RIPA buffer [Tris-HCl, pH 7.4 (50mM), NaCl (150mM), NP-40 (1%), Sodium deoxycholate (0.5%), SDS (0.1%), EDTA (5mM), protease inhibitor cocktail (COMPLETE, Roche)]. The cell suspension was then lysed by vortexing and the homogenate was spun at 13,500rpm in a microfuge to remove unbroken cells and cells debris.

5.5 Protein quantification

Total protein concentrations were measured from the cell lysates. The protein quantification assay was performed with the Lowry method which is based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, using the kit DC Protein Assay (BioRad) in accordance with the manufacturer's instructions. Here, a standard curve was constructed using bovine serum albumin between the concentrations 0 to 1mg/mL. Absorbance readings were taken at 750nm, and all samples were measured in triplicate [96].

5.6 PAK1 immunoprecipitation from cell lysates

For the immunoprecipitation of PAK1, 100µg of total protein were made up to a total volume of 100µL with immunoprecipitation buffer then the supernatant was pre-cleared with 30µL of 50% Protein A agarose bead slurry and incubated at 4°C for 2h, 1µL (1µg/µL) of the anti-myc antibody (Cellsignal). The supernatant was removed and the beads were washed 3 times with immunoprecipitation buffer and resuspended in 200uL of immunoprecipitation buffer [97].

5.7 SDS-PAGE

To determine whether PAK1 immunoprecipitation was successful, 10µL of the immunoprecipitation material were migrated on SDS-polyacrylamide gels. The resolving gel was prepared with the following final concentrations: Acrylamide 10%, Bis-acrylamide 1%, Tris base 375mM pH=8.8 and 0.1% SDS. To achieve polymerization of the gel was added 0.05% ammonium persulfate and 0.003% TEMED. Regarding the stacking gel the final concentrations were: Acrylamide 4%, Bis-acrylamide 0.1%, Tris base 125mM pH=6.8 and SDS 0.1%, 0.05% ammonium persulfate and 0.005% TEMED. After both phases of the gel (the stacking and resolving) were polymerized and prior to initiating the run, 6X Laemmli buffer (Tris-HCl 375mM pH=6.8, 9% SDS, 50% glycerol, 9% beta-mercaptoethanol, 0.03% bromophenol blue) was added to immunoprecipitated material at a final concentration of 1X, and then boiled at 96°C for 5 min and loaded directly onto the SDS-PAGE gel. Electrophoresis was started using running buffer (Tris base 25mM, Glycine 192mM, 0.1% SDS) and the run was performed at a constant voltage of 120V [98]. Once the migration was completed, the gel was removed from the electrophoresis chamber and Western blot was performed.

5.8 WESTERN BLOT

The proteins separated electrophoretically in an SDS-PAGE gel are transferred to a PVDF membrane as was described by Towbin [99]. To perform the blotting briefly, prepare a transfer sanchwich as follows: first a sponge previously moistened in transfer buffer, then two Whatman papers also moistened in the same buffer, then the gel, then placed the membrane followed by two Whatman papers and sponge. When assembling this sandwich was taken care of not leaving bubbles that could interfere with the transfer of the proteins to the membrane. Protein transfer was performed from the negative pole (gel) to the positive pole (PVDF membrane). This sandwich was introduced into the transfer chamber which was filled with transfer buffer to the indicated limit. To prevent the buffer from overheating and interfering with the amperage, the transfer was performed in an ice bath for 60 min to 400mA. Upon completion of the transfer and to verify transfer of the proteins, the membrane was stained with 0.5% Ponceau red solution in 2% acetic acid for 5 min at room

temperature and washed in ddH_2O. Once the transfer of the proteins was corroborated, the membranes were blocked 5% nonfat dry milk in TBS-Tween 0.1% (50 mM Tris base, pH=7.5, 150 mM NaCl) for 1h at room temperature, and incubated with the primary antibody [anti-myc 1:30,000 (Abcam), anti-PAK1 1: 1,000) (Cellsignal)] in blocking solution overnight at 4°C. The next day, the membrane was washed 3 times every 15 min with TBS-Tween 0.1%. After the last wash the membrane was incubated with the secondary antibody, anti-mouse or anti-rabbit antibody (1: 10,000) conjugated to hot radish peroxidase for 2h at room temperature. Finally, 3 further 15 min washes were performed with TBS-Tween 0.1%. The membranes were immersed in 1mL of Clarity Western ECL Substrate (BIO-RAD) solution. After this time the membranes were exposed to an "HyBlot ES® Autoradiography Film (Denville scientific Inc.)"[99].

5.9 GST-CaMKIIγ-212-317aa recombinant protein

To obtain GST-CaMKIIγ-212-317aa recombinant protein we amplified the DNA fragment corresponding to the open reading frame (ORF) of the sequence encoding the amino acid region of position 212 to 317 of the CaMKIIγ protein and we use DNA that are complementary (cDNA) to messenger RNAs (mRNAs) from the BT474 cell line.

5.10 RNA extraction

BT474 cells were plated in T75 flask with an 80% of confluence and the culture medium was removed and scraped into 1mL TRIzol® (Invitrogen) and incubated for 5 min at room temperature. 200mL of chloroform was added and the cells were inverted for 15 seconds before a further 3 min incubation. Mixture was then spun at 13500rpm for 15 min at 4°C to separate the RNA fraction in the upper aqueous phase. The aqueous phase was recovered in a new tube and 500μL of isopropanol were added. The RNA was precipitated for 30 min at 4°C, and the mixture was centrifuged at 12,500rpm for 10 min at 4°C. The RNA pellet was washed with 1mL of 70% ethanol and centrifuged at 7,500rpm for 5 min and allowed to dry almost completely at room temperature, and then resuspended in 30μL RNA-free water

treated with diethylpyrocarbonate (DEPC). Samples were stored at -80°C before use.

5.11 Retrotranscription and Polymerase Chain Reaction (RT-PCR)

To obtain the cDNA of the gene encoding the CaMKIIγ protein an RT-PCR assay was performed. For the RT-PCR reaction the DNA of the sample was first removed according to the protocol described for the "RNA-free" kit. Briefly, 10µg of RNA from the BT474 cell line were treated with 3U of DNAsa I (Thermo) for 1h at 37°C. The reaction was carried out in the presence of 1X DNAse I buffer. Once the RNA was free of DNA, its integrity was evaluated for which an electrophoresis was performed under denaturing conditions. Briefly 3µg of total RNA were mixed with an equal volume of formamide (1:1) subsequently heated at 65°C for 2 min and centrifuged at 12,500rpm/1 min, then loaded onto a 2% agarose gel, 0.56% of guanidine thiocyanate at 110 V for 15 min and 60 min at 80V, once electrophoresis was completed in a transilluminator observing an intensity of 28S/18S ribosomal RNA. To perform reverse transcription (RT) according to the protocol described for the "SuperScript II Reverse Transcriptase" kit (Invitrogen). Briefly, 5µg of total RNA and 500ng of Oligo (dT) 12-18 were incubated at 65°C for 5 min. Subsequently, 10mM of each of the deoxynucleotides, 4µL of 5X RT Buffer, 2µL of 100mM dithiothreitol (DTT) were added and this mixture was incubated for 2 min at 42°C. Finally 2.5U of Superscript II reverse transcriptase were added and incubated for 50 min at 42°C, then at 70°C for 15 min and finally at 4°C. Once we obtained the first cDNA strand, it was used as a template for PCR reactions.

5.12 Amplification and cloning of the gel fragment of the CaMKIIγ protein 212-317aa.

To obtain the recombinant CaMKIIɣ 212-317aa protein fragment we amplified the DNA fragment corresponding to the open reading frame of the CaMKII gene from the messenger RNA of the CAMKII gene. For this purpose, specific oligonucleotides were designed whose sequence was as follows: CaMKIIγ BamHI Forward 5 'CGCGGATCCGCGCCTCCCTTCTGGGATGAGGA 3', CaMKIIγ EcoRI Reverse 5 'CCGGAATTCCGG TGGCAGCTGAGAAGTTCCTG 3'. To amplify this fragment the

following PCR conditions were employed: final concentrations: 1μM oligonucleotides, 1.25U GoTaq DNA polymerase (Promega), 1X Green GoTaq® Reaction Buffer (1.5mM MgCl2), 0.2mM of each of the dNTPs and 500 ng of the first cDNA strand. The reaction was performed in a final volume of 50μL under the following conditions: 1 initial denaturation cycle of 95°C for 2 mi, 35 cycles (denaturation at 95°C for 1 min, alignment at 56°C for 1 min, extension at 72°C for 30 seconds, a final extension step at 72°C for 5 min and the reaction was maintained at 4°C. To corroborate the amplification of the fragment the PCR product was resolved on a 0.8% agarose gel in TAE buffer 0.5X and stained with ethidium bromide.

5.13 Extraction of DNA from agarose gels

The PCR fragment corresponding to the amino acid coding sequence from 212 to 317 (336bp), were purified using the GeneJET extraction kit (Thermo Scientific) and cloned into the BamHI and EcoRI sites of the prokaryotic expression vector pGEX6P-1. Briefly, once resolved on an agarose gel, the fragment was cut from the gel and introduced into an eppendorf tube for each 100mg of agarose were added 100μL of binding buffer. This mixture was incubated for 10 min at 65°C mixing the tube by inversion from time to time. Subsequently, 100μL of isopropanol were added to the solubilized gel solution and vigorously mixed. This mixture was transferred to a GeneJET purification column and centrifuged at 13,500rpm for 1 min. After this time the column was washed twice with 700μL of wash buffer and centrifuged at 13,500rpm for 1 min. Finally the purification column was transferred to a 1.5mL eppendorf tube and 50μL of elution buffer were added to the center of the column membrane and centrifuged for 1 min. The amount and quality of DNA recovered were monitored on a 2% agarose gel. Oligonucleotides that were used to amplify the CaMKIIγ fragment contained restriction sites for the endonucleases EcoRI and BamHI. Thus, once this fragment was amplified and purified, 1μg of the fragment was incubated with 2U of BamHI and 1U of EcoRI, 2X buffer Tango (Thermo Fisher) for 1h at 37°C.

5.14 Cloning of the CaMKIIɣ 212-317aa (325pb) fragment into the vector pGEX6P-1

The DNA fragment encoding amino acids 212 to 317 of the CaMKIIγ protein was cloned into the eukaryotic expression vector pGEX6P-1 (it has the characteristic that fuses a GST flag at the amino terminal end of the desired recombinant protein). To clone this fragment into vector pGEX6P-1 (pre-digested with the same restriction enzymes as the PCR product under the same conditions) the amount of insert and vector required for the ligation reaction was calculated using the following formula:

$$Required\ insert\ DNA\ mass = \frac{(Insert\ DNA\ length\ )(Vector\ DNA\ mass)}{(Vector\ DNA\ length)}$$

The PCR product size was 325 bp and the size of the vector is 4984 bp. Once obtained the value of X multiplied by 3 to obtain a final ratio of 1: 3 (vector: insert), yielding a result of 9.57ng of insert. For the ligation reaction the following mixture was performed: 50ng of the digested vector, 9.57ng of the purified and digested insert, 2µL of 10x ligation buffer (PROMEGA), 3U T4 DNA ligase (PROMEGA) and water molecular biology grade for a final volume of 20µL. This mixture was incubated for 16h at a temperature of 16°C. Subsequently competent cells of *E. coli* DH5α cells were transformed with 10µL of the ligation mixture. Transformation was performed under the following protocol: 10ul of the ligation was contacted with 50µL of competent bacteria, this mixture was incubated on ice for 30min in order for the plasmid to be introduced to the bacteria. Subsequently they were given a heat shock at 42°C for 2 min and immediately placed on ice for 3 min then 1 mL of sterile Luria Bertani medium (LB) was added and incubated at 37°C for 1h under stirring. After bacterial growth was complete, the bacteria were centrifuged at 5,000rpm for 2 min, the supernatant was removed and the bacterial pellet was resuspended in 100µL of sterile LB medium and inoculated into LB agar plate ampicillin-supplemented (100µg/mL) at 37°C overnight for the growth of bacterial colonies.

5.15 Positive colony scrutiny using colony PCR

In order to know which of the colonies had the construction was carried out a colony PCR. Briefly, 10 colonies were selected and taken with the aid of a white micro-tip

and used to replicate in a dish of LB-Amp medium, which was incubated at 37 °C until bacterial growth was observed, the remainder of the colony was dissolved in a reaction mixture for PCR in a final volume of 10ul. The mixture was composed of 0.25 U GoTaq DNA polymerase (Promega), 1X Green GoTaq® Reaction Buffer (1.5mM MgCl2), 0.2mM of each of the dNTPs under the following conditions: 1 initial denaturation cycle of 95°C for 2 min, 35 cycles (denaturation at 95°C for 60 seconds, alignment at 56°C, extension at 72°C for 30 seconds, one final extension step at 72°C for 5 min and the reaction was maintained at 4°C. Subsequently the amplified products of each colony were resolved on a 0.8% agarose gel stained with 3μL of ethidium bromide.

5.16 Plasmid Isolation (Mini prep)

Nine of ten colonies that were positive in the PCR were placed in 5 mL of LB-Amp medium (100μg/mL) and allowed to grow for 15h under continuous stirring at 37°C to extract the plasmid DNA. The GenJeT Plasmid miniprep kit (Thermo) was used. Following bacterial growth, 3mL were centrifuged in an eppendorf tube at 8,000rpm for 2 min and the supernatant discarded. The pellet was resuspended in 250μL of resuspension buffer which was supplemented with RNase A, vortexed and ubsequently lysed in 250μL lysis buffer which contained NaOH and SDS in such a manner that exposure of the samples to SDS Solubilized phospholipids and cell wall proteins and allowed lysis and release of cellular contents, while NaOH denatured chromosomal DNA and proteins, resulting in the release of the plasmid to the supernatant. 350μL of neutralization buffer were added, the tube were mixed and centrifuged at 13,500rpm for 30 min at room temperature. The supernatant containing the plasmid was recovered and this was added directly to the column and centrifuged at 13.5000 pm for one min to remove the remaining liquid. Subsequently 700μL of wash solution were added to the column, in order to remove the excess of detergent from the sample and centrifuged under the above conditions. The column was centrifuged for additional min. Finally 50μL of elution buffer were added to the column and incubated for 2 min at room temperature. The column was centrifuged

at 13,500rpm within 1 min. Integrity of the plasmid was evaluated by migrating 100ng on a 0.8% agarose gel.

5.17 Determination of the plasmid DNA concentration.

The concentration of the plasmid DNA was measured by EPOCH BIOTEK spectrophotometer. 1µl of elution buffer was used as blank. 1µl of the plasmid sample was used for the measurement.

5.18 Digestion of the vector pGEX6P-1-CaMKIIγ-212-317

To corroborate that the pGEX6P-1-CaMKIIγ-212-317 construct contained the cloned fragment, the DNA from two colonies were digested with BamHI and EcoRI, briefly: 1µg of the construct was digested with 1U EcoRI, 2U BamHI, 4µL Buffer Tango 10X (Thermo Scientific) in a final volume of 20µL, this mixture was incubated 37°C for 1 hour, the released fragment was determined by migrating the digestion on a 1% agarose gel.

5.19 Sequencing of the purified plasmids.

The sequence of the purified plasmid was determined by sequencing using the pGEX Fwd 5'. Sequencing only in one direction. The samples were sent to UBIPRO at Faculty of Superior Studies Iztacala-UNAM.

5.20 Expression and purification of GST-CaMKIIγ-212-317aa recombinant protein.

The vector pGEX6p-1-CaMKIIγ-212-317aa was transformed into *E. coli* BL-21DE3 cells. A single colony is picked from the plate and incubated in a starting culture (5 ml) of LB-Ampicillin media (100µg/mL). After incubation overnight with shaking at 37°C the starting culture was used to seed a larger culture (100mL). The culture was incubated at 37°C with shaking until an optical density (OD) 600nm of 0.6–0.8 was obtained (as blanked by fresh LB media). The protein expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to the mixture to a final concentration of 0.4mM, and the bacterial suspension incubated for 4h at 37°C. After

IPTG induction of expression, the bacteria was pelleted by centrifugation at 5000rpm.

The pellet from a 100mL bacterial culture was resuspended in 500µL 6X Laemmli sample buffer, then boiled at 96°C for 5 min and centrifuged at 13.5000rpm for 30 min, the supernatant was recovered and migrated into a gel 12% SDS-PAGE. Once the gel migration was completed, a lane was stained with Coomassie Brillian Blue to visualize the enrichment of a protein band corresponding to GST-CaMKIIγ-212-317aa (37kDa) and this was cut with a sterile protease-free surgical scalpel blade. The GST-CaMKIIγ-212-317aa protein contained in the aclamide fragments was passively eluted in 250Ul of 1X PBS/2X protease inhibitor cocktail (Roche) overnight at 4°C in rotation. The next day it was centrifuged at 13,500 rpm for 15 min and carefully recovered the supernatant containing the recombinant protein. To monitor recovery of the recombinant protein we quantified by the Lowry method, a 12% SDS-PAGE gel was migrated stained with coomassie brillian blue and blotted with anti GST antibody 1:1,000 (Cellsignal) and anti-CaMKIIγ antibody 1:2,500 (Abcam).

5.21 Expression and purification of GST-CABL-601-720aa and GST recombinant protein.

The vector pGEX6P-3-GST-CABL-601-720aa and pGEX6P1 was transformed *into E. coli* BL-21DE3 cells. Purification of these recombinant proteins was performed as described by Fragioni, et al [100]. Colonies containing the plasmid pGEX6P-3-GST-CABL-601-720 and pGEX6P1 respectively, were grown overnight at 37°C with constant agitation in LB medium supplemented with ampicillin (100µg/mL). The next day 10mL of culture was taken overnight and added in 100mL of LB-Amp medium. These cells were allowed to grow for 4h at 37°C with constant agitation and constant monitoring of the optical density between 0.5 and 0.7, IPTG was added to a final concentration of 1mM, in order to induce expression of the GST-CABL-601-720aa and GST proteins. The bacterial culture was allowed to grow for an additional 4 h at 37°C under agitation. At this time the cells were centrifuged at 5,000rpm for 10 min to recover while the supernatant was discarded. The bacterial pellet from a 100mL bacterial culture was resuspended in 10mL of 1X PBS and resuspended by

continuous pipetting. To the mixture was added 100mM DTT-PBS to a final concentration of 5mM. Cells were lysed by sonication using an ultrasonic bath for 30 min. Samples were centrifuged at 10,000rpm for 30 min at 4°C to remove cellular debris. After centrifugation the supernatant was recovered and Triton X-100 was added in PBS to a final concentration of 2%. Because GST binds reversibly and with an affinity for glutathione, we purified the GST-CABL 601-702aa and GST proteins by incubating the bacterial lysate supernatant with the glutathione sepharose 4B beads (Amersham) overnight at 4°C. In this way only the recombinant protein GST-ACBL 601-702aa and GST were adhered to the resin. While the other proteins from the bacteria were removed during 3 times with 10 min washes with 1X PBS with continued agitation. Elution of the recombinant proteins was performed using 500uL of a 10mM "reduced glutathione" (SIGMA) solution dissolved in 50mM Tris-HCL pH=8.0 and this mixture was left 4h at 4°C in continuous rotation. To obtain the recombinant proteins the beads were centrifuged at 3,000rpm at 4°C for 5 min. The corresponding 500µL of the supernatant were quantified and their identity was checked by SDS-PAGE-Coomassie-Western blotting and Western blotting using the anti-GST antibody.

5.22 PAK1 Kinase assay

PAK1 kinase assays were performed by incubating a PAK1 alone and with substrates (2µg of GST-CaMKIIγ 212-319aa/ 10ug of GST as negative control and 10ug of GST-cAbl recombinant as positive control) in kinase buffer 1X (10mM MgCl2, 40mM Hepes, pH=7.4) supplemented with 5µM ATP for 30 min at 30°C in a reaction volume of 25µL. The reaction was terminated with 1X SDS sample buffer, followed by SDS-PAGE and then blotted with anti Phospho-Threonine Antibody (1:1,000) (Zymed®) and for positive control with anti Phospho-Serine Antibody (1:1,000) (Zymed®).[101]

5.23 Site directed Mutagenesis

Point mutations in plasmids were generated using the QuikChange II Site-Directed mutagenesis Kit according to the manufacturer's instructions. Briefly, 10 ng of DNA template was mixed with forward and reverse primers containing the relevant

mutations (Table 1) with the appropriate buffers after which the Pfu DNA polymerase was added to the reaction mixture. The mixture (50µL) was subject to PCR reaction with the conditions as follows: 95°C for 30 seconds, 18 cycles of 95°C for 30 seconds, 55°C for 1 min and 68°C for 6 min. After cycling a final extension step of 5 min at 68°C is applied. The mixture incubated with 1µL of DpnI endonuclease at 37°C for 1h to digest the methlyated template DNA (which has been purified from bacteria). The digested mixture is then transformed into *E. coli* DH5α competent cells according to manufacturer's protocols. The DNA is then purified using the Qiagen mini-prep kit and the mutations confirmed by DNA sequencing. Once the mutations were confirmed we proceeded to the expression and purification of mutant GST-CaMKIIγ-212-317aa recombinant proteins.

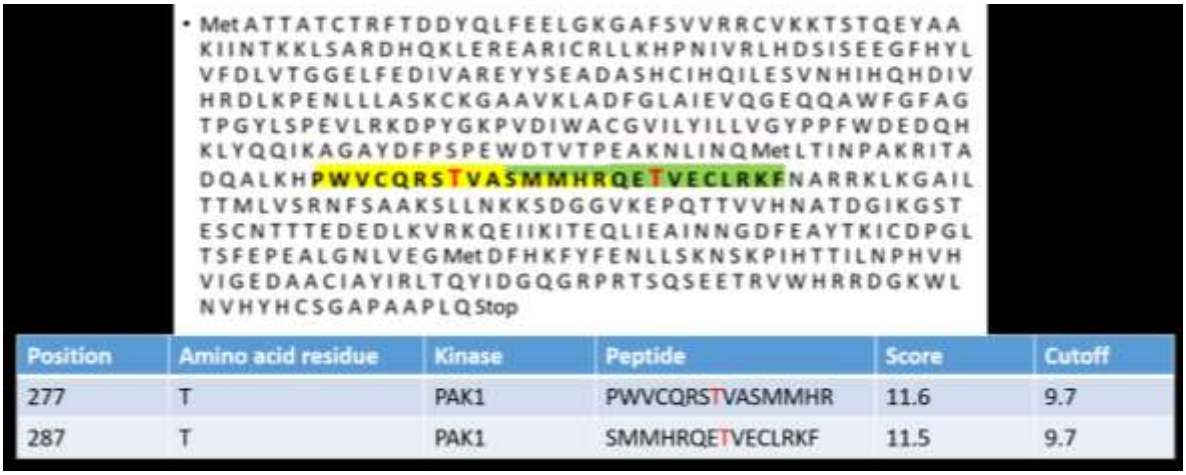
5.24 PAK1 kinase assay with mutant gst-camkiiy-212-317aa recombinant proteins

PAK1 kinase assays were performed by incubating a PAK1 alone and with substrates (10µg of GST-CaMKIIγ WT, 10µg of GST-CaMKIIγ Thr277A, 10µg of GST-CaMKIIγ Thr287A, 10µg of GST-CaMKIIγ Thr277/287A, 10µg of GST as negative control and 10µg of GST-cABL recombinant as positive control) in 1X kinase buffer (10mM MgCl2, 40mM Hepes, pH=7.4) supplemented with 5µM ATP for 30 min at 30°C in a reaction volume of 25µL. The reaction was terminated with 1X SDS sample buffer, followed by SDS-PAGE and then blotted with anti Phospho-Threonine Antibody (1:1,000) (Zymed®) and for positive control with anti Phospho-Serine Antibody (1:1,000) (Zymed®).

6. RESULTS

6.1 The *in silico* analysis identified CaMKIIγ as a possible direct substrate of PAK1

As a first step in trying to identify whether PAK1 has the ability to phosphorylate CaMKIIγ, an *in silico* analysis was performed using the Group Based Prediction System 3.0 (GPS 3.0) bioinformatic program. To this purpose, the CaMKIIγ primary sequence was introduced as a query sequence, and analyzed in order to determine if it contained putative PAK1 phosphorylation sites. The software identified two threonine residues at positions 277 and 287, both of them located in the regulatory domain of CaMKII, as PAK1 phosphorylation sites, with scores of 11.6 and 11.5, respectively. (Figure 9).



Position	Amino acid residue	Kinase	Peptide	Score	Cutoff
277	T	PAK1	PWVCQRSTVASMMHR	11.6	9.7
287	T	PAK1	SMMHRQETVECLRKF	11.5	9.7

Figure 9. Schematic representation of predicted kinase-specific phosphorylation sites of CaMKIIγ by PAK1 using GPS 3.0 (Group-based Prediction System) software.

To corroborate if PAK1 can effectively phosphorylate the regulatory domain of CaMKIIγ we performed an *in vitro* kinase assay using the following recombinant proteins: PAK1 (which has the catalytic activity only), the regulatory domain of CaMKIIγ as a substrate, a positive control (GST-CABL-601-720aa) and a negative control (GST).

6.2 Transfection and immunoprecipitation of recombinant PAK1

For the development of this project we obtained the recombinant PAK1 from the expression vector pCMV6M-PAK1 L107F donated by Dr Jonathan Chernoff which has a cytomegalovirus promoter followed by a myc-tag and the sequence encoding the PAK1 protein, which has the substitution of a Leu to a Phe at position 107, which allows the protein to be constitutively active. As a first step we performed a digestion of the vector pCMV6M-PAK1 L107F in order to verify the identity of the vector (Figure 10A). Once the plasmid identity was verified, we transfected this vector into the HEK293T cell line and the expression of the myc-tagged PAK1 protein was assessed by Western blot using anti-myc and anti-PAK1 antibodies (Figure 10B). As expected only when the cell line was transfected with the vector, the recombinant myc-PAK1 protein was expressed at an estimated molecular weight of 70 kDa. Finally, the myc-tagged PAK1 protein was immunoprecipitated using an anti-myc antibody (Figure 10C).

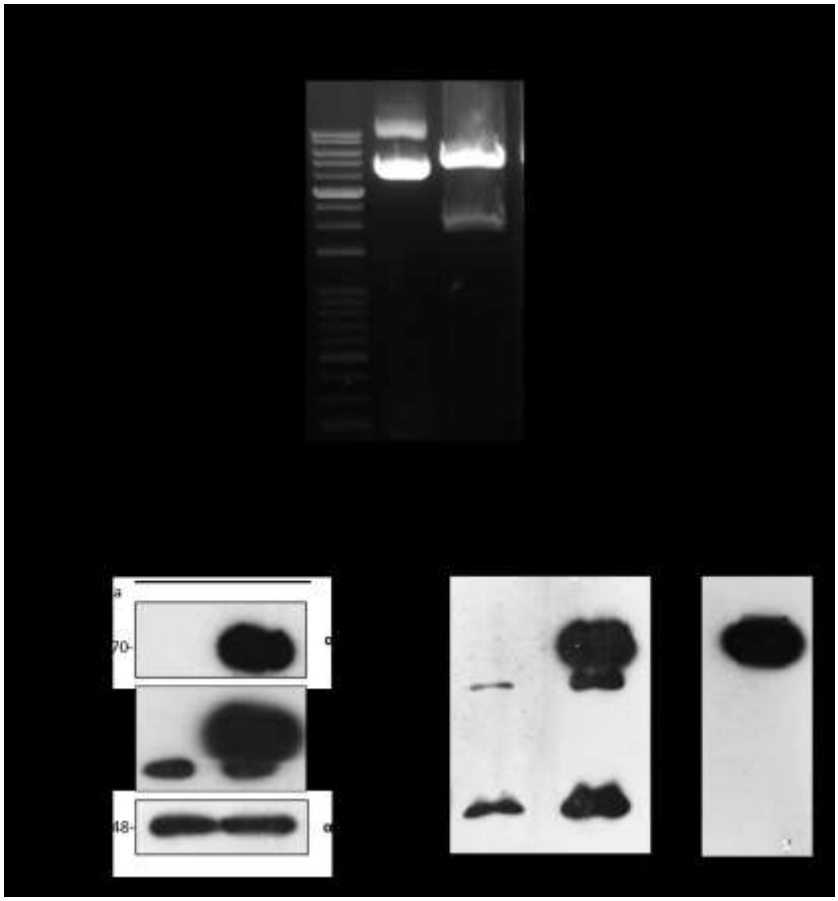


Figure 10. Obtention of the myc tagged PAK1-L107F recombinant protein. A) Digestion of the pCMV-6MPAK1L107F vector with EcoRI and BamHI. B) Transfection and immunodetection of Myc-PAK1-L107F (70Kda). C) Immunoprecipitation and immunodetection of Myc-PAK1-L107F

6.3 Cloning and purification of GST-CaMKIIγ-212-317aa recombinant protein.

Another important tool for the development of this project was to obtain the CaMKIIγ recombinant protein, however, it was necessary to obtain only the regulatory domain (corresponding approximately to amino-acid residue 212 to 317) because, since the protein has kinase activity, the complete form would not allow us to discriminate a phosphorylation mediated by PAK1 or an autophosphorylation phenomenon (Figure 11A).

For this purpose, we amplified by PCR a 336bp fragment containing the open reading frame of the regulatory domain of CaMKIIγ from cDNA (Figure 11B and C).

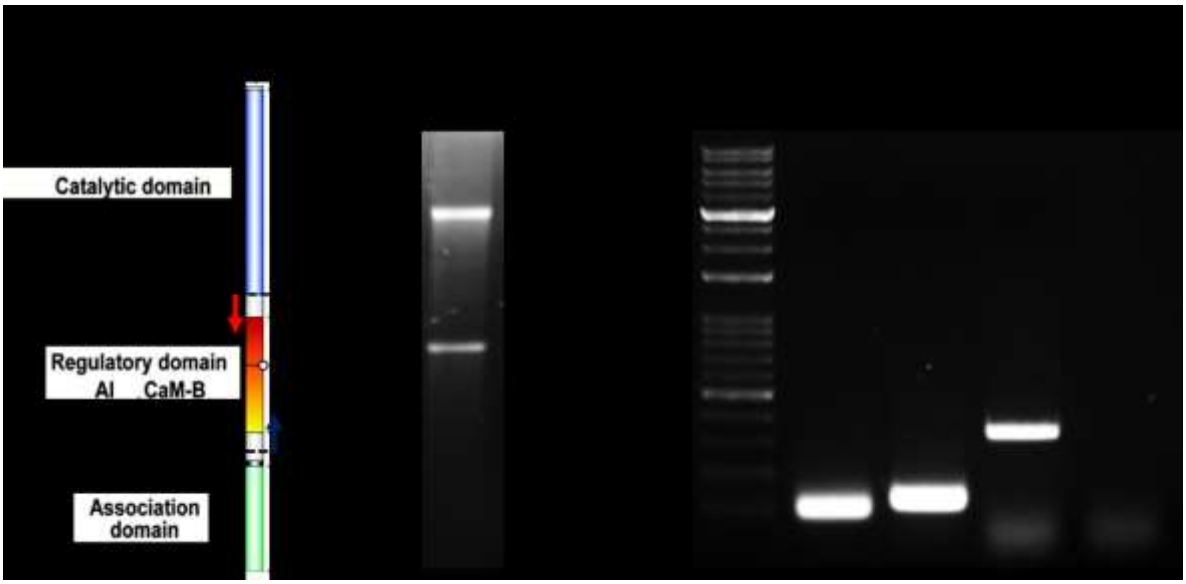


Figure 11. Amplification of the regulatory domain of the CaMKIIγ protein. A) Schematic representation of regulatory domain that we cloned, where the arrows represent the oligonucleotides used to amplify the open reading frame of this gene. B) Denaturing gel electrophoresis of RNA isolated from BT474 cell line. C) RT-PCR fragment of the regulatory domain of CaMKIIγ.

The oligonucleotides used to amplify this sequence were designed according to the sequence published in NCBI/Primer-BLAST, a restriction site for BamHI was added

to the forward oligonucleotide, and a restriction site for EcoRI was added to the reverse oligonucleotide. Once this fragment was amplified, it was purified and digested with both enzymes, and subsequently cloned in the expression vector pGEX6P-1 which has the sequence encoding the GST protein adjacent to the cloning site (Figure 12A). The ligation mix was transformed into *E. coli* strain DH5α. Colonies containing the plasmid with our insert were identified by colony PCR using the oligonucleotides used for amplification. The result of this PCR is shown in figure 12B where again the positive colonies amplified a fragment of 336bp. One of the positive colonies was selected to obtain the plasmid DNA and digested with the restriction enzymes BamHI and EcoRI to check for release of the previously cloned fragment (Figure 12C).

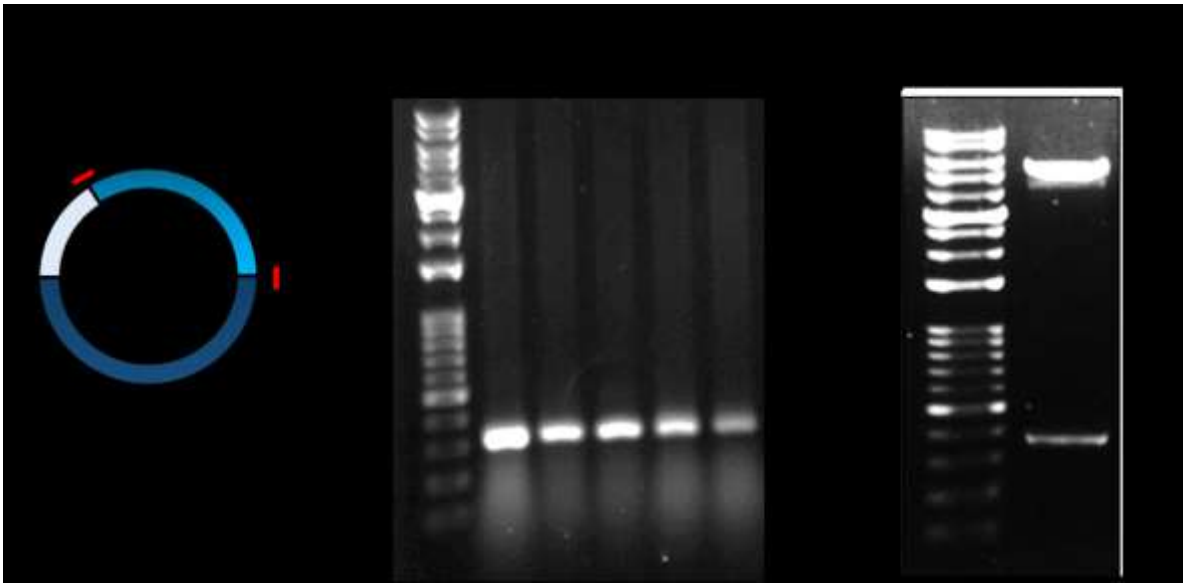


Figure 12. Cloning of the regulatory domain of CaMKIIγ protein. A) Schematic representation of the pGEX6P-1-CaMKIIγ-212-317 where the oligonucleotides flanking the vector cloning site are indicated by red lines. B) PCR of the candidate colonies, the specific oligonucleotides used for amplification of the insert were used. C) Digestion of the vector pGEX6P-1-CaMKIIγ-212-317 with the restriction enzymes BamHI and EcoRI where liberation of the cloned insert is observed.

This plasmid was then sequenced to verify that the cloned fragment encoding the regulatory domain of CaMKIIγ was in open reading frame with respect to GST. Sequencing indicated that the open reading frame of the regulatory domain of

CaMKII was intact and further that the insert had not undergone changes in its sequence indicating that we already had the construct pGEX6P-1 GST-CaMKIIγ 212-317 (Figure 13).

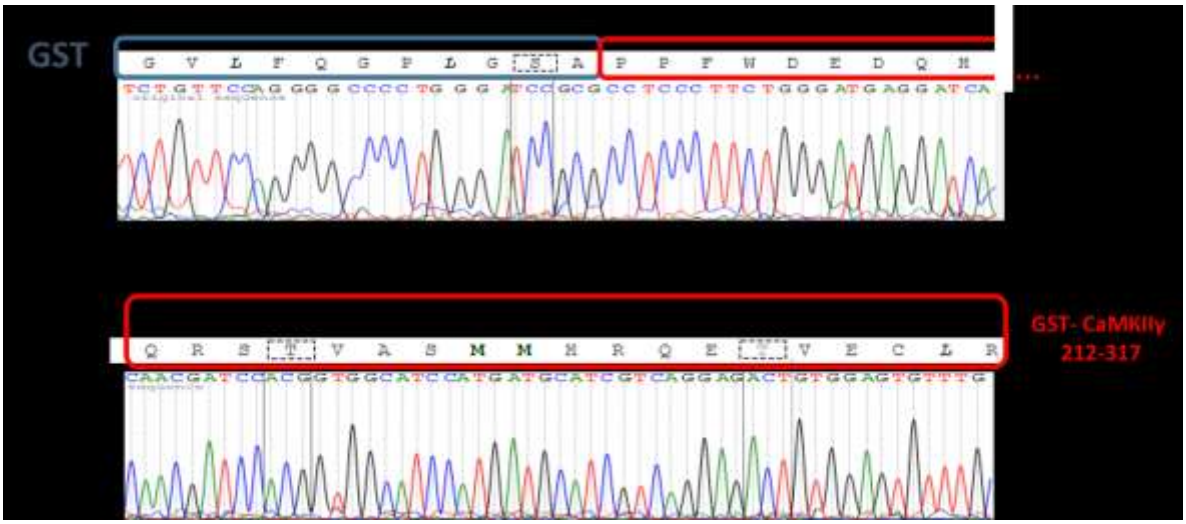


Figure 13. Electropherogram of the plasmid pGEX6P-1-GST-CaMKIIγ-212-317.

This construct was then employed to induce the expression of the GST-CaMKIIγ-212-317 fusion protein, this protein showed the expected molecular weight of about 37 kDa where 26 kDa correspond to the GST protein and the remaining 11 kDa are from regulatory domain of CaMKIIγ (Figure 14A, lane 2 and 3).

Figure 14 shows that only when the culture was induced with IPTG the recombinant protein was expressed (Figure 14A, lane 2) and this was not observed in non-transformed bacteria induced with IPTG (Figure 14A, lane 1). Once we had induced the expression of our GST-tagged recombinant protein, we attempted to purify the soluble and insoluble fractions by affinity chromatography with Glutathione Sepharose 4B beads, which have high affinity for the GST. However it was impossible to achieve a good purification, so we decided to perform a passive elution of the protein from the polyacrylamide gels, and we observed an almost total elimination of other bacterial proteins and an enrichment of GST-CaMKIIγ-212-317 (Figure 14A, lane 3).

In order to verify the identity of the protein, we performed a Western blot analysis using antibodies directed against GST and against CaMKII, which allowed us to

identify the recombinant protein GST-CaMKIIγ-212-317aa (37 kDa) (Figure 14B and C, lane 3), this recognition was not observed when non-transformed *E. coli* extracts were used (Figure 14B and C, lane 2).

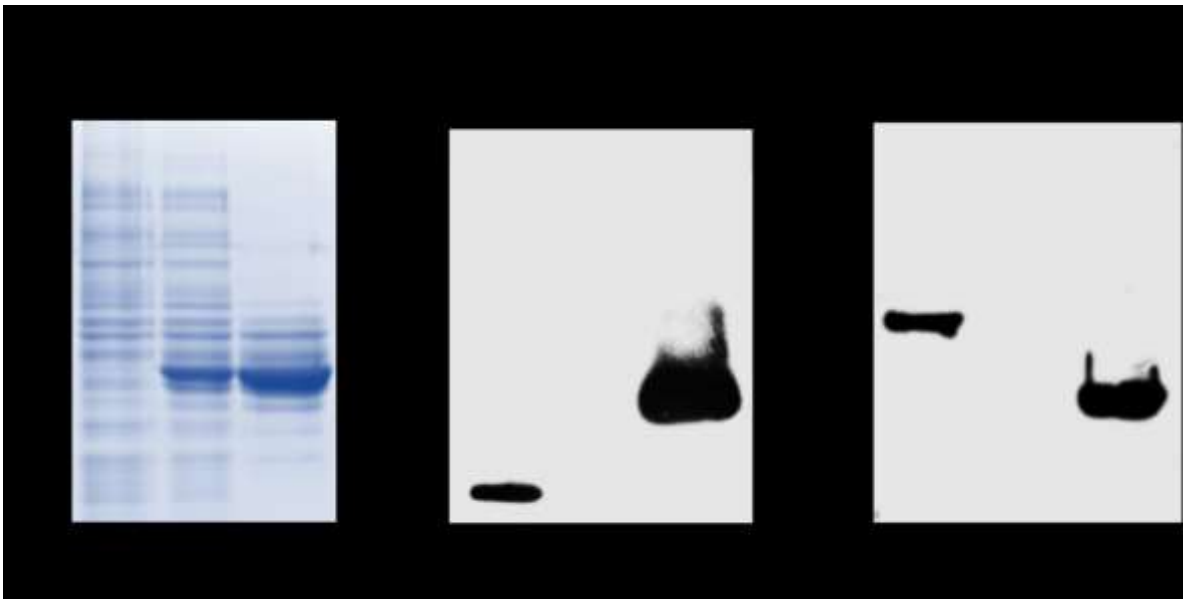


Figure 14. Induction, purification and immunodetection of GST-CaMKIIγ-212-317aa protein. A) The proteins were resolved on a 12% SDS-PAGE gel and stained with Coomassie bright blue. Total proteins from uninduced *E. coli* that were transformed with the plasmid containing our insert (lane 1), after induction with IPTG (lane 2), after purification (lane 3). B) Western blot using the anti-GST antibody, using GST as a positive control (lane 1), total extracts of uninduced *E. coli* (Lane 2), the recombinant GST-CaMKIIγ-212-317aa protein. C) Western blot using the anti-CaMKII antibody, using total protein extracts from the HEK-293T cell line as a positive control (lane 1), total extracts from no induced *E. coli* (Lane 2), and the GST-CaMKIIγ-212-317aa protein.

6.4 Expression and purification of GST-CABL-601-720aa and GST recombinant protein.

As a positive control for the *in vitro* kinase assay we used the GST-CABL-601-720aa fragment protein, which is a reported PAK substrate, and as a negative control the GST protein. The vectors pGEX6P-3-GST-ACBL-601-720 and pGEX6P-1 were then employed to induce the expression of fusion proteins, these proteins showed the expected molecular weight 42 kDa where 26 kDa correspond to the GST protein and the remaining 16 kDa are from CABL (Figure 15A, lane 2 and 3) and GST (26Kda) (Figure 15B, lane 2 and 3)

Figure 15 shows that only when the culture was induced with IPTG the recombinant protein was expressed (Figure 15A and B, lane 2) and this was not observed in the absence of IPTG (Figure 15A and B, lane 1) which indicated that our construction was functional. Once we had induced the expression of our recombinant protein, we proceed to purify it by affinity chromatography with Glutathione Sepharose 4B beads, which have high affinity for the GST.

With the purpose of verify the identity of the protein a Western blot analysis was performed using antibodies against GST, which allowed us to identify the recombinant protein GST-CABL-601-720 (42 kDa) (Figure 15A and B, lane 3), this recognition was not observed when extracts of non-induced *E. coli* were used (Figure 15A and B, lane 2).

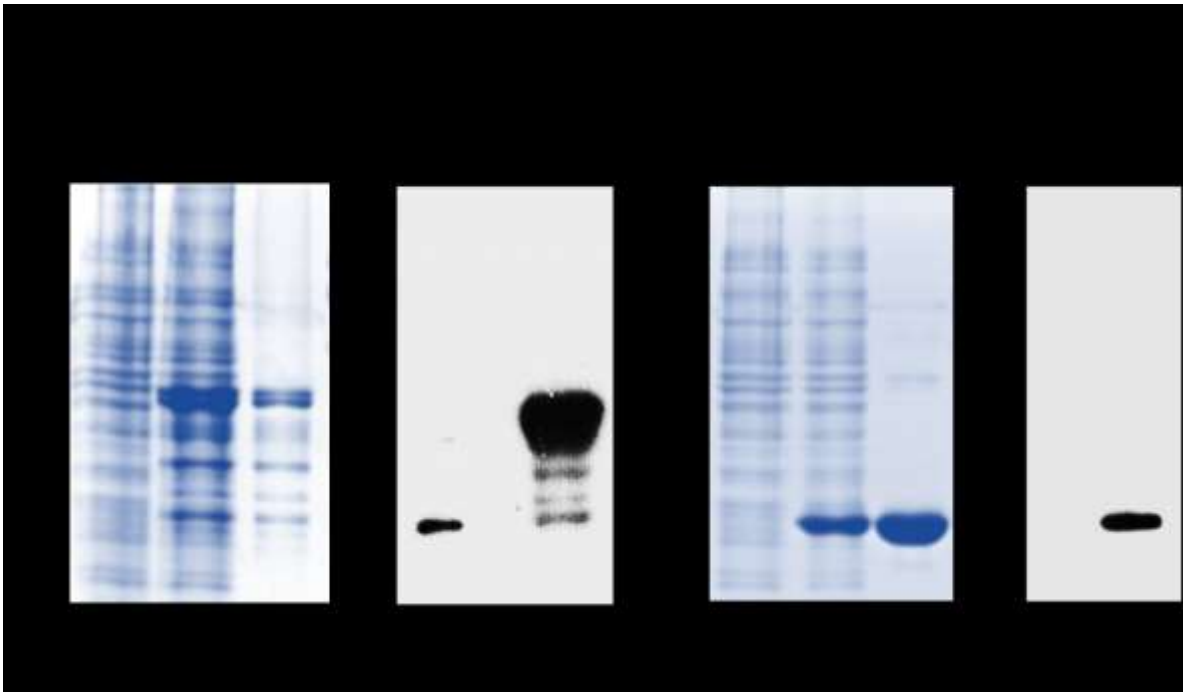


Figure 15. Induction, purification and immunodetection of positive control (GST-CABL-601-720aa protein and negative control (GST) A) Positive control (GST-CABL-601-720).The proteins were resolved on a 12% SDS-PAGE gel and stained with coomassie bright blue. Total *E. coli* proteins uninduced that were transformed with the plasmid pGEX6p-3-GST-CABL(lane 1), after induction with IPTG (lane 2), after purification (lane 3) and Western blot using the anti-GST antibody B) Negative control (GST) The proteins were resolved on a 12% SDS-PAGE gel and stained with coomassie bright blue. Total *E. coli* proteins uninduced that were transformed with the plasmid pGEX6p-1-GST (lane 1), after induction with

IPTG (lane 2), after purification (lane 3) and Western blot using the anti-GST antibody.

6.5 PAK1 directly phosphorylates the regulatory domain of CaMKII in an *in vitro* kinase assay

Once that we purified our recombinant proteins we proceeded to evaluate whether PAK1 was able to phosphorylate the regulatory domain of CaMKII. To this end, we performed an *in vitro* kinase assay using PAK1 as a kinase in the absence or presence of the recombinant GST-CaMKII-212-317aa protein (2µg), as well as in the presence of the recombinant GST-CABL-601-720aa protein (10µg). Our results showed that there is no phosphorylation signal in the absence of a PAK1 substrate (Figure 16, upper panel, lane1). In contrast, in the presence of the GST-CaMKII-212-317aa protein, we observed a signal resulting from immunodetection (Figure 16, upper panel, lane 2). As expected, no signal was observed in the absence of PAK1 kinase (Figure 17, upper Panel, lane 3). In order to demonstrate that the phosphorylation observed was in the regulatory domain and not in the GST-tag, we performed an *in vitro* kinase assay using GST protein as a substrate (Figure 16, upper panel, lane 4). As expected the GST protein was not phosphorylated by PAK1. This result indicated that PAK1 phosphorylates CaMKIIγ in the regulatory domain and confirms the data obtained in the *in silico* analysis. Finally, we observed that our positive control, GST-cABL-601-aa protein, was phosphorylated by PAK1 (Figure 16, upper panel, lane 5). The lower panel shows the Coomassie brilliant blue staining to confirm equal amounts of protein were used.

Finally, to demonstrate that the phosphorylation observed was in the regulatory domain and not to the GST fusion protein, we performed an *in vitro* kinase assay using the GST protein as a substrate (Figure 16, upper panel, lane 4). As expected the GST protein was not phosphorylated by PAK1. This result indicated that PAK1 phosphorylates CaMKIIγ in the regulatory domain and confirms the data obtained in the *in silico* analysis.

Figure 16. PAK1 phosphorylates CaMKIIγ-212-317aa *in vitro*. 5µg of GST-CaMKIIγ-212-317aa, 10µg of GST and 10µg of GST-cABL proteins (substrates) were incubated with 5µM of ATP and 2µg of constitutively active myc-PAK1 L107F for 30 min at 30°C. GST was used as a negative control and GST-cABL was used as a positive control. The reaction was separated by 10% SDS-PAGE, and phosphorylated proteins were detected and probed with the anti-phosphothreonine antibody (upper panel). 2µg of myc-PAK1 L107F, 5µg of GST-CaMKIIγ-212-317aa, 10µg of GST and 10µg of GST-cABL proteins were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue (lower panel).

6.6 PAK1 phosphorylates threonine 277 but not threonine 287 in the regulatory domain of CaMKIIγ in vitro.

Once we had determined that PAK1 phosphorylated the regulatory domain of CaMKII we proceeded to identify the phosphorylation sites. Initially we proceeded to generate 3 mutant versions of the GST-CaMKIIγ-212-317: GST-CaMKII-Thr277Ala, GST-CaMKIIγ-Thr287Ala and GST-CaMKIIγ-Thr277/287Ala protein. To this purpose, the plasmid pGEX6P-1-GST-CaMKIIγ-212-317 was used as template in a PCR-directed site mutagenesis reaction using oligonucleotides containing the desired mutations. The reaction was digested with DpnI enzyme and transformed into *E. coli* competent cells. The plasmids were purified and sequenced to verify that they contained the desired mutation (Figure 17).

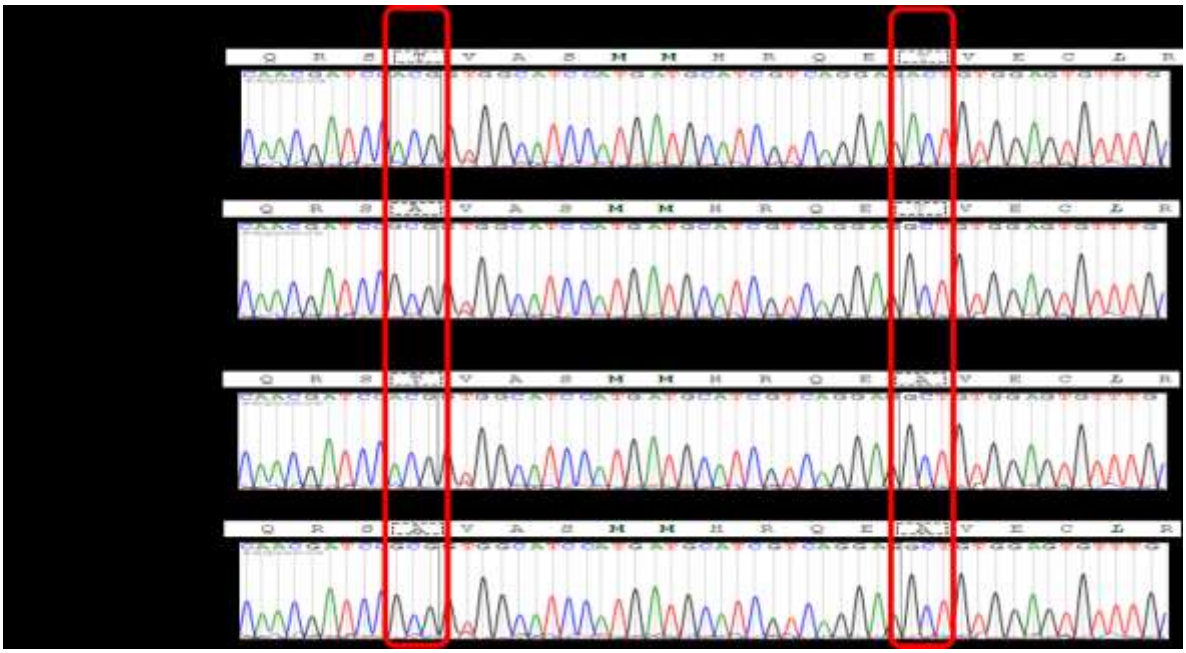


Figure 17. Electropherogram of the mutant plasmids pGEX 6P-1-GST-CaMKIIγ-212-317 (Thr277Ala, Thr287Ala, Thr277/287Ala)

Subsequently the induction and purification of the mutant proteins was carried out, and they were used as substrates in an *in vitro* kinase assay. The results showed that as expected, no signal is detected in the absence of substrate (Figure 18 lane 1), whereas when PAK1 was incubated with GST-CaMKIIγ-212-317 aa a signal is detected, which is concordant with our previous results (lane 2). Interestingly, when PAK1 was incubated with GST-CaMKIγI-Thr277Ala (Lane 3) a very important decrease in signal intensity was observed, but when we incubated PAK1 with GST-CaMKIIγ-Thr287Ala (Lane 4) there were no changes in signal intensity. Finally, when the double mutant GST-CaMKIIγ-Thr277/287Ala was used as a substrate (Lane 5), a decrease in signal intensity, similar to that observed when GST-CaMKIIγ-Thr277Ala was used as substrate, was noted. The lower panel shows the Coomassie brilliant blue staining to confirm equal amounts of protein were used. These results altogether suggest that PAK1 phosphorylates threonine 277 but not threonine 287 and that there is also another threonine, which is phosphorylated by PAK1 and was not identified in the *in silico* analysis.

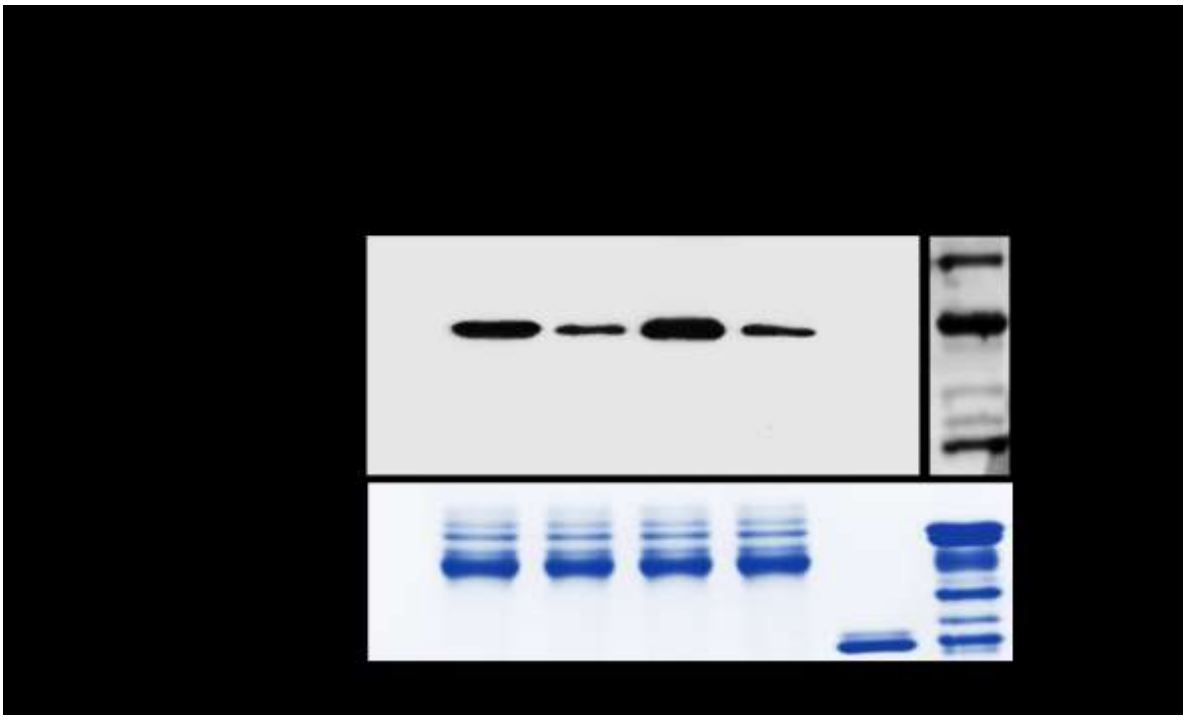


Figure 18. PAK1 phosphorylates GST-CaMKIIγ-212-317aa in the Thr277 *in vitro*. Purified GST-CaMKIIγ-212-317aa and mutants (Thr277, Thr287, Thr277/287Ala) as indicated in the figure were incubated PAK1 with 5μM of unlabeled ATP for *in vitro* kinase assay for 30 min at 30°C. GST was used as a negative control and GST-CABL was used as a positive control, the reaction was separated by 10% SDS-PAGE, and phosphorylated proteins were detected and probed with the anti-phosphothreonine antibody (upper panel). 2μg of myc-Pak1-L107F, 10μg of GST-CaMKIIγ-212-317aa and 10ug of mutants, 10μg of GST and 10 mg of GST-CABL-601-720 proteins were applied to 10% SDS-PAGE and stained with Coomassie Brilliant Blue (lower panel).

7. DISCUSSION

In Mexico, breast cancer ranks first in incidence and mortality due to malignant neoplasms in women older than 25 years [4]. Among breast cancers in particular, it is known that approximately 25-30% of breast cancer patients present tumors that are characterized by being “ErbB2/HER2-enriched”, meaning that the gene that encodes the epidermal growth factor receptor 2, is highly expressed [102]. Therapies targeting ErbB2/HER2 with the monoclonal antibody trastuzumab were initially promising, but, as commonly seen with targeted agents, were subsequently shown to be effective in some patients but not in others [38,40]. Because the therapies targeting the ErbB2/HER2 pathway are only sometimes effective, there is a need to further delve into the activities and mechanisms downstream to ErbB2/HER2 to elucidate the key signaling pathways triggered by over-active ErbB2/HER2 responsible for tumorigenesis. It is well documented that ErbB2/HER2 signaling promotes the stimulation of several downstream protein kinase cascades, including the Ras/RAF/MEK/ERK, PI3K/Akt and Wnt/β-catenin pathways, known to affect both tumor cell growth and migration [69]. Signaling through all these pathways can be influenced by Group I p21-activated kinases (PAKs), a family of effectors of the Rho GTPases Rac and Cdc42 [69].

Recently, *PAK1* gene amplification has been reported to be particularly relevant in breast cancer since it has been shown that such amplification is associated with resistance to tamoxifen treatment and decreased survival [46]. In addition, PAK1 overexpressing tumors are "addicted" to PAK1 signaling, and are highly sensitive to PAK inhibition. However a more detailed study is needed in order to identify the most relevant PAK1 substrates involved in cancer initiation and/or progression. Experiments with phospho-antibody microarrays facilitated the identification of additional signaling molecules that could be responsible for the defects observed in PAK1 deficient cells. Some of the hypophosphorylated proteins identified in these studies, included some well characterized PAK1 substrates with pro-survival and anti-apoptotic functions, such as c-ABL, RAF and BAD, and also some other proteins that could be regulated directly or indirectly by PAK1, including FAK, JAK2, VAV1 and CaMKII, which has recently been associated with breast cancer progression

[69]. In this work, we focused in CaMKIIγ, a multifunctional serine/threonine kinase whose activity is regulated through Ca^{2+} signaling and is an activator of several signaling pathways involved in cell proliferation and cell cycle progression.

Here, we demonstrate for the first time that PAK1 phosphorylates the regulatory domain of CaMKIIγ in the threonine 277 *in vitro.* The importance of this phenomenon stands in the classical CaMKIIγ activation mechanism, in which a first step of Ca^{2+}/CaM complex binding to the regulatory domain is necessary, this interrupts the interaction between the autoinhibitory sequence and the catalytic domain found in the N-terminal to later allow the phosphorylation of their substrates. Activation also catalyzes autophosphorylation within the autoinhibitory domain in threonine 287. It is that this phosphorylation that gives CaMKIIγ its ability to remain active even after Ca^{2+}/CaM dissociation, since this modification prevents the reassociation of the CaMKIIγ autoinhibitory domain, even in the absence of Ca^{2+}/CaM. However, several reports have demonstrated that the mutation of threonine 287 for alanine (T287A) does not prevent the protein from remaining active even in the absence of Ca^{2+}/CaM, suggesting that other modifications in the catalytic domain could be necessary to sustain its activity. Under this premise several investigations have been performed in order to demonstrate if other post-translational modifications that could occur in the regulatory domain are relevant for the activation of CaMKIIγ.

Some post-translational modifications, which are relevant for CaMKIIγ function are oxidation and O-GlcNac. It has been reported that CaMKIIγ oxidation at methionine 281 and/or 282 in a reactive oxygen species (ROS) dependent fashion, considerably enhances its kinase activity. In addition, in some animal models for hyperglycemia and diabetes, the O-GlcNac of serine 279 activates the kinase autonomously, creating a molecular memory even after calcium concentrations decay. In contrast, it has been reported that autophosphorylation of threonine 306 and 307 in the regulatory domain inhibits CaMKIIγ activation by blocking its binding to Ca^{2+}/CaM [73, 74, 78, 79, 103].

Interestingly enough, not all post-translational modifications in the regulatory domain affect the activity of CaMKIIγ, but they may alter its cellular distribution and the affinity

and/or selectivity for its substrates. Recently, has been shown that when CaMKIIγ is phosphorylated at threonine 253, it form molecular complexes with other binding proteins, which is translated into the phosphorylation of different substrates, and consequently into modifications on its biological function [82].

Remarkably, the biological effect of CaMKIIγ phosphorylation at threonine 277 has not been studied, and since this kinase displays pleiotropic effects in several solid tumors, sometimes favoring cell cycle progression and others inducing cell cycle arrest, it would be important to determine if PAK1 mediated phosphorylation is involved in this phenomenon. The effects of CaMKII on cell proliferation are dependent on post-translational modifications involved in the activation of multiple signaling cascades. However in ErbB2/HER2+ breast cancer cells these phenomena has been poorly studied. One possibility that should be addressed in the future, is if the phosphorylation of threonine 277 mediated by PAK1 creates a Ca2+/CaM autonomous activation state in CaMKIIγ, an inactive state, or is not involved on its activation, but in the selectivity of different substrates *in vivo* (Figure 19).

Figure 19. Schematic representation of our hypothesis for PAK1 mediated phosphorylation of CaMKIIγ. The phosphorylation of threonine 277 of CaMKIIγ mediated by PAK1 creates a Ca2+/CaM autonomous activation state, an inactive state, or is not involved on its activation, but in the selectivity of different substrates and this phenomenon could be involved in the arrest or progress of the cell cycle in ErbB2/HER2+ breast cancer.

8. CONCLUSIONS

- PAK1 phosphorylates the regulatory domain of CaMKIIγ *in vitro*.
- PAK1 phosphorylates CaMKIIγ directly at Thr277, but not at Thr287.
- There is at least another threonine residue in the regulatory domain of CaMKIIγ that is phosphorylated by PAK1, and that was not identified by the *in silico* analysis

9. REFERENCES

1. Cooper, G.M. and R.E. Hausman, *The cell: a molecular approach. Washington, D.C.* ASM Press, 2009.
2. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer Statistics, 2017.* CA Cancer J Clin, 2017. **67**(1): p. 7-30.
3. Organization, W.H., *Breast cancer: prevention and control.* 2013: p. http://www.who.int/cancer/detection/breastcancer/.
4. SINAIS/SINAVE/DGE/SALUD/, *Perfil epidemiológico de los tumores malignos en México.* México, DF, 2011.
5. Weigelt, B. and J.S. Reis-Filho, *Histological and molecular types of breast cancer: is there a unifying taxonomy?* Nat Rev Clin Oncol, 2009. **6**(12): p. 718-30.
6. Eroles, P., *et al.*, *Molecular biology in breast cancer: intrinsic subtypes and signaling pathways.* Cancer Treat Rev, 2012. **38**(6): p. 698-707.
7. Perou, C.M., *et al.*, *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
8. Rivenbark, A.G., S.M. O'Connor, and W.B. Coleman, *Molecular and cellular heterogeneity in breast cancer: challenges for personalized medicine.* Am J Pathol, 2013. **183**(4): p. 1113-24.
9. Guarneri, V. and P. Conte, *Metastatic breast cancer: therapeutic options according to molecular subtypes and prior adjuvant therapy.* Oncologist, 2009. **14**(7): p. 645-56.
10. Kennecke, H., *et al.*, *Metastatic behavior of breast cancer subtypes.* J Clin Oncol, 2010. **28**(20): p. 3271-7.
11. Paik, S., *et al.*, *A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer.* N Engl J Med, 2004. **351**(27): p. 2817-26.
12. Marchio, C., *et al.*, *The genomic profile of HER2-amplified breast cancers: the influence of ER status.* J Pathol, 2008. **216**(4): p. 399-407.
13. Parker, J.S., *et al.*, *Supervised risk predictor of breast cancer based on intrinsic subtypes.* J Clin Oncol, 2009. **27**(8): p. 1160-7.

14. Prat, A. and C.M. Perou, *Deconstructing the molecular portraits of breast cancer.* Mol Oncol, 2011. **5**(1): p. 5-23.
15. Gianni, L., *et al.*, *Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: a 4-year follow-up of a randomised controlled trial.* Lancet Oncol, 2011. **12**(3): p. 236-44.
16. Slamon, D.J., *et al.*, *Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2.* N Engl J Med, 2001. **344**(11): p. 783-92.
17. Bosch, A., *et al.*, *Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research.* Cancer Treat Rev, 2010. **36**(3): p. 206-15.
18. Livasy, C.A., *et al.*, *Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma.* Mod Pathol, 2006. **19**(2): p. 264-71.
19. Smid, M., *et al.*, *Subtypes of breast cancer show preferential site of relapse.* Cancer Res, 2008. **68**(9): p. 3108-14.
20. Nielsen, T.O., *et al.*, *Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma.* Clin Cancer Res, 2004. **10**(16): p. 5367-74.
21. Sorlie, T., *et al.*, *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10869-74.
22. Graus-Porta, D., *et al.*, *ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling.* EMBO J, 1997. **16**(7): p. 1647-55.
23. Sibilia, M., *et al.*, *The epidermal growth factor receptor: from development to tumorigenesis.* Differentiation, 2007. **75**(9): p. 770-87.
24. Gassmann, M., *et al.*, *Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor.* Nature, 1995. **378**(6555): p. 390-4.
25. Riethmacher, D., *et al.*, *Severe neuropathies in mice with targeted mutations in the ErbB3 receptor.* Nature, 1997. **389**(6652): p. 725-30.

26. Lee, K.F., *et al.*, *Requirement for neuregulin receptor erbB2 in neural and cardiac development.* Nature, 1995. **378**(6555): p. 394-8.
27. Meyer, D. and C. Birchmeier, *Multiple essential functions of neuregulin in development.* Nature, 1995. **378**(6555): p. 386-90.
28. Hynes, N.E. and H.A. Lane, *ERBB receptors and cancer: the complexity of targeted inhibitors.* Nat Rev Cancer, 2005. **5**(5): p. 341-54.
29. Shih, C., *et al.*, *Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts.* Nature, 1981. **290**(5803): p. 261-4.
30. van de Vijver, M., *et al.*, *Amplification of the neu (c-erbB-2) oncogene in human mammmary tumors is relatively frequent and is often accompanied by amplification of the linked c-erbA oncogene.* Mol Cell Biol, 1987. **7**(5): p. 2019-23.
31. Guy, C.T., R.D. Cardiff, and W.J. Muller, *Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease.* Molecular and Cellular Biology, 1992. **12**(3): p. 954-961.
32. Pittius, C.W., *et al.*, *A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice.* Proceedings of the National Academy of Sciences of the United States of America, 1988. **85**(16): p. 5874-5878.
33. Slamon, D.J., *et al.*, *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene.* Science, 1987. **235**(4785): p. 177-82.
34. Hung, M.C., *et al.*, *Molecular cloning of the neu gene: absence of gross structural alteration in oncogenic alleles.* Proceedings of the National Academy of Sciences of the United States of America, 1986. **83**(2): p. 261-264.
35. Moasser, M.M., *The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis.* Oncogene, 2007. **26**(45): p. 6469-87.

36. Moasser, M.M., *The oncogene HER2; Its signaling and transforming functions and its role in human cancer pathogenesis.* Oncogene, 2007. **26**(45): p. 6469-6487.
37. Kute, T., *et al.*, *Development of Herceptin resistance in breast cancer cells.* Cytometry A, 2004. **57**(2): p. 86-93.
38. Kast, K., *et al.*, *Trastuzumab and survival of patients with metastatic breast cancer.* Arch Gynecol Obstet, 2017. **296**(2): p. 303-312.
39. Iqbal, N. and N. Iqbal, *Human Epidermal Growth Factor Receptor 2 (HER2) in Cancers: Overexpression and Therapeutic Implications.* Mol Biol Int, 2014. **2014**: p. 852748.
40. Baselga, J., *et al.*, *Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer.* N Engl J Med, 2012. **366**(2): p. 109-19.
41. Cobleigh, M.A., *et al.*, *Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease.* J Clin Oncol, 1999. **17**(9): p. 2639-48.
42. Zahreddine, H. and K.L. Borden, *Mechanisms and insights into drug resistance in cancer.* Front Pharmacol, 2013. **4**: p. 28.
43. Gajria, D. and S. Chandarlapaty, *HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies.* Expert Rev Anticancer Ther, 2011. **11**(2): p. 263-75.
44. Manser, E., *et al.*, *A brain serine/threonine protein kinase activated by Cdc42 and Rac1.* Nature, 1994. **367**(6458): p. 40-6.
45. Bokoch, G.M., *Biology of the p21-activated kinases.* Annu Rev Biochem, 2003. **72**: p. 743-81.
46. Whale, A., *et al.*, *Signalling to cancer cell invasion through PAK family kinases.* Front Biosci (Landmark Ed), 2011. **16**: p. 849-64.
47. Arias-Romero, L.E. and J. Chernoff, *A tale of two Paks.* Biol Cell, 2008. **100**(2): p. 97-108.
48. Radu, M., *et al.*, *PAK signalling during the development and progression of cancer.* Nat Rev Cancer, 2014. **14**(1): p. 13-25.

49. Kumar, R. and D.Q. Li, *Chapter Four - PAKs in Human Cancer Progression: From Inception to Cancer Therapeutic to Future Oncobiology*, in *Advances in Cancer Research*, D.T. Kenneth and B.F. Paul, Editors. 2016, Academic Press. p. 137-209.
50. Baskaran, Y., *et al.*, *Group I and II mammalian PAKs have different modes of activation by Cdc42.* EMBO Rep, 2012. **13**(7): p. 653-9.
51. Ha, B.H., *et al.*, *Type II p21-activated kinases (PAKs) are regulated by an autoinhibitory pseudosubstrate.* Proc Natl Acad Sci U S A, 2012. **109**(40): p. 16107-12.
52. Adam, L., *et al.*, *Heregulin regulates cytoskeletal reorganization and cell migration through the p21-activated kinase-1 via phosphatidylinositol-3 kinase.* J Biol Chem, 1998. **273**(43): p. 28238-46.
53. Brown, L.A., *et al.*, *Amplification of EMSY, a novel oncogene on 11q13, in high grade ovarian surface epithelial carcinomas.* Gynecol Oncol, 2006. **100**(2): p. 264-70.
54. Ormandy, C.J., *et al.*, *Cyclin D1, EMS1 and 11q13 amplification in breast cancer.* Breast Cancer Res Treat, 2003. **78**(3): p. 323-35.
55. Begum, A., *et al.*, *Identification of PAK4 as a putative target gene for amplification within 19q13.12-q13.2 in oral squamous-cell carcinoma.* Cancer Sci, 2009. **100**(10): p. 1908-16.
56. Radu, M., *et al.*, *Pak Signaling in the Development and Progression of Cancer.* Nature reviews. Cancer, 2014. **14**(1): p. 13-25.
57. Whale, A.D., *et al.*, *PAK4 kinase activity and somatic mutation promote carcinoma cell motility and influence inhibitor sensitivity.* Oncogene, 2013. **32**(16): p. 2114-20.
58. Parsons, D.W., *et al.*, *Colorectal cancer: mutations in a signalling pathway.* Nature, 2005. **436**(7052): p. 792.
59. Huang, Z., J.A. Traugh, and J.M. Bishop, *Negative Control of the Myc Protein by the Stress-Responsive Kinase Pak2.* Molecular and Cellular Biology, 2004. **24**(4): p. 1582-1594.

60. Jakobi, R., *et al.*, *Molecular cloning and sequencing of the cytostatic G protein-activated protein kinase PAK I.* J Biol Chem, 1996. **271**(11): p. 6206-11.
61. Huang, Z., J. Ling, and J.A. Traugh, *Localization of p21-activated protein kinase gamma-PAK/Pak2 in the endoplasmic reticulum is required for induction of cytostasis.* J Biol Chem, 2003. **278**(15): p. 13101-9.
62. Balasenthil, S., *et al.*, *p21-activated kinase-1 signaling mediates cyclin D1 expression in mammary epithelial and cancer cells.* J Biol Chem, 2004. **279**(2): p. 1422-8.
63. Wang, Z., *et al.*, *p21-Activated Kinase 1 (PAK1) Can Promote ERK Activation in a Kinase-independent Manner.* The Journal of Biological Chemistry, 2013. **288**(27): p. 20093-20099.
64. Wang, Z., *et al.*, *Rac1 is crucial for Ras-dependent skin tumor formation by controlling Pak1-Mek-Erk hyperactivation and hyperproliferation in vivo.* Oncogene, 2010. **29**(23): p. 3362-73.
65. Higuchi, M., *et al.*, *Scaffolding function of PAK in the PDK1-Akt pathway.* Nat Cell Biol, 2008. **10**(11): p. 1356-64.
66. Arias-Romero, L.E., *et al.*, *A Rac-Pak signaling pathway is essential for ErbB2-mediated transformation of human breast epithelial cancer cells.* Oncogene, 2010. **29**(43): p. 5839-49.
67. Dummler, B., *et al.*, *Pak protein kinases and their role in cancer.* Cancer Metastasis Rev, 2009. **28**(1-2): p. 51-63.
68. Wang, R.A., *et al.*, *PAK1 hyperactivation is sufficient for mammary gland tumor formation.* Oncogene, 2006. **25**(20): p. 2931-6.
69. Arias-Romero, L.E., *et al.*, *Pak1 kinase links ErbB2 to beta-catenin in transformation of breast epithelial cells.* Cancer Res, 2013. **73**(12): p. 3671-82.
70. He, H., *et al.*, *P-21 activated kinase 1 knockdown inhibits beta-catenin signalling and blocks colorectal cancer growth.* Cancer Lett, 2012. **317**(1): p. 65-71.

71. Hook, S.S. and A.R. Means, *Ca(2+)/CaM-dependent kinases: from activation to function.* Annu Rev Pharmacol Toxicol, 2001. **41**: p. 471-505.
72. Hoelz, A., A.C. Nairn, and J. Kuriyan, *Crystal structure of a tetradecameric assembly of the association domain of Ca2+/calmodulin-dependent kinase II.* Mol Cell, 2003. **11**(5): p. 1241-51.
73. Wang, Y.Y., R. Zhao, and H. Zhe, *The emerging role of CaMKII in cancer.* Oncotarget, 2015. **6**(14): p. 11725-34.
74. Pellicena, P. and H. Schulman, *CaMKII inhibitors: from research tools to therapeutic agents.* Front Pharmacol, 2014. **5**: p. 21.
75. Swaminathan, P.D. and M.E. Anderson, *CaMKII inhibition: breaking the cycle of electrical storm?* Circulation, 2011. **123**(20): p. 2183-6.
76. Erickson, J.R., *Mechanisms of CaMKII Activation in the Heart.* Front Pharmacol, 2014. **5**: p. 59.
77. Erickson, J.R., *et al.*, *A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation.* Cell, 2008. **133**(3): p. 462-474.
78. Erickson, J.R., *et al.*, *Diabetic hyperglycaemia activates CaMKII and arrhythmias by O-linked glycosylation.* Nature, 2013. **502**(7471): p. 372-376.
79. Hanson, P.I., *et al.*, *Expression of a multifunctional Ca2+/calmodulin-dependent protein kinase and mutational analysis of its autoregulation.* Neuron, 1989. **3**(1): p. 59-70.
80. Liang, D., *et al.*, *BAFF activates Erk1/2 promoting cell proliferation and survival by Ca2+-CaMKII-dependent inhibition of PP2A in normal and neoplastic B-lymphoid cells.* Biochem Pharmacol, 2014. **87**(2): p. 332-43.
81. Si, J. and S.J. Collins, *Activated Ca2+/calmodulin-dependent protein kinase IIgamma is a critical regulator of myeloid leukemia cell proliferation.* Cancer Res, 2008. **68**(10): p. 3733-42.
82. Hoffman, A., *et al.*, *Dephosphorylation of CaMKII at T253 controls the metaphase-anaphase transition.* Cell Signal, 2014. **26**(4): p. 748-56.
83. Cohen, M.B. and O.W. Rokhlin, *Mechanisms of prostate cancer cell survival after inhibition of AR expression.* J Cell Biochem, 2009. **106**(3): p. 363-71.

84. Villarreal-Garza, C., *et al.*, *Breast cancer in young women in Latin America: an unmet, growing burden.* Oncologist, 2013. **18**(12): p. 1298-306.
85. Holm, C., *et al.*, *Association between Pak1 expression and subcellular localization and tamoxifen resistance in breast cancer patients.* J Natl Cancer Inst, 2006. **98**(10): p. 671-80.
86. Tharakan, R., *et al.*, *Phosphorylation of estrogen receptor alpha, serine residue 305 enhances activity.* Mol Cell Endocrinol, 2008. **295**(1-2): p. 70-8.
87. Rayala, S.K. and R. Kumar, *Sliding p21-activated kinase 1 to nucleus impacts tamoxifen sensitivity.* Biomed Pharmacother, 2007. **61**(7): p. 408-11.
88. Roig, J., *et al.*, *Functional interaction between c-Abl and the p21-activated protein kinase gamma-PAK.* Proc Natl Acad Sci U S A, 2000. **97**(26): p. 14346-51.
89. Stam , K., *et al.*, *Evidence of a New Chimeric bcr/c-abl mRNA in Patients with Chronic Myelocytic Leukemia and the Philadelphia Chromosome.* New England Journal of Medicine, 1985. **313**(23): p. 1429-1433.
90. Greuber, E.K., *et al.*, *Role of ABL family kinases in cancer: from leukaemia to solid tumours.* Nat Rev Cancer, 2013. **13**(8): p. 559-71.
91. Srinivasan, D. and R. Plattner, *Activation of Abl tyrosine kinases promotes invasion of aggressive breast cancer cells.* Cancer Res, 2006. **66**(11): p. 5648-55.
92. Britschgi, A., *et al.*, *Calcium-activated chloride channel ANO1 promotes breast cancer progression by activating EGFR and CAMK signaling.* Proc Natl Acad Sci U S A, 2013. **110**(11): p. E1026-34.
93. Xue, Y., *et al.*, *GPS 2.0, a Tool to Predict Kinase-specific Phosphorylation Sites in Hierarchy.* Molecular & Cellular Proteomics : MCP, 2008. **7**(9): p. 1598-1608.
94. Sells, M.A., *et al.*, *Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells.* Curr Biol, 1997. **7**(3): p. 202-10.
95. Martick, M., *et al.*, *A discontinuous hammerhead ribozyme embedded in a mammalian messenger RNA.* Nature, 2008. **454**(7206): p. 899-902.

96. Lowry, O.H., *et al.*, *Protein measurement with the Folin phenol reagent.* J Biol Chem, 1951. **193**(1): p. 265-75.
97. Harlow, E. and D. Lane, *Immunoprecipitation: purifying the immune complexes.* CSH Protoc, 2006. **2006**(4).
98. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
99. Towbin, H., T. Staehelin, and J. Gordon, *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979.* Biotechnology, 1992. **24**: p. 145-9.
100. Frangioni, J.V. and B.G. Neel, *Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins.* Anal Biochem, 1993. **210**(1): p. 179-87.
101. Ye, D.Z., *et al.*, *p21-Activated kinase 1 (Pak1) phosphorylates BAD directly at serine 111 in vitro and indirectly through Raf-1 at serine 112.* PLoS One, 2011. **6**(11): p. e27637.
102. Jan, C.I., *et al.*, *Tid1, CHIP and ErbB2 interactions and their prognostic implications for breast cancer patients.* J Pathol, 2011. **225**(3): p. 424-37.
103. Colbran, R.J., *Inactivation of Ca2+/calmodulin-dependent protein kinase II by basal autophosphorylation.* J Biol Chem, 1993. **268**(10): p. 7163-70.

APPENDIX A. LIST OF ANTIBODIES

Antibody	Primary antibody dilution	Secundary antibody dilution
Anti-myc (Cellsignal) #2276	1:30,000	Anti-mouse 1:10,000
Anti-Pak1 (Cellsignal) #2602	1:1,000	Anti-rabbit 1:10,000
Anti-GST (Cellsignal) #2622	1:1,000	Anti-rabbit 1:10,000
Anti-CaMKII (Abcam) ab52476	1:1,000	Anti-rabbit 1:20,000
Anti-phospho threonines (Zymed)	1:500	Anti-rabbit 1:10,000
Anti-phospho serines (Zymed)	1:500	Anti-mouse 1:10,000