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Protein Kinase C as an Apoptosis Regulator and a Potential Prognostic Factor in Breast Cancer

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LUND UNIVERSITY
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Abstract

Several protein kinase C (PKC) isoforms have been suggested as potential targets for breast cancer therapy. To explore the role for PKC isoforms in processes that facilitate malignant progression and the utility of PKC isoforms as biomarkers in breast cancer, we designed a study where we evaluated the expression of PKCα, PKCδ, and PKCε in primary breast cancers as well as in breast cancer cell lines. We found that PKCα levels correlated to estrogen and progesterone receptor negativity, tumor grade, and proliferative activity, whereas PKCδ and PKCε did not correlate to any clinicopathological parameters investigated. Moreover, patients with PKCα-positive tumors showed poorer survival than patients with PKCα-negative tumors independently of other factors. We also observed that PKCα favored proliferation and migration in cultured breast cancer cells. Since mechanisms that mediate apoptosis resistance are attractive therapeutic targets for cancer, we also investigated the involvement of PKC isoforms in survival and apoptosis of breast cancer cells. Protein kinase Cδ is considered to be a pro-apoptotic factor in many cell types. In breast cancer, however, it has shown both pro-survival and pro-apoptotic effects. We have found that down-regulation of PKCδ per se leads to apoptosis of MDA-MB-231 cells, a breast cancer cell lines with constitutive activation of the ERK1/2 pathway due to activation mutations of Ras and Raf. The apoptosis induced by PKCδ silencing was found to be accompanied by further increased MEK1/2 and ERK1/2 phosphorylation as well as reduced levels of the ERK1/2 phosphatase MKP3. These results suggest that PKCδ favors survival by suppressing the ERK1/2 pathway upstream and downstream of ERK1/2.

We also show that PKCδ is involved in other aspects of apoptosis regulation since it interacts with Smac in breast cancer cells. The interaction is mediated via the C1b domain in the regulatory domain of PKCδ and depends on the N-terminus of Smac. Treatment with lethal triggers such as paclitaxel cause dissociation of the interaction, which is accompanied with release of Smac from the mitochondria. Activation of PKC by phorbol esters rescues the interaction during paclitaxel exposure concomitant with a suppression of cell death. Taken together, we have identified a previously unrecognized interaction and suggest that the PKCδ-Smac association may prevent apoptotic effects of Smac.

Key words: Protein kinase C, breast cancer, prognostic marker, apoptosis, Smac, ERK1/2, MKP3

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For my Family

”We don’t know a millionth of a per cent about anything”
-Thomas A. Edison

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This thesis is based on the following papers, referred to in the text by their respective Roman numerals.

Paper I

Lønne, GK., Masoumi, KC., Lennartsson, J., Larsson, C.
PKCδ supports survival of MDA-MB-231 breast cancer cells by suppressing the ERK1/2 pathway.
J. Biol. Chem. 2009; ***284****:33456-33465*

Paper II

Lønne, GK., Cornmark, L., Zahirovic, IO., Landberg, G., Jirström, K., Larsson, C.
PKCα expression is a marker for breast cancer aggressiveness.
Mol. Cancer 2010; ***9****:76*

Paper III

Masoumi, KC., **Lønne, GK**., Cornmark, L., Hellman, U., Larsson, C.
Identification of a novel PKCδ-SMAC complex that is dissociated during apoptosis.
Submitted for publication

Publication not Included in the Thesis

Lønne, GK., Larsson, C. In: *Protein Kinase C in Cancer Signaling and Therapy.* Introduction: PKC isozymes in the control of cell function. *Kazanietz, M. G. Ed., Springer 2010; p107-115*.

Abbreviations

Ala	alanine
AP-1	activating protein-1
Apaf-1	apoptosis protease-activating factor-1
aPKC	atypical PKC
Arg	arginine
Asp	aspartate
ATP	adenosine-5'-triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2–associated X protein
Bcl-2	B cell CLL/lymphoma-2
BCSS	breast cancer-specific survival
Bid	BH3-interacting domain death agonist
Bik	Bcl-2-interacting killer
BIR	baculovirus IAP repeat
BH	Bcl-2 homology
Bmf	Bcl-2-modifying factor
bp	basepair
C1-C4	conserved regions 1-4
CD	catalytic domain
CDC42	cell division control protein 42
cFLIP	cellular FLICE-like inhibitory protein
cIAP	cellular inhibitor of apoptosis
cPKC	classical PKC
C-terminal	carboxy-terminal
Cys	cysteine
DAG	diacylglycerol
DD	death domain
DED	death effector domain
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
DUSP	dual-specificity phosphatase
FADD	Fas-associated death domain
EGF	epidermal growth factor
EGFR	EGF receptor
ER	estrogen receptor
ERK	extracellular-signal-regulated kinase
Glu	glutamate
Grb2	growth factor receptor bound protein 2
GDP	guanosine-5'-diphosphate
G-phase	growth phase
GTP	guanosine-5'-triphosphate
HER2	human epidermal growth factor receptor 2
His	histidine
HSP	heat shock protein
Hrk	activator of apoptosis harakiri
IBM	IAP binding motif

ILE	isoleucine
JNK	c-Jun N-terminal kinase
Lys	lysine
MAPK	mitogen-activated protein kinase
MCL-1	induced myeloid leukemia cell differentiation protein
MDR1	multidrug resitance 1
MEK	MAPK/ERK kinase
MKP	MAPK phosphatase
MOMP	mitochondrial outer membrane permeabilization
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
mTORC2	mTOR complex 2
NAIP	neuronal apoptosis inhibitory protein
NF-κB	nuclear factor-κB
NHG	Nottingham histological grade
NLS	nucleus localization signal
Noxa	latin for *damage*
nPKC	novel PKC
N-terminal	amino-terminal
Par6	partitioning defective 6
PB1	Phox Bem1
PDGFR	Platelet-derived growth factor receptor
PDK-1	phosphoinositide-dependent kinase-1
P-glycoprotein	permeability glycoprotein
Phe	phenylalanine
PHLPP	pleckstrin homology domain and leucine rich repeat protein phosphatase
PI3K	phosphoinositide-3 kinase
$PI(3,4,5)P_3$	phosphatidylinositol 3,4,5-triphosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
PR	progesterone receptor
Pro	proline
PS	phosphatidylserine
Puma	p53 up-regulated modulator of apoptosis
RACK	receptor for activated C kinase
RasGRP	Ras guanyl nucleotide-releasing protein
RINCK	RING-finger protein that interacts with C kinase
RING	really interesting new gene
RIP	receptor-interacting protein
ROCK-1	Rho-associated, coiled-coil containing protein kinase 1
RTK	receptor tyrosine kinase
Ser	serine
siRNA	small interfering RNA
Smac	second mitochondria-derived activator of caspases
Sos	son of sevenless
S-phase	synthesis phase
Thr	threonine
TMA	tissue microarray
TNF	tumor necrosis factor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis inducing ligand
Tyr	tyrosine
V1-V5	variable regions 1-5
Val	valine
VGFR	vascular growth factor receptor
wt	wild-type
XIAP	X-chromosome-linked inhibitor of apoptosis

Cancer

Introduction to Tumor Progression

Transformation of normal cells to malignant cells is thought to depend on certain alterations in cell physiology including self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, and resistance to programmed cell death. As cancer is considered as a disease of genes, genetic alterations such as mutations, amplifications, deletions, and epigenetic events are thought to be responsible for the physiological changes that eventually lead to tumor progression[1,2]. Genomic aberrations that drive the malignant progression are associated with activation (*ie.* gain of function) of oncogenes and inactivation (*ie.* loss of function) of tumor suppressor genes. Whereas oncogene activation occurs if a single allele is genetically altered, both alleles of tumor suppressor genes must be lost, mutated, or epigenetically altered for the phenotype to be apparent[3,4]. In normal cells, sporadically occurring mutations are corrected by different DNA repair mechanisms. However, the DNA repair machinery occasionally bypasses mutations, which in some cases can alter the function of the gene product. Mutations resulting in growth advantage over adjacent normal cells can cause clonal expansion of the cell and further genetic and epigenetic changes, eventually causing tumor formation. This theory of cancer propagation is referred to as the clonal evolution model and is based on the assumption that cancer originate from a single cell that has acquired multiple genetic aberrations[1]. Human solid tumors frequently have a pronounced heterogeneity of both neoplastic and normal cells. According to the clonal evolution model, heterogeneity within a tumor arises from additional genetic alterations affecting the offspring of the cell of origin. Moreover, in this model, resistance to therapy is believed to arise from natural selection by clonal evolution[5,6].

The clonal evolution model has recently been challenged by the cancer stem cell hypothesis, which proposes that only a specific subset of tumor cells that has or has acquired stem cell properties are capable of driving tumorigenesis. The cancer stem cells (also known as tumor-initiating cells) are thought to arise from normal stem cells or progenitor cells that have acquired stem cell-like characters. These cells have the ability to self-renew indefinitely and differentiate, supporting the idea that the tumor stem cells are responsible for tumor maintenance and metastatic spread[7-9]. According to this model, tumor heterogeneity is achieved by the potential of a cancer stem cell to give rise to different cancer cell types within a tumor, much like organ-specific stem

cells that differentiate into various cell types within the respective tissue[10]. The cancer stem cells have also been suggested to play a critical role in treatment failure and tumor recurrence, conceivably due to intrinsic drug resistance in the cancer stem cell pool[11]. The cancer stem cell and the clonal evolution hypotheses have more differences than similarities. For further reading, see REF 12, which compares these two hypotheses describing the origin of cancer.

Breast Cancer

Breast cancer is the most common malignancy in women and one of the leading causes of female cancer-related death[13]. The majority of breast cancers develop sporadically, and risk factors include age, hormonal exposure, and lifestyle. However, approximately 5-10% of all breast cancers are considered to be hereditary, mainly through mutation of the tumor suppressor genes *BRCA1* or *BRCA2*[14]. The majority of breast cancers are thought to arise from luminal epithelial cells in the terminal duct lobular unit; hence, the histological subtyping into ductal and lobular breast carcinomas reflects differences in cell morphology rather than the site of origin[15]. The progression from normal breast tissue to metastic cancer is believed to start with the development of non-invasive carcinoma *in situ*, where the cancer cells are still separated from the stroma by a continuous layer of organized myoepithelial cells and the basement membrane. Further progression into invasive carcinoma and finally metastatic cancer is accomplished by disruption of the myoepithelial cell layer and the basement membrane and eventually invasion into the surrounding tissue[16].

The specific histological types of invasive breast cancers are determined on the basis of growth pattern. Invasive ductal carcinomas and invasive lobular carcinomas are the most common subtypes, accounting for approximately 75% and 15% of all breast cancers, respectively. The remaining subtypes include medullary, tubular, and mucinous cancers[17].

Prognostics

Established prognostic factors for breast cancer are tumor size, nodal status, distal metastases, histological grade, human epidermal growth factor receptor 2 (HER2) status, age, progesterone receptor (PR) status, and proliferation rate (Swedish Breast Cancer Group, 2010). The Nottingham histological grade (NHG) classification is based on the assessment of the tubular formation, nuclear atypia, and mitotic count. The sum of the scored parameters defines the aggressiveness of the tumor, where grade I, II, and III corresponds to well, moderately, and poorly differentiated breast cancers, respectively[18].

The TNM classification is a staging system used in Sweden for further determining the progression of the cancer, where primary tumor size (T), lymph node status (N),

and metastatic spread (M) are taken into account. These parameters are subdivided into different stages, which are finally combined to give an overall stage (Swedish Breast Cancer Group, 2010).

Since breast cancer is a heterogenous disease for which established clinicopathological parameters are not sufficient for prognostication and treatment prediction, gene expression profiling has been given much attention in the last years[19,20]. This information can be helpful in designing more tailored treatment protocols for individual patients, and several prognostic tests are now available[21].

Therapy

Treatment of primary breast cancer includes surgery, radiotherapy, chemotherapy, and endocrine therapy. Depending on the local extension of the tumor, surgical removal can be achieved by either mastectomy or by breast conserving surgery. In addition, sentinel node dissection is performed to examine if the cancer has spread to the lymph system, without causing severe side effects such as lymphedema[22]. Radiotherapy is used postoperatively in patients treated with breast conserving surgery and is associated with reduced risk of recurrence and increased patient survival[23]. Treatment-predictive factors for systemic therapies include HER2 and estrogen receptor (ER) status, which predict the sensitivity of the tumor cells to the HER2 antibody Trastuzumab (Herceptin) and endocrine treatment, respectively (Swedish Breast Cancer Group, 2010). Adjuvant systemic endocrine therapy and/or chemotherapy is given to breast cancer patients to further minimize the risk of relapse[24,25]. Chemotherapeutic agents affect the cell cycle machinery and are toxic to healthy cells as well as to cancer cells[25,26]. Endocrine therapy is based on inhibiting the estrogen effect, either by blocking ERα or by inhibiting estrogen synthesis[24,27].

Signaling Pathways in Cancer

Cellular processes driving cancer progression are regulated by a large number of extra- and intracellular signaling pathways. A comprehensive review of all, or even most, signaling pathways and cellular processes regulating cancer cell behavior is far beyond the scope of this thesis. However, those that are of particular importance to the present investigation is discussed in this and the following sections.

Protein Kinase C

The major focus of this work has been to investigate the role of kinase C (PKC) isoforms in different breast cancer cell processes. PKC was first discovered by Nishizuka and colleagues in 1977[28,29] and is a family of Ser/Thr kinases comprises at least ten members. Some years later, Nishizuka's group showed that tumor promoting phorbol esters activate PKC[30,31], which initiated extensive investigations on the involvement of PKC in signal transduction and oncogenesis. However, the large number of PKC family members and their generally overlapping substrate specificities have made it difficult to point out individual functions of the different isoforms. Although redundancy has been suggested, increasing evidence supports isoform-specific functions of PKC. A myriad of studies have revealed that the functions of PKC are cell type and isoform-specific and include regulation of cell proliferation, migration, differentiation, and cell death[32], processes that may have opposite effects on cancer progression. Except for a rare mutation in PKCα (Asp294Glu) found in some cases of pituitary and thyroid tumors, causing impaired membrane binding of PKCα[33-35], mutations of PKC have not been observed. De-regulation of PKC in cancer is thus a consequence of de-regulation of proteins upstream of PKC, causing changes in expression levels, post-translational modifications, and spatiotemporal localizations. An increased knowledge on how PKC isoforms regulate carcinogenesis in specific cancers and cancer subtypes is of importance for the development of more cancer type-specific personalized therapies.

Structure and Function

All PKCs contain an N-terminal regulatory domain linked by a proteolytically labile

hinge region to a highly conserved C-terminal catalytic domain. These domains are further divided into four conserved regions (C1-C4) separated by five less homologous variable regions (V1-V5). The PKC family is divided into three groups based on the structure of the regulatory domain and thus regulation by second messengers[36] (Figure 1a). The classical PKCs (cPKCs; α, βI and the alternative splicing variant βII, and γ) and the novel PKCs (nPKCs; δ, ε, θ, and η) contain typical C1 domains and are thus activated by diacylglycerol (DAG)[36,37]. Typical C1 domains also bind phorbol esters[38], which are functional DAG analogs that are widely used in research to mimic DAG action[39]. Whereas cPKCs also bind Ca^{2+} due to possession of a C2 domain, the C2-like domain of nPKCs is Ca^{2+}-insensitive[36]. In addition, it was recently reported that the C2 domain of cPKCs also binds phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$)[40,41]. Atypical PKCs (aPKCs; ζ and PKCι/λ) respond neither to DAG nor to Ca^{2+}[42,43]. However, unlike nPKCs and cPKCs, aPKCs also possess a Phox Bem1 (PB1) domain and can therefore be allosterically activated by interaction with other PB1-containing proteins, including the PAR6-CDC42 complex, p62, and MEK5[44].

The Regulatory Domain

The Pseudosubstrate

In all PKCs, the regulatory domain possesses a pseudosubstrate sequence resembling a substrate except that it has an Ala instead of a Ser or Thr in the phosphoacceptor site. In inactive PKC enzymes, the pseudosubstrate binds the substrate binding cleft in the C4 domain keeping PKC in an auto-inhibited state and blocking substrate binding[46,47] (Figure 1b). Mutation of an Ala to Glu in the pseudosubstrate region of PKCα creates a protein with increased effector-independent kinase activity [48].

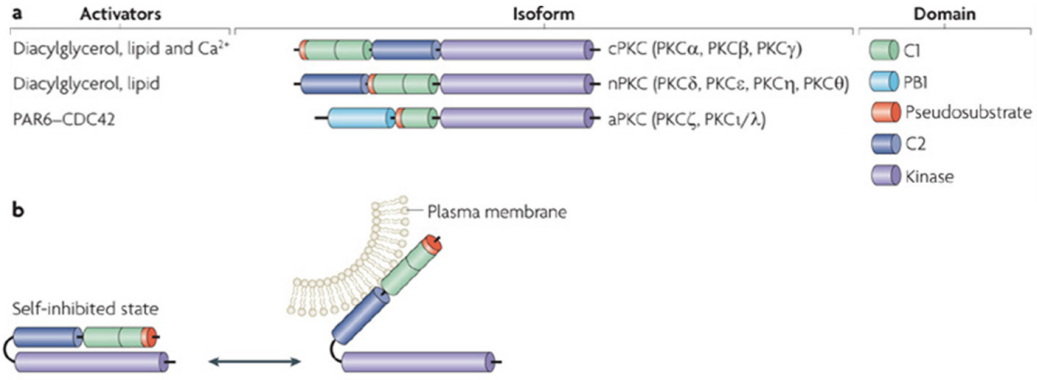

FIGURE 1. **A schematic overview of the protein kinase C family members.** **A**, The PKC family is divided into three groups according to their regulatory domains. These are the classical, novel, and atypical isoforms. **B**, Activation of PKC as exemplified by a classical PKC. Mature, inactive PKC is locket in a self-inhibited state through binding of the pseudosubstrate to the substrate-binding site. This inhibition is relieved upon binding of indicated activators, recruiting PKC to the plasma membrane and expelling the pseudosubstrate. Adapted by permission from Macmillian Publishers Ltd: Nature Reviews Molecular Cell Biology[45] © 2010.

Activation of PKC by tethering of the membrane-targeting modules provides energy to release the pseudosubstrate from the kinase core enabling substrate binding [49]. The pseudosubstrate, which is rich in basic amino acid residues, can when exposed participate in stabilizing the enzyme by interacting with the negative head groups of phospholipids in the membrane[50].

Membrane-Targeting Modules

The C1 domain is a conserved Cys-rich globular structure that possesses two β sheets and a C-terminal α helix, where the coordination of two Zn^{2+} ions, each by one His and three Cys, at the end of the β sheets is required for proper folding of the domain[51-53]. The β sheets form a hydrophilic ligand-binding pocket surrounded by hydrophobic residues. The positively charged residues of the C1 domain initially bind acidic phospholipids, such as phosphatidyl serine (PS) in the membrane. This creates a contiguous hydrophobic surface and positions the C1 domain for further penetration of the membrane bilayer and binding of DAG, which is located deeper in the membrane structure[54]. The electrostatic interactions between PKCs and negatively charged phospholipids in the membrane and the preference for PS over other lipids vary between the PKC isoforms and the individual C1 domains[55-58]. cPKCs and nPKCs contain tandem repeats of DAG-sensitive typical C1 domains; termed C1a and C1b[59]. aPKCs, on the other hand, contain only one atypical C1 domain (Figure 1a). It is the intrinsic structure of the atypical C1 domain that determines its insensitivity to DAG and phorbol esters, rather than the number of copies in aPKCs, since a single typical C1 domains can bind phorbol esters[60-62]. The N-terminal half of the atypical C1 domain contains several basic residues (Arg and Lys). By substituting four Arg in PKCζ to the corresponding residues in PKCδ-C1b, the atypical C1 domain converts to 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-sensitive[42]. Both typical and atypical C1 domains are found in other proteins as well as in PKC. Typical C1 domains are also found in other kinases such as protein kinase D and DAG kinases and in non-kinase proteins such as chimaerins, RasGRPs, and Munc13s, while atypical C1 domains are, in addition to aPKCs, found in several proteins, including DAG kinases, Vav1-3, and c-Raf[54,63]. Moreover, there is evidence for cross-talk between PKC and other C1 domain-containing proteins[64].

The plasma membrane is not the sole target of C1 domains. They can also locate to other organelles such as the Golgi apparatus, mitochondria, and nuclear membranes[65-68]. In contrast to transient activation of PKC at the plasma membrane due to transient DAG availability, DAG is sustained at the Golgi, resulting in sustained activation of PKC at this organelle[69]. The C1b domain of nPKCs has higher affinity for DAG than the C1b domain of cPKCs. Moreover, nPKCs are primarily recruited to the Golgi apparatus, in which the membrane is rich in DAG[69,70]. cPKCs, on the other hand, are mainly recruited to the plasma membrane, likely due its higher concentration of $PI(4,5)P_2$ and PS[71-73].

In addition to the DAG-binding C1 domain, the classical isoforms also contain a Ca^{2+}-binding C2 domain which facilitates membrane translocation by increasing the affinity of the C2 domain for negatively charged phospholipids such as PS[29] (Figure 1a). The C2 domain constitutes approximately 130 residues and comprises eight anti-parallel β strands connected by Ca^{2+}-binding loops that bind two or three Ca^{2+} ions via conserved Asp residues at the tip of the domain[74-76]. The positively charged Ca^{2+} ions then neutralize the negatively charged heads of PS and form bridges between PS and the C2 domain[77]. Neither nPKCs nor aPKCs respond to Ca^{2+}. The C2-like domain of the novel isoforms lacks the critical Ca^{2+}-coordinating Asp residues and is thus Ca^{2+}-insensitive. aPKCs, on the other hand, lacks the entire C2 domain[36]. The C2 domain is also a site for intra- and intermolecular protein-protein interactions. It functions as an interaction site for receptors for activated kinases (RACKs) and V5[78-82], which is discussed later.

The Catalytic Domain

The substrate binding site, the ATP binding site, and one of the priming phosphorylation sites, termed the activation loop, are located in the conserved regions (C3 and C4) of the catalytic domain[36]. The ATP-binding site is located in C3 and replacement of one invariant Lys by Arg leads to a catalytically inactive kinase[83]. The substrate-binding site is located in C4. The hydrophilic nature of the substrate-binding site is conceivably the reason why it has high affinity for basic peptides[84]. C4 also contain the activation loop[85] that is phosphorylated by phosphoinositide-dependent kinase-1 (PDK-1)[86-88], an AGC kinase family member possessing a pleckstrin homology domain with high affinity for $PI(3,4,5)P_3$[89]. Phosphorylation of the activation loop enables phosphorylation of the turn motif and the hydrophobic motif and thus maturation of PKC. In PKCδ, however, Glu500 is positioned close to the activation loop (Thr505) creating the negatively charge necessary for its catalytic activity. Hence, PKCδ does not need to be phosphorylated in the activation loop to become mature[90].

Variable Regions

Of the variable regions of PKCs, V3 and V5 have conferred crucial roles for regulation of PKC. V3 is a hinge region that is a target for proteolysis, protein-protein interactions, and Tyr phosphorylation. Proteolytic cleavage has been identified in several PKC isoforms in response to apoptotic stimuli and creates a constitutively active catalytic fragment due to its release from the auto-inhibitory regulatory domain[91]. Proteolytic cleavage of PKCζ and PKCδ has been shown to be involved in long-term memory and apoptosis, respectively[92,93]. Caspase-dependent proteolytic cleavage of PKCδ is discussed in the cell death section.

Two phosphorylation sites in PKC, termed the turn motif (generally Pro-flanked)

and the hydrophobic motif (flanked by hydrophobic Phe residues), are located in the V5 region[85]. These sites are sequentially phosphorylated after PDK1-dependent phosphorylation of the activation loop. aPKCs, however, are not phosphorylated in the hydrophobic motif, since instead of a Ser or Thr at this position, they have a Glu that mimics phosphorylation[90]. Both the turn motif and the hydrophobic motif have long been thought to be auto-phosphorylation sites. Recent studies, however, have shown that the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) is crucial for phosphorylation of the turn motif[94,95]. Moreover, the PKC enzymes are more unstable and prone to degradation in cells lacking mTORC2 due to unphosphorylated turn motif[94,96]. It is still not known whether mTORC2 mediates phosphorylation of the hydrophobic motif as well[94,97-100]. Noteworthy, mTORC2 is not able to phosphorylate PKC *in vitro*[94], and the mechanism by which it facilitates PKC-phosphorylation remains to be delineated. Novel and classical PKCs also bind HSP90 via a PXXP (where P is Pro and X is any amino acid) motif in V5, and this interaction facilitates the phosphorylation of the hydrophobic motif[101]. V5 is also the interaction site for PDK-1, which binds newly synthesized, unphosphorylated PKC in this region to catalyze the phosphorylation of the activation loop[86,102,103]. Yeong *et al* have shown that the very distal part of the V5 region of PKCα is necessary for activation loop phosphorylation and thus for activation of the enzyme. Truncation of the last ten amino acids renders the protein unphosphorylated and essentially inactive[104]. Similar results have also been reported for PKCε[105]. The V5 region can also interact with the C2 domain. Our group recently reported that acidic residues in V5 and a Lys-rich cluster in C2 contribute to a locked, DAG-insensitive conformation of PKCα[78]. Finally, V5 is the site for a nucleus localization signal (NLS) in PKCδ. In some cell types, nuclear import of PKCδ is required for apoptosis, and the NLS direct rapid import of PKCδ into the nucleus. Swapping the V5 of PKCε with PKCδ-V5 locates PKCε to the nucleus, a translocation that does not occur in *wt*PKCε under the same conditions[106].

Regulation

PKCs are regulated by phosphorylations that prime the protein for catalysis, by cofactor binding that allosterically activates the enzyme, and by interaction with other proteins targeting PKC to its regulators and substrates. Removal of second messengers or dephosphorylation and subsequent down-regulation of PKC terminates the PKC signaling.

Maturation and Activation

Newly synthesized PKC is associated to the membrane in an open conformation enabling PDK-1 to initiate the priming phosphorylations of PKC[86,102,103]. The maturation of PKC by phosphorylation of the activation loop, turn motif, and

hydrophobic motif is essential for the stability and the catalytic competence of the protein[107,108]. The traditional model of PKC activation is derived from studies of cPKCs that localizes in the cytosol as self-inhibited phosphorylated mature enzymes in resting cells. Phospholipase C-dependent hydrolysis of $PI(4,5)P_2$ generates membrane bound DAG and soluble inositol 1,4,5-trisphosphate[109] that promote Ca^{2+} release from the endoplasmatic reticulum. PKC binds Ca^{2+} via its C2 domain and translocates to the membrane[110,111] where it binds PS that increases the affinity of PKC for and enables binding of DAG[112,113]. The anchoring of PKC to the membrane causes conformational change that expels the auto-inhibitory pseudosubstrate from the substrate-binding site leading to activation of the protein (Figure 1b)[91,114,115].

For cPKCs, the C2 domain does not need to be fully engaged at the membrane if sufficiently tight binding is achieved by the C1 domain. Intracellular increase of Ca^{2+} is thus not essential for activation of cPKCs[36]. However, Mosior *et al* showed that DAG alone was not able to repel the pseudosubstrate from the substrate-binding site of cPKCs. Phorbol esters, on the other hand, have higher affinity for C1 domains, and activate mature cPKCs in the absence of Ca^{2+} [116].

Phosphorylation

In addition to the activation loop, turn motif, and hydrophobic motif, PKC is regulated by other more isoform-specific phosphorylations as well. Tyr phosphorylation of PKCs can regulate catalytical competence and PKC function. PKCδ is the most extensively Tyr-phosphorylated isoform, and several phosphorylation sites are found throughout the protein. Generally, phosphorylation of the Tyr residues close to the activation loop has been linked to regulation of catalytic activity. Phosphorylation of the Tyr residues located in the regulatory domain is though to be more important for regulation of gene expression and responses such as growth and apoptosis[91]. Tyr phosphorylation has been implicated in the regulation of PKCδ during apoptosis and in the PKCδ effect on apoptosis. See further discussion in the cell death section.

Anchoring

Due to the broadly overlapping substrate specificity of PKCs, mediation of unique PKC functions is governed by anchoring of PKCs to specific locations and in the vicinity of their substrates. Members of a well studied family of membrane-associated proteins, RACKs, act as scaffold proteins for activated PKCs and position them in close proximity to their substrates and allosterically activators[117,118]. Binding of RACK to PKC increases the catalytic activity of PKC several-fold[119,120]. Mochly-Rosen and colleagues have suggested that individual PKC isoforms have a specific RACK-binding sequence as well as a sequence mimicking RACK, termed pseudoRACK or ψRACK. The ψRACK can participate in intramolecular interactions that keep PKC in an inactive conformation[121]. To date, RACK proteins for PKCβII (RACK1) and PKCε

(RACK2 or β-COP) have been identified, and the RACK-binding sites of PKCβII, PKCδ, and PKCε are located in the C2 domain[79-81]. For PKCβII, an additional RACK-binding site is found in the V5 region[122]. The location of ψRACK, on the other hand, has although intensively studied not yet been identified. However, good candidates are found in C2 and in the variable regions [119,123-125]. The Mochly-Rosen group has, on the basis of this model, developed RACK-mimicking peptides that inhibit PKC translocation to RACK and ψRACK-mimicking peptides that compete with ψRACK. The ψRACK-mimicking peptides therefore break the intramolecular interactions between the RACK-binding sequences and ψRACKs, causing an active open conformation of PKCs and increase their binding to true RACKs[124,126,127].

In addition to RACK, there exist many other scaffold proteins for all conformations of PKC, from never-phosphorylated, mature but inactive, active, to dephosphorylated PKC.

Dephosphorylation and Degradation

PKC signaling is terminated by removal of second messengers. However, prolonged activation of PKC by *eg.* phorbol esters results in down-regulation of the protein, which has been used as a research tool to deplete cells for PKC. Since mature inactive PKCs are relatively resistant to dephosphorylation, the half-life of unstimulated PKCs is long (days for cPKCs in cultured cells). However, upon allosteric activation, which opens up the locked conformation, PKC becomes more sensitive to dephosphorylation. The Ser/Thr-specific phosphatase PHLPP initially dephosphorylates the hydrophobic motif, and the turn motif is subsequently dephosphorylated by an ocadaic-sensitive phosphatase[128,129]. The precise mechanisms for degradation still remain to be elucidated. However, an E3 ubiquitin ligase for PKC, termed RINCK, has already been identified[130].

It is also possible that dephosphorylated PKC can be rephosphorylated on the priming motifs by interacting with the HSP70 chaperone via the turn motif on PKC, and thereby prolong the signaling lifetime of PKC[131,132].

PKC and Breast Cancer

During mammary gland development, the expression levels of most PKC isoforms are high during pregnancy (α, δ, ε, η, ζ) and early involution (α, ε, η)[133-135]. These are periods of intense proliferation and massive apoptosis, respectively, indicating that PKC is involved in both of these processes that can facilitate cancer progression if de-regulated.

Several PKC members have been implicated in promoting malignant features of breast cancer cells. Most of the preclinical studies on the role of PKC in proliferation of breast cancer cells have been done utilizing MCF-7 cells. Over-expression of PKCα

in this cell line leads to increased proliferation and down-regulation of ERα[136]. We have recently shown that T47D cells, which hardly express PKCα under basal growth conditions, proliferate slower than other breast cancer cell lines expressing more PKCα, and that PKCα is crucial for proliferation under harsh conditions[137]. Clinical studies suggest that patients with PKCα-negative tumors respond better to endocrine treatment and have generally better prognosis compared to patients with tumors expressing PKCα[137-139]. On the contrary, PKCα levels are reduced in breast cancer compared to normal breast tissue[140,141]. Another cPKC associated with proliferation of breast cancer cells is PKCβ. Transfection with a constitutively activated PKCβI or II increases proliferation of MCF-7, MDA-MB-231, and BT474 breast cancer cells accompanied by enhanced transcription of cyclin D1 and c-Fos[142]. PKCα is also implicated in processes that facilitate metastasis. Mouse xenografts of MCF-7 cells over-expressing PKCα increased the number of metastasis compared to xenografts of control cells[136]. Moreover, we showed that down-regulation of PKCα reduced the ability of MCF-7 cells to migrate[137]. Regulation of cell motility by PKCα may involve regulation of integrin trafficking, since PKCα has been shown to interact with βI integrin in MCF-7 cells[143]. In line with this, the V3 region of PKCα interacts with βI integrin facilitating chemotaxis toward epidermal growth factor (EGF)[144]. Although PKCα seems to have pro-migratory and metastatic effects in breast cancer cell lines, no correlation between PKCα levels and metastasis has been shown in clinical studies[137].

The novel PKC isoforms PKCδ and PKCε are both considered to favor breast cancer progression. The expression levels of PKCδ were increased in highly metastatic mammary tumors compared to less metastatic parental cell lines[145,146]. This study also showed that over-expression of PKCδ in immortalized mammary epithelial cells induced anchorage-independent growth and enhanced cell survival. Moreover, it has been reported that PKCδ positively regulates proliferation of MCF-7 cells by mediating activation of ERK1/2[147]. PKCδ has also been implicated in positive regulation of invasion, since it mediates the platelet-effect on invasion of MCF-7 cells by facilitating MMP9 secretion[148]. Furthermore, PKCδ mediates invasion downstream of Akt in HER2 over-expression mammary epithelial cells[149]. PKCδ has also conferred pro-survival effects in breast cancer, however, this is discussed elsewhere.

PKCε has been assigned pro-survival effects in breast cancer as well as in many other cancer types. Down-regulation of this PKC isoform in MDA-MB-231 cells reduced the tumor growth and metastatic capacity in mice[150], in line with our recent results showing that PKCε silencing induces cell death in this cell line[151]. Importantly, PKCε has received much attention as an anti-apoptotic protein in breast cancer cells. Basu's group has reported that whereas over-expression of PKCε protects breast cancer cells against apoptosis, its down-regulation makes the cells more sensitive to lethal triggers[152-155].

Mitogen-Activated Protein Kinase Pathways

The mitogen-activated protein kinases (MAPKs) are a family of Ser/Thr kinases that utilize sequential kinase activation to amplify signals from the extracellular environment. The signaling cascades are initiated by stimuli that activate a G-protein, which in turn activates the Ser/Thr kinases, MAPK kinase kinases (MAPKKKs). MAPKKKs can then phosphorylate and activate MAPKKs. MAPKKs are dual specificity kinases, *i.e* they are capable of phosphorylating their substrates on Ser/Thr residues as well as on Tyr residues, and hence can activate the MAPKs by phosphorylating both a Tyr and a Ser in their activation loop. Activated MAPKs phosphorylate a number of substrates, including both cytoplasmic proteins and nuclear transcription factors. Therefore, the responses to MAPK activation include changes in gene transcription as well as alterations in protein activities. MAPK signaling is associated with diverse cellular responses, such as proliferation, differentiation, apoptosis, migration, and inflammation[156]. The most extensively characterized MAPK pathways are those resulting in activation of extracellular-signal-regulated kinase 1 and 2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK).

Stress-Activated MAPKs

Cancer cells are exposed to various stresses, such as hypoxia, detachment from substrate, inflammation, and metabolic stress that, in addition to cytokines, can activate the p38 (α, β, γ, δ) and JNK (1, 2, 3) pathways. The function of p38 and JNK depends on stimuli and cell type[157]. A major substrate of JNK is c-Jun, and JNK-mediated phosphorylation of c-Jun on Ser63 and Ser73 results in heterodimerization with c-Fos and thus enhanced transcriptional activity of this so-called activating protein-1 (AP-1). Moreover, JNK-mediated c-Jun phosphorylation is thought to be crucial for Ras-induced transformation. However, JNK is also associated with tumor suppressive effects by mechanisms such as phosphorylation and inactivation of anti-apoptotic Bcl-2 family members[158,159]. p38 is mainly linked to tumor suppressive effects, which has *eg.* been demonstrated by the increased oncogene-induced tumor formation in p38 knock-out mice. Furthermore, p38 negatively regulates cell cycle progression and is involved in the activation of p53 and p53-induced apoptosis[157].

The ERK1/2 Pathway

The ERK1/2 pathway is classically initiated by growth factors or mitogens binding to transmembrane receptor tyrosine kinases (RTKs) such as EGF receptors (EGFRs). Ligand binding causes dimerization and Tyr phosphorylation of the receptor and recruitment of the adaptor protein Grb2 and the guanine nucleotide exchange factor Sos. Sos in turn facilitates the exchange of GDP for GTP, promoting conformational change of and activation of the small GTPase, Ras[160], that is located on the cytosolic leaflet of cellular membranes[161]. GTP-bound Ras recruits Raf to the membrane,

enabling it to phosphorylate MEK1/2 on Ser218 and Ser222 in the activation loop. Activated MEK1/2 then phosphorylate ERK1/2 on Tyr185 followed by Thr183 in a conserved Thr-Glu-Tyr motif in the activation loop of its kinase domain[157]. Activated ERK1/2 phosphorylate a variety of different substrates on Ser or Thr residues that are followed by a Pro[162]. Examples of ERK1/2 substrates are the transcription factors c-Jun, c-Fos, Ets1/2, and Elk; which are all crucial for regulation of cell proliferation and oncogenic transformation[163,164].

Ras can also crosstalk with other signaling pathways. One example is the phosphoinositide-3 kinase (PI3K) pathway, in which Ras interacts with the catalytic domain of PI3K and thus recruits it to the membrane leading to conformational changes and activation of PI3K[165,166].

The ERK1/2 Pathway and Cancer

The ERK1/2 pathway is generally thought to promote cellular processes that favor cancer progression. For example, several early studies showed that antisense oligonucleotides and pharmacological inhibitors of this pathway attenuated proliferation of NIH3T3 cells[167-169]. Furthermore, ERK1/2 signaling has been linked to the cell cycle, since it regulates the G1- to S-phase transition, where it promotes cell cycle entry. However, too strong ERK1/2 signaling can cause reversible or permanent cell cycle arrest[170]. Another cancer promoting feature of ERK1/2 signaling is the facilitation of cell migration. Downstream targets of ERK1/2 such as myosine light chain kinase, calpain, focal adhesion kinase, and AP-1, which regulates the transcription of several metalloproteinases, are likely to be involved in ERK1/2-mediated cell migration and invasion[171,172]. The ERK1/2 pathway has also been implicated in survival and apoptosis regulation. The general view is that ERK1/2 positively regulate survival of cancer cells *eg*. by interfering with the Bcl-2 family proteins, where they exert their anti-apoptotic effect by mechanisms, such as prevention of BH3-only protein binding to anti-apoptotic Bcl-2 proteins, inhibition of *de novo* synthesis of anti-apoptotic Bcl-2 proteins, and phosphorylation of pro-apoptotic Bcl-2 proteins leading to degradation[173]. However, under certain conditions aberrant ERK1/2 activation can facilitate apoptosis induction. Cell death induced by DNA-damaging agents and other anti-tumorigenic compounds has been shown to require ERK1/2-dependent activation of the intrinsic apoptotic pathway. These conclusions are mainly drawn on the basis that the apoptosis-induction is reduced when cells are treated with the MEK inhibitors PD98059 or U0126[174].

The ERK1/2 pathway is de-regulated in approximately 1/3 of human cancers, and most of the abnormalities causing constitutively activated ERK1/2 occur at the early steps of the pathway. On the receptor level, over-expression of EGFR is seen in more than 50% of all carcinomas[157], and HER2 is amplified and over-expressed in 20-30% of breast cancers[175]. Mutation of the downstream protein Ras is also a common feature

of many cancers, and about 30% of all human cancers harbor mutant *RAS* genes[161]. *RAS* was among the first recognized oncogenes[176], and *RAS*-mutations have been found in codon 12, 13, and 61. Mutations of Ras prevent efficient GTP hydrolysis, rendering Ras in an active, GTP-bound state[161]. The Ras target B-Raf is also frequently mutated in cancer. The majority (63-66%) of melanomas possesses the most common mutation (90%) of B-Raf, Val600Glu (previous terminology Val599Glu), which means that the Val at this position is exchanged with a Glu, mimicking phosphorylation in the activation loop and causing constitutive activation of the kinase[177,178]. *BRAF* is considered to be an oncogene and the Val600Glu mutation is found in several other cancers as well[179].

Many approaches have been developed to block ERK1/2 signaling for the purpose of restraining cancer progression. Several inhibitors of RTKs upstream of Ras have been developed. Trastuzumab (Herceptin) is a humanized HER2 antibody that targets HER2 by binding to its ligand binding pocket, preventing receptor dimerization and, hence, blocks signaling[160]. Phase III trials have revealed this compound to be effective in treatment of metastatic breast cancers with amplified *ERBB2* (the gene that encodes HER2)[180], and thus Trastuzumab is now used in breast cancer therapy. In melanoma, B-Raf has received much attention as a target for therapy since the majority of these tumors have the Val600Glu-mutation of B-Raf, as previously mentioned. Treatment of melanoma cells with siRNA targeting B-Raf or the Raf-inhibitor sorafenib (BAY43-9006, Nexavar) reduces ERK1/2 activity and induces apoptosis of cultured cells and growth delay *in vivo*[181]. These findings argue for the development of compounds targeting B-Raf signaling in cancers possessing Val600Glu mutation, and sorafenib is now in clinical trials with promising results. However, monotherapy with this compound has little or no effect in patients with the Val600Glu mutation, hence, trials evaluating sorafenib combination therapies are ongoing[182]. Sorafenib is also considered to be a promising drug for other cancers as well, and it is already approved for the treatment of certain cases of renal cell carcinomas and hepatocellular carcinomas [183]. Noteworthy, sorafenib is a multikinase inhibitor and target VEGFR and PDGFR as well as Raf[184].

Although MEK1/2 are not oncogenes, constitutive activation provides them with transforming activities[185]. Furthermore, since MEK1/2 have central roles in the ERK1/2 pathway, they are believed to be promising targets for cancers with elevated activity of the ERK1/2 pathway. The most widely used MEK inhibitors in preclinical studies are PD98059 and U0126[169,186]. However, two other compounds, PC0325901 and ARRY-142886, are more ideal clinical candidates for cancer treatment in patients partially due to higher MEK1/2-selectivity (U0126 and PD98059 also inhibit MEK5), and clinical trials with these compounds are ongoing[187].

ERK1/2 Signaling in Breast Cancer

As mentioned above, 20-30% of human breast cancers over-express HER2, resulting

in enhanced activity of the ERK1/2 pathway and poor clinical outcome with reduced survival rates[175]. Moreover, the existence of tumor-initiating breast cancer cells was demonstrated by Clarke and colleagues in 2003[188], and these cells were found in HER2-induced mammary tumors and could be derived from cultured mammary cells following the activation of ERK1/2 pathway and EMT induction[189,190]. However, evidence points toward both pro- and anti-tumorigenic nature of ERK1/2. In clinical studies, elevated ERK1/2 levels and high ERK1/2 phosphorylation have been linked with both better[191-193] and poorer[194-196] prognosis. *In vitro* studies using breast cancer cell lines also show different functions of the ERK1/2 pathway. For example, the Raf-inhibitor, sorafenib, has been shown to attenuate MEK1/2 and ERK1/2 phosphorylation, angiogenesis, and tumor growth in xenografts of MDA-MB-231 breast cancer cells possessing mutated *KRAS* and *BRAF* (Glu463Val for B-Raf)[184], indicating that the constitutively activated ERK1/2 signaling pathway is vital for this cell line. However, ERK1/2 activation is also required for apoptosis induced by Resveratrol and Genistein in this cell line[197,198]. Moreover, combination-treatment with retinoic acid and PKC inhibitors (GF109203X or Rottlerin) or siRNA targeting PKCα or PKCδ induced cell death and increased ERK1/2 phosphorylation in MDA-MB-231 cells[199]. Noteworthy, studies with Rottlerin should be interpreted with caution, since Rottlerin has been reported to exert multiple effects in a PKCδ-independent manner[200]. We have shown that down-regulation of PKCδ *per se* enhances ERK1/2 phosphorylation and apoptosis in MDA-MB-231 cells[151]. Apoptosis induced by PKCδ silencing was abrogated by inhibition of MEK1/2 or Raf, suggesting that, when unrestrained, the ERK1/2 pathway has pro-apoptotic effects in this cell line. On the contrary, inhibition of ERK1/2 signaling in MCF-7 breast cancer cells (with lower basal ERK1/2 activity) slightly enhanced basal cell death, decreased TNFα-induced autophagy, and enhanced caspase activation and TNFα-sensitivity[151,201]. Finally, whereas transient activation of the ERK1/2 pathway resulted in increased proliferation, sustained ERK1/2 activation caused reduced cell growth of MCF-7 cells[202].

MAPK Phosphatases

There exist several possibilities for regulation of ERK1/2, including localization, upstream and downstream scaffolding, and inhibition of ERK1/2 signaling[203]. One way is to regulate its own activity by so-called positive or negative feedback loops. An example of negative feedback signaling is the ERK1/2-mediated up-regulation of the dual specificity phosphatases, MAPK phosphatases (MKPs, also known as DUSPs) that dephosphorylate both the phospho-Tyr and phospho-Thr in the activation loop of MAPKs, and thus inhibit their catalytic activity. MKPs possess an N-terminal MAPK binding domain and a C-terminal DUSP domain[204,205]. Binding of phosphorylated MAPKs to MKPs alters the conformation of the DUSP domain and greatly enhances its catalytic activity[206,207].

MAPK Phosphatases in Cancer

The MKPs are divided into three groups based on their sequence similarities, protein structures, substrate specificities, and subcellular localizations. The proteins in the first group, including MKP1, are encoded by highly inducible genes and are located in the nucleus. MKP1 is a broad spectrum MKP and dephosphorylates p38, JNK, as well as ERK1/2[204,205], and its expression is up-regulated by several cellular stresses including oxidative stress, DNA-damaging agents, and hypoxia[208,209]. Although the MKP1 gene is a transcriptional target of p53[210], indicating a tumor suppressive role of MKP1, it has mainly been associated with tumor promoting effects. For example, primary mouse embryonic fibroblasts derived from mice lacking MKP1 exhibit increased sensitivity to stress-induced caspase 3 cleavage and apoptosis due to hyperactivation of p38 and JNK[211]. Clinical studies have shown that MKP1 levels are increased in poorly differentiated and late stage breast cancers, and this is associated with decreased JNK activity[212,213]. In line with this, over-expression of MKP1 in MDA-MB-231 breast cancer cells reduced JNK levels as well as apoptosis induced by chemotherapeutic agents such as paclitaxel (Taxol)[214]. In glioma cells, phosphorylation of PKCδ on Tyr64 and Tyr187 induces ERK1/2 phosphorylation and apoptosis via down-regulation of MKP1[215]. Furthermore, PKCδ triggers proteasomal degradation of MKP1 during glutamate-induced cell death of neuronal cells[216]. These and other findings support the tumor-promoting effect of MKP1.

The next group includes MKP3 and consists of cytoplasmic MKPs that bind and selectively dephosphorylate ERK1/2[205]. MKP3 can be regulated by ERK1/2 themselves, as phosphorylation of MKP3 on Ser159 and Ser197 by ERK1/2 results in proteasomal degradation of MKP3[217]. It was recently shown that TPA-induced ERK1/2 activation resulted in increased MKP3 transcription in MCF-7 cells, and simultaneous treatment with a PKC inhibitor abolished the TPA-effect[202]. Furthermore, over-expression of MKP3 in ERα-positive breast cancer cells rendered them resistant to tamoxifen. Here, tamoxifen treatment reduced MKP3 activity and thus enhanced ERK1/2 signaling[218]. In line with a report showing that MKP3 was up-regulated in MCF10A breast epithelial cells stably expressing activated H-Ras[219], we found MKP3 to be exclusively expressed in MDA-MB-231 breast cancer cells harboring mutated *KRAS* and *BRAF*, compared with other breast cancer cell lines with lower basal ERK1/2 activity. Moreover, PKCδ silencing in MDA-MB-231 cells led to increased ERK1/2 phosphorylation and apoptosis partially due to decreased MKP3 levels. Finally, down-regulation of MKP3 *per se* slightly enhanced cell death[151], indicating a crucial role for MKP3 in restraining the constitutively activated ERK1/2 pathway in MDA-MB-231 breast cancer cells.

The final group consists of MKPs that are localized in both nucleus and cytoplasm and have selectivity toward p38 and JNK. These MKPs will not be discussed in this thesis, however, for reviews regarding these MKPs, see REFS 204, 205, and 220.

Cell Death

Cell death can be classified into apoptosis, necrosis, and autophagy-associated cell death which are distinguished by differences in mode of death and morphological, biochemical, and molecular characteristics[221]. The existence of genetically controlled programmed cell death was suggested in the 1960s[222] and mostly refers to apoptosis. The concept of programmed cell death also includes autophagy that is a survival mechanism in which the cells recycle their own organelles and macromolecules to generate energy and metabolites. However, unrestrained autophagy can result in cell death due to depletion of organelles and critical proteins[223-225]. Necrosis is usually considered a non-programmed form of cell death, with characteristics like swelling of the cell and its organelles and leakage of intracellular contents into the surroundings. Nevertheless, accumulating evidence suggests that necrosis can be induced in a programmed fashion as well as accidental[226,227], which is discussed in the following section.

Necroptosis

It was long thought that apoptosis was the sole form of programmed cell death. However, emerging evidence speaks for the possibility that necrosis can be induced in a controlled manner as well. This phenomenon is called necroptosis and is initiated by death receptors, such as tumor necrosis factor (TNF) receptor 1 (TNFR1). Furthermore, it generally requires receptor-interacting protein 1 (RIP1) and RIP3 kinase activities[228]. Necroptosis can be induced by activation of several death receptors. However, the most extensively studied necroptosis-triggering receptor is TNFR1. TNFα-binding can induce nuclear factor-κB (NFκB)-mediated survival, caspase 8-mediated apoptosis, or caspase-independent necroptosis, depending on the intracellular context. In the presence of cellular inhibitors of apoptosis (cIAPs), RIP1 is ubiquitylated on Lys63 and act as a scaffold for transforming growth factor-β activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2), and TAB3, which initiate the canonical NFκB pathway. In the absence of cIAPs, RIP1 and RIP3 are cleaved and inactivated by caspase 8, which induces the classical caspase cascade and eventually apoptosis. However, by deleting caspase 8 or blocking caspase 8 activity, RIP1 and RIP3 become phosphorylated and induce necroptosis[228-230]. Second mitochondria-derived activator of caspases (Smac) mimetics has been reported to drive TNFα

signaling toward induction of apoptosis or necroptosis, instead of NFκB activation, due to their ability to facilitate auto-degradation of cIAPs[231,232]. Moreover, necrostatin 1 and 3 has been identified as inhibitors of RIP1 activity, and therefore inhibits necroptosis[233,234]. Execution of necroptosis involves disintegration of mitochondrial, lysosomal, and plasma membranes[228]. Fully necrotic cells are mainly cleared by internalization by macrophages through macropinocytosis[235,236]. However, in some cases PS externalization occurs before plasma membrane permeabilization, facilitating phagocytic removal of the necrotic cells[237-239].

Apoptosis

In 1972 the phenomenon apoptosis was described by Kerr, Wyllie, and Currie as programmed cell death with characteristics including condensation of nucleus and cytoplasm, nuclear fragmentation, and membrane blebbing. Apoptosis is a crucial instrument of cellular homeostasis and enables organisms to remove damaged or potentially dangerous cells. The apoptotic cell death is a controlled mechanism and takes place in two discrete stages involving the formation of apoptotic bodies and thereafter phagocytosis and degradation by other cells[240]. Exposure of ligands, such as PS on the outer plasma membrane of apoptotic cells enables phagocytes and macrophages to recognize and engulf them[241]. The apoptotic cells can therefore be eliminated with their plasma membrane intact, which prevents the release of cellular content into the surroundings and thus minimizes the damage of neighboring cells[242]. This is in contrast to necrosis that often causes inflammation of the surrounding tissue due to release of proteins, so-called alarmins[243], that can trigger the immune response[244-246].

Since the discovery of apoptosis 38 years ago, the molecular mechanisms of apoptosis have been extensively studied. Three main signaling pathways have been described; the receptor-mediated extrinsic, the mitochondria-mediated intrinsic, and the granzyme pathway. These apoptotic pathways are all leading to activation of a group of proteins, called caspases, that execute the apoptotic process[242].

The Extrinsic Pathway

The extrinsic pathway is activated by binding of extracellular death ligands, such as FasL and TNFα, to their cognate transmembrane death receptors (Figure 2). The receptors themselves do not possess enzymatic activity. However, activated death receptors oligomerize and thereby form the death-inducing signaling complex (DISC) on their intracellular parts. This is accomplished by recruiting adaptor proteins via their death domains (DD) and subsequently initiator caspases, such as pro-caspase 8, via their death effector domain (DED) by so-called homotypic DD and DED interactions[247]. The formation of the DISC induces proximity of the bound pro-caspases, leading to

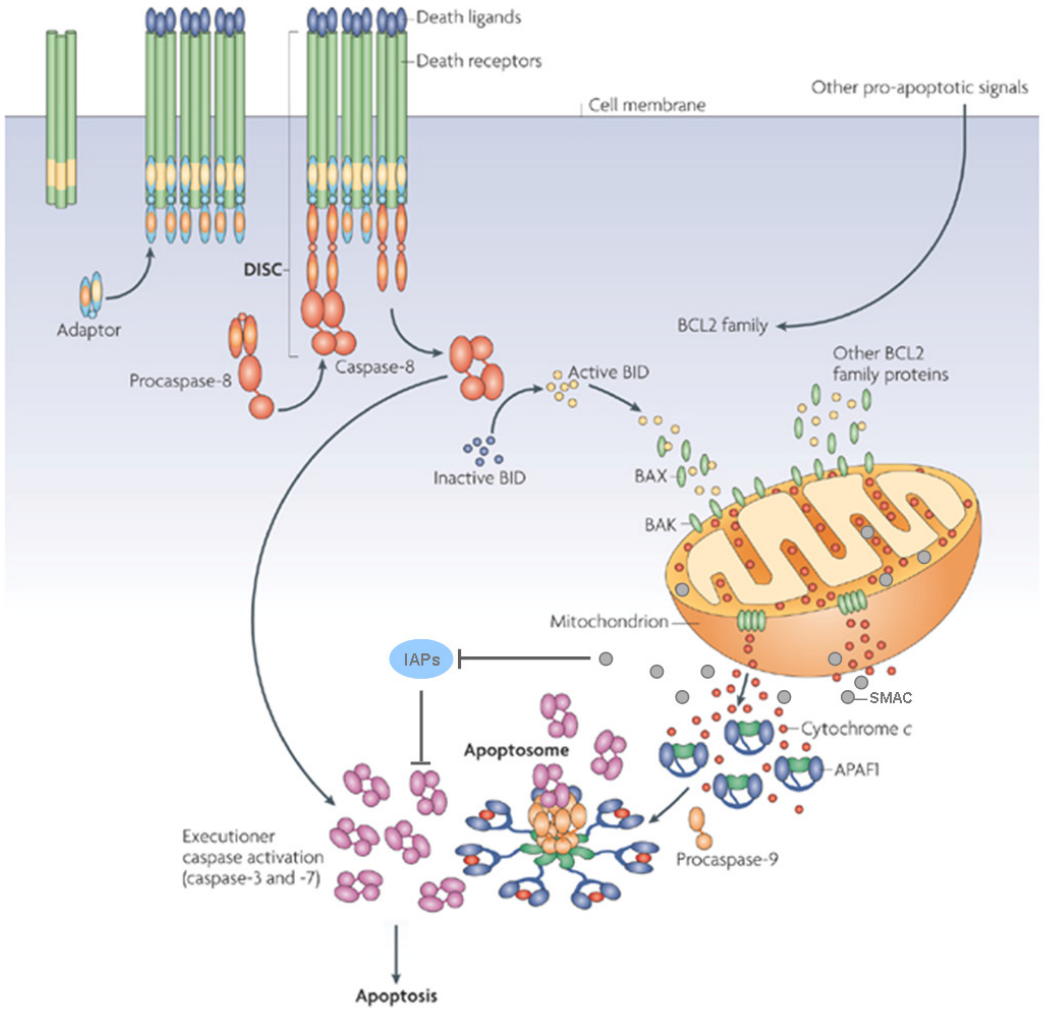

FIGURE 2. **Activation of the extrinsic and intrinsic apoptotic pathways.**
The extrinsic pathway is initiated upon binding of an extracellular death ligand that triggers oligomerization of its death receptor and assembly of the DISC. The intrinsic pathway is activated by stimuli that lead to mitochondrial outer membrane permeabilization, subsequent release of pro-apoptotic proteins from the mitochondrial intermembrane space, and eventually formation of the apoptosome in the cytosol. The DISC and the apoptosome activate their respective initiator caspases, which leads to activation of the executioner caspases and eventually apoptosis. Adapted by permission from Macmillian Publishers Ltd: Nature Reviews Molecular Cell Biology[254] © 2008.

auto-proteolysis, release of activated caspases into the cytosol, subsequent initiation of the apoptotic proteolytic cascade, and eventually cell death[248-250].

The connection between the extrinsic and the intrinsic apoptotic pathways has been recognized by the ability of caspase 8 to cleave the B cell CLL/lymphoma-2 (Bcl-2) family member Bid into its active truncated form, tBid. Translocation of tBid to the mitochondria facilitates loss of mitochondrial outer membrane permeabilization (MOMP) and thereby activation of the intrinsic pathway[251,252] (Figure 2).

The Intrinsic Pathway

Stresses such as DNA damage and cytokine deprivation activate the mitochondrial intrinsic pathway, leading to permeabilization of the outer mitochondrial membrane and thereby loss of mitochondrial membrane potential and release of proteins such as cytochrome *c*[253] from the intermembrane space of the mitochondria into the cytosol[254] (Figure 2). The loss of mitochondrial integrity is promoted or inhibited by many key regulators of apoptosis, substantiating the importance of the mitochondria in apoptosis induction. Once released into the cytosol, mitochondria-derived cytochrome *c* binds the WD40-repeat region on the apoptosis protease-activating factor-1 (Apaf-1) and thereby triggers the oligomerization of this protein into a caspase-activating platform termed the apoptosome. This in turn leads to recruitment of pro-caspase 9 via the caspase-recruitment domains on Apaf-1 and pro-caspase 9, resulting in activation of caspase-9 and subsequent induction of apoptosis[255,256].

The Bcl-2 Family

The survival role of the Bcl-2 was first demonstrated in 1988 by Vaux *et al*[257], and Bcl-2 was the first identified cell death regulator. It was soon revealed that this protein belonged to a family of proteins functionally categorized as either pro-apoptotic or anti-apoptotic. The members of the Bcl-2 family are structurally classified depending on their constitution of BH domains. The anti-apoptotic Bcl-2 proteins contain four BH domains (BH1-4) and include Bcl-2, Bcl-2w, A1, Bcl-xL, and MCL-1, which are generally integrated within the outer mitochondria membrane, maintaining the mitochondrial integrity by directly inhibiting the pro-apoptotic Bcl-2 proteins. The pro-apoptotic members of the Bcl-2 family are divided into the effector proteins (Bax and Bak), containing BH1-3, and the BH3-only proteins. All BH3-only proteins can inhibit anti-apoptotic Bcl-2 proteins. However, according to their ability to interact with the effectors, the BH3-only proteins are further divided into direct activators (Bid, Bim) and sensitizers/de-repressors (Bad, Bik, Bmf, Hrk, Noxa, and Puma)[258,259], where Bid and Bim can directly induce oligomerization of the effector proteins and subsequently MOMP[260-262]. The Bcl-2 family members regulate additional cell functions beyond apoptotic MOMP induction. These include autophagy[258] and the mitochondrial dynamics in healthy cells as well as in apoptotic cells[263,264]. Furthermore, the Bcl-2 family members are regulated by other proteins outside the Bcl-2 family, *eg.* by cytosolic p53, which has been shown to directly activate the effectors and thus mediate MOMP[265,266].

The Granzyme Pathway

Granzymes are a family of Ser proteases that have extraordinarily limited tissue specificity and are found in the cytosolic granules of activated cytotoxic T-lymphocytes and natural killer cells. In addition to granzymes, a major component of cytotoxic

granules is the pore-forming protein perforin. The combination of perforin and granzymes promotes rapid apoptotic cell death when delivered to the target cell. The most studied isoforms are granzymes A and B, where granzyme B is an Aspase that can trigger apoptosis directly by activating the executioner caspases 3 and 7 or via the intrinsic apoptotic pathway by activation of Bid. Granzyme A acts differently from granzyme B and induces cell death by targeting the nucleus, eventually resulting in single-strand DNA nicks and weakened structural integrity of the nucleus[267,268].

Caspases and Execution of Apoptosis

Caspases are a family of widely expressed homologous Cys proteases that cleave preferentially after Asp residues in the substrate. The cleavage of proteins by caspases is a process of limited proteolysis where a small number of cuts are made and cause activation or inactivation of the substrate protein[269,270]. Caspases involved in apoptosis include the initiator caspases 8, 10 (extrinsic pathway) and 9 (intrinsic pathway) and the executioner caspases 3, 6, and 7[271] (Figure 2). Initiator caspases occur as inactive monomers and require homodimerization for activation, which is facilitated by the activation platforms DISC (extrinsic pathway) or apoptosome (intrinsic pathway). The executioner caspases dimerize shortly following their synthesis, and reside as inactive zymogen dimers. Cleavage within the linker segment in the catalytic domain is required for activation[272-274]. Following activation, the caspases are further matured by cleavage (in the case of initiator caspases) and removal of the pro-domain[271].

Upon activation, executioner caspases cleave numerous cellular proteins[275] leading to fragmentation of the nucleus and cellular organelles. Caspase-dependent activation of ROCK-1, a regulator of the actin cytoskeleton dynamics, causes the membrane blebbing seen during early apoptosis[276]. Moreover, cleavage of the major constituents of the cytoskeleton; actin microfilaments, several microtubular proteins, and intermediate filaments[277] as well as activation of focal adhesion kinase[278], account for the rounding up and loss of substratum contact. Caspase-mediated cleavage of the lamins A, B, and C leads to fragmentation of the nucleus[279]. Caspase activation causes DNA hydrolysis creating numerous DNA fragments down to multiples of 50-200 bp[280], which is seen as a DNA ladder when separated by DNA agarose gel elecrophoresis. This is a well known tool for distinguishing between apoptosis and necrosis, since necrosis results in a smear of DNA fragments due to random DNA degradation[281]. Moreover, caspase activation is conceivably also responsible for releasing nucleotides from apoptotic cells serving as find-me signals for macrophages[282]. Finally, the caspases shut down the functions of many proteins involved in essential housekeeping functions of the cell by targeting the transcriptional and translational machineries[242]. Since proteolysis is an irreversible process, caspase activation is strictly regulated by mechanisms including caspase inhibition, decoy inhibition, and caspase degradation[271].

The caspases possess other functions in cellular responses beyond apoptosis. These

include cell migration, survival, proliferation, and differentiation and involve proteolytic activity and non-proteolytic functions of the caspases[283,284].

Smac, IAPs, and Other Regulators of Caspase Activity

As mentioned above, caspase activation is tightly regulated by mechanisms such as inhibition and degradation of the mature caspase protein. The inhibitors of apoptosis (IAPs) are a family of caspase inhibitors with the capacity of targeting caspases to proteasomal degradation or inhibit their enzymatic activity. Any protein containing a Baculovirus IAP repeat (BIR) motif is defined as member of the IAP family, which includes the human proteins X-chromosome-linked IAP (XIAP) and cIAP 1 and 2, ILP2, ML-IAP, NAIP, survivin, and BRUCE, where the most extensively studied isoforms are XIAP and cIAPs[285]. Most IAPs also contain a RING domain, which binds E2 ubiquitin conjugating enzymes, giving the IAPs E3 ubiquitin ligase properties that enables them to ubiquitylate their bound substrates as well as themselves[286]. The interaction between caspases and IAPs are mediated by the BIR motifs on IAPs[287] (Figure 3), and it was initially thought that the three BIRs of both XIAP and cIAP were sufficient and critical for the caspase inhibition[288,289]. Moreover, it was also shown that these IAPs specifically interact with and inhibit the initiatior caspase 9 via BIR3 and the effector caspases 3 and 7 via BIR2[288-290]. These observations indicate that XIAP and cIAPs can block the Apaf-1 mediated apoptosis as well as downstream effector caspases of all apoptotic pathways. However, more recent studies have demonstrated that although cIAPs can bind caspases, they are, unlike XIAP, weak inhibitors of caspases. This can probably be explained by the notion that cIAPs lack certain residues that are present in XIAP BIR motifs and are crucial for caspase inhibition[291,292]. Moreover, Salvesen and colleagues have predicted that the other mammalian IAPs are not caspase inhibitors, since caspase inhibition requires a BIR motif containing both of the caspase-binding elements found in XIAP and the IAP binding motif (IBM) interacting groove[287]. Nevertheless, survivin and BRUCE have been suggested to inhibit caspases through other mechanisms[293,294].

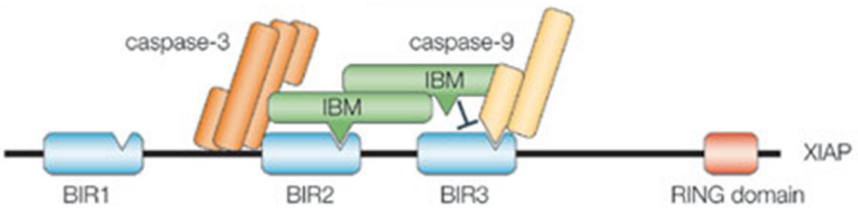

FIGURE 3. **A schematic model of the X-chromosome-linked IAP interactions.**
X-chromosome-linked IAP (XIAP) binds and inhibits processed caspase 3 and caspase 9 via its BIR2 and BIR3 domains, respectively. IAP binding motif (IBM)-containing proteins binds the IBM interaction groove within BIR2 and BIR3 and thereby compete with binding of the IBM of caspases. Adapted by permission from Macmillian Publishers Ltd: Nature Reviews Molecular Cell Biology[286] © 2005.

All IAP inhibitors contain a conserved IBM and antagonize the functions of IAPs by promoting IAP degradation or by directly binding BIR domains and thus displacing bound caspases[286]. An exception from this rule is the apoptosis-inducing factor that interacts with BIR2 of XIAP despite lacking an IBM[295]. Inhibitors of IAPs include the pro-apoptotic protein Smac[296-299], which is synthesized as a full-length precursor with the N-terminal residues serving as a mitochondrial signal sequence. Upon translocation of Smac to the mitochondrion, the mitochondrial signal sequence is cleaved off exposing the N-terminal tetrapeptide AVPI (Ala, Val, Pro, Ile) crucial for IAP-binding[297,300,301]. Following activation of the mitochondrial apoptotic pathway, mature Smac molecules are released into the cytosol, where they can inhibit IAPs by interacting with their BIR domains[298-300] (Figure 2 and 3).

Since IAPs are E3 ligases, they can facilitate ubiquitylation of Smac. However, although XIAP promotes ubiquitylation of Smac, only ubiquitylation by cIAPs results in degradation of Smac[302-304], indicating that the ligation of polyubiquitin mediated by cIAPs are linked via Lys48, while XIAP-mediated ubiquitylation is multi-monounbiquitylated or non-Lys48 ubiquitylated. Smac has also been shown to antagonize the E3 ligase activity of XIAP, without being ubiquitylated[305,306].

Another well-known caspase inhibitor is cFLIP, which is a DED-containing decoy inhibitor of the caspases 8. When highly expressed, cFLIP competes with caspase 8 for binding to the DISC and thereby inhibits the activation of caspase 8. In lower concentrations, however, cFLIP have shown pro-apoptotic properties, where it promotes caspase 8 activation, probably through heterodimerization between caspase 8 and cFLIP at the DISC[307].

Apoptosis in Cancer and Cancer Therapy

Being able to evade apoptosis is one of the fundamental hallmarks of cancer, as described by Hanahan and Weinberg in 2000[2]. De-regulation of pro- and anti-apoptotic proteins is a common feature of cancer cells, and among the best characterized abnormalities in the apoptotic machinery are enhanced expression of pro-survival Bcl-2 family members and mutation of the tumor-suppressor gene *TP53*, encoding p53, which initiates apoptosis in response to DNA damage[258,308,309]. Ionizing radiation and chemotherapy lead to cell death by induction of primarily the intrinsic apoptotic pathway. However, these therapies hardly discriminate between normal and cancer cells and thus also affect normal high proliferating cells[310]. Therefore, more specific therapeutic agents are needed to optimize tailored treatment strategies for individual patients. Our increasing knowledge on the apoptotic machinery is an important tool for the development of apoptosis-inducing agents for cancer treatment. As mentioned above, many cancer cells escape apoptosis by up-regulating anti-apoptotic Bcl-2 proteins. Therefore, much attention has been given to these proteins as targets for cancer therapy, and clinical trials of several compounds are ongoing[311]. During the last few years, the family of IAPs

and the IAP inhibitor Smac have been of increasing interest for potential therapeutic strategies. The structural information of the interaction between Smac and IAPs has been used for development of Smac mimetics, which are proteolytically stable small molecules mimicking the Smac AVPI-motif and thus target the BIR domains of IAPs[312]. A pro-apoptotic role for Smac mimetics has been revealed by preclinical profiling studies showing numerous examples where they potentiate cell death induction in the presence of lethal triggers such as TNFα and TNF-related apoptosis inducing ligang (TRAIL)[313-316], and is further highlighted by the finding that Smac mimetics suppress growth of cell line xenografts[315,317,318]. Recent studies have linked the effect of Smac mimetics to the TNFR1 and the noncanonical NFκB pathway leading to TNFα-dependent cell death. Smac mimetic-induced degradation of cIAPs leads to release of RIP1 from the TNFR1 complex and subsequent activation of the caspase 8 complex, as previously described in the necroptosis section. An alternative route for Smac mimetic-induced cell death is by activation of the noncanonical NFκB pathway due to stabilization of NIK as a result of cIAP loss[313,319]. Activation of the noncanonical NFκB pathway induced by Smac mimetics leads to transcription of its target genes, including TNFα, which in combination with XIAP silencing or inhibition, can facilitate cell death[231,313,318-320]. Nevertheless, two Smac mimetics have reached phase I clinical development, and several others are expected to enter human clinical testing in near future[321].

Other receptors transferring death stimuli are also under investigation for development of possible therapeutic compounds, and several death receptor agonists are currently in clinical trials for treatment of a number of cancers. For further reading see REF 322.

Protein Kinase C and Apoptosis

The members of the PKC family exert diverse effects on the apoptotic machinery, which are dependent on the PKC isoform and cell type. Unfortunately, early studies regarding PKC and apoptosis do not distinguish between individual PKC isoforms. This is due to usage of broad spectrum PKC activators and inhibitors that, at most, only discriminate between the subgroups of the PKC family. However, more isoform-specific approaches, such as siRNA, have been developed, adding more detailed information to the role of PKC in apoptosis regulation.

Classical PKCs

Classical PKCs have been connected with anti-apoptotic roles in the extrinsic apoptotic pathway, since they prevent Fas-mediated cell shrinkage[323], FasR aggregation, Fas-associated death domain (FADD) recruitment, DISC formation,[324,325], and TRAIL-induced apoptosis via death receptor 5 inhibition[326]. Moreover, a recent paper has shown that PKCα and PKCβ mediate phosphorylation of cFLIP on Ser193 which regulates its auto-ubiquitylation and therefore affects the intracellular levels of cFLIP[327]. The

most extensively studied classical PKC isoform, PKCα, has also been associated with the intrinsic pathway due to its ability to mediate expression and phosphorylation of Bcl-2, leading to increased anti-apoptotic function of this protein[328-330]. Moreover, PKCα also suppress drug-induced apoptosis by increasing MDR1 promoter activity and thus P-glycoprotein stability and expression[331,332]. These findings suggest a pro-survival role of PKCα in most cell systems. However, in some cell types, such as prostate cancer and renal cells, PKCα has conferred pro-apoptotic effects[333,334].

Novel PKCs

Of the novel PKC isoforms, PKCδ and PKCε are the best studied isoforms. These two isoforms have generally been assigned opposite apoptotic effects[335]. PKCε is considered to be anti-apoptotic, since its inhibition or silencing makes cancer cell lines more susceptible to apoptotic insults such as TRAIL[154,336,337]. Furthermore, over-expression or activation of PKCε protects against apoptosis by mechanisms including prevention of caspase 8 and Bid activation, down-regulation of Bid, phosphorylation and thus inhibition of Bad, and up-regulation of Bcl-2[154,338,339]. One survival signaling pathway of PKCε has been suggested to be conducted via the pro-survival protein Akt[338,340,341].

Protein Kinase C δ

PKCδ has long been thought to have tumor suppressor properties. One reason for this is the notion that the human PKCδ gene is located on chromosome 3p in a region that is characterized by loss of heterozygsity in many tumors[342]. Moreover, the pro-apoptotic effect of PKCδ has been extensively studied in many cell types, supporting an anti-tumorigenic nature of this PKC isoform. However, PKCδ affect apoptosis differently depending on cell type and stimulus, and the effect of PKCδ is usually associated with caspase-dependent cleavage, subcellular localization, and Tyr phosphorylation of PKCδ.

Proteolytic Cleavage

Proteolytic activation of PKCδ by caspases is directly linked with apoptosis, since it is cleaved in the V3 region during apoptosis and since transfection with its isolated cofactor-independent catalytic domain induces apoptosis[92,343]. A positive feedback loop has been suggested because PKCδ can mediate caspase 3 activation[344-346].

Localization

Subcellular localization of PKCδ apparently decides the outcome of the apoptotic stimulus, where nuclear and mitochondrial localizations of PKCδ are the strongest promoters of apoptosis. Reyland and colleagues have shown that PKCδ possesses a

nuclear localization signal (NLS), which is required for nuclear import of PKCδ and apoptosis of C5 rat parotid salivary acinar cells[106]. The same group also showed that nuclear accumulation of full length PKCδ was required for caspase 3-mediated PKCδ cleavage and occurred before nuclear localization of caspase 3 and cleavage of PKCδ. Moreover, forced localization of PKCδ to the nucleus increased apoptosis[347]. Brodie's group demonstrated that forced localization of PKCδ to both mitochondria and nucleus promoted apoptosis to a greater extent than cytosolic PKCδ, and depending on its localization, PKCδ affected different apoptotic signaling pathways[348]. PKCδ mediates phosphorylation of several nuclear proteins, such as Lamin B[349], which is a component of the nuclear lamina, and DNA-dependent protein kinase[350] and Rad9[351], which are involved in DNA repair. PKCδ also phosphorylates p53 on Ser46 to induce apoptosis[352]. In the mitochondria PKCδ can cause increase in MOMP and release of cytochrome *c*, via mechanisms such as facilitation of Bad dephosphorylation[353-356]. Mochly-Rosen's group showed that using a peptide derived from the PKCδ-binding site on Annexin V sequestered PKCδ due to increased Annexin V-binding. This led to the inhibition of mitochondria translocation of PKCδ and reduced apoptosis[357], which is in line with the abovementioned effects of mitochondrial PKCδ.

Tyrosine Phosphorylation

Tyr phosphorylation is another way of regulating the apoptotic effect of PKCδ, as PKCδ undergoes Tyr phosphorylation in response to a number of lethal triggers. Brodie's group has published several reports concerning Tyr phosphorylation of PKCδ. They found that Tyr phosphorylations in both the regulatory domain (Tyr64) and the catalytic domain (Tyr187) are essential for cleavage of PKCδ by caspase 3 and for etoposide-induced apoptosis in glioma cells[345]. They also showed that cisplatin induced phosphorylation of PKCδ on Tyr332, and this was crucial for PKCδ cleavage[358]. Moreover, phosphorylation of PKCδ on Tyr155 was shown to be required for PKCδ cleavage, which protected glioma cells from TRAIL-induced apoptosis[359]. Tyr phosphorylation seems to act as a molecular switch, since mutation of PKCδ on Tyr155 to Phe sensitized glioma cells to TRAIL-induced apoptosis[359]. c-Abl has been identified as a PKCδ Tyr kinase that interacts with and phosphorylates PKCδ upon apoptotic stimuli such as DNA damage and oxidative stress[360-362]. Another Tyr kinase that also has been shown to phosphorylate PKCδ upon apoptotic stimuli is Src[358].

PKCδ and Apoptosis in Breast Cancer

Although PKCδ is pro-apoptotic in most cell systems, PKCδ has mainly been assigned anti-apoptotic effects in breast cancer. In ER-positive MCF-7 cells, for example, resistance to tamoxifen, irradiation, and TRAIL involves PKCδ[363-365]. PKCδ also confers resistance to irradiation in ER-negative MDA-MB-231 cells[363]. We have recently shown that down-regulation of PKCδ *per se* induces apoptosis of this cell

line [151]. Furthermore, our unpublished results suggest a connection of PKCδ with the mitochondrial apoptotic pathway, since PKCδ interacts with Smac in several breast cancer cell lines. This interaction conceivably prevents the apoptotic effect of Smac, as sustained interaction was accompanied with reduced apoptosis (unpublished data). In addition, PKCδ might interact with the mitochondrial pathway via Bcl-2 family members to suppress apoptosis, since stable over-expression of PKCδ in murine mammary epithelial cells results in up-regulation of Bcl-2 as well as increased phosphorylation of, and thus inactivation of, Bad. Finally, PKCδ over-expression increases the activity of the pro-survival proteins Akt and NFκB[366]. However, there are also a few studies using breast cancer cells showing pro-apoptotic effects of PKCδ in response to UV light[367,368]. Taken together, evidence from the literature suggests that the effect of PKCδ on the apoptotic response might be determined by the upstream lethal trigger, since UV radiation exerts opposite effects of most apoptotic stimuli. Therefore, the role of PKCδ as an apoptosis regulator needs to be further investigated before it can be validated as a therapeutic target in breast cancer.

The Present Investigation

Aims

The general aim of this thesis was to understand the role of PKC isoforms in cellular processes that promote malignant progression of breast cancer. An additional objective was to evaluate different PKC isoforms as prognostic markers in breast cancer.

Specific Aims

- Identify cancer-promoting cellular processes regulated by PKC isoforms.

  An initial finding was that PKCδ supports breast cancer cell survival.
  Therefore, subsequent aims were generated:

- Characterize signaling pathways involved in PKCδ-mediated survival of breast cancer cells.
- Identify novel binding partners of PKCδ and analyze the impact of these interactions on apoptosis-regulation.

- Identify PKC isoforms suitable as breast cancer biomarkers and elucidate their prognostic value. In relation to this, we wanted to analyze associations between PKC expression, proliferation, and migration in cultured cells as well as in patient material.

Results and Discussion

PKCδ is a Survival Factor in Breast Cancer Cells (Paper I and III)

Identification of mechanisms that mediate apoptosis resistance is highly valuable in the search for novel therapeutic targets. It has previously been shown that PKCδ protects breast cancer cells against apoptotic insults such as endocrine treatment and radiation[363,364]. On the other hand, UV-light-induced apoptosis is mediated via PKCδ[367,368]. Thus, the role of PKCδ in breast cancer cell survival is still not elucidated. Paper I and III shed light on this issue, where we propose that PKCδ is a pro-survival protein in breast cancer cells and regulates apoptosis on different levels.

PKCδ Regulates MDA-MB-231 Breast Cancer Cell Survival by Suppressing the Constitutively Activated ERK1/2 Pathway (Paper I)

In paper I we investigated the role of individual PKC isoforms in breast cancer cell survival and observed that down-regulation of PKCδ with siRNA *per se* induced apoptosis of MDA-MB-231 cells. One reason that this is a previously unrecognized phenomenon may be that a rather long period of reduced PKCδ levels is necessary for the apoptosis to be detectable. The apoptosis induction was measured with annexin V and nuclear morphology analyses. Moreover, the absence of possible off-target effects affecting the results was strengthened since we obtained similar results with an additional siRNA targeting PKCδ. The apoptosis induced by PKCδ silencing was abolished by addition of a pan-caspase inhibitor and PKCδ silencing resulted in cleavage of caspase 3, confirming that the cell death induced by down-regulation of PKCδ was apoptotic.

We further found that PKCδ favors survival of MDA-MB-231 cells by regulating the ERK1/2 pathway. MDA-MB-231 cells possess a mutation in codon 13 of the *KRAS* gene[369] and a B-Raf mutation (Glu463Val)[183] resulting in constitutive activation of the ERK1/2 pathway. MDA-MB-231 was the breast cancer cell line with highest basal ERK1/2 phosphorylation, and inhibition of the ERK1/2 pathway by two different MEK inhibitors (PD98059 or U0126) abrogated the apoptosis induced by PKCδ depletion. Inhibiting MEK1/2 in MCF-7 or MDA-MB-468 cells, having lower basal activity of ERK1/2, on the other hand, slightly enhanced, rather than decreased, the death of these cells. A reduction in apoptosis induced by PKCδ silencing in MDA-MB-231 cells was also seen if cell were treated with the Raf inhibitor, sorafenib. Inhibition of p38 and JNK, on the contrary, had no influence on the apoptotic response. A link between PKCδ and survival of cells containing mutated constitutively activated Ras has been reported in mouse fibroblasts as well. In this cell line PKCδ was shown to induce activation of Akt[370]. Moreover, the PI3K pathway was recently shown to be negatively regulated by the ERK1/2 pathway in several breast cancer cell lines, including MDA-MB-231 cells[371]. However, in our case, reduced Akt signaling can not explain the apoptosis induced by down-regulation of PKCδ, since we did not observe a decrease

in Akt phosphorylation after PKCδ silencing. Another observation was that PKCδ silencing in MDA-MB-231 cells resulted in increased phosphorylation of ERK1/2, in line with a previous study showing that treatment with a PKC inhibitor induces ERK1/2 phosphorylation in MDA-MB-231 cells[199]. To prevent progression toward malignancy, failsafe mechanisms, such as senescence and apoptosis, can be activated as a response to oncogene-induced accelerated proliferation. However, enhancement of pro-survival signaling enables cells to evade this[372,373]. Since enhanced ERK1/2 activity as a result of PKCδ depletion is accompanied with increased apoptosis, PKCδ may favor cell survival by suppressing Ras oncogene-induced failsafe mechanisms.

As mentioned earlier, ERK1/2 are activated upon phosphorylation of the Thr and Tyr residues at their kinase activation loop by their specific dual specificity kinases, MEK1/2[156]. Inactivation of ERK1/2 is achieved by dephosphorylation of the regulatory Thr and Tyr residues by dual specificity MKPs[374]. We found the increased ERK1/2 phosphorylation induced by PKCδ silencing to be regulated both upstream and downstream of ERK1/2. Down-regulation of PKCδ caused increased MEK1/2 phosphorylation and decreased expression of MKP3, an ERK1/2 specific MKP[375-377]. Moreover, although not to the same extent as for PKCδ silencing, down-regulation of MKP3 *per se* slightly increased death of these cells. We also observed that MKP3 was uniquely expressed in MDA-MB-231 cells, the cell line that possess a mutated KRAS, and not in any of the other cell lines investigated. This is in accordance with a previous report showing that MKP3 was found to be up-regulated in MCF10A breast epithelial cells stably expressing activated H-Ras[219]. Furthermore, it was recently reported that TPA induced sustained ERK1/2 activation and increased MKP3 expression levels in MCF-7 cells. Simultaneous inhibition of PKC with GF109203X (an inhibitor of cPKCs and nPKCs) abolished this TPA-effect[202]. These findings suggest that PKC activation induces MKP3 expression, but a remaining question is whether the TPA-effect is mediated by PKCδ.

Since the expression levels of MKP3 are mainly regulated by ubiquitylation and subsequent proteasomal degradation[220] and we saw that proteasome inhibition (by MG132) led to increased MKP3 levels, we search for potential E3 ubiquitin ligases for MKP3. We found the E3 ubiquitin ligase Nedd4 to be up-regulated in MDA-MB-231 cells void of PKCδ when compared to control cells, and as for MKP3, Nedd4 was uniquely expressed in MDA-MB-231 cells. Furthermore, silencing of Nedd4 resulted in enhanced MKP3 levels, suggesting that Nedd4 facilitates MKP3 degradation.

Taken together, PKCδ is a pro-survival factor in MDA-MB-231 cells and probably prevents activation of Ras oncogene-induced failsafe mechanisms by inhibiting MEK1/2 and stabilizing MKP3, which result in suppressed ERK1/2 signaling.

The Isolated Catalytic Domains of PKCδ and PKCε Induce Apoptosis of Breast Cancer Cells (paper I)

The results from paper I and III implicate PKCδ as a survival factor in breast cancer cells. However, in many cell types, PKCδ is considered to be pro-apoptotic due the ability of its catalytic domain (CD) to facilitate caspase cleavage and activation and because it is cleaved in the V3 region by caspases during apoptosis[92,343]. In order to evaluate whether the isolated CD of PKCδ is pro-apoptotic also in breast cancer cells, the CD of PKCδ was over-expressed in MCF-7 and MDA-MB-231 cells. As commonly seen for the CD of PKCδ, its over-expression did induce apoptosis in these cell lines. Interestingly, we also observed increased apoptosis following expression of the free CD of PKCε. Although PKCε has generally been assigned pro-survival effects[335], cleavage at Asp383 by caspases creates an active catalytic fragment, and depending on the cell type and context, the free CD of PKCε can promote or protect against apoptosis[152,338,378-380], possibly explaining this finding. Full-length PKCδ and PKCε, on the contrary, did not influence survival, indicating that the catalytic domains of PKCδ and PKCε, which are more conserved among the isoforms compared to the regulatory domains [36], can induce apoptosis when not spatiotemporally or allosterically regulated by their N-terminal regulatory domains. Moreover, the finding that the free CDs of PKCδ and PKCε induces apoptosis and at the same time down-regulation of the endogenous proteins has the same effect, can probably explain the sometimes opposing effects of these PKC isoforms in apoptosis regulation.

PKCα Expression is Associated with Aggressive Tumors and Poor Prognosis (paper II)

In breast cancer, *in vitro* and *in vivo* studies have suggested that PKCα, PKCδ, and PKCε facilitate processes that favor malignant progression. Hence, these PKC isoforms could be potential prognostic markers and future candidates for targets in breast cancer therapy. However, to date there are only a few reports investigating the potential of different isoforms as treatment-predictive and prognostic markers in breast cancer. The study in paper II was therefore designed to contribute to the knowledge on this issue and sheds light on the utility of PKCα, PKCδ, and PKCε as markers of tumor aggressiveness.

To achieve accurate and representative results, a substantial validation of the PKC antibodies was undertaken in this paper. Cross reaction is a notorious problem for immunohistochemical analyses of PKC isoforms, and several batches of antibodies were tested to identify isoform-specific antibodies. Initially a tissue microarray (TMA) of a cohort that originally consisted of breast cancer tumors from 114 patients (cohort I) was analyzed for the expression levels of PKCα, PKCδ, and PKCε. When present in a tumor, the PKC isoforms were generally cytoplasmic and expressed in all of the tumor cells. Stainings were therefore assessed based on cytoplasmic staining intensity and scored as absent, low, moderate, or intense staining. The majority of the tumors lacked PKCα, in line with the notion that PKCα is down-regulated in breast cancer

tissue compared to normal breast tissue[140,141]. However, elevated PKCα expression correlated significantly with tumor grade (NHG), proliferation rate (Ki67), and ER and PR negativity, which are all parameters that are associated with increased breast cancer aggressiveness. On the contrary, PKCα expression did not correlate with lymph node spread or metastasis. PKCδ and PKCε did not correlate to any clinicopathological parameter investigated.

We continued our analyses of PKCα in a larger cohort of originally 512 consecutive breast cancer cases (cohort II) and the results corroborated the finding in the cohort I. The negative correlation between PKCα and the ER is in accordance with a previous study[138] and firmly establish the relationship between PKCα and ER. Our results also suggest that PKCα expression predicts for poorer survival of the patients, since patients with PKCα-negative tumors had a significantly improved 10-year breast cancer-specific survival (BCSS) compared to patients with PKCα positive tumors. Moreover, a multivariate analysis revealed that the association between PKCα expression and poor 10-year BCSS was independent of established prognostic parameters, such as age at diagnosis, tumor size, tumor grade, node status, and ER expression. This result indicates that PKCα is an independent prognostic factor in breast cancer.

Although no correlations were seen between PKCε and the parameters analyzed in cohort I, we further investigated PKCε as well since our results somewhat contradict an already published study suggesting that PKCε is a marker of aggressive breast cancers[150]. However, neither in cohort II did we observe correlations between PKCε and clinicopathological parameters. Furthermore, survival of breast cancer patients was not significantly associated with PKCε expression.

PKCα Supports Proliferation and Migration of Cultured Breast Cancer Cells (paper II)

In paper II we also investigated whether different breast cancer cell lines can represent the PKC isoform expression pattern in tumors. To substantiate this analysis, we measured the expression levels of PKC in four different cell lines, the ER positive cell lines T47D and MCF-7 and the ER negative cell lines MDA-MB-231 and MDA-MB-468. There were no major differences in PKCδ and PKCε levels between the cell lines, except for slightly higher levels of PKCδ in MCF-7 cells. Expression levels of PKCα, on the other hand, differed more between the cell lines. Whereas PKCα was undetectable in T47D cells, it was highly expressed in the MDA-MB-231 cells compared to in MCF-7 and MDA-MB-468 cells.

In line with the positive correlation between PKCα and the proliferation marker Ki-67 observed in the patient material, T47D cells had lower percentage Ki-67-positive cells and slower growth rate under normal culturing conditions compared to the other cell lines investigated. However, proliferation of MDA-MB-231 cells was only marginally increased compared to MCF-7 and MDA-MB-468 cells and did not reach

statistical significance. This may be explained by the notion that the fraction Ki-67-positive cells was high (97%) and could not be further increased. Treatment with TPA in serum-free conditions or inhibition of classical PKCs by Gö6976 in complete medium did not influence cell growth, indicating that PKC activity is not crucial for the PKCα effect. However, TPA treatment led to reduced PKCα levels, making these results difficult to interpret. It was recently reported that the PKCα protein, but not its activity, is crucial for glioma cell proliferation[381]. We found that silencing of the PKCα protein resulted in reduced percentage of MDA-MB-231 cells in S-phase when cells were grown under serum-free conditions, suggesting that, as in glioma cells, the PKCα protein supports proliferation.

In addition to its involvement in breast cancer cell proliferation, PKCα has also been associated with increased migratory effects since over-expression of this protein has been shown to facilitate migration and metastasis of breast cancer cells[136,143,382]. Based on this, we aimed at investigating the significance of endogenous PKCα for breast cancer cell motility. Inhibition of classical PKCs significantly reduced the ability of MDA-MB-231 cells to migrate. On the contrary, silencing of PKCα in the same cell line did not influence migration. We speculated that since MDA-MB-231 cells have high basal PKCα expression, the remaining PKCα after siRNA treatment was sufficient for the migratory capacity of the cells. For this reason, we silenced PKCα in MCF-7 cells, where basal PKCα levels are similar to the PKCα levels in MDA-MB-231 obtained after PKCα silencing, and observed that PKCα is indeed crucial for migration of MCF-7 cells. In accordance with previous over-expression studies, these findings indicate that although we did not observe any correlations between PKCα and lymph node status or metastasis in the TMAs, PKCα activity does facilitate breast cancer cell migration *in vitro*.

Conclusions

In this thesis we have gained insight into the PKCδ-mediated regulation of breast cancer cell survival, as well as identified PKCα as a marker of breast cancer aggressiveness.

We could conclude that:

- PKCδ is a survival factor in breast cancer cells.

- PKCδ favors survival of MDA-MB-231 cells by regulating the ERK1/2 pathway through MEK1/2 and MKP3.

- The free catalytic domains of PKCδ and PKCε induce apoptosis in breast cancer cells.

- PKCα is a marker of breast cancer aggressiveness and a predictor of worse patient outcome.

- PKCα supports proliferation and migration of cultured breast cancer cells.

Popularized Summary in Norwegian

For at alle kroppens funksjoner skal fungere er det viktig at cellene kan kommunisere med hverandre. Derfor har hver eneste celle et komlekst nettverk av signaleriseringsmolekyler som gjør at de kan ta imot informasjon fra utsiden av cellen og videreføre den til cellens kjerne. Informasjonen som kommer hit avgjør hvilke proteiner som skal produseres, og det er proteinene i sin tur som bestemmer hvilke prosesser cellen skal gjennomgå eller om den skal sende ut ny informasjon til omgivelsene. Hos kreftceller fungerer ikke denne kommunikasjonen som den skal, fordi de har ulike genetiske forandringer som mutasjoner, gen-amplifisering, gen-tap, og/eller DNA-modifikasjoner. Disse genetiske forandringene leder til endring i proteinproduksjon eller proteinaktivitet og er med på å gi kreftcellene de egenskaper de trenger for å dele seg uhemmet, overleve og forflyttes i kroppen.

Brystkreft er den vanligste kreftformen blant kvinner. Det finnes mange varianter av brystkreft, og pasientene får behandling på grunnlag av et fåtall parametere. Dessverre er det sånn at pasienter med tilsynelatende like brystkrefttyper svarer ulikt på samme type behandling, og det er derfor viktig å utvikle enda mer skreddersydde behandlinger.

Protein kinase C (PKC) er en enzymfamilie som viderefører signaler i cellen ved å fosforylere andre proteiner. Familien består av ti medlemmer (isoformer) navngitte etter det greske alfabetet. PKC deltar i mange ulike cellulære prosesser, og visse PKC isoformer anses å være potensielle mål for behandling av brystkreft. Derfor har hensikten med mitt forskningsarbeid vært å analysere hvilken rolle ulike PKC isoformer spiller i overlevelsesmekanismer og andre cellulære prosesser hos brystkreftceller og om PKC kan brukes som prognostisk markør for brystkreft.

I delarbeid I har vi sett at PKCδ er avgjørende for visse brystkreftcellers overlevelsesevne, for om man hemmer produksjon av PKCδ dør cellene i en nøye kontrollert programmert celledød som kalles apoptose. En av cellelinjene vi studerte har en mutasjon i genet som koder for Ras, noe som gjør at dette proteinet er hyperaktivt. Ras ligger høyt oppe i og aktiverer en signaliseringvei som fremmer celledeling. I normale celler bruker overdrevet celledelingsfremmende signalerisering å lede til celledelingsstopp eller apoptose. Kreftceller derimot har utviklet evnen til å unnvike apoptose ved å produsere andre proteiner og aktivere andre signaliseringsveier som fremmer kreftcellenes overlevelse. Vi har sett at PKCδ bremser aktiviteten og fremmer degradering av proteiner lenger ned i Ras-signaliseringsveien slik at det gis beskjed om

at cellen skal dele seg i stedet for å dø. Når vi hemmer produksjonen av PKCδ i disse cellene, fjerner vi dermed den PKCδ-medierte reguleringen av signaleriseringsveien, og cellene dør sannsynligvis på grunn av for høyt celledelingsstimuli. Selv om ytterligere studier kreves for å bedre forstå hvordan dette virkelig går til og om dette er tilfelle for andre Ras-muterte cellelinjer, foreslår vi på grunnlag av resultatene i dette delarbeidet at PKCδ kan være et potensielt mål for behandling av brystkreft med overaktiv Ras-signalisering.

En celle blir programmert til å dø når de pro-apoptotiske proteinene overvinner de anti-apoptotiske proteinene og dermed aktiverer en gruppe proteiner som kalles caspaser. Caspaser utfører selve apoptosen ved å klippe i stykker en rekke vitale proteiner og aktivere proteiner som kutter opp DNAet. Second mitochondria-derived activator of caspase (Smac) er et pro-apoptotisk protein som frigjøres fra mitokondriene ut til cytosolen når apoptose induseres. Smacs viktigste oppgave er da å binde til medlemmer av en gruppe anti-apoptotiske caspase-hemmere som kalles inhibitorer av apoptose proteiner (IAP). I delarbeid III fortsetter vi å studere hvilken rolle PKCδ spiller i regulering av apoptose i brystkreftceller. Her viser vi at PKCδ kan regulere apoptose på flere nivåer i og med at den kan binde til Smac. Vi har sett at PKC-aktivering stabiliserer interaksjonen mellom PKCδ og Smac, og at celler som vanligvis dør når de behandles med cellegift, forblir levende om PKC aktiveres. Vi har i tillegg funnet ut at PKCδ binder inn til samme del av Smac som også er bindingssetet for IAP. Derfor har vi formulert en hypotese om at interaksjonen mellom PKCδ og Smac forhindrer Smac i å binde og hemme IAP og dermed forhindrer apoptose. For øyeblikket har utforskning av denne hypotesen og eventuelt andre mekanismer som ligger bak effekten av bindingen mellom PKCδ og Smac fått høy prioritet i vår pågående forskning.

I delarbeid II studerte vi forekomsten av PKC i brystkreftsvulster hos flere hundre pasienter hvor små biopsier er ordnet i mikromatriser som tillater at man kan analysere omtrent hundre vevsprøver på et og samme objektglass. En liten gruppe pasienter hadde svulster med veldig høye nivåer av PKCα. Disse pasientene hadde generelt sett kortere levetid og mer aggressive svulster med egenskaper som høyfrekvent celledeling. På laboratoriet observerte vi at brystkreftceller var avhengige av PKCα for å kunne dele seg hyppig og for å kunne forflytte seg. Videre så vi at brystkreftceller med høyt PKCα-nivå delte seg oftere enn brystkreftceller med mindre PKCα. Disse resultatene indikerer at PKCα fremmer cellulære prosesser som kan lede til brystkreft og at PKCα er en prognostisk markør for brystkreft.

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