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Regulation of cellular growth and identification of stromal gene signatures in breast cancer

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Regulation of Cellular Growth and Identification of Stromal Gene Signatures in Breast Cancer

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Abstract

Normal tissue is tightly controlled to keep a balance between reproduction and elimination of cells. In cancer, these regulated processes are disrupted, resulting in uncontrolled cell growth. Regulation of RNA stability and turnover is important to maintain cellular homeostasis and can be controlled by various mechanisms. During stress, the cell can form cytoplasmic complexes of proteins and RNAs, called stress granules, to inhibit translation of proteins unnecessary for the cell during harmful conditions and focus translation on stress-related proteins. In neuroblastoma and breast cancer cell lines, we have found that the protein kinase Cα (PKCα) isoform can influence stress granule formation in a stress inducer-specific way. Depletion of PKCα led to a delayed stress response along with an initial loss of eIF2α phosphorylation in heat shock, but not arsenite, treated cells. G3BP proteins are well-known stress inducers and we identified a direct interaction between PKCα and the G3BP2 isoform.

G3BP2 belongs to a family of three homologous proteins with RNA-regulating capacities. With the aim to identify specific RNA targets, we performed a gene expression analysis and detected a negative regulation of the peripheral myelin protein (PMP22) by the G3BP1 isoform. The previously reported growth suppressing effects by PMP22 was here verified in breast cancer cells and we could show that G3BP1 influences growth regulation by reducing PMP22 expression, although not through mRNA destabilizing mechanisms.

Another RNA-regulating mechanism that can promote or prevent tumor progression are mRNA silencing through miRNA. We analyzed miR-34c and its function in breast cancer and identified impaired cell growth, induced apoptosis and cell cycle G2/M arrest, which might be due to regulation of the anaphase-promoting complex protein CDC23.

The tumor is not a homogenous compartment, but consists of various different cell types, both among the cancer cells as well as in the surrounding stroma. We have developed a methodological procedure for isolation and characterization of cancer- and stroma-specific genes using laser capture microdissection on FFPE triple negative breast cancers. Gene expression microarrays of these samples revealed compartment-specific gene expression and enabled identification of stromal-specific gene signatures with tumor-predictive capacity.

Key words: Breast cancer, cellular stress, proliferation, PKCα, G3BP, PMP22, miR-34c, gene expression profiles, stroma

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PLoS One 2012, 7:e35820.

Paper II

Regulation of PMP22 mRNA by G3BP1 affects cell proliferation in breast cancer cells
Sofia Winslow, Karin Leandersson, Christer Larsson
Mol Cancer 2013, 12:156.

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Expression of miR-34c induces G2/M cell cycle arrest in breast cancer cells
Chandrani Achari[1], **Sofia Winslow**[1], Yvonne Ceder, Christer Larsson
[1] Equal contribution
Manuscript

Paper IV

Identification of stromal gene signatures in breast cancer
Sofia Winslow, Karin Leandersson, Anders Edsjö[1], Christer Larsson[1]
[1] Equal contribution
Manuscript

Abbreviations

AGO	Argonaute
APC	Anaphase promoting complex
ARE	AU-rich element
ATP	Adenosine triphosphate
BRCA	Breast cancer gene
C1-4	Conserved region 1-4
CAF	Cancer-associated fibroblast
CD	Cluster of differentiation
CDC23	Cell division cycle homolog 23
CK	Cytokeratin
CMT	Charcot-Marie-Tooth disease
CpG	Cytosine - phosphate - guanine
DAG	Diacylglycerol
DCIS	Ductal carcinoma in situ
DCP	Decapping protein
DGCR8	DiGeorge Syndrome Critical Region 8
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
eIF	Eukaryotic translation initiation factor
EMP	Epithelial membrane protein
ER	Estrogen receptor
FFPE	Formalin-fixed paraffin-embedded
FMRP	Fragile X mental retardation protein
G3BP	Ras-GTPase-activating protein SH3 domain-binding protein
GAS	Growth arrest-specific protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HER2	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen
HMSN	Hereditary motor and sensory neuropathy
HNPP	Hereditary neuropathy with liability to pressure palsies
HRI	Heme-regulated inhibitor
HSF	Heat shock factor
HSP	Heat shock protein
HuR	Human antigen R
IG	Immunoglobulin
IGF2BP	Insulin-like growth factor 2 mRNA-binding protein
INSS	International neuroblastoma staging system
IP3	Inositol triphosphate
IRES	Internal ribosome entry site
MAG	Myelin-associated glycoprotein
MAP	Mitogen-activated protein
MBP	Myelin basic protein
MHC	Major histocompatibility complex
miRNA	micro ribonucleic acid
MMP	Matrix metalloproteinase
MPZ	Myelin protein zero
mRNA	messenger ribonucleic acid
MYCN	v-myc myelocytomatosis viral-related oncogene, neuroblastoma-derived (avian)
NHG	Nottingham histological grade
NTF2	Nuclear transport factor 2
PABP	PolyA-binding protein
PB	Processing body
PB1	Phox-Bem1
PDK-1	Phosphoinositide-dependent kinase-1
PERK	PKR-like ER kinase

PIP_2	Phosphatidylinositol-4,5-bisphosphate
piRNA	Piwi-interacting ribonucleic acid
PKC	Protein kinase C
PKR	Protein kinase R
PLCβ	Phospholipase Cβ
PMP22	Peripheral myelin protein 22
PNS	Peripheral nervous system
PR	Progesterone receptor
RACK	Receptor for activated C kinase
RasGAP	Ras-GTPase-activating protein
RBP	RNA-binding protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RRM	RNA recognition motif
SELEX	systemic evolution of ligands by exponential enrichment
SG	Stress granule
SH3	SRC homology 3 domain
siRNA	small interfering ribonucleic acid
TCGA	The Cancer Genome Atlas
TDLU	Terminal ducts lobular unit
T_H	T helper cell
TIA-1	T cell intracellular antigen-1
TMP	Tumor-associated membrane protein
TPA	12-O-tetradecanoylphorbol-13-acetate
TTP	Tritetraprolin
UPR	Unfolded protein response
UTR	Untranslated region
UV	Ultraviolet
V1-5	Variable region 1-5
VEGF	Vascular endothelial growth factor

Cancer introduction

Cancer is a collective name of more than 100 diseases, having in common an uncontrolled and abnormal cell growth. In a healthy tissue, the balance of cell division and cell death is tightly regulated for the organ to keep its structure and function. If an imbalance in any of these processes occurs, there is an increased risk of developing cancer.

Genetic mutations frequently occur as the DNA replicates during cell division. Most errors will not result in permanent modifications due to advanced repair mechanisms within the cell. Yet, those mutations that cause advantageous properties of the cell will remain and eventually, these might lead to cancer development. The mutations can result in sustained proliferation by creating self-sufficiency in growth-promoting signals or by avoiding growth suppressive signals. In addition, escape from apoptosis, induced immortality, sustained angiogenesis, tissue invasion and metastatic spread are characteristics that have been linked to cancer progression [1]. These Hallmarks of cancer have later been updated to further include tumor-promoting inflammation, ignorance of immune cell mediated destruction, genome instability and mutations as well as deregulation of energy metabolism in the cell [2].

In addition to the genetic events mentioned above, initiation of cancer can also be facilitated by epigenetic events such as hyper- or hypomethylation [3, 4]. Tumor suppressor genes in cancers can display hypermethylation in the promotor region and consequential silenced expression (*e.g.* of *BRCA1* [5] and *VHL* [6]), but also hypomethylation, and thus activation of cancer associated genes, has been reported [7].

Breast cancer

Epidemiology and Etiology

Breast cancer is the most common form of cancer in women and the second leading cause of cancer-related deaths in women after lung cancer [8]. In Sweden, over 8000 new cases are diagnosed every year, accounting for approximately 30% of all cancer diagnoses in women [9, 10]. The breast cancer incidence is still increasing, but better treatment and earlier diagnosis have led to reduced mortality and the five-year survival rate is now almost 90% [10]. The risk of developing breast cancer depends on both hereditary and non-hereditary factors such as hormonal factors, age, smoking, diet, infections and genetic predisposition. In addition, women with early menarche, late menopause and late first birth have also showed an increased risk of breast cancer [8]. Although most breast cancers arise sporadically, about 5-10% of all cases depend on hereditary factors and some of the most prominent ones are mutations in the tumor suppressor genes *BRCA1* and *BRCA2* [10].

Breast cancer progression

The breast tissue comprises a branched glandular structure surrounded by supportive connective tissue. The gland consists of an inner epithelial layer of luminal cells forming the ducts and terminal lobules, and a surrounding layer of basal myoepithelial cells responsible for communication with the adjacent stroma and maintenance of tissue polarity (Figure 1a). The functional compartments of the breast gland are the milk secreting lobules, formed by a cluster of alveoli, gathered as terminal duct lobular units (TDLU), which through the ducts subsequently drain into the nipple. The TDLUs are the sites where most breast lesions arise, starting with a benign modification, often followed by progression into more malignant states called atypical hyperplasia and ductal carcinoma in situ (DSIC) (Figure 1b) [11]. DCIS accounts for about 20% of all breast cancer cases and is characterized by ductal cell invasion into the lumen of the duct [12]. In some cases the tumor cells invade the surrounding connective tissue and can as invasive breast cancer eventually metastasize

to the lymph node and distant metastatic sites, such as brain, bone, liver and lung [13].

Histological classification, grading and staging

The histological classification of breast cancers is based on cellular characteristics and morphology of the lesion, and thus reflects the growth pattern of the tumor. The most common form is the invasive ductal carcinoma comprising of about 75% of all cases, whereas the invasive lobular carcinoma is the second most common (approximately 15%) [14, 15]. At least 17 histological types of breast cancer have been identified, although most of them show a low prevalence, such as medullary, mucinous, tubular and papillary breast lesions. The best prognosis is found among patients with carcinoma in situ, where the lesion still resides within the basement membrane [12].

Histological grading of tumors has shown better prognostic and predictive values than histological classification and is routinely used in the clinic. Nottingham histological grade (NHG) is a classification system based on the aggressiveness of the tumor where three histological characteristics, glandular/tubular differentiation, nuclear pleomorphism and mitotic count, are being evaluated. Each feature is graded from I-III, with grade III being least differentiated, and a summary of the scores defines the grade of the tumor [16].

To define the progression and estimate the outcome of the tumor, the staging system TNM is used, which is an abbreviation of tumor, node and metastasis. The tumors are classified from T0 to T4 dependent on size and N0-N3, with N0 implying no tumor cells present in the adjacent lymph nodes and higher number indicate more lymph node involvement. Metastasis is only classified as M0 or M1 depending on absence or presence of distant tumor metastasis [17].

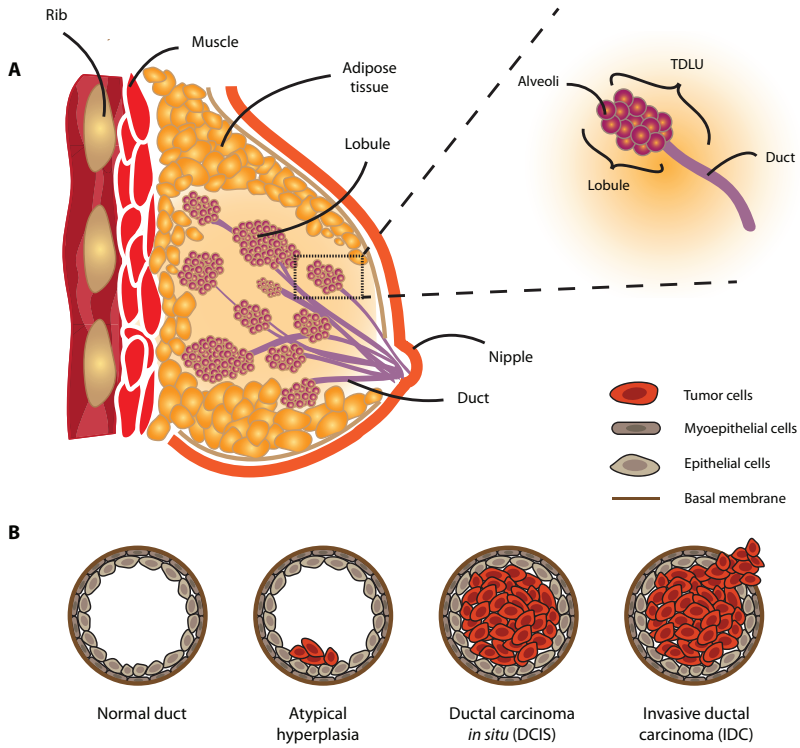
Immunohistochemical analysis

In clinical diagnostics of breast tumors, immunohistochemical examination for the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2, also known as ERBB2) as well as the proliferation marker Ki67 is routinely performed. In case of HER2 positive staining, in situ hybridization (FISH) is additionally performed to evaluate a potential presence of

ERBB2 amplification. The expression of these markers can indicate the prognostic outcome and is important for therapeutic decisions [17].

Molecular subtypes

Analysis of breast tumor specimens using gene expression microarrays was initiated in the beginning of 21st century to identify gene expression patterns that could



Figur 1. Schematic illustration of the breast structure and breast cancer progression. **(A)** The normal breast comprises ducts and lobules of the glandular structure surrounded by adipose and stromal cells in the connective tissue. Terminal duct lobular unit (TDLU) is the functional unit of the breast, responsible for the milk production. **(B)** Breast cancer typically arises in the TDLU, where initial abnormal cell growth can cause atypical hyperplasia with irregular cell morphology. Continuous proliferation of the luminal epithelial cells results in formation of ductal carcinoma in situ (DCIS) with cells filling the lumen of the duct. Invasive ductal carcinoma (IDC) arises as the basement membrane degrades and cells invade the surrounding microenvironment

correspond to the phenotypic diversity identified among breast tumors. These results described distinctive molecular portraits of each tumor, which further was used to identify intrinsic subtypes within the breast cancers. Initially, four biologically distinct and clinically relevant classes were identified, although this has later been refined [18, 19]. Today, tumors are classified as luminal A, luminal B, HER2-enriched, basal-like, claudin-low or normal-like, even though the latter has been questioned and novel subgroups have been proposed [20-26].

A majority of the breast cancers are classified as either luminal A (50-60%) or luminal B (10-20%) tumors with high ER expression and/or PR expression. The luminal B tumors have a higher expression of the proliferation marker Ki67 and have showed a higher grade and worse prognosis than luminal A [14, 27]. HER2-enriched tumors are characterized by a high expression or gene amplification of the *ERBB2* gene (the HER2 encoding gene) and account for approximately 15-20% of all breast tumors. These tumors are associated with poor prognosis, although HER2 targeting therapies such as trastuzumab have improved the survival of this subgroup [27, 28]. The normal-like subtype is poorly characterized, but resembles the expression pattern of normal breast samples and displays an intermediate prognosis [27]. Basal-like breast cancers, composing 15% of all breast carcinomas, are high-grade tumors with an overall bad prognosis [27]. They frequently lack the expression of estrogen and progesterone receptors as well as HER2-amplification and are hence called triple-negative tumors. However, not all basal-like tumors are triple-negative and only 70% of tumors lacking ER, PR and HER2 expression are clustered as basal-like [29]. One subtype that originally was included among the heterogeneous basal-like tumors is the claudin-low group (12-14%). They are mainly triple-negative, but differ from the basal tumors by displaying a low expression of adhesion molecules such as E-cadherin and claudin -3, -4 and -7 along with having a stem cell like phenotype ($CD44^+/CD24^-$) and these tumors often have a high immune cell infiltration [24].

Basal-like tumor cells resemble the features of the basal myoepithelial cells surrounding the mammary ducts and share a common elevated expression of high molecular cytokeratins (*e.g.* CK5 and CK17) [20, 27]. Yet, basal-like tumors are heterogeneous and further characterization of these tumors is essential for improved therapeutic possibilities since the lack of hormonal receptors disables the use of hormone-based or anti-HER2 therapies. Today, patients with basal-like tumors mainly receive neoadjuvant treatment, such as anthracyclines, and those who experience a pathologic complete response have an improved prognosis. However, patients that do not gain any improvement instead display a significantly worse survival [30, 31]. Novel studies indicate an advantage of using additional chemotherapy blocking DNA repair mechanisms, such as platinum drugs and PARP inhibitors, especially in BRCA1 deficient basal tumors [21, 32].

Tumor microenvironment in breast cancer

Tumors were long believed to consist of a homogenous collection of cancer cells and the main focus in cancer research was restricted to investigating the genetic alterations resulting in tumorigenic transformation within the cell. Later studies have identified tumor promoting capacities also in the surrounding tumor microenvironment and elucidation of how the cancer and stromal cells communicate and promote tumorigenic events may be important for diagnosis and therapy improvement in malignant diseases [33-37].

In normal breast tissue, the ductal epithelium is surrounded by supportive stroma that mediates tissue homeostasis and provides signals for epithelial cell differentiation and tissue organization. This connective tissue is composed of extracellular matrix (ECM) and various types of stromal cells, including fibroblasts, pericytes, endothelial cell, adipocytes and immune cells [38-40]. One of the most abundant cell types of the stroma are the fibroblasts, mainly functioning by producing ECM components and by regulating inflammation, wound healing and epithelial differentiation. The ECM balance is regulated by production of collagen fibers for maintenance of a stable architecture, but also proteases such as matrix metalloproteinases for ECM degradation. Resting fibroblasts can be activated upon tissue injury to produce ECM and generate a platform for additional cells that can assist in wound healing [41].

Activation of tumor stroma

Survival and progression of tumor cells are initially counteracted by fibroblasts, macrophages and cytotoxic immune cells from the tumor microenvironment to prevent tumor growth [42]. However, the tumor-associated stroma can be influenced by tumor cells to rearrange the microenvironment to promote tumor growth. This process resembles the activation of stroma during wound healing with increased number and activation of fibroblasts, enhanced production of ECM components, newly formed capillaries and inflammatory infiltrate as a consequence [41] (Figure 2).

Angiogenesis

The tumor stroma is composed of a variety of non-malignant cells and the composition and proportion of stromal compartment in relation to tumor mass varies extensively between tumors, influencing the profound tumor heterogeneity. Activated tumor stroma cells promote an angiogenic switch of endothelial cells, in particular through production of vascular endothelial growth factors (VEGF), which leads to an activation of endothelial cells and induced formation of new blood vessels [2]. The increased need for oxygen and nutrients makes the growing tumor extremely sensitive to altered angiogenesis, and inhibition of novel blood vessel formation cannot only reduce tumor progression through insufficient oxygen supply but also diminish possible vascular routes for tumor metastasis [43-45]. Surrounding the endothelial cells of blood vessels, pericytes provide a stabilizing and growth-regulatory effect of blood vessels and assist in blood flow regulation in non-active stromal tissue. In tumor stroma, blood vessels have a reduced coverage of pericytes, which has been shown to have effects on both metastatic rate and survival in breast cancer [46-49].

Cancer-associated fibroblast

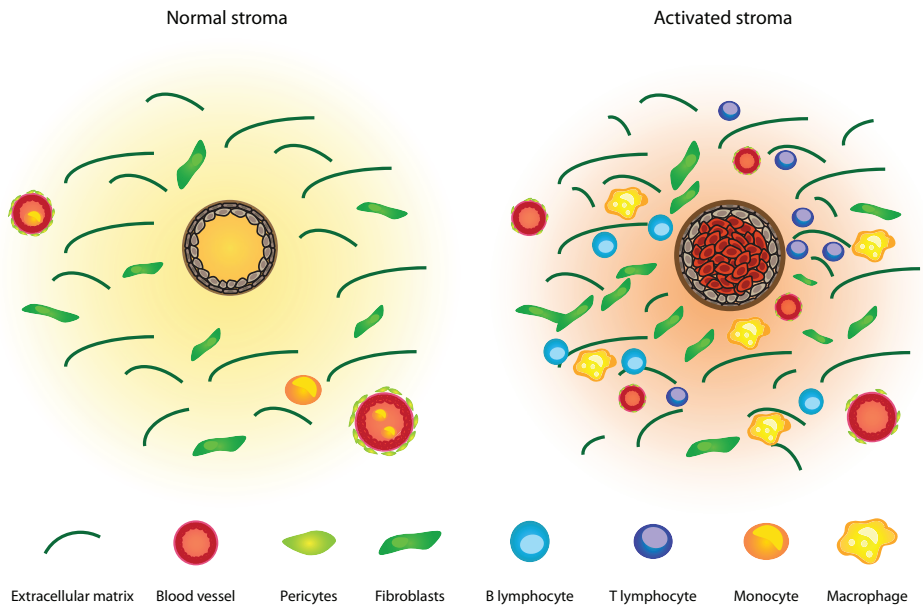
Cancer-associated fibroblasts (CAFs or myofibroblasts) become more abundant as a response to stroma activation and have in addition been characterized to proliferate faster than regular fibroblasts. Growth factors and cytokines, released by the tumor cells, recruit CAFs to the tumor site and promote CAF activation. In return, CAFs release growth-promoting signals for the adjacent epithelial cells, cytokines for immune cell recruitment and various extracellular matrix proteases, such as matrix metalloproteinases (MMP), to rearrange the ECM and promote tissue invasion [45, 50]. Several molecular markers for CAFs have been identified, although none of them are exclusively expressed in fibroblasts or expressed in all fibroblasts. Some of the most prominent proteins in fibroblasts are α-smooth-muscle-actin (α-SMA), fibroblast specific protein-1 (FSP-1), fibroblast-activated protein (FAP), but the variation in marker expression both within and between different tissues may indicate the presence of fibroblast subtypes [41, 51, 52].

Cancer and inflammation

Immune cell infiltrates are heterogeneous and can vary both in location and composition even within the same tumor type. In some cancers, a chronic inflammation is a prerequisite for tumor induction, *e.g.* human papillomavirus in cervical carcinoma and *Helicobacter pylori* in gastric carcinoma [53, 54]. Upon an

innate immune reaction, leukocytes are recruited and various mediators like cytokines and proteases are produced to eliminate the source of the infection. Identification of foreign antigens by dendritic cells will stimulate clonal expansion of adaptive immune cells, such as T cells, for elimination of the pathogen. This is followed by induction of cell death to remove damaged cells as well as induction of cell proliferation to reestablish the tissue morphology. Sustained stimulation results in chronic inflammation, genomic instability and an altered microenvironment, which will provide growth survival advantage for neoplastic cells [55, 56]. In other cancers, transformed cells can produce inflammatory mediators and thus create an inflammatory microenvironment themselves without an underlying inflammatory cause. This pro-tumorigenic inflammation provides cancer cells with growth factors and stroma-remodeling molecules, promoting further tumor development [57, 58].

T cells are common infiltrating lymphocytes identified as various subpopulations with different regulatory functions. Cytotoxic CD8+ T cells are prone to killing tumor



Figur 2. Tumor stroma activation. Normal stroma contains a tightly packed extracellular matrix making up a supportive network for the resting fibroblasts, pericyte-covered blood vessels and circulating and resident monocytes. In activated stroma, fibroblasts differentiate into cancer-associated fibroblasts and together with increased angiogenesis and lymphocyte and macrophage recruitment, this tumor microenvironment further promotes tumor progression.

cells with assistance of CD4+ T helper 1 (T_H1) cells and the presence of both of these immune cell types was strongly associated with good prognosis in an analysis of 20 different cancer types [59]. Presence of most other T cells, such as T_H2 and regulatory T cells, is on the other hand often associated with worse prognosis in breast cancer [59, 60], although the opposite has also been reported for T_H2 cells [61]. T cell receptors expressed on most T cells recognize antigens presented on either major histocompatibility complex I (MHC-I) for intracellular peptides or MHC-II for foreign proteins. MHC complexes are encoded by HLA (human leukocyte antigen) genes on antigen presenting cells, where HLA-A, -B and –C belongs to the MHC-I family and HLA-D to MHC-II. HLA-D exist in three variants HLA-DR, -DP and –DQ that are upregulated in response to hormonal or cytokine stimulation and HLA-DR as well as HLA-DM, responsible for peptide loading onto HLA-DR, are associated with a T_H1 profile and improved survival [62].

B cells are identified in the invasive margin and stroma of tumors and are the main inflammatory component in ductal carcinoma in situ and invasive breast tumors [63]. Infiltrating B cells were initially associated with good prognosis in breast cancer [64], although later mouse model studies have demonstrated tumor-promoting roles for B cells and immunoglobulins in skin cancer [58, 65] and increased lung metastasis in breast cancer [66]. However, a B cell signature, consisting of clusters of heavy and light chains, was identified among 200 invasive breast carcinomas to associate with metastasis-free survival among highly proliferating tumors [67] and immunoglobulin kappa chain (IGKC) was identified as a single biomarker for better prognosis and expression correlated with complete chemotherapy response in breast cancer [68].

Extracellular matrix

ECM is the supportive tissue in the stroma, responsible for structural organization of the tissue and cellular polarization and contains various combinations of proteins (*e.g.* collagen, laminin), glycoproteins (*e.g.* fibronectin, osteopontin), proteoglycans (*e.g.* decorin, lumican) and polysaccharides (*e.g.* hyaluronic acid) [69]. The ECM is identified to be a dynamic structure that reorganizes depending on the surroundings, in particular in response to stromal and immune cell influences. Under pathological conditions, the loosely packed matrix stiffens and collagen deposition increases, resulting in upregulated integrin signaling which can promote a variety of tumor promoting effects, including cell survival, proliferation and lymphocyte infiltration. In addition, thickening of collagens is often found at sites of tissue invasion, on which cancer cells can migrate. Regulation of ECM is also regulated by MMPs, expressed by branching endothelial cells to promote angiogenesis [70, 71]. In breast cancer,

stromal expression of especially the gelatinase subgroup MMP-2 and MMP-9 has been associated with poor prognosis [72, 73].

Identification of ECM gene signatures has been shown to provide breast cancer classification with implications for clinical outcome. These clusters did not completely overlap the molecular subtypes of breast cancer, but instead identified patients with a bad prognosis that otherwise reside in a "good prognosis" subtype. In this study, high collagen expression correlated with lymphocyte infiltration, high adhesion molecule expression and poor survival [74].

Stromal gene expression profiles

The phenotypic changes in the activated stroma highlight the importance of the tumor microenvironment for cancer induction and progression. Evaluations of the tumorigenic capacities of the stroma have indicated abilities both to induce and reduce neoplastic changes in mammary epithelium [75, 76]. The connective tissue is continuously remodeling to meet the needs in the tissue and miss-regulation can affect the adjacent epithelium and possibly induce neoplastic transformation [77-79]. Stromal gene expression profile analyses have been performed in various ways and tissues to delineate how the stroma influences the tumor tissue. In breast cancer, studies have identified profiles based on altered stromal expression in cancer and non-cancer tissue, but also signatures from different stages of progressing breast cancer [80-82]. In addition, these profiles could be useful in predicting clinical outcome [33] and response to neoadjuvant therapies [83]. Although, the studies vary with regards to tissue preparation and isolation techniques, all could produce stroma-specific profiles. However, the profile outcome differs to some extent between the studies indicating that more studies are necessary to improve stromal-based tumor classification and establish prognostic and therapeutic implications.

Neuroblastoma

During early neural development, the outermost ectoderm germ cell layer folds into a groove formation creating a neural tube, which later will develop into the central nervous system and neural crest cells. Depending on stimulation and site of migration, these cells can mature into melanocytes or various peripheral nerve cells including glial cells and sensory, sympathetic or parasympathetic neurons [84, 85]. Cromaffin cells, residing in the medulla of the adrenal gland, originate from the same common sympathoadrenal progenitor cell as the neurons of the sympathetic nervous system and function by secreting "fight or flight" catecholamine hormones, adrenaline and noradrenaline, directly into the circulation [86].

Neuroblastoma is the most common solid childhood malignancy, arising from immature neural crest cells in the sympathetic nervous system, with the primary tumor residing in the adrenal gland or along the sympathetic ganglia [87]. In Sweden, neuroblastoma accounts for about 6% of all childhood tumors and around 20 children are diagnosed every year [88] with a median age of diagnosis of 18 months [87].

The survival of neuroblastoma patients has improved during the last decades due to better treatment options with an overall survival of about 75%. However, neuroblastoma is a heterogenous disease and patients with less differentiated tumors have shown a worse prognosis. Most neuroblastomas arise sporadically (98%) with amplification of *MYCN* gene (>10 copies) being one of the most prevalent chromosomal aberrations [87]. MYCN encodes a transcription factor that has been implicated to affect proliferation and differentiation of neural crest cells [89] and is associated with rapid progression, aggressive phenotype and poor prognosis n neuroblastoma [87]. In rare cases (2%) neuroblastoma are familial and can depend on germline mutations in *ALK* [90] or *PHOX2B* genes amongst others [91].

According to the International neuroblastoma staging system (INSS) the tumors can be divided into five stages based on clinical, radiographical and surgical evaluations which can be used for characterization and treatment prediction. Stage 1 tumors are localized, well differentiated and often show a good prognosis, whereas patients with metastatic stage 4 tumors have a worse outcome [92]. The fifth group, called 4S, is less characterized, but the patients are diagnosed before the age of 1 year and show a

restricted metastatic pattern to the liver, skin and bone. These patients show a favorable prognosis, mainly due to spontaneous regression [93]. Tumors in low-risk patients of stage 1-3 are often surgically removed, whereas patients with intermediate tumors and established lymph node involvement receive chemotherapy in addition to surgery. More aggressive metastatic tumors are treated with a combination of the above along with radiotherapy [94].

Protein kinase C

Protein kinases are regulatory proteins with enzymatic activity, mainly functioning as signaling molecules in the cell by adding a phosphate group to serine, threonine or tyrosine residues of the substrate. One large group of serine/threonine kinases is the AGC kinases, named after the most prominent members, protein kinase A (PKA), PKG and PKC, and characterized for the similarities in the catalytic domain sequence [95-98].

PKC isoforms and their structure

PKC is a family of serine/threonine kinases, consisting of 10 isoforms that arise from nine different genes [99]. The family members are divided into three different groups (classical, novel and atypical) depending on structure and activation (Figure 3a). Most isoforms contain four conserved subdomains (C1-C4), with C1 and C2 residing in the class-specific regulatory domain in the N-terminal region and C3 and C4 in the catalytic domain localized in the C-terminal region. PKCs can all be activated by the interaction with phosphatidylserine, but where the novel PKCs only need additional diacylglycerol (DAG) for full activation, classical PKC activation is dependent also on Ca^{2+} ions. The atypical PKCs differ from the other classes in that they are insensitive to both DAG and Ca^{2+}.

Regulatory domain

The classical (or conventional) group of PKCs consists of the isoforms α, βI/βII and γ, where βI and βII are alternatively spliced variants of the same gene [100]. The structure of classical PKCs consists of two C1 domains, denoted C1a and C1b, in the N-terminal region, involved in the interaction of DAG [101] or phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) [102]. Both C1 domains share a similar sequence and function, but have shown differences in affinity for DAG and phorbol esters under certain circumstances and for different isoforms [103, 104]. A cysteine-rich region in the C1 domain creates a binding pocket for DAG and phorbol

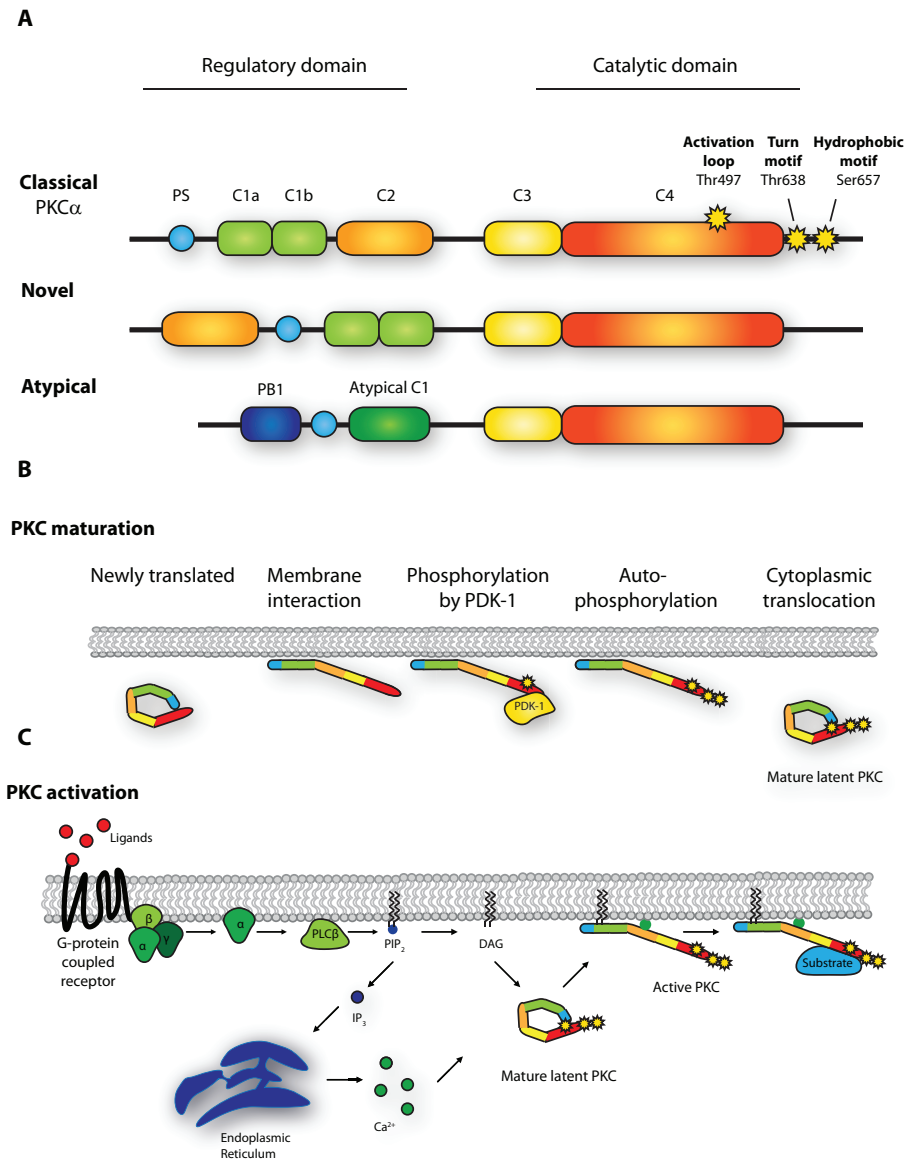
esters, enabling hydrophobic residues in the C1 domain to penetrate into the membrane and create a stable interaction [105, 106]. The DAG interaction also mediates a conformational change of PKC, resulting in a release of the pseudosubstrate from its binding with the catalytic site and enabling access for PKC substrates. The classical C2 domain binds phospholipids in a calcium-dependent manner, mediating the interaction of PKC to the membrane. Aspartic acid residues interact with phosphatidylserine of the cellular membrane, a process that is necessary for subsequent C1 domain binding to DAG [107]. PKC localization to the membrane has been suggested to be mediated by receptors for activated C kinases (RACKs), through interaction with the regulatory domain, although this has not been verified for all isoforms [108, 109].

The novel PKCs (δ, ε, η and θ) display a different alignment of the subdomains in the regulatory domain, with a calcium-insensitive C2 domain in the N-terminal region followed by tandem C1 domains. Although not sensitive to calcium, this C2-like domain is still important for PKC activation. It can interact with proteins, such as RACKs, for cellular translocation, but also mediate membrane anchorage by interacting with phosphatidic acid [110]. In addition, the C2-like domain has been suggested to have an auto-inhibitory effect by blocking the DAG binding to the C1 domain and removal of the C2 domain in novel isoforms has been shown to increase protein translocation to the plasma membrane [111] [112].

The structure of atypical PKCs (ζ and ι/λ) contains a C1-like domain, without the residues important for DAG-binding, and lacks a C2 domain. These proteins are thereby neither activated by DAG interaction nor by calcium stimulation. Instead, the atypical isoforms carry a phox-Bem1 (PB1) domain which mediates protein-interactions and thereby activation of the protein [113].

Catalytic domain

The catalytic domain is located in the C-terminal region and shares a common conserved sequence among the PKC isoforms with high similarity [114]. The ATP-binding site resides in the C3 domain, from which the PKC catalyzes hydrolysis of ATP, enabling transfer of a phosphate group to the substrate and subsequent downstream signaling. Point mutation of a lysine residue in the ATP-binding site results in a catalytically inactive PKC as a consequence of the abrogation of its phosphotransfer function, and is often experimentally used [115, 116]. The PKC signaling transduction through substrate phosphorylation is enacted at the substrate-binding site in the C4 domain. A large number of proteins have been shown to be



Figur 3. Schematic overview of the PKC structure and regulation. (A) The PKC isoforms can be divided into three classes depending on structure and function; the classical, novel and atypical isoforms. Phosphorylation sites, depicted on PKCα are important for PKC maturation. (B and C) Newly translated PKC is translocated to the cellular membrane for phosphorylation and maturation, before residing in the cytoplasm in a mature latent state. Upon stimulation, PKC translocates back to the membrane for fully activation. Maturation and activation steps are exemplified by PKCα.

phosphorylated by PKC, such as transcription factors, kinases, growth factor receptors, cytoskeletal proteins, eukaryotic initiation factors and RNA binding proteins [117], [118, 119]. Optimal isoform-specific substrate sequences have been identified, showing an importance of basic amino acids among the serine and threonine residues [120, 121].

Five variable regions (V1-V5) surrounding the conserved domains of the PKC structure have been identified and are, as the name implies, variable in size and structure between the family members. They can provide specificity for the isoform, such as V3 in the hinge region between the regulatory and catalytic domain, sensitive for caspase-dependent proteolytic cleavage during apoptosis and the C-terminal V5 region that can influence the protein translocation pattern [122] [123].

Regulation of PKCs

Maturation

Maturation of PKC by post-translational modifications is necessary for the protein to become catalytically competent and includes phosphorylation on three conserved positions. Newly translated PKC directly translocates and interacts with anionic lipids in the cellular membrane through its C1 and C2 domains as well as the newly released pseudosubstrate (Figure 3b) [124, 125]. This open conformation enables phosphoinositide-dependent kinase-1 (PDK-1) to bind to the unphosphorylated hydrophobic motif and initiate phosphorylation at the activation loop (T497 in PKCα). In classical and novel PKCs, this site is located close to the active site in C4 and contains a threonine residue. As phosphorylation is completed, PDK-1 is released, which opens up for additional phosphorylation at other residues [126].

The following maturation steps include phosphorylation at two additional positions in the V5 region of the catalytic domain, namely the turn motif (T638 in PKCα) and the hydrophobic motif (S657 in PKCα). Phosphorylation of the hydrophobic motif is mediated by autophosphorylation [127], whereas the turn motif probably depends on additional kinase activity, such as the mammalian target of rapamycin complex 2 (mTORC2), containing the serine/threonine kinase mTOR [128, 129]. Phosphorylation on these two sites is believed to have a stabilizing, yet not activating, effect [130].

Even though many PKCs require the same activators, localization and protein conformation are important factors that regulate which isoforms will be activated. For

example, phosphorylation by PDK-1 only affects PKCs in a membrane bound state, when the pseudosubstrate is released. As PKC becomes mature, it adopts a closed conformation that is more resistant to phosphatases, proteinases and variations in temperature. This latent state involves binding of the pseudosubstrate to the substrate-binding pocket as well as interaction of phosphorylated hydrophobic motif to a phospho-hydrophobic site binding pocket [131]. Mature latent PKC is released from the membrane and diffuses into the cytosol until stimulating signals like DAG and Ca^{2+} facilitate PKC activation and possible substrate interaction [98]. Additional phosphorylation of PKC is believed to fine-tune the function of the enzyme in its substrate selection [132]. Unphosphorylated PKCs on the other hand are unstable and will undergo immediate degradation [133].

Activation

Upon extracellular ligand-binding to G protein-coupled receptors or tyrosine kinase receptors a signaling cascade, that enables activation of PKC, is initiated through phospholipase Cβ (PLCβ) or PLCγ signaling, respectively (Figure 3c). PLC hydrolyzes the membrane bound phosphatidylinositol-4,5-bisphosphate (PIP_2) resulting in two products; the membrane bound DAG and the soluble inositol triphosphate (IP3). IP3 can thus mediate release of Ca^{2+} from the endoplasmic reticulum (ER) into the cytoplasm [134]. The Ca^{2+} ions can then bind to the C2 domain of mature and competent classical PKCs and increase attraction to the cellular membrane by altering the electrostatic potential of PKC [124, 135]. The lipid binding to C2 of classical and novel PKCs is enhanced by DAG interaction with C1 domains, leading to a conformational change and release of the pseudosubstrate from the substrate-binding site [136].

Membrane-bound, active PKCs are sensitive to dephosphorylation and as the need for further signaling is lost, the molecule gets degraded via poorly understood mechanisms, although both endosomal and proteasomal degradation have been suggested [137, 138]. In addition, chronic stimulation of phorbol esters can result in increased degradation of PKC [138]. Heat-shock proteins (HSP) have been shown to influence the PKC turnover, either through increased phosphorylation, through HSP90, or by protecting dephosphorylated turn motifs through HSP70, and thus enabling re-phosphorylation [139].

PKCα in cancer

PKCα is in general suggested to be a pro-tumorigenic protein, since PKCα can induce tumor growth, progression and invasion [140] as well as inhibit apoptosis both i*n vivo* and *in vitro* [97, 141, 142], *e.g.* through regulation of the anti-apoptotic Bcl-2 protein [143]. On the contrary, PKCα-deficient mice show elevated intestinal tumor formation with earlier onset and more aggressive tumors [144]. This reflects the great diversity of PKC functions, which not only depends on the isoform expressed, but also on tissue distribution, subcellular localization and condition of the cell.

PKCα is abundantly expressed in many tissues, but even though PKCα is coupled to tumorigenic events there are no general conclusions to be drawn from its expression pattern in tumor tissue. PKCα has been reported to be highly expressed in high-grade urinary bladder and endometrial cancers [145, 146], whereas hepatocellular carcinoma and colon tumors display decreased levels [147, 148]. This complex picture is further corroborated by high-grade glioma cell lines that demonstrate a high expression of PKCα, whereas tumors rarely show any variation in expression compared to normal brain tissue [149].

In breast cancer, the expression of PKCα is generally lower compared to non-malignant tissues [150], but in relation to tumor grade, reports have shown both positive and negative correlations [151, 152]. The expression of PKCα has been coupled to estrogen receptor negative tumors [153-156] and increased activity correlates with HER2 amplification [157]. In addition, increased PKCα expression can lead to a loss of ER-positivity in MCF-7 cells along with other features that can be coupled to a more aggressive phenotype, such as increased proliferation [158]. As a consequence of the increase in hormonal receptors, PKCα-low tumors respond better to endocrine treatment and are associated with a better prognosis [152, 159, 160].

PKCα has recently been identified as a marker for cancer aggressiveness [152] and has been shown to induce migration in breast cancer cell lines [152, 161]. In concordance with this, inhibition of PKCα with an isotype-specific V5-region peptide (αV5-3), led to reduced intravasation and metastasis through reduction of matrix metalloproteinase 9 (MMP9) in a mammary tumor-bearing mouse model [162]. PKCα has also been reported to be highly expressed and activated specifically in $CD44^{hi}/CD24^{lo}$ mammary epithelial cells. Inhibition of PKCα in these cells induces depletion of stem-like cells and decreased tumor growth, indicating that PKCα may function as a potential therapeutic target for elimination of cancer stem cells within the tumor [156].

Therapeutics

The work of discovering PKC-specific drugs has been a difficult task because of the non-specificity both regarding targeting a certain isoform and for distribution to the right cellular compartment. The expected effect by using protein kinase inhibitors targeting the kinase domain with ATP-competitive compounds (*e.g.* Staurosporin, Rottlerin, Enzastaurin), and thereby inhibiting downstream signaling, was shown to be unspecific due to the presence of ATP-binding sites in all isoforms. As a consequence, development of novel PKC-targeting drugs has focused on more divergent regions and inhibitors affecting protein interactions or substrate binding have been established, mainly targeting the C2 domain, since it is the least conserved region among the isoforms [163, 164].

In PKCα-targeted therapy, aprinocarsen (LY900003) was a promising drug for tumor reduction in several cancers, especially non-small cell lung carcinoma. By using anti-sense oligonucleotides that complementary bind to the 3' UTR of PKCα mRNA (*PRKCA*), aprinocarsen could block translation. However, randomized phase III studies showed no differences in metastasis or survival compared to control samples and no clinical trials are ongoing as of today [165, 166]. Few other therapeutic agents against PKCα have reached clinical trials. The lactone bryostatin 1 showed a modulating effect on PKC activation, where long exposure could induce loss of membrane interaction and hence diminished activity [167]. However, in phase II studies, bryostatin 1 could only show minimal anti-tumor effects and is therefore not a part of any ongoing studies [168]. Other clinical trials have involved the ATP-competitive midostaurin and enzastaurin, which have shown promising effects on single malignancies, such as leukemia and advanced brain lesions, alone or in combinational therapies [163].

RNA metabolism

The central dogma of molecular biology was postulated by Francis Crick in 1958 (revised in 1970) and comprised how the flow of genetic information, residing in the DNA is transcribed into mRNA molecules that serve as templates for the ribosomal protein translation [169]. Even though this is still the fundamental principle, later studies have pointed out more complex regulation processes and even non-coding RNAs are now well known regulators of RNA expression through a process called RNA interference (discussed below) [170].

RNA regulation is important, not only for spatiotemporally-specific transcription and degradation of the molecules, but also for taking care of errors that occur during mRNA processing. The RNA metabolism comprises all the regulatory steps affecting the RNA, including transcription and translation as well as post-transcriptional modifications such as mRNA processing, localization and stabilization of RNA [171]. Balance of these processes is of importance and defects can result in various oncogenic features [172].

The half-life of mRNAs varies between minutes and days and a precise regulation of mRNA turnover is important for maintaining the steady-state gene expression levels of the cell [173]. Eukaryotic mRNAs are protected by a 7-methylguanosine cap in the 5' end along with a poly-A tail in the 3' end and the conventional decay pathway is initiated by deadenylation of the poly-A tail by specific enzymes. Once the deadenylation is initiated, processing of the 5' cap by the decapping enzymes Dcp1/2 will follow, enabling exonucleolytic degradation by Xrn1 in a 5' to 3′direction or by exosomes in a 3' to 5' direction. For unstable short-lived mRNAs, an endoribonuclease-mediated decay process is active, binding directly to the mRNA body or the 3' UTR without initial deadenylation [174, 175].

Translation initiation

One of the most important regulatory steps for RNA metabolism is the translational initiation, but this is also one of the most deregulated processes in cancer. Eukaryotic translation initiation is a precisely regulated process required for the onset of protein

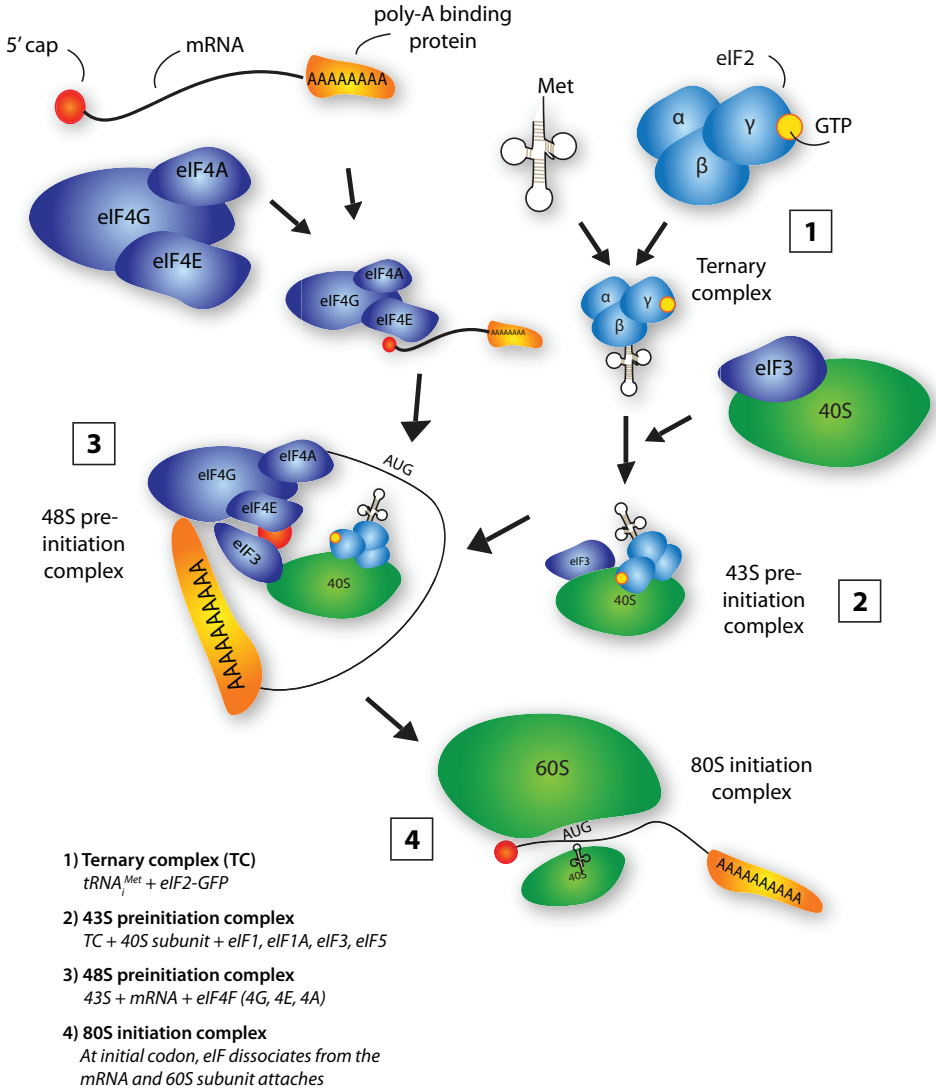
synthesis for most 5'cap mRNAs, although internal ribosome entry sites (IRES) identified in the middle of the mRNA sequence can be used by RNA viruses or cells in mitosis to ignore or even repress regular translation and induce production of specific proteins [176]. Initiation of 5' cap mediated translation requires the binding of the ternary complex (eIF2-GTP-$tRNA_i^{Met}$) to the small ribosomal subunit 40S, creating a 43S pre-initiation complex, followed by subsequent recruitment to mRNAs by a complex of eukaryotic translation initiation factors (eIFs) called eIF4F. The 43S complex scans the mRNA for identification of the initial codon and not until then is the eIF2-bound GTP hydrolyzed, initiation factors dissociated and the 60S ribosomal subunit attached for translation initiation (briefly summarized in Figure 4) [176, 177].

Cellular stress response

To survive stressful conditions, the cells have evolved an ability to regulate protein translation and alter the translational balance to produce more stress-protective proteins, in a process called stress response. Precise regulation of these processes is of importance for the cell and modifications in any direction can have disease promoting effects as described both for Parkinson´s disease [178] and cancer [179]. Described below are some of the most common stress responses.

Heat shock response is induced in cells exposed to elevated temperatures (3-5°C above the physiological level), but also oxidative stress and heavy metals can activate this response. Under normal conditions heat shock factors (HSFs) are maintained in an inactive state by HSP90. Upon stress, unfolded proteins compete with HSFs for HSP90, which releases the HSFs and enables them to function as transcription factors for certain protective genes, such as HSP27 and HSP70. These proteins function as chaperones, remodeling denatured unfolded proteins and preventing protein aggregation and subsequent cell death [180, 181].

Unfolded protein response (UPR) is a consequence of stress affecting the endoplasmic reticulum (ER), so called ER stress. Accumulation of unfolded proteins, due to lack of post-translational modifications such as glycosylation, will induce an activation of the three stress-related transmembrane receptors in the ER, PKR-like ER kinase (PERK), inositol-requiring protein-1 (IRE1) and activating transcription factor 6 (ATF6). Together, these proteins cause a repressed protein translation and induce ER-specific protein degradation [182].



Figur 4. Brief description of translational initiation. A ternary complex, consisting of the initial methionine tRNA (Met-tRNAi) and the carrying eIF2 associates with the small ribosomal subunit 40S creating a 43S preinitiation complex. This complex is recruited to the mRNA by an eIF4F complex forming a 48S complex. The eIF4F consist of the 5' cap-binding eIF4E, the scaffolding eIF4G and the eIF4A helicase protein promoting a structure necessary for the 60S ribosomal subunit to associate and translation to initiate. Poly-A binding protein (PABP) bound to the poly-A tail of the mRNA interacts with the eIF4G, forming a stable circularized structure favoring ribosomal cycling and enhance translation.

Moreover, stress inducing external factors can cause cellular damage and possible cell death upon sustained stimulation. Irradiation, ultraviolet (UV) light or chemotherapeutic agents can all result in DNA single or double strand breaks which leads to induced DNA damage response in the cell [183]. Oxidative stress caused by a disruption in the balance between the production of free radicals and the cells ability to create antioxidants can be triggered by induced reactive oxygen species (ROS) or hydrogen peroxide (H_2O_2) within the cell, resulting in cell death unless the stress is relieved [184]. Autophagy (self-eating) is a cellular response to metabolic stress such as growth factor deprivation, causing a lysosomal degradation of cytoplasmic organelles [185].

Several of these stress responses are connected and one effector does not only result in one kind of response. Many of the environmental stress factors can induce phosphorylation of the eIF2α subunit at Ser51 and block the eIF2B-mediated exchange of GDP to GTP, thereby preventing the formation of the ternary complex and subsequent translational arrest [186, 187]. This is one of the major events for promoting stress granule (SG) formation. The increased phosphorylation status can depend on multiple upstream kinases, including heme-regulated inhibitor (HRI), protein kinase R (PKR), general control nonderepressible 2 (GCN2) and PKR-like ER kinase (PERK), during various kinds of stress. In general, GCN2 is induced during amino acid deprivation, PKR is active in response to viral infections, HRI is influenced during heat shock and PERK is induced upon ER stress [188]. Viruses have evolved mechanisms to succeed with infection also in translationally repressed cells. Some viruses completely repress SG formation, whereas others can trigger initial phosphorylation of eIF2α, but later repress this stress response by disrupting stress factors such as RasGAP-binding protein (G3BP) [189, 190].

Stress granules and Processing bodies

In response to the various stress factors, cells block the translation of house-keeping mRNAs, leaving the mRNAs in a “ready to go”-state. Once the stress factor disappears, the ribosomal unit will reunite and translation resume. This stress-induced translational arrest was first identified during heat-shock in tomato cell cultures that demonstrated a formation of cytoplasmic aggregates consisting of mRNA and protein complexes called stress granules (SG) [191]. Even though the SGs in this initial finding in tomatoes later was shown not to contain any mRNA [192], the stress response was proven to be a well conserved phenomenon among species and in 1999, Kedersha et al. identified mammalian SGs [193]. SGs are large complexes containing translationally repressed 48S-preinitiation complexes including the ribosomal

subunits, eukaryotic initiation factors, RNA-binding proteins and the attached mRNA, indicating that this formation is due to an inhibiting process of the protein translation initiation [194]. These complexes function as a protective mechanism preventing the mRNA from being degraded.

In most cases, SG-formation is initiated by phosphorylation of the ternary complex member eIF2α at residue Ser51 (as described above), although other reports have suggested the existence of an alternative eIF2α-independent mechanism for stress granule formation by inhibiting the recruitment of eIF4A and eIF4F with pateamine or hippuristanol [195, 196] or through H_2O_2 stimulation [197]. SG assembly can as well be promoted by RNA-binding proteins, such as T-cell intracellular antigen-1 (TIA-1) and its receptor TIAR [198], Fragile X mental retardation protein (FMRP) [195], tristetraprolin (TTP) [199] and G3BP [200].

Processing bodies (PBs) are other dynamical cytoplasmic structures formed under normal conditions when required, for maintaining a balance between mRNA translation and degradation. They are small, round structures containing proteins involved in mRNA decay and silencing, including the decapping proteins (DCP1 and DCP2) and 5'-3' exonucleases (Xrn1). In addition, most of the proteins involved in microRNA (miRNA) repression by the RNA-induced silencing (RISC) complex (GW182 and Argonaute) reside in the PB [201]. Translationally inactive mRNAs can assemble into PB to undergo translational repression, degradation or translocalization depending on circumstances [202]. Although PBs are sites for mRNA decay, these turnover processes can occur also in the absence of PB molecules [203, 204] and release of mRNA from these complexes to return to translation has been reported [205] demonstrating a complicated and yet not fully understood picture of mRNA decay.

RNA-binding proteins

Ribonucleoprotein (RNP) complexes consist of mRNA molecules and associating RNA-binding proteins (RBP). RBPs function by regulating several aspects of the mRNA such as capping, splicing, deadenylation, transport, stabilization and translation. Several different RNA-binding domains have been identified, among which the RNA recognition motif (RRM) is the most common [206].

AU-rich elements (ARE) in the 3' UTR of the mRNA are target sites for many RBPs and provide for rapid regulation of mRNA expression. ARE are 50-150 nucleotides long sequences that can be divided into three classes depending on their composition and how they are regulated [207]. Activation of classical signaling pathways, such as the mitogen-activated protein (MAP)-kinase cascade, can lead to phosphorylation of RBPs which influences their regulatory effect in mRNA decay and hence affect the level of ARE-containing mRNAs [208]. Whereas most RBPs promote mRNA degradation during normal conditions (*e.g.* TTP and TIA-1) others have shown a stabilizing effect (*e.g.* human antigen R (HuR)). A major class of RBPs is the PolyA-binding proteins (PABPs), which can, through binding to the 3' polyA tail of most mRNAs, both protect the mRNA from degradation and mediate translation initiation. RRMs in the PABP sequence mediate the interaction with at least 12 adenosines of the polyA tail and since this protective tail contains up to 250 adenosines, several PABPs bind to a single mRNA. For proper translation initiation, polyA-bound PABPs bind to the initiation factor eIF4G to circularize the mRNA and create a possibility for rapid translation as the ribosomes can be recycled. In addition, PABP can affect other metabolic processes of mRNA, such as polyadenylation, mRNA transport and degradation [209].

G3BP

G3BP and its structure

G3BP is an abbreviation of Ras-GTPase-activating protein SH3 domain-binding protein and was named because of its ability to bind the Ras-GTPase-activating protein (RasGAP) [210]. Ras is a family of small GTPases, a main signaling transducer in the cell, that in an active GTP-bound state initiates downstream signaling by activating serine/threonine kinases, *e.g.* Raf. Inactivation of Ras is mediated by hydrolysis of the Ras-bound GTP molecule to a GDP with assistance of RasGAPs and will result in inhibition of further signaling [211]. G3BP was suggested to interact with the SH3 domain of RasGAP in growing cells or cells with GTP-activated Ras and thus interfere with the Ras signaling [210, 212, 213], although recent studies have failed to repeat these results [214].

G3BP is a family of three homologous proteins, G3BP1, G3BP2a and the splice variant G3BP2b, with the size of 52, 54 and 50 kDa, respectively. Human G3BP1 is encoded by a gene located on chromosome 5, whereas the G3BP2 variants are located on chromosome 4 [212, 215]. The three isoforms share structural similarities, with the presence of four distinct regions; the nuclear transport factor (NTF2)-like domain, the acidic- and proline-rich region, the RRM and the arginine and glycin-rich box (RGG box) (Figure 5). The main structural difference between the G3BP2 splice variants is located in the proline-rich region, where G3BP2b lacks residues [215].

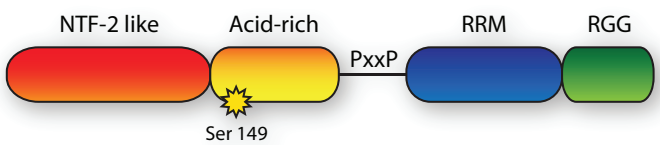


Figure 5. Schematic overview of the G3BP structure. G3BP family comprises three different variants called G3BP1, G3BP2a and G3BP2b. They all share four domains, NTF2-like, Acid-rich, RRM and RGG, and vary in sequence mainly in the proline-rich area (PxxP).

The NTF2-like domain resembles both the structure and function of the small NTF2 protein, involved in nuclear transport through nuclear pore complexes [216]. The presence of both G3BP1 and G3BP2 in the nucleus upon serum stimulation supports the nuclear-transporting functions of the NTF2-like domain [217, 218], however in a study of mutations in G3BP2a, the NTF2-like domain was more important for distribution to the nuclear envelope and not actual nuclear translocation [219]. The NTF2-like domain has, in addition, been shown to mediate protein interaction [210, 220] and can facilitate G3BP dimerization [200].

The center of the G3BP sequence, where a conserved acid- and proline-rich region (PxxP) resides, has been suggested to influence protein binding to aromatic amino acids in target SH3 domains [221] and was hence believed to mediate the RasGAP interaction [210, 212]. Although the protein interaction mediated by this region has been questioned [214], it has been indicated to mediate G3BP binding to *BART* mRNA [222]. G3BP2a contains four conserved PxxP regions, whereas G3BP2b, due to the alternative splicing, has five of them [212]. G3BP1 only contains one PxxP motif, which might limit its protein interacting capacity [223].

The RRM contains two consensus sequences named RNP1 and RNP2, which in their three-dimensional structure, create a platform of alpha helixes and beta sheets for interaction with a RNA sequence of 2-8 nucleotides [212, 224]. The RNP1 and RNP2 are very similar between the G3BP isoforms, with the main variance in the RNP2 region, which may influence the RNA-binding specificity [212]. In addition to its RNA-binding function, RRM can interact with proteins which may influence the specificity of RNA-RRM interaction [225].

RGG box is a sequence of closely located arginine-glycine-glycine residues, hence the name RGG, which has been identified in many RBPs. It often has an undefined structure due to the larger polar amino acids, such as arginine, that surrounds the glycine residues. This open structure influences interaction with proteins or RNA and enables post-translational modifications [226]. Modification through methylation of arginine residues might affect protein and RNA interactions by blocking the hydrogen-bonds important for binding [227]. Arginine methylation of heterogeneous nuclear RNP (hnRNP) A1 has been shown to induce nuclear export [228], whereas in G3BP1, methylation at R435 in the RGG-region (Uniprot: Q13283) could regulate the stability of *CTNNB1* (β-catenin) mRNA in a Wnt-dependent manner [229]. Interaction with the 3' UTR of mRNA has been proposed to be mediated by a structure of four guanine residues (G-quartet) in the RGG boxes, as described for FMRP [230]. This structure has also been identified in nucleolin-binding and subsequent repression of *c-MYC* mRNA [231], although no such binding has been reported for G3BP proteins. In addition to RNA- and protein-interactions, RGG

regions might influence an ATP- and Mg^{2+}-dependent RNA/DNA helicase activity in G3BP [232].

G3BP functions

All G3BP isoforms are expressed in most normal cells, although some isoform-specific tissue expression has been identified for G3BP1 in lung and kidney, G3BP2a in brain and G3BP2b in small intestine [212]. Over-expression has also been detected in many different tumor types *e.g.* breast [217, 218, 233], pancreas [222], thyroid, colon, head and neck tumors [233] as well as in several cancer cell lines [222, 233]. The expression of all three G3BP variants is primarily cytoplasmic [210], although differences in distribution has been indicated between the isoforms. G3BP2 can localize to the nucleus in serum-stimulated cells [217], whereas G3BP1 may reside in nucleus also in quiescent cells, probably due to phosphorylation at Ser149 [213, 234].

Functional studies have indicated a role for G3BP proteins in cellular proliferation. G3BP1 was found to be highly expressed in proliferating retinal pigment epithelial cells [235] and in fibroblast cells, G3BP-mutants, lacking the RNA-binding domain, lose their ability to induce cell cycle progression, mainly as a result of impaired S-phase entry [233]. In addition, G3BP1-deficient mice showed fetal growth retardation during embryonic stages and, although viable at birth, these mice displayed a severe neuronal cell death, resulting in embryonic lethality [236]. Fibroblasts from these $G3BP1^{-/-}$ knock-out mice displayed reduced proliferation further supporting the role of G3BP1 as a growth inducing agent. Yet, the reports regarding effects on cell cycle distribution remain unclear since later studies of $G3BP^{-/-}$ fibroblasts were unable to detect any cell cycle arrest [236]. Deficiency of the Drosophila G3BP-homolog Rasputin (so named because of its connection to the Ras-signaling pathway), only resulted in defects in photoreceptor recruitment in the development of the eyes [237]. Altogether, these results indicate growth-inducing capacities by G3BP.

G3BP and mRNA interaction

Studies of G3BP proteins have to a large extent focused on their roles as RNA-binding proteins, although to date no direct RNA targets for G3BP2 have been identified. G3BP1, however, was early identified to interact with and regulate the expression of *c-MYC* mRNA [213]. In a phosphorylation dependent manner, G3BP

displayed endonuclease activity resulting in cleavage in the 3' UTR. This degrading effect has later been reported by others [234, 238] and can be attributed to other mRNAs as well; *BART* [222], *CTNNB1* [229], *IGF-II* and *GAS5* [236]. In rare cases, G3BP has been implicated in mRNA-stabilization *e.g.* for *TAU* [239] and *CDK7* [240]. Since unphosphorylated G3BP result in cell proliferation, whereas phosphorylated G3BP displays endoribonuclease activity, it has been proposed that the phosphorylation status of G3BP may function as a cell growth switch, where phosphorylated G3BP can induce degradation of growth-related mRNAs and thus reduce cellular proliferation [215].

Several of the G3BP-regulated mRNAs have demonstrated effects on cell growth both in a positive (*c-MYC* [213] and *CDK7* [240]) and a negative way (*GAS5* [236]). Using an in vitro SELEX (systemic evolution of ligands by exponential enrichment) technique, Tourriere et al. identified a best guess consensus site for substrate specificity (ACCC(A/C)(U/C)(A/C)(C/G)GC(C/A)(G/C). G3BP interaction to this target site mediates for cleavage at CA dinucleotide-rich regions in the 3' UTR [234]. The RNA-binding specificity of G3BP has in several cases been indicated to be influenced by protein interactions. CD24 interaction with G3BP can inhibit *BART* mRNA decay which leads to increased invasion capacities of pancreatic cancer cells [222] and Caprin-1 can interact with G3BP1 and affect its localization to stress granules [220].

G3BP and stress granule formation

The activity of G3BP proteins seems to vary depending on the condition of the cell, even though the general function may be to influence proliferation and survival. Both G3BP1 and G3BP2 can associate with polysome-associated mRNP complexes [239, 241] and the main function for this may be to protect mRNAs from translation initiation and induce stress granule assembly.

The dynamic shuttling of mRNPs between translating polysomes and translationally arrested compartments such as stress granules (SGs) might be a consequence of mRNA sorting. SG formation is induced due to exposure of stressful agents, but both duration and the form of stress may influence the components of the SG. Some of the most common stress-inducing agents are heat shock and arsenite, which through various ways can block translation initiation. Arsenite is a chemical compound derived from arsenic and a toxic agent causing cellular stress and subsequent cancer and neuropathy development, in part by inducing signaling cascades such as the MAP kinase pathway [242]. Stress induction with arsenite results in an oxidative stress

response and HRI activation, which causes a phosphorylation of eIF2α and subsequent translational arrest [243]. Heat shock response leads to a rapid production of protective proteins and subsequent activation of HRI [244]. Both G3BP1 and G3BP2 have been identified in eIF2α-induced SGs (G3BP1 [200], G3BP2, shown in Paper I), but they have also been reported to induce stress granules independently [245]. G3BPs are recruited to SGs in a dephosphorylation dependent manner indicating that phosphorylation status of G3BP might influence the fate of the mRNA by protecting it from degradation during cellular stress. Under normal conditions, G3BP is phosphorylated, causing mRNA degradation, whereas upon cellular stress, unphosphorylated G3BP can oligomerize and bring mRNA to the stress granule. In concordance with this, arsenite leads to dephosphorylation of G3BP at Ser149 and subsequent SG formation [200].

In addition to the stress inducing agents, overexpression of several RBP such as TIA-1, CPEB1, cold-inducible RNA-binding protein and G3BP as well as inhibition of components of the initiation complex [195, 196] can induce SG formation [198, 200, 246]. Although many stress factors are known to induce eIF2α phosphorylation with subsequent translational repression, induction of stress granules by G3BP was independent of this initial phosphorylation and could instead influence the translational complex later by inducing a PKR-mediated eIF2α phosphorylation [238]. Yet other reports have shown that overexpression of the C-terminal G3BP region can induce eIF2α phosphorylation [222] and there have been contradictory reports regarding whether these G3BP-induced SGs colocalize with the SG marker TIA1 [200, 238, 247].

G3BP as a cancer marker and drug target

The overexpressed levels of G3BP in human cancers, the reported functions in growth-related signaling pathways and the role in stress granule assembly indicate that G3BP proteins could be a potential target in anticancer therapy, although no relationships have been reported between expression and clinicopathological parameters [217]. G3BP-RasGAP interaction was previously suggested to occur in proliferating cells in a RasGAP-dependent manner and since G3BP-interacting RasGAP-derived peptides showed cytotoxic effect on tumor cells but not normal cells [248], a study was conducted to evaluate the targeting potential of G3BP. The peptide GAP161 did compete with RasGAP for G3BP-interaction and led to a reduced G3BP protein expression. This further triggered an apoptotic response in colon carcinoma cells and suppressed cell growth in tumor-bearing mice, supporting the idea of targeting G3BP for novel therapies [249]. Yet, it is important to bear in

mind the neuronal cell death and embryonic lethality detected in G3BP-deficient mice [236] and to realize that targeting G3BP is not a simple task. The essential roles for RNA-binding proteins in gene expression regulation and the defects in regulation processes during cancer development, demonstrate the importance of further studies.

RNA interference

During the past decades, research has identified novel functions for RNA molecules. In addition to its role as an intermediate protein coding template, RNA molecules themselves have a regulatory role in its untranslated form [250]. The human genome encodes for RNA transcripts of long (>200 nucleotides (nt)) and small (~20-30 nt) non-coding RNA that can interfere with other RNA molecules and affect the gene regulation program [251, 252]. The small non-coding RNAs can be grouped as small interfering RNA (siRNA), Piwi-interacting RNA (piRNA) or microRNA (miRNA) depending on biogenesis and function [253]. Both siRNA and miRNA are processed by the RNA III ribonuclease Dicer and obtain a silencing function through interaction with Argonaute proteins, but whereas miRNAs have an endogenous origin, siRNAs are most often derived from exogenous sources such as viral infections [254]. In addition, miRNAs differ from siRNAs in the transcript structure they are derived from, where the longer hairpin structure of siRNA transcripts can give rise to a greater diversity of small RNAs [255]. PiRNA also function in a similar way, but regulate RNA silencing in germ cells through interaction with a certain group of Argonaute protein called piwi proteins [256].

Introduction to microRNAs

The first miRNA discovered (lin-4) was found in *Caenorhabditis elegans* 1993 [257], but it was not until 2001 that researchers identified miRNAs in other organisms [258-260] and introduced the term microRNA.

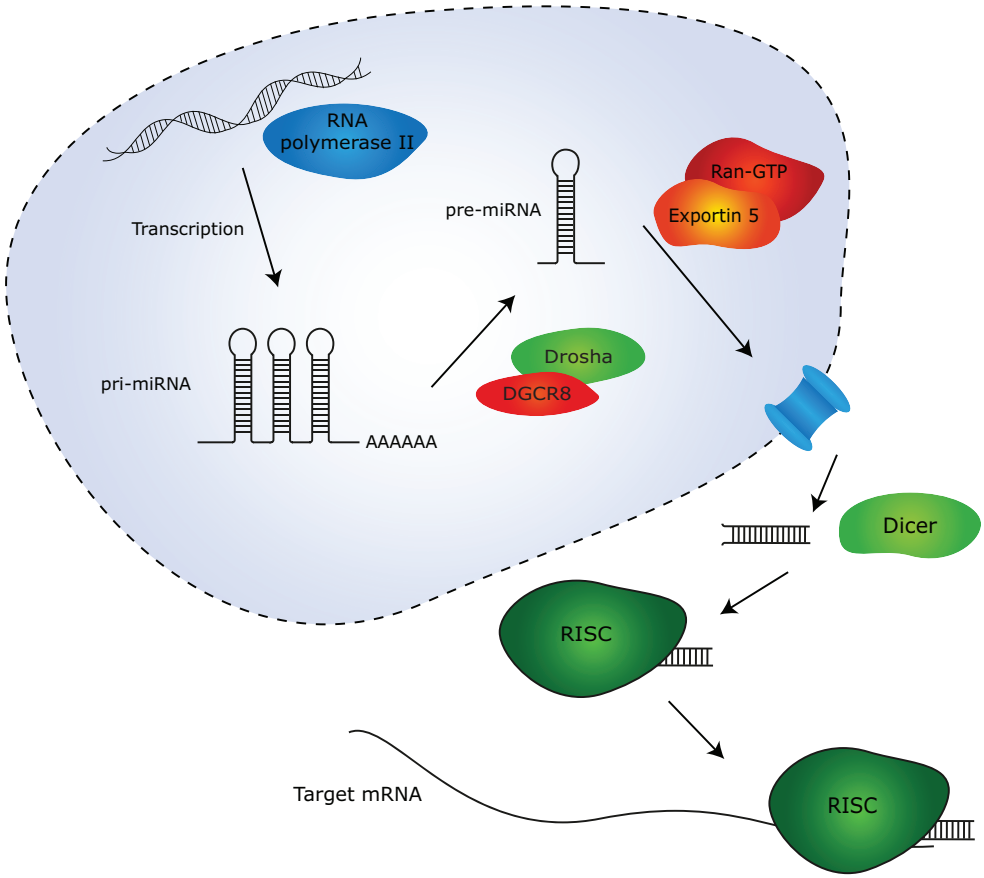
Today, more than 2000 miRNAs have been identified (according to the miRNA database (miRBase) [261]) and each miRNA can influence more than hundreds of target mRNAs. The miRNAs are 19-25 nucleotides long single-stranded RNAs, encoded by genes localized in the introns or in rare cases exons of both protein-coding and non-coding genes [262]. The miRNA encoding genes are in general transcribed by RNA polymerase II [263] resulting in capped and polyadenylated pri-miRNAs (Figure 6). These stem-loop formed transcripts are recognized by the RNA-binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) which forms a complex

with the nuclear RNase III enzyme Drosha and mediates processing of the pri-miRNA to a shorter pre-miRNA structure [264]. The hairpin formed pre-miRNAs are transported to the cytoplasm by Exportin 5 and Ran-GTP through nuclear pore complexes [265] where they are processed by the RNAse III enzyme Dicer creating double stranded ~22-nt miRNAs [266]. As the dsRNA unwinds, only the strand with the most thermodynamically stable 5' terminal will mature and associate with Argonaue (AGO) proteins, forming the RNA–induced silencing complex (RISC). The unstable passenger strand, previously denoted miRNA*, will most often be degraded [267, 268], although recent studies have identified miRNA characteristics also for the miRNA* [269]. This has led to an improved nomenclature where the guide strand has gained the suffix -5p and the passenger strand -3p [261]. The 5p strand will then guide the RISC complex to its target mRNA and bind to a complementary sequence in the 3' UTR that matches the seed region of the miRNA. The seed region is a conserved sequence of the first 2-7 nucleotides in the 5' end of the miRNA and depending on perfect or imperfect base pairing to the mRNA, the interaction will induce degradation or inhibit translation, respectively [255, 270].

The RISC consists of a variety of AGO proteins (AGO1-4 in mammals) that harbor a function of binding the 3' end of the miRNA to its PAZ domain (Piwi - Argonaute - Zwille) and the 5' end to its MID domain. Some AGO proteins have endonuclease activity (AGO2 in mammals) and upon perfect complementary miRNA-mRNA binding these mRNAs are directly cleaved [271]. Partially complementary miRNA binding requires assistance of both AGO proteins along with GW182 proteins to mediate translational repression and mRNA deadenylation [272, 273]. The function of GW182 is not fully known, but recent reports have indicated a competitive binding between GW182 and eIF4G to PABPC1, where the presence of GW182 results in a linearized and repressed mRNA [274]. As the deadenylation proceeds, decapping enzymes (DCP1 and DCP2) initiate the following degradation.

The miR-34 family

The miR-34 family consists of three homologous miRNAs located at chromosome 1p36 (miR-34a) and chromosome 11q23 (miR-34b/c). They are 22 (34a) and 23 (34b/c) nucleotides long, respectively, and share 82-86% homology. Especially the important 8 nucleotide long "seed region" in the 5'-terminal, responsible for identification of target mRNAs, is identical between the different family members [275, 276], which causes these miRNAs to control the similar sets of target genes [277].



Figur 6. Schematic of miRNA processing. Transcription of miRNA coding genes by RNA polymerase II results in production of multi hairpin formed pri-miRNA. This structure is processed by Drosha, forming a single-looped pre-miRNA that is exported via Exportin-5 to the cytosol for further processing by Dicer. Dicer removes the hairpin structure which mediates for unvinding of the double-stranded miRNA and incorporation of the single-stranded guide miRNA into the RISC complex. The seed region of the miRNA will identify target mRNA and induce degradation and/or translational repression by the catalytically active Argonaute proteins in the RISC complex.

Expression of miR-34 in cancer

The regions, in which the miR-34 genes reside, are frequently mutated in various malignancies. Deletions in 11q22-q23 have, for example, been identified in human chronic lymphocytic leukemia (CLL), prostate, lung, breast and colorectal cancers [278-282]. In addition, loss of heterozygosity in 1p36 was early identified in neuroblastoma [283], but has later been identified in various tumor types such as ovarian and breast cancer [284, 285]. In concordance with this, CpG methylations of the miR-34 promotors are frequently identified in tumor tissues, with displayed effects on the miR-34 expression as well as subsequent downstream processes, such as tumor growth, motility and metastasis [286-291]. In breast cancer, genetic aberrations in both these loci have been associated with invasiveness [288, 292]. Corresponding to these reports, miR-34 expression is reduced in a broad range of tumor types, such as prostate [293], ovarian [294], colon [295], lung [296], neuroblastoma [297] and breast [298]. The miR-34-dependent effects on such a variety of cancer types indicate the importance of miR-34 in tumor suppression.

Targets of miR-34

Due to the homology between the miR-34 family members, they to a large extent regulate similar targets [277]. These targets are often proto-oncogenic and a tightly controlled regulation of these genes enables the execution of tumor suppressor effects by miR-34. More than 30 target genes have been postulated for miR-34 and to gain the miR-induced phenotype, several of these targets must be regulated simultaneously [276]. Some of the distinct miR-34-regulated pathways affect cell cycle progression, cell senescence and apoptosis. The G1/S cell cycle arrest [277, 299] can be explained by the targeting of the cell growth related genes *CCND1*, *CCNE2*, *CDK4*, *CDK6* and *E2F3* [277, 291, 300, 301] and proliferation inhibition can further be mediated by regulation of the signaling molecules MET and Myc [302-304]. Myc is constitutively active in many tumors and can thus induce gene expression and promote oncogenic signaling. In this matter, Myc can also regulate the expression of miRNAs, both by inducing oncogenic miRNAs such as miR-17-92 and by inhibiting tumor-suppressive miRNAs like miR-34a [305-307]. The miR-34 family members can also repress *BCL2* and *SIRT* and thus induce apoptosis [308-310]. On the contrary, miR-34c may protect lung cancer cells from paclitaxel-induced apoptosis, further proving the complexity of miRNA regulation on gene expression [311].

Regulation of miR-34 by p53

The transcription factor p53 is a well-studied tumor suppressor protein, mainly responsible for inducing cell cycle arrest and eliciting apoptosis. Through DNA binding, it induces several downstream proteins, such as p21, a cyclin-dependent kinase inhibitor (CKI) that blocks the cell cycle progression in the G1/S transition phase. Several miRNAs have also been identified as p53-targets and the miR-34 family belongs to one of the most studied. Although most studies report of the p53-induced effect through miR-34a [312], also miR-34b and miR-34c can be regulated in a p53-dependent manner [277, 294, 299, 313]. On the other hand, miR-34-deficient mouse models displayed no impact on p53-regulated cell cycle inhibition or apoptosis and no increased tumor take [314], indicating a complex and probably context-dependent regulation of miR-34 by p53. In addition, miR-34a can in a p53-independent manner induce a G2/M arrest with increased abundance of mitotic cells and a subsequent mitotic catastrophe response rather than DNA damage response due to X-ray irradiation [315]. During mitosis, the E3 ubiquitin ligase, anaphase-promoting complex (APC), plays a pivotal role in the metaphase-anaphase stages and the separation of the two sister chromatids [316]. One member of the APC is the cell division cycle homolog 23 (CDC23), which was reported to be highly expressed in thyroid cancer cells [317]. Moreover, *CDC23* has been indicated to be a target of miR-34a in colon carcinoma and was significantly downregulated in a miR-34c-dependent manner in prostate cancer cells and in breast cancer cells as reported in paper III [302, 318].

Implication of miR-34 in replacement therapy

Among miR-based therapies, anti-miR, where a complementary anti-sense nucleotide strand will bind and reduce the efficacy of the miRNA, is the most widely used approach and has showed promising results for many miRNAs [319]. However, this method is only applicable when the miRNAs are overexpressed. To counteract the frequent miR deficiencies in cancer cells, replacement therapies have shown to be a new promising aspect. By delivering miR-34a systemically with nanoparticles or adeno-associated viruses, animal models carrying various tumor types have shown a reduced tumor burden along with increased apoptotic features, reduced proliferation as detected by Ki67 and reduction in expression of common miR-34a targets [276]. However, this needs the presence of tissue specific promotors or addition of tumor-targeting antibodies. In an ongoing phase I clinical trial of primary liver cancers, a miR-34a mimic (MRX34) will be delivered through a pH-sensitive liposome that will adhere to tumor cells where the pH tends to be lower. It is important to note that a

larger difference in expression between tumor and normal tissue is necessary for accurate results. This is why miR-34a, highly expressed in normal tissue, can be suitable for novel miRNA replacement therapy [168, 276].

Peripheral nervous system and its composition

The peripheral nervous system (PNS) consists of nerve cells that transmit signals from the central nervous system (*e.g.* brain and spinal cord) to limbs and organs in the body. Input signals from several dendrites are gathered in the cell body (soma) and only signals strong enough will be passed along the neural axon and transmitted to adjacent neurons (Figure 7). The PNS is subdivided into the somatic and autonomic nervous system, responsible for voluntary and involuntary body movements, respectively. The axons of both peripheral and central neurons are surrounded by non-neuronal glial cells; oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. These cells are wrapped around the axons creating a fatty matter called myelin, which insulates the axons and mediates increased speed of electrical impulses in the axons [320, 321]. Along the axons, the myelin sheaths, also denoted internodal segments, are interrupted by unmyelinated gaps called nodes of Ranvier. They contain a high density of voltage-gated sodium channels, which mediate sodium intake, generating the action potential and transmission of electrical impulses. In between the node of Ranvier and the internodal segments resides a region called the paranodal region, with a high expression of proteins forming a tight complex with the axon and participates in isolating the nodal region [322].

Myelin proteins

The myelin composition is mainly characterized by a high proportion of water and lipids, whereas only a proportion of about 15-30% are proteins with specific expression regions. The most abundant myelin protein (about 50%) is the myelin protein zero (MPZ or P0), a glycoprotein that has been postulated to maintain the compact structure of the myelin sheath through interaction with other myelin-associated proteins. In addition, frequently expressed myelin proteins are myelin basic

protein (MBP), myelin-associated glycoprotein (MAG) and peripheral myelin protein (PMP22) [323].

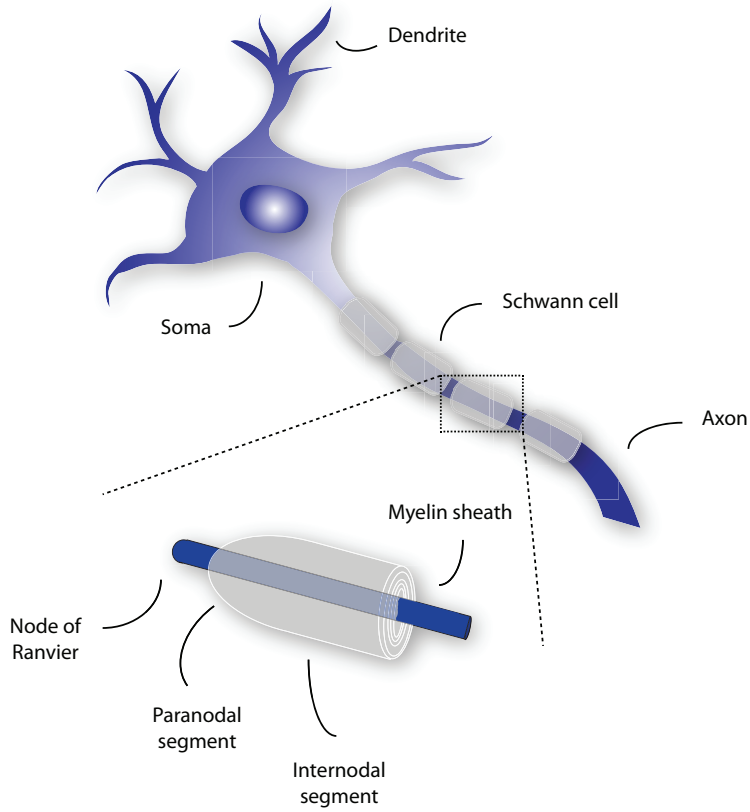


Figure 7. Schematic structure of a peripheral nerve cell. Myelinating Schwann cells enwrap their membrane around the axon to promote signal transduction. In between each internode resides the node of Ranvier, carrying important voltage gated channels. The paranodal region is located in the terminal part of the internodes and contains proteins enabling tight complex formation with the axon.

Peripheral myelin protein 22

PMP22 is a tetraspan 22 kDa protein, first identified as PASII from bovine peripheral myelin [324], but has later also been designated SR13 [325], pCD25 [326] and GAS3, because of its growth-arrest specific capacities (Figure 8) [327, 328]. PMP22 is one of the main proteins in peripheral nerve fibers (2-5%), but only scantily expressed in central neurons [329], and although dysfunctional PMP22 mainly has been coupled to peripheral neurological diseases, PMP22 expression has been identified in other tissues, including muscle, lung and intestine [329-332].

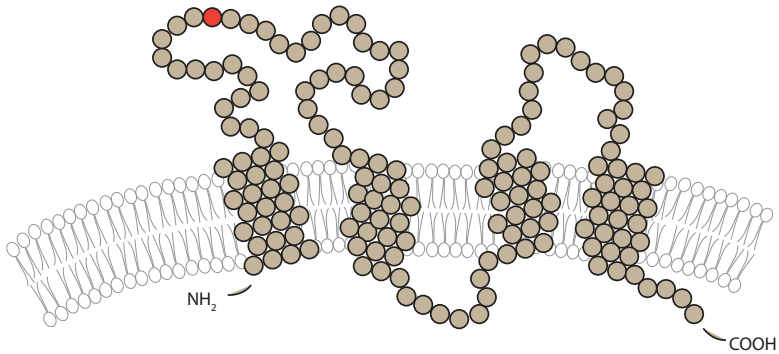
Expression of PMP22 has been reported to be initiated by the small GTPase Rho [333] as well as by cAMP [334], although exact mechanisms for these regulations remain unknown. In addition, IL-6 stimulation of Schwann cells can initiate PMP22 production in a Jak2 dependent manner, a process that can be counteracted by distribution of ascorbic acid [335]. Also post-transcriptional regulation can affect the expression of PMP22 both through 5' and 3' UTR interactions [328, 336] and proliferation-specific miR-29a expression induces PMP22 degradation in cultured Schwann cells [337]. Transcription of PMP22 can start at two alternative promotors (1A and 1B), giving rise to almost identical transcripts that only differ in the 5' untranslated region [330]. The PMP22 transcripts seem to be expressed in various tissues and under different conditions, with 1A expressed mainly in peripheral tissue and 1B in non-neuronal tissue [338]. Post-translational modification of PMP22 through N-glycosylation of the asparagine residue (N41) in the first extra cellular loop is necessary for protein stabilization as well as for membrane translocation [339]. In addition, N-glycosylation is required for cell growth regulatory capacity, but not for apoptotic induction [333].

PMP22 belongs to a tumor-associated membrane protein (TMP) family of tetra-transmembrane glycoproteins, including the epithelial membrane proteins EMP1, EMP2, EMP3 and the genetically more distant lens membrane protein MP20 [331, 340]. Although EMP and MP20 have not been identified to have any function in peripheral nervous system, they all seem to be important for growth, differentiation and apoptosis [341]. In addition, PMP22 has structural similarities with claudin proteins and PMP22 has been proven to be a component of intracellular junctions in epithelial cells of colon and liver [342].

Expression, neural damage and neurodegenerative disorders

Schwann cell development is dependent on axonal contact for initial proliferation and subsequent differentiation [343]. During the first postnatal weeks, as Schwann cells stop dividing and begin to differentiate and myelinate the axons, PMP22 expression increases distinctly [329]. Upon neural damage of peripheral fibers, Schwann cells lose their axonal connection and a dedifferentiation process, called Wallerian degeneration, is initiated. This results in fragmentation and degradation of the distal part of the axon as well as the surrounding myelin sheaths and a recruitment of macrophages that together with Schwann cells mediate clearance of the nerve crush debris [344]. Under these circumstances the levels of PMP22 and other myelin proteins decline in the distally located Schwann cells [326, 329]. As the non-myelinating Schwann cells get in contact with the wounded axons, they enter the cell cycle and initiate novel Schwann cell proliferation that can constitute a path for regenerating axon fibers [345]. Not until the Schwann cell proliferation ceases, and myelin production re-occur, does the PMP22 expression increase [329].

The gene of PMP22 is located at chromosome 17p11.2, where genetic aberrations are frequent. Duplications, deletions or missense mutations of PMP22 have been encountered in various hereditary demyelinating neuropathies [346-348]. Hereditary motor and sensory neuropathy (HMSN) is a group of related diseases with early onset where the patients develop muscle atrophy and sensory neuropathy in the distal limbs with characteristic foot drop and hammer toe [349]. Charcot-Marie-Tooth disease type 1 (CMT1) is the most common HMSN caused by chromosomal duplication in either the minor locus 1q22-q23 (CMT1B) or the major locus 17p11.2-p12 (CMT1A). The most frequent type CMT1A affects 1/5000 people [350] and results in increased PMP22 expression and as a consequence, myelin deficiency and reduced conduction of neuronal signaling [351]. Deletions in the same region results in a more mild neurological disease known as Hereditary neuropathy with liability to pressure palsies (HNPP), where pressure on the nerves can cause sensation loss or pain that can last up to a year [352]. Point mutations on the other hand, results in a more severe variant of neuropathy, Dejerine-Sottas syndrome, with early onset and inability to walk as one of the worst symptoms [353]. More than 30 mutations have been identified in the PMP22 gene resulting in demyelination of peripheral neurons and studies have shown that some of these mutations can lead to accumulation of intracellular PMP22 and hence a non-functional protein [333, 347]. It is hypothesized that the myelin-associated proteins form complexes between or within the myelin sheaths and that mutated proteins lack the interacting ability and



Figur 8. Representative structure of PMP22. PMP22 is mainly identified in the myelin membrane and has been suggested to be a four transmembrane protein. Depicted in red is the N-glycosylation site on the asparagine residue (N41).

prevent the sheaths from forming compact structures. This is supported by reports demonstrating that PMP22 does not actually affect the initiation of myelination, but instead the thickness of the myelin sheaths [354].

Two spontaneous mouse mutants named Trembler and Tremble-J are available, which carry autosomal dominant point mutations in the PMP22 gene. These models demonstrate myelin defects and continued Schwann cell proliferation. In addition, their characteristics resemble the symptoms for CMT1, including limb paralysis and tremor, although functional diverseness is also observed. Furthermore, PMP22 overexpressing mouse models have been established, but these only show a pathological phenotype when carrying a high copy number in comparison to the trisomy of PMP22 in CMT1A [355].

PMP22 and Cell growth

In addition to the putative role in myelination, PMP22 has also been suggested to have a regulatory function in cell growth. Already early investigations proposed this, as PMP22 was highly expressed at growth arrest, but attenuated after serum stimulation, in Schwann cells [356] as well as in fibroblasts [327, 328, 331]. In multiple studies, either reduction or overexpression of PMP22 showed an inverse correlation between PMP22 levels and cellular proliferation [354, 356-358] and elevated levels of PMP22 lead to delayed G0/G1 to S-phase transition as seen in various types of cells [356, 359]. Concomitantly, fibroblasts overexpressing PMP22

show an apoptotic phenotype [332, 357, 360] and mutated PMP22 displays impaired cell death inducing capacities [333].

PMP22 and Cancer

PMP22 has been thoroughly studied within the field of neuroscience and its association with cell growth control. However, little has been investigated regarding its role in human cancers and the few existing studies give a complex picture. Reduced expression of PMP22 has been identified in invasive breast cancer cells and is associated with development of lung cancer in mice [360]. In addition to this, in a retrospective study of sporadic primary breast cancers, PMP22 was pointed out as an independent prognostic factor for disease-free and overall survival and showed an inverse correlation with differentiation grade [361, 362].

On the other hand, enhanced expression of PMP22 has been detected in osteosarcoma [363], pancreatic tumors [364] and glioblastoma [365] and PMP22 has been suggested to be an oncogene in human osteosarcoma due to the frequent amplification in the 17p11.2-p12 chromosomal region [366]. These reports indicate that a potential role in tumor progression may be tissue type-specific, but since both deletion and duplication of PMP22 can lead to neuropathies, this just further indicates that an exact regulation of PMP22 seems to be important.

The present investigation

Aims

The general objective of this thesis was to study cellular mechanisms affecting cell growth and survival in breast cancer and to investigate expression patterns in the surrounding stroma in breast tumors.

The specific aims were:

-To identify novel interaction partners for PKC and elucidate the role of PKCα in cellular stress response.

-To investigate the role of G3BP proteins in breast cancer cell proliferation.

-To evaluate how miRNA-34 can influence cell growth and survival of breast cancer cells.

-To establish a method for obtaining RNA with adequate quality for global gene expression analysis from microdissected formalin-fixed paraffin-embedded breast tumors.

-To delineate expression patterns in various compartments of the tumor and identify gene signatures that can be valuable for prediction of clinical outcome.

Paper I

PKCα binds G3BP2 and regulates stress granule formation following cellular stress

Previous investigations in our group have revealed an importance of the PKCε isoform in neurite outgrowth. To identify novel interaction partners, a mass spectrometry analysis was performed, which revealed several proteins bound to a neurite-inducing PKCε construct [367]. Among these, we identified the RNA-binding proteins PABPC1, IGF2BP3 and G3BP2. The interaction with the identified proteins could not be verified with endogenous PKCε in the neuroblastoma cell line SK-N-BE(2)C, however, a complex formation was detected with the PKCα isoform.

The interaction between PKCα and two of the proteins, IGF2BP3 and PABPC1, was disrupted in the presence of RNase, indicating a non-direct RNA-dependent interaction. The direct binding to G3BP2 was confirmed in an *in vitro* pull-down assay, which also verified interaction with the G3BP1 isoform. The regulatory domain of PKCα, especially the C1a domain, was important for G3BP2 interaction, whereas G3BP2 needed its RNA-binding domain for proper binding. In addition, PKCα was found to phosphorylate G3BP2, in particular the NTF2-like domain, indicating that G3BP2 may be a putative substrate for PKCα. Since G3BP2 mainly pulled down a more slowly migrating PKCα variant, we analyzed possible post-translational modifications of PKCα and found G3BP2 to mainly interact with a mature form of PKCα, phosphorylated in both the turn motif (T638) and the hydrophobic motif (S657).

The interaction of PKCα with three RNA-binding proteins led us to explore a potential role for PKCα in RNA regulation. PABPC1 as well as G3BP are localized in stress granules upon stress response induction in the cell [368] and we could detect that PKCα co-localized with stress granule proteins in response to both heat-shock and arsenite treatment. Upon heat shock, we identified the C1a domain of PKCα, responsible for G3BP2 interaction, to interact with the stress granule component TIAR, indicating that the presence of PKCα in stress granules is mediated by the C1a domain. On the other hand, no co-localization with P-bodies was seen for PKCα, indicating that PKCα is, presumably not, involved in the RNA decay process.

To evaluate if PKCα is a driver or a bystander in stress granule formation, the protein was depleted with three different siRNAs in MDA-MB-231 breast cancer cells. Knockdown of PKCα significantly reduced the stress granule formation capacity to almost 50%, which was not seen for PKCε, but prolonged stress still induces stress

granules in PKCα-depleted cells to the same extent as in control cells, suggesting that removal of PKCα may only delay the stress response. This effect was seen to be stress-inducer specific since no delay occurred upon arsenite stimulation. Removal of the stress factor led to a rapid stress granule disassembly also in the absence of PKCα, indicating that the importance of PKCα in stress granule regulation mainly involves the initiation process. In addition to the effect on stress induction, we could detect a delayed eIF2α phosphorylation when PKCα was depleted. We further analyzed if this effect could be a consequence of lost expression of any of the previously recognized eIF2α kinases. Out of the two kinases evaluated, only the HRI, mainly responsible for inducing stress response during heat shock, showed a tendency to lower expression upon PKCα-depletion, indicating that other mechanisms probably also are involved in the PKCα-regulated stress response.

Paper II

Regulation of PMP22 mRNA by G3BP1 affects cell proliferation in breast cancer cells

In Paper II, we investigated the role of G3BP proteins in breast cancer cell proliferation. G3BP proteins have been shown to be highly expressed in several tumor types [217, 218, 222, 233] and have been indicated to both induce cell growth and reduce apoptosis in various cell types [213, 222]. We could detect a decreased proliferation upon depletion of G3BP proteins in four different breast cancer cell lines, as indicated both in a [^{3}H]-thymidine incorporation assay as well as when quantified as the number of viable cells. Since G3BPs have been reported to be pro-survival factors, we analyzed if the decreased cell growth corresponds to an increase in cell death. However, knockdown of the G3BP proteins did not influence cell death, as evaluated by nuclear morphology or analyzed in an Annexin V assay, indicating that the G3BP1 effect on cell growth is not dependent on cell death regulation.

Since G3BP is a well-known RNA-binding protein, we performed a global gene expression analysis on G3BP1- and/or G3BP2-depleted MCF-7 cells to identify genes with altered expression in response to G3BP. Depletion of G3BP1resulted in altered expression of several genes, but among the genes that were most affected, only *PMP22* displayed a corresponding change in mRNA expression in a qPCR analysis. G3BP has been reported to regulate mRNA stability and hence expression of several genes, such as *c-MYC*, *CTNNB1*, *IGF-II* and *GAS5* [213, 229, 234, 236]. In our study, none of these genes were found to have a significantly altered expression, which may indicate that the difference in G3BP-regulated mRNA turnover is time- and cell

type-dependent. Our results, with only a few genes being regulated by G3BP1, correspond to other reports such as in G3BP1-deficient mouse epithelial fibroblast (MEF) cells, where neither of the previously reported G3BP1-regulated genes were detected [236].

PMP22 was initially identified as a growth arrest-specific protein (GAS3) [327, 328] and in concordance with this, we could see that depletion of PMP22 significantly increased cell growth in MCF-7 and MDA-MB-231 cells. To evaluate if the growth reduction by siG3BP1 was due to increased expression of PMP22, G3BP1 and PMP22 were simultaneously downregulated and the results indicated that depletion of PMP22 partially reversed the decreased proliferation seen by siG3BP1. PMP22 depletion in MDA-MB-231 cells did in concordance with the result in MCF-7 cells, cause an increased proliferation. The decreased effect after knockdown of G3BP1, on the other hand, was not seen in these cells. This could be due to the difference in basal expression levels between the two cell lines investigated. Since MDA-MB-231 cells express higher levels of PMP22, depletion of G3BP1 may not be able to induce it further, which would explain the lack of effect on cell proliferation by G3BP1. Unfortunately, we could not evaluate PMP22 expression on Western blot, probably due to difficulties in antibody detection since the N-glycosylation on the N-terminal extracellular loop has been suggested to influence antibody binding [332, 333]. To evaluate the functional effect of PMP22 on cell growth, we conducted an experiment with overexpressed PMP22 in MCF-7 cells (Figure 1). Cell proliferation was reduced in response to increased PMP22 expression, further supporting a growth inhibiting effect of PMP22 in breast cancer cells.

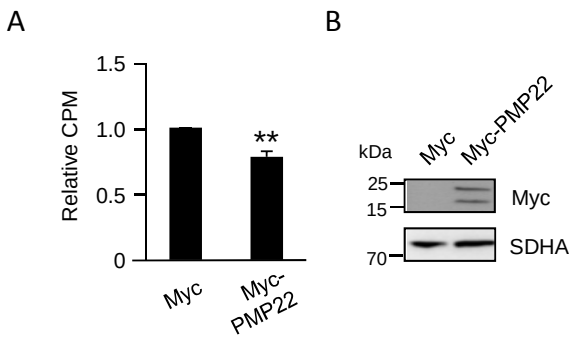


Figure 1. **PMP22 decreases cell proliferation**. MCF-7 cells were transiently transfected with expression vectors encoding Myc-tagged PMP22 for 24 hours prior to [^{3}H]-thymidine incubation of 6 hours **(A)**. Western blot confirms PMP22 overexpression **(B)**. Data (mean ± SEM, n = 3) are expressed as CPM relative to control. **< 0.01, according to Student´s *t*-test.

To investigate if G3BP1 regulates PMP22 expression through destabilization of the mRNA, MCF-7 cells were treated with Actinomycin D to block novel transcription. Analysis of the remaining PMP22 mRNA showed no change in mRNA half-life, indicating that G3BP1 does not regulate *PMP22* expression by destabilizing its mRNA.

Both G3BP1 and G3BP2 have been reported to be up-regulated in various tumor types, but only G3BP1 has been shown to influence cell growth both in tumorous [249] and non-tumorous cells [235]. In concordance, depletion of G3BP2 did not have an effect on cell proliferation in our cells and did not lead to altered gene expression, further indicating a difference between G3BP1 and G3BP2 functions. Yet, overexpression of both G3BP1 and G3BP2 proteins results in an increased proliferation and a decreased PMP22 expression, suggesting that G3BP2 still may have a functional role in cell growth regulation when expressed at sufficient levels.

Paper III

Expression of miR-34c induces G2/M cell cycle arrest in breast cancer cells

The aim of Paper III was to investigate the role of miR-34c in the regulation of cell growth in breast cancer cells. The genomic regions for the miR-34 family members are frequently deleted in solid tumors, including breast cancer [292] and deletions or silencing of these regions have been associated with increased tumor growth and metastasis [288, 290]. In previous findings, our group demonstrated that PKCα expression is correlated to breast cancer aggressiveness, poor prognosis and ER and PR negativity [152] and PKCα has been shown to be regulated by miR-34c [302]. In five different miR-target prediction programs, we identified PKCα to be a possible target for miR-34c, which led us to investigate if miR-34c can influence PKCα levels and if it has any tumor suppressive roles in breast cancer.

To investigate the expression and potential importance of miR-34 family members in breast cancer, we utilized the publicly available TCGA (The Cancer Genome Atlas) dataset. Expression data from 658 breast tumors and 86 normal breast tissue samples were analyzed. As expected, a clear correlation was detected between miR-34b and miR-34c expression levels as these miRNAs reside at the same loci. On the other hand, miR-34c, but not miR-34a or miR-34b, showed a lower expression in basal-like tumors compared to other breast cancer types as well as normal breast tissue. The result that miR-34a expression does not vary between cancerous and non-cancerous tissue was not in line with previous results, since miR-34a has previously been reported to be reduced in breast tumors compared to the adjacent non-malignant

breast tissue [308]. In addition, only miR-34b and miR-34c were shown to have a prognostic value, as lower expression correlates with the incidence of new tumor events in breast cancer.

The lower expression of miR-34c in basal-like breast tumors and the worse prognosis for patients with tumors that had low expression levels, led us to investigate if the presence of miR-34c can provide tumor suppressive functions in basal-like breast cancer cells, as has been described for other cancer cells [302, 369]. Overexpression of miR-34c led to a significant decrease in proliferation, as analyzed with thymidine incorporation, and resulted in a changed cell cycle distribution with reduced number of cells in G1 cell cycle phase along with an increase in G2/M phase. This is not in line with several other studies, which have reported that miR-34c induces G1/S arrest [277, 304]. Instead we found that miR-34c induces a G2/M arrest in breast cancer cells. It has been reported that miR-34a can inhibit the transition through the mitotic checkpoint inducing a mitotic catastrophe [315] and a miR-34c-mediated G2/M arrest was identified in pancreas cancer [370]. The reason for this difference in cell growth regulation is not clear, but could be a consequence of diversity in miR-34 target genes, which is presumably a cell type and context dependent function. Furthermore, miR-34c increased the fraction of cells in the sub-G1 phase, which may indicate an increased cell death. This was validated with an Annexin V analysis, where a two-fold increase in the number of apoptotic cells was detected in response to miR-34c expression.

In order to elucidate the mechanisms behind the miR-34–induced growth suppression we analyzed the expression of common miR-34 targets. The levels of cyclin D1, CDK4 and CDK6 were all reduced in the presence of high miR-34c expression, but since they generally influence the G1/S transition, they are probably not responsible for the G2/M arrest. The mRNA levels of the predicted miR-34c target PRKCA (PKCα) were only reduced in one (MDA-MB-231) out of three cell lines evaluated and induced in another (MDA-MB-468). This might be a consequence of the alternative expression levels of both miR-34c and PKCα. Basal B cell lines (MDA-MB-231 and BT-549) have a lower basal level of miR-34c and a more prominent PKCα expression compared to basal A cell lines (MDA-MB-468) [152, 371], which may explain why MDA-MB-231 cells are more sensitive to miR-34c. However, since no effect was seen on protein levels, PKCα is most likely not the effector on miR-34c mediated growth suppression.

In addition, we analyzed the involvement of CDC23, a member of the anaphase-promoting complex (APC) that has been reported to be a target of miR-34a [318]. In concordance with the reduced mRNA expression in prostate cancer cells upon miR-34c expression [302], *CDC23* expression also decreased in breast cancer cells. Furthermore, the protein levels were lower in all three cell lines, indicating that

CDC23 may be a potential target of miR-34c and thus function as an effector molecule for miR-34c growth suppression in breast cancer cells.

Paper IV

Identification of stromal gene signatures in breast cancer

In paper IV, we aimed at identifying characteristic gene expression profiles from isolated breast tumor compartments and analyze if they can be used as prognostic markers. To identify novel breast cancer subtypes, molecular similarities and differences between tumors have previously been studied with gene expression analyses. However, this has mainly been performed on whole tumor homogenates [18, 24, 372]. Since the tumor consists of not only malignant cells, but also various amount of different stromal cells, we wanted to investigate the characteristics of epithelial and stromal compartments in breast tumor samples. For this purpose, we utilized formalin-fixed paraffin-embedded (FFPE) breast tumor tissue, since this is the routine procedure for conservation and diagnosis of tumors after surgery and there is a vast amount of FFPE tissue available for analysis. The preservation of tissue samples with formalin fixation has a negative impact on RNA quality and reliable transcriptome analyses on FFPE material have previously been difficult to perform [373].

To isolate tumor compartments, laser capture microdissection was used and preparation of tissue sections for this procedure was performed with highest possible purity to maintain the RNA quality. Optimization was performed to obtain adequate amount of RNA with sufficient quality and preserved tissue morphology for compartment identification. Analysis of collected material revealed that tumors with non-inflammatory stroma did not yield sufficient amount of RNA for performing a global gene expression analysis, implying that only tumors with inflammatory stroma could be used. In this study, we have analyzed triple-negative breast tumors, a subgroup associated with poor prognosis and for which specific therapy is missing [29].

Since the RNA obtained from FFPE samples is partially degraded, we used novel techniques for amplification, labeling and hybridization of our samples with primers and probes detecting the whole transcript and not only the 3' UTR. By selecting genes with high expression levels (>7) and variance (>0.15) of log2 normalized data in all samples, we analyzed the expression differences by using the limma package of R and identified compartment specific genes in the stroma and epithelium. Even though the analysis of the stromal compartment identified specific stromal markers,

the choice of material (FFPE samples) and method (laser microdissection) is not optimal for accurate assessment of the gene expression, due to poor RNA quality. The identified stromal genes might reflect the composition of the tumor microenvironment and to identify stromal-specific gene signatures, we chose to expand the list of identified genes with breast tumor data from the TCGA dataset. Based on the expression levels, genes, from the dataset with a correlation coefficient above 0.85 to at least one of the original genes, were included for further analysis. Iterative correlation analysis of the identified genes identified nine clusters, where the expression level of all genes within a cluster had a correlation coefficient above 0.89 with all the other genes in that cluster. In particular, we identified gene sets with extracellular matrix (gene set 1 and 2), endothelial (gene set 3 and 4) or immune cell/inflammation (gene set 5-9) related genes. Based on the TCGA data we found basal-like breast tumors to have a lower expression in the extracellular matrix and vasculature gene sets and higher in the immune profiles. The importance of several of the cluster-specific genes has been reported in other studies [81, 374, 375], supporting a prognostic value of these genes.

Evaluation of the prognostic value of these gene sets highlighted gene set 5, 7 and 8 in a multivariate Cox proportional hazard analysis. Higher expression of gene set 5 and lower of 7 and 8 correlated with an increase in new tumor events in breast cancer patients. The same stromal signatures could be used to predict new tumor events and survival in kidney and ovarian carcinoma, respectively. Yet, neither of the gene sets had alone any effect on new tumor events or survival, further indicating that various factors in the tumor stroma are important for influencing tumor progression and that a certain composition of stromal cells can alter the prognosis for breast cancer patients.

Conclusions

In this thesis, we have identified novel mediators of stress response and cell growth regulation in breast cancer cells. In addition, we have identified stromal gene signatures that can be a valuable tool for prediction of outcome in breast cancer as well as other cancer forms.

We can conclude that:

- G3BP proteins are direct interaction partners of PKCα.
- PKCα can regulate stress response by causing a delay in stress granule assembly and affect eIF2α phosphorylation.
- G3BP1, but not G3BP2, has growth promoting effects and mRNA regulating capacities in breast cancer cells.
- PMP22 has a growth inhibiting function in several breast cancer cells.
- G3BP1 can reduce PMP22 mRNA expression, which may explain the increased cell growth induced by G3BP1 expression.
- Expression of miR-34c has a growth suppressive effect and induces a block of the G2/M cell cycle transition in breast cancer cells.
- G2/M cell cycle arrest might be affected by a miR-34c-mediated repression of CDC23.
- The developed methodological procedure for isolation and characterization of compartment-specific genes, using LCM on FFPE triple negative breast cancers, enables identification of stromal-specific gene signatures.
- The stromal gene signatures, obtained from LCM breast tissue, can predict new tumor event or death in breast, kidney or ovarian cancer.

Populärvetenskaplig sammanfattning

Cancer är ett samlingsnamn för flera hundra sjukdomar som har gemensamt att de har fått en okontrollerad celldelning till följd av genetiska förändringar. Celldelning sker ständigt i vår kropp när gamla celler måste bytas ut och nya kopior bildas. Vid dessa processer sker det ofta mutationer i vårt DNA så att informationen förändras, vilket resulterar i att skadan kopieras till den nya cellen som därmed inte blir identisk med sin föregångare. Dessa fel förhindras i det flesta fall genom reparationsmekanismer i cellen, men ibland blir de nya förändringarna för starka och de felaktiga cellerna kan börja dela sig okontrollerat.

All grundinformation om hur cellen ska bli finns i cellens DNA, den så kallade arvsmassan. Informationen i arvsmassan läses av under kontrollerade former och ger då upphov till en ny molekyl som kallas RNA. Denna process heter transkription och efterföljs i många fall av translation, där informationen från RNA-molekylen fungerar som mall för bildandet av proteiner. Celler skickar signaler både mellan och inom sig för att styra olika processer och de flesta av dessa processer regleras av proteiner. På senare tid har man dock förstått att även RNA-molekyler är viktiga för att sköta regleringen i cellerna och i *artikel I* och *artikel III* har vi studerat två mekanismer som påverkar cellens RNA-nivåer.

Under cellulär stress, vilket uppstår i cellen när de normala förhållandena förändrats, t.ex. vid förhöjd temperatur eller minskad syrenivå, reagerar cellerna genom att utveckla olika skyddsmekanismer. En av dessa försvarsmekanismer omstrukturerar balansen för bildandet av nya proteiner, så att cellen enbart producerar proteiner som kan reparera skadan. De övriga RNA-molekylerna som redan var producerade och redo för att bli proteiner tas nu omhand av cellen i ett skyddskomplex som kallas stressgranula. Där förvaras RNAt till dess att stressen försvinner. Om cellen fortfarande har behov av de skyddade RNA-molekylerna kan cellen snabbt starta proteinproduktionen istället för att börja om från DNA-nivån, och de RNA-molekyler som inte behövs bryts ner. I *artikel 1* har vi studerat ett protein som heter proteinkinas Cα (PKCα). Vi har sett att detta protein kan vara viktigt för bildandet av stressgranula och därmed skyddande av cellen. Vi har dessutom sett att PKCα kan binda till en familj av välkända stress-skyddande proteiner som kallas G3BP. Denna bindning skulle kunna vara orsaken till att PKCα kan påverka skyddandet vid stress, men exakt hur det här är reglerat är inte klart.

G3BP är RNA-bindande proteiner som finns i höga nivåer i flera olika typer av cancer och man har trott att dessa kan påverka cellernas förmåga att överleva och föröka sig, bland annat genom att reglera RNA-nivåerna av kända tillväxtproteiner. I *artikel II* visar vi att G3BP kan leda till ökad celldelning hos bröstcancerceller genom att minska nivåerna av det tillväxthämmande proteinet PMP22.

Cellens RNA-nivåer kan även regleras av en speciell sorts RNA-molekyl som kallas microRNA. Dessa microRNA binder till RNA-sekvenser och förhindrar att RNA translateras till protein. I *artikel III* undersökte vi hur ett visst microRNA, miR-34c, kan påverka celldelning i bröstcancer eftersom detta microRNA visat sig skydda mot celldöd och öka nyproduktion av celler i andra cancerformer. I bröstcancerceller kunde vi se att miR-34c hade en negativ effekt på ett specifikt steg i celldelningsprocessen när den viktiga separationen mellan de två nyproducerade cellerna sker. Under celldelningsprocessen finns flertalet kontrollsteg och miR-34c verkar påverka åtminstone ett av dessa, förmodligen genom att minska RNA-nivåerna av CDC23. Vi såg dessutom att patienter med höga nivåer av miR-34c hade en bättre prognos än patienter med låga nivåer.

Inom tumörbiologiforskning tittar man mestadels på cancerceller och hur de beter sig, men tumörer består inte bara av cancerceller. Runt tumören finns omgivande celler som kan påverka cancercellernas utveckling, till exempel fibroblaster och inflammatoriska celler. I normala fall har de som uppgift att hindra felaktiga celler från att finnas kvar i kroppen. Till sin hjälp har de ett strukturellt stöttande extracellulärmatrix som tillsammans med blodkärl bildar en omgivning som kallas stroma, eller när man pratar om tumörer, tumörcellsmikromiljö. Cancercellerna kan lära sig att undvika signaler från det omgivande stromat och därmed främja tumörutvecklingen. I *artikel IV* har vi utvecklat en metod för att kunna isolera cancerområden och stromadelar separat med laserteknik och utforska dessa delars genuttryck. Analyser av genuttrycken, det vill säga de RNA som finns uttryckta i vävnaden, visade tydliga mönster från de olika tumördelarna. Mönster från stromadelarna kunde sedan användas för att analysera och bedöma prognos för bröstcancerpatienter, men de visade sig även vara viktiga för bedömning av patienter med andra tumörformer.

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